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PROGNOSTIC AND PREDICTIVE BIOMARKERS OF GLIAL TUMORS OF THE CENTRAL NERVOUS SYSTEM IN THE CONTEXT OF PERSONALIZED MEDICINE

The dissertation

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Abstract

The glial tumors, so called gliomas, represent the largest group of the primary central nervous system malignancies. Gliomas remain generally an incurable disease progressing from the lower grades of malignancy to the more aggressive tumors in the course of time. This finally leads to the rapid patient's clinical deterioration and eventually the death. Recently there has been a significant expansion of knowledge in the neuro-oncology domain regarding the onset and development of neoplastic disease at the genetic as well as epigenetic level. Novel prognostic and predictive molecular genetic biomarkers are emerging that can be used for more precise diagnosis, for more accurate assessment of a patients´ prognosis, or for better selection of therapy and prediction of therapeutic response. The fundamental view of the histological-based classification of central nervous system tumors is gradually changing and the molecular biomarkers are incorporating in addition to histopathology to refine the diagnoses of many tumor entities at the moment. The recent findings from molecular genetics of gliomas together with the results from clinical trials incorporating the various biomarkers are discussed in this thesis.

In the first study the biomarker isocitrate dehydrogenases 1 (IDH1) R132H mutation was examined in the tumor tissue from patients with glioblastoma multiforme and the results were correlated with the clinical characteristics of patients. The prognostic value of this biomarker was proved. Patients with IDH1 R132H mutation in the tumor tissue had significantly longer survival than patients with IDH1 wild-type tumors. The presented results were included into the large recently published meta-analysis that confirmed positive prognostic effect of the IDH mutations on both overall survival and progression-free survival in patients with gliomas.

The second study examined the chromosomal aberration 1p/19q co-deletion in patients with anaplastic oligodendroglioma who were treated with the combined radiotherapy and chemotherapy (procarbazine, lomustine and vincristine regime - PCV). The results were correlated with the clinical characteristics of patients. The prognostic value of 1p/19q co-deletion was proved. The strong positive predictive value of this biomarker for overall survival was also shown for patients with co-deletion treated with neurosurgery and radiotherapy plus PCV chemotherapy by comparison with neurosurgery and radiotherapy alone.

The enormous advances in the molecular genetics of central nervous system tumors especially gliomas bring completely new opportunities for the optimization of the treatment strategies for an individual patient with these diagnoses. The analysis of molecular genetics in central nervous system tumors is now recommended in order to implement the principles of personalized medicine into the clinical management of these malignancies.

Preface

This work was made up at the Department of Histology and Embryology, Faculty of Medicine in Pilsen, Charles University, under the supervision of Doc. MUDr. Mgr. Zbyněk Tonar, Ph.D. and with the help of valuable advices of Prof. MUDr. Ondřej Hes, Ph.D.

I declare that I wrote this thesis by myself and all the literary resources are properly cited. The present work was not used to obtain another or the same scientific degree. The text of this thesis comprised the parts of the author´s scientific articles, whose full texts are available in the attachments to this work.

In Pilsen …… ……

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List of abbreviations

2-HG	2-hydroxyglutarate
ABL	abelson murine leukemia viral oncogene homolog 1
AODG	anaplastic oligodendroglioma
ATRX	alpha-thalassemia/mental retardation syndrome
BCR	breakpoint cluster region
CI	confidence interval
CIC	homolog of the drosophila gene capicua
c-KIT	mast/stem cell growth factor receptor
CNS	central nervous system
COSMIC	catalogue of somatic mutations in cancer
CpG	cytosine-guanine
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DNA	deoxyribonucleic acid
DNET	dysembryoplastic neuroepithelial tumors
ECOG	eastern cooperative oncology group
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
EORTC	European organization for research and treatment of cancer
FDA	Food and Drug Administration
FFPE	formalin-fixed, paraffin-embedded
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FUBP1	far upstream element binding protein
GBM	glioblastoma multiforme
G-CIMP	glioma cytosine-guanine islets methylator phenotype
HGG	high grade glioma
HLA	human leukocyte antigen
HR	hazard ratio

CHT	chemotherapy
IDH1/2	isocitrate dehydrogenases 1 and 2
IGF	insulin-like growth factor
IgG1	immunoglobulin G1
IL-12	interleukin 12
KPS	Karnofsky performance status
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGG	low grade glioma
MGMT	O-6-methylguanine-methyltransferase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MYC	myelocytomatosis viral oncogene homolog
NADP+	nicotinamide adenine dinucleotide phosphate
NCCTG	north central cancer treatment group
NOS	non-otherwise specified
ODG	oligodendroglioma
OS	overall survival
PARP	poly(ADP-ribose) polymerase
PCR	polymerase chain reaction
PCV	procarbazine, lomustine and vincristine chemotherapy
PD-1	programmed cell death 1
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PD-L1	programmed cell death 1 ligand
PET/CT	positron emission tomography and computed tomography
PFS	progression-free survival
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PTEN	phosphatase and tensin homolog
RNA	ribonucleic acid
RT	radiotherapy
RTOG	radiation therapy oncology group

SD	standard deviation
STAT3	signal transducer and activator of transcription 3
TCGA	the cancer genome atlas research network
TERT	telomerase reverse transcriptase
TP53	tumor protein p53
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WHO	world health organization
WT1	Wilms tumor 1
α-KG	alpha-ketoglutarate

1. Theoretical introduction

1.1 Biomarkers and personalized medicine in neurooncology

Personalized medicine represents new model of an individual patient's medical care [1,2]. The main goal of personalized medicine is the shift from the concept of "one medicine fits to all patients with the same disease" to individual treatment of each patient - "the right treatment to the right patient in the right time" [3-5]. Personalized medicine is based on the evolving knowledge about the human genome, gene functions as well as the genetic basis of the individual differences in responses to a treatment. The main strategy of personalized medicine is to provide an individualized approach to each patient, based on his/her personal genetic profile and combining information from omics disciplines (genomics, epigenomics, proteomics, transcriptomics, metabolomics and others) with innovative preventive and therapeutic strategies that are more efficient, safe and cost-effective [6-9].

Central nervous system (CNS) tumors account for about 2% of all cancers with the annually incidence 9.5 cases out of 100,000 people [10,11]. The widely used World Health Organization (WHO) classification from 2007 recognized more than 130 different histopathological units of primary CNS tumors [12]. This represents a very extensive and markedly heterogeneous group of diseases, with individual types of tumors exhibiting various biological behaviors.

Recently there has been a significant expansion of knowledge in the neuro-oncology domain regarding the onset and development of neoplastic disease at the genetic as well as epigenetic levels [13]. Novel prognostic and predictive biomarkers are emerging and the fundamental view of the histological-based classification of CNS tumors is gradually changing. Moreover, even in the given histopathological units, further segmentation is starting to establish based on molecular genetic profiles resulting from the international integrative multiplatform studies of the CNS tumors [14-16]. The huge progress in genetic and epigenetic findings led to the very recent update of WHO CNS tumors classification in 2016 [17]. For the first time, the molecular biomarkers are incorporated in addition to histopathology to refine the diagnoses of many tumor entities. The updated classification presents a new perspective for how CNS tumor diagnoses should be structured in the era of molecular medicine.

The largest group among the primary CNS tumors (about 50%) are formed from supporting glial cells and are called gliomas [12,18]. Gliomas are the most diverse group of CNS tumors differing in their typical localization, age predisposition, morphology, grade and the inclination to progression. To date, gliomas are classified mainly based on their histopathological characteristics. The most important classes are astrocytomas, oligodendrogliomas, ependymomas and mixed type of gliomas such as oligoastrocytomas.

Gliomas can be also categorized according to morphological features of anaplasia into the grade of malignancy with a range of WHO grades I to IV. This classification is closely linked to the distinct disease behavior, ranging from slow progression in lower grade tumors, to extremely poor prognosis for patients with WHO grade IV glial tumors (glioblastoma multiforme - GBM). However, it is not time independent during the disease course. Low-grade tumors (WHO grade II) progress to high-grade (anaplastic) gliomas (WHO grade III) and finally also to secondary GBM over time, which is now explained in detail on molecular genetic level [19]. The progression to GBM leads to rapid clinical deterioration and eventually to the patient's death within 15 months despite the complex treatment [20,21]. The only exception are WHO grade I gliomas (the most important representative - pilocytic astrocytoma) representing biologically entirely different type of tumors also called "circumscribed". These tumors are potentially curable with surgical resection only and do not progress to the higher grades over the disease time course [22]. Schematically CNS gliomas are subdivided into the lower grade tumors (low grade glioma - LGG) representing the WHO grades I and II tumors and high grade tumors (high grade glioma - HGG) with the WHO grade III and IV tumors (anaplastic gliomas and GBM). This sub-classification has strong clinical significance because of the substantial differences in treatment strategies.

In the near future it is likely to be necessary to integrate various molecular genetic biomarkers together with the principles of personalized medicine into standard clinical care for patients suffering from neurological cancers. The most recent and clinically relevant examples of the use of personalized medicine approaches in the management of the glioma patients will be discussed in this work focusing on glioblastoma multiforme, oligodendroglioma and the group of low grade gliomas.

1.2 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumor in adults with an incidence of 3-4/100,000/year [23,24]. GBM is extremely invasive and difficult to treat surgically, characterized by intense and aberrant vascularization and high resistance to radiotherapy (RT) and chemotherapy (CHT). The current standard of care for patients with newly diagnosed GBM is neurosurgery followed by fractionated external beam RT and CHT with systemic temozolomide [25]. The median survival of GBM patients is 12.1-14.6 months and only 3-5% of patients survive longer than 3 years [26]. The progress in the knowledge of GBM genetics over the past 10 years has revealed several abnormalities in a diversity of mutated genes and cellular signaling pathways. The importance of the GBM microenvironment, especially of tumor angiogenesis, has also been studied. New knowledge regarding the diversity of GBM on molecular and genetic level could lead to the deep analysis of the tumor and the refinement of management personalized to the individual patient in the near future.

1.2.1 Histopathology of glioblastoma

The application of histopathology together with molecular genetics is required for so called "integrated diagnosis" of GBM according to the WHO 2016 classification of CNS tumors [17]. GBM represent primary brain malignancy originating from glia, the brain tissue which provides supportive functions to neural cells (nutrients, oxygen supply, mechanical support, guidance in development and immune functions) but also acts in very complex processes (signal transduction and neurotransmission). GBM is the most common form of high-grade glial tumor defined by specific histopathological criteria such as hyper-cellularity, necrosis, pleomorphism, vascular proliferation and pseudopallisading [20,27].

GBM can be categorized into two subgroups - primary and secondary. Primary GBM are diagnosed as advanced cancer, whereas secondary cases have clinical, radiological or histopathological evidence of progression from a pre-existing lower-grade tumors [17,28]. There are substantial clinical differences between these two groups. Secondary GBM occur less frequently (<10% of all GBM), among younger patients (with a median age of 44 years), and have longer median overall survival (OS) by comparison with primary GBM (31 vs. 15 months, respectively). However, distinction between primary and secondary GBM based on

the histopathological findings alone is not possible [29]. On the other hand, there are fundamental differences between primary and secondary tumors at the genetic level that might allow their differentiation [17,19].

1.2.2 Molecular genetics of glioblastoma

The origin of cancer is currently understood as the accumulation of hereditary and/or somatic alterations (mutations) in genes that control critical biological processes, such as the regulation of apoptosis, the cell cycle progression and proliferation [30,31]. This could be manifested by the activation of oncogenes or by the silencing of tumor suppressor genes, which leads to the different gene expression profile of cancer cells. However, not only genetic alterations are immediately essential for malignant transformation. Epigenetic mechanisms of modification of gene expression, such as DNA methylation status, imprinting, chromatin changes, and the role of micro-RNAs, are also being discussed [30,31].

Comprehensive analysis of genetic and epigenetic alterations in glial tumors by comparison with normal brain tissue is now very important. This molecular approach could provide novel targets for diagnostic, prognostic or therapeutic purposes. It could also help with the identification of subgroups of patients who have better prognosis on standard therapy or preferentially respond to certain single or combined novel targeted therapies.

Some of the pioneer genetic studies of malignant gliomas described the presence of an extra copy of chromosome 7 in the cancer cells and an amplification of the receptor of epidermal growth factor (EGFR) gene was identified [32]. Further karyotype and loss of heterozygosity studies identified the positions of tumor suppressor genes on chromosomes 9, 10 and 17 [33]. The main gene which was altered on chromosome 17 in GBM was identified as tumor suppressor TP53, which has a critical role in the inspection of the genome for DNA damage and can arrest the cell cycle and trigger apoptosis. Owing to further progress in genetics, the loss of tumor suppressors from chromosomes 9 (p16 cell-cycle inhibitor) and chromosome 10 (phosphatase and tensin homolog - PTEN) were described in 1994 and 1997, respectively [34,35]. The role of p16 is to arrest cell cycle progression, whereas PTEN is a negative regulator of the phosphoinositide 3-kinase (PI3K) cell signaling pathway [36].

The unprecedented progress of recent years in all "omics" disciplines (such as genomics, transcriptomics, proteomics and others) together with improvements in bioinformatics technologies have provided new opportunities for the current brain cancer research. One of the most important genome-wide analyses of 20,661 protein-coding genes in GBM was completed in 2008. This study examined 22 tumors´ genome samples and identified the most important alterations at the genetic level that drive glioblastoma formation and progression [16]. Most of the common alterations in DNA were identified, such as point mutations, small insertions and deletions, as well as larger copy number changes, genetic amplifications and deletions.

Another exciting work in this area is conducted by the cancer genome atlas research network (TCGA). The TCGA consortium is carrying out research in more than 20 types of human cancers including GBM. A total number of 500 specimens of primary untreated GBM were utilized for the DNA (gene copy number, gene sequencing, epigenetic modification), mRNA (gene expression profile), and microRNA (regulation of expression) analyzes [15].

The alterations of several important cell signaling pathways involving in GBM development and growth were uncovered from these and others large multiplatform studies [15,16,19,37]. Among the most important ones are i) KRAS and PI3K oncogenic pathways (88% of GBM), ii) the p53 pathway (87% of GBM), iii) cell-cycle regulatory pathway (78% of GBM), and iv) the newly discovered alterations in metabolic pathways including isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) mutations (10% of GBM, in the vast majority the secondary). The alterations in IDH1/2 could also serve as independent prognostic factor that will be discussed hereafter in this section [38].

Based on these high-throughput sequencing studies evaluating large groups of tumors, GBM could be divided into different subtypes. By this approach, GBM still remain one pathological unit but are subdivided by their genetic alterations and expression profiles. The novel four subgroups of GBM explored from the TCGA data are called Classical, Mesenchymal, Proneural and Neural, named because of the function of overexpressed so called "signature" genes across these classes [39]. This subdivision has an important clinical relevance as the proneural group has the better prognosis, whereas the GBM with mesenchymal gene expression pattern have the worse OS of less than 12 months. Novel molecular classifications of GBM could be also useful for defining important molecular

alterations within each group, eventually suitable for therapeutic intervention by personalized targeted anticancer therapy.

1.2.3 Prognostic and predictive glioblastoma biomarkers

The huge progress in the genetics as well as epigenetics of gliomas in the recent years revealed some particularly important molecular biomarkers that significantly change the approach to clinical management of patients with these primary CNS tumors. The most important examples of prognostic and/or predictive biomarkers and their clinical relevance in the treatment of GBM patients is discussed in this section, such as the mutations in isocitrate dehydrogenases 1 and 2 (IDH1/2), the glioma cytosine-guanine (CpG) islets methylator phenotype (G-CIMP) or the promoter methylation status of the O-6-methylguanine-methyltransferase (MGMT) gene.

1.2.4 Mutations in IDH1/2 as a glioblastoma biomarker

The isocitrate dehydrogenases mutations are the important glioma biomarkers close to clinical application that are able to contribute to determining the patient's prognosis. IDH is an important Krebs cycle enzyme that converts isocitrate into alpha-ketoglutarate (α-KG) and reduces nicotinamide adenine dinucleotide phosphate (NADP+) to the reduced form NADPH. IDH thus acts in one of the critical steps of carbohydrate, lipid as well as amino acid metabolism [40]. Human IDH enzyme has three different isoforms - IDH1 (found in the cytoplasm and peroxisomes) and IDH2 and 3 (presented in the mitochondria).

Recurrent mutations in IDH were systematically described in patients with GBM, although only in about 5-10% of the tumors (predominantly secondary GBM). In contrast, IDH1/2 mutations were subsequently found with high frequency in diffuse astrocytomas (70-80%) and anaplastic astrocytomas (up to 50%) [41,42]. Mutations in IDH1 show conservative amino acid substitution R132H in 90%. R132C, R132G, R132S and R132L substitutions are also known but uncommon. Mutations in IDH2 are much rarer and primarily involve R172 amino acid substitution [42,43].

The real breakthrough in the understanding of IDH1/2 mutations for glioma oncogenesis was the discovery of completely new function of the mutant enzyme. Instead of NADP+ dependent production of α-KG, mutant IDH catalyzes the NADPH-dependent reduction of

α-KG to 2-hydroxyglutarate (2-HG). Gliomas with IDH1/2 mutations therefore contain the high concentration of 2-HG, unlike tumors without such mutations [44]. Potential onco-metabolite 2-HG is closely related to cancer initiation and progression. 2-HG serves as an potent inhibitor of alpha-ketoglutarate-dependent dioxygenases, which leads to genome-wide epigenetic changes [45]. Cells with mutations in IDH1/2 thus undergo massive epigenetic alterations including DNA and histone hypermethylation that leads to chromatin remodeling and extensively influences gene expression [46-48].

From the perspective of personalized medicine the marked impact of these mutations on GBM prognosis is especially important, regardless of the therapy intervention. Patients with GBM and mutations in IDH1/2 are generally younger and have a significantly longer median OS than patients without these mutations (IDH-wild type). Across several studies, better prognosis for patients with IDH1/2 mutated GBM than IDH-wild type GBM were observed with the longer median OS of 3.8 vs. 1.1 years, 2.6 vs. 1.3 years, 2.3 vs. 1.2 years and 3 vs. 1 year [16,43,49,50]. Even more significant differences in OS were found in patients with anaplastic astrocytomas; 5.4 vs. 1.7 years, 6.8 vs. 1.6 years and 7 vs. 2 years [43,49,50] as well as diffuse astrocytoma 12.6 vs. 5.5 years [49]. Recent meta-analysis of 55 observational studies has shown that patients with gliomas positive for IDH1/2 mutations have improved both overall survival and progression-free survival [38].

The growing importance of IDH mutations in clinical practice also requires the development of standardized and validated methods for analyzing of this biomarker in the tumor tissues with high sensitivity and specificity. IDH mutations can be assessed by immunohistochemistry or molecular biology techniques from the resected tumor tissue or biopsy [51-54]. These can be complemented or even replaced with non-invasive in-vivo determination of onco-metabolite 2-HG in the tumor tissue by MRI-spectroscopy [55-58]. This approach detecting the resulting product of mutated enzyme is also independent from sequential type of IDH1/2 mutations. It represents unique case in oncology when the specific mutation in the tumor tissue can be assessed by accessible radiology method with high sensitivity and specificity.

Further research will clarify the potential therapeutic effect of inhibition of mutated enzyme or depletion of onco-metabolite 2-HG accumulated in glial tumors. Inhibition of mutant IDH

shows promise in phase I/II clinical trials with hematologic malignancies and further development is ongoing in solid tumors including gliomas [59].

1.2.5 MGMT promoter methylation as a glioblastoma biomarker

The current standard of care for GBM patients includes neurosurgery, RT and the use of the temozolomide-based chemotherapy. Temozolomide is an oral alkylating agent that causes DNA damage by alkylation of the 0-6 position of guanine and the production of DNA interstrand cross-links [25]. In a large, randomized, phase III trial in newly diagnosed patients with GBM conducted by Roger Stupp, RT and concurrent daily temozolomide followed by adjuvant temozolomide provided a median survival benefit of 2.5 months and the proportion of 2-year survivors increased from 10.4% to 26.5% in comparison with RT alone. 5-year OS was also higher in combined treatment arm (9,8 vs. 1,9%) [60]. The Stupp's regime has become a gold standard of care in the treatment of patients with newly diagnosed GBM and is still valid today. There exists a subset of patients who have better response to temozolomide, but the majority of GBM patients become rapidly resistant.

One of the strongest predictive biomarkers for the chemotherapy response is the alteration in the MGMT gene [61]. The enzyme MGMT is able to repair the DNA damage caused by temozolomide. The presence of MGMT leads to reduction in the effect of temozolomide-based chemotherapy. The silencing of MGMT can be caused by epigenetic mechanisms, such as the DNA hypermethylation of CpG islands in the promoter region. This alteration leads to a decrease in the transcription of MGMT and to worse ability of tumor to repair damage caused by temozolomide which means a better therapeutic response [62]. Methylation of the MGMT promoter was observed in more than 40% of patients with GBM (more in the subgroup with secondary GBM) [63,64].

The subset analysis of the Stupp´s clinical trial showed that the patients with hypermethylated MGMT promoter had a significantly longer median OS after therapy with RT plus temozolomide compared with RT alone (21.7 vs. 15.3 months) [25,63]. There was no statistically significant difference in OS between the treatment arms in the subgroup without methylation of the MGMT promoter. In another study, MGMT promoter hypermethylation was predictive for a better response to RT independently of treatment with

temozolomide [65]. Therefore, the MGMT methylation status could be potentially considered as a general biomarker of better therapeutic response in GBM.

But what is the real predictive value of MGMT promoter methylation in everyday clinical practice? The substantial limitation of the use of this biomarker in choosing the most appropriate therapy for an individual patient is the lack of an alternative effective treatment for patients with newly diagnosed GBM. Moreover, the randomized phase III clinical trial radiation therapy oncology group (RTOG) 0525 which compared dose-intense temozolomide (75-100 milligrams per square meter of body surface on days 1 to 21 of a 28-day cycle) versus standard dose temozolomide (150-200 milligrams per meter squared on days 1 to 5 of a 28-day cycle) didn´t reveal a benefit of dose-intense regime overall, or in the subgroups of MGMT hypermethylated or unmethylated patients [66]. However the prognostic effect of this biomarker was also proven in this trial.

The MGMT promoter methylation could be incorporated into clinical practice as a predictive biomarker in some particular scenario, such as in the treatment of patients with the higher age and/or poorer performance status. Patients with age of more than 65 years and/or Karnofsky performance status (KPS) less than or equal to 60 often develop significant toxicity which limits the applicability of the standard treatment regime with RT and temozolomide.

Two independent clinical trials in elderly patients with GBM (NOA-08 and Nordic trial) randomized subjects into the RT alone (standard RT vs. hypofractionated RT in Nordic trial) versus temozolomide alone (dose-intense temozolomide in NOA-08) arms as an initial treatment [67,68]. Patients with MGMT promoter methylated tumors showed better outcome with temozolomide in both trials. Whereas those with MGMT unmethylated tumors had reduced survival when treated with temozolomide by comparison with RT alone. These results strongly support the predictive role of MGMT biomarker for the choosing of the optimal therapy in elderly GBM patients who are not commonly eligible for the combined modality treatment [13]. Currently, the optimal treatment strategy for elderly patients with GBM should be selected in a multi-disciplinary setting taking into account the KPS, extent of tumor resection and MGMT promoter methylation status. Based on the results from clinical trials mentioned above, it is now recommended to use temozolomide monotherapy after surgery in GBM patients with age more than 70 years and/or KPS less than or equal to 60 with tumor positive for MGMT promoter methylation also in the Czech Republic [60].

The prognostic as well as predictive role of MGMT biomarker has a close relation to the presence or absence of IDH mutations in the tumor tissue. In the recent study with 98 GBM patients the combined analyses of IDH mutations together with MGMT promoter methylation outperforms either IDH1 mutations or MGMT methylation assessment alone in predicting survival [69]. The best prognosis was observed for those patients with IDH mutated MGMT methylated tumors followed by IDH mutated MGMT unmethylated and IDH wild-type MGMT methylated GBM. The worst prognosis was found in patients with IDH wild-type MGMT unmethylated tumors. The subanalyses of 183 anaplastic glioma patients from the NOA-04 clinical trial revealed the predictive effect of MGMT promoter methylation for benefit from alkylating agent chemotherapy only in patients with IDH1-wild-type, but not IDH1-mutant tumors [70]. The analysis of various biomarkers and their combinations will probably become the gold standard in the treatment planning for GBM patients in the near future.

1.2.6 G-CIMP as a glioblastoma biomarker

Another molecular genetic biomarker with possible clinical relevance for GBM patients is the glioma cytosine-guanine (CpG) islets methylator phenotype (G-CIMP). Hypermethylation of the CpG islets in glioma genome was studied mainly as a prognostic biomarker for GBM patients. The subanalysis of 272 GBM from the TCGA dataset demonstrated that patients with G-CIMP positive tumors were of younger age and experienced significantly improved OS [71]. Moreover, the vast majority of the G-CIMP positive tumors had also IDH1 mutations and belonged to proneural pattern of gene expression. The direct relationship between the mutations in IDH1/2 and occurrence of G-CIMP in tumor tissue was subsequently found [72]. The presence of IDH1/2 mutations and an accumulation of onco-metabolite 2-HG seems to be the sufficient factor for the establishment of G-CIMP in glioma genome.

1.2.7 The perspectives of novel therapies for patients suffering from GBM

The standard therapeutic options for the treatment of GBM as well as other types of high-grade glioma have only limited benefits. The novel group of anticancer drugs so called targeted therapies are directed against certain tumor features such as altered signaling and metabolic pathways, aberrant tumor vessels, angiogenesis and the tumor microenvironment

[30,36]. Recent genome-wide studies and the molecular characterization of GBM has enabled the identification of potential new targets in cancer cells together with the development of novel therapeutic small molecules and monoclonal antibodies and initiation of clinical trials with these drugs [24,73]. Also new approaches of targeted immunotherapy could bring a fundamental breakthrough into the treatment of gliomas [74].

However, there is a wide molecular diversity and heterogeneity associated with the aberrant GBM signaling pathways. Recent study identified distinct mutation profile of recurrent glioma that varied from the initial mutation analyses in the same patient [75]. The exomes of 23 initial low-grade gliomas and recurrent tumors resected from the same patients were sequenced and the mutation profiles were compared each other. In 43% of cases, at least half of the mutations presented in the initial tumor were not found at recurrence. Moreover, the mutational profile of GBM is also affected by chemotherapy as the recurrent tumors exhibit temozolomide-induced damage to the DNA mismatch repair system resulting in a hypermutated phenotype [76]. Another study revealed the possibility of GBM proneural gene expression pattern transition to a mesenchymal pattern at recurrence which could also affects the effectiveness of new drugs used at the beginning of the treatment in newly-diagnosed disease [37].

These and others mechanisms such as the lack of tumor dependence on proposed target, failure of drug penetration into CNS, or clonal evolution and antigen escape of tumor after effective therapeutic intervention, could be the reasons for the relative lack of success of new targeted approaches in the treatment of gliomas. Only a small clinical benefit has been demonstrated with the novel therapeutics so far which is discussed in more detail in the following text.

Overcoming of these barriers will probably require the use of individualized molecular profiling of each GBM tumor at the initial diagnosis and also at the recurrence and application of personalized medicine principles in combination of targeted therapies with other types of treatment for high-grade gliomas.

1.2.8 Inhibitors of growth factors and their receptors, inhibitors of intracellular signaling pathways

This is a group of relatively new molecules that are able to specifically affect (inhibit) various aberrantly activated cell signaling pathways leading to the formation and progression of cancer [24,36]. Such effect can be achieved by inhibition of specific growth factors and their receptors including epidermal growth factor family (EGF) and their receptors (EGFR), platelet-derived growth factors (PDGF) and their receptors (PDGFR), insulin-like growth factors (IGF), fibroblast growth factor (FGF) and their receptors and others. These receptors and their ligands are overexpressed or mutated in high proportion of GBM [16,77].

The molecular aberrations in EGFR comprising mutations or gene overexpression are described in approximately 50% of GBM [15]. Therefore aberrantly activated EGFR could be possible therapeutic target, similar to the situation common in other tumor types. One of the approved drugs directed against EGFR is gefitinib. The progression-free survival (PFS) at 6 months was 13% and the median OS was 10 months in early phase II clinical trial of recurrent GBM treated with gefitinib [78]. There were more recent studies with gefitinib as monotherapy or in combinations for GBM treatment with results of only very limited efficacy compared to standard treatment [79-81]. Another EGFR inhibitor also examined as a possible treatment for GBM is erlotinib. Number of phase II trials of erlotinib as a single agent showed only minimal benefit for GBM treatment and modest survival benefit in combination with other agents [82-84]. Another promising drug with EGFR inhibitory activity is lapatinib. This drug was tested in multiple clinical trials in patients with GBM but again with very limited antitumor effect [85-87]. The newer irreversible EGFR inhibitor afatinib has been recently evaluated as a monotherapy or in combination with temozolomide in phase I/II study with recurrent GBM patients [88]. Afatinib had a manageable safety profile but only very limited activity. Cetuximab is a chimeric monoclonal antibody which can also inhibit EGFR. This drug was tested in the small group of GBM patients but with poor results [89]. Some improvement was observed in the phase II study evaluating the combination of cetuximab, irinotecan, and bevacizumab. However, the efficacy data were not superior compared to results with bevacizumab and irinotecan alone [90]. The observed effects of EGFR inhibitors in the treatment of GBM patients are still generally weak. Better

results could be possibly achieved by stratification of patients eligible to the treatment by presence of overexpression or specific mutations of EGFR in the tumor tissue [91-93].

The PDGFR is another receptor on cell surface frequently overexpressed and activated in GBM, especially in the proneural subtype [15,39]. The aberrant activation of PDGFR assists in the transition from lower grade glioma to GBM and the PDGF ligand is able to stimulate tumor growth and angiogenesis [94,95]. Imatinib is a kinase inhibitor of PDGRF, c-KIT, and oncogene fusion protein BCR-ABL that was extensively examined also in patients with GBM. The PFS 16% at 6 months was observed in one phase II trial of patients with the recurrent disease [96]. Further multicenter phase II studies confirmed that imatinib as a monotherapy or in combinations failed to improve PFS or OS in patients with GBM [97,98]. Multikinase inhibitors influencing tumor angiogenesis sunitinib, sorafenib, or vandetanib have also inhibitory effect on PDGFR. These substances were evaluated in the treatment of GBM as well [99,100]. Nevertheless, more recent multicentric randomized phase II clinical trial of RT and temozolomide with or without vandetanib in newly diagnosed GBM patients showed no significant OS benefit of combination compared with the parallel control arm, which led to early termination of the study [101]. Newer multikinase inhibitors affecting PDGFR such as dasatinib or nintedanib also failed to improve OS in patients with recurrent GBM [102-104]. Based on the results from these and other clinical trials with various targeted drugs inhibiting overexpressed PDGFR in the tumor tissue, this approach unfortunately does not seem to be an effective therapeutic strategy for patients with GBM at the moment.

Intracellular components in signaling pathways mediate the response of cells to the growth factors and their interactions with cell surface receptors. Inhibition of such aberrant signaling components is a promising targeted therapeutic approach for the treatment of many types of cancer including high-grade glioma [24,36]. Activation of protein kinase C (PKC) contributes to the signal propagation from several growth factors, such as EGF and PDGF, which stimulate glioma cell proliferation. The examples of drugs that inhibit PKC and were evaluated in patients with GBM were tamoxifen [105,106] or enzastaurin [107,108]. Again, the minimal or no benefit was observed. Mammalian target of rapamycin (mTOR) is another intracellular protein-kinase involved in cell growth signaling. It transduces the signals from PI3K as well as the KRAS pathway [36]. Overexpression of growth factors or deletion of PTEN increases the mTOR activation in GBM [77,109]. Selective mTOR inhibitors were

examined in GBM settings as well. The small molecule sirolimus was not effective as a single agent and had limited efficacy in a phase II trial in combination with erlotinib [84,110]. Another mTOR inhibitor everolimus showed no clear clinical benefit in combination with gefitinib for recurrent GBM [80]. The recent phase II study evaluating everolimus, temozolomide, and radiotherapy in patients with newly diagnosed glioblastoma showed no appreciable survival benefit of the combination compared with historical controls treated with conventional therapy [111]. The newer selective inhibitor of PI3K showed low overall response rate with median PFS at 6 months only 17% in the phase II study with recurrent GBM patients. However, 21% of participants had durable stable disease even if the association between stable disease and molecular biomarkers was not seen [112]. There are many other targeted therapeutics affecting various aberrantly activated intracellular signalizations of cancer cells that were examined in the GBM settings, such as PARP inhibitors, STAT3 inhibitors and others [24,73,113-115].

Unfortunately, despite enormous advances in the research of personalized medicine and targeted oncological therapy during the past decade, none of these therapeutics have proved the significant PFS or OS benefit in the well-designed phase III clinical trial for patients with newly diagnosed or recurrent disease as a monotherapy or in combination with standard treatment regime so far.

1.2.9 Inhibition of angiogenesis in glioblastoma

The cancer research increasingly highlights the fundamental role of tumor microenvironment together with pathological angiogenesis and tumor neovascularization for the development and progression of malignant diseases [30,116]. The processes of pathological angiogenesis and possible mechanisms to their therapeutic inhibition have been extensively studied also in the case of GBM [117-119]. The central position in tumor angiogenesis hold the vascular growth factors, especially vascular endothelial growth factor (VEGF) and its variant VEGF-A, primarily through its interactions with the VEGFR1 and VEGFR2 receptors found on endothelial as well as cancer cells. Excessive microvascular proliferation and VEGF overexpression were identified in the tumor tissue from GBM patients. The higher intra-tumor as well as plasma VEGF concentrations were associated with rapid disease progression and presence of early recurrence [119-124]. Therefore a great

effort is being made with the evaluation of antiangiogenic and anti-VEGF agents in GBM settings.

One of the most commonly used inhibitors of angiogenesis in cancer treatment is bevacizumab, which is a humanized IgG1 monoclonal antibody against VEGF-A. Bevacizumab was extensively examined in clinical trials for treatment of recurrent as well as newly diagnosed GBM, as a single agent and in various combinations with chemotherapy and other targeted therapeutics [118,125-130]. Bevacizumab gained accelerated approval by the US Food and Drug Administration (FDA) for the use in recurrent GBM in 2009 based on a high radiographic response rate and prolonged PFS [131]. The multicenter phase 2 BELOB trial undertaken in 14 hospitals in the Netherlands suggested the possible OS benefit for patients with recurrent GBM treated with the combination of bevacizumab plus lomustine versus bevacizumab or lomustine alone [132]. However, the well-designed phase III European organization for research and treatment of cancer (EORTC) 26101 clinical trial failed to confirm the OS benefit of bevacizumab plus lomustine by comparison with lomustine alone (9.1 vs. 8.6 months, HR 0.95; CI 0.74 - 1.21, P = 0.65) in patients with first progression of GBM [133]. Whereas PFS was longer in the combination arm by comparison with lomustine alone arm (4.2 vs. 1.5 months, HR 0.49; CI 0.39 - 0.61). The combinations of bevacizumab and standard treatment for newly diagnosed GBM were also examined with encouraging results in initial phase II studies [134-136]. Based on the results from the previous studies, there were designed two large phase III clinical trials AVAglio (NCT00943826) and RTOG-0825 (NCT00884741) evaluating bevacizumab-containing regimes for patients with newly diagnosed GBM. Unfortunately, neither trial demonstrated a benefit in OS for the combination of bevacizumab with standard RT plus temozolomide treatment compared to standard regimen alone [137,138]. Both studies demonstrated PFS survival benefit of combination but it reached prespecified statistical significance only in AVAglio trial (10.6 vs. 6.2 months, P < 0.001). Also the baseline health-related quality of life and performance status were maintained longer and the glucocorticoid use was lower in the bevacizumab arm in AVAglio trial but with more grade 3 and 4 adverse events (66.8% vs. 51.3%). The retrospective analysis of molecular biomarkers in AVAglio trial showed that patients with both IDH1 wild-type tumors and proneural pattern of gene expression may have derived 4.3 months OS benefit with the addition of bevacizumab to standard regimen [139]. Because of the post-hoc nature of this analysis the predictive effect in relation to

bevacizumab treatment must be interpreted with caution. Recent meta-analysis examined clinical trials that compared bevacizumab plus combined RT and temozolomide with RT and temozolomide alone in patients with newly diagnosed GBM [126]. The meta-analysis included 1,738 patients from three well-designed clinical trials. The result failed to demonstrate OS benefit (HR 1.04; CI 0.84 - 1.29, P = 0.71) but identified increased PFS (HR 0.74; CI 0.62 - 0.88; P = 0.0009) for combined treatment with bevacizumab. Moreover, there was no increase in the 6-month survival (P = 0.13) and the rate of serious adverse events was higher in the bevacizumab compared with the placebo group. Based on the results from AVAglio and RTOG-0825 trials, bevacizumab was not approved for the treatment of patients with newly diagnosed GBM and remains the treatment alternative only in the recurrent setting in the USA and in Canada.

There is a substantial number of studies evaluating other angiogenesis inhibitors in the treatment of recurrent as well as newly-diagnosed GBM. The VEGFR tyrosine kinase inhibitor cediranib showed activity in the phase II clinical trial as a monotherapy in patients with recurrent GBM [140]. Despite the promising results, cediranib demonstrated no PFS benefit as a monotherapy (HR = 1.05; CI 0.74 - 1.50, P = 0.9) or in combination with lomustine (HR = 0.76; CI 0.53 - 1.08, P = 0.16) versus lomustine alone in patients with recurrent GBM in phase III clinical trial [141]. Cilengitide is an inhibitor of αvβ3 and αvβ5 integrin receptors that also blocks pathological tumor angiogenesis. Cilengitide was evaluated with promising results in phase II study as a monotherapy in patients with recurrent GBM [142]. However, the phase III clinical trial evaluating cilengitide combined with standard treatment compared to standard regime alone failed to show significant OS benefit of the combination (26.3 vs. 26.3 months, HR 1.02; CI 0.81 - 1.29, P = 0.86) in patients with newly-diagnosed MGMT methylated GBM [143]. Another angiogenesis inhibitor aflibercept, a recombinant produced fusion protein that scavenges both VEGF and PDGF, was studied in recurrent setting but demonstrated minimal evidence of single-agent activity in GBM patients with PFS at 6 months of only 7.7% [144]. Unfortunately, apart from bevacizumab no other inhibitor of angiogenesis has been approved for the treatment of patients with newly-diagnosed nor recurrent GBM so far.

1.2.10 Immunotherapy of glioblastoma

Immunotherapy represents a very promising area of multimodal anticancer treatment for many types of cancers at the moment [145-148]. There was also a great progress in immunotherapy research in GBM over the past few years. There are many various approaches currently being evaluated in GBM clinical trials, including passive immunotherapy with antibodies, utilization of autologous stimulated lymphocytes and cytokines, oncolytic virotherapy, or active immunotherapy with vaccine strategies based on tumors, peptides, or dendritic cells (DC) [74,149,150].

More than 40% of GBM carry the unique deletion mutant variant of EGFR called EGFRvIII that is characterized by a deletion of 267 amino acids in the receptor extracellular domain [151,152]. This mutation causes constitutive ligand independent receptor activation and signal propagation that leads to the cancer cell proliferation. The enhanced proliferation of EGFRvIII positive cancer cells together with the lack of EGFRvIII expression in normal non-cancerous cells makes it an ideal candidate for targeted therapy and the use of personalized medicine in the GBM treatment. Rindopepimut is a peptide-based vaccine (containing 13 EGFRvIII-specific amino acid sequences) targeted against EGFRvIII surface antigens. The phase I/II multicenter study evaluating rindopepimut in patients with newly diagnosed GBM showed promising results with a median PFS of 15.2 months and an OS of 23.6 months [153]. Subsequent phase II clinical trial (ACT III) examined rindopepimut in combination with standard RT and temozolomide in 65 patients with newly diagnosed GBM overexpressing EGFRvIII [154]. The median OS was 21.8 months. Patients with unmethylated MGMT promoter had an OS of 20.9 months, whereas those with methylated MGMT had longer OS of 40 months. Based on the promising results from early clinical trials, the double-blinded randomized multicenter phase III ACT IV study of rindopepimut in patients with newly diagnosed GBM (study number NCT01480479) was designed and started the enrollment of patients [155]. However, rindopepimut combined with temozolomide failed to improve OS during the interim analysis by comparison with the standard treatment (20.4 vs. 21.1 months; HR 0.99) and the study was discontinued in March 2016, which was a real disappointment. At the same time this example showed the importance of verification of promising preliminary results from early drug development in the well-designed randomized placebo controlled phase III clinical trials. Rindopepimut is still evaluating in the phase II ReACT clinical trial in combination with bevacizumab in

patients with recurrent EGFRvIII-positive GBM (NCT01498328). There are other peptide vaccines targeting tumor antigens, such as the HLA-restricted Wilms tumor 1 (WT1) 9-mer peptide vaccine, which was examined in patients with recurrent GBM in phase II clinical trial [156]. The median PFS was 20 weeks and the PFS at 6 months was 33.3%. More recent phase II clinical trial with an autologous heat-shock protein-peptide vaccine HSPPC-96 (vitespen) showed promising results in recurrent GBM patients with the median OS of 42.6 weeks [157].

Dendritic cell (DC) vaccines use autologous tumor lysates or common tumor antigens to induce immune response against the cancer. These strategies were evaluated in early-phase clinical trials in newly diagnosed GBM [158-161]. The DC vaccine loaded with autologous tumor lysate was examined in phase I clinical trial with 56 relapsed GBM patients. The results showed the median PFS of 3 months and the median OS of 9.6 months with a 2-year OS 14.8% [162]. The same group investigated the integration of the DC vaccine into the standard treatment of patients with newly diagnosed GBM and achieved the median OS of 24 months [163]. Another large double-blinded randomized phase II DC vaccine (DCVax-L) trial in patients with newly diagnosed GBM showed encouraging results with a median OS of 3 years, with 4-year survival reaching 33%, and 27% of patients exceeding 6-years survival from initial surgery [164,165]. However, the clarification of these promising results with DC vaccine strategies are essential in the well-designed randomized phase III clinical trials, such as the DCVax-L phase III study (NCT00045968) which is now ongoing and the final results are eagerly awaited.

There is a dramatic success in the treatment of various advanced solid tumors such as melanoma, renal cancer, lung cancer, head and neck cancer and other tumor types with the novel class of immunomodulatory anticancer agents called immune checkpoint inhibitors [145-148,166-168]. These therapeutics are able to block inhibitory molecules and their receptors on effector immune cells which leads to a robust T cell response against the tumor. At the moment, there are approved monoclonal antibodies directed against distinct inhibitory molecules such as ipilimumab targeting cytotoxic T lymphocyte antigen 4 (CTLA-4) and nivolumab or pembrolizumab targeting programmed cell death 1 (PD-1) and many others are in development. The great success of immune checkpoint inhibitors in a number of advanced solid tumors also led to the examination of these compounds in CNS gliomas [169,170]. There is a comprehensive pre-clinical research supporting a role for immune

checkpoint inhibitors in the treatment of GBM. In the study with murine glioma model, the treatment with CTLA-4 blockade effectively reversed glioma-induced changes to the CD4+ T cells compartment and enhanced antitumor immunity [171]. Another study with mouse GBM model showed the synergic effect of combined treatment with systemic CTLA-4 blockade together with intratumoral IL-12 application leading to the tumor eradication even at advanced disease stages [172]. The effectivity of combined CTLA-4 with PD-1 ligand (PD-L1) and indoleamine 2, 3 dioxygenase 1 blockade was studied in the study of mouse GBM model. It was shown that 100% of mice survived the triple combination therapy for a long time [173]. More recent study with murine GBM model showed that anti-CTLA-4 plus anti-PD-1 therapy was able to cure 75% of the animals, even with advanced and later-stage tumors [174].

The rationality for the use of immune checkpoints inhibitors in the treatment strategies for GBM patients comes also from some substantial clinical findings. First, these drugs are effectively overcoming the blood-brain barrier and are active in CNS. CTLA-4 inhibitor ipilimumab showed activity in patients with melanoma brain metastases without significant CNS toxicity [175,176]. Also the PD1 inhibitor pembrolizumab was active in the treatment of brain metastases in patients with melanoma or non-small-cell lung cancer with an acceptable safety profile [177,178]. Next, PD-L1 expression level in the tumor tissue was associated with the likelihood of clinical benefit with the PD-1 inhibitors in non-small-cell lung cancer as well as other tumor types [179,180]. The recent study showed that there is a robust and diffuse expression of PD-L1 assessed by immunohistochemistry in newly diagnosed as well as recurrent GBM specimens (88% vs. 72.2%, respectively) which is a relatively high percentage compared to other cancers including melanoma [181]. Higher expression of PD-L1 in tumor tissue correlated with worse outcome in another study with 94 GBM patients [182]. Because of the promising pre-clinical experiments, proven activity in the CNS and the presence of targets in tumor tissue, clinical trials with specific immune checkpoint inhibitors are warranted in GBM patients in newly diagnosed as well as recurrent settings.

The clinical trials evaluated immune checkpoint inhibitors including ipilimumab, nivolumab, and pembrolizumab in GBM patients are currently being conducted. The phase II CheckMate 143 study (NCT02017717) is evaluating nivolumab alone and nivolumab plus ipilimumab versus bevacizumab as an active comparator in patients with recurrent GBM.

The updated results observed the high activity of nivolumab arm with the 12-month OS of 40% [183]. Nivolumab alone was also the best tolerated arm with no new safety signals. Another currently running phase II study (NCT02337491) validates the combination of pembrolizumab and bevacizumab in recurrent GBM patients. Very preliminary results from 6 patients treated with combination showed median OS 6.8 months with two patients remain alive long-term (327 and 328 days) [184]. Pembrolizumab is also examined in combination with bevacizumab and hypofractionated stereotactic irradiation in phase I/II study (NCT02313272) in patients with recurrent GBM. Preliminary results from 6 patients who were treated with the combination showed durable disease control in all 3 patients evaluable for response [185]. The phase II CheckMate 548 (NCT02667587) study is examining the combination of standard RT plus temozolomide treatment with nivolumab or placebo in patients with newly-diagnosed GBM. Moreover, the phase III CheckMate 498 (NCT02617589) clinical trial is evaluating the head to head comparison of nivolumab to temozolomide both in combination with standard RT in newly-diagnosed GBM patients with unmethylated MGMT promoter in tumor tissue. The preliminary results from both of these studies are eagerly awaited. Pembrolizumab is also being examined in newly-diagnosed setting in combination with standard RT plus temozolomide regime in phase I/II (NCT02530502) clinical trial. The results have not been published yet. The newer checkpoint inhibitors are evaluating for the treatment of GBM patients as well, such as the phase II study of PD-L1 inhibitor durvalumab in newly-diagnosed as well as recurrent settings (NCT02336165).

Immune checkpoint inhibitors represent a significant breakthrough in the treatment of various advanced tumors (such as melanoma, renal cancer, lung cancer, head and neck cancer, urinary bladder cancer and others) in recent years that changed dramatically the prognosis of cancer patients [145-148,166-168]. In some of them the therapy with immune checkpoint inhibitors means long-term disease control or hopefully the cure. Even if the preliminary results of these drugs are promising and generally encouraging also in GBM patients, it is necessary to wait for the results from multiple phase III clinical trials that are expected to be final in 2019 and beyond.

1.3 Oligodendroglioma

Oligodendrogliomas (ODG) represent approximately 5% of primary brain tumors. They have more favorable response to radiotherapy and chemotherapy than other types of CNS gliomas [186]. According to the updated 2016 WHO classification of CNS tumors, they are characterized by a histopathological finding with an oligodendroglial component together with the presence of distinct molecular genetic profile [17].

The huge progress in the research of ODG molecular genetics offers new knowledge in the diagnosis and treatment of these tumors that has, together with recent results from clinical trials, the direct impact on the management of ODG patients. The analysis of molecular genetics in ODG and the use of specific biomarkers are now well-established and recommended as an important part of treatment-decision algorithms in clinical practice, as will be discussed in this section.

1.3.1 Histopathology of oligodendroglioma

Oligodendroglial tumors can be differentiated by degree of malignancy into grade II and grade III tumors - anaplastic oligodendrogliomas (AODG). Only about 30% of oligodendroglial tumors have anaplastic characteristics in the histopathological image, such as nuclear atypia, increased cellularity, increased proliferation activity and increased cell mitosis. AODG comprise about 0.5-1.2% of primary brain tumors [23,187]. The highest incidence of AODG is between 45 and 50 years of age. In contrast, grade II ODG afflicts patients from seven to eight years younger. It is presumed that this difference corresponds to the progression from grade II to grade III tumor. Typical ODG histopathological findings are round nuclei with a light or empty cytoplasm in the vicinity (perinuclear “halo” effect) and the presence of microcalcifications [12,17].

1.3.2 Standard treatment of oligodendroglioma

The majority of ODG present with the epileptic seizures. The most frequent other symptoms affect the frontal and, in some cases, the temporal brain regions. Infiltrative growth and poorly defined perifocal edema cause later symptoms of intracranial hypertension.

Neurosurgery is the fundamental treatment modality for patients with ODG. The best results are obtained with the total resection of the tumor. Sophisticated diagnostic preoperative and perioperative methods (magnetic resonance imaging - MRI, use of 5-aminolevulinic acid, MRI tractography, perioperative ultrasound and MRI, awake surgical method, hybrid positron emission tomography and computed tomography - PET/CT) and navigated microsurgical techniques are important parts of surgical treatment [188-190]. Targeted biopsy of the tumor is reserved for cases where the resection is not feasible.

A postoperative MRI is required to confirm the extent of tumor resection which was found to be an independent positive prognostic factor [191,192]. Favorable prognostic factors include young age, good overall medical condition (KPS), extent of tumor resection and combined oncological treatment [193]. The role of chemotherapy and RT in the ODG treatment in relation to the use of molecular biomarkers is discussed in detail in the following sections.

1.3.3 Co-deletion of 1p/19q as a oligodendroglioma biomarker

Oligodendroglial tumors are characterized by frequent co-deletions of chromosome 1p and 19q (1p/19q co-deletion). This chromosomal aberration was discovered in 1994 and became the first biomarker in neuro-oncology [194]. 1p/19q co-deletion means the loss of genetic material from both the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q). The unbalanced translocation t(1;19)(q10;p10) and formation of derived chromosome 1p/19q was identified later as the mechanism of this aberration [195]. 1p/19q co-deletion is present almost exclusively in oligodendroglial tumors (80% to 90% of grade II ODG; 50% to 70% of AODG) [196,197].

Mutations in two important tumor suppressor genes, CIC (a homolog of the Drosophila gene capicua) located on 19q13.2, and far upstream element binding protein (FUBP1) on the 1p chromosome, were recently discovered in the majority of ODG with 1p/19q co-deletion (50-70% and 15% for CIC and FUBP1 mutations, respectively) [198,199]. Mutations in these genes are involved in the ODG formation and progression. CIC protein binds to regulatory regions and blocks gene transcription. FUBP1 mutations are closely related to a myelocytomatosis viral oncogene homolog (MYC) activation. Currently, 1p/19q co-deletion

serves as an important diagnostic, prognostic and predictive biomarker in oligodendroglial tumors, as is discussed in detail further in this section.

1.3.4 Other oligodendroglioma biomarkers

Recurrent IDH1/2 mutations were first demonstrated in GBM. However, the frequent occurrence of mutations in IDH1 and IDH2 genes were also reported in ODG (up to 69%-94% tumors) [14,43,200]. The presence of the IDH1/2 mutations is a significant positive prognostic biomarker for patients with glioma including ODG [49,186]. Patients with ODG positive for both the 1p/19q co-deletion and IDH1/2 mutations experienced the best prognosis that shows the necessity of incorporating a combination of multiple biomarkers in the management of glioma patients [14,201].

The MGMT promoter methylation was discovered as a significant prognostic as well as predictive biomarker in patients with glioblastoma. This aberration was also found in 80% of AODG and in 73% of anaplastic oligoastrocytomas [202,203]. MGMT promoter methylation serves mainly as a positive prognostic biomarker for ODG patients treated with chemotherapy as was proven in the EORTC 26951 as well as NOA-4 clinical trials [204,205].

Hypermethylator phenotype of cytosine-guanine islets in the glioma genome is another important molecular characteristic of ODG. Positivity for G-CIMP is not an entirely independent biomarker as it is closely related to the presence of the IDH1/2 mutations also in ODG [71,72]. G-CIMP is approximately two-times more frequently presents in oligodendrogliomas (93%) than astrocytomas (45%) and is an important positive prognostic factor for all types of glioma including ODG [71].

The aberrations of certain other oncogenes as well as tumor-suppressor genes were identified in ODG such as mutations in PI3K, amplification of EGFR, or loss of PTEN tumor-suppressor, even if in rare cases. These alterations generally correlated with a worse prognosis in ODG patients [36,206].

1.3.5 The relevance of biomarker 1p/19q co-deletion in the clinical management of oligodendroglioma

The 1p/19q co-deletion status can be used in clinical practice as an important diagnostic, prognostic, as well as predictive biomarker in patients with oligodendroglial tumors. According to the WHO 2016 classification of CNS tumors, the diagnosis of oligodendroglioma is supported by the presence of 1p/19q co-deletion in tumor tissue, especially in cases where the histological findings are atypical or non-conclusive [17,207]. There are other tumor types that can mimic oligodendrogliomas by histopathological diagnosis such as dysembryoplastic neuroepithelial tumors (DNET), neurocytomas, clear cell ependymomas, and small cell anaplastic astrocytomas. Unlike ODG, these tumors do not have 1p/19q co-deletion, so as this biomarker is a useful diagnostic aid in these cases [207].

The 1p/19q co-deletion also has a role as an important positive prognostic ODG biomarker. Retrospective and prospective studies showed that ODG patients with 1p/19q co-deletion treated with standard therapy had significantly better survival outcomes than patients without 1p/19q co-deletion [207,208].

The 1p/19q co-deletion also acts as an important predictive biomarker for patients with ODG, especially AODG, in relation to combined treatment with RT plus chemotherapy. As early as 1998 it was found that patients with AODG positive for 1p/19q co-deletion are more sensitive to chemotherapeutic regimen containing the combination of procarbazine, lomustine and vincristine (PCV regime) [209]. The evidence-based proof of the significantly longer survival in patients with oligodendrogliomas and 1p/19q co-deletion treated with combined chemotherapy and radiotherapy did not exist for a long time. However, the long-term follow-up of two important phase III randomized clinical trials that incorporated 1p/19q co-deletion analyses (RTOG 9402 and EORTC 26951) evaluating RT and PCV regime in patients suffering from AO brought substantial results and led to a paradigm shift of the AODG treatment [210,211].

The RTOG study 9402 randomized 291 anaplastic oligodendroglial tumors (anaplastic oligodendrogliomas and oligoastrocytomas) into two treatment arms: PCV with follow up RT, and RT-alone. In the EORTC 26951 study, 368 patients with anaplastic oligodendroglial

tumors (anaplastic oligodendrogliomas and oligoastrocytomas) were randomized into two arms: RT-alone and RT followed by PCV chemotherapy. The 1p/19q status was determined through fluorescent in situ hybridization in both studies. In RTOG 9402 study, 1p/19q co-deletion was found in 46% of the patients. Over the course of the study, 80% of the patients randomized for radiotherapy subsequently received PCV therapy due to the progression of the disease. After a minimum three-year follow-up in 2006, the median PFS was different for the RT plus PCV arm compared with the RT alone arm (2.6 vs. 1.7 years, P = 0.004). However, the median OS was similar in both study arms (4.9 vs. 4.7 years, P = 0.26). The OS in both treatment arms was not significantly different based on the presence of 1p/19q co-deletion, therefore the positive predictive effect of this biomarker in relation to PCV chemotherapy was not proven [191]. The absence of superiority of combined therapy on the OS and the occurrence of serious adverse effects of PCV in more than 65% of the patients led to skepticism in regard to PCV treatment for AODG.

Similar results were observed from EORTC 26951 study in 2006 after an average five-year follow up. 25% of patients had tumors positive for 1p/19q co-deletion. The median PFS was different for the RT plus PCV arm compared with the RT alone arm (23 vs. 13.2 months, P = 0.0018). However the medial OS was similar in both study arms (40.3 vs. 30.6 months, P = 0.23) [192]. Patients with 1p/19q co-deletion had longer OS than patients without co-deletion, irrespective of the therapy arm. The results of both studies were considered rather negative in 2006. They did not prove the significance of 1p/19q co-deletion as a predictive biomarker in relation to chemotherapy, but rather showed the significance of 1p/19q co-deletion as a prognostic biomarker.

However, the decisive results came in 2013 following the long-term patient monitoring when the positive effect of combined oncological treatment (RT plus PCV) for anaplastic oligodendroglial tumors was proven. In the RTOG 9402 study, the median OS in patients without 1p/19q co-deletion remained similar to the results in 2006 in both groups receiving RT plus PCV and RT alone (2.6 vs. 2.7 years, P = 0.39) [210]. On the contrary, patients with 1p/19q co-deletion had significantly longer median OS in the RT plus PCV arm than in the RT alone arm (14.7 vs. 7.3 years respectively, P = 0.03). In multivariate analysis including co-deletion status, the OS for all patients was prolonged by RT plus PCV treatment (HR = 0.67; CI 0.50 to 0.91; P = .01). Likewise in the EORTC 26951 trial after more than 10 years' follow up, the OS of patients without 1p/19q co-deletion in tumor tissue was similar in the

groups receiving RT plus PCV and RT alone (25 vs. 21 months, P = 0.19) [211]. However, the median OS was not reached for patients with co-deletion in the RT plus PCV arm, whereas it was just 9.3 years in patients primarily receiving only RT.

The benefit in OS resulting from combined oncological treatment (RT plus PCV) in patients with 1p/19q co-deletion positive tumors was present in both clinical studies, irrespective of which type of therapy was started first. Even in patients who, due to the occurrence of adverse effects to therapy, received lower doses of PCV than planned. These results led to an important paradigm shift in the treatment algorithm of patients with AODG tumors positive for 1p/19q co-deletion. Nevertheless, the positive effects of combined treatment is negatively impacted by the adverse effects such as late radiotherapy toxicity (post-radiation necrosis, dementia) or toxic effects of PCV chemotherapy [212,213]. It is necessary to carefully monitor patients and detect the toxic effects of the treatment as early as possible.

Another important clinical question is the administration of combined oncological treatment in patients with anaplastic oligodendroglial tumors that do not have 1p/19q co-deletion. The results from the RTOG 9402 and EORTC 26951 studies showed that RT plus PCV treatment had a positive effect on PFS even among patients without 1p/19q co-deletion. To answer this question the phase III CATNON study randomized patients with anaplastic gliomas without 1p/19q co-deletion to the RT alone treatment or RT plus temozolomide in three different regimens (RT with concurrent daily 75 mg/m2 temozolomide, RT followed with 12 cycles of 150-200 mg/m2 adjuvant temozolomide, and RT with both concurrent temozolomide and 12 cycles of adjuvant temozolomide). The primary endpoint was OS. Recent interim analysis showed the OS benefit for patients in the temozolomide arms by comparison with RT alone arm (HR 0.645; CI 0.450 - 0.926, P = 0.0014) [214]. The 5-year OS rate was 56% when temozolomide was added to RT compared with 44% survival rate in patients treated with RT alone. The analysis of another glioma biomarker MGMT promoter methylation showed that patients with tumors positive for this biomarker had the OS advantage (HR 0.54; CI 0.38 - 0.77, P = 0.001). However, MGMT promoter methylation did not predict improved outcome with adjuvant temozolomide as was previously determined in GBM.

To evaluate the effect of temozolomide on treatment of AODG patients with 1p/19q co-deletion, the CODEL study (NCT00887146) was opened with three parallel arms: RT plus temozolomide, RT alone, and temozolomide alone. Based on the results of RTOG 9402 and EORTC 26951 trials, the RT-alone arm was abolished and the study is continuing in a two-

arm design comparing the RT plus temozolomide with RT plus PCV regimes. The final results are planned up to 2018 that should give definitive answer for the best therapeutic strategies in patients with 1p/19q co-deletion positive anaplastic oligodendroglial tumors.

The 1p/19q co-deletion status is currently recommended to be determined in all patients with AODG [186,215]. The PCV chemotherapeutic regimen in combination with RT should be implemented for all patients with AODG positive for 1p/19q co-deletion. The analysis of molecular genetics in ODG is now recommended as an important part of the management of these tumors and together with the novel chemotherapeutic regimes means a paradigm shift in current clinical practice in neurooncology, which demonstrates another example of the integration of the personalized medicine principles and molecular biomarkers into the management of glioma patients.

1.4 Low grade gliomas

Low grade gliomas (LGG) form a heterogeneous group of neuroepithelial tumors of the CNS. LGG primarily consist of astrocytomas, oligodendrogliomas, oligoastrocytomas and a rare group of mixed glioneural tumors. LGG are histologically characterized by hypercellularity, nuclear atypia, pleomorphism and the lack of significant mitotic activity [17,216]. These tumors also have lower proliferative index and don´t comprise necrosis and vascular proliferation as gliomas of higher degrees of malignancy.

LGG occur mainly at a younger age with a maximum between the third and fourth decade [217]. The clinical manifestations are mostly epileptic seizures (80%), less frequently changes in cognition, behavior, focal neurological symptoms or headaches. Neurological symptoms significantly impair patient´s quality of life. LGG may also be asymptomatic with an incidental diagnosis with imaging methods indicated for another reason. They grow infiltrative and often affect eloquent areas of the brain parenchyma. Although LGG are considered relatively benign tumors they progress gradually to the higher grade and the median OS of patients after diagnosis is only 7.5 years [217,218]. Therefore an intensive LGG research is needed in order to optimize the clinical management and improve the quality of life and prolong survival of patients.

1.4.1 Molecular genetics of low grade gliomas

Also in patients with LGG both the IDH1/2 mutations as well as 1p/19q co-deletion are the most important molecular aberrations in relation to clinical practice. IDH1 is mutated in high portion of diffuse astrocytomas (70-80%) and grade II oligodendrogliomas (up to 80%). IDH2 mutations are rare, occurring in 1-2% of diffuse astrocytomas and in 4.5% of grade II oligodendrogliomas [48,219,220]. Mutations in IDH1/2 detected in tumor tissue significantly correlate with better prognosis of patients with gliomas across all grades of malignancy including LGG [38,43,49,50,221].

The 1p/19q co-deletion was detected in 80-90% of low grade ODG and up to 10% of low-grade astrocytomas [197,222]. The recent meta-analysis showed prognostic and predictive significance of this biomarker in patients with gliomas [223]. The data from 28 studies were analyzed including 3408 patients with glial tumors of which 898 (26.3%) patients had

confirmed diagnosis of LGG. Compared with patients with wild-type tumors, co-deletion of 1p and 19q was associated with a better PFS (HR = 0.63; CI 0.52-0.76) and OS (HR = 0.43; CI 0.35-0.53) irrespective of the grades and subtypes of gliomas. Isolated 1p deletion had positive prognostic significance particularly in patients with LGG. Independent 19q deletion was not related to the patients´ survival. 1p/19q co-deletion was also demonstrated to be a positive predictive biomarker of responses to combined RT and chemotherapy (PCV regime) in patients with anaplastic oligodendroglioma and anaplastic oligoastrocytoma (grade III tumors) as was discussed in detail in the previous section. However, similar relation of this biomarker to treatment response in patients with LGG has not been confirmed yet. Thus 1p/19q co-deletion is the strong positive prognostic biomarker in patients with glial tumors including LGG.

The interrelations of individual LGG molecular genetic biomarkers seems to be more important for the clinical practice. Recently it has been shown that there exist at least three genetically as well prognostically heterogeneous groups of gliomas (having significant homogeneity within the groups) that can be distinguished by the presence of IDH1/2 mutations, 1p/19q co-deletion and mutual combination of these biomarkers in the tumor tissue [14]. The international consortium TCGA conducted an extensive multi-platform analyses of 293 patients with grade II and III gliomas. Data processing by Cluster of Clusters analysis and OncoSign integrated methods revealed three genetically distinct categories of gliomas. These categories strongly correlated with tumor subtypes determined based on the presence of IDH1/2 mutations, 1p/19q co-deletion and their combinations, but only weakly correlated with the histological type of tumors (R = 0.79 vs. R=0.19, respectively).

Gliomas in the first group were characterized by the presence of both IDH1/2 mutations and 1p/19q co-deletion. Activating mutations in the telomerase reverse transcriptase (TERT) gene promoter region, also identified in primary GBM, occurred in 96% of tumors classified into this group [14,224]. Other frequent aberrations identified in this glioma group were activating mutations in PI3K (20%), or inactivating mutations in tumor suppressor genes CIC (62%) and FUBP1 (29%) that were identified previously in 1p/19q co-deleted ODG [199]. This group mostly comprised of gliomas with oligodendroglial component (82% of oligodendrogliomas and 16% of oligoastrocytomas). The patients exhibited the best prognosis with the longest median OS of 8 years. It is necessary to emphasize that in this group of patients with the most favorable prognosis, there were 43% of patients with grade

III gliomas who should have a significantly worse prognosis if classified by the histopathological criteria alone without the use of molecular genetic biomarkers (especially IDH 1/2 mutations and 1p/19q co-deletion).

The second group included patients with gliomas positive for IDH1/2 mutations, but without the presence of 1p/19q co-deletion [14]. Moreover, 94% of the tumors had inactivating mutations in the tumor suppressor gene p53 and 86% in alpha - thalassemia/mental retardation syndrome (ATRX) gene. Tumors in this group comprised various gliomas without a clear predominance in the histological type and patients in this category had worse prognosis with shorter median OS of 6.3 years.

The last group comprised gliomas without the presence of IDH1/2 mutations, so-called IDH 1/2 wild-type tumors [14]. None of these tumors had 1p/19q co-deletion. Molecular genetic profile and biological behavior of these tumors were considerably closer to the primary GBM. Likewise the survival of patients with a median OS of just 1.7 years was similar to GBM. More than a half of these tumors were astrocytoma (56%). It is necessary to emphasize that almost one quarter of patients (24%) had histopathologic diagnosis of grade II gliomas that should expect a much better prognosis. Therefore the molecular genetic biomarkers incorporated into the classification of CNS gliomas provide an additional information to simple histopathological diagnosis that could improve the clinical care of patients with these tumors.

However, TCGA study was not the only one that tried to subdivide gliomas including LGG into prognostically different subcategories using several molecular genetic biomarkers and their combinations. The research group from Mayo Clinic/University of California San Francisco analyzed 1,087 patients with gliomas (grades II-IV) and defined five distinct subgroups of tumors according to the combination of three molecular genetic biomarkers (IDH1/2 mutations, 1p/19q co-deletion and mutations in TERT promoter region) [225]. Patients with grade II and III tumors had significant differences in median OS among the groups, which was not the case for GBM. The worst prognosis among patients with grade II and III gliomas had TERT positive and IDH and 1p/19q-negative tumors, where the OS was similar with GBM patients. On the contrary, the best prognosis was observed in the group of patients with IDH and TERT positive tumors.

There are also other studies trying to classify gliomas into different subgroups according to combinations of various biomarkers. For example Japanese research group subdivided 332 grade II and III gliomas using the IDH1/2 mutations and 1p/19q co-deletion [226], or German study of 405 adult patients with gliomas which analyzed IDH1 mutations 1p/19q co-deletion and ATRX expression [227] and others [228].

1.4.2 The treatment of low grade gliomas

Therapeutic strategies for patients with LGG involves the combination of neurosurgical intervention, radiotherapy and chemotherapy. However, there has not been set explicit criteria for determining the extent of treatment and the combination of different modalities in individual patient so far. The definitive consensus on therapeutic approach to patients with LGG in the light of new findings from recent prospective clinical trials is still missing.

1.4.3 Surgical treatment of low grade gliomas

Neurosurgical intervention remains a crucial part of LGG treatment [229]. Besides the cytoreduction, neurosurgery enables the acquisition of tumor tissue for histopathological diagnosis and determination of molecular genetic characteristics. For the ethical reasons, it was impossible to realize a prospective study that would compare the outcomes of neurosurgical treatment in relation to the extent of tumor resection. However, the maximum possible tumor resection was associated with the better prognosis of LGG patients in a number of retrospective studies [230]. The retrospective study of 216 LGG patients showed positive prognostic effects of radiologically confirmed greater extent of resection of tumor tissue (≥90% vs. <90%) on five-year OS (97 vs. 76%) as well as eight-year OS (91 vs. 60%). The Norwegian retrospective study compared an early resection versus a biopsy and careful monitoring (watchful waiting) in the surgical treatment of LGG patients [231]. The median OS was prolonged in the group with an early tumor resection when compared with the group with a biopsy and watchful waiting (9.7 vs. 5.6 years, P = 0.047). In another study comprising a retrospective analysis of 1509 patients with LGG, the extent of resection together with the volume of postoperative tumor residues represented independent prognostic factors for PFS and OS [232].

The maximization of the extent of tumor resection is currently enabled owing to the advances in imaging methods, neurosurgical techniques as well as perioperative monitoring [217,229]. The tumor localization in eloquent or surgically inaccessible areas allows only a partial tumor resection or navigated tumor biopsy.

1.4.4 Radiotherapy in low grade gliomas treatment

The favorable effect of radiotherapy for patients with LGG has been repeatedly demonstrated. The various doses of photon radiation were compared fractionated in the range of 45-64.8 Gy [233,234]. However, high doses of radiation did not improve neither PFS nor OS of patients by comparison with lower and middle doses.

Patients with LGG in phase III EORTC 22844 clinical trial did not benefit from a higher dose of radiation compared with the lower dose (59.4 vs. 45 Gy) [235]. The five-year survival difference was not statistically significant between the two arms of the study, while in the higher radiation dose arm patients had worse quality of life in long-term monitoring. Likewise NCCTG/RTOG/ECOG phase III study failed to demonstrate neither longer PFS nor OS in patients with LGG treated with high-dose RT (64.8 Gy) by comparison with medium-dose RT (50.4 Gy) [236]. Moreover, there was a greater incidence of radiation necrosis grades 3 - 5 in the high-dose RT arm (5 vs. 2.5%).

Therefore a lower radiation dose brings a comparable benefit in PFS and OS of patients as the higher dose, but with a substantial decrease in treatment toxicity. Currently, the preferred total radiation dose for the LGG treatment is 54 Gy.

1.4.5 Chemotherapy in low grade gliomas treatment

A number of chemotherapeutic agents has been evaluated so far in the treatment of LGG such as carboplatin, vincristine, etoposide, a combination of PCV, or temozolomide. However, the studies had substantial limitations that were especially given by the small number of patients, the lack of a control group, the inhomogeneity of observed groups of patients and tumors. Therefore the results were not conclusive. There was also concern about the toxicity of chemotherapy, particularly in the younger patients with potentially long-term survival (chemotherapy-induced cognitive impairment, leukoencephalopathy, myelodysplastic syndrome or leukemia) [229,237,238].

In 1998 the phase III clinical trial RTOG 9802 was started evaluating the combined RT plus PCV regime in high risk LGG patients. The study randomized 254 patients with high risk LGG defined as the age under 40 years with postoperative radiographic residuum or age over 40 years after any surgical intervention. Patients were randomized to adjuvant treatment with either RT alone (the total dose of 54 Gy over 6 weeks) or RT followed by 6 cycles of combined PCV chemotherapy. Initial results published in 2012 didn´t demonstrate statistically significant difference in survival between patients in both treatment arms [239].

On the other hand, the recently published results after the median follow up of 11.9 years clearly demonstrated a significant benefit of combined therapy RT plus PCV compared with RT alone, both with respect to PFS and OS [240]. Patients in the combined treatment arm achieved significantly longer median PFS compared with RT alone arm (10.4 vs. 4 years, $P < 0.001$). Also five year survival without disease progression reached more patients treated with RT plus PCV than RT alone (61 vs. 44%). Even more important was the difference in PFS after ten years of follow-up (51 vs. 21%). The difference in PFS remained significant in the subanalysis for individual histological types of LGG. Patients with the tumors positive for IDH1 R132H mutation had significantly longer PFS than patients without the mutation irrespective of the selected therapy. Nevertheless, also in IDH1 mutated tumors there was seen a PFS benefit of the combined therapy RT plus PCV over RT alone ($P <0.001$).

The significant benefit of combined therapy was demonstrated in OS of patients as well. Results from the long term follow up of RTOG 9802 study showed a substantial difference in OS of 5.5 years for patients treated with RT plus PCV compared with RT alone (13.3 vs. 7.8 years, $P = 0.003$) [240]. 20 % more patients survived after 10 years of follow-up in the combined treatment arm (60 vs. 40%). These results were achieved despite the fact that more patients in the RT alone arm received the salvage chemotherapy in case of tumor progression. Although the incidence of adverse events was higher in the RT plus PCV arm, no significant difference in cognition was observed in patients in both arms, nor was the difference in the incidence of leukemia or myelodysplasia [240,241].

The difference in OS remained significant also in the subanalysis for individual histological types of LGG. Once again, patients with the tumors positive for IDH1 R132H mutation had significantly longer OS than patients without the mutation irrespective of the selected therapy (13.1 vs. 5.1 years, $P = 0.02$). Nevertheless, also in IDH1 mutated tumors there was seen OS benefit of the combined therapy RT plus PCV over RT alone ($P = 0.02$). The

analysis of 1p/19q co-deletion was performed in RTOG 9802 study too. However, the sufficient sample of tumor tissue to determine this biomarker was available in only 63 patients which didn't allow the verification of predictive value of 1p/19q co-deletion in relation to PCV treatment for patients with LGG.

The different situation occurs for patients with low risk LGG. The careful clinical monitoring and regular examinations by imaging methods (MRI) until disease progression can still be the appropriate procedure of postoperative care for patients with these tumors [233,242]. The significance of combined treatment with chemotherapy and radiotherapy in patients with low risk LGG has not been prospectively examined so far.

Currently, the high risk group of LGG considered patients who have at least three of the following six factors: age $\geq$ 40 years; KPS < 70%; astrocytic component in the tumor histology; size of the tumor $\geq$ 6 cm in diameter; tumor beyond the median line and the presence of neurological deficit before the surgery. The adjuvant therapy, preferably the combination of RT plus PCV, should be initiated immediately after the surgery in these patients. The use of 1p/19q co-deletion for the prediction of better effect of combined therapy in LGG still remains the open question. Another perspective can be brought by CODEL phase III clinical trial comparing RT followed by PCV to RT with concomitant and subsequent administration of temozolomide in patients with glioma grade II and III positive for the 1p/19q co/deletion. The final results of this study planned until 2018 should definitively answer the question of appropriate adjuvant chemotherapy in 1p/19q co-deleted gliomas [65].

1.5 The 2016 WHO classification of the CNS tumors

Until recently the valid WHO classification of tumors of the CNS from 2007 has been based mainly on concepts of histogenesis. The CNS tumors has been classified according to their microscopic similarities with distinct putative cells of origin and their assumed levels of differentiation [12]. The histopathological diagnosis of the CNS tumors has been primarily dependent on light microscopy techniques based on the hematoxylin and eosin-stained sections in combination with immunohistochemical assessment of an expression of lineage-associated proteins.

The past two decades of an intensive research elucidated the molecular genetic basis of the multistage cancirogenesis process of various CNS tumors, bringing the possibility that such findings may contribute to classification of these tumors [243]. Some of the molecular genetic characteristics were already known during the preparation of WHO classification in 2007, but the findings were mostly preliminary and didn't enable to incorporate molecular biomarkers into the routine clinical practice.

In 2014 the meeting of experts was held under the patronage of the International Society of Neuropathology in the Dutch city of Haarlem that set out recommendations for future incorporation of molecular genetic biomarkers into the CNS tumors´ diagnostic process [244]. The Haarlem consensus was followed by the intensive work of 117 addressed experts from 20 countries and a three-day conference of 35 world-leading neuropathologists, neurooncologists and clinical scientists from 10 countries in Heidelberg. As the result of this effort, the updated WHO classification of CNS tumors was formulated and published in May 2016 [17].

For the first time the WHO classification of CNS tumors includes molecular genetic biomarkers in addition to histopathological diagnosis to define various tumor entities. For the gliomas, the updated classification now includes the most important biomarkers that were discussed in detail in the previous sections of this work. All the diffuse gliomas are now subdivided into the IDH1/2 mutated or wild-type entities. This subdivision reflects the distinct biological behavior of these tumors and substantial differences in patients' prognosis and survival. The diagnoses of oligodendrogliomas and anaplastic oligodendrogliomas now include the assessment of 1p/19q co-deletion representing the oligodendroglial component

of the tumor. This biomarker is also important for the prediction of better effect of combined RT plus PCV treatment in anaplastic oligodendrogliomas as was discussed in ODG section.

On the other hand, the routine assessment of CNS tumors´ molecular genetics is still not the case for all hospitals and pathological departments. Moreover, there can be the insufficient amount of tumor tissue for the analyses or the results are inconclusive. Therefore the 2016 WHO classification comprises also the non-otherwise specified (NOS) group of tumors (such as NOS-diffuse astrocytomas or NOS-oligodendrogliomas) that are diagnosed by the histopathological findings only, as was the standard in the old 2007 classification scheme.

The subsequent progress in the fundamental findings in molecular genetics of CNS tumors together with the improvements in diagnostic assays are likely to further improve the diagnostic accuracy of CNS tumors and to better asses the patients prognosis together with the selection of the best therapeutic approach for the individual tumor type in context of personalized medicine.

2 Thesis objectives and hypotheses

The fundamental aim of this thesis was to obtain and discuss new knowledge on the molecular genetics and biological behavior of the most common CNS tumors - gliomas in relation to their clinical management. These findings should be applied for the optimization of the treatment strategies for an individual patient with this diagnosis. Molecular genetic biomarkers should be used to more accurately determine patients´ prognosis or to predict better the treatment efficacy and outcome.

The practical part of this thesis contains two studies dealing with the important molecular genetic biomarkers in patients with two types of CNS gliomas. The experimentally obtained data were statistically analyzed and discussed in relation to clinical characteristics and outcome of patients.

In the first study the occurrence of the biomarker IDH1 R132H mutation was examined in the tumor tissue from patients with glioblastoma multiforme who were treated with the standard protocol and subsequently monitored in the Faculty Hospital in Pilsen. The mutation was assessed by the quantitative real time polymerase chain reaction (PCR) method and the results were correlated with the clinical characteristics of GBM patients.

In the second study the chromosomal aberration 1p/19q co-deletion was observed in patients with anaplastic oligodendroglioma who were treated with the combined radiotherapy and chemotherapy (procarbazine, lomustine and vincristine - PCV regime) and subsequently monitored in the Faculty Hospital in Pilsen. The 1p/19q co-deletion was assessed by the fluorescence in situ hybridization (FISH) method and the results were correlated with the patients´ clinical characteristics.

Hypotheses:

1. The IDH1 R132H mutation will be observed in a subset of patients with glioblastoma multiforme, predominantly with secondary glioblastomas.
2. Patients with the IDH1 R132H mutation detected in tumor tissue will have a better prognosis and longer survival than patients with wild-type tumors. Therefore this mutation will serve as a positive prognostic biomarker for patients with glioblastoma multiforme.

3. Patients with anaplastic oligodendroglioma positive for the chromosomal aberration 1p/19q co-deletion will have a better prognosis with longer survival than patients with wild-type tumors. Therefore this mutation will serve as a positive prognostic biomarker for patients with anaplastic oligodendroglioma.
4. Patients with anaplastic oligodendroglioma positive for the chromosomal aberration 1p/19q co-deletion will have a better response to the combined RT plus PCV treatment with longer survival than to the RT alone. Therefore this mutation will serve as a positive predictive biomarker for the treatment with combined RT plus PCV regimen in this subset of patients.

3 Materials and methods

3.1 The assessment of IDH1 R132H mutation in tumor tissue from patients with glioblastoma multiforme

3.1.1 Study participants

The study enrolled 44 patients diagnosed with WHO grade IV astrocytoma - glioblastoma multiforme (GBM) in the Faculty Hospital in Pilsen, who had available complete clinical data as well as tissue samples of the tumors. There were 22 males and 22 females among the patients. The median age of the entire study group was 64.3 years. Patients were treated (total or subtotal tumor resection or tumor biopsy, radiotherapy, chemotherapy with temozolomide) in the Faculty Hospital in Pilsen between the years 2009 and 2011. The formalin-fixed, paraffin-embedded (FFPE) tissue samples were obtained from the archives of the Sikl´s institute of pathology, Faculty of Medicine in Pilsen and Faculty Hospital in Pilsen. The complete clinical data were obtained from the medical information system of the Faculty Hospital in Pilsen. The study protocol was approved by the ethics committee. Written informed consent was obtained from all participants in this study. The description of the entire study group with important patients´ clinical characteristics is given in Table 1.

Table 1 - The study group demographics and clinical characteristics

Patients characteristics	
Sex	
Male-to-female ratio	1
Male	22
Female	22
Age, years	
Median	64.3
Range	35 - 87
KPS	
Median	77.5
Range	30 - 100
Postoperative treatment	
RT (±CHT)	29
CHT alone	1
None	15
Abbreviations KPS, Karnofsky performance score; RT, radiotherapy; CHT, chemotherapy	

3.1.2 DNA isolation

DNA was extracted from 10 μm FFPE sections following macrodissection of tumor tissue and normal brain tissue using the QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany). The 10 μm sections corresponded to the representative hematoxylin eosin slide with tumor tissue verified by pathologist.

3.1.3 Mutation detection

For detection of mutant allele IDH1 c.395G>A (p.R132H, COSMIC ID 28746) the TaqMan® Mutation Detection Assays (Assay Name: IDH1 28746 mu and IDH1 rf) was used with the TaqMan® Mutation Detection IPC Reagent Kit (Life Technologies, Carlsbad, California, USA). Mutant allele detection was performed in the laboratory of the department of biology at the Faculty of Medicine in Pilsen according to the recommended procedure and reaction conditions found in the manual. For the amplification the Stratagene Mx3000P real-time PCR system instrument was used (Agilent Technologies, Inc., Santa Clara, California, USA). Detection of mutant alleles was performed in duplicates in a reaction volume of 20 μl. Likewise detection of reference gene. Detection of samples with high values of cycle threshold (Ct) of the reference gene were repeated. The analyses of the normal brain tissue samples were done for detection of cut-off amplification curve before analyzes of tumor samples. No amplifications of mutant allele were present in normal brain tissue samples. On the base of these results and the shape of amplification curve of positive tumor samples the 25 deltaCt cut-off value was determined.

3.1.4 Statistical analysis

Overall survival (OS) was defined as the time between the diagnosis and death or last follow up. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow up. Kaplan-Meier survival curves were plotted and the survival distributions were compared with the use of the Wilcoxon test. Reported P values are two-sided. P values of less than 0.05 were considered to indicate statistical significance. All statistical analyzes were performed in software SPSS Statistics (IBM, Armonk, New York USA).

3.2 The examination of chromosomal aberration 1p/19q co-deletion in tumor tissue from patients with anaplastic oligodendroglioma

3.2.1 Study participants

The study enrolled 23 patients diagnosed with WHO grade III oligodendroglioma - anaplastic oligodendroglioma (AODG) in the Faculty Hospital in Pilsen, who had available complete clinical data as well as tissue samples of the tumors. There were 13 males and 10 females among the patients. The median age of the entire study group was 55.4 years. Ten patients were treated with the neurosurgery followed by radiotherapy (RT) plus chemotherapy (procarbazine, lomustine and vincristine - PCV regime), thirteen patients were treated with the neurosurgery followed by RT alone. The formalin-fixed, paraffin-embedded (FFPE) tissue samples were obtained from the archives of the Sikl´s institute of pathology, Faculty of Medicine in Pilsen and Faculty Hospital in Pilsen. The complete clinical data were obtained from the medical information system of the Faculty Hospital in Pilsen. The study protocol was approved by the ethics committee. Written informed consent was obtained from all participants in this study. The description of the entire study group with important patients´ clinical characteristics is given in Table 2.

Table 2 - The study group demographics and clinical characteristics

Patients Characteristics	
Sex	
Male-to-female ratio	1.3
Male	13
Female	10
Age, years	
Median	55.4
Range	25 - 72
mRS	
Median	3.35
Range	0 - 6
Postoperative treatment	
RT alone	10
RT + CHT (PCV)	13
Abbreviations mRS, modified Rankin Scale; RT, Radiotherapy; CHT, Chemotherapy	

3.2.2 Mutation detection

Deletion of 1p and 19q in FFPE tumor tissue samples were primarily determined by the fluorescence in situ hybridization (FISH) with locus-specific probes (10µl mixture) LSI 1p36/1q25 or LSI 19q13/19p13 (Vysis/Abbott, Downers Grove, IL, USA) in the laboratory of the Sikl´s institute of pathology, Faculty of Medicine in Pilsen and Faculty Hospital in Pilsen. The positive result for the 1p/19q co-deletion was assessed as the loss of 1p36 or 19q13 signal in more than 50% of nuclei (±3 SD in negative control).

3.2.3 Statistical analysis

Overall survival (OS) was defined as the time between the diagnosis and death or last follow up. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow up. Kaplan-Meier survival curves were plotted and the survival distributions were compared with the use of the Wilcoxon test. Reported P values are two-sided. P values of less than 0.05 were considered to indicate statistical significance. All statistical analyzes were performed in software SPSS Statistics (IBM, Armonk, New York USA).

4 Results and discussion

4.1 The assessment of IDH1 R132H mutation in tumor tissue from patients with glioblastoma multiforme

4.1.1 Results

The mutation IDH1 R132H was observed in 20 from 44 GBM patients´ tumor samples. Therefore the IDH1 mutation was identified in more than 45.4% of glioblastomas. The separation of primary and secondary glioblastomas (GBM that progressed from the low-grade glioma) was done on the basis of clinical information, where possible. The IDH1 R132H mutation occurred in 4 from 26 primary GBM (15.3%). Whereas the majority 16 from 18 (89.9%) of secondary GBM was mutated (Table 3).

Table 3 - The representation of IDH1 R132H mutation in primary versus secondary glioblastomas.

Glioblastoma type	**Primary GBM (n=26)**	**Secondary GBM (n=18)**
Mutation status	**[n]**	**[n]**
IDH1 R132H	4 (15.3 %)	16 (89.9 %)
IDH1 wild-type	22 (84.7 %)	2 (11.1 %)

The significant relations between the IDH1 mutation status and clinical characteristics such as PFS and OS were also observed (Table 4). Patients with IDH1 R132H mutation had longer PFS than patients with wild-type IDH1 (136 vs. 51 days, $P < 0.021$, Wilcoxon test) (Figure 1). Significantly longer OS was observed as well for patients with IDH1 R132H mutation than for patients without the mutation (270 vs. 130 days, $P < 0.024$, Wilcoxon test) (Figure 2).

Table 4 - Results for progression-free survival and overall survival differences in patients with GBM in the relation to IDH1 mutation status.

Glioblastoma patients results	n	Median [days] (95% Cl)	P (Wilcoxon)
Overall Survival (OS)			
IDH1 R132H	20	270 (139-400)	0.024
IDH1 wild-type	24	130 (87-172)	
Progression-free Survival (PFS)			
IDH1 R132H	20	136 (22-249)	0.021
IDH1 wild-type	24	51 (19-82)	

Figure 1 - Progression-free survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H mutation (P = 0.021, Wilcoxon test).

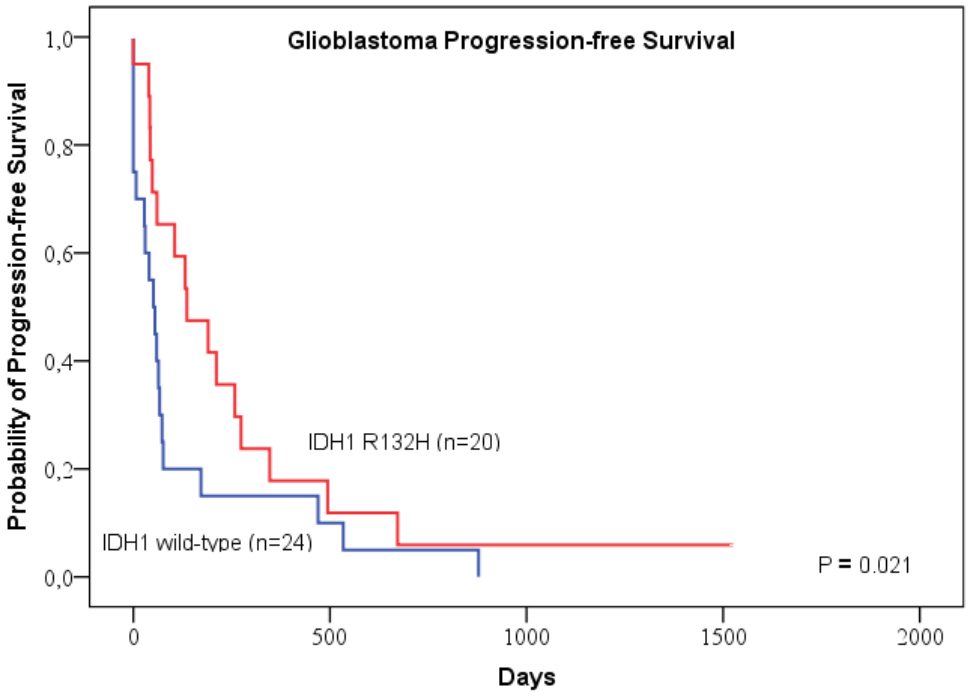
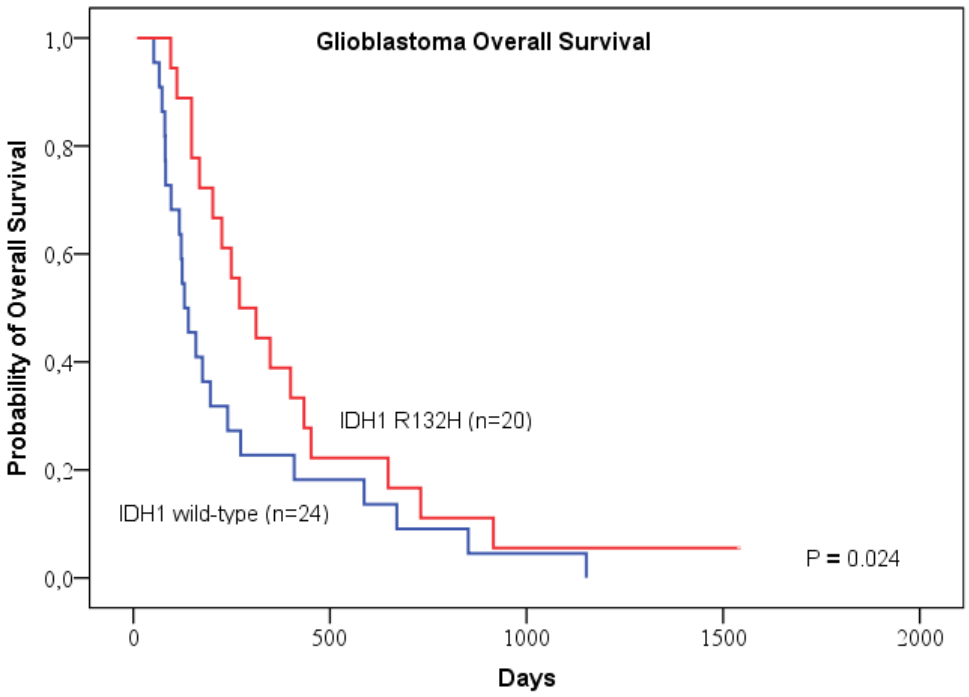


Figure 2 - Overall survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H mutation (P = 0.024, Wilcoxon test).



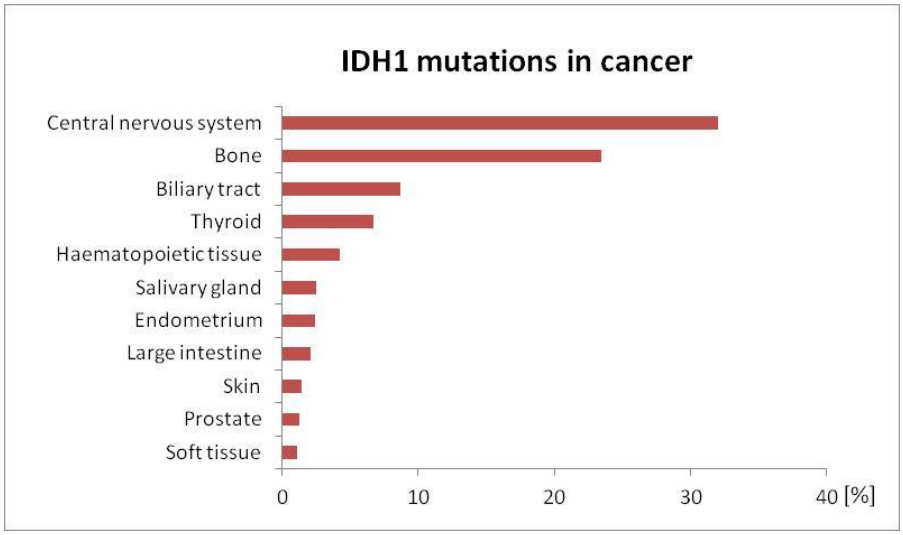
4.1.2 Discussion

Recurrent IDH1/2 mutations and their role in oncogenesis and tumor progression were systematically described first in GBM [16]. This observation led to new insights into the biology of cancer including GBM. Alterations in cancer cell metabolism are now well accepted as one of the principal hallmarks of the process of cancerogenesis and tumor progression [30].

Mutations in IDH1 were also identified in other tumor types. The data from the Sanger Institute Cancer Genome Project - Catalogue of Somatic Mutations in Cancer (COSMIC) revealed the presence of IDH1 mutations in more than 32% of central nervous system tumors, 23% of bone tumors, 8% of biliary tract tumors, 6% of thyroid cancer and many other tumor types [220] (Figure 3). In the primary brain tumors, IDH1 mutations are presented mostly in diffuse astrocytomas, anaplastic astrocytomas, glioblastomas or oligodendrogliomas as was discussed in detail in the theoretical part of this thesis [220]. The R132H amino acid substitution is the most common form of IDH1 mutations with the

prevalence of 90% among IDH1-mutant tumors. Less common mutants such as R132C, R132G, R132S, and R132L are also known [42,43].

Figure 3 - The representation of IDH1 mutations in various types of cancer [220].



The fundamental shift in the understanding of mutated IDH and its role in cancer progression came with the observation of the neomorphic function of the mutated enzyme. Instead of the production of alpha-ketoglutarate, mutated IDH produced novel onco-metabolite 2-hydroxyglutarate (2-HG) that were highly accumulated in the cancer cells [44]. It was subsequently discovered that 2-HG inhibits the functions of the alpha-ketoglutarate dependent superfamily of dioxygenases. These enzymes have diverse cellular functions including, but not limited to histone demethylation and demethylation of hypermethylated DNA [45,46]. Moreover, IDH mutations and 2-HG production were identified to be sufficient steps in the process leading to glioma hypermethylator phenotype. That observation was important for understanding of glioma oncogenesis and highlighted the interplay between genetic and epigenetic changes in human cancers [72,245].

Mutations in IDH are important also for their clinical consequences as was discussed in more detail in the theoretical part of this thesis. Recent studies revealed the important role of mutated IDH in the assessment of astrocytoma patient prognosis. Across several studies, the better prognosis for patients with IDH 1/2 mutated GBMs were observed with the longer OS of 3.8 vs. 1.1 years, 2.6 vs. 1.3 years, 2.3 vs. 1.2 years and 3 vs. 1 year [16,43,49,50]. Even more significant differences in OS were found in patients with anaplastic astrocytomas; 5.4 vs. 1.7 years, 6.8 vs. 1.6 years and 7 vs. 2 years [43,49,50] as well as diffuse astrocytoma

12.6 vs. 5.5 years [49]. These data highlighted the major impact of IDH1/2 mutation status on glioma patient survival and support the incorporation of this biomarker into the clinical management. Mutations in IDH1/2 and production of onco-metabolite 2-HG could be used as well for therapeutic intervention in the near future [246].

The results from this study also support the IDH1 R132H mutation to be the strong prognostic biomarker for patients with GBM. However, the differences in median PFS and OS between patients with IDH1 mutated and IDH1 wild-type tumors were not as big as in other studies. The reason for the relatively small differences in median survival between both groups could be the heterogeneity of the treatment protocols. The standard treatment with neurosurgery and concomitant chemo-radiotherapy with temozolomide was implemented in only 29 patients. 1 patient had radiotherapy alone and 15 patients were treated neither with radiotherapy nor with chemotherapy. The proportion of IDH1 mutated tumors was also higher than in other similar studies. The IDH1 mutations in glioblastomas were originally identified predominantly in secondary GBM that progressed from the low grade tumors [247]. The distinction between the primary and secondary GBM in this study was done on the basis of clinically relevant information from the patient history, although it was not possible absolutely exactly. Only 5 patients had previously assessed low-grade glioma (surgery in 2 cases, tumor biopsy in 3 cases). Other patients with tumor´s corresponding neurological symptomatology (epileptic seizures, focal neurological deficit) present at least 6 month before the final diagnosis were considered as likely secondary GBM. Moreover, the primary-like glioblastomas could be in fact secondary without the symptoms of low-grade tumors.

The recent study of mutations in the promoter of TERT gene has revealed the high incidence of these aberrations in a large portion of primary GBM (about 80%) [248]. In the further research the TERT promoter mutations will be used in addition to clinically relevant information for the separation of primary and secondary glioblastomas. The assessments of other IDH1 mutations as well as the analysis of IDH2 mutations are also planned together with their quantification using digital PCR methods (digital droplet PCR).

Despite the drawbacks of this study, IDH1 R132H mutation still served as a strong prognostic biomarker for the patients with GBM treated in the Faculty Hospital in Pilsen. The results from this work became a part of an important and recently published meta-analysis of 55 observational studies that confirmed improved both overall survival and

progression-free survival in patients with gliomas positive for IDH1/2 mutations and helped with the incorporation of IDH1/2 mutations status into the updated 2016 WHO classification of CNS tumors [17,38].

4.2 The examination of chromosomal aberration 1p/19q co-deletion in tumor tissue from patients with anaplastic oligodendroglioma

4.2.1 Results

The biomarker 1p/19q co-deletion was identified in 12 out of 23 patients´ tumor samples (52.2%) (Table 5). Patients with tumors positive for co-deletion had a significantly longer median OS than patients without 1p/19q co-deletion (587 vs. 132 weeks, P = 0.012, Wilcoxon test) (Figure 4). There was also the trend for longer median PFS in patients with 1p/19q co-deleted tumors than in those without this biomarker (321 vs. 43 weeks, P = 0.075, Wilcoxon test) (Figure 5).

Table 5 - Progression-free survival and overall survival differences in patients with anaplastic oligodendroglioma in relation to 1p/19q co-deletion.

Anaplastic oligodendroglioma patients´ results	**n**	**Median [weeks] (95% Cl)**	**P (Wilcoxon)**
Overall survival			
1p/19q co-deleted	12	587 (466 - 707)	0.012
1p/19q negative	11	132 (0 - 271)	
Progression-free survival			
1p/19q co-deleted	12	321 (21 - 620)	0.075
1p/19q co-deletion-negative	11	43 (0 - 150)	

Figure 4 - Overall survival of patients with anaplastic oligodendroglioma in relation to 1p/19q co-deletion status (P = 0.012, Wilcoxon test).

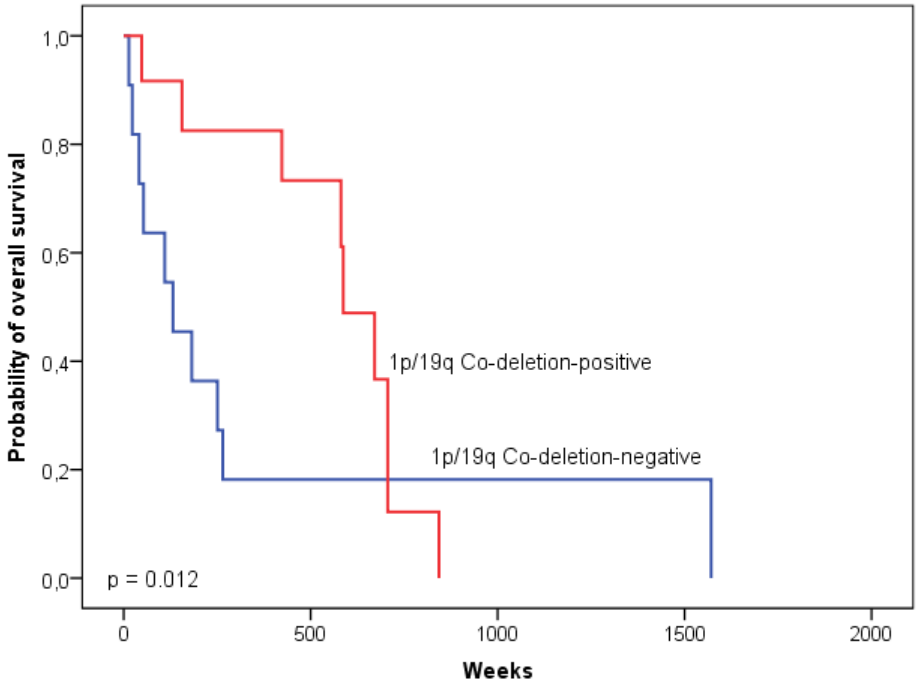
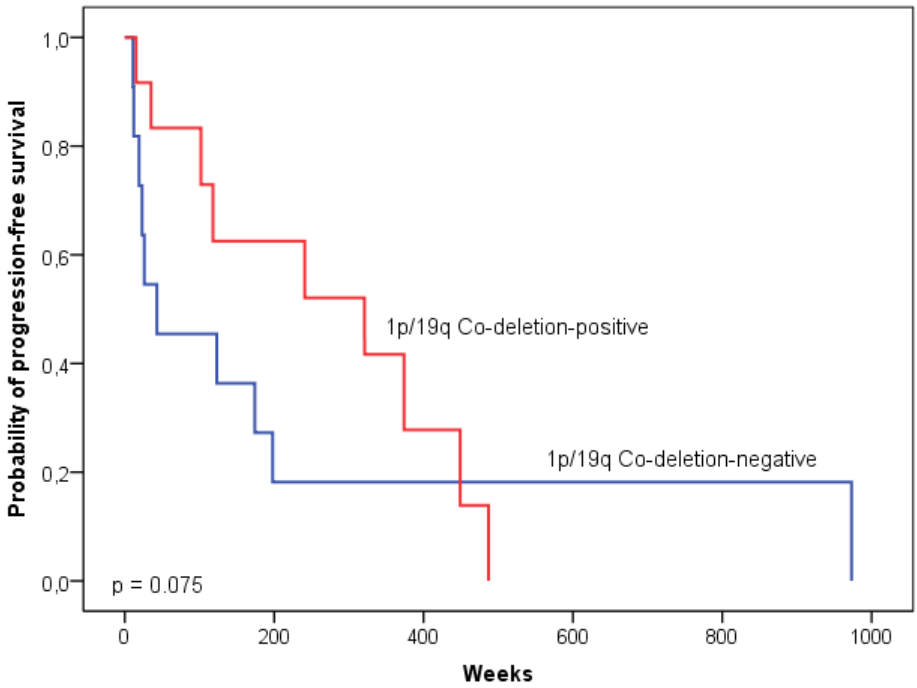


Figure 5 - Progression-free survival of patients with anaplastic oligodendroglioma in relation to 1p/19q co-deletion status (P = 0.075, Wilcoxon test).



In the subgroup of patients with 1p/19q co-deleted tumors (n=12), the median OS was significantly longer in those treated with neurosurgery plus RT and PCV (n=7) by comparison with patients that were treated with neurosurgery followed by RT alone (n=5) (706 vs. 423 weeks, P = 0.008, Wilcoxon test) (Table 6 and Figure 6). On the other hand, there was no significant difference in median PFS in the subgroup of patients treated with neurosurgery plus RT and PCV vs. those treated with neurosurgery plus RT alone (374 vs. 321 weeks, P = 0.626, Wilcoxon test) (Table 6 and Figure 7).

Table 6 - Progression-free and overall survival differences in patients with anaplastic oligodendroglioma treated with neurosurgery plus radiotherapy (NRT) or with neurosurgery plus radiotherapy and procarbazine, lomustine and vincristine (NRT-PCV) in relation to 1p/19q co-deletion.

1p/19q co-deletion status	**Median OS [weeks] (95% Cl)**	**P (Wilcoxon)**	**Median PFS [weeks] (95% Cl)**	**P (Wilcoxon)**
Co-deletion (n=12)				
NRT-PCV (n=7)	706 (675 - 736)	0.008	374 (129 - 618)	0.626
NRT (n=5)	423 (0 - 996)		321 (67 - 574)	
Without co-deletion (n=11)				
NRT-PCV (n=6)	182 (12 - 351)	0.223	43 (0 - 224)	0.523
NRT (n=5)	53 (0 - 117)		26 (10 - 41)	

Figure 6 - Overall survival of patients with anaplastic oligodendroglioma positive for 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)] (P = 0.008, Wilcoxon test).

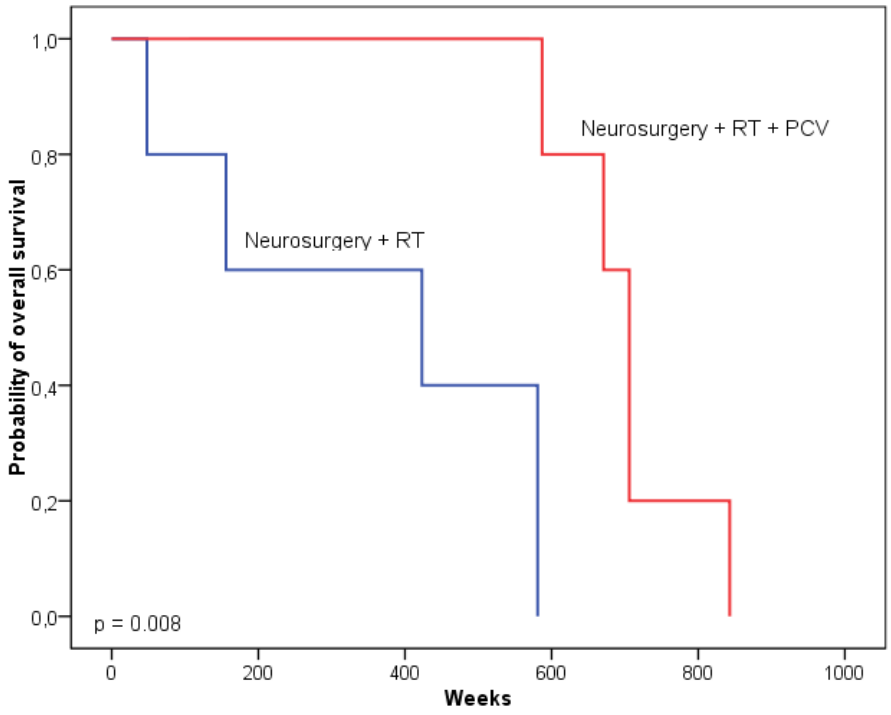
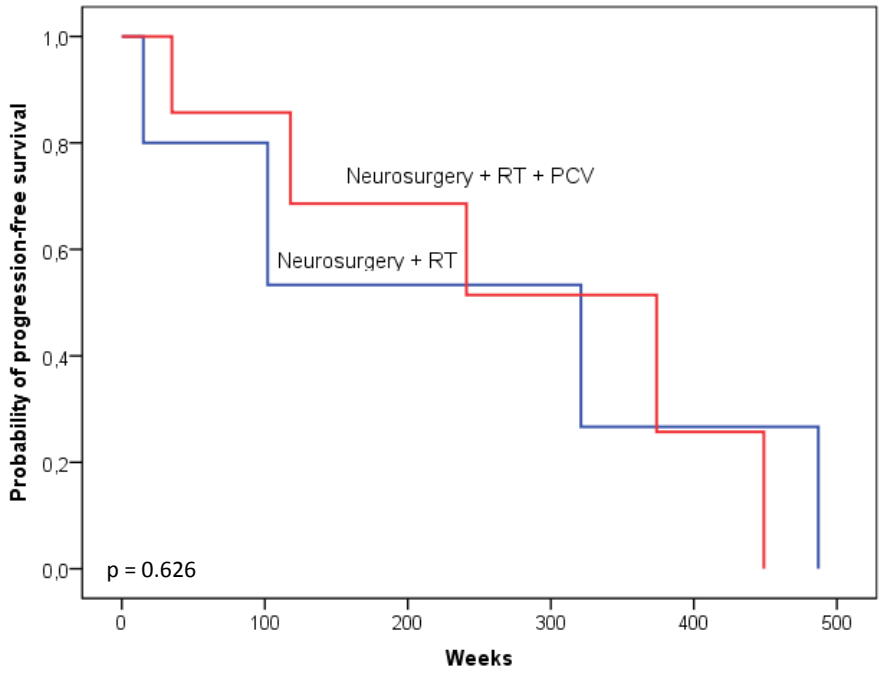


Figure 7 - Progression-free survival of patients with anaplastic oligodendroglioma positive for 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)] (P = 0.626, Wilcoxon test).



In contrast, in the subgroup of patients without 1p/19q co-deletion (n=11) the median OS was not significantly different in those treated with neurosurgery plus RT and PCV (n=6) by comparison with those treated with neurosurgery followed by RT alone (n=5) (182 vs. 53 weeks, P = 0.223, Wilcoxon test) (Table 6 and Figure 8). There was also no significant difference in the median PFS in this subgroup of patients (43 vs. 26 weeks, P = 0.523) (Table 6 and Figure 9).

Figure 8 - Overall survival of patients with anaplastic oligodendrogliomas without 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)] (P = 0.223, Wilcoxon test).

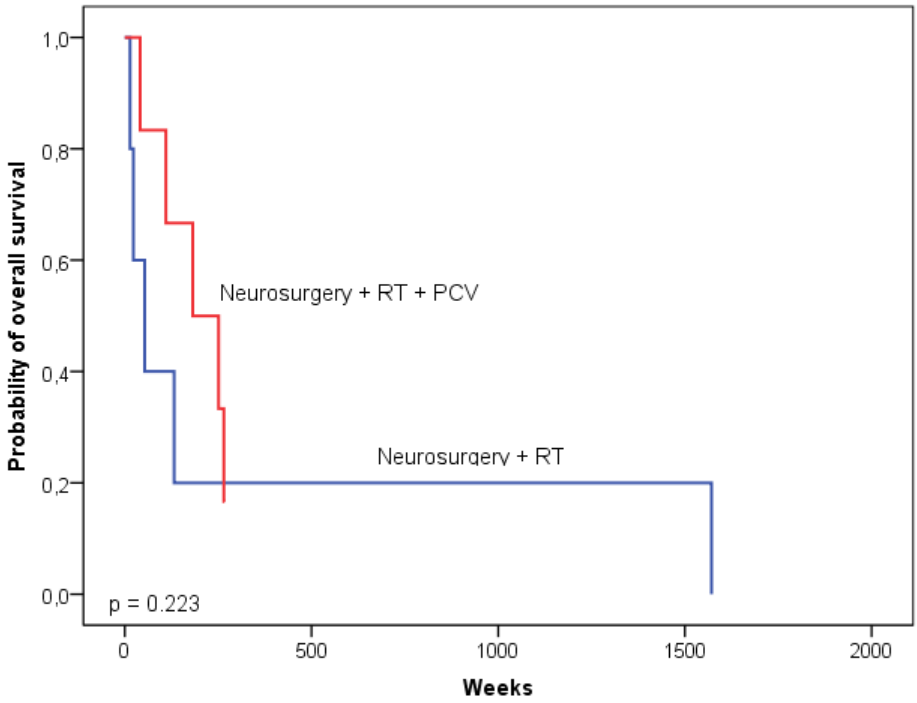
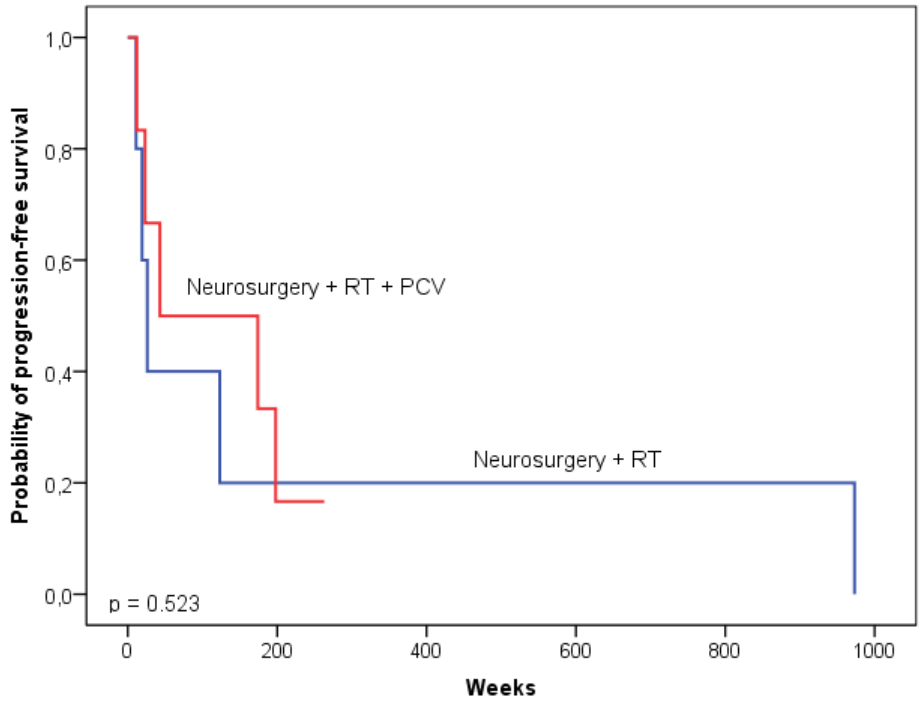


Figure 9 - Progression-free survival of patients with anaplastic oligodendrogliomas without 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)] (P = 0.523, Wilcoxon test).



4.2.2 Discussion

The molecular genetic characteristic of oligodendroglial tumors is the frequent co-deletion of chromosome 1p and 19q. This chromosomal aberration was identified in 1994 and became the first biomarker in neuro-oncology [194]. The mechanism of 1p/19q co-deletion is the unbalanced translocation t(1;19)(q10;p10) [195]. Recently, the mutations in two important tumor-suppressor genes, capicua homolog drosophila (CIC) located on 19q13.2, and far upstream element-binding protein (FUBP1) on the 1p chromosome, was discovered in the majority of oligodendrogliomas with 1p/19q co-deletion [198,199]. Mutations in these genes are probably involved in the formation and progression of oligodendrogliomas. However, their true significance in neoplastic diseases remains to be verified.

Co-deletion of 1p/19q appears almost exclusively in oligodendroglial tumors (80% to 90% of grade II oligodendrogliomas and 50% to 70% of AO) [196,197]. This chromosomal

aberration can be used in clinical practice as an important diagnostic, prognostic, as well as predictive biomarker. From the diagnostic point of view, the presence of 1p/19q co-deletion supports the diagnosis of oligodendroglioma, especially in cases where the histological findings are not clear.

The 1p/19q co-deletion is also an important positive prognostic biomarker of the disease. Several studies found significantly better survival outcome for patients with oligodendroglioma with 1p/19q co-deletion than for those without [186,191,192,207,208,210,211].

Co-deletion of 1p/19q was found to have substantial clinical significance also as a strong predictive biomarker for patients with anaplastic oligodendroglial tumors. Its detection predicts longer survival of patients with the combined RT plus PCV treatment by comparison with RT alone that was recently shown by the long-term follow-up of two important phase III clinical trials RTOG 9402 and EORTC 26951 [210,211]. These trials produced substantial results and led to a paradigm shift in anaplastic oligodendroglioma treatment as was discussed in detail in the theoretical part of this thesis.

In this study, the 1p/19q co-deletion served as the strong prognostic biomarker for OS for all patients with anaplastic oligodendroglioma irrespective of the treatment regime. Moreover, the positive predictive value of 1p/19q co-deletion was demonstrated for the subgroup of patients treated with the combination of neurosurgery and RT plus PCV. These results are in concordance with the results from the recently published long-term follow up of two phase III clinical trials RTOG 9402 and EORTC 26951. The major weakness of this work remains the relatively small number of patients and the retrospective study design. The limited number of patients in this study is caused mainly by the rare incidence of anaplastic gliomas among cancer patients. The future research will expand the assessment of other molecular biomarkers in the patient cohort such as mutations in IDH1/2 or the PI3K signaling pathway and the correlation of these mutations with 1p/19q co-deletion and patients´ clinical characteristics and outcome.

5 Conclusions

The IDH1 R132H mutation was observed in the interestingly higher number of patients with glioblastoma multiforme that was previously published. On the other hand the majority of mutated tumors in the cohort were probably secondary glioblastomas. The prognostic value of the IDH1 R132H mutation was also observed. Patients with this mutation in the tumor tissue had significantly longer PFS as well as OS than patients with IDH1 wild-type tumors.

The presented results were included into the large recently published meta-analysis that confirmed positive prognostic effect of the IDH mutations on both overall survival and progression-free survival in patients with CNS gliomas. These findings helped with the incorporation of IDH mutations assessment into the updated 2016 WHO classification of CNS tumors.

The prognostic value of 1p/19q co-deletion in the cohort of patients with anaplastic oligodendroglioma was proved. The strong positive predictive value of this biomarker for OS was also shown for patients with co-deletion who were treated with neurosurgery and RT plus PCV by comparison with neurosurgery and RT alone. Patients with anaplastic oligodendrogliomas who have tumor positive for 1p/19q co-deletion should be treated intensively with combined RT and chemotherapy (PCV) regime.

The results of this thesis were also practically applied during the formation of the multidisciplinary neurooncology center, which was set up in the Faculty of Medicine in Pilsen and Faculty Hospital in Pilsen under the patronage of the neurooncology section of the Czech oncological society. The main objective of this center is to help to patients as well as treating physicians with the decision-making process during the whole treatment procedure.

The enormous advances in the molecular genetics of CNS tumors and especially gliomas that were made over the past decade bring completely new opportunities for the optimization of the treatment strategies for and individual patient with these diagnoses. The important molecular biomarkers were discovered and validated in the clinical studies. These biomarkers can be used in the clinic for more precise diagnosis, for the more accurate assessment of a patients´ prognosis, or for the better selection of therapy and prediction of therapeutic response.

Together with the implementation of the molecular genetics in the recently updated 2016 WHO classification of CNS tumors it will likely be necessary to integrate molecular biomarkers and personalized medicine principles into standard clinical care of patients suffering from neurological cancers.

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8 List of attachments

Original articles

Attachment I

Polivka J Jr, Polivka J, Repik T, Rohan V, Hes O, Topolcan O. Co-deletion of 1p/19q as prognostic and predictive biomarker for patients in West Bohemia with anaplastic oligodendroglioma. Anticancer Res. 2016; 36(1):471-6. **(IF = 1.895)**

Attachment II

Polivka J, Polivka J Jr, Rohan V, Pesta M, Repik T, Pitule P, Topolcan O. Isocitrate dehydrogenase-1 mutations as prognostic biomarker in glioblastoma multiforme patients in West Bohemia. Biomed Res Int. 2014; 2014:735659. **(IF = 2.134)**

Attachment III

Polivka J Jr, Polivka J, Karlikova M, Topolcan O. Pre-graduate and post-graduate education in personalized medicine in the Czech Republic: statistics, analysis and recommendations. EPMA J. 2014; 5(1):22.

Review articles

Attachment IV

Polivka J Jr, Polivka J, Rohan V, Priban V. Current view on management of central nervous system low-grade gliomas. Cesk Slov Neurol N. 2016; 79/112(5):1-7. **(IF = 0.209)**

Attachment V

Polivka J Jr, Pesta M, Janku F. Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? Expert Rev Mol Diagn. 2015; 15(12):1631-44. **(IF = 3.333)**

Attachment VI

Polivka J, Polivka J Jr, Krakorova K, Peterka M, Topolcan O. Current status of biomarker research in neurology. EPMA J. 2016; 7:14.

Attachment VII

Karlikova M, Polivka J Jr, Strojil J, Topolcan O. A road towards better education in personalized medicine at universities and beyond. Pers Med. 2015; 12(3):259-267. **(IF = 1)**

Attachment VIII

Polivka J Jr, Polivka J, Rohan V, Topolcan O. New treatment paradigm for patients with anaplastic oligodendroglial tumors. Anticancer Res. 2014; 34(4):1587-94. **(IF = 1.895)**

Attachment IX

Polivka J Jr, Janku F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther. 2014; 142(2):164-75. **(IF = 11)**

Attachment X

Polivka J, Polivka J Jr, Rohan V, Priban V. Anaplastic oligodendrogliomas - The age of personalized medicine has arrived? Cesk Slov Neurol N. 2014; 77(4):428-434. **(IF = 0.209)**

Attachment XI

Polivka J, Polivka J Jr, Rohan V, Topolcan O. Glioblastoma multiforme - A review of pathogenesis, biomarkers and therapeutic perspectives. Cesk Slov Neurol N. 2013; 76(5):575-583. **(IF = 0.209)**

Attachment XII

Polivka J Jr, Polivka J, Rohan V, Topolcan O, Ferda J. New molecularly targeted therapies for glioblastoma multiforme. Anticancer Res. 2012; 32(7):2935-46. **(IF = 1.895)**

Articles out of the main topic of the thesis

Attachment XIII

Holubec L, Polivka J Jr, Safanda M, Karas M, Liska V. The role of cetuximab in the induction of anticancer immune response in colorectal cancer treatment. Anticancer Res. 2016; 36(9):4421-6. **(IF = 1.895)**

Attachment XIV

Rohan V, Baxa J, Tupy R, Cerna L, Sevcik P, Friesl M, Polivka J Jr, Polivka J, Ferda J. Length of occlusion predicts recanalization and outcome after intravenous thrombolysis in middle cerebral artery stroke. Stroke. 2014; 45(7):2010-7. **(IF = 5.787)**

Attachment XV

Polivka J, Rohan V, Sevcik P, Polivka J Jr. Personalized approach to primary and secondary prevention of ischemic stroke. EPMA J. 2014; 5(1):9.

Attachment XVI

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Attachment XVII

Tonar Z, Eberlova L, Polivka J, Daum O, Witter K, Kralickova A, Gregor T, Nedorost L, Kochova P, Rohan E, Kalusova K, Palek R, Skala M, Glanc D, Kralickova M, Liska V. Stereological methods for quantitative assessment of hepatic microcirculation. Current Microscopy Contributions to Advences in Science and Technology. Vol. 1. Microscopy Book Series - 2012 Edition. Formatex Research Center, Badajoz, Spain, pp. 737-748. ISBN 978-84-939843-6-6.

Attachment XVIII

Rohan V, Polivka J, Sevcik P, Polivka J Jr. Current approach to the options of primary and secondary prevention of ischemic cerebrovascular accident. Kardiol Rev. 2013; 15(4):218 223.

Attachment XIX

Karas M, Steinerova K, Lysak D, Hrabetova M, Jungova A, Sramek J, Jindra P, Polivka J, Holubec L. Pre-transplant quantitative determination of NPM1 mutation significantly predicts outcome of aIlogeneic hematopoietic stem cell transplantation in patients with normal karyotype AML in complete remission. Anticancer Res 2016; 36. ***Article in press.*** **(IF = 1.895)**

Attachment I

Polivka J Jr, Polivka J, Repik T, Rohan V, Hes O, Topolcan O. Co-deletion of 1p/19q as prognostic and predictive biomarker for patients in West Bohemia with anaplastic oligodendroglioma. Anticancer Res. 2016; 36(1):471-6. **(IF = 1.895)**

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Co-deletion of 1p/19q as Prognostic and Predictive Biomarker for Patients in West Bohemia with Anaplastic Oligodendroglioma

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Abstract. *Background: Anaplastic oligodendrogliomas (AO) are rare tumors. Two phase III clinical trials (RTOG 9402 and EORTC 26951) proved favorable effects of radiotherapy (RT) with chemotherapy (procarbazine, lomustine and vincristine; PCV) in patients with AO carrying chromosomal mutation of co-deletion1p/19q even if it was not the primary endpoint of these studies. We assessed 1p/19q co-deletion as a prognostic and predictive biomarker for our patients with AO. Materials and Methods: 1p/19q co-deletion was assessed by fluorescence in situ hybridization in tumor samples from 23 patients and correlated with progression-free (PFS) and overall (OS) survival for the entire cohort and for the subgroups of patients with different treatment (neurosurgery plus RT alone vs. RT plus PCV). Results: 1p/19q co-deletion was identified in 12 out of 23 tumors (52.2%). Patients with co-deletion had longer OS (587 vs. 132 weeks, p=0.012) and a trend for longer PFS (321 vs. 43 weeks, p=0.075). Patients with co-deletion treated with neurosurgery and RT plus PCV vs. neurosurgery and RT alone also had longer OS (706 vs. 423 weeks, p=0.008). There was no survival difference for patients without 1p/19q co-deletion in relation to treatment. Conclusion: The prognostic value of 1p/19q co-deletion in our patients with AO was verified. The strong positive predictive value of this biomarker for OS was also shown for patients with co-deletion treated with neurosurgery and RT plus PCV vs. neurosurgery and RT alone.*

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Key Words: anaplastic oligodendroglioma, 1p/19q co-deletion, prognostic biomarker, predictive biomarker.

Anaplastic oligodendroglial tumors (oligodendrogliomas and oligoastrocytomas grade III; AO) are rare types of cancer that represent only 0.5-1.2% of all primary brain tumors (1, 2). The highest incidence of AO is between 45 and 50 years of age. The major symptoms are epileptic seizures, focal symptoms that affect the frontal and the temporal regions of the brain, or later the symptoms of intracranial hypertension. The standard therapy for AO comprises neurosurgery followed by radiotherapy (RT) and chemotherapy. RT is administered to a total dose of 54 to 60 Gy. Chemotherapy consists of a triple combination of procarbazine, lomustine and vincristine (PCV) or temozolomide (3, 4).

Oligodendrogliomas are known to respond better to RT and chemotherapy than other types of malignant primary brain tumors. Their sensitivity to RT was discovered as early as the 1980s (5), and the positive effect of chemotherapy, PCV and temozolomide, was found later (6-8). Research into molecular genetics of oligodendrogliomas offers new knowledge in the diagnosis and treatment of these tumors and has an impact on their management. The very frequent genetic aberration of oligodendroglial tumors is the co-deletion of chromosome 1p and 19q. This 1p/19q co-deletion means the loss of genetic material from the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) in the tumor and became the first biomarker in neuro-oncology discovered in 1994 (9). 1p/19q Co-deletion was found to be an important positive prognostic biomarker of this disease (4, 10-14).

Recently, the long-term results of two large independent phase III clinical trials, the Radiation Therapy Oncology Group (RTOG) 9402 and European Organization for Research and Treatment of Cancer (EORTC) 26961 trials, also demonstrated the strong predictive role of 1p/19q co-deletion for patients with AO treated with chemotherapy. Patients with tumors with1p/19q co-deletion that were

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treated with RT and PCV had a significantly longer median overall survival (OS) than patients treated only with RT. There was no significant difference in median OS for patients without 1p/19q co-deletion that were treated either by RT alone or RT plus PCV (12, 13).

The aim of this study was to analyze the 1p/19q status in tumors of patients with AO treated in West Bohemia. We assessed the prognostic role of this biomarker for the entire patient cohort, as well as the ability to predict the better OS and PFS for patients treated with RT plus chemotherapy.

Patients and Methods

Patients. We performed a study of 23 patients with a diagnosis of WHO grade III oligodendroglioma, anaplastic oligodendroglioma (n=23; 13 males and 10 females; mean age=55.4 years) who were treated with the standard protocol at the Faculty Hospital Plzen, Czech Republic (neurosurgery plus RT alone, n=10; neurosurgery plus RT and PCV, n=13) (Table I).

Mutation detection. Deletion of 1p and 19q in tumor tissue samples were primarily assessed with fluorescence *in situ* hybridization (FISH) with locus-specific probes (10 μl mixture) LSI 1p36/1q25 or LSI 19q13/19p13 (Vysis/Abbott, Downers Grove, IL, USA). A positive result for 1p/19q co-deletion was assessed as the loss of 1p36 or 19q13 signal in more than 50% of nuclei.

Statistical analysis. OS was defined as the time between the diagnosis and death or last follow-up; PFS was defined as the time between the diagnosis and recurrence or last follow-up. Kaplan–Meier survival curves were plotted and the survival distributions were compared with the use of the Wilcoxon test. Reported *p*-values are two-sided; *p*-values of less than 0.05 were considered to indicate statistical significance.

Table I. *Patients' demographics and clinical characteristics*.

Characteristic	
Gender	
Male	13
Female	10
Age, years	
Median	55.4
Range	25-72
mRS	
Median	3.35
Range	0-6
Postoperative treatment	
RT alone	10
RT + PCV	13

mRS, Modified Rankin Scale; RT, radiotherapy; PCV, procarbazine, lomustine and vincristine.

Table II. *Results for progression-free survival and overall survival differences in patients with anaplastic oligodendroglioma in relation to 1p/19q co-deletion*.

Variable	N	Median (±SD), weeks	*p*-Value*
Overall survival			
1p/19q co-deleted	12	587 (±61.3)	0.012
1p/19q negative	11	132 (±71)	
Progression-free survival			
1p/19q Co-deleted	12	321 (±152.8)	0.075
1p/19q Co-deletion-negative	11	43 (±55)	

*Wilcoxon test.

Results

1p/19q Co-deletion was detected in 12 out of 23 patient tumor samples (52.2%). Patients with tumors with co-deletion had a significantly longer median OS than patients without 1p/19q co-deletion (587±61.3 *vs*. 132±71 weeks, respectively, $p=0.012$) (Table II and Figure 1A). There was also the trend for better median PFS in patients with tumors with co-deletion than in those without (321±152.8 *vs*. 43±55 weeks, respectively, $p=0.075$) (Table II and Figure 1B).

In the subgroup of patients with tumors with co-deletion of 1p/19q (n=12), the median OS was significantly longer in those treated with neurosurgery plus RT and PCV (n=7) in comparison to patients that were treated with neurosurgery followed by RT alone (n=5) (706±15.7 *vs*. 423±292.5 weeks, respectively, $p=0.008$) (Table III and Figure 2A). On the other hand, there was no significant difference in median PFS in the subgroup of patients treated with neurosurgery plus RT and PCV *vs*. those treated with neurosurgery plus RT alone (374±124.5 *vs*. 321±129.5 weeks, respectively, $p=0.626$) (Table III and Figure 2B).

In contrast to the previous results, in the subgroup of patients without 1p/19q co-deletion (n=11), the median OS was not significantly different in those treated with neurosurgery plus RT and PCV (n=6) in comparison to those treated with neurosurgery followed by RT alone (n=5) (182±86.3 *vs*. 53±32.8 weeks, respectively, $p=0.223$) (Table III and Figure 3A); there was also no significant difference in the median PFS (43±92.5 *vs*. 26±7.7 weeks respectively, $p=0.523$) (Table III and Figure 3B).

Discussion

The molecular genetic characteristic of oligodendroglial tumors is frequent co-deletion of chromosome 1p and 19q. This genetic aberration was identified in 1994 and became the first biomarker in neuro-oncology (9). The mechanism of 1p/19q co-deletion is the unbalanced translocation t(1;19)(q10;p10) (15). Recently, the presence of mutations in two important tumor-suppressor genes, capicua (Drosophila)

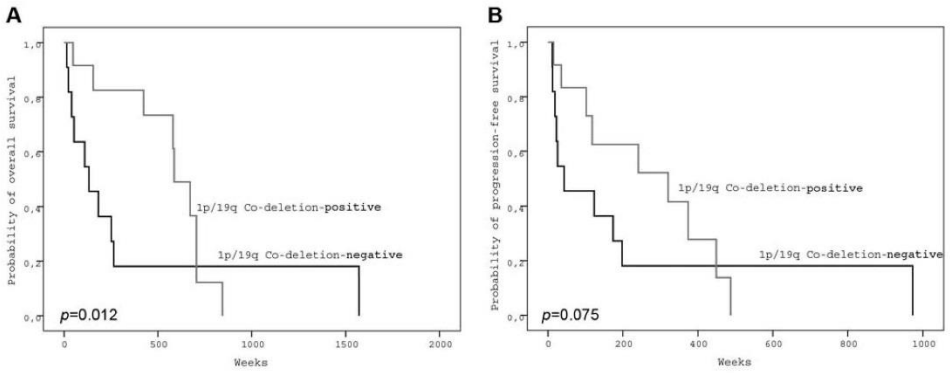

Figure 1. *Overall (A) and progression-free (B) survival of patients with anaplastic oligodendroglioma in relation to 1p/19q co-deletion status.*

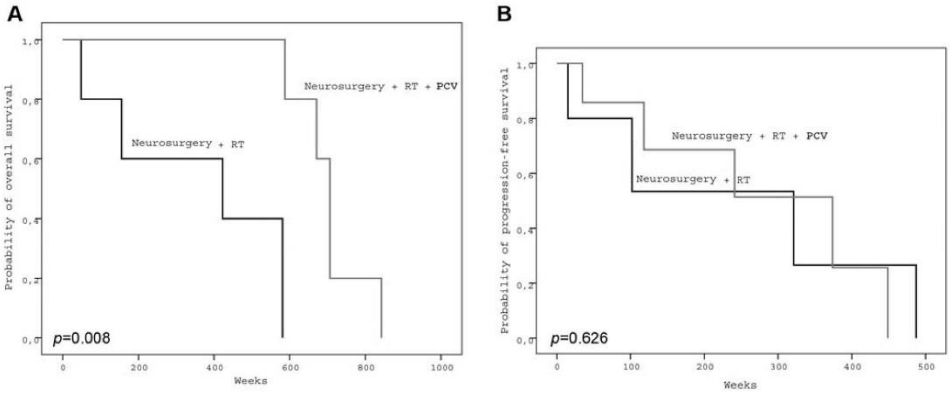

Figure 2. *Overall (A) and progression-free (B) survival of patients with anaplastic oligodendroglioma and 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)].*

homolog (*CIC*) located on 19q13.2, and far upstream element-binding protein (*FUBP1*) on the 1p chromosome, was discovered in the majority of oligodendrogliomas with 1p/19q co-deletion (16, 17). Mutations in these genes are probably involved in the formation and progression of oligodendrogliomas. However, their true significance in neoplastic diseases remains to be verified.

Co-deletion of 1p/19q appears almost exclusively in oligodendroglial tumors (80% to 90% of grade II oligodendrogliomas and 50% to 70% of AO) (18, 19). This chromosomal mutation in oligodendrogliomas can be used in clinical practice as an important diagnostic, prognostic, as well as predictive biomarker. From the diagnostic point of view, the presence of 1p/19q co-deletion supports the diagnosis of oligodendroglioma, especially in cases where the histological findings are not clear. Some other tumor types may also mimic oligodendrogliomas, such as dysembryoplastic neuroepithelial tumors, neurocytomas, clear cell ependymomas and small cell anaplastic astrocytomas. These tumors usually do not have 1p/19q co-deletion and this biomarker is a useful diagnostic aid in these clinical cases (10).

The presence of 1p/19q co-deletion has a role as an important positive prognostic biomarker of the disease.

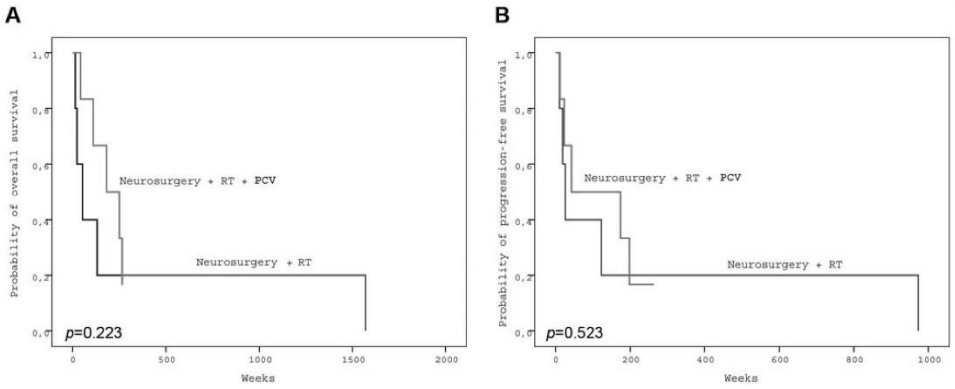

Figure 3. *Overall (A) and progression-free (B) survival of patients with anaplastic oligodendrogliomas without 1p/19q co-deletion in relation to the treatment protocol [neurosurgery plus radiotherapy (RT) vs. neurosurgery plus RT and procarbazine, lomustine and vincristine (PCV)].*

Table III. *Results for progression-free (PFS) and overall (OS) survival differences in patients with anaplastic oligodendroglioma treated with neurosurgery plus radiotherapy (NRT) or with neurosurgery plus radiotherapy and procarbazine, lomustine and vincristine (NRT-PCV) in relation to 1p/19q co-deletion.*

1p/19q Co-deletion status	Median OS (±SD), weeks	*p*-Value*	Median PFS (±SD), weeks	*p*-Value*
Co-deletion (n=12)				
NRT-PCV (n=7)	706±15.7	0.008	374±124.5	0.626
NRT (n=5)	423±292.5		321±129.5	
Without co-deletion (n=11)				
NRT-PCV (n=6)	182±86.3	0.223	43±92.5	0.523
NRT (n=5)	53±32.8		26±7.7	

*Wilcoxon test.

Retrospective and prospective studies found significantly better survival outcome for patients with oligodendroglioma with 1p/19q co-deletion than for those without (4, 10-14, 20).

Co-deletion of 1p/19q was found to have substantial clinical significance as a strong predictive biomarker for patients with anaplastic oligodendroglial tumors. Its detection predicts longer survival with PCV and RT in comparison to RT alone, as recently shown by the long-term follow-up of two important phase III randomized clinical trials, RTOG 9402 and EORTC 26951 (12, 13). These trials are producing substantial results and leading to a paradigm shift in disease treatment (11-14). In the RTOG 9402 study, the median OS for patients with anaplastic oligodendroglial tumors without 1p/19q co-deletion was similar in both groups receiving PCV plus RT or RT alone (2.6 and 2.7 years, respectively) (12). On the other hand in patients with 1p/19q co-deletion, the OS was significantly longer in the PCV plus RT arm than in the RT-alone arm (14.7 *vs.* 7.3 years, respectively, $p=0.03$) (12). Similar results were found in the EORTC 26951 trial (13). After more than 10 years' follow-up, the median OS in patients with anaplastic oligodendroglial tumors and without 1p/19q co-deletion was similar in the group receiving PCV plus RT and that receiving RT alone (25 and 21 months, respectively, $p=0.19$). However, the median OS was not reached for patients with co-deletion in the PCV plus RT arm, whereas it was just 9.3 years in patients primarily receiving RT alone (13). The positive effect of combined oncological treatment (PCV plus RT) in patients with 1p/19q co-deletion was present in both clinical studies, irrespective of which type of therapy was started first. Both studies demonstrated that neither radiotherapy nor chemotherapy alone is sufficient in AO treatment.

There are also other molecular biomarkers that could be used to better determine the prognosis for patients with oligodendrogliomas such as mutations in the genes for isocitrate dehydrogenase 1 and 2 (*IDH1/2*). A high frequency of mutations in the *IDH1* and *IDH2* (up to 69%-94%) was found in patients with oligodendroglioma (21, 22). The presence of the *IDH1/2* mutations is a significant positive prognostic biomarker for patients with various types of glioma (22–25). The alteration of certain other well-known pro-oncogenes and tumor-suppressor genes in patients with AO was identified, such as mutations in phosphatidylinositiol 3-kinase (*PI3K*), amplification of epidermal growth factor receptor (*EGFR*) or loss of the phosphatase and tensin homolog (*PTEN*) tumor suppressor. These alterations are associated with the poorer prognosis of AO (26, 27).

In our study, we found 1p/19q co-deletion to be a strong prognostic biomarker for OS for all patients with AO, irrespectively of their treatment regimen. Moreover, the predictive value of 1p/19q co-deletion was demonstrated for the subgroup of patients treated with the combination of neurosurgery and RT plus PCV *vs*. those treated with neurosurgery and RT alone. Our results are in concordance with the results from the recently published long-term follow-up of two phase III clinical trials RTOG 9402 and EORTC 26951. Although the follow-up of our patients is sufficient to prove the positive prognostic value of 1p/19q co-deletion in relation to combined therapy with PCV, the major weakness of this work remains the relatively small number of patients and the retrospective study design. The small number of patients in our study is mainly because of the rare incidence of anaplastic gliomas.

In our future work, we will expand the assessment of other molecular biomarkers in our patient cohort such as mutations in *IDH1/2* or the PI3K signaling pathway and we will correlate these alteration with 1p/19q co-deletion and patient clinical characteristics and outcome.

Conclusion

The importance of 1p/19q co-deletion in anaplastic oligodendrogliomas as a diagnostic, prognostic and predictive biomarker was shown and its presence should be also tested in all low-grade gliomas. Patients with anaplastic oligodendrogliomas who have tumors with 1p/19q co-deletion should be treated intensively with combined RT and chemotherapy (PCV).

Conflicts of Interests

The Authors declare that they have no conflict of interests regarding the publication of this article.

Acknowledgements

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Attachment II

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Research Article

Isocitrate Dehydrogenase-1 Mutations as Prognostic Biomarker in Glioblastoma Multiforme Patients in West Bohemia

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Introduction. Glioblastoma multiforme (GBM) is the most malignant primary brain tumor in adults. Recent whole-genome studies revealed novel GBM prognostic biomarkers such as mutations in metabolic enzyme IDH—isocitrate dehydrogenases (IDH1 and IDH2). The distinctive mutation IDH1 R132H was uncovered to be a strong prognostic biomarker for glioma patients. We investigated the prognostic role of IDH1 R132H mutation in GBM patients in West Bohemia. *Methods*. The IDH1 R132H mutation was assessed by the RT-PCR in the tumor samples from 45 GBM patients treated in the Faculty Hospital in Pilsen and was correlated with the progression free and overall survival. *Results*. The IDH1 R132H mutation was identified in 20 from 44 GBM tumor samples (45.4%). The majority of mutated tumors were secondary GBMs (16 in 18, 89.9%). Low frequency of IDH1 mutations was observed in primary GBMs (4 in 26, 15.3%). Patients with IDH R132H mutation had longer PFS, 136 versus 51 days ($P < 0.021$, Wilcoxon), and OS, 270 versus 130 days ($P < 0.024$, Wilcoxon test). *Summary*. The prognostic value of IDH1 R132H mutation in GBM patients was verified. Patients with mutation had significantly longer PFS and OS than patients with wild-type IDH1 and suffered more likely from secondary GBMs.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumor in adults with an incidence of 3-4/100,000/year. The median survival of patients with GBM is 12.1–14.6 months [1] and only 3–5% of patients survive longer than 3 years [2, 3]. The progress in genomics of GBM has revealed several abnormalities in signaling pathways and a diversity of mutated genes. One of great importance among them is isocitrate dehydrogenase (IDH) [4, 5]. Isocitrate dehydrogenases (three isoforms IDH1, IDH2, and IDH3) catalyze the oxidative carboxylation of isocitrate to alpha-ketoglutarate and reduce nicotinamide adenine dinucleotide phosphate (NADP) to NADPH, which is necessary for the regeneration of reduced glutathione that serves as the main antioxidant [6]. The genes for IDH1 and IDH2 carry specific mutations in 70%–80% of low-grade gliomas, in approximately 50% of anaplastic gliomas, and in more than 5% of glioblastomas [7, 8]. The mutations are involved in 90% single amino acid substitution—R132H in the IDH1 active site that leads to the loss of regular enzyme function—and are predominantly heterozygous. Mutations in IDH2 occurred rarely in brain tumors [7, 9]. The aberrant function of mutated IDH1 is the conversion of alpha-ketoglutarate to the novel oncometabolite 2-hydroxyglutarate (2-HG), which leads to genome-wide epigenetic changes in human gliomas [10]. Tumors with mutated IDH1 and corresponding epigenetic changes demonstrated better prognosis than gliomas with wild-type IDH1. This association was observed also for GBM [4, 6, 7, 11, 12]. The aim of this study was to assess the prognostic role of IDH1 R132H mutation in the relation

Table 1: Glioblastoma patient demographics and clinical characteristics.

Patient characteristics	
Sex	
Male to female	1
Male	22
Female	22
Age, years	
Median	64.3
Range	35–87
KPS	
Median	77.5
Range	30–100
Postoperative treatment	
RT (±CT)	29
CT alone	1
None	15

KPS: Karnofsky performance score; RT: radiotherapy; CT: chemotherapy.

to progression-free survival (PFS) as well as overall survival (OS) of our GBM patients in West Bohemia.

2. Patients and Methods

2.1. Patients. We performed a retrospective study of 44 patients with a diagnosis of WHO grade IV astrocytoma—GBM ($n = 44$; 22 males and 22 females; mean age 64.3 years) who were treated (total or subtotal tumor resection or tumor biopsy, radiotherapy, and chemotherapy with temozolomide) in the Faculty Hospital in Pilsen between the years 2009 and 2011. The study protocol was approved by the ethics committee (Table 1).

2.2. DNA Isolation. DNA was extracted from 10 μm FFPE sections following macrodissection of tumor tissue and normal brain tissue using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). The 10 μm sections corresponded to HES-representative with tumor tissue verified by pathologist.

2.3. Mutation Detection. For detection of mutant allele IDH1 c.395G>A (p.R132H, COSMIC ID 28746), we use TaqMan Mutation Detection Assays (assay name: IDH1 28746 mu and IDH1 rf) with the TaqMan Mutation Detection IPC Reagent Kit (Life Technologies, Carlsbad, California, USA). Mutant allele detection we performed according to recommended procedure and reaction conditions is found in the manual. For the amplification, we used the Stratagene Mx3000P real-time PCR system instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). Detection of mutant alleles was performed in duplicate in a reaction volume of 20 μL. Detection of reference gene was also performed in duplicate. The analysis of the positive samples was repeated. Before analysis of our collection of tumor samples, we analyzed samples of normal brain tissue for detection of cut-off amplification curve. No amplification of mutant allele was present in normal brain tissue. On the basis of these results and the shape of amplification curve of positive tumor samples, we determined the ΔCt cut-off 25 value.

Table 2: The representation of IDH1 R132H mutation in primary versus secondary glioblastomas.

Glioblastoma type	Primary GBM ($n = 26$)	Secondary GBM ($n = 18$)
Mutation status [n]		
IDH1 R132H	4 (15.3%)	16 (89.9%)
IDH1 wild-type	22 (84.7%)	2 (11.1%)

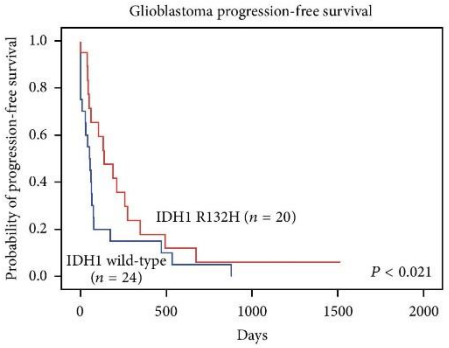

Figure 1: Progression-free survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H gene mutation.

2.4. Statistical Analysis. Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Kaplan-Meier survival curves were plotted and the survival distributions were compared with the use of the Wilcoxon test. Reported P values are two-sided. P values of less than 0.05 were considered to indicate statistical significance.

3. Results

The examined mutation IDH1 R132H was observed in 20 of 44 GBM-patient tumor samples. Therefore we identified the IDH1 mutation in more than 45.4% of glioblastomas. The separation of primary and secondary glioblastomas (GBM that progressed from the low-grade glioma) was done on the basis of clinically relevant information, where possible. The IDH1 R132H mutation occurred in 4 of 26 primary GBMs (15.3%), whereas the majority, 16 of 18 (89.9%) were of secondary glioblastomas mutated (Table 2). The significant relation between IDH1 mutation status and clinical parameters such as PFS and OS was also observed (Table 3). Patients with IDH1 R132H mutation had longer PFS than patients with wild-type IDH1-136 versus 51 days ($P < 0.021$, Wilcoxon test) (Figure 1). Significantly longer OS was observed as well for

Table 3: Results for progression-free survival and overall survival differences in patients with GBM in relation to IDH1 mutation status.

Glioblastoma results	N	Median [days] (95% Cl)	P (Wilcoxon)
Overall survival (OS)			
IDH1 R132H	20	270 (139–400)	0.024
IDH1 wild-type	24	130 (87–172)	
Progression-free survival (PFS)			
IDH1 R132H	20	136 (22–249)	0.021
IDH1 wild-type	24	51 (19–82)	

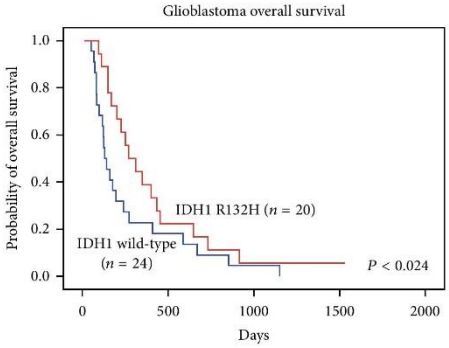

Figure 2: Overall survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H gene mutation.

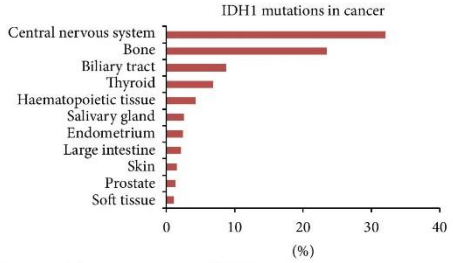

Figure 3: The representation of IDH1 mutations in various cancers [14].

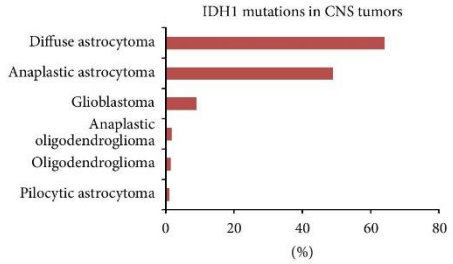

Figure 4: The representation of IDH1 mutations in various types of central nervous system tumors [14].

patients with IDH1 R132H mutation than for patients without the mutation-270 versus 130 days ($P < 0.024$, Wilcoxon test) (Figure 2).

4. Discussion

Recurrent IDH mutations and their role in oncogenesis and tumor progression were described for the first time in GBM [4]. This observation has led to new insights into GBM and cancer biology. Alterations in cancer cell metabolism are now well accepted as one of the principal hallmarks of the cancerogenesis and tumor progression [13]. Mutations in IDH1 were also identified in substantial portion of other tumor types. The data from the Sanger Institute Cancer Genome Project-Catalogue of Somatic Mutations in Cancer revealed the presence of IDH1 mutations in more than 32% of central nervous system tumors, 23% of bone tumors, 8% of biliary tract tumors, 6% of thyroid cancer, and many other tumor types [14] (Figure 3). In the primary brain tumors group, IDH1 mutations are presented mostly in diffuse astrocytomas (64%), anaplastic astrocytomas (49%), glioblastomas (9%), or oligodendrogliomas (2%) [14] (Figure 4). The R132H amino acid substitution is the most common form of IDH1 mutations with the prevalence of 90% among IDH1-mutant tumors. Less common mutants such as R132C, R132G, R132S, and R132L are also known [7, 9].

The fundamental shift in the understanding of mutated IDH and its role in cancer progression came with the observation of the neomorphic function of the mutated enzyme. Instead of the production of alpha-ketoglutarate, mutated IDH1 produced novel oncometabolite 2-hydroxyglutarate (2-HG) that was highly accumulated in the cancer cells [15]. It was subsequently discovered that 2-HG inhibits the functions of the alpha-ketoglutarate dependent superfamily of dioxygenases. These enzymes have diverse cellular functions including, but not limited to, histone demethylation and demethylation of hypermethylated DNA [16, 17]. Moreover, IDH1 mutations and 2-HG production were identified to be sufficient steps in the process leading to glioma hypermethylator phenotype. That observation was important for understanding of glioma oncogenesis and highlighted the interplay between genomic and epigenomic changes in human cancers [10, 18].

Mutations in IDH1 are important also for their clinical consequences. Recent studies revealed the important role of mutated IDH1 in the assessment of astrocytoma patient prognosis. Therefore IDH1 mutations could serve in the near future as the standard prognostic biomarkers for patients with grade II, III, and IV astrocytomas. The differences in OS between IDH1-mutant and IDH1 wildtype GBM were 3.8 versus 1.1 years [4], 2.6 versus 1.3 years [7], 2.3 versus 1.2 years [6], and 3 years versus 1 year in several studies [11]. Similar OS differences in IDH1-mutant versus IDH1-WT tumors were observed for anaplastic astrocytomas, such as 5.4 versus 1.7 years [7], 6.8 versus 1.6 years [6], and 7 versus 2 years [11] as well as for low-grade gliomas [19]. Recent meta-analysis also confirmed the prognostic role of IDH1/2 mutations in gliomas [20]. These data highlight the major impact of IDH1 mutation status on glioma patient survival and support the incorporation of this biomarker into the clinical assessments. Mutations in IDH1/IDH2 and production of oncometabolite 2-HG could be used as well for therapeutic intervention in the near future [21].

The results from our study also support the IDH1 mutation R132H to be the strong prognostic factor for patients with GBM. Although the differences in median PFS and OS between patients with IDH1 mutated and IDH1 wild-type tumors are not as big as in other studies, they are statistically significant. One reason for the relatively small differences in median PFS and OS between both groups could be the heterogeneity of the treatment protocols. The standard treatment with neurosurgery and concomitant chemo-radiotherapy with temozolomide was implemented in 29 patients and 1 patient had only radiotherapy and 15 patients were treated neither with radiotherapy nor with chemotherapy (Table 1). The proportion of IDH1 mutated tumors is also higher in our study than in other similar studies. The IDH1 mutations in glioblastomas were formerly identified predominantly in secondary GBM that progressed from the low grade tumors [22]. In our study, we tried to distinguish between the primary and secondary glioblastomas on the basis of clinically relevant information from the patient history. However, the distinction between primary and secondary GBM was not possible exactly. Only 5 patients had previously assessed low grade glioma (surgery in 2 cases, tumor biopsy in 3 cases). Patients with tumor corresponding neurological symptomatology (epileptic seizures, focal neurological deficit) present at least 6 months before the tumor diagnosis was considered as likely secondary GBM. Moreover the primary-like glioblastomas could be in fact secondary without the symptoms of low grade tumors.

The recent study of mutations in telomerase reverse transcriptase (TERT) gene promoter has revealed the high incidence of these aberrations in a large portion of primary GBMs (about 80%) [23]. In the perspectives of our further research, we will use TERT promoter mutations in addition to clinically relevant information for the separation of primary and secondary glioblastomas. The assessment of other IDH1 mutations than R132H as well as the analysis of mutations in IDH2 is also planned.

Despite the drawbacks of our study mentioned above, IDH1 R132H mutation still serves as a strong prognostic biomarker for our patients with GBM.

5. Summary

The IDH1 R132H mutation was observed in the interestingly higher number of patients with GBM that was previously published by other groups. On the other hand, the majority of mutated GBMs in our cohort are probably secondary glioblastomas. The prognostic value of the IDH1 R132H mutation was also observed. Patients with this mutation had significantly longer PFS as well as OS than patients with wild-type IDH1. The IDH1 mutation status could be used as a strong prognostic factor for patients with GBM, but further validation of this biomarker in large prospective clinical trials is urgently needed.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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Attachment III

Polivka J Jr, Polivka J, Karlikova M, Topolcan O. Pre-graduate and post-graduate education in personalized medicine in the Czech Republic: statistics, analysis and recommendations. EPMA J. 2014; 5(1):22.

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The EPMA Journal

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Pre-graduate and post-graduate education in personalized medicine in the Czech Republic: statistics, analysis and recommendations

Jiri Polivka Jr[1,2*], Jiri Polivka[3], Marie Karlikova[4] and Ondrej Topolcan[4]

Abstract

The main goal of personalized medicine is the individualized approach to the patient's treatment. It could be achieved only by the integration of the complexity of novel findings in diverse "omics" disciplines, new methods of medical imaging, as well as implementation of reliable biomarkers into the medical care. The implementation of personalized medicine into clinical practice is dependent on the adaptation of pre-graduate and post-graduate medical education to these principles. The situation in the education of personalized medicine in the Czech Republic is analyzed together with novel educational tools that are currently established in our country. The EPMA representatives in the Czech Republic in cooperation with the working group of professionals at the Faculty of Medicine in Pilsen, Charles University in Prague have implemented the survey of personalized medicine awareness among students of Faculty of Medicine in Pilsen—the "Personalized Medicine Questionnaire". The results showed lacking knowledge of personalized medicine principles and students' will of education in this domain. Therefore, several educational activities addressed particularly to medical students and young physicians were realized at our facility with very positive evaluation. These educational activities (conferences, workshops, seminars, e-learning and special courses in personalized medicine (PM)) will be a part of pre-graduate and post-graduate medical education, will be extended to other medical faculties in our country. The "Summer School of Personalized Medicine in Plzen 2015" will be organized at the Faculty of Medicine and Faculty Hospital in Pilsen as the first event on this topic in the Czech Republic.

Keywords: Predictive, Preventive and personalized medicine, Statistics, Training, Educational tools, EPMA, Recommendations

Review

Personalized medicine (PM) is the novel model of individual patient's medical care [1,2]. The main goal of PM is the shift from the concept of "one medicine fits to all patients with the same disease" to individual treatment of each patient—"the right treatment to the right patient in the right time" [3-6].

Personalized medicine is based on the evolving knowledge about the human genome, gene functions and the genetic basis of the individual differences in responses to a treatment. However, without a "societal stimulus", the evolution of the personalized medicine would not be most probably occur. The basis of this "societal stimulus" was the alarming finding, in the 90th years of the last century, that the disease incidence and mortality induced by the treatment intolerance and complications (adverse drug reaction) are superior to the disease incidence and mortality caused by civilization diseases [6]. Consequently, a boom occurred in the use of genomics, metabolomics, proteomics and other omics methods for the prediction of the adverse drug reactions.

The strategy of PM is to provide an individualized approach to each patient, based on his/her personal genetic profile and combining information from omics disciplines with innovative preventive and therapeutic strategies that are more efficient, safe and cost-effective [7-9]. The philosophy of PM has become a reality with the sequencing of human genome and the development of novel technologies including laboratory diagnostics, advances in genetics and genomics, new methods of medical imaging and implementation of various biomarkers into the medical care

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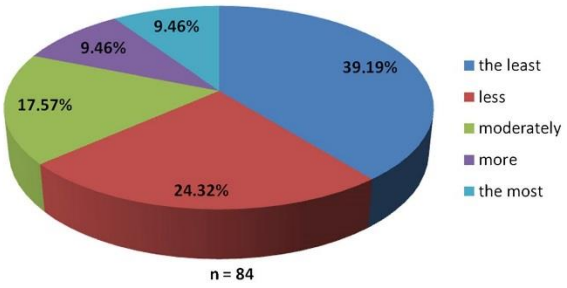

Figure 1 The PM questionnaire results 1. The PM questionnaire results—the question "Have you ever heard the term personalized medicine?"

[10-13]. The process of personalization of the health care includes an efficient prevention and screening, more complex and targeted diagnostics, prediction of possible adverse health effects of prescribed drugs, individualization of therapy and treatment monitoring. This can be achieved by means of prediction, prevention or diagnostics biomarkers, genetic testing of individuals, advances in pharmacogenomics and so on.

The personalized medicine has already proved its usefulness in clinical practice and will be the most important trend in the future medicine. The potential of genetics and genomics to provide new horizons for prevention, diagnosis and treatment of disease is immense, but in order to use this appropriately, and to prevent misuse, before the vision of a personalized medicine can be fully realized, health professionals as well as medical trainees must be given the proper educational foundation [14,15].

The role of education in personalized medicine

Traditional medical education needs to be modified in order to prepare medical fellows and health professionals to the challenges of personalized medicine implementation. Several issues to be considered are listed:

1. As personal genetic information will become a current component of a patient's record, it is crucial that medical students be trained to use and interpret this information appropriately and responsibly [16]. *Fundamental training in genetics and genomics*, along with the attendant legal, ethical and psychosocial issues, should fall within the purview of medical school education.
2. *Pharmacogenetics* and *pharmacogenomics* should be incorporated in medical curricula. There is a growing need to prepare clinicians and health providers to the anticipated arrival of pharmacogenomics diagnostics tools (companion diagnostics) [17,18].
3. Diseases are complex; they originate from a combination of genetic and environmental factors. Also, patients are complex entities resulting from the integration of environmental elements, genetic characteristics, and individual mutations. Recent omics technologies produce a large amount of data.

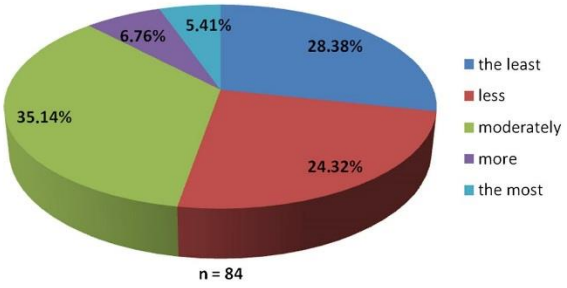

Figure 2 The PM questionnaire results 2. The PM questionnaire results—the question "Would you be able to explain, what does the personalized medicine mean?"

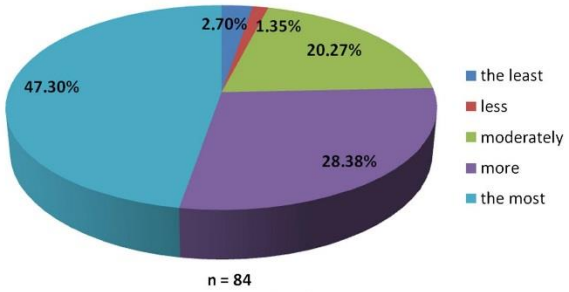

Figure 3 The PM questionnaire results 3. The PM questionnaire results—the question "Do you consider PM to be important?"

> A complex approach is needed, with the use of databases and models (bioinformatics), and students should be trained to work *in a multidisciplinary team* [19].

Over the world, a great number of universities already offer undergraduate and graduate education in molecular medicine, in some of which personalized medicine is also discussed. However, the majority of medical schools have not yet incorporated genetic or genomic courses into their curricula. Yet, there are a few exceptions, such as programs dedicated to personalized medicine at Duke University (NC, USA) and at Mount Sinai School of Medicine (NY, USA) [20]. Also, some schools have updated their curricula by including genomic medicine, such as the Harvard Medical School (MA, USA). Some of these courses are designed for e-learning, for example, the US National Coalition for Health Professional Education in Genetics has developed a series of web-based medical education programs discussing the influence of genetics on various diseases [21]. Similarly, in the UK, the National Genetics Education and Development Centre has developed evidence-based learning objectives and competencies in genetics for health professionals [20].

There is also a debate about the most efficient educational models that could be deployed. At Stanford School of Medicine, a novel hands-on genomics course was developed in 2010 that provided students the option to undergo personal genome testing as part of the course curriculum [22]. Authors had hypothesized that the use of personal genome testing in the classroom would enhance the learning experience of students. After the course, authors concluded that undergoing personal genome testing and using personal genotype data in the classroom enhanced students' self-reported and assessed knowledge of genomics.

Education in PM in the Czech Republic

The medical pre-graduate and post-graduate educational system in the Czech Republic has very long history. It was oriented mainly toward the traditional medicine. During the past decade, the system changed due to the step by step reveal of new findings in biosciences (mentioned above). However, a complex approach

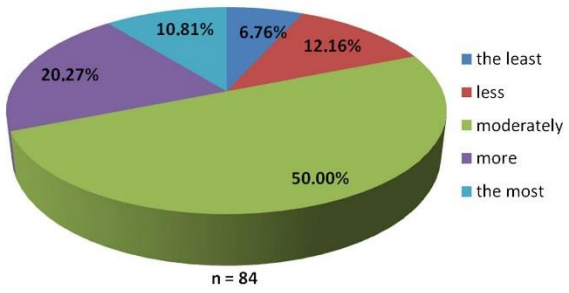

Figure 4 The PM questionnaire results 4. The PM questionnaire results—the question "Do you think personalized medicine should be studied as an independent discipline or through the various disciplines separately?"

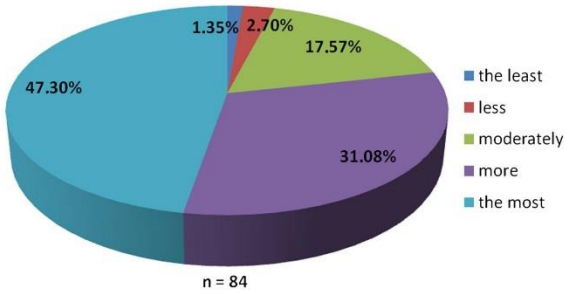

Figure 5 The PM questionnaire results 5. The PM questionnaire results—the question "Do you consider the role of personalized medicine will increase with the progress of knowledge?"

to the PM education focused on its principles does not exist yet. Therefore, the implementation of PM into the common medical education as well as into the clinical practice is essential. The education is thought to be most effective among young physicians and medical students.

In the Czech Republic, there are eight faculties of medicine attached to four universities, faculties of health sciences (for nurses, health care, and laboratory staff) and one faculty of pharmacy. The faculties of medicine (and also faculties of natural sciences) have already incorporated issues linked with personalized medicine in their curricula. The courses dealing with personalized medicine, molecular medicine, systems biology or pharmacogenomics are facultative and offer specialized lectures; however, their quality differs among universities/faculties, they are scarcely interconnected with the overall curriculum, and practical training is missing.

In the next paragraphs, the road to an implementation of the PM education into the curriculum at the Faculty of Medicine in Pilsen is presented.

Survey of PM awareness among students of the Faculty of Medicine in Pilsen

The working group of professionals at the Faculty of Medicine in Pilsen, Charles University in Prague and in the Faculty Hospital in Pilsen has been established with the goal to implement the principles of PM into the pre-graduate and post-graduate education at these institutions. The first step was to ascertain the real situation—how intense was the knowledge of PM among medical students. For this reason, the "Personalized Medicine Questionnaire" was prepared and addressed to medical students at the Faculty of Medicine in Pilsen. Students had to answer eight nominal questions about the PM. Each question had the response range from 1 to 5 (from "the least" up to "the most"). There were 84 responders, mainly students from the fourth year of medical school. The distributions of responses for each question are represented on the Figures 1, 2, 3, 4, 5, 6, 7 and 8. The summary of the means of responses for each question is represented in Figure 9 and Table 1. The results showed lacking knowledge of PM principles and students' will of education in PM.

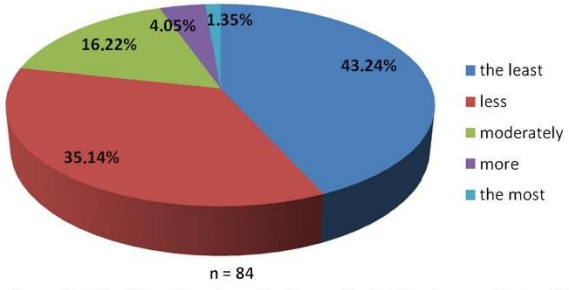

Figure 6 The PM questionnaire results 6. The PM questionnaire results—the question "Is tuition in personalized medicine sufficient in pre-graduate education in medical school?"

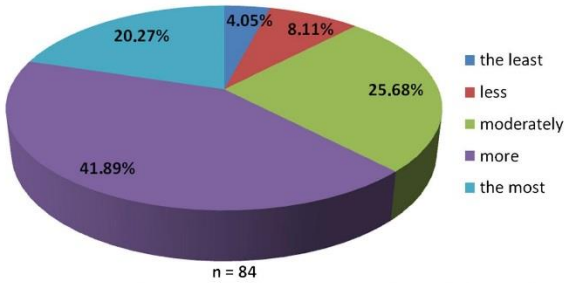

Figure 7 The PM questionnaire results 7. The PM questionnaire results—the question "Do you think personalized medicine should be implemented into the pre-graduate education of medicine for all students?"

As the results of the PM questionnaire show, the awareness about PM among students is quite weak (more than 39% have not even heard the term "personalized medicine"), although most students (more than 75%) recognize the importance of PM and would welcome its implementation into the pre-graduate education.

Educational activities in PM at the Faculty of Medicine in Pilsen

Several educational activities (workshops, conferences, seminars, new optional courses) addressed particularly to medical students and young physicians at our institution were realized in last 5 years.

Since 2009, the presentations concerning PM and its applications have been included in the scientific program of the Immunoanalytical Days—the congress with an international participation, organized every year by the Czech Society of Nuclear Medicine. Since 2011, PM topics have their independent section at this congress. In 2011, a two-day conference "Personalized medicine—from bench to bed" was held at the Faculty of Medicine in Pilsen. The topics of presentations were varying from strategic ones (Horizon 2020 presented by Dr. Patrick Kollar from the European Commission, and Innovative Medicine Initiative) through PM overviews from the USA experts, to the examples of research topics and case studies from cardiology, oncology, neurology and so on.

In 2013, a conference "Genes determine treatment" was organized with the cooperation of the EPMA. Several members of EPMA including the President of EPMA Dr. V. Costigliola and EPMA Secretary-General Prof. Dr. O. Golubnitschaja participated at this event and students from the Faculty of Medicine have a first-hand opportunity to learn about the activities of the association and its members. The presentations embraced many issues, including bioinformatics, systems biology, company diagnostics and so on. The presentation of special topics on the Czech personalized medicine website was the next step in the support of education. This website is widely accessible and free of charge.

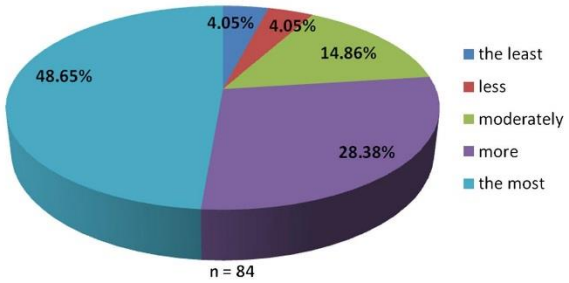

Figure 8 The PM questionnaire results 8. The PM questionnaire results—the question "Do you think personalized medicine should be implemented into the pre-graduate education of medicine as credit course?"

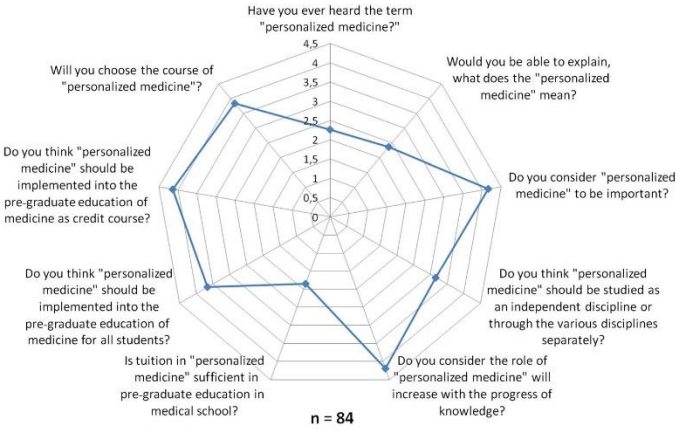

Figure 9 The PM questionnaire results 9. The PM questionnaire results—means of responses for particular questions.

Perspectives

One of the main activities of the working group of professionals in the personalized medicine domain at the Faculty of Medicine in Pilsen and in Faculty Hospital in Pilsen is the organization of the first course of the "Summer School of Personalized Medicine in Pilsen 2015", the first event in the Czech Republic on this topic. During two weeks of the courses, the theoretical background and clinical application of PM across the variety of the fields of medicine will be presented and discussed. The summer school will be open for the pre-graduate as well as post-graduate students of medicine from the Charles University and from other medical schools in the Czech Republic and for medical students abroad.

Recommendations

PM has become an increasingly important topic for physicians, health-care organizations, and their patients. The knowledge and education in PM is crucial for the efficiency of future medical care. The best and the most effective way to achieve this goal is to educate the students of medicine together with young health-care professionals in this field. The PM education should be an essential part of medical study, should be comprehensive and complex, respect mutual interrelations, and should be based on the most advanced knowledge of molecular genetic cause of diseases and their sophisticated scientific management. Various forms of educational activities are optimal, such as conferences, workshops, e-learning and special courses in PM.

Table 1 The summary of the means of responses for each question in the personalized medicine questionnaire

Question in personalized medicine questionnaire	The mean of responses (1–5)
Have you ever heard the term personalized medicine?	2.26
Would you be able to explain, what does the personalized medicine mean?	2.36
Do you consider personalized medicine to be important?	4.16
Do you think personalized medicine should be studied as an independent discipline or through the various disciplines separately?	3.16
Do you consider the role of personalized medicine will increase with the progress of knowledge?	4.2
Is tuition in personalized medicine sufficient in pre-graduate education in medical school?	1.85
Do you think personalized medicine should be implemented into the pre-graduate education of medicine for all students?	3.66
Do you think personalized medicine should be implemented into the pre-graduate education of medicine as credit course?	4.14

Each question had the response range from 1 to 5 (from "the least" up to "the most").

Conclusions

The concept of personalized medicine is one of the most perspective trends in medicine at present. PM assures the individual approach to each patient with tailored therapy and distinctive medical care. The education in this field is the keystone for understanding and application of PM principles. The educational activities of the working group of professionals in the PM at the Faculty of Medicine in Pilsen, Charles University in Prague and in the Faculty Hospital in Pilsen are unique through the country and will be extended among pre-graduate and post-graduate medical students in the Czech Republic.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PJ Jr. and PJ conceived the review and coordinated the drafting of the manuscript. PJ Jr., KM and PJ participated in the design of the review, performed literature searches and identified relevant studies. PJ and TO provided content expertise. All authors read and approved the final manuscript.

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Attachment IV

Polivka J Jr, Polivka J, Rohan V, Priban V. Current view on management of central nervous system low-grade gliomas. Cesk Slov Neurol N. 2016; 79/112(5):1-7. **(IF = 0.209)**

Aktuální pohled na management nízkostupňových gliových nádorů centrálního nervového systému

Current View on Management of Central Nervous System Low-grade Gliomas

Souhrn

Diagnóza gliomu centrálního nervového systému stupně malignity II (nízkostupňový gliom, Low-Grade Glioma; LGG) znamená vždy významný zásah do života nemocných, neboť i přes zřetelný pokrok v terapii se nadále jedná o nevyléčitelné onemocnění. Léčebné možnosti zahrnují neurochirurgický zásah, radioterapii a chemoterapii. Dosud však nejsou stanovena jednoznačná kritéria pro určení rozsahu jednotlivých léčebných metod, jejich kombinací nebo vhodného načasování. Teprve výsledky dlouhodobého sledování v klinické studii fáze III RTOG 9802 prokázaly příznivý účinek kombinované onkologické léčby radioterapie (54 Gy) a chemoterapie (režim prokarbazin, lomustin a vinkristin; PCV) u nemocných se zvýšeným rizikem (věk > 40 let s reziduálním pooperačním radiografickým nálezem nebo věk ≥ 40 let po jakémkoli operačním zásahu). Nezodpovězena prozatím zůstává otázka významu molekulárně-genetických biomarkerů (kodelece 1p/19q, mutace *IDH1/2* a dalších) ve vztahu k predikování účinku kombinované léčby. Očekává se, že detailní molekulárně-genetická analýza nádoru bude součástí rutinní klinické péče o nemocné s gliomy všech stupňů malignity vč. LGG. Kombinovaná radioterapie a chemoterapie režimem PCV následující po neurochirurgickém zásahu by měla být v současné době preferovaným přístupem k léčbě nemocných s vysoce rizikovým LGG.

Abstract

The diagnosis of grade II central nervous system glioma (Low-Grade Glioma; LGG) always significantly impacts on the lives of patients, as, despite clear progress in therapy, LGG continues to be an incurable disease. Treatment options include neurosurgical intervention, radiotherapy and chemotherapy. So far, however, no clear criteria have been set to determine the effect of individual treatments, their combinations or their timing. The results of a long-term follow up of the phase III RTOG 9802 trial demonstrated better effect of combined radiotherapy (54 Gy) and chemotherapy (procarbazine, lomustine and vincristine – PCV) treatment in patients with high risk disease (age > 40 years with postoperative radiographic residuum or age ≥ 40 years after any surgical intervention). The question of the role of molecular genetic biomarkers (co-deletion 1p/19q, IDH1/2 mutations and others) in predicting the effects of combined treatment remains unanswered. It is expected that detailed molecular genetic analysis of each tumor will become a part of routine clinical care of patients with gliomas of all stages of malignancy, including LGG. Combined radiotherapy and chemotherapy with PCV following neurosurgical intervention should be the preferred approach to treatment of patients with high-risk LGG.

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Key words

low-grade glioma – chromosomal codeletion 1p/19q – *IDH1/2* mutations – personalized medicine

Úvod

Gliomy II. stupně malignity (synonyma nízkostupňové gliomy, Low-Grade Glioma; LGG) tvoří heterogenní skupinu neuroepitelových nádorů centrálního nervového systému. Jejich členění vychází z dosud užívané WHO (World Health Organization) klasifikace z roku 2007 [1]. Jedná se především o astrocytomy, oligodendrogliomy, oligoastrocytomy a skupinu vzácných smíšených glioneuronálních nádorů. Typickými histopatologickými charakteristikami LGG jsou hypercelularita, nukleární atypie, pleomorfizmus a chybění významné mitotické aktivity. U této skupiny nádorů je také nalézán nižší proliferační index a nejsou přítomny nekrózy a vaskulární proliferace jako u gliomů vyšších stupňů malignity [1,2].

LGG se vyskytují převážně v mladším věku, s maximem ve 3. a 4. dekádě [3]. Projevují se nejčastěji epileptickým záchvatem (80 %), méně často změnami kognice, chování, fokálními neurologickými příznaky nebo bolestmi hlavy. Mohou být také asymptomatické, kdy k nalezení choroby zobrazovacími metodami dochází náhodně. Rostou infiltrativně a postihují často elokventní oblasti. Přítomná neurologická symptomatologie podstatně zhoršuje kvalitu života. Významným důsledkem růstu LGG je epilepsie, která může být refrakterní. Ačkoli jsou LGG považovány za relativně benigní nádory, progredují postupně do vyšších stupňů malignity a medián přežití léčených LGG po stanovení diagnózy je 7,5 roku [3,4]. LGG jsou z těchto důvodů závažná skupina nemocí, u které probíhá intenzivní výzkum s cílem optimalizovat jejich management pro zlepšení kvality života a prodloužení přežití nemocných.

Diagnostika

Pro diagnostiku LGG je kromě klinické úvahy zásadní magnetická rezonance (MR). V T1 vážených sekvencích je obvykle homogenní hyposignální oblast, v T2 a FLAIR sekvencích je nádor hyperintenzitní. Sycení kontrastní látkou je nevýznamné nebo malé, stejně jako okolní edém. U oligodendrogliálních nádorů mohou být (mikro)kalcifikace. Významným přínosem je MR spektroskopie a pozitronová emisní tomografie (PET). Nálezy výpočetní tomografie (CT) odhalují hypodenzní oblast obvykle bez expanzivního chování a okolního edému nesytící se kontrastní látkou a mohou snadněji uniknout pozornosti. Finální diagnostika je histopatologická [5–7].

Molekulární genetika a biomarkery LGG

Kromě histopatologických nálezů nabývají u gliomů stále větší význam molekulárně-genetické charakteristiky nádorové tkáně. Uplatňují se v upřesnění diagnostiky a v managementu LGG, některé také slouží jako prognostické a prediktivní biomarkery. V posledních letech je velká pozornost věnována zejména dvěma molekulárně-genetickým charakteristikám nacházejícím se u gliomů vč. LGG. Jedná se o kodeleci 1p/19q a mutace genů izocitrát dehydrogenázy 1 a 2 (*IDH1/2*).

Kodelece 1p/19q

Kodelece 1p/19q je kombinovaná ztráta genetického materiálu z krátkého raménka chromozomu 1 a dlouhého raménka chromozomu 19. Vyskytuje se u 75–80 % nízkostupňových oligodendrogliomů a také u malého počtu nízkostupňových astrocytomů (do 10 %) [8]. Metaanalýza publikovaná v roce 2014 prokázala prognostický i prediktivní význam této kodelece [9]. Zpracována byla data z 28 studií, do nichž bylo zařazeno 3 408 nemocných s gliovými nádory, z nichž 898 pacientů mělo potvrzenou diagnózu LGG. Izolovaná delece 1p rovněž měla zejména u pacientů s LGG příznivý prognostický význam. Nemocní s kodelecí měli delší dobu do progrese choroby (Progression-Free Survival; PFS; HR = 0,63; 95% CI 0,52–0,76) a delší dobu celkového přežití (Overall Survival; OS; HR = 0,43; 95% CI 0,35–0,53). Samostatná delece 19q nebyla významná ve vztahu k OS. Kodelece 1p/19q byla tedy potvrzena jako silný a nezávislý prognostický biomarker u nemocných s gliovými nádory. Nemocní s anaplastickými oligodendrogliomy a oligoastrocytomy (st. III) pozitivními na přítomnost kodelece 1p/19q profitovali v dlouhodobém sledování z kombinované léčby radioterapií a chemoterapií (režimem prokarbazin, lomustin-CCNU, vinkristin; PCV) [10–13]. Podobný vztah k léčebné odpovědi u pacientů s LGG zatím jednoznačně potvrzen není.

Mutace genů izocitrát dehydrogenázy 1 a 2

Izocitrát dehydrogenáza (*IDH*) patří mezi významné enzymy Krebsova cyklu uplatňující se v jednom z klíčových kroků sacharidového, lipidového i aminokyselinového metabolizmu. Lidská *IDH* má tři izoformy, a to *IDH1* (vyskytuje se v cytoplazmě a peroxizomech), *IDH2* a *IDH3* (výskyt v mitochondriích). Mutace genů *IDH1* a *IDH2* se uplatňují při vzniku a progresi mozkových nádorů [14]. Mutace jsou téměř vždy pouze v jedné alele, *IDH1* vykazuje v 90 % aminokyselinovou substituci argininu na histidin na 132. pozici proteinu (R132H), známé jsou také substituce argininu na cystein (R132C), argininu na glycin (R132G), argininu na serin (R132S) a argininu na leucin (R132L). Mutace v *IDH2* jsou mnohem vzácnější. Jedná se především o aminokyselinovou substituci argininu na pozici 172 proteinu (R172) [15,16]. Četnost mutací *IDH1* je vysoká především u difuzních astrocytomů (76 %), anaplastických astrocytomů (62 %) a sekundárních glioblastomů (76 %). Naopak nízká je u primárních glioblastomů (6 %) a velmi vzácně se vyskytuje též u pilocytárních astrocytomů (0,01 %). Mutace genu *IDH2* jsou vzácnější. Vyskytují se u difuzních astrocytomů (1,6 %), anaplastických astrocytomů (1,4 %) a u glioblastomů se téměř neobjevují. Mutace genů *IDH1/2* se nacházejí také u oligodendrogliomů stupně II (78 % mutace *IDH1*, 4,5 % mutace *IDH2*) a oligodendrogliomů stupně III (67,5 % mutace *IDH1*, 5,7 % mutace *IDH2*) [17–20]. Mutace *IDH1/2* zjištěné v nádorové tkáni jsou významným pozitivním prognostickým biomarkerem gliomů v podstatě napříč všemi stupni malignity. Nemocní s gliomy II.–IV. stupně s přítomností mutací *IDH1/2* vykazovali v mnoha studiích signifikantně delší PFS a OS než nemocní bez mutací [15,21–26]. Rozsáhlá metaanalýza rovněž potvrdila významný pozitivní prognostický efekt přítomnosti *IDH1/2* mutací v gliomech ve vztahu k OS i PFS [27]. Mutační stav *IDH1/2* v nádoru může být stanoven postupy molekulární biologie [28,29], imunohistochemicky [30,31] a také neinvazivním stanovením onkometabolitu 2-hydroxy-glutarátu produkovaného mutovaným enzymem *IDH1/2* metodami MR spektroskopie [32–34].

Vzájemná souvislost genetických alterací LGG

Z klinického pohledu se zdá být mnohem důležitější vzájemná souvislost jednotlivých molekulárně-genetických biomarkerů LGG. Ukazuje se totiž, že existují minimálně tři geneticky i biologickým chováním a klinickou prognózou do značné míry heterogenní skupiny LGG (naopak s významnou homogenitou uvnitř jednotlivých skupin), které je možné vzájemně odlišit při znalosti mutačního stavu *IDH1/2*, kodelece 1p/19q a jejich kombinace v nádorové tkáni [35].

Mezinárodní konsorcium The Cancer Genome Atlas Research Network (TCGA) provedlo rozsáhlou multiplatformovou analýzu 293 pacientů s gliomy stupně II a III. Analýza integrovaných dat metodami Cluster of Clusters analysis (CoC) a OncoSign odhalila tři navzájem molekulárně-geneticky rozdílné kategorie gliomů, které silně korelovaly se subtypem nádoru stanoveném na bázi mutací *IDH1/2*, kodelece 1p/19q a jejich kombinace, a naopak velmi slabě korelovaly s histologickým typem gliomu (R = 0,79 vs. R = 0,19).

Gliomy v první skupině byly charakterizovány mutacemi v *IDH1/2* a zároveň přítomnou kodelecí 1p/19q. Aktivační mutace promotoru genu *TERT* identifikované také u primárního glioblastomu se vyskytovaly v této skupině u 96 % nádorů [36]. Časté byly rovněž aktivační mutace *PIK3CA* (20 %) nebo inaktivační mutace tumor supresorových genů CIC (62 %) a *FUBP1* (29 %) identifikovaných již dříve u 1p/19q deletovaných oligodendrogliomů [37]. Tato skupina gliomů obsahovala převážně nádory s oligodendrogliální složkou (82 % oligodendrogliomů a 16 % oligoastrocytomů) a vykazovala nejlepší prognózu s nejdelším mediánem OS osm let. Nutné je zdůraznit, že v této prognosticky nejpříznivější skupině stanovené na základě dvou molekulárně-genetických biomarkerů se vyskytovalo 43 % pacientů s gliomy stupně III, kteří by dle pouhého histopatologického zařazení měli mít významně horší prognózu.

Do druhé skupiny byly zařazeni pacienti s gliomy pozitivními na *IDH1/2* mutace, ale bez přítomnosti 1p/19q kodelece. Zároveň 94 % nádorů mělo inaktivační mutaci v tumor supresorovém genu *TP53*, 86 % pak v genu *ATRX*. Nádory v této skupině obsahovaly různé histologické typy gliomů bez jasné predominance a pacienti zařazení do této kategorie měli horší prognózu s kratším mediánem OS 6,3 roku.

Poslední skupina zahrnovala nádory bez přítomnosti *IDH1/2* mutací, tzv. *IDH* wild-type tumory. Žádný z nádorů neměl kodeleci 1p/19q. Molekulárně-genetickým profilem i biologickým chováním se významně přibližovaly primárnímu glioblastomu. Rovněž přežití pacientů s mediánem OS pouhého 1,7 roku bylo podobné glioblastomu. Více než polovina těchto tumorů byly astrocytomy (56 %). Nutné je opět zdůraznit, že téměř čtvrtina pacientů (24 %) zařazených do této kategorie měla dle histopatologické diagnostiky nádor stupně II, a tedy byla u nich očekávána mnohem lepší prognóza.

Studie TCGA ale nebyla jediná, která se snažila o rozčlenění gliomů vč. LGG do prognosticky rozdílných podkategorií za pomoci několika molekulárně-genetických biomarkerů a jejich kombinací. Například výzkumná skupina z Mayo Clinic/University of California San Francisco provedla analýzu 1 087 gliomů (stupně II–IV), u nichž definovala pět navzájem rozdílných skupin nádorů dle kombinací tří molekulárně-genetických biomarkerů (*IDH* mutace, 1p/19q kodelece a mutace promotoru *TERT*) [38]. Pacienti s gliomy stupně II a III měli mezi jednotlivými skupinami signifikantní rozdíly v mediánu OS, což však neplatilo pro nádory stupně IV, tedy glioblastomy. Nejhorší prognózu mezi gliomy stupně II a III měla skupina TERT pozitivních a *IDH* a 1p/19q negativních nádorů, kde se OS blížilo pacientům s glioblastomem. Naopak nejlepší prognózu měla skupina pacientů s *TERT* a *IDH* pozitivními nádory. Existují však i další studie klasifikující gliomy do různých skupin dle kombinací různých biomarkerů, jako např. japonská analýza 332 gliomů stupně II a III využívající také *IDH* mutace a 1p/19q kodeleci [39], německá studie 405 dospělých pacientů s gliomy sledující *IDH1* mutace, 1p/19q kodeleci a expresi *ATRX* [40] a další [41].

Ačkoliv molekulárně-genetické biomarkery prokazatelně přinášejí aditivní informaci k současné diagnostice gliomů založené na pouhém histopatologickém nálezu, nebyl dosud učiněn jednoznačný konsenzus nad jejich optimální kombinací využitelnou v klinické praxi. I přes to byla původní WHO klasifikace nádorů CNS z roku 2007 recentně updatována a využívá nyní spolu s histopatologickými kritérii také dobře známé a probádané biomarkery, jakými jsou např. mutace *IDH1/2* nebo kodelece 1p/19q. To povede alespoň k přesnějšímu zařazení gliových nádorů do užších klasifikačních skupin, což pravděpodobně přinese též zpřesnění pacientovy prognózy [42].

Management LGG

Léčba LGG zahrnuje kombinaci neurochirurgického zásahu, radioterapie a chemoterapie. Dosud však nejsou stanovena jednoznačná kritéria pro určení rozsahu jednotlivých léčebných metod, jejich kombinací nebo vhodného načasování. Definitivní konsenzus nad terapeutickým přístupem k pacientům s LGG se ve světle nových poznatků z recentních prospektivních klinických studií stále ještě hledá, jak bude dále podrobně probíráno.

Chirurgická léčba LGG

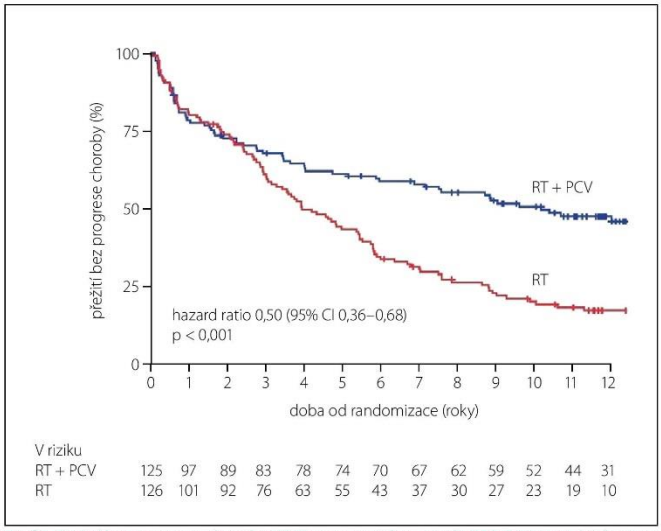
Neurochirurgický zásah zůstává i nadále naprosto zásadní součástí léčby LGG [5]. Kromě cytoredukce umožňuje získání tkáně pro stanovení histopatologické diagnózy a molekulárně-genetických charakteristik nádoru. Podle rozsahu odstranění nádoru se může jednat o maximální/totální resekci, subtotální resekci, parciální resekci nebo o nádorovou biopsii. Z etických důvodů nebylo a není možné realizovat prospektivní studii, která by porovnávala výsledky chirurgické léčby LGG ve vztahu k velikosti resekce nádorové tkáně. Z retrospektivních studií je považován za prognosticky nejpříznivější maximální možný rozsah resekce [43]. Retrospektivní studie zahrnující 216 nemocných s LGG prokázala příznivý vliv radiologicky potvrzeného většího rozsahu resekce nádorové tkáně (≥ 90 vs. < 90 %) na pětileté (97 vs. 76 %) i osmileté OS (91 vs. 60 %) [44]. Norská retrospektivní studie srovnávala dva přístupy k chirurgické léčbě pacientů s LGG, a sice časnou resekci ve srovnání s biopsií a pečlivým sledováním (watchfull waiting) [45]. Medián OS byl delší ve skupině s časnou resekcí nádoru (9,7 vs. 5,6 roku; p = 0,047). V další studii zahrnující retrospektivní analýzu 1 509 pacientů s LGG představoval rozsah resekce spolu s objemem pooperačního rezidua nádorové tkáně nezávislý prognostický faktor pro PFS i OS nemocných [46].

Maximalizace rozsahu chirurgické léčby je v současné době umožněna pokroky v zobrazovacích metodách, neurochirurgických operačních technikách a peroperačním monitorování [3,5]. Lokalizace nádoru v eloквentních nebo chirurgicky nepřístupných oblastech umožňuje pouze částečné odstranění nádoru nebo navigovanou biopsii.

Radioterapie LGG

Příznivý efekt radioterapie (RT) na redukci objemu tumoru při léčbě pacientů s LGG byl opakovaně prokázán. Porovnávány byly především různé dávky fotonového záření, frakcionovaně v rozmezí 45–64,8 Gy [6,47]. Vysoké dávky záření ale nepřinesly zlepšení PFS ani OS ve srovnání s dávkami nižšími a středními.

Nemocní s LGG zařazení ve studii fáze III EORTC 22844 neprofitovali z vyšší radiační dávky ve srovnání s dávkou nižší (59,4 vs. 45 Gy). Rozdíl v pětiletém PFS i OS mezi oběma rameny studie nebyl statisticky signifikantní, naopak ve skupině s vyšší dávkou záření měli pacienti v dlouhodobém sledování zhoršenou kvalitu života [48]. Rovněž



Graf 1. Přežití bez progrese choroby (PFS) nemocných ve studii RTOG 9802 v závislosti na použitém léčebném režimu následujícím po neurochirurgickém zásahu, a to kombinované terapie PCV + RT (modře) nebo RT samotné (červeně).
Statisticky významný rozdíl v PFS byl prokázán. Upraveno dle [52].

studie fáze III NCCTG/RTOG/ECOG neprokázala delší PFS ani OS (p = 0,48) u pacientů s LGG léčenými vysoko dávkovanou (64,8 Gy) ve srovnání se středně dávkovanou RT (50,4 Gy) [49]. Navíc v rameni s vyšší dávkou RT byl častější výskyt radiační nekrózy stupně 3–5 (5 vs. 2,5 %).

Nižší radiační dávka tedy přináší srovnatelný benefit v PFS i OS pacientů v porovnání s dávkou vyšší, avšak při nižší toxicitě léčby. V současné době je v léčebných schématech LGG preferována celková radiační dávka 54 Gy.

Chemoterapie v léčbě LGG a význam studie RTOG 9802

V léčbě LGG byla testována řada chemoterapeutik, např. karboplatina, vinkristin, etoposid, kombinace PCV nebo temozolomid. Limitací studií u LGG však byla jejich nedostatečná validita daná především malými počty zařazených pacientů, chyběním kontrolního souboru, nehomogenitou sledovaných skupin nemocných i nehomogenitou samotných nádorů. Výsledky proto nebyly přesvědčivé. Zároveň byla obava z nežádoucích účinků a toxicity chemoterapie, a to zejména u mladší populace s potenciálně dlouhodobým přežíváním (chemoterapií indukované kognitivní poruchy, leukoencefalopatie, myelodysplastický syndrom, leukemie) [6,50,51].

V roce 1998 byla zahájena prospektivní klinická studie fáze III RTOG 9802. Bylo do ní zařazeno 254 nemocných s LGG s vysokým rizikem definovaným jako věk pod 40 let s reziduálním pooperačním radiografickým nálezem, nebo věk nad 40 let po jakémkoli operačním zásahu. Nemocní byli randomizováni k adjuvantní léčbě buď samotnou radioterapií (celková dávka 54 Gy v průběhu šesti týdnů), nebo k radioterapii následované šesti cykly kombinované chemoterapie režimem PCV (prokarbazin, lomustin, vinkristin). Předběžné výsledky sledování z roku 2012 neprokázaly statisticky signifikantní rozdíl v OS pacientů mezi oběma větvemi studie [52].

Naproti tomu recentně publikované výsledky dlouhodobého sledování pacientů zařazených do studie RTOG 9802 (medián sledování 11,9 roku) jednoznačně prokazují podstatný benefit kombinované léčby (RT + PCV) oproti samotné RT, a to jak ve vztahu k PFS tak OS [53]. Pacienti ve větvi RT + PCV dosahovali významně delšího mediánu PFS ve srovnání s větví samotné RT (10,4 vs. 4 roky; p < 0,001) (graf 1). Pětiletého přežití bez progrese choroby dosáhlo více pacientů léčených RT + PCV než RT samotnou (61 vs. 44 %). Ještě podstatnější byl rozdíl v PFS po 10 letech sledování (51 vs. 21 %). To ukazuje, že se křivky přežití bez progrese dále rozestupují se vzrůstající dobou sledování, a tedy že benefit kombinované terapie se postupem času od léčebného zásahu ještě zvyšuje. Rozdíl v PFS zůstal významný také při subanalýze jednotlivých histologických typů LGG. Pacienti s přítomností *IDH1 R132H* mutace v nádoru měli signifikantně delší PFS než pacienti bez této mutace a to bez ohledu na zvolenou terapii, nicméně také u *IDH1* mutovaných nádorů byl patrný profit kombinované terapie RT + PCV nad RT samotnou (p < 0,001).

Významného benefitu kombinované terapie bylo dosaženo také u celkového přežití. Studie prokázala podstatný rozdíl 5,5 roku v OS nemocných léčených RT + PCV oproti samotné RT (13,3 vs. 7,8 roku; p = 0,003) (graf 2). Deset let přežívalo o celých 20 % nemocných léčených kombinovanou terapií více (60 vs. 40 %). Těchto výsledků bylo dosaženo i přes fakt, že záchrannou chemoterapii při progresi nádoru dostalo více nemocných ve větvi samotné RT oproti větvi s RT + PCV. I když toxické projevy léčby byly větší ve větvi RT + PCV, byly léčitelné a nebyl pozorován signifikantní rozdíl v kognici pacientů sledovaných v obou větvích, ani nebyl zaznamenán výskyt myelodysplázie nebo leukemie [53,54]. Rozdíl v OS zůstal významný také při subanalýze jednotlivých histologických typů LGG s výjimkou astrocytomů, kde je patrný trend k delšímu OS bez statistické významnosti dané pravděpodobně omezeným počtem pacientů a omezenou délkou dalšího sledování (p = 0,31).

Při subanalýze dle přítomnosti mutace *IDH R132H* v nádoru bylo zjištěno podstatně delší OS nemocných s mutací než bez ní, a to bez ohledu na zvolenou terapii (13,1 vs. 5,1 roku; p = 0,02). Nemocní s *IDH* mutovanými nádory nicméně opět profitovali z kombinované léčby RT + PCV ve srovnání s RT samotnou (p = 0,02).

Otázkou zůstává možné využití dalšího biomarkeru s velmi častým výskytem u LGG (kodelece 1p/19q) k predikci vyšší účinnosti kombinované terapie RT + PCV u 1p/19q pozitivních nádorů. Taková souvislost byla nalezena u dvou nezávislých studií fáze III (RTOG 9402 a EORTC 26951) u pacientů s anaplastickými oligodendrogliomy a oligoastrocytomy [10,11]. Analýza kodelece 1p/19q byla ve studii RTOG 9802 rovněž provedena, nic-

méně dostatek nádorové tkáně ke stanovení byl dostupný pouze u 63 pacientů a nebylo možné statisticky ověřit prediktivní význam tohoto biomarkeru. Prozatím tedy nebyla prokázána role kodelece 1p/19q jako pozitivního prediktivního biomarkeru vyšší účinnosti kombinované terapie RT + PCV u pacientů s LGG a na definitivní závěr si bude třeba ještě počkat.

Příznivé výsledky kombinované terapie RT + PCV ve srovnání s RT samotnou získané ze studie RTOG 9802 přinášejí další otázky v managementu LGG. Jde např. o volbu optimálního časování léčby, výběr nemocných dle molekulárně-genetického profilu (přítomnost kodelece 1p/19q, mutací *IDH*) nebo možnost použití alternativní chemoterapie (temozolomid).

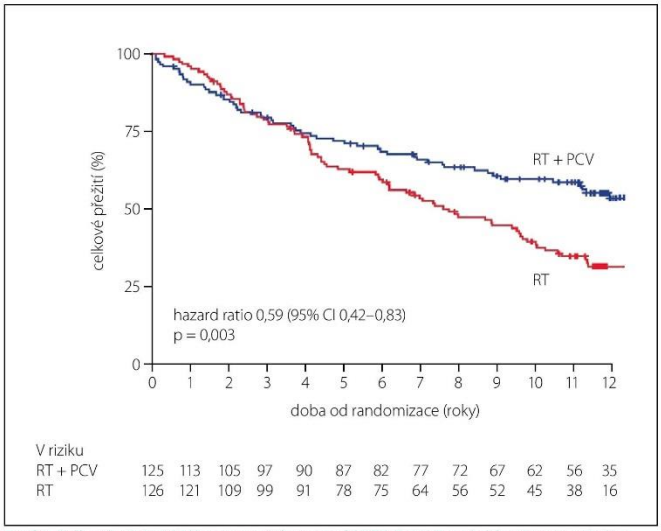
Časování léčby LGG – „timing"

Optimální časování léčby, která následuje po operačním výkonu, je důležitá otázka v managementu LGG. Ta však není dosud jednoznačně zodpovězena. Historicky byl uplatňován převážně postup pečlivého sledování (watchfull waiting) pro všechny nemocné s LGG. V 90. letech 20. století byla realizována klinická studie EORTC 22845 sledující účinnost časné a odložené RT na dobu PFS a OS u 314 nemocných s LGG. Ačkoliv PFS bylo delší u časné RT, rozdíl v OS u obou skupin nebyl signifikantní (7,2 roku u časné RT vs. 7,4 roku u odložené RT; p = 0,872) [55]. Na základě výsledků této studie bylo za optimální strategii považováno zahájení RT až při manifestaci klinických příznaků, radiografické progresi nádoru nebo výskytu refrakterní epilepsie. Odložení RT je výhodné i vzhledem k jejím dlouhodobě hendikepujícím následkům, jako např. SMART (Stroke-like Migraine Attacks after Radiotherapy), PIPG (Peri-Ictal Pseudoprogression) nebo ALERT (Acute Late-onset Encephalopathy after Radiotherapy [56]. Publikována byla také analýza 111 pacientů zařazovaných do studie RTOG 9802, kteří měli LGG s nízkým rizikem relapsu (věk < 40 let a zobrazovacími metodami ověřenou kompletní resekci nádoru) [57]. Tato skupina byla pouze sledována bez další adjuvantní terapie. Pětileté PFS dosahovalo 48 % a delší PFS korelovalo s předoperační velikostí nádoru < 4 cm v průměru, histologickým typem oligodendrogliomu a přítomností pooperačního reziduálního nálezu < 1 cm dle MR.

Klinické sledování a pravidelné kontroly zobrazovacími metodami (MR) až do progrese choroby mohou být i nadále odpovídajícím postupem pooperační péče u nemocných s nízkorizikovým LGG [6,58]. Význam kombinované léčby chemo- a radioterapie nebyl dosud u nízkorizikových LGG prospektivně sledován. V současné době jsou za vysoce rizikovou skupinu považováni pacienti, u kterých jsou přítomny alespoň tři z následujících šesti faktorů:

- věk ≥ 40 let;
- Karnofského skóre (Karnofsky Performance Status; KPS) < 70 %;
- histologický nález astrocytární složky nádoru;
- velikost nádoru ≥ 6 cm v průměru;
- nádor přesahující střední čáru;
- přítomnost neurologického deficitu před operací [59,60].

U těchto nemocných by měla být adjuvantní terapie zahájena bezprostředně po chirurgickém výkonu, nejlépe kombinací RT a PCV.



Graf 2. Celkové přežití (OS) nemocných ve studii RTOG 9802 v závislosti na použitém léčebném režimu následujícím po neurochirurgickém zásahu, a to kombinované terapie PCV + RT (modře) nebo RT samotné (červeně).
Statisticky významný rozdíl v OS byl prokázán. Upraveno dle [52].

Volba vhodné chemoterapie LGG

Ne zcela dořešená zůstává otázka volby nejvhodnějšího chemoterapeutického režimu u pacientů s LGG, kteří vyžadují adjuvantní terapii. Režim PCV byl zaveden do léčby mozkových nádorů v 80. letech 20. století. Stal se populárním zejména pro efektivitu při léčbě rekurentních oligodendrogliomů [61]. Postupně byl režim PCV v léčbě mozkových nádorů nahrazován novějším alkylačním chemoterapeutikem temozolomid, zvláště vzhledem k jeho příznivějšímu profilu nežádoucích účinků a jednodušší dostupnosti [62]. Temozolomid se stal nedílnou součástí terapie pacientů s gliomy vyšších stupňů malignity, převážně multiformního glioblastomu, kde prokázal významný benefit v mediánu OS v kombinaci s RT ve srovnání s RT samotnou pro jinak neselektovanou skupinu pacientů (14,6 vs. 21,1 měsíců; p < 0,001) [63]. Dvouleté OS bylo více než dvojnásobné v rameni kombinované terapie (26,5 vs. 10,4 %).

Poněkud jiná situace nastává při užití temozolomidu u LGG. Klinická studie fáze III EORTC 22033-26033 srovnávala účinnost léčby samotným temozolomidem oproti RT u pacientů s vysoce rizikovým LGG bez přítomnosti 1p/19q kodelece v nádorové tkáni [64]. Nádor s vysokým rizikem byl definován pomocí kritérií: věk nad 40 let, přítomnost klinických symptomů nebo radiologicky ověřená progrese choroby po úvodním chirurgickém výkonu. Po sledování v délce 45,5 měsíců bylo podobné PFS

v obou větvích studie (47 vs. 40 měsíců pro RT, resp. temozolomid), medián OS nebyl ve větvi samotné RT dosažen (74 měsíců pro temozolomid). Výsledky tedy nebyly konečné a vyžadují další sledování.

Na tomto místě je nutné zdůraznit, že dosud nejsou dostupná data dlouhodobého sledování podobného rozsahu jako ve studii RTOG 9802, která by hodnotila efektivitu temozolomidu u pacientů s LGG ať už v kombinaci s RT nebo bez ní. Další pohled může přinést studie fáze III CODEL, která srovnává RT následovanou PCV s RT s konkomitantním a následným podáním temozolomidu u pacientů s gliomy stupně II a III s přítomností kodelece 1p/19q. Výsledky této studie by měly přinést definitivní odpověď na otázku vhodné adjuvantní chemoterapie u 1p/19q deletovaných gliomů [65]. Nyní je tedy prokázána účinnost kombinované terapie RT + PCV u nemocných s vysoce rizikovým LGG a tento chemoterapeutický režim by měl být dle zásad medicíny založené na důkazech preferován. V České republice je však situace komplikována omezenou dostupností režimu PCV pouze na individuální dovoz (procarbazin není v České republice registrován, CCNU je v České republice nedostupný a je nahrazován BCNU) a je zde tedy dosud preferován temozolomid.

Závěr

Diagnóza LGG znamená vždy významný zásah do života nemocných, neboť i přes zřetelný pokrok v terapii se nadále jedná o nevyléčitelné onemocnění. Recentně publikované výsledky dlouhodobého sledování pacientů zařazených v klinické studii RTOG 9802 však pravděpodobně způsobí významnou změnu v managementu léčby LGG. Nyní je prokázáno, že nemocní s LGG se zvýšeným rizikem (věk pod 40 let s reziduálním pooperačním radiografickým nálezem nebo věk nad 40 let po jakémkoli operačním zásahu) výrazně profitují z kombinované onkologické léčby s radioterapií v celkové dávce 54 Gy v kombinaci s chemoterapeutickým režimem PCV, ve srovnání se samotnou radioterapií (medián celkového přežití o 5,5 roku delší ve skupině kombinované léčby). Nemocní mladší 40 let, nemocní s kompenzovanou epilepsií jinak asymptomatičtí a nemocní s náhodně zjištěným LGG mají být po chirurgickém zásahu sledováni klinicky a zobrazovacími metodami k časnému záchytu progrese onemocnění. Nezodpovězena prozatím zůstává otázka kodelece 1p/19q a mutací *IDH1/2* v nádorové tkáni jako pozitivních prediktivních biomarkerů kombinované radiochemoterapie (RT + PCV) LGG. Očekává se, že detailní molekulárně-genetická analýza nádoru bude součástí rutinní klinické péče o nemocné s gliomy všech stupňů malignity včetně LGG. Dosud nezodpovězené otázky zodpoví až výsledky probíhajících a nově designovaných prospektivních klinických studií.

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Attachment V

Polivka J Jr, Pesta M, Janku F. Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? Expert Rev Mol Diagn. 2015; 15(12):1631-44. **(IF = 3.333)**

EXPERT REVIEWS

Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet?

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The optimal choice of cancer therapy depends upon analysis of the tumor genome for druggable molecular alterations. The spatial and temporal intratumor heterogeneity of cancers creates substantial challenges, as molecular profile depends on time and site of tumor tissue collection. To capture the entire molecular profile, multiple biopsies from primary and metastatic sites at different time points would be required, which is not feasible for ethical or economic reasons.

Molecular analysis of circulating cell-free DNA offers a novel, minimally invasive method that can be performed at multiple time-points and plausibly better represents the prevailing molecular profile of the cancer. Molecular analysis of this cell-free DNA offers multiple clinically useful applications, such as identification of molecular targets for cancer therapy, monitoring of tumor molecular profile in real time, detection of emerging molecular aberrations associated with resistance to particular therapy, determination of cancer prognosis and diagnosis of cancer recurrence or progression.

KEYWORDS: Liquid biopsy • cell-free DNA • advanced cancer • targeted therapy • personalized medicine

Despite significant progress in modern oncology, efficacy of treatment for advanced cancer remains poor; the majority of advanced tumors become resistant to available therapies and the patient ultimately succumbs to advancing metastatic disease.[1] This is mainly due to clonal evolution of the disseminated tumor and acquired resistance to cancer therapies, even if fitted to the known molecular profile.[2,3] In the current era of personalized medicine, the optimal choice of therapy depends upon detailed analysis of the cancer genome and identification of the targetable aberrations for each individual patient.[4] This approach is substantially limited by the considerable spatial and temporal intratumor heterogeneity of advanced disease. The cancer-related aberrations in the original tumor can differ among tumor regions and distinct disease sites.[5]

Molecular testing of tumor samples obtained by surgical procedures or biopsies remains the standard of care.[6] However, this approach has significant limitations because of the temporal and spatial tumor heterogeneity, which would mandate multiple biopsies from primary and metastatic sites at multiple time points. This is not feasible because of the medical condition of patients with advanced cancer, the risk of complications, and various economic and logistic considerations. To overcome these limitations, novel minimally invasive methods to detect pertinent molecular changes in tumors are being developed. Mandel and Métais in 1948 noticed the presence of cell-free nucleic acids (cfNA) in human blood. [7] However, it took several decades before reports emerged on oncogenic mutations in blood-derived cell-free DNA (cfDNA) of patients with cancer [8] or fetal cfDNA in

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pregnant women.[9] cfDNA also was investigated in prediction of outcome after brain trauma,[10] myocardial infarction [11] and stroke.[12,13]

Fragments of cfNA such as DNA, messenger RNA or microRNA can be detected in plasma, urine, cerebrospinal fluid (CSF) and other body fluids. In cancer patients, these cfDNA fragments can be used for detection of underlying cancer-related molecular abnormalities.[8,14] Such approaches, which have become known as liquid biopsies, can be used to monitor a cancer molecular profile in real time with minimal invasiveness. It is assumed that fragments of cfDNA are released to blood from diverse tumor sites and perhaps better represent prevailing molecular abnormalities than single-site biopsies. In addition, molecular testing of cfDNA can be used to evaluate response to therapy, disease progression or recurrence and emergence of molecular abnormalities that drive resistance to systemic therapy.

The biology of cfDNA

Fragments of cfDNA can be detected in extracellular fluids, such as blood (plasma or serum), urine, CSF or even ascites, of patients with cancer,[15-21] and increased levels of cfDNA can be associated with unfavorable outcome.[22,23] It has been demonstrated that patients with advanced cancer have higher levels of cfDNA than patients with localized cancer or individuals without cancer.[24-34]

DNA can enter the circulation by several distinct mechanisms, including release of nuclear and mitochondrial DNA from dying cells during either apoptosis or necrosis (Figure 1). Other mechanisms of DNA release include autophagy and necroptosis.[35,36] cfDNA structural characteristics differ substantially by type of release mechanism. Apoptosis is a programmed and well-controlled process of cellular destruction, and fragments of DNA released from apoptotic cells

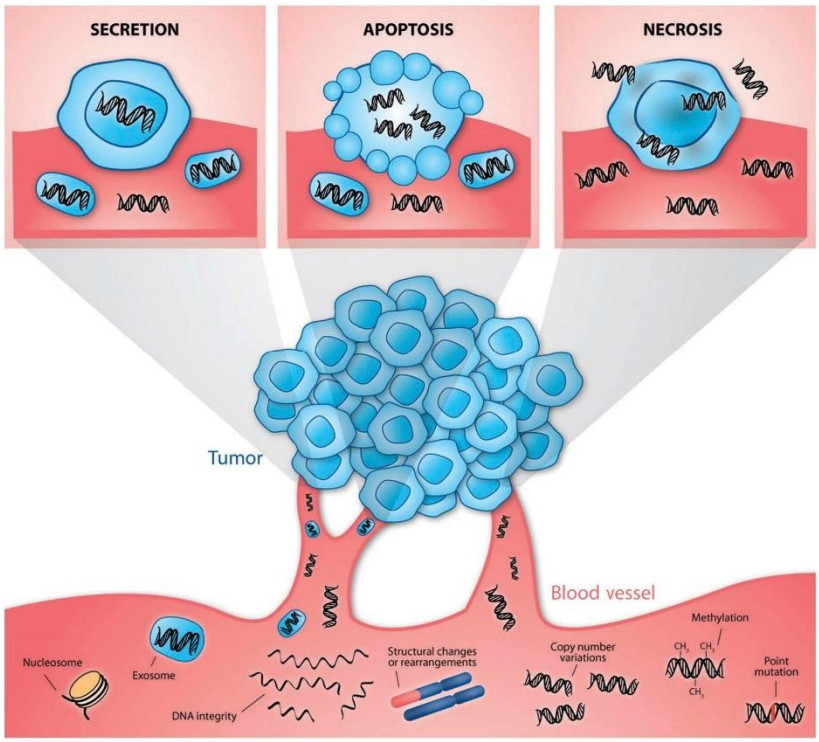

Figure 1. Passive (from apoptotic and necrotic cells) and active release of DNA fragments from tumor cells into the circulation. This cell-free DNA can be used for testing of tumor-specific aberrations.

average around 160–180 bp in length.[37,38] In contrast, necrosis is a pathological process, and the fragments of DNA are generated more randomly and usually are longer. The average lengths of cfDNA fragments from apoptotic and necrotic processes and their ratio may be assessed as an important element of the DNA integrity index, which may have prognostic implications.[39] Thierry *et al.* described experimental system for studying the cfDNA characteristic based on the nude mice xenografted with human HT29 or SW620 colorectal cancer cells.[40] The discrimination of cfDNA fractions from normal (murine) cells and from *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) *V600E*-mutated and nonmutated tumor (human) cells was possible and the concentration of tumor (human mutated and nonmutated) but not mouse cfDNA increased significantly with tumor burden ($P > 0.001$ and $P < 0.05$, respectively). The higher cfDNA fragmentation was also observed in mice with bigger tumors as the integrity index decreased with tumor size. The study confirmed the predominance of mononucleosome-derived cfDNA fragments in plasma from xenografted animals and of apoptosis as a source of tumor cfDNA.[40]

It has been proposed that plasma cfDNA can be also involve in the oncogenesis via the uptake of nucleic acids originating from tumor cells by susceptible healthy cells that consequently underwent malignant transformation, the process referred to as "genometastasis".[41,42] In the *in vitro* study with cultures of NIH-3T3 cells treated with plasma from colorectal cancer patients, the transfer of human DNA were observed and the NIH-3T3 cells were oncogenically transformed, as shown by the development of carcinomas in nonobese diabetic–severe combined immunodeficient mice after the injection of such cells.[43]

The cfDNA fragments are cleared from the circulation by the liver and kidney, with half-lives ranging from 15 min to a few hours.[15,44]

Technologies for cfDNA analysis

Sample collection and processing can have significant impacts on cfDNA assessment.[45] Most often the circulating DNA is extracted from plasma; plasma is preferred to serum because of serum has higher levels of noncancerous cfDNA due to lysis of normal leukocytes. Timely processing is paramount for success.[46] Cell-stabilizing streck tubes, which allow sample processing to be delayed for several days, have become increasingly popular for collection of blood samples intended for cfDNA analysis.[47,48] Other materials, such urine or CSF, are less cellular and should be less prone to DNA degradation.[17,19] At the moment, specimen collection protocols vary considerably in details such as use of ethylenediaminetetraacetic acid or even streck tubes.

The optimal pre-analytical handling conditions for the collected blood sample were described by Messaoudi *et al.*.[45] Blood samples must be drawn carefully and agitation should be avoided to prevent any hemolysis. The sample may be kept at room temperature or +4°C and must be processed within 4 h to prevent changes in cfDNA concentration and fragmentation. The two-step centrifugation (1200–1600 g for 10 min and 16,000 g for 10 min) is highly recommended to eliminate any cells from the plasma. The second step can be done after the storage of plasma sample at –20or –80°C. Plasma as well as cfDNA extracts are sensitive to freeze-thaw cycles. Plasma must be stored at –80°C up to maximum of 9 months before the final cfDNA analysis. The extracts of cfDNA must be stored at –20°C for up to 3 months for the concentration and fragmentation analysis or up to 9 months for specific mutations analysis.[45]

The techniques for the quantification of total amount of cfDNA include fluorescence-based methods (such as Hoechst dye and PicoGreen staining), spectrophotometric-based methods (ultraviolet spectrometry) or quantitative real-time polymerase chain reaction (PCR) methods (such as SYBR Green and TaqMan).[35,49,50] The study with plasma samples collected from 10 non-small-cell lung cancer patients compared PicoGreen staining to real-time PCR methods for the quantification of cfDNA.[51] The results from PicoGreen method correlated with both the SYBR Green ($R = 0.87$, $P < 0.0001$) and TaqMan probe approach ($R = 0.94$, $P < 0.0001$). The results from another method for the cfDNA quantification using the fluorescent SYBR Gold staining without prior DNA extraction and amplification showed the high correlation with the conventional quantitative PCR assay of beta-globin ($R = 0.9987$, $P < 0.001$).[52] Therefore fluorescence-based methods could be the rapid, accurate and inexpensive alternatives to real-time PCR for total cfDNA quantification.

The tumor-specific fraction of the total cfDNA can be identified by the presence of cancer-specific alterations. Epigenetic modifications such as methylation patterns also are being investigated as signature markers to differentiate tumor-specific cfDNA fraction.[53] The tumor-specific fraction can vary in plasma from 0.01% to more than 90%.[36,54] Lower-stage tumors have lower levels of cfDNA than advanced disease.[28] Therefore, highly sensitive methods are required for detection of cfDNA in early disease.

Various PCR approaches were used originally to detect tumor-related aberrations in cfDNA. These included methods such as ARMS (amplification refractory mutation system)-Scorpion PCR, PCR-SSCP (single-strand conformation polymorphism), ME (mutant-enriched)-PCR, MASA (mutant allele-specific amplification)-PCR, PAP-A amplification (pyrophosphorolysis-activated polymerization allele-specific amplification) or RFLP (restriction fragment length polymorphism)-PCR.[55-60] Even higher sensitivity is required, however, for detection of ctDNA from tumors in which specific mutations occur in very low allele fractions. For this reason, novel methods using digital PCR were introduced into cfDNA assays. Digital PCR methods include droplet-based systems,[61] the use of beads, emulsions, amplification and magnetics (BEAMing),[62] or microfluidic assays.[63,64]

Next-generation sequencing (NGS) techniques, which allow detection of multiple alterations across wider regions of the

Table 1. Comparison of PCR-based and next generation sequencing-based approaches for cfDNA analysis and their possible applications.

Methods	PCR-based approaches	Next-generation sequencing
Important features	Sensitive	Less sensitive in most cases
	Short turnaround	Longer turnaround times
	Tested and validated	Tested and validated
	Available in the CLIA-certified environment	Available in the CLIA-certified environment
	Limitations in terms of multiplexing	Need for bioinformatics, higher cost
Possible applications	Molecular diagnosis in well-defined situation when limited mutation panel is adequate	Molecular diagnosis in clinical setting
	Monitoring of mutation load as a surrogate for response to therapy	Discovery research
	Diagnosis of emergent resistant clones in malignancies with well-defined mechanisms of resistance	Malignancies with undefined patterns of resistance to targeted therapy

cfDNA: Cell-free DNA; CLIA: Clinical laboratory improvement amendments; PCR: Polymerase chain reaction.

cancer genome, also can be used for testing of cfDNA. The specific regions of cfDNA are analyzed by using targeted deep-sequencing techniques such as TAm-Seq (tagged amplicon deep sequencing),[63] Ion AmpliSeq,[65] Safe-Seq (safe-sequencing system) [66] or CAPP-seq (cancer personalized profiling by deep sequencing).[67] The latest and most comprehensive approaches to cfDNA analyses that do not require knowledge of preexisting mutations include whole-exome [68] as well as whole-genome sequencing of plasma samples.[69,70] The NGS techniques and unbiased whole-exome and whole-genome assays might dominate the future of cfDNA research. The advantages of PCR-based and NGS-based approaches are summarized in Table 1.

Clinical application of cfDNA in cancer management

Identification of molecular targets

The feasibility of identifying molecular targets in cfDNA as well as the level of concordance between mutations detected in tumor tissue and plasma samples are important attributes for future routine clinical use of cfDNA liquid biopsy techniques (Table 2). In a pilot study of 18 patients with metastatic colorectal cancer who were candidates for surgical resection or radiofrequency ablation, oncogenic mutations (*APC* (adenomatous polyposis coli gene), *TP53* (tumor protein p53), *PIK3CA* (catalytic domain p110α of the class I phosphatidylinositol 3-kinase) and *KRAS* (kirsten rat sarcoma viral oncogene homolog)) were assessed by direct sequencing in tumor tissue.[15] At least one mutation was identified in each of the tumors. The unique molecular signature of each tumor was used for detection and quantification of tumor-derived cfDNA by the BEAMing PCR-based technology. This study demonstrated that cfDNA can be isolated from plasma samples and used to identify oncogenic mutations in cancer patients.

In a cohort of 49 patients with advanced breast cancer, there was 100% concordance (34 of 34 cases) between BEAMing-detected *PIK3CA* mutations in plasma cfDNA and in tumor tissues obtained at the same time.[62] However, the concordance decreased to 79% in an additional cohort of 60 patients when tumor samples and plasma cfDNA were obtained at different time points.

In a study of 157 patients with advanced cancer that progressed on systemic therapy who were referred for treatment with experimental targeted therapies, a panel of 21 oncogenic mutations in the *BRAF, EGFR* (epidermal growth factor receptor), *KRAS* and *PIK3CA* genes was assessed in plasma cfDNA by BEAMing technology. The results demonstrated acceptable concordance (*BRAF*, 91%; *EGFR*, 99%; *KRAS*, 83%; *PIK3CA*, 91%) with results of standard-of-care mutation analysis of primary or metastatic tumor tissue obtained during clinical care.[77]

Thierry *et al.* [71] assessed the mutation status of *KRAS* and *BRAF* by using allele-specific quantitative PCR of cfDNA in 106 plasma samples from patients with metastatic colorectal cancer and compared it to the mutations detected in tissue (primary or metastatic) tested by standard-of-care methods. The cfDNA analysis showed 100% specificity and sensitivity for the *BRAF V600E* mutation and 98% specificity and 92% sensitivity for the *KRAS* mutations, with a concordance value of 96%.

The *BRAF V600E* mutation was recently detected in plasma and urine cfDNA samples obtained from individuals with Erdheim–Chester disease and Langerhans cell histiocytosis.[18] These patients have a high prevalence of *BRAF V600E* mutations and a good response to BRAF inhibitors. There was 100% concordance between tissue and urinary cfDNA genotypes assessed by droplet-digital PCR assay (ddPCR) in samples from 30 treatment-naive patients. The targetable mutation *BRAF V600E* was also analyzed in plasma- and serum-derived cfDNA samples from 221 patients with advanced melanoma. [73] Assay sensitivity for mutation detection was 44% in serum and 52% in plasma. Test specificity was 96% in both matrices.

Panka *et al.* [74] developed blood-based assay for the detection of *BRAF V600E* mutation and used it in the study with 128 patients with stage II–IV melanoma. The high 96% sensitivity and 95% specificity of the assay were observed for the subset of 42 stage IV patients. The area under the receiver operator curve (ROC) was 0.9929 demonstrating an excellent ability to discriminate *BRAF*-mutant melanoma patients. Pupilli *et al.* [78] investigated the role of *BRAF V600E*-mutated allele in plasma cfDNA from 103 patients with papillary thyroid

Table 2. The summarization of the studies that examined the concordance between mutations detected in tumor tissue samples and cfDNA.

Cancer type	Mutated genes	Cohort no.	Results of the concordance between cfDNA and tumor tissue	Reference
Colorectal cancer	*KRAS, BRAF*	106	100% specificity and sensitivity for the *BRAFV600E* mutation and 98% specificity and 92% sensitivity for the *KRAS* mutations detection	Thierry *et. al.* [71]
Breast cancer	*PIK3CA*	49 + 60	100% concordance if tumor and cfDNA samples collected at the same, 79% in another cohort if collected at different time points	Higgins *et al.* [62]
	PIK3CA	29	93.3% sensitivity and 100% specificity for the mutations detection in presurgery plasma samples	Beaver *et al.* [61]
	50 selected genes (NGS)	17	76% concordance between the analysis of primary or metastatic tumors and matched plasma samples	Rothe *et al.* [72]
Melanoma	*BRAF*	221	Sensitivity for *BRAFV600E* mutation detection in serum was 44%, in plasma 52%. Specificity was 96% in both matrices	Aung *et al.* [73]
	BRAF	128	96% sensitivity and 95% specificity for *BRAFV600E* mutation detection in the subset of 42 stage IV patients	Panka *et al.* [74]
Pancreatobiliary carcinomas	54 selected genes (NGS)	26	90.3% of mutations detected in tumor biopsies were also detected in cfDNA. 92.3% sensitivity and 100% specificity across the five most frequently mutated genes	Zill *et al.* [75]
ECD/LCH	*BRAF*	30	100% concordance of *BRAFV600E* mutation between tissue and urinary cfDNA	Hyman *et al.* [18]
Advanced cancers	*BRAF, EGFR, KRAS, PIK3CA*	157	Acceptable concordance (*BRAF*, 91%; *EGFR*, 99%; *KRAS*, 83%; *PIK3CA*, 91%) with standard-of-care mutation analysis of primary or metastatic tumor tissue	Janku *et al.* [76]

cfDNA: Cell-free DNA; ECD/LCH: Erdheim–Chester disease and Langerhans cell histiocytosis; NGS: Next generation sequencing techniques.

carcinoma (PTC) as a marker for the diagnosis and follow-up. Patients with PTC showed a higher percentage of circulating *BRAF V600E* mutation (P = 0.035) compared to those with benign histology (n = 16) and healthy controls (n = 49). The assay diagnostic sensitivity and specificity were 80 and 65%, respectively.

Zill *et al.* [75] assessed the mutation status of 54 genes by NGS in the tumor tissue and corresponding cfDNA in plasma samples from 26 patients with pancreatobiliary carcinomas (18 pancreatic ductal adenocarcinoma cases and 8 biliary cancer cases). 90.3% of mutations detected in tumor biopsies were also detected in cfDNA. Across the five most frequently mutated genes in tumor tissue biopsies (*KRAS, TP53, APC, FBXW7* and *SMAD4*), the assay sensitivity for the detection of such mutations in cfDNA was 92.3%, specificity was 100% and the diagnostic accuracy was 97.7%.

Forshew *et al.* [79] reported on using the TAm-Seq method for identification and monitoring of oncogenic mutations in plasma cfDNA. They screened 5995 genomic bases in coding regions of *TP53* and *PTEN* (phosphatase and tensin homolog) and selected regions in *EGFR, BRAF, KRAS* and *PIK3CA* for low-frequency mutations. The assay was able to detect mutations in cfDNA with sensitivity and specificity of >97%. In one patient with synchronous primary cancers of the bowel and ovary, moreover, disease relapse was identified as being derived from the original ovarian tumor. At relapse, analysis of the plasma cfDNA detected the *TP53* mutation (p.R273 H) originally found in the ovarian primary tumor, whereas the bowel-associated mutations were not detected.

Beaver *et al.* [61] showed the possibility of identifying *PIK3CA* mutations in plasma samples from 29 patients with early-stage breast cancer. The same mutations identified in primary tumors were detected in presurgery plasma samples by ddPCR with high sensitivity and specificity (93.3 and 100%, respectively). Residual disease was successfully identified by detection of the mutations in cfDNA from postoperative plasma samples. In another study of 17 patients with metastatic breast cancer, analysis of primary or metastatic tumors together with matched plasma samples for mutations in 50 selected genes by NGS yielded a concordance of 76%.[72]

Bettegowda *et al.* [66] evaluated the possibility to detect the cfDNA point mutations and genetic rearrangements that were originally found in tumor tissue biopsies from 640 patients with various cancer types. Tumor-derived cfDNA was detected in >75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular and head and neck cancers. In patients with localized tumors, cfDNA was detected in 73, 57, 48 and 50% of patients with colorectal cancer, gastroesophageal cancer, pancreatic cancer and breast adenocarcinoma, respectively.[66]

Newman *et al.* [67] developed CAPP-Seq, an ultrasensitive method for quantifying tumor-derived plasma cfDNA by targeting recurrently mutated regions in the cancer of interest. In patients with non-small-cell lung cancer, the CAPP-Seq method

was able to detect cfDNA in 100% of patients with stage II–IV disease and 50% of patients with stage I disease. The method specificity was 96% for mutant allele fractions as low as 0.02%.

Assessment of prognosis

The quantification of total and/or mutant cfDNA has been studied for prognosis assessment in various tumor types. Some studies demonstrated that, in cancer patients, higher levels of cfDNA are associated with higher risk of disease recurrence and progression.[15,28,30,66,80–82] In a study by Diehl *et al.* [15] of 18 colorectal cancer patients, the absence of cfDNA in plasma during the first follow-up visit after surgical resection was associated with 100% recurrence-free survival.

Early limited data suggested that persistence of *TP53* mutation in plasma cfDNA of patients with stage II or III breast cancer that was in remission was associated with higher likelihood of disease recurrence; however, the small sample size precluded any definitive conclusion.[56]

The amount of mutant cfDNA has been found to be of prognostic significance. Spindler *et al.* [80] demonstrated the prognostic value of the amount of total cfDNA and *KRAS* mutant cfDNA in a study of 108 patients with metastatic colorectal cancer treated with third-line cetuximab and irinotecan. Patients with higher cfDNA levels had shorter progression-free survival (PFS; 2.1 vs. 4.4 months; P = 0.0015) and overall survival (OS; 3.6 vs. 10.4 months; P < 0.0001) than patients with lower cfDNA levels. Similarly, patients with higher levels of *KRAS*-mutant cfDNA had shorter PFS (1.8 vs. 2.3 months; P = 0.008) and OS (2.1 vs. 5 months; P = 0.0005) than patients with lower levels of *KRAS*-mutant cfDNA.

The mutated fraction of plasma cfDNA (mutation in codon 12 or 13 of *KRAS)* was assessed in another study of 206 patients with metastatic colorectal cancer.[66] Concentration of mutated cfDNA was found to provide added value in survival prediction (likelihood ratio test, P = 0.00253, df = 3) to the model of well-known prognostic factors (age, Eastern Cooperative Oncology Group performance status, and level of carcinoembryonic antigen). Also, holding other predictors constant, the 2-year survival rate steadily decreased as the plasma concentration of mutated cfDNA increased.

The study already mentioned [77] of the panel of 21 mutations in *BRAF, EGFR, KRAS* and *PIK3CA* assessed by BEAMing technology in plasma cfDNA of 157 patients with advanced cancer also examined the prognostic impact of the amount of mutated plasma cfDNA. A higher percentage of mutant cfDNA (>1% [n = 67 patients] vs. ≤1% [n = 33 patients]), irrespective of type of mutation, was associated with a shorter OS (5.5 vs. 9.8 months; P = 0.001), which was confirmed in a multivariable analysis. Similarly, 41 patients with >1% of *KRAS* mutant (codon 12 or 13) cfDNA had a shorter median OS than 20 patients with ≤1% of *KRAS* mutant cfDNA (4.8 vs. 7.3 months; P = 0.008). The significant differences in OS were not observed for mutations in other examined genes, probably because of the smaller sample size.

In another study of 246 patients with advanced non-small-cell lung carcinoma treated with platinum and vinorelbine chemotherapy, the patients with detectable plasma *KRAS* mutant (codon 12 or 13) cfDNA had a shorter median OS (4.8 vs. 9.5 months; P = 0.0002) and shorter median PFS (3.0 vs. 5.6 months; P = 0.0043) than patients whose cancer expressed wild-type *KRAS*.[81] A multivariate analysis confirmed the independent prognostic value of *KRAS* mutant cfDNA in OS but not in PFS. Wang *et al.* [83] showed the negative prognostic effect of *KRAS* mutation (codon 12 or 13) in plasma cfDNA of 273 patients with advanced non–small-cell lung cancer. The median PFS of patients with a plasma *KRAS* mutation was 2.5 months, while that of patients with wild-type *KRAS* was 8.8 months (P < 0.001).

In a study of 44 pancreatic cancer patients, the 1-year survival rate was 0% in those with *KRAS* codon-12 mutation in cfDNA and 24% in those with *KRAS* wild-type in cfDNA (P < 0.005), and plasma *KRAS* mutation was the only independent prognostic factor (odds ratio, 1.51; 95% confidence interval [CI], 1.02 to 2.23).[60] In 103 patients with melanoma receiving biochemotherapy,[84] those with a *BRAF* mutation in serum cfDNA had significantly shorter OS than those that did not have the *BRAF* mutation in serum cfDNA (13 vs. 30.6 months, P = 0.039).

The negative prognostic impact of increased levels of mutant cfDNA was supported by other studies in breast cancer,[85] colorectal cancer,[86,87] ovarian cancer [88] and other tumor types. Furthermore, the presence of other tumor-related genomic cfDNA aberrations was associated with poor prognosis. Detection of loss of heterozygosity and microsatellite instability in cfDNA was associated with worse prognosis for patients with breast cancer,[89] ovarian cancer,[90] melanoma,[91] lung cancer [92] or other tumor types.

Epigenetic alterations detected in cfDNA can also help determine patient prognosis. The aberrant DNA methylations were detected in cfDNA of patients with breast, lung as well as liver cancer.[93–95] Hypermethylated promoter regions of *BRCA1* in serum cfDNA from 100 primary invasive ductal breast cancer patients was associated with poor DFS (14.2 months; P ≤ 0.0001) as well as poor OS (24.3 months; P = 0.0001).[96] Similarly cfDNA promoter hypermethylation of *GSTP1* was associated with poor DFS (24.2 months; P = 0.03). Another study with 336 primary invasive breast cancer patients showed worse OS rate at 100 months (78 vs. 95%; P = 0.002) for patients with serum cfDNA hypermethylation in promoter regions of *GSTP1, RASSF1A* and *RARβ2* than those with negative findings.[97] In the study with 428 primary breast cancer patients, the detection of methylated *PITX2* and *RASSF1A* in plasma cfDNA determined shorter OS in multivariate analysis (low vs. high methylation; HR 3.4, P = 0.021 and HR 3.4, P = 0.002 respectively).[98] For distant DFS only *RASSF1A* showed prognostic significance (low vs. high methylation; HR 3.4, P = 0.002). The aberrant methylation of selected genes in cfDNA was associated with poor prognosis also in colorectal cancer,[99] gastric cancer,[100] hepatocellular carcinoma [101] and other tumor types.

Prediction of response to therapy

The liquid biopsy could provide an easy way to assess predictive biomarkers for targeted therapy as well as a minimally invasive way to monitor therapy response in real time [8,14] (Table 3).

In a prospective study of 52 patients with metastatic breast cancer, the plasma cfDNA was monitored to qualitatively and quantitatively assess disease progression and treatment response and compare with levels of circulating tumor cells (CTC) and tumor marker cancer antigen 15-3 (CA15-3) and computed tomography (CT) imaging.[63] The cfDNA was detected by identification of the same *PIK3CA* and *TP53* mutations and structural variations as were found in the tumor tissues. The levels of cfDNA in plasma generally correlated well with the treatment response assessed by CT imaging (as defined by Response Evaluation Criteria in Solid Tumors). However, two patients in this study had discordant correlations. In 10 of the 19 patients who experienced disease progression, the cfDNA levels increased at one or more consecutive time points, on average 5 months before progressive disease was observed on imaging. Moreover, the cfDNA was found to be a more accurate biomarker for monitoring metastatic disease than CTCs, CA 15-3 (cancer antigen 15-3) or CT imaging.

Another study in 72 patients with advanced non-small-cell lung cancer examined the dynamic changes in cfDNA *EGFR* mutations as a predictor of response to EGFR tyrosine-kinase inhibitor (EGFR-TKI) targeted therapy.[103] Failure to clear plasma *EGFR* mutations after EGFR-TKI was an independent predictor for shorter PFS (hazard ratio [HR] 1.97, P = 0.001) and OS (HR 1.82, P = 0.036). The *EGFR* mutations were detected by ddPCR in serial plasma samples of non-small-cell lung cancer patients treated with erlotinib. [104] The study demonstrated the disappearance of *EGFR* mutations in exon 19 and 21 and the emergence of *EGFR T790M* resistance mutation several weeks before radiographic disease progression.

Similarly, *EGFR* mutations were detected in primary tumors and corresponding plasma samples in a study of 1060 patients with advanced lung cancer treated with gefitinib.[102] Objective response rates were 76.9% (95% CI, 65.4–85.5) for patients with detected mutations in both tumor and plasma and 59.5% (95% CI, 43.5–73.7) for patients with mutation in the tumor but not in plasma. Median PFS was 9.7 months (95% CI, 8.5–11.0) for patients with mutation in the tumor sample only and 10.2 months (95% CI, 8.5–12.5) for patients with mutation in both tumor and plasma samples. This demonstrated that *EGFR* mutation status could be assessed in cfDNA and serve as a positive predictive biomarker for targeted therapy.

Another study [76] assessed *BRAF* mutations in plasma cfDNA from 160 patients with advanced cancer and known *BRAF* status from archival tumor samples. Patients whose formalin-fixed paraffin-embedded (FFPE) tumor had a *BRAF V600* mutation (n = 51) received therapy with a BRAF and/or MEK (mitogen-activated protein kinase) inhibitor. The time to treatment failure (TTF) of 13 patients with a *BRAF V600* mutation in the tumor but not in plasma obtained before therapy was significantly longer than that of 38 patients whose baseline plasma cfDNA had a *BRAF V600* mutation (13.1 vs. 3.0 months; P = 0.001). The absence of *BRAF V600*–mutant cfDNA also was associated with longer TTF (HR, 0.31; P = 0.004) in multivariate analysis.

Detection of resistance to targeted therapy

The implementation of personalized medicine principles and targeted therapy into routine oncology practice is bringing an important shift in the treatment of advanced cancers. In metastatic disease, a chronic course is no longer unusual, and patients can survive for many years.[105] However, despite the significant initial therapeutic effect of targeted therapy, the vast majority of patients eventually develop resistance and experience tumor progression. The tumor resistance results from

Table 3. Examples of studies that assessed cfDNA for response prediction and/or therapeutic monitoring.

Cancer type	Mutated genes	Cohort no.	Therapeutic intervention	Results	Reference
Lung cancer	EGFR	1060	gefitinib	ORR were 76.9% (95% CI, 65.4–85.5) for EGF-mutant tumors and mutated cfDNA, and 59.5% (95% CI, 43.5–73.7) for *EGFR*-mutant tumors and wt cfDNA	Douillard *et al.* [102]
	EGFR	72	EGFR-TKIs	Failure to clear *EGFR*-mutant cfDNA after EGFR-TKI was an independent predictor of shorter PFS (HR 1.97, P = 0.001) and OS (HR 1.82, P = 0.036)	Tseng *et al.* [103]
Breast cancer	PIK3CA, TP53, structural variations	52	Various systemic therapeutics	The levels of cfDNA in plasma generally correlated well with the treatment response assessed by CT imaging	Dawson *et al.* [63]
Melanoma	BRAF	160	BRAF and/or MEK inhibitors (n = 51)	TTF of 13 patients whose baseline cfDNA samples (but not tissue samples) did not have *BRAFV600* mutation was significantly longer than that of 38 patients with baseline cfDNA-mutated samples (13.1 vs. 3.0 months; P = 0.001)	Janku *et al.* [76]

cfDNA: Cell-free DNA; CT: Computed tomography; HR: Hazard ratio; ORR: Overall response rate; OS: Overall survival; PFS: Progression-free survival; TKIs: Tyrosine kinase inhibitors; TTF: Time to treatment failure; wt: Wild type

acquisition of mutations in the targeted genes or signaling pathways of cancer cells under therapeutic selective pressure (i.e., secondary resistance). The mutations causing resistance also can be present in the infrequent subclones of pretreatment tumor cells and can predict the further failure of targeted therapy (i.e., primary resistance).[5,14,106]

The mechanisms of resistance are often known; however, since routine multiple sequential biopsies are not performed, we have no tools to describe these mechanisms at the level of an individual patient. Both intrinsic and adaptive resistance can occur because of pre-existing or acquired molecular abnormalities, such as gatekeeper mutations in the *BCR-ABL* (breakpoint cluster region-abelson murine leukemia viral oncogene homolog) kinase domain, which cause resistance to imatinib and other TKIs in chronic myelogenous leukemia.[107] Similarly, emergence of *KRAS* mutations plausibly causes resistance to EGFR monoclonal antibodies in metastatic colorectal cancer, [108] and emergence of *EGFR T790M* mutation causes resistance to EGFR-TKIs in non-small-cell lung cancer.[109,110] Last but not least, *ALK* (anaplastic lymphoma kinase) mutation *L1196M* or *C1156Y* mediates adaptive resistance to crizotinib in non-small-cell lung cancer with *ALK* rearrangement,[111] and mutations in *NRAS* (neuroblastoma RAS viral oncogene homolog), *MEK* and *BRAF* amplification indicate resistance to *BRAF* inhibitor vemurafenib in *BRAF*-mutant melanoma.[112] Because liquid biopsies can be obtained at low cost at multiple time points, they offer a useful tool for monitoring molecular changes associated with resistance to certain cancer therapies (Table 4).

For instance, PCR detection with the BEAMing approach in patients with advanced non-small-cell lung cancer demonstrated *EGFR T790M* mutation in cfDNA from 10 of 23 patients who experienced disease progression while receiving an EGFR-TKI. [113] In a different study, digital PCR detection of the *EGFR T790M* resistance mutation in pretreatment cfDNA plasma samples from 135 patients with advanced non-small-cell lung cancer treated with an EGFR-TKI was associated with a shorter median PFS (8.9 vs. 12.1 months; P = 0.007) and OS (19.3 vs. 31.9 months; P = 0.001) than no *T790M* mutation.[114]

Another example of emerging resistance mutations to targeted therapy with high clinical relevance is the acquisition of tumor *KRAS* mutations in codon 12, 13 or 61 in patients with advanced colorectal cancer treated with anti-EGFR monoclonal antibodies cetuximab or panitumumab.[16,20] Two landmark studies have shown the possibility of detecting and monitoring these emerging *KRAS* mutations in such patients in cfDNA by using BEAMing technology.[16,20] Testing of serum cfDNA from 28 colorectal cancer patients receiving panitumumab showed that 9 of 24 patients whose tumor and cfDNA were initially *KRAS* wild-type had developed detectable cfDNA *KRAS* mutations.[16] Interestingly, multiple *KRAS* cfDNA mutations were detected in three individuals. The appearance of mutations generally occurred between 5 and 6 months following initiation of treatment. In the second study, emergence of *KRAS* aberrations was found in tumor tissue samples from metastatic sites obtained after initiation of therapy.[20] Corresponding plasma samples also showed emergence of *KRAS* mutation in cfDNA, which could have happened as early as 10 months before radiographic progression.[20] Furthermore, a group from MD Anderson Cancer Center, using BEAMing technology, reported acquired *KRAS* and/or *EGFR* ectodomain mutations in 44% (27/62) and 8% (5/62) of plasma samples from patients with advanced colorectal cancer treated with cetuximab or panitumumab, respectively.[115] *KRAS* codon 61 and 146 mutations were predominant (33 and 11%, respectively).

Even if the candidate-gene techniques to monitor emerging resistance mutations to various targeted therapeutics provide

Table 4. Examples of studies that assessed cfDNA as the biomarker to monitor resistance to the administrated therapy.

Cancer type	Resistance mutation	Cohort no.	Therapeutic intervention	Results	Reference
Lung cancer	*EGFR T790M*	23	EGFR-TKIs	*EGFR T790M* mutation was detected in 10 of 23 patients with progression on EGFR-TKI	Taniguchi *et al.* [113]
	Pretreatment *EGFR T790M*	135	erlotinib, gefitinib	Pretreatment detection of mutation in plasma cfDNA-predicted lower PFS (8.9 vs. 12.1 months, P = 0.007) and OS (19.3 vs. 31.9 months, P = 0.001) compared to patients without mutation	Wang *et al.* [114]
Colorectal cancer	*KRAS* mutations	28	panitumumab	Serum cfDNA *KRAS* mutations were detected in 9 of 24 originally *KRAS* wild-type patients	Diaz *et al.* [16]
	KRAS and/or *EGFR* ectodomain mutations	62	cetuximab, panitumumab	Acquired KRAS and/or EGFR ectodomain mutations were detected in 44% (27/62) and 8% (5/62) plasma samples after treatment	Morelli *et al.* [115]
Breast, ovarian, and lung cancers	Plasma whole-exome sequencing	6	Various therapeutics	Activating mutations in *PIK3CA* after paclitaxel, *RB1* after cisplatin, *MED1* after tamoxifen and trastuzumab, and *GAS6* after lapatinib; *T790M EGFR* mutation after gefitinib	Murtaza *et al.* [68]

OS: Overall survival; PFS: Progression-free survival; TKIs: Tyrosine kinase inhibitors

promising results, such approaches have substantial drawbacks, most notably the requirement for prior knowledge of mechanisms of resistance and corresponding mutations. Application of unbiased approaches for detection of emergence of resistant cancer cell subclones using NGS technologies directly on the plasma samples could overcome these limitations. A proof-of-principle study by Murtaza *et al.* [68] monitored cancer clonal evolution and the acquisition of secondary resistance mutations to various anticancer treatments in serial plasma samples from six patients with advanced breast, ovarian or lung cancer using unbiased whole-exome sequencing. Follow-up intervals were 1–2 years, and the exome sequencing was performed on two to five plasma samples in each patient. The results revealed emergence of distinct secondary mutations, such as an activating mutation in *PIK3CA* after paclitaxel, a truncating mutation in *RB1* (retinoblastoma gene) after cisplatin, a truncating mutation in *MED1* (mediator complex subunit 1) after tamoxifen and trastuzumab and a splicing mutation in *GAS6* (growth arrest-specific 6) after subsequent treatment with lapatinib in the same patient and a *T790M EGFR* mutation after treatment with gefitinib. The results of this study established that exome-wide analysis of cfDNA could complement standard biopsy to detect mutations associated with acquired resistance to therapeutic agents in advanced cancers. However, it should be noted that the detected mutant allele fractions for the aberrations were rather high (3–45%), which can limit the applicability of such an approach to a limited subset of patients.

Overall, liquid biopsy-guided detection of clonal evolution and acquired mechanisms of resistance can be an attractive tool in cancer therapy. However, its utility needs to be tested in future prospective clinical trials that use liquid biopsies as a tool for therapeutic decision making (Table 5).

Expert commentary

Liquid biopsy utilizing cfDNA is an attractive tool in oncology for identification of molecular targets, determination of prognosis, assessment of response to anticancer therapy and real-time monitoring of cancer molecular profile. However, the clinical utility of molecular profiling in cfDNA remains to be proven in prospective studies. Retrospective observations demonstrated that changes in the amount of mutant cfDNA can indicate response to anticancer therapy and that emergence of certain molecular abnormalities can predict emergent therapeutic resistance; however, it will remain unclear whether this offers any clinical advantage or alters therapeutic decisions until it is tested in prospective controlled clinical trials. Even though most cfDNA technologies have demonstrated high concordance with molecular testing of tumor tissue, there is still uncertainty whether molecular profile from cfDNA can replace tissue testing, at least in situations when the tissue is in short supply. Furthermore, cfDNA consists of both nonmalignant and tumor DNA, unlike tumor tissue, increasing the need for high sensitivity and limiting the use of technologies such as whole-genome or whole-exome NGS. Also, cfDNA occurs in short fragments, which can further complicate molecular analysis.

Table 5. Overview of active clinical trials with cfDNA as their primary or secondary objective.

Cancer type	Clinical trials with cfDNA as primary or secondary objective [ClinicalTrials.gov Identifier]			
	Assessment of prognosis	Prediction and monitoring of response to therapy	Detection of disease recurrence	Detection of resistance to therapy
Breast cancer	NCT00899548, NCT02306096	NCT01617915, NCT00899548, NCT02109913, NCT01160211, NCT02318901	NCT01617915, NCT02318901	NCT01884285
Lung cancer	NCT02245100	NCT02169349, NCT01930474, NCT02186236, NCT01884285, NCT02281214	NCT02169349	NCT02169349, NCT01930474, NCT01884285, NCT02281214, NCT00997334
Colorectal cancer	NCT01198743	NCT01983098, NCT01212510, NCT01943786	NCT01983098, NCT01943786, NCT01198743	
Melanoma		NCT02251314, NCT02171286, NCT02071940		NCT02251314, NCT02133222, NCT02071940
Pancreatic cancer	NCT02072616, NCT02331251	NCT02331251	NCT02331251	
Hepatocellular carcinoma	NCT02036216	NCT02036216	NCT02036216	
Gastrointestinal stromal tumor		NCT01462994	NCT02331914	NCT02331914
Lymphoma		NCT02339805	NCT02339805	
Prostate cancer		NCT01884285		NCT01884285
Thyroid cancer		NCT01723202		

Five-year view

In the next 5 years, cfDNA-based liquid biopsies will be implemented in clinical studies and drug development. Such studies will provide real-time evaluation of pertinent biomarkers as well as the technology itself. Liquid biopsy approaches have been selected for testing as exploratory endpoints in national molecular matching initiatives such as the multi-arm NCI MATCH clinical trial. Furthermore, randomized studies exploring whether liquid biopsy approaches can be used for biomarker detection and subsequent treatment allocation in lieu of tumor tissue are being designed. Liquid biopsies will likely become an integral part of diagnostics in oncology; however, they are not expected to entirely replace tumor biopsies since they cannot address many important factors such as changes in and interactions with the tumor microenvironment. Furthermore, novel liquid sources of DNA will be tested and validated, including CTC and exosomes.[116–120]

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Key issues

- Identification of oncogenic aberrations provided key insight into cancer biology and led to discovery of new targeted therapies.
- Tumor-specific aberrations are usually tested in archival tumor tissue, and limitations or absence of such tissues can preclude molecular analysis and limit the use of personalized therapy.
- Small fragments of cancer cell-free DNA are released into the circulation and can be detected in blood, urine or other biologic materials.
- Testing for oncogenic mutations in cell-free DNA (cfDNA), which is not all from the tumor, requires highly sensitive methods capable of detecting one mutant allele in 1000–10,000 wild-type background alleles.
- PCR-based technologies are highly sensitive but do not allow testing for a broad spectrum of aberrations in cfDNA. Next-generation sequencing can detect multiple aberrations, but with somewhat lower sensitivity than PCR.
- Detection of oncogenic aberrations in cfDNA demonstrated high though not absolute concordance with tumor tissue and can be used plausibly for treatment selection.
- Quantity of mutant cfDNA seems to be of prognostic value in predicting survival.
- Dynamic tracking of molecular aberrations in cfDNA has potential to be used for monitoring of treatment response in lieu of standard imaging.
- Emergence of molecular aberrations in cfDNA can provide insight into mechanism of resistance at the individual patient level and can be investigated as a plausible tool for treatment guidance.

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Attachment VI

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REVIEW Open Access

Current status of biomarker research in neurology

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Abstract

Neurology is one of the typical disciplines where personalized medicine has been recently becoming an important part of clinical practice. In this article, the brief overview and a number of examples of the use of biomarkers and personalized medicine in neurology are described. The various issues in neurology are described in relation to the personalized medicine and diagnostic, prognostic as well as predictive blood and cerebrospinal fluid biomarkers. Such neurological domains discussed in this work are neuro-oncology and primary brain tumors glioblastoma and oligodendroglioma, cerebrovascular diseases focusing on stroke, neurodegenerative disorders especially Alzheimer's and Parkinson's diseases and demyelinating diseases such as multiple sclerosis. Actual state of the art and future perspectives in diagnostics and personalized treatment in diverse domains of neurology are given.

Keywords: Biomarker, Personalized medicine, Neuro-oncology, Stroke, Alzheimer's disease, Parkinson's disease, Multiple sclerosis, Predictive preventive personalized medicine

Background

The term "personalized medicine" (PM) was first explained in detail in Kewal K. Jain's Textbook of Personalized Medicine, published in 1998. The first reference made to PM in the MEDLINE database dates back to 2000. It describes the predicted effect of albuterol in asthma sufferers based on their DNA makeup. This was the first example of personalized treatment based on human genome sequencing [1]. Personalized medicine is closely related to pharmacogenetics and pharmacogenomics, and the field primarily grew in the period after the complete human genome was mapped in 2000 [2].

It is difficult to offer a precise definition of personalized medicine. Sometimes other terms are used, such as therapy according to diagnosis, genomic medicine, genotype-based therapy, individualized or individual medicine, omics-based medicine, predictive medicine, rational drug selection, and tailored therapy.

In this review, we provide the overview and a number of examples where personalized medicine, or an individualized approach to therapy, has been recently applied in neurology domain. Our article conforms with the recommendations of the "EPMA White Paper" [3].

Biomarkers and personalized medicine in neuro-oncology

In recent times, there has been a significant expansion of knowledge in the field of neuro-oncology regarding the onset and development of neoplastic disease at the genomic and epigenomic levels. New prognostic and predictive biomarkers for the disease are appearing and the basic view of the histological typing of central nervous system (CNS) tumors is changing. In the near future, it will likely be necessary to integrate personalized medicine into standard clinical care for patients suffering from neurological cancer. The current World Health Organization (WHO) typing from 2007 recognizes more than 130 different histopathological units of primary CNS tumors [4]. This represents a very extensive and markedly heterogeneous group of diseases, with individual types of tumors exhibiting various biological behaviors. Moreover, even in the given histopathological units, further segmentation is starting to establish itself that is based on molecular genetics profiles resulting from international groups' current whole exome and whole genome sequencing studies, which focus on the

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genomics and epigenomics of neoplastic diseases. An ambitious project that may serve as an example is a tumor atlas of selected cancer diagnoses, The Cancer Genome Atlas (TCGA), sponsored by the National Institutes of Health (NIH) in the United States. In a sample of 500 previously untreated patients, the NIH was the first in the world to clarify changes in the most frequent and most malignant primary brain tumor, glioblastoma multiforme (GBM), at the DNA, mRNA and short non-coding microRNA's levels [5]. This led to the new division of what till then had been a homogenous group, GBM, into four subtypes according to dissimilar gene expression profiles with differing responses to conventional chemotherapy. In the future, this may contribute to the further personalization of tumor therapy for this type of disease. Despite the marked diversity of primary CNS tumors, the absolute majority are tumors of neuroepithelial tissue, specifically the astrocytoma group. It is further divided according to growing malignancy potential into four groups of gliomas, with GBM having the highest representation. Despite the limited options for choosing standard glioma therapy for now, new prognostic and predictive biomarkers have recently appeared that will soon allow for therapy to be "tailored" to each patient with the aim of achieving longer survival and better quality of life [6, 7].

The forecast of a more favorable prognosis as well as the prediction of a better response to the therapy administered are both important elements in the basic principles of personalized medicine. In this regard, several predictive CNS tumor biomarkers are important and it is expected that they will be included in clinical practice. The predictive biomarker in GBM patients probably closest to practice is the status of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation [8–10]. The MGMT enzyme is able to effectively repair the DNA damage caused by temozolomide, a standard chemotherapy administered to patients. MGMT-promoter methylation reduces the production of the active enzyme and the patient's response to temozolomide therapy is higher, as has also been reflected in the longer overall survival periods of GMB patients at 21.7 vs. 15.3 months [11]. The status of MGMT-promoter methylation may also serve as a predictive biomarker in relation to radiotherapy [12].

The isocitrate dehydrogenases (IDH) mutation is an important glioma biomarker near clinical application that is able to contribute to determining the patient's prognosis. IDH is an important Krebs cycle enzyme and has three different isoforms—IDH1 (found in the cytoplasm and peroxisomes) and IDH2 and 3 (in the mitochondria) [13]. Recurrent mutations in IDH were first systematically described in patients with GBM, though only in about 5 % of the patients [14]. In contrast, gene mutations for IDH1 and IDH2 were found with high frequency in diffuse astrocytomas (70–80 %) and anaplastic astrocytomas (up to 50 %) [15]. Mutations in IDH1 show conservative substitution of R132H in 90 %; R132C, R132G, R132S and R132L are also known. Mutations in IDH2 are far more rare and primarily involve R172 substitution [16]. In terms of personalized medicine in neurological cancer patients, the marked impact of these mutations on the disease prognosis is especially important, regardless of the therapy used. It has been found that GBM patients with IDH1/2 mutations have a significantly longer median of overall survival than patients without these mutations. Several different papers have shown 3.8 vs. 1.1 years, 2.6 vs. 1.3 years, 2.3 vs. 1.2 years, and 3 vs. 1 year of overall survival [14, 15, 17–20]. Even more significant differences in overall survival were found in patients with anaplastic astrocytomas: 5.4 vs. 1.7 years, 6.8 vs. 1.6 years and 7 vs. 2 years [15, 17, 18]. Similarly, diffuse astrocytoma has a far better prognosis if there is a mutation in IDH1/2: 12.6 vs. 5.5 years [17]. Recent meta-analysis of 55 observational studies has shown that patients with gliomas positive for IDH1/2 mutations have improved both overall survival and progression-free survival, especially patients with WHO grade III and grade II-III tumors [21]. Moreover, the combination of two biomarkers (IDH1 mutation and MGMT methylation status) outperforms either IDH1 mutations or MGMT methylation alone in predicting survival of glioblastoma patients [22].

Oligodendrogliomas are also important representatives of neuroepithelial tumors of the CNS. WHO grade III anaplastic oligodendrogliomas (AO) are among those with a higher malignancy potential [4, 23]. The median overall survival of AO patients is reported as between 2 and 6 years with standard treatment. Conventional radiotherapy may be augmented with a combination regimen of PCV (procarbazine, lomustine and vincristine) chemotherapy, though the effect of combined radiotherapy and chemotherapy on the overall survival of newly diagnosed AO patients has not been sufficiently proven for a non-selected population [24]. A certain breakthrough in regards to adjuvant chemotherapy in the treatment of AO occurs only with the application of the principles of personalized medicine and predictive biomarkers. Molecular changes in a certain group of AOs, the co-deletion of the short arm of chromosome 1 (1p), and the long arm of chromosome 19 (19q) in neoplastic tissue, have been known for a relatively long time [25, 26]. Following several dramatic responses to AO therapy with a combination PVC regimen and radiotherapy in the 1990s, two international phase III clinical trials of combination chemo-radiotherapy for AO patients were launched: Radiation Therapy Oncology

Group (RTOG) trial 9402 and European Organization for Research and Treatment of Cancer (EORTC) trial 26951. In these trials, the co-deletion of 1p/19q was monitored as a potential predictive biomarker of response to treatment. An ongoing analysis of the results of both studies in 2006 did not find a statistically significant relationship between the overall survival of patients who received radiotherapy alone or radiotherapy in combination with PCV and the presence of 1p/19q co-deletion [27, 28]. However, data from the long-term monitoring of both independent studies now clearly show a significant increase in the overall survival of patients with proven 1p/19q co-deletion in the neoplastic genome that were treated with combined radiotherapy and PCV chemotherapy. With a median patient monitoring period of 11.3 and 11.7 years in RTOG 9402 and EORTC 26951, respectively, the increase in the overall survival of AO patients was found to be 14.7 vs. 7.3 years (HR = 0.47, $P < 0001$) and NR (median overall survival not reached) vs. 9.3 years (HR = 0.56, $P = 0.0594$) for patients with 1p/19q co-deletion who received combined therapy [28, 29]. This clinical trial clearly demonstrates the predictive significance of the 1p/19q co-deletion biomarker in newly diagnosed AO patients and its effect on long-term survival for decades from the start of combined therapy.

These clinically very significant findings are successful examples of the integration of the principles of personalized medicine into modern neuro-oncology and will certainly soon become an important addition to standards in decision algorithms regarding care for these patients [10, 30–32] (Table 1).

Biomarkers and personalized medicine in cerebrovascular diseases

Care for patients suffering from cerebrovascular diseases is highly sophisticated, based on high-quality diagnostics that allow physicians to determine the cause and extent of stroke and select the optimal treatment. In addition to clinical examinations and basic laboratory parameters, imaging methods (CT, CT perfusion, CT angiography, or MRI) are needed. In cases of acute cerebrovascular accidents, the rapid administration of the target treatment is essential to success. What else can biomarkers and concept of personalized medicine offer to this field?

The determination of blood biomarkers is an area that offers promise but as yet little applicability [33–35]. The ideal blood biomarker should be highly specific and sensitive, able to differentiate stroke mimics, determine the type and extent of stroke and have a predictive value for serious stroke complications, such as the risk of malignant edema or risk of hemorrhagic transformation of ischemic stroke.

However, such a single biomarker does not exist. In spite of this, the field is being carefully studied and certain partial successes have been described. Ischemic and hemorrhagic stroke lead to rapid changes in the signaling pathways and metabolic processes. Brain damage, ischemic cascade, activation of the immune system and blood-brain barrier dysfunction lead to an expression of biomarkers and the possible detection of these markers in peripheral blood.

The ischemic cascade includes the activation of glia, oxidative stress, the release of inflammatory mediators and neuron damage [36]. Biomarkers with relative specificity towards these processes could be detected in blood [37]. Biomarkers for glial activation include S100 beta, glial fibrillary acidic protein and myelin basic protein. S100 beta is also marker of astrocyte activation and brain tissue injury with low specificity for ischemic stroke. Glial fibrillary acidic protein differed in hemorrhagic stroke compared with ischemic stroke ($p < 0.0001$) within 4.5 h of symptom onset [38]. Myelin basic protein is one of the main component of CNS myelin and could be found in cerebrospinal fluid (CSF) and blood within first hours after stroke onset. Determination in blood is sufficient. The release of these biomarkers after stroke is associated with the volume of brain lesions. PARK-7 and malondialdehyde are biomarkers of oxidative stress. Their potential clinical application is in early diagnosis of stroke and in prediction of stroke prognosis. Biomarkers

Table 1 Examples of molecular biomarkers in gliomas and their clinical relevance

Molecular biomarker	Assessment method	Biomarker relevance		
		Diffuse gliomas (grade II)	Anaplastic gliomas (grade III)	Glioblastoma (grade IV)
1p/19q co-deletion	FISH, PCR	Positively prognostic	Positively prognostic for RT or CHT Predictive for PCV and RT	Very rare, unclear
IDH1/2 mutations	RT-PCR, IHC, sequencing	Positively prognostic	Positively prognostic	Positively prognostic, rare Distinguishing secondary GBM
MGMT promoter methylation	Methylation-specific PCR	Unclear	Positively prognostic	Predictive for temozolomide
G-CIMP	Methylation-specific PCR	Positively prognostic	Positively prognostic	Positively prognostic

Abbreviations: *IDH1/2* Isocitrate dehydrogenase 1 and 2, *MGMT O6* methylguanine, *DNA* methyltransferase, *G-CIMP* Hypermethylator phenotype of cytosine-phosphate-guanine islands in gliomas genome, *GBM* Glioblastoma multiforme, RT Radiotherapy, CHT Chemotherapy, FISH Fluorescent in situ hybridization, RT-PCR Real time polymerase chain reaction, IHC Immunohistochemistry

of inflammation include C-reactive protein, matrix metalloproteinase (MMP) 9, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-alpha). Namely MMP 9 have been widely investigated for its role in disruption of the blood-brain barrier and extracellular matrix following stroke [37, 39].

The main biomarkers of neuronal damage are neuron specific enolase and *N*-methyl-D-aspartate receptor (NMDA-R). D-dimmer, fibrinogen, fibronectin, von Willebrand factor and thrombomodulin are biomarkers of endothelial dysfunction. Additional biomarkers are lipoprotein-associated phospholipase A2 and brain natriuretic peptide (BNP). Blood biomarkers can help in distinguishing the etiology of stroke (BNP in cardioembolic stroke), in predicting early neurological deterioration and clinical outcome (S100 beta, MMP, IL-6, TNF-alpha), and in predicting hemorrhagic transformation (cellular fibronectin, MMP 9) [37, 40, 41]. However, most of these biomarkers do not have a sufficient level of sensitivity, specificity or both. Moreover the heterogeneity of different cell populations in the brain and their ischemia tolerance and distributions within the central nervous system, the complexity of the ischemic cascade, and presence of the blood-brain barrier cause that no single biomarker has ever been demonstrated to be clinically useful. For this reason, batteries of biomarkers are described that offer greater predictive value when applied. For example, a panel of biomarkers for the ischemic cascade can distinguish patients with acute stroke from age and gender-matched control subjects with a sensitivity and specificity of 90 % [42].

Another prospect is the use of biomarkers that signify changes in the gene expressions that occur in minutes and hours after the onset of stroke. These include capturing changes in certain mRNA in the peripheral blood and circulating leukocytes [43] or determining several circulating microRNA [44, 45].

The clinical application of blood and gene biomarkers in the acute phase of stroke has thus far run up against technical limitations, speed of detection and especially high cost. However, they may offer valuable additional information about the type and prognosis of stroke.

Biomarkers can also be used in the field of stroke prevention. Clopidogrel is transformed into an active metabolite with a significant anti-platelet effect by cytochrome P-450. Carriers of at least one of the transformed allele of the enzyme CYP2C19 (about 30 % of the population) have an increased risk of vascular accidents. In the TRITON TIMI-38 study, they had a 53 % greater risk of stroke, heart attack, and cardiovascular death when treated with clopidogrel [46]. Dicumarol is used for a 30 % reduction in the relative risk of cardioembolic stroke. Its individually transformed effect is tied to polymorphisms in the genes VKORC1 and CYP2C9 [47, 48]. Statins are used for the relative reduction of around 20 % in the onset of stroke. Statin-induced myopathy is a risk associated with their use. This effect is tied to rs4149056 polymorphism in the SLCO1B1 gene located on chromosome 12. Persons with one variant allele have a 4.5 times greater risk of statin-induced myopathy. Homozygotes with both variant allele (2.1 % of the investigated population) have as much as a 17-times greater risk of statin-induced myopathy [49].

Biomarkers and personalized medicine in neurodegenerative diseases

Biomarker research in neurodegenerative disease is a rapidly advancing area in personalized medicine. The good biomarker should have specificity more than 80 % and the same level of sensitivity (more than 80 %). The role of these markers is not only diagnostic; they have also prognostic potential or role in development of new treatment [50, 51]. A large number of molecules have been evaluated and associated with different neurodegenerative disorders, but only several of them are validated and well-established. Current status of the development of new biochemical biomarkers for Alzheimer's disease and Parkinson's disease, two most common neurodegenerative disorders, is discussed.

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder with prevalence from 2 % in seventh decade to 25 % in ninth decade of life [52]. Diagnosis is difficult especially at early stages before all of sings meeting criteria of AD are presented. So, there is a great field for exploration of novel specific and sensitive biochemical markers which do not constitute discomfort for the patient and which are cost-effective. Useful candidates have been found in blood and in CSF [53]. Potential and already used biomarkers of AD can be divided according to assumed mechanisms of pathogenesis into markers related to the amyloidogenic pathway and cholesterol metabolism, markers of oxidation, markers of immunologic mechanism and inflammation, markers associated with microvascular changes and proteome-based plasma biomarkers [54].

Major CSF biomarkers that are used in clinical practice are tau proteins (T-tau, P-tau) and amyloid β (especially Aβ40, Aβ42). Amount of T-tau correlates with the intensity of neuroaxonal degeneration, level of P-tau reflects tangle pathology and Aβ correlates with plaque pathology [55–57]. The specificity and sensitivity of these biomarkers is between 80 and 90 % [58]. However, the lumbar puncture is relatively invasive practice, especially repeated and in elderly

patients. More comfortable tests are searched especially in blood and plasma.

Several studies deal with antibodies against amyloid β as a biomarker of AD. Du at al. describe significantly lower titres of Aβ antibodies in patients with AD [59], but not in another study [60]. It was hypothesized that more relevant target provides detection of low molecular weight oligomeric cross linked Aβ protein species (CAPS) and anti-CAPS antibodies [61]. Anti-CAPS are significantly reduced in AD patients. These results suggest possibility of using anti-CAPS as a plasma biomarker of AD and promising possibility of therapeutic use in the future.

Further very interesting results provides a research of amyloid precursor protein (APP). This protein is present in central nervous system, but it is also expressed in peripheral tissues such as in circulating cells. The isoforms of APP can be detected in platelets membrane. The intact 150 kDa weight APP is divided into two forms after platelet activation [62]. The ratio of forms with molecular weight 120–130 kDa and of 110 kDa weight are called "platelet APP isoform ratio," and it is decreased in AD and mild cognitive impairment (MCI) not in other dementias [63, 64].

Markers related to cholesterol metabolism are total cholesterol plasma level, CSF and plasma level of 24S-hydroxycholesterol, plasma level of apolipoprotein E and apolipoprotein E genotype. There are three major human apolipoprotein E isoforms—ε2, ε3, and ε4; they are encoded by different alleles with different risk for development of the AD [65–67]. These all provide different and ambiguous results and the interpretation for clinical use remains to be clarified in future studies [68]. The promising biomarker would be 24S-hydroxycholesterol that is elevated in AD patients' CSF and plasma [69]. New studies demonstrate a sensitive and a powerful specific biomarker for early and easy AD diagnosis—desmosterol. Desmosterol was found to be decreased in AD plasma versus controls and more significant in females [70].

Promising but also inconsistent results provided studies of oxidation and immunologic biomarkers. AD and vascular dementia are associated with decrease of plasma and serum levels of vitamins A, C, E, and dietary intakes of the three antioxidants can lower the risk of AD [71]. Sano et al. found that supplementation of vitamin E delayed progression of AD [72] and elevated levels of tocopherol and tocotrienol forms are associated with reduced risk of cognitive impairment in older adults [73]. A significant association between AD and low levels of vitamin D has been demonstrated [74]. Plasma level of isoprostane 8,12-iso-iPF2α-VI as a specific and sensitive marker of lipid oxidation is increased in AD and correlates with level of cognitive and activities of daily living impairment [75]. Other results show that plasma or urine level of this marker do not accurately reflect situation in the central nervous system [76]. Controversial data have been published about α1—Antichymotrypsin (ACT)—which is one of the components of senile plaque. High plasma levels of ACT would be associated with an increased risk of AD [77].

The next candidate biomarker is Alzheimer-associated neuronal thread protein (AD7c-NTP) which can be detected in CSF, brain-tissue extracts, cortical neurons, and urine. Its level also positively correlated with degree of dementia [78].

Neuroimaging techniques can disclose signature abnormalities of brain morphology and function many years before AD symptoms appear. A number of neuroimaging candidate markers are promising, such as hippocampus, amygdala, and entorhinal cortex volumes, basal forebrain nuclei or atrophy of the grey matter of the medial temporal and dorsolateral frontal lobes [58, 79, 80]. Sabuncu et al. examined a total of 317 participants with baseline cerebrospinal fluid biomarker measurements and 3 T1-weighted magnetic resonance images obtained within 1 year. Their results show that AD-specific cortical thinning and hippocampal volume loss are consistent with a sigmoidal pattern, with an acceleration phase during the early stages of the disease [81]. Fluorodeoxyglucose positron emission tomography has shown a specific pattern of regional hypometabolism. Hippocampal glucose metabolism reduction was found in both mild cognitive impairment and Alzheimer disease and contributes to their diagnostic classification [82, 83]. Fleisher et al. used positron emission tomography (PET) and florbetapir F18 to image cortical amyloid in patients with mild cognitive impairment or dementia due to Alzheimer disease. Their analysis confirmed the ability of florbetapir-PET to characterize amyloid levels in clinically probable AD and mild cognitive impairment [84, 85].

Parkinson's disease

Parkinson's disease (PD) is another most common neurodegenerative disease in human population with prevalence of about 1 % after the sixth decade. It is expected a doubling of prevalence until 2030 [86]. Cardinal motor symptoms of the disease (tremor at rest, rigidity, bradykinesia, and postural instability) are presented after more than 50 % of dopaminergic nigral cells are damaged [87]. PD is not just motor disorder and its non-motor symptoms often precede the motor ones. These premotor markers include olfactory and autonomic dysfunction, sleep disorder, depression, and cognitive disturbances [88, 89]. Biomarkers of PD are required for detection persons at risk, for recognition of PD before clinical

symptoms are presented, prediction of disease progression, for stratification of success of treatment or for distinguishing PD from parkinsonism. Unfortunately, no validated diagnostic biomarker of PD is available.

Similar to AD, perspective biomarkers of PD can be divided into biomarkers belonging to oxidative stress, dopamine metabolism, α synuclein, auto antibodies against α synuclein and inflammatory markers. Novel approach is also demonstrated by research in the field of metabolomic profiling.

The most promising results are provided by the research of α synuclein which is one of the main component of Lewy bodies and has been detected in serum, plasma, saliva and CSF [86]. Studies of Mollenhauer and Devic showed decreased level of α synuclein in CFS in PD and in parkinsonism [90, 91]. Measurements of α synuclein and phosphorylated α synuclein concentrations can distinguish PD from multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) [92]. MSA is a rare neurodegenerative disorder previously called Shy-Drager syndrome. It is classified into two types: parkinsonian and cerebellar phenotypes. It is characterized by abnormal accumulation of α-synuclein but in contrast to PD with mainly accumulation in glial cytoplasmic inclusions [93]. And PSP is a neurodegenerative syndrome that is clinically characterized by progressive postural instability, supranuclear gaze palsy, parkinsonism, and cognitive decline [94]. El Agnaf reported that oligomeric soluble forms of α synuclein are significantly elevated in plasma of PD patients [95]. Also auto antibodies against α synuclein are elevated in 90 % of familiar PD cases and 51 % sporadic cases [96].

Several studies show abnormalities of inflammatory markers. Chen reported that higher level of IL-6 is associated with greater risk of PD [97] and Scalzo found that higher levels of soluble TNF receptor-1 are connected with early onset of disease [98]. Interesting role in pathogenesis of PD plays increased oxidative stress. For example, significant reductions of mitochondrial complex I was found in platelets membrane in PD patients [99] but these results were not confirmed in another study [100]. Coenzyme Q10 (CoQ10) related to PD is also studied. Platelet CoQ10 redox ratio (reduced CoQ10 to oxidized CoQ10) was significantly decreased in de novo PD patients. Redox ratio was not correlated to disease severity [101]. Schwarzschild reported that high level of serum urate is connected with slower progression of PD and therefore urate is the first molecular factor linked directly to the progression of typical PD [102].

Recent studies in personalized medicine of neurodegenerative diseases are focused on metabolomic biomarkers. That means identification and quantification of intracellular metabolites, small changes in mRNA and exploration of small molecules in tissues, cells and body fluid that can be significant for specific disease or process including PD. A lot of another studies investigate a role of different molecules in PD. Chen discovered that low level of epidermal growth factor in plasma is linked with cognitive function and can be used as a marker of cognitive decline in patients with PD [103].

Over 25 genetic factors have also been shown to constitute risk factor for PD [104]. For example, homozygous and heterozygous mutations of the glucocerebrosidase gene are a major risk factor for PD [105]. The mutations in the gene for α-synuclein in familial forms of Parkinson's disease have led to the belief that this protein has a central role and is associated with more rapid disease progression; dementia or hallucinations [106]. Newly Azuma et al. reported mutation of the cyclic nucleotide phosphodiesterase 8B gene as one of the causal gene mutation of this disease [107].

Magnetic resonance imaging (MRI) positron emission tomography, transcranial sonography or single-photon emission tomography (SPECT) allow the non-invasive tracking of molecular targets of relevance to neurodegeneration [108]. MRI can provide information about disease-induced changes in the structure and nigral abnormalities and about reduction of brain regional *N*-acetyl-aspartate that is biomarker of neuronal loss [109, 110]. Fibrillar amyloid load can be quantitfied in vivo with PET [111]. Next PET biomarkers include e.g., F-18 fluorodeoxyglucose uptake for mitochondrial bioenergetics [112, 113], F-18 DOPA uptake which is associated with an increased risk for later motor complications and comprises a disease-intrinsic predisposing factor for their development [114] or a dopamine transporter marker [(11) C] CFT and [(11) C] (R)-PK11195 to investigate changes in microglial activity [115]. Siderowf et al. tried to evaluate the relationship between [99mTc] TRODAT-1 SPECT imaging, odor identification skills, and motor function in patients with early PD and they found that olfactory function is highly correlated with dopamine transporter imaging abnormalities [116] and also that it correlated with anxiety and depression symptoms [117]. Impulse control disorders including compulsive gambling, buying, eating, and hypersexuality are relatively frequent especially in younger male PD patients, especially in those treated with dopamine agonists. It may cause catastrophic consequences, including financial ruin, divorces, loss of employment, and others. Pharmacological treatment should be individualized based on patient's unique neuropsychiatric profile, social support, medical comorbidities, tolerability, and motor symptoms [118, 119].

Several markers have shown the potential of effective biomarkers but they need verification in further studies. It is likely that a single measure and one biomarker are

not sufficient and that only combination of various biomarkers as well as the clinical relevant patient characteristics can provide complex and useful information on disease.

Biomarkers and personalized medicine in demyelinating diseases

The current interest in the field of demyelinating disease focuses on multiple sclerosis (MS), not just for its frequency of occurrence, but also because it is a disease that disables young working-age population.

Recently, the diagnosis of this disease was very carefully worked up and also simplified, especially through the use of MRI. The MRI of brain and spinal cord is currently used as the main supporting diagnostic method [120, 121]. Among other supportive parameters in MS diagnosing belongs the testing for cerebrospinal fluid-restricted oligoclonal bands (OCB) by isoelectric focusing, which is used to detect intrathecally produced total IgG. Another characteristic findings in patients with MS is the polyspecific intrathecal B cell response against neurotropic viruses, specifically against measles virus, rubella virus, and varicella zoster virus, also known as an MRZ (Measles antibody index, Rubella antibody index, Zoster antibody index) reaction and abnormalities in visual, auditory, somatosensory, and motor-evoked potentials [122–126].

In the last few, years the treatment of MS achieved a huge progress with the arrival of disease-modifying drugs (interferons and glatiramer acetate, natalizumab or fingolimod and lately also alemtuzumab, dimethyl fumarate, teriflunomid). Moreover new oral and parenteral drugs are already on the verge of clinical use, which can bring more hope in the treatment of MS. Currently there is a number of drugs that differs in their efficiency and safety profile, due to this fact the problem is how to select patients according to their susceptibility to treatment with specific drug and how to prevent or minimize the adverse effects. The timing of the treatment is crucial for the patient's prognosis. The best is to start when only clinically isolated syndrome (CIS) is present. But not only early treatment is important, huge role plays also the choice of the most suitable drug according to the clinical and MRI findings, the presence of underlying diseases and other related aspects. The aim is to stabilize the process of this disease and minimize the adverse effects. That is the goal of personalized medicine in patients with MS.

Personalized medicine in the field of MS is based on couple of aspects of the disease. These are demyelination and progression of inflammation, neurodegeneration (axonal loss), progression of disability, and therapeutical response. It is very important to keep all these aspects in mind when choosing the best therapy.

The key question seems to be how to determine the risk of conversion from clinically isolated syndrome to clinically definitive diagnosis of MS. The answer to this question brings the multicentre studies published in 2015. The results showed the higher risk of conversion in patients with the presence of OCB in CSF, higher number of lesions on MRI and younger age patients. Low level of vitamin D has also showed a small predictive value to conversion to clinically definite multiple sclerosis (CDMS), but this parameter is still the subject of investigation. On the other hand other observed parameters such as sex, smoking, CSF cytology, type of clinical presentation of CIS, the presence of IgG antibodies against EB virus or IgG antibodies against CMV, did not show any predictive value for conversion from CIS to CDMS. Multivariable regressive analysis has shown that accumulation of single risk parameters leads to increasing risk of conversion from CIS to CDMS and malignant course of diseases [127]. Another recent study dealt with similar topic, specifically focusing on predictive factors for conversion from CIS to CDMS. The results came out of long term data collection already since 1995. Clinical status of the patients was thoroughly examined in the interval from 3 to 6 months and brain MRI was done after 12 months and then every 5 years. Based on this analysis the risk variables were established for developing CDMS and expanded disability status scale (EDSS) 3.0—the count of lesions on brain MRI, the presence of oligoclonal bands in CSF, type of clinical presentation of CIS, sex, and age. Thanks to all these variables it was possible to analyse the risk of developing CDMS or risk of reaching EDSS 3.0 for every patient with CIS. It is a very dynamic model, which is able to valorize the risk again after 12 months based on the presence of relapses, new T2 lesions on MRI and type of treatment in the last 12 months. Regarding all the results it is possible to re-analyze the risk of progression of the disease and therefore change the treatment if necessary [128].

Another biomarker that has been recently followed in patients with MS is vitamin D. Its role in bone metabolism and calcium homeostasis is already well-known, but recently it has been proved also its immunomodulatory, anti-inflammatory and neuroprotective effect. Therefore, many studies now focus on the influence of vitamin D to the development and course of autoimmune diseases such as MS. Many epidemiologic, preclinical and clinic data showed that low level of vitamin D had proven to be one of the risk factors in developing MS and is often linked with higher activity and progression of the disease [129–131]. In 2014, Kimbourgh et al. published the results of a study examining the risk factors of transversal myelitis reoccurring. Low level of

vitamin D during the first attack of transversal myelitis was proven among the highest risk factors of developing another attack [132].

In 2013 a team Sormani published a new modified Rio score, which helps to identify patients with positive response to treatment with interferon beta. Those patients are called responders. This score analyses the presence of new T2-weighted lesions on MRI, the number of relapses after a 1 year of treatment with interferon beta. Based on those results patients are divided into three groups; first group involves patients with the lowest risk of progression of the disease and therefore patients with the best response to treatment—no relapse and max. five new T2-weighted lesions on MRI after 12 months of treatment. Patients with moderate risk of progression so-called partial responders belong to the second group. Those patients had only one relapse and max. five new lesion on MRI in the past year or they had no relapse at all but more than five new T2-weighted lesions on MRI. The last group contains patients with the highest risk of progression, so called non-responders to interferon beta, they showed more than two relapses in 1 year and max. five new lesions on MRI or 1 or 2 relapses and more than five new T2-weighted lesions on MRI. This scoring system comes from the original Rio et al. score published in 2009 with the addition of new parameter the progression of disability evaluated with EDSS [133, 134]. Stangel et al. suggested another scheme which includes more parameters that should be followed in patients with MS, regarding the aim of "no evidence of disease activity". New parameters such as depression, anxiety, fatigue, quality of life and cognitive function were added to the already existing parameters (relapses, disability progression, and new lesions on MRI). It was proven as a very broad and sensitive tool, which helps to follow the disease progression even at the very beginning. These tools are nowadays very important not only for examining the stability of the disease but also for deciding about treatment escalation [135, 136].

The measuring of retinal nerve fibre layer thickness (RNFL) in the peripapilar area using the optical coherence tomography (OCT) is another very useful method with great potential. It is used for tracking the disability progression in patients with MS [137]. This method is non-invasive and can be relatively quickly and easily performed. Studies comparing the findings in the peripapilar area RNFL in patients with MS, neuromyelitis optica (NMO) and NMO spectrum disorders (NMOSD) showed a more severe infliction in patients with NMO and NMOSD. In patients with MS subclinical decrease of RNFL using the OCT can be found but this method still cannot be used as an independent method to differentiate MS and NMOSD in clinical praxis [138, 139]. One possible cause of not responding to treatment with interferon beta is the production of neutralizing antibodies (NAbs). These antibodies are bonded directly to the epitope of interferon beta and that disables its binding to the receptor. Up to 42 % of patients has shown the occurrence of these antibodies which usually form after 6 months of therapy. Their appearance after 2 years of therapy is very rare. NAbs are non-direct biomarkers and their presence only rises the possibility of decreased efficiency of interferon beta [140–142].

Commonly used direct biomarker in clinical practice is the production of MxA mRNA in patients treated with interferon. It is a protein produced by mononuclears due to stimulation of interferon protein. The evidence of MxA is based on determination of mRNA using PCR (polymerase chain reaction) method. Its transcription correlates with the efficiency of the drug [143–145].

Biomarkers that would currently seem as possible predictors of disease progression and that could warn against high risk of malignant course of disease are cerebral atrophy, atrophy of brain gray matter, diffusion tensor imaging (DTI) abnormalities, corpus callosum DTI abnormalities, upper cervical cord atrophy (UCCA), and early MR spectroscopy abnormalities. Based on the presence of these parameters the treatment of MS should be led the most effectively from the disease diagnosis [146–149].

Very crucial complication in using one of the most efficient drug natalizumab for treating the patients with MS is the occurrence of progressive multifocal leukoencephalopathy (PML). This important side-effect has appeared already during the treatment with other immunomodulatory drugs such as fingolimod and dimethyl fumarate, but now it is the center of attention in treatment with natalizumab. There are three main parameters to optimize the risk of PM occurrence—the duration of treatment, former immunomodulatory treatment, and seropositive tests to PML. There was an effort to find some other parameters, which could be used to select patients with low risk of PML during the treatment with natalizumab and also some parameters which could draw the attention to new or increasing risk during the treatment. One of the new parameters found is the antibody JCV (John Cunningham virus) index. The risk of PML increases with the increasing level of JCV antibodies. Also, antibodies seroconversion showed higher risk of PML occurrence. Another parameter is low count of T-lymphocytes expressing L-selectin (CD62L) which also leads to higher risk of PML appearance [150–153].

In last few years, the diagnosis of neuromyelitis optica drew big attention. The interest increased especially in 2004, when a highly specific serum antibody IgG was found. This antibody is aimed against aqua channel aquaporin 4 (AQP4) occurring especially in astrocytes.

This key finding together with former findings of humoral pathogenic mechanisms led to distinguishing this diagnosis from MS even though the clinical picture and paraclinical findings often overlap. Sensitivity of this method is about 80 % combined with specificity reaching over 99 %. Couple recent studies have also proven the presence of antibodies against myelin oligodendrocyte glycoprotein (MOG-Ab) in patients with NMOSD. However, clinical meaning of these antibodies in the field of CNS demyelinating diseases remains uncertain. The highest profit is hoped to be in seronegative patients with NMOSD [154–157].

Conclusions

The role of biomarkers and personalized medicine in neurology is becoming extensively important. The actual state of knowledge in several domains of neurology (neuro-oncology, cerebrovascular, neurodegenerative, and demyelinating diseases) was discussed in this article. A huge amount of perspective biomarkers could be routinely used in the neurological practice in many distinct settings. Especially in more precise diagnostics, better determination of patient prognosis or in prediction of treatment response. Future perspectives in neuro-oncology will bring the concurrent assessment of IDH1/2 mutations and MGMT promoter methylation status for glioblastoma and 1p/19q co-deletion for oligodendroglioma. In cerebrovascular diseases, the panels of blood biomarkers would be widely accessible and will serve especially for the outcome prediction. The anti-CAPS antibodies and β amyloid as well as amyloid precursor protein markers are promising in Alzheimer's disease and α-synuclein in Parkinson's disease. In demyelinating diseases, the goal for the future is to implement biomarkers that could help to distinguish patients with high risk of serious course from patients with potentially benign course of disease. Nevertheless, further validation of these biomarkers is necessary before their incorporation into standard clinical decision-making algorithms. Personalized medicine will certainly play the crucial role in the more effective, cheaper, and better tailored treatment of various neurological diseases in the near future.

Abbreviations

ACT, α1-antichymotrypsin; *AD*, Alzheimer's disease; *AD7c-NTP*, Alzheimer-associated neuronal thread protein; *AO*, anaplastic oligodendroglioma; *APP*, amyloid precursor protein; *AQP4*, aqua channel aquaporin 4; *BNP*, brain natriuretic peptide; *CAPS*, cross-linked Aβ protein species; *CIS*, clinically isolated syndrome; *CDMS*, clinically definite multiple sclerosis; *CNS*, central nervous system; *COX-1*, cyclooxygenase-1; *CSF*, cerebrospinal fluid; *CT*, computed tomography; *DTI*, diffusion tensor imaging; *EDSS*, expanded disability status scale; *EORTC*, European Organization for Research and Treatment of Cancer; *GBM*, glioblastoma multiforme; *IDH*, isocitrate dehydrogenases; *IL-6*, interleukin 6; *JCV*, John Cunningham virus; *MCI*, mild cognitive impairment; *MGMT*, o6-methylguanine-DNA methyltransferase; *MOG-Ab*, antibodies against myelin oligodendrocyte glycoprotein; *MRI*, magnetic resonance imaging; *MS*, multiple sclerosis; *Nabs*, neutralizing antibodies; *NIH*, National Institutes of Health; *NMDA-R*, *N*-methyl-D-aspartate receptor; *NMO*, neuromyelitis optica; *OCB*, oligoclonal bands; *OCT*, optical coherence tomography; *PCR*, polymerase chain reaction; *PCV*, procarbazine, lomustine, and vincristine; *PD*, Parkinson's disease; *PM*, personalized medicine; *PML*, progressive multifocal leukoencephalopathy; *RNFL*, retinal nerve fiber layer thickness; *RTOG*, Radiation Therapy Oncology Group; *SNP*, single-nucleotide polymorphisms; *TCGA*, The Cancer Genome Atlas; *TNF-alpha*, tumor necrosis factor alpha; *UCCA*, upper cervical cord atrophy; *WHO*, World Health Organization

Authors' contributions

PJ, and PJ Jr. conceived the review and coordinated the drafting of the manuscript. PJ, PJ Jr., KK, and PM participated in the design of the review, performed literature searches and identified relevant studies. PJ, PJ Jr., and TO provided content expertise. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Attachment VII

Karlikova M, Polivka J Jr, Strojil J, Topolcan O. A road towards better education in personalized medicine at universities and beyond. Pers Med. 2015; 12(3):259-267. **(IF = 1)**

Review

Personalized Medicine

A road toward better education in personalized medicine at universities and beyond

Personalized medicine is likely to become a future direction of medicine. There is increased knowledge about gene functions in human health and disease and a rapid advance of biotechnologies. Personal genetic testing is available outside the medical room, as direct-to-consumer testing. There is concern about genetic literacy of general public and healthcare professionals which are to handle genetic results and their clinical interpretation. Education and training in personalized medicine and genetic/genomics/pharmacogenomics issues at different levels (high school, university, continuing medical education) is needed. Examples of innovated educational tools and curricula over the world are presented. The educational initiatives in the field of personalized medicine in the Czech Republic are followed from the very beginning.

Keywords: education • genetics • genomics • personal genome testing • personalized medicine • pharmacogenomics

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Personalized medicine is likely to be a future direction of healthcare. Rapidly increasing knowledge about human genes and their role in our health and diseases (an important milestone was the completion of the Human Genome Project in the year 2003) along with fast development of novel molecular tools and biotechnology methods such as oligonucleotide-based microarrays or next-generation sequencing supports this vision. The knowledge of our individual DNA, together with environmental factors, is supposed to enable more accurate predictions of whether an individual is developing an illness or will develop it in the future, or it now, will respond positively to treatment, or will suffer a serious adverse reaction to a drug [1]. In the light of these facts, clinicians and other healthcare providers should focus on the prevention, prediction of treatment effects, targeted care and screenings in order to earlier detect a disease, even before its manifestation. However, such shift from classical reactive medicine (treating symptoms) to the active approach to human health often fails to be promoted in the education of future healthcare specialists.

Recently, many research activities focused on issues such as genetics, genomics, pharmacogenomics, discovery of new prediction and prognosis biomarkers, etc., are going on, and to date a number of success stories of implementation of personalized medicine, especially in oncology, have been reported (f.i., the use of the *Her2/neu* gene as a predictor of breast cancer patients' responses to a drug called Herceptin) [2–4]. One important development is the rise of personal genome testing on the basis of genetic profiling: the testing of multiple genetic variants simultaneously for the prediction of common multifactorial diseases. The personal genome testing is available not only at the clinician's prescription but has also become commercially available as direct-to-consumer testing and a number of companies (23 and Me, deCODE, Athleticode,…) offer the genome sequencing to the customers. The cost of sequencing a single human genome has dropped over

Future Medicine part of fsg

the last decade from $100 million in 2001 to less than $10,000 in 2014 [5] and therefore genetic tests are getting more affordable. Such tests, however, often lack clinical validity and have problematic clinical utility and raise many questions concerning interpretation and proper counseling [6,7]. Consumers then consult the results with their physicians which, as studies suggest [8], are ill-equipped for the role of preliminary genetic consultancy. There is growing concern about the genetic literacy of clinicians and how they will communicate information about personalized genomics to their patients [9,10].

In order to prepare society and especially healthcare professionals for the era of personalized medicine and genome testing, an educational requirement for different groups (high school students, medical students, healthcare professionals) has been expressed [9–11]. The aim of this paper is to point out the need of the education with the focus on students and healthcare professionals.

Genetics & genomics for students & in continuing medical education

Education in genetics is important already at the high school level since each individual should be able to make informed decisions about his or her own DNA on the basis of the knowledge about human genes, genotype and phenotype and their role in inheritance [12–14]. Studies demonstrated that general public and high school students often misunderstand basic genetic concepts [12,13,15,16]. These findings are troubling as it has been reported that such misconceptions influence how patients make decisions about genetic testing and treatment [17].

Since 2006, the American Society of Human Genetics annually organizes the National DNA Day Essay Contest intended to challenge students to examine, question and reflect on important concepts in genetics [15]. An analysis of a sample of 500 submitted essays, performed over years 2006 and 2007, found that several significant misconceptions about basic genetic concepts (such as 'one gene is always responsible for one trait or one gene with one mutation always causes one disease' or confusing 'genetic' with 'hereditary') could be identified in more than half of sampled essays.

Several authors estimate that genetics curricula at high schools and universities are often out-of-date and do not prepare their students for the era of genetic testing [14,18,19] and call for the revision of curricula. A way to overcome this problem could be the integration of genetic education throughout the entire medical school curriculum [20,21]. Dhar *et al.* [21] described a novel elective Genetics Track Curriculum for all 4 years of the undergraduate medical curriculum at Baylor College of Medicine to enhance genetic and genomic education.

A particular example of innovation of a university course is reported by Redfield [19]. The 'old' second-year course in genetics (part of biology curriculum) followed classical textbooks with the history of genetics providing the organizing framework. Unfortunately, students finishing such course did not seem to understand much genetics. For instance, most students mistakenly believed that alleles are intrinsically either dominant or recessive, had no idea what makes one allele dominant to another. The author and collaborators have designed a new course leaving behind traditional but not really necessary topics (like Mendel's laws and Punnett squares) and underlining topics and connections that students will need to know in the era of genetic testing (like personal genomics, natural genetic variation in populations and others). Moreover, according to the author, similar course design could be suitable as well for high school education in genetics. Similarly, Dougherty *et al.* [13] suggested a reorganization of genetic curricula by inverting the sequence of topics and emphasizing complex traits instead of the classical methods following historical consequences.

However, the reorganization of lectures alone will probably not enhance students' understanding of genetics and especially their motivation. Professor Robin Wright, the 2014 Awardee of the Genetics Society of America's Elizabeth W. Jones Award for Excellence in Education, has transformed the undergraduate education of biology and genetics at the University of Minnesota into 'doing biology' classrooms and has a simple proposition for biology teachers: instead of preparing exhaustive lectures, teach as if the only thing that matters is what your students are able to do, or do better, at the end of the course than at the beginning [22].

Genomics is a young and quickly evolving area, so it is hard to anticipate either the genomic knowledge or the clinical application of that knowledge that will be common in the working years of today's medical students and trainees [8]. It is the field of medicine that demands a lifelong learning. An important goal in educating healthcare professionals in genomics is to enable them to understand and utilize genetic-based probability and risk assessment, and to communicate effectively about them.

Along with the activities of individual institutions, concerted efforts exist as well. In 2006, a set of core competences in genetics and genomics for the Europe was defined by the Education Committee of the European Society of Human Genetics and

other experts of the EuroGentest [23]. The proposed competences differ for different groups of healthcare professionals (general practitioners, nurses, medical specialist in fields other than genetics, specialist dentists) and provide a framework for genetics education of health professionals across national boundaries. The suggested learning outcomes are available to manage development of curricula that are applicable to the national context, educational system and healthcare setting of the professional involved. However, it depends on each nation how it will take responsibility for development of professional and educational standards in genetic healthcare that apply to local needs.

Education of pharmacogenomics: need for medical students & physicians

Pharmacogenomics, or its predecessor pharmacogenetics, have been a part of medical curricula in some sort for over half of a century, ever since difference in drug response based on genetic predisposition was first reported [23,24,25,26]. However, with the rapid expansion of pharmacogenetic and pharmacogenomic information in the recent decades it has become nearly impossible for physicians to keep up with current knowledge and for curricula to include all the possibly relevant information in their courses.

The translation of pharmacogenomic information from the labs to bedside has been somewhat slow, ranging from clear and universal successes (trastuzumab and *HER2/neu*, imatinib and the Philadelphia chromosome, abacavir and *HLA-B*5701*) to intermediate progress (warfarin and *CYP2C9* and *VKORC1*) to virtually no clinical relevance in daily practice (statins and *SLCO1B1* polymorphism).

A study performed in the USA by the American Medical Association surveyed over 10,000 doctors and found that a vast majority of them (98%) believed patients' genetic makeup affects pharmacotherapy, yet only 10% felt educated enough to feel comfortable adopting this genetic information in their patient practice [27]. Other surveys have confirmed these results, with oncologists reporting being better informed about advances in personalized therapy and only about 50% of recent graduates having received any training in genomic-based therapy; this number decreased for graduates from more than 5 years ago [28,29]. The conclusion of these and other studies was similar: most doctors see molecular tests as an important an asset in patient care, however, a minority of surveyed doctors feel up to date on genetic tests and feel very familiar and confident with current findings in genomics – it can be called an 'awareness–information gap.' Moreover, a distinct difference exists in baseline awareness, knowledge and adoption between oncologists, cardiologists and primary care physicians. The education should be provided with respect to these differences, instead of one-fits-to-all approach [29].

Many schools now include instruction on pharmacogenomics either as part of basic and/or clinical pharmacology courses, or as a stand-alone elective course in their medical doctor (MD) program [30,31,32]. Pharmacy schools have had a certain head start on medical schools in implementing pharmacogenomics information in their curricula as compared with medical curricula which is overcrowded by an increasing body of information in which pharmacotherapy and prescribing is just one part competing for time and resources. Reports on pharmacists' confidence in pharmacogenomic prescribing are similar to those in physicians mentioned above [34,33].

With the increasing number of drugs that need to be taught to students and therapy getting more fragmented as treatment gets more personalized, teaching of pharmacology and pharmacogenomics will face a problem of selecting only the most relevant (and future-proof) pharmacogenomic information to include in instruction of all medical students. Details on specific drugs relevant to particular medical specializations will be a vital component of continuing medical education for which a medical graduate should come prepared with understanding of pharmacogenomics concepts and most relevant examples that illustrate them.

Personal genetic testing: learning by doing

An efficient way to increase students' motivation and encourage learning of genetics and genomics can be to provide them with their own practical experience with personal genetic testing [34–36]. Several academic institutions in the USA, mainly with the support of commercial direct-to-consumer providers, have considered offering courses for medical students including personal genetic testing and published their experiences and recommendations.

A positive experience was reported by Sansgiry and Kulkarni [34] from the Stanford School of Medicine (CA, USA). In the elective course GENE 210 [36], students used personal genotype data in the classroom; testing was voluntary and anonymous. According to the survey among participating students, this experience enhanced their self-reported and assessed knowledge of genomics, and did not appear to cause significant anxiety.

To evaluate students' attitude toward personal genetic testing as an educational tool, Vernez *et al.* [37]

conducted individual interviews with students who chose to undergo personal genotyping in the context of an elective genetics course. Overall, students stated that personal genotyping enhanced their engagement with the course content, although they expressed skepticism over the clinical utility of some of the test results. At the same time, students did not report utilizing genetic counseling, despite feeling strongly that the 'general public' would need these services. The authors concluded with the recommendation that before incorporating personal genotyping into coursework, institutions should lead multidisciplinary discussion to anticipate issues and incorporate teaching mechanisms that engage the ethical, legal, and social implications of personal genotyping. Boguski *et al.* [38] added a suggestion that specialists with expertise in clinical laboratory tests should be included as members of multidisciplinary curriculum development and delivery teams to assure the preanalytical and analytical issues of the test results.

An analysis on the ethical concern raised by the educational genotyping was published by Callier *et al.* [39]. The publication lists several educational DNA testing initiatives and considers them in terms of who performs the testing (e.g., a commercial laboratory, university laboratory or classroom laboratory instructor), the format for returning results, the number of single-nucleotide polymorphisms tested, and the scope and goals of different lessons. The authors underline the benefits of educational DNA testing together with pointing out that students should receive information on the ethical issues and the risks associated with genetic testing.

Ethical, legal & social issues

It is increasingly important that physicians have a thorough understanding not only of the basic science of human genetics and genomics but also of the ethical, legal and social implications associated with genetic testing and counseling. As has been already suggested, physicians will often be the first healthcare professionals to advise patients about genetic testing or assist with result interpretation. Due to the demanding curriculum requirements of medical schools and the need for medical students to complete many courses, adding extra courses to the medical school curriculum can be difficult. A possible solution is the development of supplemental course modules that can then be accessed online. Metcalf *et al.* [40] developed a series of interactive web-based courses including clinical case studies for medical students on these topics. A pilot trial of the courses indicated that the courses have a statistically significant positive effect on knowledge, attitude, intended behavior and self-efficacy related to genetic testing. However, we do not yet know the long-term effects of these modules on medical student knowledge and behavior.

Examples of education at academic institutions & national levels

Over the world, especially in the North America and the Europe, a number of universities already offer undergraduate and graduate education in genomics, pharmacogenomics, molecular medicine or simil21-ar topics, in some of which personalized medicine is also discussed.

Programs dedicated to personalized medicine exist at the Duke University (NC, USA) [41] and at Mount Sinai School of Medicine (NY, USA). Mount Sinai School of Medicine was the first university to offer to the students the possibility of personal sequencing of the whole genome [42]. Also, some medical schools have updated their curricula by including genomic medicine, such as Harvard Medical School (MA, USA). The University of California, San Francisco Medical School, launched in 2001 a graduate PhD program in Pharmaceutical Sciences and Pharmacogenomics [43]. At the Tel Aviv University Faculty of Medicine (Tel Aviv, Israel), pharmacogenetics has been incorporated to the MD teaching curriculum since 2001 and offered as an elective class for graduate students since 2003 [31]. The education for health professionals is provided by the Golden Helix Institute (Greece) [44]. At the Karolinska Institute (Sweden) there is a master program in molecular medicine, and at the University of Bonn (Germany) an International Masters Course in Molecular Biology and Biotechnology is going on .

Some of the courses are designed for e-learning, for example, the US National Coalition for Health Professional Education in Genetics [45] has developed a series of web-based medical education programs discussing the influence of genetics on various diseases. Similarly, in the UK the national genetics education and development center [46] has developed evidence-based learning objectives and competencies in genetics for health professionals.

In Europe, at least two associations are involved in the education in personalized medicine: the European Association for Predictive, Preventive and Personalized Medicine (EPMA) [47,48] which publishes a series of educational books, and the European Personalized Medicine Association (EPEMED) which together with EuroBioForum offers Education and Training in Personalized Medicine for Healthcare Professionals in the form of webinars [49].

First steps: experience from the Czech Republic

A Chinese proverb says that 'a journey of a thousand miles begins with a single step.' A journey toward the education in personalized medicine in the Czech Republic has also begun with few steps or, more precisely, with few activities conducted by few enthusiastic people.

Most research and educational activities in the area of personalized medicine are concentrated at the Charles University of Prague – the Faculty of Medicine in Pilsen and the Faculty Hospital in Pilsen [50].

Since 2007, lectures and workshops on personalized medicine for healthcare professionals and medical students have been included in the program of the international congress Immunoanalytical Days and CECH-TUMA [51], organized every year under the auspices of the Charles University – the Faculty of Medicine in Pilsen and the Faculty Hospital in Pilsen. At the beginning, these lectures were provided by invited experts from the USA; recently Czech researchers and academics report their growing experience with personalized medicine. Additional conferences focused on the application of personalized medicine in different clinical specializations take place every year. In June 2014, professionals from the Faculty of Medicine in Pilsen had an honor to co-organize the third International Congress on Personalized Medicine Up Close and Personalized in Prague [52].

In 2010, a working group of professionals was established at the Faculty of Medicine in Pilsen with the goal to implement principles of personalized medicine into the undergraduate and postgraduate education. They assessed, with the help of a survey questionnaire, the awareness about personalized medicine among medical students. The 'Personalized Medicine Questionnaire' was prepared and addressed to fourth-year medical students; 80 responders were involved. Students had to answer nine nominal questions. The summary of the average of responses for each question is presented in Table 1.

As the results of the questionnaire imply, the awareness about personalized medicine among students is quite weak (more than 39% have not even heard the term 'personalized medicine'), although most students (more than 75%) recognize the importance of personalized medicine and would welcome its implementation into the medical education [53].

In the frame of several EU-funded projects, several elective courses were introduced in undergraduate and graduate curricula of the Faculty in 2012. Examples of courses are presented in Figure 1.

In the region of Olomouc, the institute of molecular and translational medicine was opened as part of the program biomedicine for regional development and human resources (BioMedReg), financed by European regional development fund (ERDF) and the national budget through the operational programme for research and development for innovation.

The Institute serves primarily as a research center with ties to the University hospital providing personalized medicine services and diagnostics to patients, but is also involved in teaching at both graduate and postgraduate level. Since 2011, the institute is involved in the European social fund project managed by the department of pharmacology which coordinates 13 different

Table 1. Summary of the responses for each question in the personalized medicine questionnaire (scoring 1–5, 1 = not at all; 5 = definitely yes).

Question	Average value (n = 80)
Have you ever heard the term 'personalized medicine'?	2.26
Would you be able to explain, what does the 'personalized medicine' mean?	2.36
Do you consider 'personalized medicine' to be important?	4.16
Do you think 'personalized medicine' should be studied as an independent discipline or through the various disciplines separately?	3.16
Do you consider the role of 'personalized medicine' will increase with the progress of knowledge?	4.2
Is tuition in 'personalized medicine' sufficient in pregraduate education in medical school?	1.85
Do you think 'personalized medicine' should be implemented into the undergraduate education of medicine for all students?	3.66
Do you think 'personalized medicine' should be implemented into the education of medicine as a required course?	4.14
Will you choose the course of 'personalized medicine'?	3.84

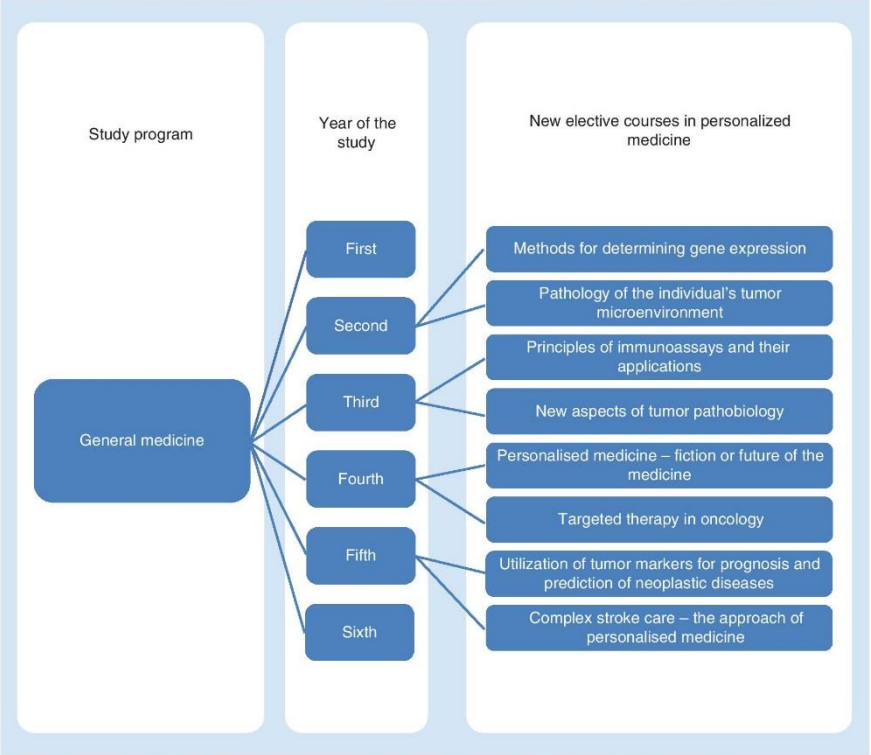

Figure 1. Examples of elective courses in personalized medicine at the Faculty of Medicine in Pilsen.

departments in an effort to update and improve teaching of personalized and laboratory medicine in the curriculum. The aims of the project are both to provide support for updating and innovating course materials of traditional 'core' subjects, from basic sciences such as biology to clinical subjects such as internal medicine or even surgery. The aim is to include up-to-date information on predictive and personalized medicine so that students are equipped for modern clinical practice once they graduate. In another part of the project, over 20 new elective courses were introduced into the curriculum of the faculty of medicine in Olomouc, aimed to provide students interested in individual subjects in more detail (the most important courses are listed in Table 2). These courses received a positive student response in the evaluation, performed by the instructor at the end of the academic year (78% of students rated the courses like 'very useful' and 'rather useful'). Most of them (65%) appraised the focus on practical applications and correct and rational indication and interpretation of individual tests, rather than on technical details of methods. Students, especially in 4th and 5th grades with more clinical exposure, realize their future role as users, rather than direct executors, of these tests.

At both faculties of medicine, the students' participation, evaluation and motivation to complete the courses in the field of personalized medicine are continuously monitored and it is anticipated that several courses will be included in the MD curricula as required ones. The authors envisage that the course portfolio could serve as a model for other academic institutions at national level.

Conclusion

Academic and other institutions proceed in the changes in their educational curricula toward genetics and genomics, and the implementation of new models and curricula takes place gradually. Different tools have been described and applied, from innovated programs and lectures to practical training through personal genetic testing. Cooperation and exchange of experiences between institutions is crucial for an efficient implementation of

Table 2. Selected list of new elective courses introduced as part of an European social fund project in the curriculum of the Faculty of Medicine in Olomouc, Czech Republic.

Year of study	Extent (h)	Title
4–6	10	Interpretation of microbiology tests in clinical medicine
4–6	15	Molecular-biology methods in microbiology and their limitations
2–6	7	Predictive methods in pathology
4–6	15	Epigenetics in oncology
4–6	8	Immunology monitoring in medicine
4–6	8	Immunology in transplant medicine
4–6	15	Interpretation of coagulation tests and management of anticoagulation
4–6	8	Case reports in laboratory medicine
4–6	9	'-omics' in medicine
4–6	9	Animal models and their use in medicine
4–6	15	Laboratory medicine

these tools. A support from educational organizations on the national level is important as well.

Future perspective

It is anticipated that in the next 5–10 years the knowledge in the field of genomics and pharmacogenomics, together with rapid advances in biotechnologies and will increase, together with the availability of personal genetic testing. At the same time, general public's awareness about genetic testing will grow, most probably also through media and commercial adds, and the role of physicians and other healthcare professionals as first hand 'counselors' will be more pronounced. The implementation of genetic data into individual's healthcare record will continue and healthcare specialists will face the necessity to get familiar with these data. More coordinated revisions of medical curricula and cooperation between academic institutions and other institutions (national societies, companies) are critical to assure competent healthcare professionals for the era of personalized medicine.

Executive Summary

Background

- Healthcare professionals are not fully prepared to the era of personalized medicine; they often lack appropriate education and training at universities.

Genetics & genomics for high school & medical students & in continuing medical education

- All individuals should have knowledge of basic genetic principles in order to be able to make decisions concerning her/his own DNA.
- Many students show misconceptions in basic genetic concepts, the classes are not up-to-date.
- Innovation lies in the revised classes and more practical work.

Education of pharmacogenomics: need for medical students & physicians

- Physicians are aware of the importance of pharmacogenomics, however they lack sufficient knowledge.
- Universities are incorporating pharmacogenomics in their curricula and it is not always easy since the medical curricula are overcrowded.

Personal genetic testing: learning by doing

- Several universities offer personal genetic testing as an educational tool, they reported a positive experience like enhanced students, motivation.
- Discussions are going on about ethical, societal and legal issues as well as about the analytical performances of such classroom tests.

Ethical, legal & social issues

- There is need to include ethical, legal and social issues of genetic testing into the curricula.

Examples of education at academic institutions

- A number of examples of innovated academic programs and courses are reported.

First steps: experience from the Czech Republic

- Faculty of Medicine in Pilsen and Faculty of Medicine in Olomouc are the most active subjects in implementing personalized medicine in university curricula.
- A survey was held to assess students' awareness and interest in personalized medicine.
- Seminars, conferences and innovative courses are ongoing.

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Attachment VIII

Polivka J Jr, Polivka J, Rohan V, Topolcan O. New treatment paradigm for patients with anaplastic oligodendroglial tumors. Anticancer Res. 2014; 34(4):1587-94. **(IF = 1.895)**

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Review

New Treatment Paradigm for Patients with Anaplastic Oligodendroglial Tumors

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Abstract. *Oligodendrogliomas are uncommon tumors in neurooncology that represent about 5% of primary brain malignancies. Their high sensitivity to radiotherapy and chemotherapy was observed a long time ago. Nonetheless, the evidence-based proof of the significantly longer survival in patients with oligodendrogliomas treated with combined chemotherapy and radiotherapy in comparison to radiotherapy-alone did not exist. The long-term follow-up of two landmark phase III clinical trials: RTOG 9402 and EORTC 26951, recently demonstrated favorable effects of combined radiotherapy and chemotherapy (procarbazine, lomustine and vincristine) in patients with anaplastic oligodendrogliomas and anaplastic oligoastrocytomas carrying the chromosomal mutation of co-deletion of 1p/19q. There is also an increasing role of other molecular biomarkers, such as mutations in the metabolic enzyme isocitrate dehydrogenase 1/2, O6-methylguanine DNA methyltransferase gene promoter methylation, or glioma genome cytosine-phosphate-guanine islands methylator phenotype. The analysis of molecular genetics in oligodendrogliomas is now recommended as an important part of the management of these tumors and together with the novel chemotherapeutic regimens means a paradigm shift in current clinical practice in neurooncology.*

This article is freely accessible online.

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Oligodendroglial tumors (oligodendrogliomas, oligoastrocytomas) represent approximately 5% of primary brain tumors. What sets them apart from other types of malignant gliomas is their more favorable response to radiotherapy and chemotherapy. According to the 2007 WHO classification of tumors of the central nervous system, they are characterized by a histopathological finding with an oligodendroglial component (1). However, the current WHO classification does not reflect on the molecular genetic characteristics of tumors. Research into molecular genetics of oligodendrogliomas offers new knowledge in the diagnosis and treatment of these tumors, and together with results from clinical studies, has an impact on management. The treatment paradigm of oligodendroglial tumors was recently changed, reflecting on the long-term results of two large independent phase III clinical trials, The Radiation Therapy Oncology Group (RTOG) 9402 and European Organisation for Research and Treatment of Cancer (EORTC) 26961. The analysis of molecular genetics in oligodendrogliomas is now well-established and recommended as an important part of treatment-decision algorithms in clinical practice. This review presents an overview of novel therapeutic approaches for patients with oligodendroglial tumors, primarily in regard to anaplastic oligodendrogliomas.

Diagnosis and Standard Treatment of Oligodendrogliomas

Oligodendroglial tumors can be differentiated by degree of malignancy into grade II and grade III oligodendrogliomas–anaplastic oligodendrogliomas (AO). Only about 30% of oligodendroglial tumors have anaplastic characteristics in the histopathological image: nuclear atypia, increased cellularity, increased proliferation activity and increased cell mitosis. Typical histopathological findings are round nuclei with a light or empty cytoplasm in the vicinity (perinuclear 'halo' effect) and the presence of microcalcification (1). AO

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comprise about 0.5-1.2% of primary brain tumors (2, 3). The highest incidence of AO is between 45 and 50 years of age; grade II oligodendroglioma afflicts patients from seven to eight years younger. It is presumed that this difference corresponds to the progression from tumor grade II to grade III. The majority of oligodendrogliomas present with an epileptic seizure. The most frequent other symptoms affect the frontal and, in some cases, the temporal regions. Infiltrative growth and poorly defined perifocal edema later cause symptoms of intracranial hypertension.

The standard therapy of oligodendrogliomas includes neurosurgery and oncological treatment: radiotherapy and chemotherapy. Radiotherapy is administered to a total dose of 54 to 60 Gy. Chemotherapy is administered in a triple combination of procarbazine, lomustine and vincristine (PCV) or temozolomide (4, 5). The sensitivity to radiotherapy of oligodendrogliomas was discovered as early as the 1980s (6), and the positive effect of chemotherapy, PCV and temozolomide, was found later (7-9).

Neurosurgery is fundamental to remove the tumor and obtain neoplastic tissue in order to make a precise diagnosis. Total resection of the tumor is considered optimal. Sophisticated diagnostic preoperative and perioperative methods (magnetic resonance imaging - MRI, use of 5-aminolevulinic acid, MRI tractography, perioperative ultrasound and MRI, awake surgical method, hybrid positron emission tomography and computed tomography - PET/CT) and navigated microsurgical techniques are important parts of surgical treatment (4, 10, 11). A postoperative MRI (24 to 72 h after surgery) is required to confirm the extent of tumor resection, found to be an independent positive prognostic factor (12, 13). Targeted-biopsy of the tumor is reserved for cases where tumor resection is impossible (11, 14). It is important to note that total biological radicality of tumor resection is still unrealistic. Favorable prognostic factors include young age, good overall medical condition (Karnofsky score), extent of tumor resection and combined oncological treatment (15).

Molecular Genetics of Oligodendrogliomas

Characteristic of oligodendroglial tumors are frequent co-deletions of chromosome 1p and 19q. This genetic aberration was discovered in 1994 and became the first biomarker in neuro-oncology (16). 1p/19q co-deletion means the loss of genetic material from the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q). The mechanism of 1p/19q co-deletion, the unbalanced translocation t(1;19)(q10;p10) and formation of derived chromosome 1p/19q, was identified later (17). It appears almost exclusively in oligodendroglial tumors. The frequency of 1p/19q co-deletion is estimated to be 80% to 90% for grade II oligodendrogliomas and 50% to 70% for AO (18, 19). Recently, the presence of mutations in two important tumor-suppressor genes, CIC (a homolog of the *Drosophila* gene *capicua*) located on 19q13.2, and far upstream element-binding protein (*FUBP1*) on the 1p chromosome, was discovered in the majority of oligodendrogliomas with 1p/19q co-deletion. The prevalence of *CIC* and *FUBP1* mutations among 1p/19q co-deleted oligodendroglial tumors are 50-70% and 15%, respectively. Mutations in these genes are probablyinvolved in the formation and progression of oligodendrogliomas. CIC protein binds to regulatory regions and blocks gene transcription. *CIC* is also negatively-regulated by the mitogen activated protein kinase (MAPK) signaling pathway. *FUBP1* mutations closely related to a myelocytomatosis viral oncogene homolog (MYC) activation. However, their true significance in neoplastic diseases remains to be verified (20, 21). Currently, 1p/19q co-deletion serves as an important diagnostic, prognostic and predictive biomarker in oligodendroglial tumors and is discussed later from the perspective of novel therapeutic approach to this disease.

Recurrent mutations of the enzymes isocitrate dehydrogenase 1 and 2 (*IDH1/2*) were first demonstrated in glioblastoma multiforme, even if the prevalence was relatively low (about 5%) (22). A high frequency of mutations in the *IDH1* and *IDH2* genes was found in low-grade glioma; in grade II and grade III oligodendrogliomas up to 69%-94% of patients (23, 24). Mutation of *IDH1/2* causes neomorphic enzyme activity with subsequent accumulation of the cancer-associated metabolite 2-hydroxyglutarate (2-HG) in the tumor tissue (25). Cells with mutations in *IDH1/2* and 2-HG accumulation undergo massive epigenetic changes (DNA and histone methylation, chromatin remodeling), which leads to an extensive impact on gene expression and likely supports the onset and progression of neoplastic disease (26, 27). The presence of the IDH1/2 mutations is a significant positive prognostic biomarker for patients with glioma (28-30). It has been found that all patients with a tumor positive for 1p/19q co-deletion also have a mutation in *IDH1* or *IDH2*. These patients have the best prognosis (31). On the other hand, there is a group of gliomas with *IDH1/2* mutations, but without the presence of 1p/19q co-deletion. Patients with these tumor types have a worse prognosis than tumors with co-deletion, but still a significantly better prognosis than gliomas without the *IDH1/2* mutations (32, 33).

The promoter methylation of the gene O^6-methylguanine DNA methyltransferase (*MGMT*) was discovered as a significant prognostic, as well as predictive, biomarker in patients with glioblastoma. Patients with a methylated *MGMT* promoter responded better to temozolomide and had significantly longer overall survival (OS) than patients with intact *MGMT* (34-36). This aberration was also found in 80% of AO and in 73% of anaplastic oligoastrocytomas (37, 38). In oligodendroglial tumors, *MGMT* promoter methylation

Table I. *Important molecular biomarkers and their relevance in glioma.*

Molecular biomarker	Assessment method	Biomarker Relevance		
		Diffuse glioma	Anaplastic glioma	Glioblastoma multiforme
1p/19q co-deletion	FISH, PCR	Positively prognostic	Positively prognostic for RT or CHT Predictive for PCV & RT	Very rare, unclear
IDH1/2 mutations	RT-PCR, IHC, sequencing	Positively prognostic	Positively prognostic	Positively prognostic, rare Distinguishing secondary GBM
MGMT promoter methylation	Methylation-specific PCR	Unclear	Positively prognostic	Predictive for temozolomide
G-CIMP	Methylation-specific PCR	Positively prognostic	Positively prognostic	Positively prognostic

IDH1/2: Isocitrate dehydrogenase 1 and 2; MGMT: O^6-methylguanine DNA methyltransferase; G-CIMP: hypermethylator phenotype of cytosine-phosphate-guanine islands in glioma genome; GBM: glioblastoma multiforme; RT: radiotherapy; CHT: chemotherapy; FISH: fluorescent *in situ* hybridization; RT-PCR: real-time polymerase chain reaction; IHC: immunohistochemistry.

serves mainly as a positive prognostic, not predictive, biomarker when the patient is treated with PCV, as was proven in the EORTC 26951 study and in current results of the NOA-4 trial (39, 40).

Another molecular genetics characteristic, as well as important prognostic biomarker for patients with glioma, is the hypermethylator phenotype of cytosine-phosphate-guanine islands (CpG) in the tumor genome (G-CIMP). Positivity for G-CIMP probably is not an entirely independent biomarker, as it is closely related to the presence of the *IDH1/2* mutations (27, 41). G-CIMP-positive grade II and III gliomas usually also have a methylated *MGMT* promoter. G-CIMP positivity is approximately two-times more frequent in oligodendrogliomas (93%) than astrocytomas (45%). G-CIMP is an important positive prognostic factor for all types of glioma, including oligodendroglioma (41). The important molecular biomarkers in glioma, together with their clinical relevance, are summarized in Table I.

The alteration of certain other known pro-oncogenes and tumor-suppressor genes in patients with AO was also identified, even if in rare cases. These alterations include mutations in phosphatidylinositiol 3-kinase (*PI3K*), amplification of epidermal growth factor receptor (*EGFR*) or loss of the phosphatase and tensin homolog (*PTEN*) tumor-suppressor and correlate with a worse prognosis of AO (42, 43).

Clinical Relevance of 1p/19q Co-Deletion in Oligodendroglioma

The 1p/19q co-deletion status can be used in clinical practice as an important diagnostic, prognostic, as well as predictive, biomarker in patients with oligodendroglial tumors. The presence of 1p/19q co-deletion supports the diagnosis of oligodendroglioma, especially in cases where the histological findings are atypical (44). However, the very presence of co-deletion is not sufficient to diagnose oligodendroglioma. As many as 20% of glioblastomas may have the oligodendroglial component, 5 to 25% of which have 1p/19q co-deletion (45). Some other tumor types may also mimic oligodendrogliomas: dysembryoplastic neuroepithelial tumors (DNET), neurocytomas, clear cell ependymomas and small cell anaplastic astrocytomas. As these tumors do not have 1p/19q co-deletion, this biomarker is a useful diagnostic aid (44).

The presence of 1p/19q co-deletion also has a role as an important and independent positive prognostic biomarker of the disease. Retrospective and prospective studies showed that when patients with 1p/19q co-deletion are given standard treatment, they have significantly better survival outcome than patients without 1p/19q co-deletion (5, 12, 13, 44, 46, 47). 1p/19q co-deletion also has substantial clinical significance as a strong predictive biomarker for patients with anaplastic oligodendroglial tumors. Its detection predicts longer survival with PCV and radiotherapy in comparison with radiotherapy alone (13, 47), as will be discussed in detail below.

Novel Treatment Paradigm for Anaplastic Oligodendroglioma

As early as 1998, it was found that patients with 1p/19q co-deletion are more sensitive to PCV (48). Nonetheless the evidence-based proof of the significantly longer survival in patients with oligodendrogliomas and 1p/19q co-deletion treated with combined chemotherapy and radiotherapy did not exist for a long time. The long-term follow-up of two important phase III randomized clinical trials with patients suffering from AO treated with PCV, namely RTOG 9402 and EORTC 26951, is bringing substantial results and leading to a paradigm shift of the disease treatment (12,13,46,47).

In the RTOG study 9402, conducted between 1994 and 2002, 291 patients with AO and anaplastic oligoastrocytomas were included and randomized into two treatment arms: PCV with follow-up radiotherapy, and radiotherapy-alone. In the EORTC, study 26951 conducted from 1996 until 2002, 368 patients with AO and anaplastic oligoastrocytomas were randomized into two arms: radiotherapy-alone and RT followed by PCV chemotherapy. The 1p/19q status was determined through fluorescent *in situ* hybridization (FISH) in both studies.

In RTOG 9402, 1p/19q co-deletion was found in 46% of the patients. Over the course of the study, 80% of the patients randomized for radiotherapy subsequently received PCV therapy due to the progression of the disease. After a minimum three-year follow-up in 2006, the median progression-free survival (PFS) was different for the PCV-plus-radiotherapy arm and the radiotherapy-only arm (2.6 and 1.7 years, p=0.004), but the medial OS was similar in both study arms (4.9 and 4.7 years, p=0.26). The OS in patients with 1p/19q co-deletion was longer than in patients without co-deletion (>7 and 2.8 years, p<0.001), but the OS in both treatment arms was not significantly different based on the presence of 1p/19q co-deletion (12). As a result, the positive predictive significance of 1p/19q co-deletion in relation to PCV-plus-radiotherapy was not proven. The absence of a positive effect of combined therapy on the OS and the occurrence of serious adverse effects of PCV in more than 65% of the patients led to skepticism in regard to PCV.

The EORTC 26951 study gave similar results after an average five-year follow-up in 2006. 1p/19q co-deletion was found in 21% of patients. The patients in the arm that received PCV and radiotherapy benefited more than those receiving radiotherapy-alone in PFS (median of 23 and 13.2 months), but the median OS was similar (40.3 and 30.6 months, p=0.23) (13). Patients with 1p/19q co-deletion had longer OS than patients without co-deletion, irrespective of the therapy arm. The results of both studies were considered rather negative in 2006. They did not prove the significance of 1p/19q co-deletion as a predictive biomarker in relation to chemotherapy, but rather showed the significance of 1p/19q co-deletion as a prognostic biomarker.

However, both studies produced decisive results in 2013 following long-term patient monitoring and proved the positive effect of combined oncological treatment (PCV plus radiotherapy) for AO tumors. In the RTOG 9402 study, the median OS in patients without 1p/19q co-deletion remained similar to the results in 2006 in both groups receiving PCV-plus-radiotherapy and radiotherapy-alone (2.6 and 2.7 years). On the other hand in patients with 1p/19q co-deletion, the OS was significantly longer in the PCV-plus-radiotherapy arm than in the radiotherapy-alone arm (14.7 *vs.* 7,3 years respectively, p=0.03). The results were similar in the EORTC 26951 trial. After more than 10 years' follow-up, the OS in patients without 1p/19q co-deletion was similar in the group receiving PCV-plus-radiotherapy and radiotherapy alone (25 and 21 months, p=0.19). However, the median OS was not reached for patients with co-deletion in the PCV plus radiotherapy arm, whereas it was just 9.3 years in patients primarily receiving only radiotherapy.

The positive effect of combined oncological treatment (PCV plus radiotherapy) in patients with 1p/19q co-deletion was present in both clinical studies, irrespective of which type of therapy was started first. The positive effect on OS was also confirmed in patients who, due to the occurrence of adverse effects to therapy, received lower doses of PCV than planned (in RTOG 9402 only 42% of patients tolerated all four intended PCV cycles; in EORTC 26951 only 30% of patients completed all four planned cycles. Both studies proved that neither radiotherapy nor chemotherapy alone is sufficient in AO treatment. These results led to an important paradigm shift in the treatment algorithm of patients with AO tumors.

However, the positive effect of treatment is negatively impacted by the adverse effects. Late radiotherapy toxicity (post-radiation necrosis, dementia) is known, occurring in as many as 10% of patients, even in cases of focused therapy (6, 49). The toxic effects of PCV are even more frequent (50). It is necessary to carefully monitor patients and detect the toxic effects of the treatment early.

Another important question is the administration of combined oncological treatment in patients with AO who do not have 1p/19q co-deletion. The results of the RTOG 9402 and EORTC 26951 studies show this treatment has a positive effect on PFS even among patients without 1p/19q co-deletion. There are probably other molecular factors that have a positive impact on patients prognosis in relation to the combined therapy (33). To answer this important clinically relevant question, the CATNON study (NCT00626990) is currently randomizing patients with AO without 1p/19q co-deletion. The study is investigating the efficacy of another chemotherapeutic agent, temozolomide, during or after radiotherapy compared to radiotherapy alone.

Temozolomide is an effective alkylating cytostatic agent more frequently used for AO than PCV. It has the advantage of oral administration *versus* the intravenous administration of PCV, has fewer adverse events and less frequent termination of treatment due to toxicity (18, 35, 51, 52). The Food and Drug Administration approved temozolomide for the treatment of AO in 1999. The negative results of RTOG 9402 and EORTC 26951 trials in 2006 contributed to its frequent use for AO. For example, in one survey among physicians, temozolomide represents up to 87% of chemotherapy used for AO (4, 53, 54). Positive results of temozolomide therapy for AO comparable to PCV have been described (55). However the study was very small and included only 20 patients. In contrast, a large retrospective analysis assessing the efficacy of PCV-plus-radiotherapy and temozolomide-plus-radiotherapy for the treatment of AO in

1,013 patients reported a median OS of 7.6 years for the PCV regimen compared to only 3.3 years for that with temozolomide (38). For second-line AO treatment in cases of relapse following the failure of PCV, temozolomide was also tested and produced promising results (56).

The German NOA-4 study randomized 318 patients with AO, anaplastic oligoastrocytoma, as well as anaplastic astrocytoma, for radiotherapy, PCV or temozolomide therapy. In cases of toxicity or progression, patients undergoing radiotherapy were randomized into PCV or temozolomide arms and *vice versa*. After the first analysis, there was no significant difference among the individual study arms in PFS or OS. However, in all arms, patients with 1p/19q co-deletion had a better prognosis and reduced relative risk of treatment failure, disease progression or death by about 50%. On the other hand, the follow-up of the study is still too short (maximum 54 months) and features frequent cross-over to other treatment arms (33). To evaluate the effect of temozolomide on oligodendroglioma with 1p/19q co-deletion, the CODEL study (NCT00887146) was planned with three parallel arms: radiotherapy plus temozolomide, radiotherapy alone, and temozolomide alone. Based on the results of RTOG 9402 and EORTC 26951 trials, the radiotherapy monotherapy arm was abolished and it is uncertain whether the study will be reopened. It is expected that the radiotherapy monotherapy arm will be replaced with the PCV plus radiotherapy (4).

Based on the results of these discussed clinical trials, it is currently recommended the 1p/19q status in all patients AO be determined as routine clinical practice as a part of the standard decision-making algorithm in the treatment planning (57). The PCV chemotherapeutic regimen in combination with radiotherapy should now be implemented for all patients with AO with 1p/19q co-deletion. These recommendations mean important changes in novel treatment strategies for patients with AO and anaplastic oligoastrocytoma.

Conclusion

Oligodendrogliomas are among the most explored tumors of the nervous system. Despite the considerable malignant potential of these tumors, a significant number has been shown to respond well to treatment. The positive effect of combined early radiotherapy and PCV chemotherapy for AO and mixed forms, anaplastic oligoastrocytomas with 1p/19q co-deletion, has recently been clearly demonstrated. An equally significant or more positive effect of frequently used temozolomide has not yet been proven. The presence of 1p/19q co-deletion in oligodendroglial tumors is important for diagnosis, and prognosis, as well as prediction of therapy outcome. *IDH1/2* mutations, *MGMT* gene promoter methylation and the hypermethylator status of G-CIMP also have positive prognostic significance. The secondary product of oligodendroglioma research is demonstration of the significance of monitoring patients over the long-term in well-designed clinical trials, in which preliminary results may be inconclusive and only the final results are decisive with regard to evidence-based medicine. The use of PCV-plus-radiotherapy regimen means a novel treatment paradigm for all patients with AO with 1p/19q co-deletion at the moment.

Conflicts of Interests

The Authors declare that they have no conflicts of interests regarding the publication of this article.

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Attachment IX

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Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway

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ABSTRACT

Aberrations in various cellular signaling pathways are instrumental in regulating cellular metabolism, tumor development, growth, proliferation, metastasis and cytoskeletal reorganization. The fundamental cellular signaling cascade involved in these processes, the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR), closely related to the mitogen-activated protein kinase (MAPK) pathway, is a crucial and intensively explored intracellular signaling pathway in tumorigenesis. Various activating mutations in oncogenes together with the inactivation of tumor suppressor genes are found in diverse malignancies across almost all members of the pathway. Substantial progress in uncovering PI3K/AKT/mTOR alterations and their roles in tumorigenesis has enabled the development of novel targeted molecules with potential for developing efficacious anticancer treatment. Two approved anticancer drugs, everolimus and temsirolimus, exemplify targeted inhibition of PI3K/AKT/mTOR in the clinic and many others are in preclinical development as well as being tested in early clinical trials for many different types of cancer. This review focuses on targeted PI3K/AKT/mTOR signaling from the perspective of novel molecular targets for cancer therapy found in key pathway members and their corresponding experimental therapeutic agents. Various aberrant prognostic and predictive biomarkers are also discussed and examples are given. Novel approaches to PI3K/AKT/mTOR pathway inhibition together with a better understanding of prognostic and predictive markers have the potential to significantly improve the future care of cancer patients in the current era of personalized cancer medicine.

Contents

Abbreviations: 4EBP1, 4E-binding protein 1; AKT, protein kinase-B; AMPK, AMP-activated protein kinase; COSMIC, Catalogue of Somatic Mutations in Cancer; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular-signal-regulated kinases; HNSCC, head and neck squamous cell carcinoma; KRAS, Kirsten rat sarcoma viral oncogene homolog; LAM, lymphangioleiomyomatosis; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; MEK, mitogen activated kinase kinase; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; PDK1, phosphoinositide-dependent kinase 1; PEComas, perivascular epithelioid cell tumors; PI3K, phosphatidylinositol 3-kinase; PIK3CA, catalytic domain p110α of the class I phosphatidylinositol 3-kinase; PIK3CB, catalytic domain p110β of the class I phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog; PTEN-Long, long variant of phosphatase and tensin homolog; RHEB, Ras homolog enriched in brain; TCGA, The Cancer Genome Atlas; TSC1/2, tuberous sclerosis complex 1/2.

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1. Introduction

The mechanisms underlying cancer are marked by complex aberrations that activate critical cellular signaling pathways in tumorigenesis. The phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling cascade is one of the most important intracellular pathways, which is frequently activated in diverse cancers (Fig. 1) (Liu et al., 2009; Janku et al., 2012). PI3K/AKT/mTOR signaling regulates cell proliferation, differentiation, cellular metabolism, and cytoskeletal reorganization leading to apoptosis and cancer cell survival. Activation of the PI3K/AKT/mTOR signaling pathway mediated through molecular aberrations is instrumental in promoting tumor development as well as resistance to anticancer therapies (Engelman, 2009; Burris, 2013). In addition, germline mutations in PI3K/AKT/mTOR signaling can cause hereditary disorders associated with a high incidence of cancers. Examples include Cowden's disease associated with loss-of-function of the phosphatase and tensin homolog (*PTEN*) gene (Aslam & Coulson, 2013), tuberous sclerosis complex caused by a mutation in either of the tuberous sclerosis complex 1/2 (*TSC1* and *TSC2*) genes (Kohrman, 2012), and Peutz–Jeghers syndrome, which is linked to a mutation in the *LKB1* gene (also known as *STK11*) (Kuwada & Burt, 2011).

Numerous efforts have been made to develop PI3K/AKT/mTOR targeted therapies for cancer treatment. Various drugs such as PI3K, AKT, or mTOR kinase inhibitors are in clinical development and allosteric inhibitors of mTOR complex 1 (mTORC1), temsirolimus and everolimus, have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treating advanced renal cell cancer (Hudes et al., 2007; Motzer et al., 2008), hormone receptor-positive, HER2-negative breast cancer in combination with hormonal therapy (Baselga et al., 2012), and neuroendocrine tumors of pancreatic origin (Table 1) (Yao et al., 2011). However, these drugs have limited efficacy as single agents. Molecular factors underlying response to them and optimal drug combinations that can act against therapeutic resistance have yet to be identified.

This review delineates the PI3K/AKT/mTOR signaling cascade and emerging molecular targets for targeted therapy in cancer.

2. The biology of the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin pathway

The PI3K/AKT/mTOR pathway can be activated by transmembrane tyrosine kinase growth factor receptors, such as ErbB family receptors, fibroblast growth factor receptors (FGFR), insulin-like growth factor 1 receptor (IGF-1R), and others (Knuefermann et al., 2003; Stern, 2008). In addition, G protein-coupled receptors such as activated RAS (Stephens et al., 1997; Zhao & Vogt, 2008a) can stimulate PI3K through its catalytic subunit (Fig. 1) (Stephens et al., 1997; Shaw & Cantley, 2006; Zhao & Vogt, 2008a). Functional PI3K is translocated to the plasma membrane, ultimately leading to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Zhao & Vogt, 2008a). This step is negatively regulated by PTEN, which dephosphorylates PIP3 to PIP2. In addition, inositol polyphosphate 4-phosphatase type II (INPP4B) converts PIP2 to phosphatidylinositol monophosphate (PIP) (Agoulnik et al., 2011). Subsequently, PIP3 activates serine/threonine kinase phosphoinositide-dependent kinase 1 (PDK1) and AKT (at threonine 308) (Alessi et al., 1997; Cantley, 2002). Phosphorylated AKT activates TSC1/TSC2. This complex serves as a GTPase activating protein (GAP) for RHEB, another important member of the pathway. In its steady state, the TSC1/TSC2 complex causes GTP hydrolysis by RHEB, which converts this protein from its active GTP-binding form to its inactive GDP-binding state. Following PI3K pathway activation, the upstream kinase AKT phosphorylates TSC2, which inhibits the TSC1/TSC2 complex and enables mTOR activation by RHEB, thus allowing signal propagation (Manning & Cantley, 2003). Activation of mTORC1 leads

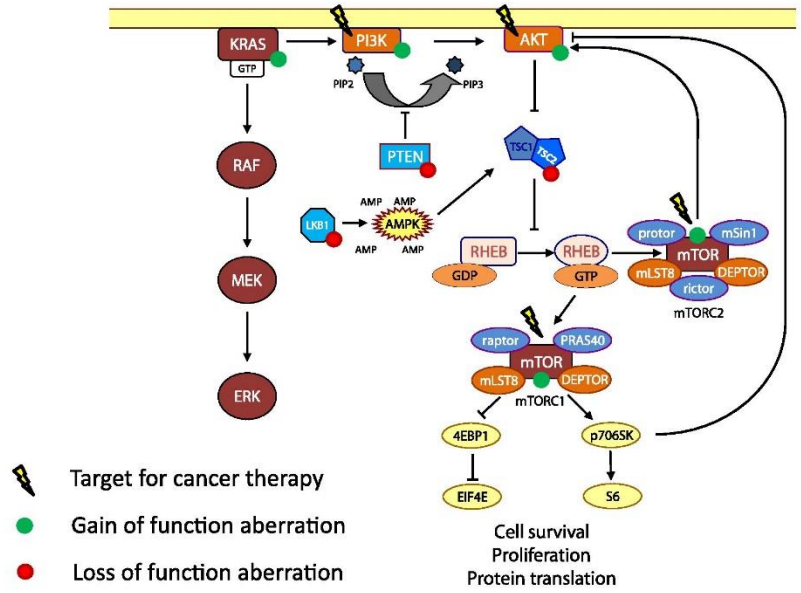

Fig. 1. The PI3K/AKT/mTOR pathway, molecular targets for anticancer therapy and most common locations for gain-of-function aberrations (green) or loss-of function aberrations (red).

Table 1
Inhibitors of the PI3K/AKT/mTOR signaling pathway under clinical development as single agents or in combination.

Target of inhibition		Compound	Phase of the clinical trials	Condition	ClinicalTrials.gov identifier
PI3K	Pan-PI3K	BKM120	I, II, III	Metastatic breast cancer, NSCLC, endometrial cancer, GBM, CRPC, advanced solid tumors	NCT01570296, NCT01550380, NCT01297452, NCT01349660, NCT01385293, NCT01633060
		XL147	I, II	Metastatic breast cancer, endometrial cancer, lymphoma, GBM, advanced solid tumors	NCT00486135, NCT01042925, NCT01082068, NCT01013324, NCT01240460, NCT01357330
		GDC 0941	I, II	Metastatic breast cancer, NHL, advanced solid tumors	NCT01918306, NCT01437566, NCT00996892, NCT00876109, NCT00876122, NCT01740336
		BAY80 6946	I, II	NHL, advanced solid tumors	NCT01460537, NCT01392521, NCT00962611, NCT01411410, NCT01660451
		PX 866	I, II	Metastatic melanoma, mCRC, mSCC, CRPC, GBM, advanced solid tumors	NCT01252628, NCT01616199, NCT01204099, NCT01331083, NCT01259869
	p110α-specific	BYL719	I, II	Metastatic breast cancer, mSCC, mCRC, advanced solid tumors	NCT01870505, NCT01791478, NCT01602315, NCT01219699, NCT01449058, NCT01719380
		INK1117	I	Advanced solid tumors	NCT01449370, NCT01899053
		GDC-0032	I	Metastatic breast cancer, advanced solid tumors	NCT01862081, NCT01296555
	p110δ-specific	CAL 101	I, II, III	Refractory hematologic malignancies	NCT00710528, NCT01306643, NCT01393106, NCT01659021, NCT01090414, NCT01569295
Dual PI3K/mTOR		BEZ235	I, II	Metastatic breast cancer, mCRC, CRPC, refractory acute leukemia, advanced solid tumors	NCT01717898, NCT00620594, NCT01482156, NCT01634061, NCT01756118, NCT01508104
		XL765	I, II	Metastatic breast cancer, GBM, advanced solid tumors	NCT01082068, NCT01240460, NCT00704080, NCT00485719
		PF-04691502	I, II	Recurrent endometrial cancer, advanced solid tumors	NCT01420081, NCT00927823, NCT01347866
		PF-05212384	I, II	Recurrent endometrial cancer, mCRC, advanced solid tumors	NCT01420081, NCT01925274, NCT01347866, NCT01920061
		GDC-0980	I, II	Metastatic breast cancer, CRPC, mRCC, NHL, advanced solid tumors	NCT01437566, NCT01254526, NCT01332604, NCT01485861, NCT01442090, NCT00854152
		SF1126	I	Advanced solid tumors	NCT00907205
		GSK2126458	I	Idiopathic pulmonary fibrosis, lymphoma, advanced solid tumors	NCT01725139, NCT01248858, NCT00972686
AKT		AZD5363	I, II	Metastatic breast cancer, CRPC, advanced solid tumors	NCT01625286, NCT01692262, NCT01353781, NCT01226316
		GDC-0068	I, II	CRPC, metastatic gastric cancer, advanced solid tumors	NCT01485861, NCT01896531, NCT01362374, NCT01562275
		GSK2141795	I, II	AML, advanced solid tumors	NCT01907815, NCT01902173, NCT00920257, NCT01138085
		MK-2206	I, II	Metastatic breast cancer, NSCLC, pancreatic cancer, CRPC, advanced solid tumors	NCT01344031, NCT01248247, NCT01147211, NCT01783171, NCT01369849, NCT01480154
mTOR	mTORC1	Everolimus	Approved	Approved for mRCC, pNET, Advanced ER+/HER2− breast cancer	
		Temsirolimus	Approved	Approved for mRCC	
		Ridaforolimus	I, II	Metastatic breast cancer, endometrial cancer, advanced solid tumors	NCT01605396, NCT01234857, NCT00770185, NCT01295632, NCT01431547, NCT01256268
	mTORC1/mTORC2	AZD2014	I, II	Metastatic breast cancer, mRCC, advanced solid tumors	NCT01597388, NCT01793636, NCT01026402
		CC-223	I, II	NSCLC, NHL, multiple myeloma, advanced solid tumors	NCT01545947, NCT01177397
		INK128	I	Refractory multiple myeloma, advanced solid tumors	NCT01351350, NCT01899053, NCT01058707, NCT01118689
		OSI-027	I	Lymphoma, advanced solid tumors	NCT00698243
		AZD8055	I	GBM, HCC, advanced solid tumors	NCT01316809, NCT00999882, NCT00973076, NCT01194193
		PP242	Preclinical		

Abbreviations: NHL Non-Hodgkin's Lymphoma, GBM Glioblastoma Multiforme, NSCLC Non-Small Cell Lung Cancer, CRPC Castration-Resistant Prostate Cancer, mCRC Metastatic Colorectal Cancer, mSCC Metastatic Squamous Cell Carcinoma, PNET Pancreatic Neuroendocrine Tumor, HCC Hepatocellular Carcinoma.

to increased protein synthesis via its effectors, eukaryotic translation initiation factor eIF4E-binding protein 1 (4EBP1) and p70S6 kinase (S6K), followed by phosphorylation of eukaryotic initiation factor 4E and ribosomal S6 protein (Engelman et al., 2006). Negative feedback also exists, including inhibition of PI3K activation by downstream S6 kinase. Phosphorylation and inhibition of the adaptor protein insulin receptor substrate 1 (IRS-1) negatively influences insulin or insulin-like growth factor 1 PI3K signaling (O'Reilly et al., 2006; Carracedo & Pandolfi, 2008). In addition, phosphorylated AKT increases cell survival by inhibiting the proapoptotic Bcl-2 family members BAD and BAX (Cantley, 2002; Engelman et al., 2006), or phosphorylation of MDM2 that antagonizes p53-mediated apoptosis.

Activation of the PI3K/AKT/mTOR pathway can be mediated by molecular aberrations in PIK3CA, PIK3R1, AKT, TSC1/2, LKB1 and PTEN; however, the most frequent aberrations in cancer are activating mutations in the helical or kinase domain of the *PIK3CA* gene (Janku, 2013).

3. Molecular targets in phosphatidylinositol 3-kinase

Most cancers driven by PI3K/AKT/mTOR signaling aberrations are marked by PI3K kinase mutations. The PI3K protein family comprises at least three different lipid kinase classes (class I, II and III). Class I PI3K contains four different isoforms, catalytic domains p110α (*PIK3CA*), p110β (*PIK3CB*), p110γ (*PIK3CG*) and p110δ (*PIK3CD*). The roles of class II PI3K, PI3K C2α (*PIK3C2A*), PI3K C2β (*PIK3C2B*) and PI3K C2γ (*PIK3C2G*), are ill defined in signal transduction and are generally not involved in human cancers (Vanhaesebroeck et al., 2010). The only class III PI3K, vacuolar protein sorting 34 (*VPS34*; also known as *PIK3C3*), is a critical regulator of autophagy, with its key role in tumorigenesis (Janku et al., 2011; Jaber et al., 2012).

Class I PI3K is the most important kinase in signal transduction (Fig. 1) (Kok et al., 2009). The kinase proteins are heterodimers, which consist of a regulatory and a catalytic subunit. In mammals, class I PI3K is divided into a class IA group with its catalytic subunits p110α, p110β and p110δ, which bind one of the p85 regulatory subunits (p85α, p55α, p50α—all splice variants of the same *PIK3R1* gene, p85β—gene *PIK3R2* and p85γ—gene *PIK3R3*), and a class IB group with its catalytic subunit p110γ, which has two associated regulatory subunits, p101 (*PIK3R5*) and p87 (*PIK3R6*) (Vanhaesebroeck et al., 2010). The p85 regulatory subunit of PI3K stabilizes and protects the p110α subunit from degradation and also inhibits its catalytic activity, thereby negatively regulating signal propagation. The oncogenic character of PI3K can theoretically be gained either by an activating mutation in the catalytic subunit of the enzyme or

by loss of function in the regulatory subunit. This mechanism was subsequently shown to have a role in many human tumors (Samuels et al., 2004; Cheung et al., 2011).

The significance in human cancer of the incidence of somatic mutations in PI3K was initially established using a high throughput sequencing approach in 297 tumor samples, most from colon cancer patients. Interestingly, all mutations were exclusively found in *PI3KCA*. Approximately 80% of *PI3KCA* mutations occur within three hotspot sites (Samuels et al., 2004). One hotspot was found in the kinase domain of the p110α catalytic subunit with its H1047R amino acid substitution. Two other hotspots were identified in the helical domain of *PI3KCA*, including E542K and E545K substitutions. It is now well established that E542K and E545K mutations in the helical domain result in the ineffective regulation of p110α kinase activity by the regulatory subunit. On the other hand, an H1047R mutation in the *PI3KCA* kinase domain induces a conformational change in the activation loop, which can imitate RAS-mediated kinase activation. Both mechanisms lead to PI3K enzyme hyperactivity, aberrantly increasing PI3K/AKT/mTOR signaling in affected cancer cells (Huang et al., 2007; Zhao & Vogt, 2008b).

Mutations in various tumor types can be found in the data of The Cancer Genome Atlas (TCGA) consortium studies that can be accessed via the cBioPortal for Cancer Genomics at http://www.cbioportal.org/public-portal/. The TCGA studies have identified mutations in *PIK3CA* in 53% of endometrial cancers, 35% of breast cancers, 23% of cervical cancers, 21% of gastric cancers, 20% of head and neck cancers, 20% of colorectal cancers, 15% of lung squamous cell cancers, and 10% of glioblastomas as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). Also mutation data from many independent studies are supplemented by the Sanger Institute Cancer Genome Project, which can be accessed via the Catalogue of Somatic Mutations in Cancer database at http://www.sanger.ac.uk/genetics/CGP/cosmic. The COSMIC database has identified mutations in *PIK3CA* in 24% of all human breast cancers, 22% of endometrial cancers, 17% of urinary tract cancers, 13% of colorectal cancers, 13% of cervical cancers, 10% of skin cancers and 9% of ovarian as well as gastric cancers and other tumor types (Fig. 2) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013).

Mutations in *PIK3R1* and *PIK3R2*, which encode the p85α and p85β regulatory subunits of PI3K, have been identified in a relatively high number of endometrial tumors (20% and 5%, respectively). Interestingly, in addition to the loss of function of the PI3K regulatory subunit, gain of function mutations in *PIK3R* were also recognized. These destabilize PTEN through disruption of p85α homodimerization (Cheung et al., 2011). This observation highlights the importance of PTEN and p85 regulatory subunit interactions in cancer. The TCGA studies identified *PIK3R1* mutations in 33% of endometrial cancers, 11% of bladder cancers, 9% of glioblastomas, 5% of gastric cancers, and 4% of colorectal cancers as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). The COSMIC database identified *PIK3R1* mutations in 28% of endometrial cancers, 6% of colorectal cancers, 3% of cervical cancers, and 2% of central nervous system tumors as well as in other tumor types, whereas *PIK3R2* mutations were recurrently observed only in 3% of endometrial cancers. *PIK3R2* mutations are sporadic in other tumor types (Fig. 3) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013).

Along with substantial growth in understanding how PIK3 functions and its role in cancer development, knowledge of the number of specific inhibitors involved is rapidly growing through preclinical studies or in early clinical trials (Rodon et al., 2013) (Table 1). The current experimental drugs that interact with PIK3s can be divided into different groups. The first group of drugs consists of reversible ATP competitive inhibitors of all class I PI3K isoforms, also known as class I PI3K selective inhibitors or pan-PI3K inhibitors, such as GDC 0941, XL147, BKM120 or BAY80 6946. An irreversible pan-PI3K inhibitor, PX 866, is currently undergoing development (Cheng et al., 2011; Wallin et al., 2011; Bendell et al., 2012; Hong et al., 2012; Reynolds et al., 2013). Another important group of experimental drugs that interact with PIK3 is composed of isoform-specific inhibitors, each of which directly interacts with a specific isoform of the catalytic domain of the enzyme. Other drugs include the p110α-selective inhibitors INK1117 and BYL719 (Furet et al., 2013; So et al., 2013), and the p110δ-selective inhibitor CAL 101 (GS-1101), which demonstrated significant activity against lymphoid malignancies in a monotherapy setting (Wiestner, 2012; Macias-Perez & Flinn, 2013). The strategy of dual PI3K and mTOR inhibition targets the pathway at two different levels with the potential to effectively overcome the feedback inhibition ordinary observed when mTORC1 inhibitors are used alone, which limits their efficacy (O'Reilly et al., 2006). These include second-generation dual pan-class I PI3K–mTOR inhibitors, which are undergoing development. These compounds effectively block both catalytic domains of mTOR and the p110 subunit of PI3K due to their structural similarities. SF1126, BEZ235 and XL765 are among the dual PI3K–mTOR inhibitory drug class now being developed and in early clinical trials (Prasad et al., 2011; Mahadevan et al., 2012; Sznol et al., 2013).

Preclinical studies demonstrated that mutations in *PIK3CA* can serve as predictive biomarkers for sensitivity to PI3K/AKT/mTOR inhibitors. These could potentially help select patients with the highest probability of response to such therapeutic agents. In preclinical experiments, head and neck squamous cell carcinoma (HNSCC) cell lines with activating *PIK3CA* (H1047R) hotspot mutations were significantly more sensitive to the dual mTOR/PI3K inhibitor BEZ235 than HNSCC cell lines with wild-type *PIK3CA*. Also, HNSCC mouse xenograft models developed from cell lines with a H1047R *PIK3CA* mutation were more sensitive to BEZ235 monotherapy and combined treatment with BEZ235 and cetuximab (Lui et al., 2013). Breast cancer cell line experiments demonstrated reduced proliferation of cells with the H1047R *PIK3CA* mutation compared to breast cancer cells with wild-type *PIK3CA* after treatment

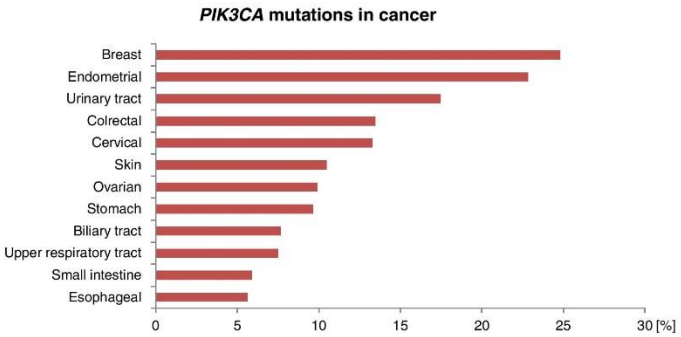

Fig. 2. Prevalence of *PIK3CA* mutations in diverse cancers.

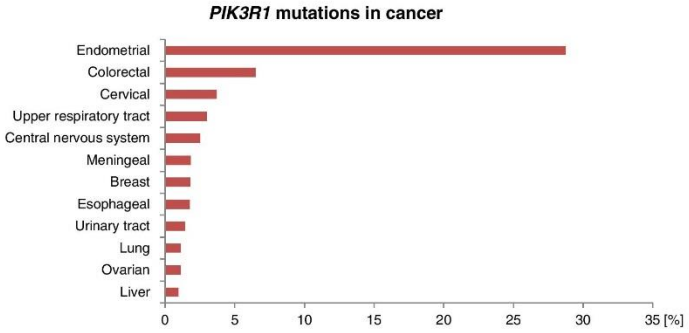

Fig. 3. Prevalence of *PIK3R1* mutations in diverse cancers.

with the mTOR inhibitor everolimus, except when there were concomitantly occurring mutations in *KRAS*, another MAPK pathway member. However, the genetic ablation of mutant *KRAS* in these cells restored the response to everolimus (Di Nicolantonio et al., 2010). In a human non-small cell lung cancer (NSCLC) mouse xenograft model, an H1047R *PIK3CA* mutation was associated with response to the BEZ235 inhibitor as assessed using PET, MRI and microscopic examination (Engelman et al., 2008). However, when the *KRAS* G12D mutation was also present, the xenograft was resistant to BEZ235, but responded to the MEK inhibitor AZD6244 or to a combination of BEZ235 and AZD6244 (Engelman et al., 2008). In another study, various cancer cell lines with simultaneous *PIK3CA* and *KRAS* mutations were resistant to the pan-PI3K inhibitor PX-866, whereas cell lines with only a *PIK3CA* mutation were sensitive to PX-866 (Ihle et al., 2009).

In early clinical trials, a response rate of 36% (6 of 17 patients) was reported in patients with *PIK3CA* mutations treated with therapies targeting PI3K/AKT/mTOR, whereas only 6% (15 of 241 patients) without documented *PIK3CA* mutations treated on the same protocols responded (Janku et al., 2011). Patients with breast and gynecologic malignancies harboring *PIK3CA* mutations treated in phase I clinical trials with PI3K/AKT/mTOR inhibitors had a significantly higher response rate (30% had a partial response rate, 9% had stable disease for more than 6 months) versus a 10% response rate in patients with wild-type *PIK3CA* (Janku et al., 2012). An important observation was that not all *PIK3CA* mutations equally predict response to PI3K/AKT/mTOR inhibitors. The results from early clinical trials demonstrated that patients with advanced cancer and an H1047R mutation in the kinase domain of the p110α catalytic subunit have a significantly higher response rate to PI3K/AKT/mTOR inhibitors than patients with other *PIK3CA* mutations (38% vs. 10%). Multivariate analysis showed that H1047R was the only independent factor predicting response (Janku et al., 2013). An early clinical trial of the p110α-selective PI3K inhibitor BYL719 tested a selected patient population with advanced solid malignancies carrying mutations in *PIK3CA*. This study differed from all clinical studies mentioned above with an unselected patient population. Preliminary data showed PET responses and/or tumor shrinkage in 8 of 17 patients and 8 patients have been on the study for at least 4 months (Juric et al., 2012). Patients with *PIK3CA* mutations and advanced cancers enrolled in early clinical trials had a significantly higher frequency of simultaneous *KRAS* mutations than patients without *PIK3CA* mutations (34% vs. 21%) (Janku et al., 2012). In accord with preclinical PI3K axis inhibitor studies, patients with simultaneous *PIK3CA* and *KRAS* mutations in codon 12 or 13 had lower response rates compared to those without simultaneous *KRAS* mutations (0% and 23%, respectively) (Janku et al., 2013). Therefore, combinatorial treatment strategies that could ensure effective inhibition of both PI3K and MAPK signaling pathways are likely to be the future standard of care, at least for those patients.

4. Phosphatase and tensin homolog alterations in cancer

The tumor suppressor gene *PTEN* (also known as *MMAC*, mutated in multiple advanced cancers) was initially observed independently by two groups in 1997 on the 10q23 chromosomal region of *PTEN*, which was known to be deleted in many advanced cancers (Li et al., 1997; Steck et al., 1997). PTEN is a lipid phosphatase that catalyzes the conversion of the second messenger PIP3 to PIP2 and thus reverses PI3K functionality in signal propagation (Fig. 1) (Maehama & Dixon, 1998). Moreover, it is now well established that PTEN has serine, threonine, and tyrosine phosphatase activity for several protein substrates, resulting in its complex functions in cellular signaling (Shi et al., 2012). PTEN protein consists of 403 amino acids divided into functional domains, the N-terminal PIP2-binding domain (PBD), phosphatase domain, C2 domain and C-terminal tail with two PEST (proline, glutamic acid, serine, threonine) domains, and a sequence governing protein–protein interactions (PDZ) (Lee et al., 1999). These different PTEN domains are relevant to its tumor suppressor function due to the large diversity of tumor-associated mutations observed across all of its domains (Chalhoub & Baker, 2009). The loss of PTEN function can be caused by various mechanisms including mutations, deletions, transcriptional silencing and epigenetic changes (Song et al., 2012). Transcriptional repression and epigenetic silencing of *PTEN* typically occurs via gene promoter hypermethylation (García et al., 2004; Goel et al., 2004).

Two major discoveries about the novel secretory function of PTEN have been observed. An important finding was that PTEN could be stored in and exported from cells in exosomes, transferred among the cells and finally internalized by recipient cells with an appropriate functional effect (Putz et al., 2012). The second discovery was the existence of the long variant of PTEN (termed PTEN-Long), that originated from an alternative translation start site on PTEN mRNA. The additional 173 amino acids in PTEN-Long were found to encode the unique signal sequence that allowed secretion of the protein outside of the cells. Subsequently, PTEN-Long could be absorbed by recipient cells and have normal tumor suppressor function (Hopkins et al., 2013). Both of these mechanisms might be physiologically important for recipient cells with endogenous PTEN deficiency. The intercellular transfer of PTEN could also have a positive impact on tumorigenesis due to the potential dissemination of mutated and ineffective PTEN into the tumor microenvironment that could interfere with the endogenous activity of wild-type PTEN in non-tumor cells. Further research is necessary to find out if it is possible to utilize

intercellular PTEN communication for cancer treatment with exogenously administered wild-type PTEN protein (Papa et al., 2013).

Mutations as well as loss of *PTEN* are found in various tumor types. The TCGA studies identified *PTEN* mutations in 63% of endometrial cancers, 30% of glioblastomas, 8% of cervical cancers, and 7% of skin cancers as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). The COSMIC database identified *PTEN* mutations in 39% of endometrial cancers, 14% of central nervous system tumors, 12% of skin cancers, 11% of colorectal cancers, 10% of prostate cancers, and 5% of cervical cancers as well as in other tumor types (Fig. 4) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013). The complete loss of *PTEN* was also observed in a high number of cases of uterine (33%), renal (27%), salivary gland (20%), colorectal (18%), breast (13%), pancreatobiliary (13%) and prostate cancers (11%) (Janku et al., 2012). Because of the numerous mechanisms underlying *PTEN* inactivation on genetic and epigenetic levels, accurate assessment of *PTEN* status in individual tumor samples remains challenging.

Simultaneous aberrations in *PIK3CA* and *PTEN* are infrequent; however, they can occur in some tumor types such as endometrial cancer (Hayes et al., 2006; Kang et al., 2008). In early clinical trials with 1656 patients with diverse tumor types, 146 (9%) patients had *PIK3CA* mutations, 150 (9%) patients had *PTEN* aberrations but only 14 (1%) patients had simultaneous *PIK3CA* mutations and *PTEN* aberrations (Janku et al., 2012). Although the prevalence of PI3K alterations in some cancer types is relatively low, identifying *PTEN* mutations or aberrations in other parts of the PI3K/AKT/mTOR pathway highlights the need for targeted therapeutic inhibition of this pathway on various downstream levels.

Preclinical models demonstrated that *PTEN* function is an important regulator of G protein-coupled receptor signaling transmitted through *PIK3CB*. *PTEN*-deficient tumors are critically dependent on *PIK3CB* activity and tumors with *PIK3CB* activation are sensitive to *PIK3CB* inhibitors (Jia et al., 2008; Ni et al., 2012). Notably, breast cancer cell lines with *PTEN* loss compared to those with *PIK3CA* mutations demonstrated resistance to treatment with mTOR complex 1 (mTORC1) targeted therapy (Weigelt et al., 2011). However, in early clinical trials, loss of PTEN expression was found in 24% of patients with various solid tumors, and 48% of heavily pretreated and PTEN loss patients treated with a PI3K/AKT/mTOR axis inhibitor experienced a partial response or stable disease greater than or equal to 4 months. This exploration suggests that matching patients with inhibitors based on PTEN loss merits further exploration (Janku et al., 2011). Moreover, early clinical experiments demonstrated that patients with PTEN loss have similar response rates to PI3K/AKT/mTOR targeted therapies, 76% of which included mTORC1 inhibitors, as do patients with *PIK3CA* mutations, or patients with a simultaneous *PIK3CA* mutation and PTEN loss (18% vs. 20% vs. 11%, respectively; $p = 0.83$) (Janku et al., 2012).

5. Molecular targets in protein kinase-B

AKT/PKB (protein kinase-B) is a family composed of three serine/threonine kinases known as AKT1, AKT2 and AKT3 (Fig. 1). These isoforms are products of three different genes, but with more than 80% structural homology. AKT is an important part of PI3K signaling as the activation of the protein is caused by PI3K and PDK1 mediated phosphorylation in the catalytic domain at threonine 308 (Alessi et al., 1997; Andjelković et al., 1997). AKT activation is involved in tumor progression through increased cell proliferation and survival, invasion, metabolism or angiogenesis (Datta et al., 1999). AKT regulates downstream targets in the PI3K pathway such as TSC2 (which leads to activation of mTORC1) and outside of the PI3K pathway such as Bcl-2-associated proteins, glycogen synthase kinase-3β (GSK3β) or MDM2 (Cheung & Testa, 2013). Therefore, the inhibition of AKT as an important node in PI3K signaling could have a significant impact on tumors that are addicted to PI3K/AKT/mTOR axis activity.

The initial observation of an AKT alteration in cancer was the amplification and overexpression of AKT2 in ovarian tumors and ovarian cancer cell lines (Cheng et al., 1992). A subsequent study of 132 ovarian and 106 breast tumors revealed AKT2 amplification in 12% and 3% of each tumor type, respectively (Bellacosa et al., 1995). Amplification and overexpression of AKT2 was also discovered in pancreatic tumors, with a 10% to 20% frequency (Miwa et al., 1996; Ruggeri et al., 1998). Another AKT family member, AKT3, is frequently amplified and overexpressed in diverse human malignancies. AKT3 amplification was observed in hepatitis C virus-associated hepatocellular carcinomas (Hashimoto et al., 2004), glioblastoma (Ichimura et al., 2008; Polivka et al., 2012) as well as in a substantial number of melanomas (Stahl et al., 2004). Activating mutations in AKT1 have been found in breast, colorectal and ovarian tumor samples. This mutation led to the amino acid substitution E17K, which affects the lipid-binding domain of AKT1. The E17K amino acid change in AKT1 alters its lipid binding capacity, which leads to constitutive membrane localization in the absence of PIP3 with a concomitant increase in the signaling function of this kinase (Carpten et al., 2007). The TCGA studies identified *AKT1* mutations in 2.5% of breast cancers, 2% of bladder cancers, 1.7% of endometrial cancers, and 1% of skin cancers as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). The COSMIC database reported *AKT1* mutations in 4% of meningiomas, 3% of breast cancers, 3% of endometrial cancers, 2% of urinary tract cancers, and 2% of thyroid cancers as well as in other tumor types (Fig. 5) (COSMIC

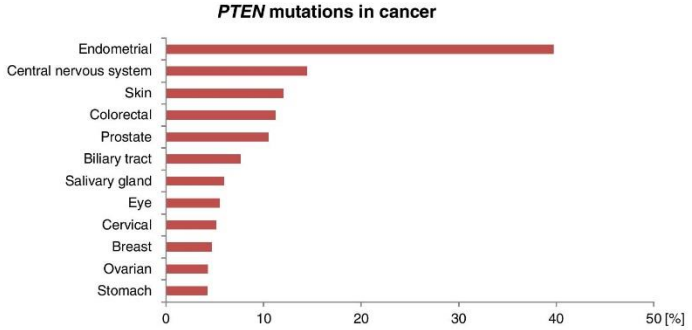

Fig. 4. Prevalence of *PTEN* mutations in diverse cancers.

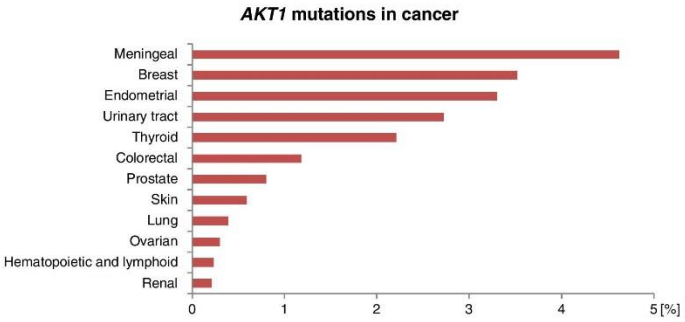

Fig. 5. Prevalence of *AKT1* mutations in diverse cancers.

(Catalogue of Somatic Mutations in Cancer), 2013). The E17K mutation was also identified in AKT3 in melanoma (Davies et al., 2008).

Preclinical studies and early clinical trials have tested various ATP-competitive selective AKT inhibitors such as AZD5363 (Addie et al., 2013), GDC-0068 (Lin et al., 2013), GSK2141795 (Pal et al., 2010) or an allosteric inhibitor of all AKT isoforms, MK-2206 (Table 1) (Yap et al., 2011). AKT inhibitors could be especially effective against cancers with AKT1 mutations and AKT2 and AKT3 amplification. However, non-AKT effectors of PI3K kinase that are hyperactivated by AKT inhibition could limit the efficacy of such drugs in a monotherapy setting (Vasudevan et al., 2009).

In a preclinical experiment, NIH 3T3 mouse embryonic fibroblast cells with the *AKT1* mutation E17K were sensitive to treatment with AKT1 ATP competitive inhibitors. On the other hand, an E17K substitution decreases the sensitivity of 3T3 cells to an allosteric kinase inhibitor. This observation could affect the further development of AKT inhibitors (Carpten et al., 2007). It is currently unknown whether mutations in *AKT* are predictive of the success of AKT targeted anticancer therapy or other inhibitors of the PI3K/AKT/mTOR axis in the clinical setting.

6. Tuberous sclerosis complex 1/2 and liver kinase B1 alterations in cancer

The activation of mTOR in the PI3K signaling pathway is inhibited by the complex of two proteins, hamartin and tuberin, (TSC1/TSC2 complex), which are products of the tumor suppressor genes *TSC1* and *TSC2* (chromosomes 9q34 and 16p13.3, respectively) (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al., 1997, p. 34). In its steady state, the TSC1/TSC2 complex inactivates RHEB. When the PI3K pathway is activated, the upstream kinase AKT phosphorylates TSC2, which inhibits the TSC1/TSC2 complex and enables mTOR activation by RHEB (Fig. 1) (Manning & Cantley, 2003). Germline mutations in *TSC1* as well as *TSC2* cause Tuberous Sclerosis Complex (TSC), a genetic disorder characterized by the formation of benign hamartomas in the kidneys, heart, lung, brain, skin and other organs (Kohrman, 2012). Sporadic *TSC1* mutations have also been seen in a small portion of bladder cancers (Pymar et al., 2008). The TCGA studies identified *TSC1* mutations in 11% of bladder cancers, 3% of endometrial cancers, 3% of lung squamous cell cancers, and 2% of skin cancers as well as in other tumor types. *TSC2* mutations were found in 5% of endometrial cancers, 4% of skin cancers, 3% of gastric cancers, and 2% of cervical cancers as well as in other cancer types (cBioPortal for Cancer Genomics, 2013). The COSMIC database identified *TSC1* mutations in 8% of urinary tract cancers, 4% of endometrial cancers, and 3% of colorectal cancers as well as in other tumor types (Fig. 6). *TSC2* mutations were found in 5% of endometrial, 5% of liver, 3% of cervical, and 3% of lung cancers as well as in other cancer types (Fig. 7) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013).

Importantly, mTOR inhibition with drugs such as everolimus is now being evaluated in various clinical trials enrolling patients with TSC disease (Kohrman, 2012). Everolimus demonstrated efficacy in subependymal giant cell astrocytomas in patients with TSC (Yalon et al., 2011; Cappellano et al., 2013) and is the first mTOR inhibitor approved by the FDA for the treatment of TSC-associated subependymal giant cell astrocytoma. Clinical benefit with

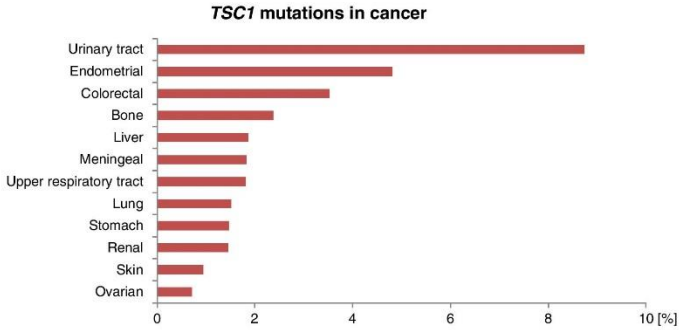

Fig. 6. Prevalence of *TSC1* mutations in diverse cancers.

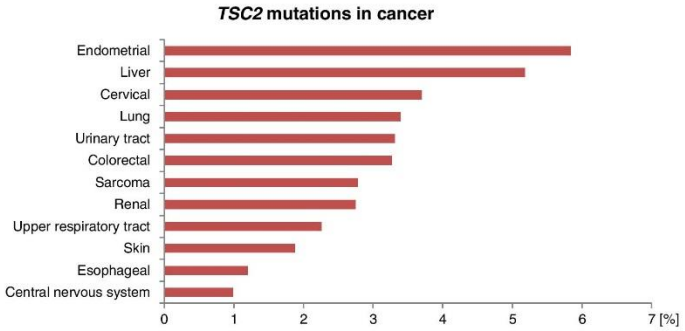

Fig. 7. Prevalence of *TSC2* mutations in diverse cancers.

mTOR targeted treatment was also seen in patients with other TSC associated diseases such as lymphangioleiomyomatosis (LAM) and perivascular epithelioid cell tumors (PEComas). LAM affects approximately 30% to 40% of women with TSC and sporadic LAM has been associated with a loss of heterozygosity in the *TSC2* region (Smolarek et al., 1998). In a clinical trial of 89 patients with LAM treated either with the mTOR inhibitor sirolimus or placebo, the patients in the sirolimus arm had a significant improvement of up to 12 months in their forced vital capacity and residual functional lung capacity of the lung as well as quality of life and functional performance (McCormack et al., 2011). A report of a patient with advanced LAM treated in a phase I clinical trial with the combination of the angiogenesis inhibitor bevacizumab and the mTOR inhibitor temsirolimus showed a sustained response with a 68% decrease in tumor volume after 18 weeks of treatment (Piha-Paul et al., 2011). Another original report of three patients with metastatic PEComas, which are rare mesenchymal neoplasms originating in perivascular epithelioid cells associated with loss of *TSC1* or *TSC2*, showed the high efficacy of sirolimus treatment, leading to radiographic responses in all three patients (Wagner et al., 2010). Of importance is the recently identified strong association between *TSC1* somatic mutations and responses to everolimus in patients with metastatic bladder cancers. Moreover, one complete response lasting for more than 2 years was seen in a patient with coincident *TSC1* and *NF2* mutations (Iyer et al., 2012). These examples illustrate the potential of TSC1/TSC2 aberrations to serve as predictive biomarkers for mTOR inhibitor-based anticancer therapy.

LKB1 is a serine/threonine kinase that phosphorylates AMP-activated protein kinase (AMPK). AMPK subsequently activates the TSC1/TSC2 complex, which negatively regulates mTOR signaling. Germline mutations in the *LKB1* tumor suppressor gene were found in more than 70% of patients with Peutz–Jeghers syndrome. This disease is characterized by multiple gastrointestinal hamartomatous polyps and a lifelong increased risk of various other types of cancers (Launonen, 2005). Interestingly, somatic *LKB1* mutations were also found in some sporadic tumors. The TCGA studies identified *LKB1* mutations in 13% of lung cancers, 2% of cervical cancers, and 1% of skin cancers as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). The COSMIC database identified *LKB1* mutations in 13% of cervical cancers, 10% of small intestine cancers, 8% of lung cancers, and 5% of skin cancers as well as in other tumor types (Fig. 8) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013).

LKB1 was also identified as an important kinase phosphorylating AMPK under various conditions of energy stress (Hardie et al., 2012). Metabolic stress can induce cell growth arrest that partly depends on AMPK activity. *LKB1* mutations led to the insufficiency of this metabolic checkpoint that resulted in rapid apoptosis in an energy stress setting (Shaw et al., 2004). In a preclinical study of LKB1-deficient NSCLC cell lines, treatment with the metabolic drug phenformin was highly effective and led to selective apoptosis in these cells. Additionally, NSCLC mouse models showed that tumors with coincident *KRAS* and *LKB1* mutations responded well to phenformin, resulting in prolonged survival. This was not the case for *KRAS* and *TP53* mutated tumors (Shackelford et al., 2013). The NSCLC cell lines with simultaneous *LKB1*/*KRAS* mutations

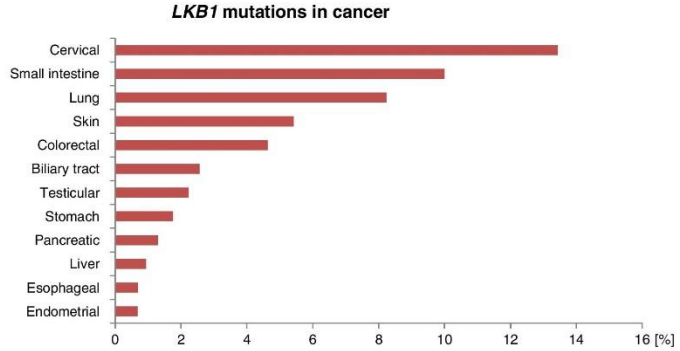

Fig. 8. Prevalence of *LKB1* mutations in diverse cancers.

were sensitive to the MEK inhibitor CI-1040 even though *LKB1* and *KRAS* mutations alone do not confer similar sensitivity. The authors concluded that LKB1/*KRAS*-mutant tumors could be found in a distinct subset of NSCLC that was additionally sensitive to treatment with the mTOR inhibitor rapamycin (Mahoney et al., 2009). The integrated genomic and proteomic profiling study with a mouse model of *LKB1*/*KRAS* mutated lung tumors revealed the additional activation of SRC kinase in those tumors. Moreover, the combination of SRC, PI3K and MEK1/2 inhibition resulted in significant tumor regression (Carretero et al., 2010). In another study, rapamycin treatment significantly slowed the progression of tumors in a mouse model of LKB1-deficient endometrial adenocarcinoma (Contreras et al., 2010). The metabolic drug metformin enhanced the cytotoxicity of gefitinib, an inhibitor of EGFR tyrosine kinase, in squamous cell carcinoma cell lines that expressed intact LKB1, but not in LKB1-deficient NSCLC and cervical cancer cells. However, both groups of cell lines demonstrated synergistic cytotoxic effects in response to the combination of lovastatin and gefitinib (Ma et al., 2012). Treatment with mTOR inhibitors such as everolimus could provide a future option in the chemoprevention of Peutz–Jeghers syndrome as well in the treatment of tumors with somatic *LKB1* mutations (Kuwada & Burt, 2011).

7. Mammalian target of rapamycin alterations in cancer

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase, which acts downstream of the PI3K pathway. It is composed of two distinct protein complexes, mTORC1 and mTORC2, that act on different levels of the pathway. mTORC1 consists of mTOR and other associated proteins such as raptor, mLST8, PRAS40 and DEPTOR (Shaw & Cantley, 2006; Guertin & Sabatini, 2007). The mTORC1 complex functions as a key regulator of cellular growth and protein synthesis. This mTOR complex stimulates S6 kinase activity as well as phosphorylating factor 4E-Binding Protein 1 (4EBP1) that leads to the release of eukaryotic initiation factor 4E (eIF4E), thus initiating translation of specific mRNAs (Engelman et al., 2006). The second mTOR complex, mTORC2, consists of mTOR and other proteins such as rictor, mLST8, DEPTOR, mSin1 and protor. Unlike the mTORC1 complex, mTORC2 positively regulates cell survival and proliferation on different signaling levels, mainly by phosphorylation of AKT (at the serine 473 residue) as well as via serum and glucocorticoid-inducible kinase (SGK) (Fig. 1) (Alessi et al., 2009; Sun, 2013).

Recently, somatic mutations in *mTOR* S2215Y (colorectal carcinoma) and R2505P (renal cell carcinoma) were found to constitutively activate mTOR signaling even under nutrient starvation conditions. These mutations led to mTORC1 complex hyperactivity that imitated RHEB activation of mTORC1, causing signal propagation (Sato et al., 2010). The TCGA studies identified *mTOR* mutations in 10% of endometrial cancers, 8% of gastric cancers, 8% of renal cancers, 6% of skin cancers, and 5% of lung cancers as well as in other tumor types (cBioPortal for Cancer Genomics, 2013). The COSMIC database identified *mTOR* mutations in 13% of endometrial cancers, 6% of renal cancers, 6% of colorectal cancers, 5% of lung cancers, and 3% of skin cancers as well as in other tumor types (Fig. 9) (COSMIC (Catalogue of Somatic Mutations in Cancer), 2013).

The therapeutic inhibition of mTOR is the only FDA- and EMA-approved approach for inhibiting PI3K/AKT/mTOR signaling. Two second generation rapamycin analogs, everolimus and temsirolimus, are available for cancer treatment. Their approved indications are renal cell carcinoma (Hudes et al., 2007; Motzer et al., 2008), advanced hormone receptor-positive, HER2-negative breast cancer (Baselga et al., 2012) and progressive neuroendocrine tumors of pancreatic origin (Yao et al., 2011). These conventional mTOR targeted drugs act as allosteric inhibitors of the mTORC1 complex through interaction with FK-binding protein 12 (FKBP12) (Markman et al., 2010). Although they are effective in cancer treatment under some circumstances, they are generally thought to have only weak activity against the mTORC2 complex (AKT activator), which can lead to AKT activation (Ogita & Lorusso, 2011).

A new generation of mTOR kinase inhibitors is expected to provide a more robust blockade of mTOR signaling via suppression of both mTORC1 and mTORC2 complexes. These ATP-competitive inhibitors of the catalytic activity of mTOR kinase are now in preclinical studies and early clinical trials, and include MLN0128/INK128 (Janes et al., 2013), AZD805, AZD2014 (Naing et al., 2012; Pike et al., 2013), PP242 (Zeng et al., 2012) or OSI-027 (Table 1) (Bhagwat et al., 2011). Unlike the currently approved rapamycin analogs temsirolimus and everolimus, novel generation of dual mTORC1 and mTORC2 inhibitors could be effective in abolishing the feedback activation of AKT induced by rapalogs. Inhibition of mTOR kinase also relieves feedback inhibition of receptor tyrosine kinases. The combined inhibition of both mTOR complexes with novel mTOR inhibitors together with inhibition of activated receptor tyrosine kinases fully abrogated AKT signaling and resulted in cancer cell death and tumor regression in vivo (Rodrik-Outmezguine et al., 2011). Another mechanism of resistance to dual mTORC1/mTORC2 inhibitors was observed in the PP242 study. The feedback activation of ERK in the MAPK pathway after mTOR inhibition was partially overcome with dual mTORC1/mTORC2 and MEK blockade (Blaser et al., 2012; Hoang et al., 2012). The multiple levels of feedback and diverse signaling pathway cross-talk underline the need for further evaluating combined inhibitory approaches in targeted anticancer therapy.

8. Conclusion

The aberrant activation of the PI3K/AKT/mTOR pathway on various levels of signaling is frequently observed in many different human malignancies. In addition to sporadic tumor development are hereditary cancer syndromes caused by hyperactive PI3K signaling, such as Cowden's disease, tuberous sclerosis complex, Peutz–Jeghers syndrome, and others. The development of novel inhibitors that could interact with

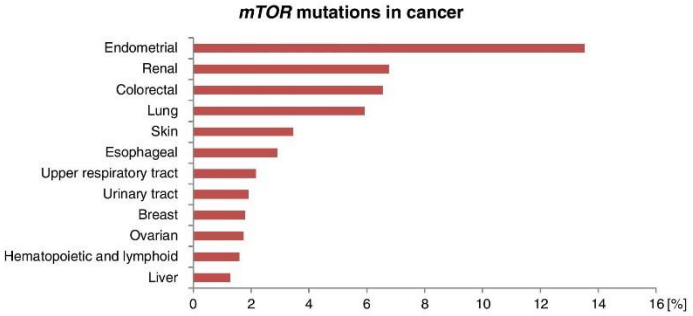

Fig. 9. Prevalence of *mTOR* mutations in diverse cancers.

distinct members of this pathway is sorely needed. To date, only a few PI3K-targeted drugs such as the mTORC1 inhibitors everolimus and temsirolimus have emerged from clinical trials with approval for the treatment of limited types of cancers. These include renal cell carcinoma, advanced hormone receptor-positive, HER2-negative breast cancer, progressive neuroendocrine tumors of pancreatic origin, or TSC-associated subependymal giant cell astrocytoma. The discovery of novel molecular targets and a better understanding of how they function in aberrant PI3K is expected to deliver novel inhibitory molecules to the clinical trial arena such as the newest generation of PI3K, AKT and mTOR inhibitors. The substantial improvement that has been made in defining specific predictive biomarkers that could be used to better stratify patients is now being translated to the clinic. For instance, combinations of PI3K and MEK inhibitors demonstrated encouraging activity in patients with advanced ovarian cancers and RAS or RAF mutations in early phase clinical trials (Janku et al., 2013). In addition, following the success with everolimus and exemestane in hormone-dependent metastatic breast cancer (Baselga et al., 2012), early clinical data with PI3K inhibitors and hormone therapy demonstrated encouraging activity in the same patient population (May et al., 2011; Juric et al., 2012; Nagaraj et al., 2012). Furthermore, combinatorial targeted therapy approaches that can affect various levels of cross-talk among many signaling pathways as well as complex networks of negative feedback will likely be required to provide truly effective anticancer treatment in the era of personalized cancer medicine.

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Conflict of interest

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Attachment X

Polivka J, Polivka J Jr, Rohan V, Priban V. Anaplastic oligodendrogliomas - The age of personalized medicine has arrived? Cesk Slov Neurol N. 2014; 77(4):428-434. **(IF = 0.209)**

Anaplastické oligodendrogliomy – nadešel čas pro personalizovanou medicínu?

Anaplastic Oligodendrogliomas – The Age of Personalized Medicine Has Arrived?

Souhrn

Oligodendrogliomy patří k vzácným, avšak nejlépe prozkoumaným nádorům v neuroonkologii. Již dlouho je známa jejich větší senzitivita na radioterapii a chemoterapii ve srovnání s ostatními gliomy. Specifickým nálezem je častá přítomnost chromozomální kodelece 1p/19q. Teprve výsledky dlouhodobého sledování nemocných ve dvou zásadních klinických studiích fáze III – RTOG 9402 a EORTC 26951 prokázaly příznivý účinek kombinované onkologické léčby radioterapie a chemoterapie v kombinaci prokarbazin, lomustin-CCNU a vinkristin (PCV) u nemocných s anaplastickým oligodendrogliomem a anaplastickým oligoastrocytomem s 1p/19q kodelecí. Přítomnost kodelece 1p/19q je důležitým diagnostickým, pozitivním prognostickým a silným prediktivním biomarkerem oligodendrogliomů. Diskutuje se o dalších molekulárně genetických charakteristikách oligodendrogliomů – mutaci metabolického enzymu Izocitrát dehydrogenázy 1 a 2 (*IDH 1/2*), metylaci promotoru genu pro O-6–metylguanin-metyltransferázu (*MGMT*), hypermetylačním stavu ostrůvků cytozin-guanin nádorového genomu (G-CIMP) a možnosti léčby těchto nádorů. Uvedeny jsou aktuální poznatky optimálního managementu anaplastických oligodendrogliomů respektující zásady personalizované medicíny.

Abstract

Oligodendrogliomas are uncommon but extensively explored tumors in neurooncology. Their superior sensitivity to radiotherapy and chemotherapy in comparison with other gliomas has been known for a long time. The chromosomal codeletion 1p/19q is frequent in this tumor type. The long-term follow up of two landmark phase III trials – RTOG 9402 and EORTC 26951 have resulted in a favorable effect of combined radiotherapy and chemotherapy – procarbazine, lomustine (CCNU), vincristine – in patients with anaplastic oligodendrogliomas and anaplastic oligoastrocytomas carrying the codeletion 1p/19q. This codeletion serves as an important diagnostic, positive prognostic and strong predictive biomarker. The role of the other molecular biomarkers (Isocitrat dehydrogenase – *IDH1*, *IDH2* mutations, methylation of the *MGMT* promoter, glioma cytosine – guanine islands methylator phenotype – G-CIMP) in oligodendroglial tumors is also discussed. All these data determine a new personalised approach to the management and treatment of anaplastic oligodendroglial tumors.

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Key words

anaplastic oligodendroglioma – chromosome deletion – biomarkers – personalized medicine

Úvod

Oligodendrogliální nádory (oligodendrogliomy, oligoastrocytomy) reprezentují asi 5 % primárních nádorů mozku. Od ostatních maligních gliomů se odlišují příznivější odpovědí na radioterapii i chemoterapii. Na základě klasifikace nádorů centrálního nervového systému dle Světové zdravotnické organizace (WHO) z roku 2007 jsou charakterizovány histopatologickým nálezem oligodendrogliální složky. Současná WHO klasifikace však nereflektuje molekulárně genetické charakteristiky nádorů. Studium molekulární genetiky oligodendrogliomů přináší nové poznatky v jejich diagnostice i léčbě a spolu s výsledky klinických studií ovlivňuje jejich management. Toto sdělení uvádí přehled aktuálních poznatků o problematice především anaplastických oligodendrogliomů.

Oligodendrogliomy – základní údaje

Oligodendrogliální nádory lze podle stupně malignity rozlišit na oligodendrogliomy grade II a oligodendrogliomy grade III – anaplastické oligodendrogliomy (AO). Pouze asi 30 % oligodendrogliálnách nádorů obsahuje v histopatologickém obraze anaplastické charakteristiky – jaderné atypie, zvýšenou celularitu, zvýšenou proliferační aktivitu, vyšší počet mitóz. Typický histopatologický nález jsou centrálně uložená kulatá jádra se světlou až prázdnou cytoplazmou v okolí (perinukleární „halo") a dále přítomnost mikrokalcifikací. Anaplastické oligodendrogliomy a vzácnější anaplastické oligoastrocytomy tvoří dvě ze čtyř skupin anaplastických gliomů (další skupiny jsou anaplastické astrocytomy a anaplastické ependymomy) [1].

AO tvoří asi 0,5–1,2 % primárních nádorů mozku [12,3]. Nejvyšší výskyt AO je mezi 45 a 50 roky věku, oligodendrogliomy II. stupně postihují osoby o 7–8 let mladší. Předpokládá se, že tento rozdíl odpovídá progresi z II. do III. stupně nádoru.

Oligodendrogliomy se většinou manifestují epileptickým záchvatem. Další symptomatologie je z nejčastěji postižené frontální, případně temporální oblasti. Infiltrativní růst a nepříliš výrazný perifokální edém způsobují až pozdější projevy nitrolební hypertenze. Oligodendrogliomy grade II jsou v CT obraze většinou hypodenzní, dobře ohraničené, s možným výskytem kalcifikací a nevelkého enhancementu. Obdobně na MR bývá obraz nehomogenního ložiska s nevelkým kolaterálním edémem a přibližně v polovině případů patrným enhancementem. Pro AO je typický CT a MR obraz infiltrativně rostoucího nehomogenního tumoru se solidními a cystickými částmi, s hypodenzními a hyperdenzními (CT), hypointenzitními a hyperintenzitními (MR) ložisky, kalcifikacemi a častým enhancementem a krvácením do tumoru [4].

Zásady léčby AO jsou obdobné jako pro ostatní gliomy; neurochirurgický zákrok a onkologická léčba – radioterapie a chemoterapie. Radioterapie (RT) se užívá v celkové dávce 54–60 Gy a chemoterapie v trojkombinaci prokarbazin, lomustin – CCNU, vinkristin (zkráceně PCV) nebo temozolomid (TMZ) [5,6].

Neurochirurgický zákrok je zásadní pro odstranění nádoru a pro získání nádorové tkáně pro přesnou diagnostiku. Za optimum je považována totální resekce nádoru. Umožňují ji sofistikované diagnostické předoperační a peroperační metody (funkční MR, užití 5–aminolevulové kyseliny – 5–ALA, peroperační ultrazvuk a MR), navigované operace a další rozvoj operačních technik. Požadovaná je pooperační MR (24–72 hod po operaci). Významné jsou i pokroky v intenzivní pooperační péči. Cílená biopsie z nádoru je vyhrazena pro případy, kdy resekce nádoru není možná. Následovat má neuroonkologická léčba [4,7–9].

Již v 80. letech 20. století byla zjištěna radiosenzitivita oligodendrogliomů [10], později také příznivý účinek chemoterapie — PCV a temozolomidu [11–13]. K prognosticky příznivým faktorům patří zejména nízký věk nemocného, jeho dobrý celkový zdravotní stav (Karnofsky skóre), radikalita odstranění nádoru a kombinovaná onkologická léčba [14]. Nutné je pečlivé sledování klinického stavu i kontrolních MR.

Molekulárně genetické charakteristiky oligodendrogliomů

Oligodendrogliální nádory jsou charakteristické častou přítomností chromozomální kodelece 1p/19q. Byla popsána v roce 1994 a jde o vůbec první biomarker v neuroonkologii [15]. Jedná se o ztrátu genetického materiálu z krátkého raménka chromozomu 1 (1p) a z dlouhého raménka chromozomu 19 (19q). Je způsobena nebalancovanou translokací t(1;19)(q10;p10) při níž vzniká derivovaný chromozom 1q/19p [16]. Kodelece 1p/19q se vyskytuje téměř výhradně u oligodendrogliálních nádorů. Většina oligodendrogliomů s kodelecí 1p/19q má také mutaci v genu *CIC*, lokalizovaném na 19q13.2, malá část má mutaci genu *FUBP-1* na chromozomu 1p [17,18]. Tyto mutované tumor supresorové geny se nejspíše mohou uplatňovat při vzniku a progresi oligodendrogliomů, jejich skutečný význam pro nádorovou chorobu bude však ještě třeba ověřit. Frekvence kodelecí 1p/19q je odhadována na 80–90 % u oligodendrogliomů grade II a na 50–70 % u AO [19,20].

Kodelece 1p/19q je považována za významný **diagnostický biomarker**. Její přítomnost podporuje diagnózu oligodendrogliomu, zejména v případech, kdy histologický nález není typický [21]. Samotná přítomnost kodelece však není dostatečná pro diagnózu oligodendrogliomu. Až 20 % glioblastomů může mít známky oligodendrogliální složky, z nich 5 až 25 % má kodeleci 1p/19q. Její význam u glioblastomů zatím není jasný [22]. Také některé další tumory mohou připomínat oligodendrogliom – dysembryoplastický neuroepiteliální nádor (DNET), neurocytom, světlobuněčný ependymom nebo malobuněčná varianta anaplastického astrocytomu. Tyto nádory nemají 1p/19q kodeleci, a její vyšetření je tudíž užitečná diagnostická pomůcka [21]. Kodelece 1p/19q a mutace tumor supresorového genu TP53 slouží také k odlišení od astrocytomů. Oligodendrogliomy s kodelecí nemají zároveň mutace *TP53*, zatímco astrocytomy s kodelecí 1p/19q mají obvykle zároveň mutovaný gen *TP53*. Mutace *TP53* se vyskytují u 5 % oligodendrogliomů, ale až u poloviny astrocytomů grade II a III [23,24].

Přítomnost kodelece 1p/19q má rovněž nezávislý pozitivní význam pro prognózu nemoci (**prognostický biomarker**). Z retrospektivních i prospektivních studií bylo zjištěno, že při standardním léčebném postupu nemocní s kodelecí 1p/19q mají lepší výsledky přežití než nemocní bez této kodelece. Přesný důvod dosud objasněn není, předpokládá se větší senzitivita takových oligodendrogliomů na radioterapii i chemoterapii [6,21,25–28].

Kodelece 1p/19q má podstatný klinický význam jakožto **prediktivní bio-**

marker. Její zjištění predikuje delší přežití při léčbě PCV+RT ve srovnání s RT samotnou [27,28], jak bude dále podrobně popsáno.

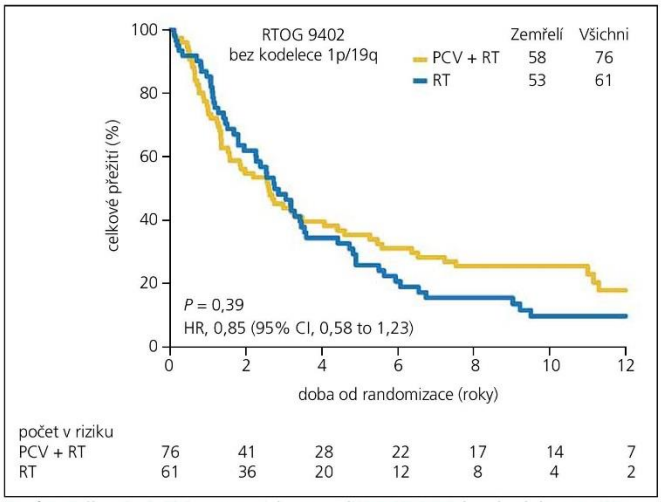
Neuroonkologická léčba anaplastických oligodendrogliomů

Již v roce 1998 bylo zjištěno, že nemocní s kodelecí 1p/19q vykazují senzitivitu vůči PCV [29]. Zásadní výsledky přinesla až realizace dvou randomizovaných studií fáze III, RTOG 9402 a EORTC 26951 [25–28].

Ve studii Radiation Therapy Oncology Group (RTOG) 9402 bylo v letech 1994–2002 zařazeno 291 nemocných s AO a anaplastickým oligoastrocytomem randomizovaných do dvou skupin – PCV s následnou radioterapií a radioterapie samotná. Ve studii European Organisation for Research and Treatment of Cancer (EORTC) 26951 bylo v letech 1996–2002 zařazeno 368 nemocných s AO a anaplastickým oligoastrocytomem randomizovaných do dvou skupin – radioterapie samotná a RT s následnou chemoterapií PCV. V obou studiích byl také stanoven status 1p/19q metodou fluorescenční in situ hybridizace (FISH).

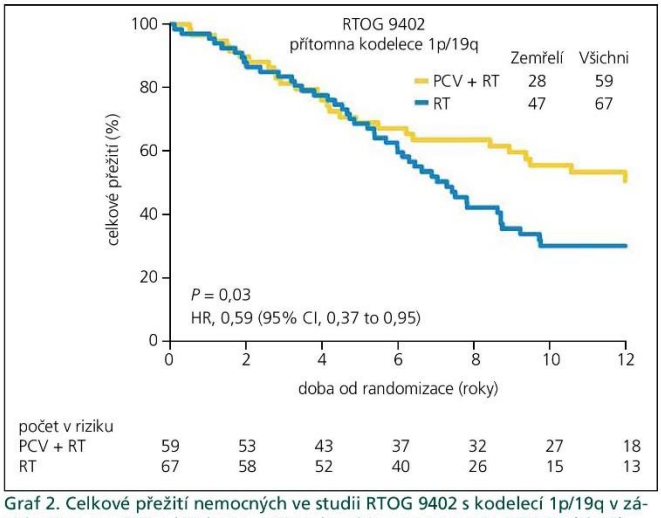
Ve studii RTOG 9402 byla zjištěna kodelece 1p/19q u 46 % nemocných. V průběhu studie bylo 80 % nemocných randomizovaných k RT následně léčeno PCV při progresi onemocnění. Po minimálně tříletém hodnocení výsledků v roce 2006 se lišil medián bezpříznakového období (Progression Free Survival, PFS) u skupiny PCV+RT a skupiny RT (2,6 a 1,7 let, p = 0,004), avšak medián doby celkového přežití (OS) byl v obou skupinách podobný (4,9 a 4,7 let, p = 0,26). Medián OS u nemocných s kodelecí 1p/19q byl delší než u nemocných bez kodelece (>7 a 2,8 let, p < 0,001), avšak medián OS v obou skupinách léčby se podle výskytu kodelece 1p/19q signifikantně nelišil [25]. Nebyl tedy prokázán pozitivní prediktivní význam 1p/19q kodelece ve vztahu k léčbě PCV+RT. Absence příznivého efektu kombinované léčby na dobu celkového přežití a výskyt závažných nežádoucích účinků PCV u 65 % nemocných vedly ke skepsi vůči PCV.

Obdobné výsledky přinesla i studie EORTC 26951 po v průměru pětiletém hodnocení v roce 2006. Kodelece 1p/19q byla zjištěna u 21 % nemocných. Profitovala skupina léčená RT+PCV oproti RT



Graf 1. Celkové přežití nemocných ve studii RTOG 9402 bez kodelece 1p/19q v závislosti na použitém léčebném režimu kombinované terapie PCV+RT (žlutě) nebo RT samotné (modře).

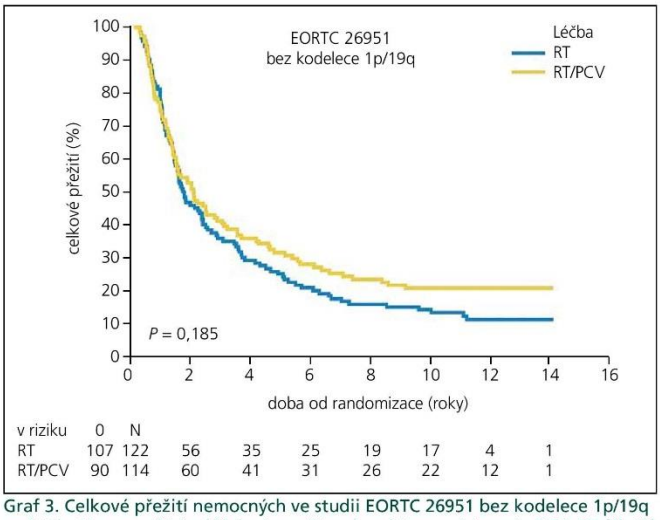
Nebyl prokázán statisticky významný rozdíl v OS. Upraveno z [25].



Graf 2. Celkové přežití nemocných ve studii RTOG 9402 s kodelecí 1p/19q v závislosti na použitém léčebném režimu kombinované terapie PCV+RT (žlutě) nebo RT samotné (modře).

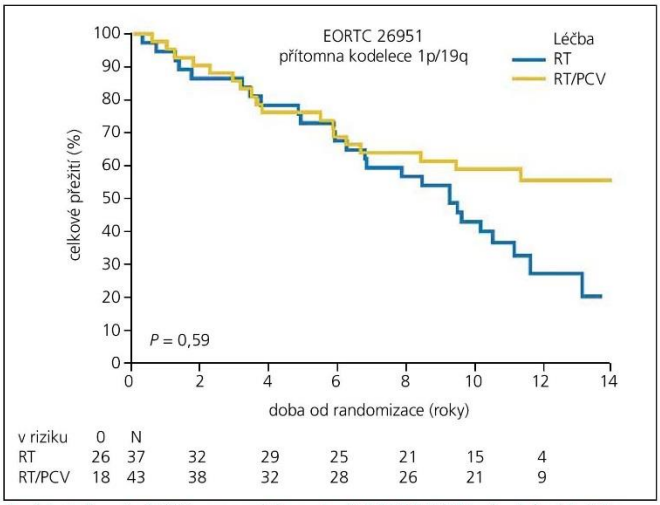
Statisticky významný rozdíl v OS byl prokázán teprve při dlouhodobém sledování nemocných. Upraveno z [25].

samotné při PFS (medián 23 a 13,2 měsíce), ale medián OS byl podobný (40,3 a 30,6 měsíců, p = 0,23) [28]. U nemocných s kodelecí 1p/19q byl nezávisle na léčbě medián OS delší než u nemocných bez kodelece. Výsledky obou stu-



Graf 3. Celkové přežití nemocných ve studii EORTC 26951 bez kodelece 1p/19q v závislosti na použitém léčebném režimu kombinované terapie PCV+RT (žlutě) nebo RT samotné (modře).

Nebyl prokázán statisticky významný rozdíl v OS. Upraveno z [26].



Graf 4. Celkové přežití nemocných ve studii EORTC 26951 s kodelecí 1p/19q v závislosti na použitém léčebném režimu kombinované terapie PCV+RT (žlutě) nebo RT samotné (modře).

Významný rozdíl v OS byl prokázán teprve při dlouhodobém sledování nemocných. Upraveno z [26].

dií v roce 2006 byly považovány za spíše negativní. Neprokázaly význam kodelece 1p/19q jakožto prediktivního biomarkeru ve vztahu k onkologické léčbě, prokázaly pouze význam kodelece 1p/19q jako prognostického biomarkeru.

Rozhodující výsledky přinesly obě studie až v roce 2012 (publikované v roce 2013) po dlouhodobém sledování nemocných. Obě studie prokázaly příznivý efekt kombinované onkologické léčby (RT+PCV) u anaplastických oligodendrogliomů.

Ve studii RTOG 9402 zůstal medián OS u nemocných bez kodelece 1p/19q obdobný jako po tříletém sledování u obou skupin léčených PCV+RT a RT samotnou (2,6 a 2,7 let), avšak u nemocných s kodelecí 1p/19q byl medián OS ve skupině léčené PCV+RT 14,7 let oproti skupině léčené pouze RT – 7,3 let (p = 0,03) (graf 1, 2). Ve studii EORTC 26951 byly výsledky obdobné. Po více než 10letém sledování byl medián OS u nemocných bez kodelece 1p/19q ve skupině léčené RT+PCV a RT samotnou podobný (25 a 21 měsíců, p = 0,19). Avšak u nemocných s kodelecí ve skupině léčené RT+PCV nebyl medián OS dosažen, zatímco ve skupině léčené primárně pouze RT byl medián OS jen 9,3 roku (část nemocných dostala později při progresi onemocnění přidánu chemoterapii PCV) (graf 3, 4).

Pozitivní efekt kombinované onkologické léčby (RT+PCV) u nemocných s kodelecí 1p/19q byl v obou klinických studiích přítomen nezávisle na tom, který typ léčby byl zahájen jako první. Příznivý účinek na OS byl i u nemocných, kteří z důvodu výskytu nežádoucích účinků léčby dosáhli nižší než plánované dávky PCV (ve studii RTOG 9402 pouze 42 % nemocných tolerovalo všechny čtyři zamýšlené cykly PCV, ve studii EORTC 26951 dosáhlo všech šesti plánovaných cyklů PCV pouze 30 % nemocných). Obě studie dále prokázaly, že ani samotná radioterapie, ani samotná chemoterapie není v léčbě AO dostatečná.

Příznivý účinek léčby je však negativně ovlivněn jejími nežádoucími účinky. Je známa pozdní toxicita radioterapie (postradiační nekróza, rozvoj poškození především kognitivních funkcí) s výskytem až u 10 % léčených i při fokusované léčbě [10,30]. Ještě častější jsou toxické účinky PCV (závažné ireverzibilní poruchy krvetvorby a polyneuropatie). Ty jsou nejvíce přičítány vinkristinu, a je tudíž posuzována otázka jeho dávky a použití [31]. Hematotoxicita stupně III nebo IV byla zjištěna u 65 % nemocných ve studii RTOG 9402 a u 47 % nemocných ve studii EORTC 26951. Použití PCV u AO vycházelo ze schémat léčby jiných malignit

a výsledků bylo dosaženo s touto kombinací léků. Je nutné pečlivé sledování nemocných, cílené vyhledávání a včasný záchyt toxických účinků léčby.

Další otázkou je podávání kombinované onkologické léčby u nemocných, kteří nemají kodeleci 1p/19q. Výsledky studií RTOG 9402 a EORTC 26951 prokazují příznivý vliv této léčby na PFS i u nemocných bez kodelece 1p/19q. Předpokládá se existence ještě jiných molekulárních faktorů, které rovněž příznivě ovlivňují léčbu a prognózu [32]. V současné době probíhá klinická studie CATNON (NCT00626990), do níž vstupují nemocní s AO bez kodelece 1p/19q. Je zkoumána účinnost temozolomidu během radioterapie nebo po ní oproti radioterapii samotné.

Temozolomid (TMZ) je účinné alkylující cytostatikum, používané u AO častěji než PCV. Má výhodu orální aplikace oproti intravenóznímu podávání PCV. Má méně nežádoucích účinků než PCV a také méně časté ukončení léčby pro toxicitu [19,33–35]. Pro léčbu AO byl schválen americkým FDA v roce 1999. K jeho častému užití u AO přispěly i relativně negativní výsledky studií RTOG 9402 a EORTC 26951 z roku 2006. TMZ například představuje až 87 % používané chemoterapie u AO [5,36,37]. Byly popsány jeho příznivé výsledky srovnatelné s PCV [38]. Avšak tato studie je malá, šlo jen o 20 nemocných. Naopak retrospektivní analýza hodnotící účinnost RT+PCV a RT+TMZ při léčbě AO u 1013 nemocných ukázala medián celkového přežití pro PCV režim 7,6 let, naproti tomu pro TMZ jen 3,3 roky. Jde však o nehomogenní zdrojová data a výsledek může být zatížen určitou chybou [39]. TMZ byl také zkoušen v další linii léčby AO v případě relapsu onemocnění po selhání PCV se slibnými výsledky [40]. Německá studie NOA-4 randomizovala 318 nemocných s AO, anaplastickým astrocytomem s anaplastickým oligoastrocytomem k léčbě RT nebo PCV anebo TMZ. V případě toxicity nebo progrese byli nemocní s RT randomizováni do PCV či TMZ větve a naopak. Po první analýze není mezi jednotlivými větvemi studie podstatný rozdíl v bezpříznakovém období ani v celkovém přežití. Nemocní s kodelecí 1p/19q měli ovšem ve všech větvích lepší prognózu, snížení relativního rizika selhání léčby a progrese nemoci nebo úmrtí přibližně o 50 %. Výsledky jsou zatíženy nedostatkem dosud krátkého sledování (maximum 54 měsíců) a častým přesmykem do jiných léčebných ramen [33]. Pro posouzení účinku TMZ u oligodendrogliomů s kodelecí 1p/19q byla plánována studie CODEL (NCT00887146) se třemi paralelními větvemi: RT+TMZ, RT samotná, TMZ samotný. Na základě výsledků studií RTOG 9402 a EORTC 26951 byla větev s RT samotnou zrušena a není jisté, zda bude studie opět otevřena. Předpokládá se, že větev samotné RT bude nahrazena větví RT+PCV [5]. Stanovení statutu 1p/19q je aktuálně doporučeno u všech nemocných s AO [41].

Další molekulárně genetické charakteristiky oligodendrogliomů

Rekurentní mutace metabolického enzymu Izocitrát dehydrogenázy 1 a 2 (IDH 1/2) byly poprvé prokázány u multiformního glioblastomu (GBM) [42]. Vyskytují se jen asi u 5 % GBM, pravděpodobně pouze sekundárních. Naproti tomu velká četnost mutací genů *IDH1* a *IDH2* byla nalezena u nízkostupňových gliomů, u oligodendrogliomů grade II a III až u 69–94 % [43,44]. Mutace *IDH1/2* způsobují neomorfní funkci enzymu s následnou akumulací onkometabolitu 2–hydroxy-glutarátu (2–HG) v nádorové tkáni [45]. Buňky s mutacemi v *IDH1/2* a akumulací 2–HG procházejí masivními epigenetickými změnami (DNA a histonové hypermetylace, remodelace chromatinu), což vede k rozsáhlému ovlivnění genové exprese a pravděpodobně podporuje vznik a progresi nádorové choroby [46,47]. Z klinického pohledu je důležité, že přítomnost mutací *IDH1/2* je významný příznivý prognostický biomarker gliomů [48,49]. Navíc bylo zjištěno, že všichni pacienti s gliomem pozitivním na 1p/19q kodeleci mají zároveň mutaci v *IDH1* nebo *IDH2* v nádorové tkáni. Tito nemocní mají také nejlepší prognózu [50]. Na druhé straně existuje skupina gliomů s *IDH1/2* mutacemi, avšak bez přítomnosti kodelece 1p/19q. Takové nádory pak mají horší prognózu než nádory s kodelecí, ale stále významně lepší prognózu než gliomy bez *IDH1/2* mutací [32,51].

Metylace promotoru genu pro O-6–metylguanin-metyltransferázu (*MGMT*) je významný prognostický a hlavně prediktivní biomarker u pacientů s GBM. Nemocní s metylovaným promotorem *MGMT* lépe reagují na TMZ a mají významně delší medián OS než pacienti s intaktním *MGMT* [35,52]. Výskyt metylace promotoru *MGMT* byl zjištěn také u 80 % AO a u 73,1 % anaplastických oligoastrocytomů [39,53]. U těch má význam především prognostický, nikoli prediktivní při léčbě režimem PCV, jak prokázala studie EORTC 26951 i dosavadní výsledky studie NOA-4 [54,55].

Další zkoumaný prognostický biomarker s možným klinickým významem pro pacienty s gliomy je hypermetylační stav ostrůvků cytozin-guanin nádorového genomu (G-CIMP). Pozitivní G-CIMP pravděpodobně není zcela nezávislý biomarker, souvisí totiž úzce s výskytem *IDH1/2* mutací [47,56]. G-CIMP pozitivní gliomy grade II a III mají většinou také metylovaný promotor genu *MGMT*, zatímco G-CIMP negativní nádory mají stejnou alteraci pouze asi v 50 % případů. G-CIMP pozitivita je přibližně dvakrát častější u oligodendrogliomů (93 %) oproti astrocytomům (45 %). G-CIMP je nezávislý příznivý prognostický faktor u všech gliomů včetně oligodendrogliomů [56]. Recentní studie zkoumající celo-genomový metylační stav 46 oligodendrogliálních nádorů odhalila možnost rozdělení G-CIMP pozitivních nádorů do dalších dvou podskupin. G-CIMP pozitivní tumory mající zároveň 1p/19q kodeleci vykazovaly nejlepší OS a byly nejblíže histopatologicky čistým oligodendrogliomům. Druhý subtyp G-CIMP pozitivních oligodendrogliálních nádorů postrádal kodeleci 1p/19q, místo toho ale obsahoval časté mutace *TP53*. Nádory náležící druhému subtypu byly častěji oligoastrocytomy a měly horší prognózu. Pozorovány byly také nádory negativní na G-CIMP. Ty pak zároveň postrádaly 1p/19q kodeleci i mutace *IDH1/2* a měly zcela nejhorší prognózu [57].

Vzácněji byly prokázány též alterace některých dalších známých proonkogenů a tumor supresorových genů u pacientů s AO. Nalezeny byly například mutace v *PI3K*, amplifikace *EGFR* či ztráta tumor supresoru *PTEN* [58,59]. Tyto nálezy zároveň korelovaly s horší prognózou AO. Zjištěn byl také negativní prognostický vliv vysoké hodnoty indexu Ki-67 (MIB-1) na PFS i OS u pacientů s AO [60].

Praktickým výstupem pro určení prognózy a optimalizaci léčby je snaha o vy-

tvoření modelů na podkladě klinických, histopatologických a molekulárněgenetických parametrů. Cílem je identifikace co nejvíce homogenních subtypů nádorů, následná personalizace léčby a další zlepšení terapeutických výsledků. Právě tyto atributy jsou určující pro tzv. personalizovanou medicínu – relativně nový směr v diagnostice, predikci i volbě individualizovaného léčebného postupu. Ideálním stavem by pak měla být správná diagnostika, správná léčba pro správného pacienta ve správném čase [6,14,61,62].

Závěr

Oligodendrogliomy patří k nejlépe prozkoumaným nádorům nervového systému. I přes jejich výraznou malignitu je prokázána citlivost na onkologickou léčbu u značné části z nich. Existuje jasný průkaz příznivého účinku kombinované časné radioterapie a chemoterapie PCV u anaplastických oligodendrogliomů i smíšených forem – anaplastických oligoastrocytomů s přítomnou kodelecí 1p/19q. Stejně významný nebo příznivější účinek nezřídka užívaného temozolomidu u těchto nádorů dosud prokázán nebyl. Často přítomná kodelece 1p/19q u oligodendrogliálních nádorů má význam diagnostický, prognostický i prediktivní. Prognosticky příznivý význam mají také mutace IDH1, metylace promotoru genu *MGMT* a hypermetylační stav ostrůvků cytozin-guanin nádorového genomu (G-CIMP). Předpokládá se význam vyšetření komplexu biomarkerů a na podkladě multifaktoriálních dat stanovení co nejvíce homogenních subtypů nádorů optimálně reagujících na léčbu. Personalizovaný léčebný postup má významný dopad etický i socioekonomický. Vedlejším produktem výzkumu oligodendrogliomů je průkaz významu dlouhodobého sledování nemocných v kvalitně založených klinických studiích, kdy předběžné výstupy mohou být neprůkazné a teprve finální výsledky jsou z hlediska evidence-based medicine rozhodující.

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Attachment XI

Polivka J, Polivka J Jr, Rohan V, Topolcan O. Glioblastoma multiforme - A review of pathogenesis, biomarkers and therapeutic perspectives. Cesk Slov Neurol N. 2013; 76(5):575-583. **(IF = 0.209)**

Multiformní glioblastom – přehled nových poznatků o patogenezi, biomarkerech a perspektivách léčby

Glioblastoma Multiforme – a Review of Pathogenesis, Biomarkers and Therapeutic Perspectives

Souhrn

Multiformní glioblastom patří mezi nejmalignější primární mozkové nádory dospělých a mortalita je velmi vysoká. Standardní terapie tohoto onemocnění zahrnuje operační léčbu, radioterapii a chemoterapii temozolomidem. Má však jen velice omezený efekt na celkové přežití nemocných. Zlepšení zatím velmi špatné prognózy pacientů s glioblastomem by mohlo být dosaženo dalším pochopením procesu vzniku a progrese nádorové choroby na molekulární a genetické úrovni, zavedením nových prognostických a prediktivních biomarkerů nebo rozšířením stávajících terapeutických schémat o nová cílená onkologická léčiva a nádorovou imunoterapii. Tato práce přináší shrnutí aktuálních poznatků v oblasti onkogeneze multiformního glioblastomu. Podrobně zvažuje roli některých nových biomarkerů pro prognózu a predikci onemocnění (mutace v genu izocitrát dehydrogenázy 1 a 2, hypermetylační stav ostrůvků cytozin-guanin nádorového genomu, metylační stav promotoru genu O-6-metylguanin-metyltransferázy). Uveden je také stručný přehled cílených terapeutických přístupů v léčbě glioblastomu, jako například inhibitorů růstových faktorů a jejich receptorů, inhibitorů abnormálních buněčných signálních drah, inhibitorů patologické angiogeneze a nádorové imunoterapie. Práce shrnuje nové poznatky o vzniku a vývoji multiformního glioblastomu a potenciálních budoucích terapeutických možnostech v kontextu personalizované medicíny.

Abstract

Glioblastoma multiforme is the most malignant primary brain tumor in adults with high mortality. Standard glioblastoma therapy consists of surgery, radiotherapy and chemotherapy with temozolomide. However, the overall survival is still very low. Further understanding of the cancerogenetic processes and implementation of novel prognostic and predictive biomarkers as well as targeted cancer therapy and cancer immunotherapy could improve this unsatisfactory situation. This review summarizes current understanding of glioblastoma cancerogenesis as well as the role of novel prognostic and predictive biomarkers (Isocitrate dehydrogenases 1 and 2 mutations, glioma cytosine-guanine island methylator phenotype, promoter methylation status of the *MGMT* gene). New targeted therapeutic approaches, such as growth factor inhibitors and their receptors, inhibitors of intracellular signaling pathways, inhibitors of pathological angiogenesis and tumor immunotherapy are briefly discussed. Novel glioblastoma treatment options are summarized in the context of predictive and personalised medicine.

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Klíčová slova

multiformní glioblastom – biomarkery – molekulární genetika – cílená léčba – personalizovaná medicína

Key words

glioblastoma multiforme – biomarkers – molecular genetics – targeted therapy – personalized medicine

Úvod

Multiformní glioblastom (GBM) je nejčastější a zároveň nejagresivnějším maligní primární mozkový nádor dospělých s incidencí 3–4/100 000/rok [1]. GBM je extrémně invazivní nádor, charakteristický značnou patologickou vaskularizací a vysokou rezistencí vůči standardní radioterapii i chemoterapii. Nově diagnostikovaný GBM je dnes léčen primárně neurochirurgicky, konkomitantní chemoradioterapií a následně standardním režimem adjuvantní chemoterapie (systémová léčba temozolomidem) [2]. Avšak i přes agresivní, správně provedenou multimodální léčbu zůstává medián celkového přežití nemocných s GBM pouze 12,1–14,6 měsíců [3], přičemž jen 3–5 % pacientů přežívá déle než tři roky [4]. Pokrok v oblasti genomiky GBM v poslední době odhaluje množství abnormalit v různých buněčných signálních drahách a značnou rozmanitost mutací jednotlivých genů účastnících se progrese onemocnění. Na významu také nabývá studium nádorového mikroprostředí GBM, především patologické angiogeneze a neovaskularizace. Je studována role nádorových biomarkerů ve vztahu k prognóze i predikci léčebné odpovědi onemocnění.

Histopatologická charakteristika

GBM je primární mozkový nádor vycházející z gliových buněk centrálního nervového systému. Jeho vysoká malignita je charakterizována hypercelularitou, nekrózami, pleomorfizmem, hypervaskularizací a pseudopalisádami [5]. Dělí se na dva typy – primární, rozvíjející se přímo jako GBM, a sekundární, vznikající transformací nízkostupňového gliomu [6]. Sekundární GBM se vyskytuje u mladších osob (medián 45 let) a představuje pouze 5 % GBM. Oba typy nelze histopatologicky odlišit [7].

Genetika gliomů

Onkogeneze je dnes chápána jako děj na genové úrovni, hereditárně a/nebo somaticky navozená porucha komplexních regulačních funkcí buňky, mimo jiné ve vztahu k regulaci buněčného cyklu, buněčné proliferace či apoptózy. Uplatňuje se aktivace onkogenů a utlumení tumor supresorových genů. Významnou roli hrají také epigenetické mechanizmy genových expresí (metylační status DNA, změny chromatinu, role mikroRNA a další). Právě tyto molekulární a genové charakteristiky jsou zásadní pro prognostické a prediktivní účely, pro léčebné intervence a hledání nových terapeutických možností.

Komplexní analýza lidského genomu z počátku tohoto tisíciletí byla brzy následována analýzou genomu vybraných nádorových onemocnění. Nejinak je tomu také u GBM. V jedné z prvních studií tohoto směru bylo zkoumáno 22 vzorků GBM, u kterých bylo stanoveno celkem 20 661 protein-kódujících genů. Zároveň byly popsány nejdůležitější genetické alterace, jež mohou být odpovědné za vznik GBM (mutace DNA, jako jsou substituce, inzerce, delece, amplifikace a další) [8]. Touto analýzou byly zjištěny mutace genů pro proteiny účastnící se důležitých buněčných signálních drah:

1. RAS a PI3K-AKT signalizace s alteracemi v EGFR/PI3K/PTEN/NF1/RAS,
2. tumor supresorová signalizace p53 s mutacemi v TP53/MDM2/MDM4/p14^{ARF},
3. regulace buněčného cyklu s mutacemi v RB1/CDK4/p16^{INK4A}/CDKN2B a
4. ovlivnění buněčného metabolizmu a metabolických kaskád s alteracemi izocitrátdehydrogenázy [8–10].

Práce přinesla první ucelený pohled na možné onkogenní genetické změny u GBM a jejich poměrné zastoupení při analýze nádorových vzorků od většího počtu pacientů s tímto onemocněním.

Nová klasifikace GBM

Glioblastom byl i jedním z prvních nádorových onemocnění, které bylo zkoumáno v rámci ambiciózního projektu nádorových atlasů pro vybrané onkologické diagnózy – The Cancer Genome Atlas (TCGA) – pod patronací The National Institute of Health (NIH) v USA. Na vzorku 500 GBM od předtím neléčených nemocných byly stanoveny změny na úrovni DNA, mRNA i krátkých nekódujících mikroRNA. Získané poznatky vedly k novému rozdělení do té doby homogenní skupiny GBM do čtyř subtypů dle rozdílných genových expresních profilů. Vzniklé dělení zahrnuje klasický, mezenchymální, proneurální a neurální subtyp GBM, přičemž zařazení konkrétního GBM je možné dle exprese určitých signaturních genů [11]. Z klinického hlediska jsou důležité odlišné reakce jednotlivých subtypů GBM na léčebné intervence. Až další výzkum ukáže, zda bude možné v budoucnu takováto i jiná dělení využít také jako pomoc při lepší personalizaci terapie a rozhodování o výběru nových cílených onkologických léčiv pro konkrétního pacienta.

Nové prognostické biomarkery pro GBM

Spolu s bouřlivým rozvojem poznatků základního výzkumu nádorů významně roste i počet nově objevených molekulárních biomarkerů těchto onemocnění. Nádorové biomarkery nacházejí stále častěji využití v upřesnění primární diagnózy, podrobnější klasifikaci onemocnění, jako prognostické ukazatele či přímo v predikci úspěšnosti konkrétního léčebného režimu. Přes obrovský počet nově popsaných molekulárních biomarkerů nádorů se však do klinické praxe dostaly jen některé. Je to způsobeno především problémy v jejich širší standardizaci a validaci.

Výzkum GBM přinesl také několik perspektivních molekulárních biomarkerů, čekajících na své definitivní ověření a zařazení do klinické praxe. Mezi jinými jsou to zejména mutace genu izocitrát dehydrogenázy (IDH), hypermetylační stav ostrůvků cytozin-guanin nádorového genomu (glioma CpG island methylator phenotype G-CIMP), nebo metylační stav promotoru genu O-6-metylguanin-metyltransferáza (MGMT).

IDH jako biomarker GBM

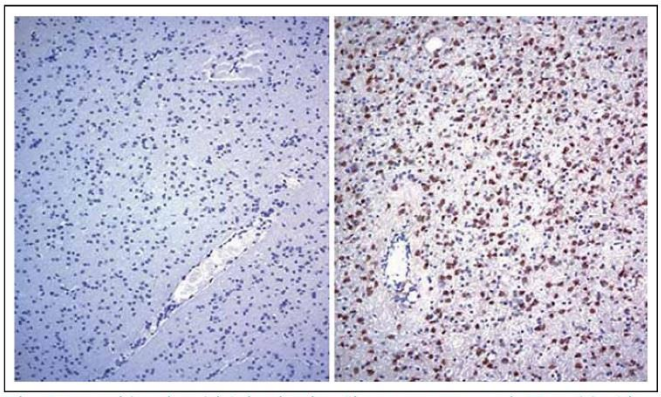
Izocitrát dehydrogenáza (IDH) patří mezi důležité enzymy Krebsova cyklu. Katalyzuje oxidativní dekarboxylaci izocitrátu na na α-keto-glutarát (α-KG), přičemž redukuje nikotinamid adenin dinukleotid fosfát (NADP+) na jeho redukovanou formu NADPH. IDH tedy působí v jednom z kritických kroků sacharidového, lipidového i aminokyselinového metabolizmu. Lidský enzym IDH má tři izoformy – IDH1 (výskyt v cytoplazmě a peroxizomech) a IDH2 a 3 (v mitochondriích) [12]. Rekurentní mutace v IDH a jejich souvislost se vznikem a progresí nádorové choroby byla poprvé popsána právě u GBM [13]. Vyskytují se však jen u 5 % GBM, pravděpodobně pouze sekundárních (s využitím nové klasifikace u proneurálního subtypu GBM). Naproti tomu velká četnost mutací genů pro IDH1 a IDH2 byla nalezena zejména u nízkostupňových gliomů (70–80 %) a anaplastických astrocytomů (až 50 %) [8,14,15]. Mutace jsou téměř vždy pouze v jedné alele, IDH1 vykazuje

v 90 % konzervativní substituci R132H, známé jsou také R132C, R132G, R132S a R132L. Mutace v IDH2 jsou mnohem vzácnější, převážně aminokyselinová substituce R172 [15,16].

Skutečným přelomem v pochopení významu identifikovaných IDH1/2 mutací pro onkogenezi gliomů bylo až zjištění, že takto mutovaný enzym nabývá zcela nové, neomorfní funkce. Místo NADP+ dependentní produkce α-KG mutovaná IDH katalyzuje NADPH dependentní redukci α-KG na 2-hydroxy-glutarát (2-HG). Gliomy s mutací v IDH1/2 pak obsahují vysoké koncentrace 2-HG, na rozdíl od nádorů bez takové mutace [17]. Potenciální onkometabolit 2-HG je dáván do přímé souvislosti se vznikem a progresí nádorového onemocnění. Onkogenní funkce 2-HG vychází pravděpodobně ze schopnosti tohoto metabolitu inhibovat mnohé α-KG dependentní dioxygenázy (enzymy mající rozličné modulující funkce v mnoha buněčných dějích, jako například demetylace histonů, demetylace DNA, metabolizmus mastných kyselin, modifikace kolagenu nebo odpověď buňky na hypoxii) [18]. Buňky s mutacemi v IDH1/2 pak procházejí masivními epigenetickými změnami, zahrnujícími DNA a histonové hypermetylace, což vede také k remodelacím chromatinu a rozsáhlému ovlivnění genové exprese [19,20,21]. Takové změny spolu s deregulovanou odpovědí buněk na hypoxii mohou být jednou z příčin vzniku a progrese nádorové choroby.

Z několika prvních prací je zřejmý značný klinický význam mutací v IDH1/2 s kumulací onkometabolitu 2-HG. Mutační stav IDH1/2 vystupuje jako silný prognostický faktor nejen u pacientů s GBM, ale i s gliomy nižších stupňů. Pacienti s GBM a mutacemi v IDH1/2 jsou zpravidla mladší a mají výrazně delší medián celkového přežití než pacienti bez mutací, napříč několika studiemi je to 3,8 vs 1,1 roku, 2,6 vs 1,3 roku, 2,3 vs 1,2 roku nebo 3 vs 1 rok [8,15,22,23]. Ještě významnější rozdíly v mediánu celkového přežití pacientů byly pozorovány u anaplastického astrocytomu, a to 5,4 vs 1,7 roku, 6,8 vs 1,6 roku a 7 vs 2 roky [15,22,23]. Podobně také u gliomu grade II, a sice 12,6 vs 5,5 let [22].

Rostoucí klinický význam mutačního stavu IDH1/2 přináší rovněž nutnost vypracovat standardizovaný postup pro stanovení tohoto biomarkeru v nádorové tkáni s co nejvyšší senzitivitou i specificitou. Základní možnosti určení mutačního stavu IDH1/2 zahrnují postupy imunohistochemie [13,24] a molekulární biologie (obr. 1) [25,26]. Ty však mohou být doplněny či zcela nahrazeny neinvazivním stanovením onkometabolitu 2-HG v nádoru metodami MR-spektroskopie [27–30]. Obrovskou výhodou takového postupu je kromě nulové invazivity také nezávislost na sekvenčním typu mutací v IDH1/2. Stanoven je až výsledek neomorfní funkce změněného enzymu, který je také pravděpodobně přímo odpovědný za onkogenní chování buněk. Takový přístup představuje v onkologii zatím zcela ojedinělý případ, kdy lze pomocí běžně dostupných zobrazovacích metod prokázat konkrétní genetickou mutaci nádoru. Teprve další výzkum odpoví na otázku, zda by mohlo být inhibicí mutované IDH1/2 nebo přímou deplecí 2-HG z nádorové tkáně dosaženo také terapeutického efektu a prodloužení přežití pacientů.



Obr. 1. Imunohistochemická detekce buněk astrocytomu grade III pozitivních na IDH1-R132H mutaci (vpravo) a vzorku nádoru bez této mutace pro srovnání (vlevo). Převzato z [21].

Sekvenační genetické studie IDH1/2 mutovaných astrocytárních a oligodendrocytárních mozkových nádorů odhalují ještě širší molekulární souvislosti [31]. IDH1 mutované astrocytomy vykazují často také aberace v TP53 apoptotické signalizaci, zatímco většina oligodendrogliomů má současně kodeleci chromozomů 1p/19q [32]. Celo-exonovým sekvenováním byly nově prokázány rekurentní mutace v genech CIC (homolog of Drosophila capicua) a FUBP1 (Far Upstream element Binding Protein) oligodendrogliomů [33]. U většiny IDH1 mutovaných astrocytomů nižších stupňů byla také zjištěna mutace v genu ATRX (Alpha Thalassemia/mental Retardation syndrome X-linked) [34]. Existují tedy minimálně dva rozdílné subtypy gliomů s IDH1/2 mutacemi. IDH1/CIC/FUBP1 mutované nádory korelují převážně s oligodendrogliálním histopatologickým typem gliomu, zatímco IDH1/ATRX/TP53 mutace odpovídají typicky astrocytomům grade II a III a sekundárním GBM [32]. Klinický význam takového rozdělení IDH1/2 mutovaných gliomů bude nutno ověřit v prospektivních studiích.

G-CIMP jako biomarker GBM

Dalším novým biomarkerem s možným klinickým významem pro pacienty s GBM je G-CIMP. Hypermetylační stav ostrůvků cytozin-guanin gliomového genomu je zkoumán převážně jako prognostický biomarker ve vztahu k celkovému přežití pacientů. G-CIMP však pravděpodobně není zcela nezávislým biomarkerem. Úzce totiž souvisí s výskytem IDH1/2 mutací [35]. Gliomy bohaté na G-CIMP je možné zařadit převážně k proneurálnímu subtypu (klasifikace GBM dle rozdílných expresních profilů), u kterého byla nalezena také naprostá většina mutací v IDH1/2. Experimentálně pak byla prokázána přímá souvislost mutací v IDH1/2 a výskytu G-CIMP. Bylo zjištěno, že samotný výskyt mutace v IDH1/2 a kumulace 2-HG je postaču-

jící faktor pro vznik G-CIMP [36]. Role G-CIMP jako prognostického nebo prediktivního biomarkeru GBM bude muset být ověřena v dalším výzkumu, který bude brát v úvahu také komplexní vztahy s mutacemi IDH1/2 i ostatní potenciální bio markery.

MGMT jako biomarker GBM

Oba zatím diskutované biomarkery (IDH1/2 mutace i výskyt G-CIMP) mohou sloužit převážně v roli prognostických faktorů. Je ovšem nutné hledat také primárně prediktivní biomarkery. Tedy takové, které pomohou předpovědět odpověď pacienta na konkrétní terapii. Jako prediktivní biomarker GBM je klinické praxi nejblíže alterace genu pro O-6-metylguanin-metyltransferázu (*MGMT*). Konkrétně pak metylační status promotoru tohoto genu. Enzym MGMT je schopen účinně opravovat poškození DNA, způsobené alkylační chemoterapií (to platí také pro temozolomid, standardně užívaný v léčebném schématu GBM). Pokud je promotor *MGMT* genu metylován, tvorba aktivního enzymu je snížena a odpověď pacientů na léčbu temozolomidem je vyšší. Pacienti s metylací promotoru genu *MGMT* měli signifikantně delší medián celkového přežití oproti pacientům bez metylace, a to 21,7 vs 15,3 měsíců [2,37]. Metylační stav promotoru genu MGMT představuje pozitivní prediktivní biomarker i ve vztahu k samotné radioterapii [38]. Byla také zjištěna silná pozitivní korelace mezi metylačním stavem *MGMT* a výskytem G-CIMP. Zvýšená metylace promotoru genu *MGMT* tak nejspíše souvisí s celkovou hypermetylací DNA u G-CIMP [39]. V dalším výzkumu bude nutné detailněji popsat vzájemné korelace diskutovaných bio markerů a jejich zastupitelnost. I tak se zdá, že tento nový biomarker najde brzy klinické využití v predikci lepší léčebné odpovědi na chemoterapii i radioterapii u pacientů s GBM.

Standard léčby GBM

Standardní léčebný postup nemocných s GBM byl formulován v roce 2005 a vychází z výsledků klinické studie fáze III prováděné European Organisation for Research and Treatment of Cancer (EORTC) a National Cancer Institute of Canada (NCIC) [2]. Zahrnuje neurochirurgickou léčbu (co nejradikálnější odstranění nádoru), radiační léčbu (60 Gy ve 2-Gy frakcích) a temozolomid (75 mg/m^2 po 42 dnů). Po konkomitantní chemoradioterapii následuje šest cyklů temozolomidu (150 až 200 mg/m^2/den po pět dnů každý 28. den cyklu). Použitím tohoto léčebného schématu došlo k signifikantnímu zlepšení mediánu celkového přežití z 12,1 na 14,6 měsíců a dvouletého přežití z 10 % na 26,5 % nemocných. Výsledky z pokračování studie prokázaly pětileté přežití ve skupině pacientů léčené radioterapií 1,9 % oproti 9,8 % ve skupině léčené radioterapií a temozolomidem. I když vstup do studie prospektivně nezohledňoval výše uvedené bio markery, bylo prokázáno, že metylační stav promotoru genu *MGMT* je významný predikční faktor příznivého průběhu nemoci u nemocných léčených temozolomidem [40]. V naší literatuře publikovali výsledky léčby souboru 86 nemocných s GBM Lakomý a spolupracovníci [41].

Optimalizace léčby a její cíle

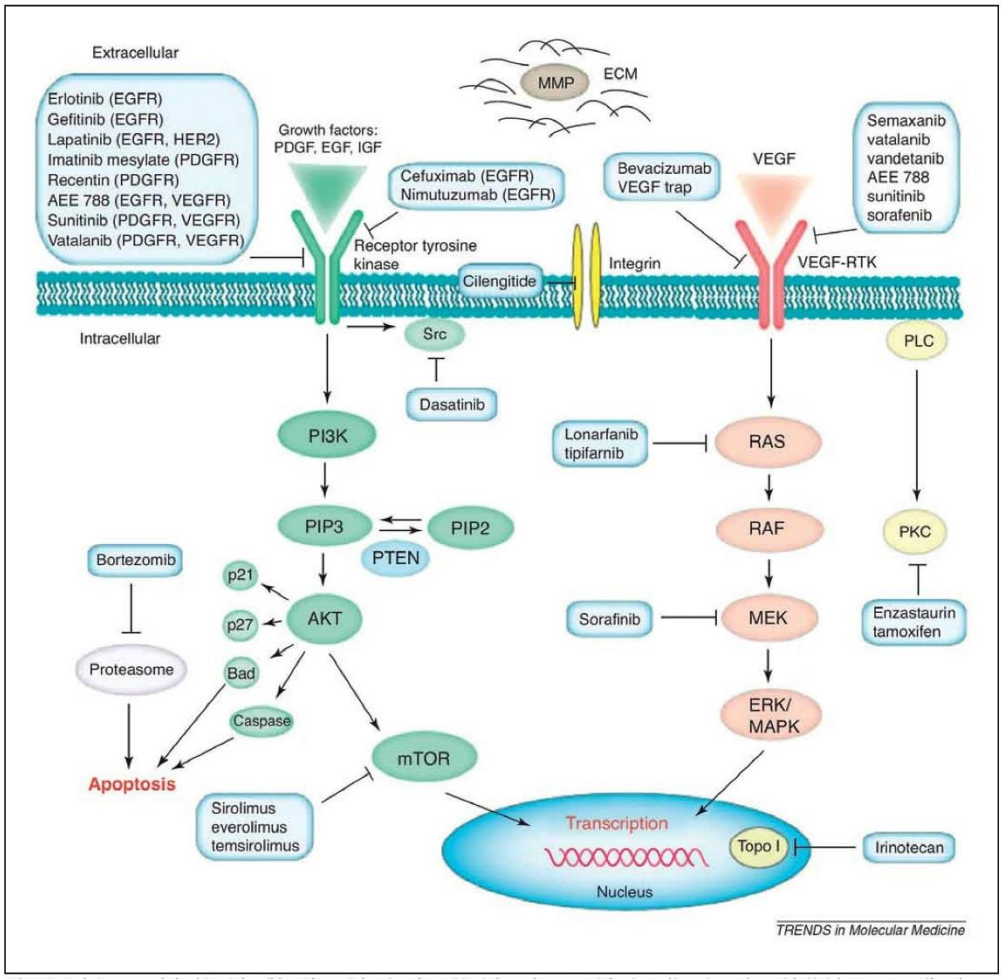
Logickým cílem v péči o pacienty s GBM je další optimalizace léčebného postupu. Té lze dosáhnout několika způsoby. Především zlepšením přesnosti neurochirurgické léčby s cílem odstranit co největší masu nádoru, a to za pomoci co nejkvalitnější předoperační a perioperační diagnostiky. Radikalitu resekce zlepšuje použití 5-aminolevulinové kyseliny – 5-ALA, umožňující fluorescenční značení nádoru. Multivarietní analýza provedená v této studii zároveň prokázala, že nejvýznamnějšími faktory pro délku přežití nemocných s GBM jsou věk, stav před operací a radikalita resekce nádoru [42]. Prognostický význam přináší také použití intraoperační magnetické rezonance, kterou lze dosáhnout větší radikality resekce [43]. Limitací je však omezená dostupnost metody. Optimalizace léčebného postupu lze dále docílit použitím uvedených biomarkerů a vytipováním nemocných, u kterých je předpoklad, že optimálně reagují na standardní léčbu, a tu jim včas poskytnout, případně ji intenzifikovat. Dále také hledáním a uplatněním nových léků a jejich kombinací se standardními terapeutickými možnostmi. V neposlední řadě hledáním a uplatněním dalších biomarkerů pro stanovení prognózy nemocných a predikce účinnosti léčby a pro monitorování léčebných výsledků.

Nové možnosti léčby glioblastomu

Genetické studie a zjištěné molekulární charakteristiky GBM umožnily identifikovat nové cíle, které bude možno v budoucnu účinně terapeuticky ovlivnit. Nové potenciální možnosti léčby GBM mohou být zaměřeny na ovlivnění změněných buněčných signálních a metabolických kaskád, na ovlivnění neovaskularizace a patologické angiogeneze, nádorového mikroprostředí nebo nádorové imunitní odpovědi. Hlavním důvodem prozatímního relativního neúspěchu takovýchto léčebných možností je významná heterogenita molekulárněgenetických aberací GBM v době diagnózy. V následujícím textu jsou stručně uvedeny příklady některých terapeutických cílů a odpovídajících nových onkologických léčiv a dosavadních výsledků jejich testování v klinických studiích pro léčbu pacientů s GBM.

Cílená onkologická léčba GBM – inhibitory růstových faktorů a jejich receptorů, inhibitory nitrobuněčných signálních kaskád

Jedná se o skupinu relativně nových látek schopných cíleně ovlivnit (inhibovat) konkrétní aberantně aktivované buněčné signalizace, vedoucí ke vzniku a progresi nádorového onemocnění. Takového efektu je dosaženo například inhibicí specifických růstových faktorů a jejich receptorů (rodina epidermálních růstových faktorů EGF a jejich receptorů EGFR, destičkové růstové faktory a jejich receptory PDGFR, inzulinu podobné růstové faktory IGF, fibroblastové růstové faktory FGF a jejich receptory a jiné) (obr. 2). Nadměrná exprese různých růstových faktorů i jejich receptorů byla popsána přibližně u 50 % GBM [9,45]. Příkladem léku v onkologii dnes standardně používaného s cíleným účinkem proti EGFR je gefitinib. Testován je také v klinických studiích pro léčbu GBM [46–48]. Dalším zavedeným inhibitorem EGFR je erlotinib, rovněž zkoušený u GBM [49–53]. Lapatinib je také inhibitorem EGFR, testovaným v klinických studiích fáze II u nemocných s GBM [54,55]. Ze skupiny monoklonálních protilátek cílených proti EGFR byl pak testován například cetuximab [56]. Pozorovaný účinek takových léků je u GBM zatím malý. Lepších výsledků by mohlo být dosaženo při



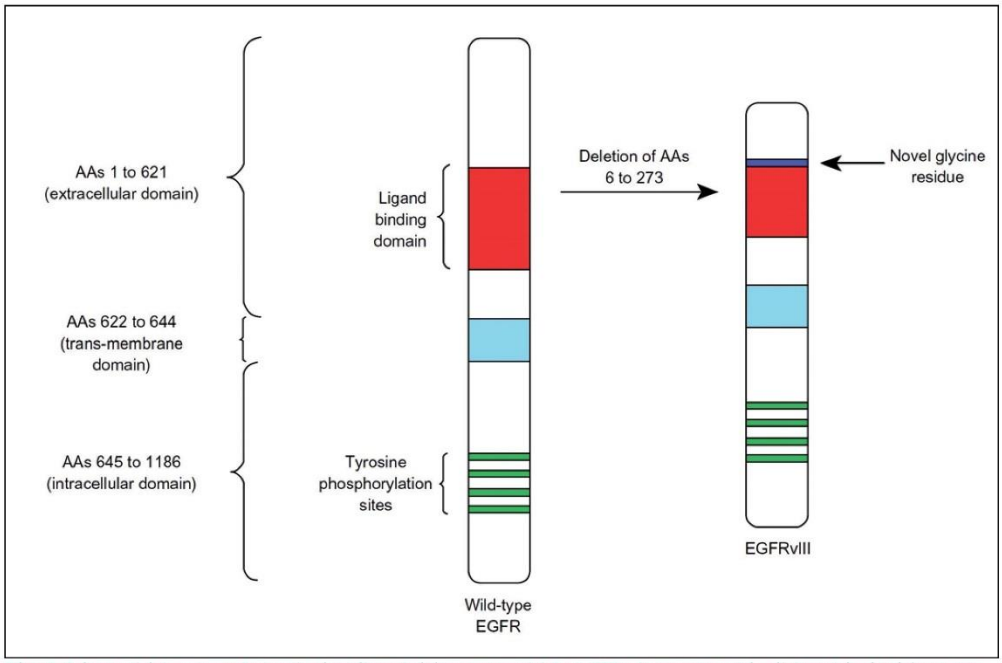
Obr. 2. Schéma molekulárních cílů některých nitrobuněčných onkogenních signalizací a odpovídajících terapeutik, zkoušených v klinických hodnoceních u GBM. Převzato z [44].

léčbě nemocných stratifikovaných podle nadměrné exprese nebo výskytu specifických mutací EGFR [57]. PDGFR je rovněž často nadměrně exprimován u GBM, zejména u proneurálního subtypu [9,11]. Ligand receptor PDGFR stimuluje mimo jiné růst a angiogenezi nádoru. Imatinib účinně inhibuje PDGFR, a je proto zkoušen také v léčbě GBM [58]. Mezi multikinázové inhibitory s účinkem na PDGFR používané rovněž k ovlivnění nádorové angiogeneze patří sunitinib, sorafenib nebo vandetanib. Také tyto látky jsou testovány v léčbě GBM [59,60].

Intracelulární signální dráhy zprostředkovávají odpověď na růstové faktory a aktivované příslušné povrchové buněčné receptory. Jejich inhibicí lze příznivě ovlivnit řadu nádorů a účinek je testován i u GBM. Příkladem léků zasahujících intracelulární aberantní signalizace jsou lonafarnib [61], tamoxifen [62,63] nebo enzastaurin [64,65]. Selektivním inhibitorem protein-kinázy mTOR (mammalian Target Of Rapamycin) je pak například sirolimus a v onkologické praxi dnes standardně používaný everolimus. Také tyto a jiné látky byly zkoušeny u GBM, opět však s minimálním klinickým benefitem [66,48].

Inhibitory nádorové angiogeneze

Současný onkologický výzkum stále více vyzdvihuje nezastupitelný význam nádorového mikroprostředí a patologické an-



Obr. 3. Schematické znázornění aminokyselinové delece na pozici 6 až 273 a inzerce nových glicinových zbytků v extracelulární doméně receptoru EGFR, čímž vzniká nový terapeutický cíl EGFRvIII. Převzato z [92].

giogeneze pro vznik a progresi maligní choroby. Úloha angiogeneze a nádorového mikroprostředí je extenzivně studována i u GBM. Nadměrná mikrovaskulární proliferace a zvýšená exprese vaskulárního endoteliálního růstového faktoru VEGF je známkou rychlé progrese a rekurence GBM [67–71]. Bevacizumab je monoklonální protilátka proti VEGF-A používaná dnes standardně v několika různých onkologických indikacích, převážně u pokročilé a metastatické choroby. Bevacizumab byl úspěšně testován i u nemocných s rekurentním GBM [72–77] a je schválen americkým FDA pro použití u rekurentního GBM jako monoterapie v USA a Kanadě [78,79]. V současné době probíhají klinické studie fáze III (AVAglio [NCT00943826] a RTOG-0825 [NCT00884741]). Obě zkoumají účinnost a bezpečnost bevacizumabu u nemocných s nově diagnostikovaným GBM.

Mezi ostatní antiangiogenní látky zkoumané u GBM patří například integrinový inhibitor cilengitide (dosaženo bylo mediánu celkového přežití 9,9 měsíce u rekurentní choroby) [80], rekombinantní fúzní protein aflibercept [81,82] nebo cediranib [83]. Žádný z těchto přípravků však dosud nebyl schválen ke klinickému použití u pacientů s GBM. Existuje také obava z mnohem agresivnějšího chování rekurentní choroby po selhání léčby antiangiogenními inhibitory [84–86], přestože další studie toto riziko nepotvrzují [87–89].

Přes všechny dosavadní úspěchy nové cílené léčby u mnohých onkologických diagnóz, v případě GBM se zatím nepodařilo přinést významné prodloužení celkového přežití nebo zlepšení kvality života nemocných oproti standardnímu léčebnému režimu s radiací a temozolomidem. Jistou roli v tomto neúspěchu může hrát snížená prostupnost léčivých látek do nádorové tkáně z důvodu přítomnosti hematoencefalické bariéry (ta je však intenzivní radiační i chirurgickou léčbou značně poškozena), ale i pozdní diagnostika GBM ve stadiu pokročilé a z molekulárněgenetického hlediska značně heterogenní choroby.

Imunoterapie GBM

Moderní imunoterapie se stává další významnou součástí léčebných režimů mnoha onkologických nemocí. V klinické praxi se již dnes s úspěchem užívá nové generace cílené imunoterapie, například u karcinomu prostaty (sipuleucel-T) nebo metastatického melanomu (ipilimumab) [90,91]. Velmi nadějným přínosem v léčbě GBM by se v blízké budoucnosti mohl stát přípravek rindopepimut (CDX-110, Celldex Therapeutics). Rindopepimut je peptidová vakcína 13 aminokyselinových sekvencí, zacílená proti povrchovému antigenu nádorových buněk EGFRvIII (receptor epidermálního růstového faktoru varianta III). Tato deleční mutanta EGFR je přítomna u více než 30 % pacientů s GBM, zatímco buňky zdravé tkáně tento antigen zpravidla neexprimují. Jde tedy o ideální molekulární cíl

vhodný pro léčebné ovlivnění (obr. 3). Prvotní klinická studie fáze I/II u nemocných s nově diagnostikovaným GBM ukázala prodloužený medián přežití bez příznaků (15,2 měsíce) i medián celkového přežití (23,6 měsíce) [92]. Další studie fáze II (ACT III) pak zkoumala účinek rindopepimutu v kombinaci se standardní chemoradioterapií u nemocných s exprimovaným EGFRvIII. Medián doby přežití byl 21 měsíců od začátku léčby a 24 měsíců od stanovení diagnózy. Nemocní byli ve studii rovněž vyšetřeni na přítomnost metylačního statusu promotoru genu *MGMT*. Pacienti s nemetylovaným a normálně funkčním *MGMT* genem měli medián doby přežití 20,9 měsíců od stanovení diagnózy, zatímco nemocní s metylovaným promotorem *MGMT* genu dosahovali medián celkového přežití 40 měsíců od stanovení diagnózy [93]. V současné době probíhá klinické hodnocení tohoto přípravku ve studii fáze III u nově diagnostikovaného GBM (ACT IV, [NCT01480479]). Studie se zúčastňují i vybraná klinická pracoviště v České republice. Jiným aktivním hodnocením rindopepimutu je pak klinická studie fáze II v kombinaci s bevacizumabem u nemocných s rekurentním GBM pozitivních na EGFRvIII [NCT01498328], probíhá však pouze v USA. Terapeutické použití rindopepmimutu je dobrým příkladem individualizovaného přístupu a uplatnění principů personalizované medicíny v léčbě pacientů s GBM.

Závěr

GBM je vysoce maligní nádor centrálního nervového systému s dosud infaustní prognózou. V současné době je stanoven jediný standardní léčebný postup tohoto onemocnění. Významnou roli zde hraje časový faktor, léčba musí být zahájena co nejdříve od stanovení diagnózy. Nejvýznamnějšími faktory pro délku přežívání nemocných jsou věk pacienta, jeho celkový stav před operací a radikalita resekce nádoru. I přes dodržení agresivního a multimodálního terapeutického postupu je léčba dosud málo účinná a medián přežití bez příznaků i medián doby celkového přežití zůstávají poměrně krátké. Vyskytují se však i relativně dlouhodobě žijící jedinci s GBM. Výzkum v molekulární genetice GBM posledních let odhaluje některé příčiny tohoto stavu (tak jak bylo prokázáno například u metylace promotoru genu *MGMT* a vyřazení funkce tohoto enzymu v opravě chemoterapií poškozené DNA nádorových buněk nebo mutace v IDH1/2 a následný hypermetylační stav nádorového genomu a zvýšený výskyt G-CIMP). Také pokroky ve výzkumu nových cílených onkologických léků přinášejí slibné perspektivy v léčbě GBM, přestože zatím neznamenaly významný převrat v léčbě tohoto onemocnění. Perspektivní by mohly být kombinace více takových terapeutik a především pak cílená imunoterapie části GBM prezentující alternativní antigeny (příklad léčby EGFRvIII pozitivního GBM vakcínou rindopepimut). Zkoumání molekulárněgenetických vlastností glioblastomu velmi pravděpodobně přispěje k odkrytí nových potenciálních terapeutických možností i dalších prognostických a prediktivních biomarkerů. To umožní personalizaci léčby pro konkrétního nemocného s dosažením nejlepších možných terapeutických efektů. V blízké době lze tak očekávat další významné pokroky v péči o nemocné s GBM.

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Attachment XII

Polivka J Jr, Polivka J, Rohan V, Topolcan O, Ferda J. New molecularly targeted therapies for glioblastoma multiforme. Anticancer Res. 2012; 32(7):2935-46. **(IF = 1.895)**

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Review

New Molecularly Targeted Therapies for Glioblastoma Multiforme

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Abstract. *Glioblastoma multiforme (GBM) is the most malignant brain tumor in adults, exhibiting high mortality. Standard therapy (surgery, radiotherapy and chemotherapy with temozolomide) has only limited effectiveness. The progress in genomics regarding GBM, in the detection of new markers of oncogenesis, abnormalities in signalling pathways, tumor microenvironment, and pathological angiogenesis over the past decade are briefly discussed. The role of novel prognostic in this review biomarkers [isocitrate dehydrogenases 1 and 2, CpG island methylator phenotype, promoter methylation status of the MGMT (O-6-methylguanine-methyltransferase) gene] is also discussed. New targeted therapeutic approaches are classified into several functional subgroups, such as inhibitors of growth factors and their receptors, inhibitors of proteins of intracellular signaling pathways, epigenetic gene-expressing mechanisms, inhibitors of tumor angiogenesis, tumor imunotherapy and vaccines. Finally novel possibilities for GBM treatment are summarized in this review.*

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumor in adults, with an incidence of 3-4/100,000/year (1). GBM is extremely invasive and difficult to treat surgically, characterized by intense and aberrant vascularization and high resistance to radiotherapy (RT) and chemotherapy. The current standard of care for patients with newly diagnosed GBM is neurosurgery, followed by fractionated external beam RT and chemotherapy with systemic temozolomide (2). The median survival of patients with GBM is 12.1-14.6 months (3) and only 3-5% of patients survive longer than 3 years (4). The progress in genomics of GBM over the past 10 years, has revealed several abnormalities in signaling pathways and a diversity of mutated genes. The importance of the microenvironment in GBM, especially of tumor angiogenesis and the role of tumor biomarkers have also been studied. The use of this new knowledge regarding the diversity of GBM on molecular and genetic levels could lead to individual patient tumor analysis and treatment management. This review focuses on novel therapeutic approaches to GBM, facilitated by these findings.

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Pathology of Malignant Glioma

The application of pathology, as well as genetics and molecular biology, is required in order for one to understand the complexity of gliomas. These tumors represent primary brain malignancies originating from glia, the brain tissue which provides supportive functions to neural cells (nutrients, oxygen, mechanical support, guidance in development and immune functions) but also acts in very complex processes (signal transduction and neurotransmission). GBM is the most common form of high-grade glial tumor, which is defined by specific histopathological criteria namely hyper-cellularity, necrosis, pleomorphism, vascular proliferation and pseudopallisading (5). GBMs can be categorized into two subgroups, as primary and secondary. Primary GBMs are diagnosed as advanced cancer,

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whereas secondary cases have clinical, radiological or histopathological evidence of progression from a pre-existing lower-grade tumor (6). There are some clinical differences between the two groups. Secondary GBMs occur less frequently (5% of GBMs) and among younger patients (with a median age of 45 years). Histopathological differentiation between primary and secondary GBMs is not possible (7). However there are distinctions between primary and secondary tumors at the genetic level (8), but none of the alterations is specific enough to distinguish between these two subgroups.

Genetics of Malignant Glioma

The origin of cancer is presently understood as the accumulation of hereditary or somatic alterations in genes that control critical biological processes, such as regulation of apoptosis, cell cycle progression and proliferation. The changes could be manifested by the activation of oncogenes, and by the silencing of tumor suppressor genes, which leads to the different gene expression profile of cancer cells. However it is not only genetic alterations that are immediately essential for malignant transformation. Epigenetic mechanisms of modification of gene expression, such as DNA methylation status, imprinting, chromatin changes, and the role of micro-RNAs, are also being frantically discussed.

Comprehensive analysis of genetic and epigenetic alterations in high grade glioma in comparison to normal brain tissue is now absolutely essential. This molecular and genomic approach could provide novel targets for diagnostic, prognostic or therapeutic purposes. It could also be helpful in the identification of subgroups of patients who have better prognosis on standard therapy or preferentially respond to certain single or combined novel targeted therapies.

Some of the first genetic studies of malignant glioma described the presence of an extra copy of chromosome 7 and an amplification of the receptor of epidermal growth factor (*EGFR*) gene was identified (9). Further karyotypic and loss of heterozygosity studies identified the positions of tumor suppressor genes on chromosomes 9, 10 and 17 (10). The main gene which was altered on chromosome 17 in GBM, was identified as tumor suppressor TP53, which has a critical role in the inspection of the genome for DNA damage and can arrest the cell cycle and trigger apoptosis (11). Owing to further progress in genetics, the loss of tumor suppressors from chromosomes 19 (p16 cell-cycle inhibitor) and chromosome 10 (phosphatase and tensin homolog, *PTEN*) were described in 1993 and 1997, respectively. The role of p16 is to arrest cell-cycle progression, whereas PTEN is a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway (12).

The unprecedented progress of recent years in all 'omics' disciplines (such as genomics, transcriptomics, proteomics and others), together with improvements in bioinformatics technologies, has provided new opportunities in current brain cancer research. The human genome was fully sequenced and the improvements of sequencing methods have lately permitted genome-wide association studies of human cancer, including those of high-grade glioma. One of the most important genome-wide analyses of 20,661 protein coding genes in GBM tumors was completed in 2008. This study examined 22 genome samples from GBM and probably identified the most important alterations at the genetic level that drive glioblastoma formation (13). Most of the common alterations in DNA were identified, such as point mutations, small insertions and deletions, as well as larger copy number changes, genomic amplifications and deletions.

The alterations of several important pathways which are involved in GBM development and growth were uncovered. Among the most important ones are i) RAS and PI3K-AKT oncogenic pathway with alterations in EGFR/PI3K/PTEN/NF1/RAS; ii) the p53 pathway with changes in TP53/MDM2/MDM4/$p14^{ARF}$ changes; iii) cell-cycle regulatory pathway, with alterations in RB1/CDK4/ $p16^{INK4A}$/CDKN2B, and iv) the newly discovered alterations in metabolic pathways including isocitrate dehydrogenases IDH1/IDH2. The alterations in IDH1/IDH2 could also serve as independent prognostic factor, which will be discussed later (13, 14).

New Classification of Human Glioblastoma

Another exciting work in this area is being conducted by The Cancer Genome Atlas (TCGA), which is sponsored by the National Institutes of Health (NIH) of the USA. This consortium studies the nature of cancer through the integration of genetic data with the gene expression profiles. The TCGA consortium is carrying out research in more than 20 types of human cancer, including GBM. A total of 500 specimens of primary untreated GBM are being utilized for the DNA (gene copy number, gene sequencing, epigenetic methylation), mRNA (gene expression profile) and microRNA (regulation of expression) assessment (15).

The current findings from this activity have uncovered some novel genetic alterations, together with the possibility that GBM can be divided into different subtypes (16). By this approach, GBMs still remain one pathological unit but are subdivided by their genetic alterations and gene expression profiles. This new division also has some clinical relevance. The novel four subgroups of GBM are called Classical, Mesenchymal, Proneural and Neural, especially because of the differently elevated expression of some 'signature' genes across the subgroups (16). This novel molecular classification of GBMs could be highly useful in the future for finding important molecular targets within each group, suitable for therapeutic intervention, as well as for the selection of the best targeted therapy for each patient.

Novel Prognostic Biomarkers for Malignant Glioma

Not only a new classification for GBMs but also novel prognostic biomarkers, have emerged in the recent years. Three of the most important markers of GBMs in relation to the prediction of clinical outcome are discussed here. These are the IDH mutations, the CpG island methylator phenotype (CIMP) and the promoter methylation status of the *MGMT* gene.

Isocitrate dehydrogenases (IDH1 and IDH2) serve as the enzymes that convert isocitrate into alpha-ketoglutarate and reduce nicotinamide adenine dinucleotide phosphate (NADP) to the reduced form NADPH. The genes for IDH1 and IDH2 were found to carry specific mutations in a significant portion of lower grade gliomas and a subset of glioblastomas (mainly the proneural type of GBM) (13, 17). The mutation is very distinctive, namely a single amino acid change – R132H, in the IDH1 active site which leads to the loss of regular enzyme function. The mutations in *IDH1* and *IDH2* are present in about 70%-80% of low-grade gliomas, in 50% of anaplastic gliomas and in approximately 5% of glioblastomas (18). The aberrant function of mutated IDH1 is the conversion of alpha-ketoglutarate to 2-hydroxyglutarate. (17). The latter is an inhibitor of alpha-ketoglutarate-dependent dioxygenases, which leads to genome-wide epigenetic changes in human glioma (19). The genome-wide changes associated with the mutated IDH1 predict a better prognosis and can be used for another subclassification of human GBMs. It would appear that there is a sequential pattern of epigenetic changes (CIMP, MGMT) regarding the *IDH1* alterations. The mutation of *IDH1* is the first step, followed by the production of 2-hydroxyglutarate, which leads to the CIMP profile, along with proneural gene expression changes (19).

A study of three different molecular alterations in low-grade gliomas (*IDH1/IDH2* point mutations, P53 expression and 1p/19q deletion status), demonstrated that only the *IDH1* mutation was an independent prognostic marker of favorable prognosis (20). In the next study, glioblastoma tissues were analyzed for prognostic markers, such as CIMP (6 CIMP markers) and *IDH1* mutations. The data came from the M.D. Anderson Cancer Center and were evaluated in the RTOG 0525 study with more than 800 newly diagnosed GBM patients. Based on multivariate analyses, both the *IDH1* mutations and the CIMP status were determined as being independent prognostic factors. The patients were subdivided into three prognostic groups according to the number of positive CIMP markers. The first group, with 0-1 CIMP (regarded as being CIMP-negative), had a median survival of 13.8 months; the second group with 2-4 CIMPs (CIMP-intermediate) had a median survival of 20.1 months, and the third group with more than 5 CIMPs (CIMP-positive) had a median survival of 90.6 months (21, 22). Naturally, there are many more studies that address the impact of *IDH* mutations in progression-free survival (PFS) and overall survival (OS) of patients with glioma (23, 24).

The current standard of care for GBM includes surgery, RT and the use of the chemotherapeutic agent temozolomide, which is the oral alkylating agent that causes DNA damage by alkylation of the 0-6 position of guanine and the production of DNA interstrand cross-links. In a large, randomized, phase III trial in newly diagnosed patients with GBM, the therapeutic interventions were divided into two subgroups: RT alone *vs.* RT and concurrent daily temozolomide followed by adjuvant temozolomide. The subgroup of patients treated with RT plus temozolomide had a median survival benefit of 2.5 months and the proportion of 2-year survivors increased from 10.4% to 26.5% (25). There is a proportion of patients who have a better response to temozolomide, but the majority of patients become rapidly resistant to this chemotherapeutic agent. One of the strongest predictive biomarkers for the chemotherapy response is the alteration in the *MGMT* gene. The enzyme *O*-6-methylguanine-methyltransferase (the product of MGMT) is able to repair the DNA damage caused by temozolomide. The presence of *MGMT* leads to reduction in the effect of temozolomide chemotherapy. The silencing of the *MGMT* gene can be caused by epigenetic mechanisms, the DNA hypermethylation of CpG islands in the promoter region of the *MGMT* gene. This alteration leads to a decrease in the transcription level of the MGMT gene and in the amount of gene product. Methylation of the *MGMT* promoter was observed in 47.7% patients with GBM (more in the subgroup with secondary GBM) (26). The subset analyses of the large, randomized, phase III trial mentioned above (25) showed that the patients with hypermethylated *MGMT* promoter had a significantly better median survival after therapy with temozolomide compared with those that did not (21.7 *vs.* 15.3 months) (25, 27). In another study, *MGMT* promoter hypermethylation was the predictive biomarker for a better response to RT independently of treatment with temozolomide. Therefore, the *MGMT* methylation status could be potentially considered as a general biomarker of better therapeutic response in GBM (28). The strong correlation between *MGMT* methylation and the CIMP profile was also observed in one study. This finding could signify that *MGMT* hypermethylation is the epiphenomenon of the genome-wide methylation status associated with the CIMP (29).

Other non-genetic prognostic biomarkers for GBM have also been reported. One study, which examined the prognostic significance of individual angiogenic factors, collected the serum samples from 36 patients with GBM and simultaneously assayed them for 48 angiogenic factors using protein microarrays. Two different subtypes of GBMs were revealed by cluster analysis and a low serum level of tissue inhibitor of metalloproteinase-1(TIMP1) was established as an independent predictor of better survival (30). Another article discussed the predictive value of serum α2-Heremans-

Schmid glycoprotein (AHSG) in patients with glioblastoma. The median survival was longer (51 *vs.* 29 weeks) in patients with normal (more than 285 mg/l) *vs.* low serum AHSG concentrations. This finding was independent of age and Karnofsky score, and the serum AHSG level inversely correlated with the Ki-67 proliferative index (31). The study of serum concentrations of extracellular matrix glycoprotein (YKL-40) and matrix metalloproteinase-9 (MMP-9) concluded that these two biomarkers could be monitored in the serum of patients with GBM and help confirm the absence of active disease. YKL-40 was also used as a predictive biomarker of overall survival in patients with high-grade glioma (32). On the other hand, a more recent study failed to prove any clinical relevance of serum MMP-9 as a biomarker of disease status or overall survival in a large group of patients with glioma (33).

Some of the previously mentioned biomarkers, together with the novel stratifications of a molecular and genetic level, could be potentially useful in the near future for treatment strategies for patients with GBM and other types of high-grade glioma. Improved insight into the therapeutic responses, as well as the biology of these tumors, are urgently needed for more effective therapeutic management of patients with high-grade gliomas.

Novel Targeted Therapies for Malignant Glioma

The standard therapeutic options for the treatment of GBM and other types of high-grade glioma have only limited benefits, as discussed earlier. The new targeted therapies which have recently emerged are directed against certain tumoral features, such as altered signaling and metabolic pathways, aberrant tumor vessels, angiogenesis and the tumor microenvironment. Recent genome-wide studies and the molecular characterization of GBM has enabled the identification of potential new targets, development of novel therapeutic small molecules and monoclonal antibodies and initiation of clinical trials with these targeted drugs. However, there is a wide molecular diversity and heterogeneity associated with the aberrant GBM signaling pathways. This could be the reason for the relative lack of success of these new approaches in the treatment of GBM. Only a small clinical benefit has been demonstrated with the novel therapeutics so far. Overcoming these barriers will require the use of individualized molecular profiling of each GBM tumor and application of personalized medicine in combinatorial targeted therapies for high-grade gliomas.

The most important molecular and genetic alterations in GBM can cause increased tumor invasiveness, cell survival, proliferation, evasion of apoptosis, angiogenesis and immune response weakening. Novel therapeutic approaches targeting such changes in high-grade glioma can be classified into several functional subgroups (34, 35).

Growth factor receptors and their inhibitors in GBM. The first subgroup, inhibitors of growth factors and their receptors, includes the therapeutics directed to the aberrant growth factor pathways presented in GBM including EGFR, platelet-derived growth factor receptor (PDGFR), insulin-like growth factor (IGF), fibroblast growth factor (FGF). These receptors and their ligands are overexpressed or mutated in high proportion of GBM (13, 14).

The amplification, as well as the overexpression, of the EGFR family are described in approximately 50% of GBM cases (14, 36). More than 40% of the tumors carry the unique deletion mutant called *EGFR*vIII. This *EGFR* gene has the deletion of exons 2-7, which causes constitutive ligand-independent constitutive receptor activation (14, 37). *EGFR*vIII could be an ideal tumor-specific target for novel therapeutics and will be discussed later. One of the new drugs directed against EGFR function is gefitinib (Iressa; AstraZeneca). In a phase II study of gefitinib, patients with GBM had partial tumor regression in 12.7% of cases (38). The PFS at 6 months was 13% and the median OS was 10 months in another study of recurrent GBM (39). There are more recent studies with gefitinib in GBM, with results of minimal efficicacy compared to standard RT/temozolomide treatment (40, 41). Another new EGFR inhibitor also examined as a possible treatment for GBM is erlotinib (Tarceva; Genentech). Some phase II trials of erlotinib as a single agent showed only minimal benefit for glioblastoma treatment and modest survival benefit in combination with temozolomide and RT, or with other agents (42, 47). A better therapeutic response to these agents was achieved by stratifying patients based on their own molecular profile (48). Another promising EGFR inhibitor is lapatinib (Tyverb; GlaxoSmithKline). According to a phase II study, lapatinib is distributed into the tumor tissue (49). However, in a subsequent trial with a small number of patients with recurrent GBM, no efficacy was observed (50). Cetuximab (Erbitux; ImClone Systems) is a chimeric monoclonal antibody which can inhibit EGFR. A small group of patients responded to this agent in a phase II study (51). In another phase II study, the patients with recurrent high-grade glioma were stratified according to amplification of the *EGFR* gene. Cetuximab had limited activity and a median overall survival of 5 months (52). Little improvement was observed in the phase II study with the combination of cetuximab, irinotecan (Camptosar; Pfizer) and bevacizumab (53).

The PDGF receptor is often overexpressed and activated in GBM, especially in the proneural subtype (14, 16). The changes leading to aberrant activation of PDGFR assist in the transition from grade II-III glioma to glioblastoma. The PDGF ligand is able to stimulate GBM growth and angiogenesis (54, 55). The kinase inhibitor of PDGRF, c-KIT and oncogene fusion protein BCR-ABL imatinib (Gleevec; Novartis Pharmaceuticals) has been extensively examined in the GBM setting. The modest response of PFS at 6 months of 15.7%

was observed in one phase II trial of patients with recurrent disease (56). A better result in PFS at 6 months of 32% was recorded in a study with stratification of patients by their PDGFR expression 57). There are more studies with imatinib in combination with hydroxyurea. After an initial promising phase II trial, further multicenter studies did not confirm the preliminary results, and other trials with combinatorial therapy are ongoing (58, 59, 60). In the most recent study, imatinib had limited activity in patients with recurrent oligodendroglioma and mixed oligoastrocytoma, with median survival of 16.6 months, but with a moderate toxicity profile (61). Another PDGFR inhibitor, tandutinib (MLN 518; Millennium Pharmaceuticals), is in phase II trials as a single agent, or in combination with bevacizumab. Among multikinase inhibitors with the potential to block PDGFR, are sunitinib (Sutent; Pfizer), sorafenib (Nexavar; Bayer and Onyx Pharmaceuticals), vandetanib (Caprelsa; AstraZeneca) and others, most of which are used in the trials as antiangiogenic drugs for GBM (62, 63).

Inhibitors of intracellular signaling pathways. Intracellular components in signaling pathways mediate the response of cells to the growth factors and their interactions with cell surface receptors. Inhibition of such aberrant signaling components is a promising targeted therapeutic approach for the treatment of many types of cancer including high-grade glioma.

Mutations of RAS protein in GBM are rare (13, 14). On the other hand, the inhibition of RAS could be effective because of its involvement in the deregulated signaling pathways through growth factor receptors. The RAS protein must be post-translationaly modified by farnesyltransferase before translocation to the cell membrane. The inhibitors of this process have also been tested in GBM. Tipifarnib (Zanestra; Johnson and Johnson) had modest activity in patients with recurrent glioblastoma, with a PFS at 6 months of 12% in a phase II trial (64). Another inhibitor of farnesyltransferase is lonafarnib (SCH66336; Schering-Plough), which was examined in a phase I study (65).

Activation of protein kinase C (PKC) contributes to the signal propagation from several growth factors, such as EGF and PDGF, which stimulate glioma cell proliferation. The targeting of PKC with the well-known anti-estrogen drug tamoxifen was examined, but with only little or no clear benefit in clinical trials for GBM (66, 67). A novel specific PKC inhibitor is enzastaurin (LY317615; Eli Lilly and Company). Its effect on recurrent malignant glioma was reported, with 22% of patients achieving radiographic response and 5% achieving stable disease (68). More recent studies of enzastaurin showed some limited efficacy in recurrent GBM (69, 70).

Another protein, (mTOR), is involved in cell growth signaling. It transduces the signals from PI3/AKT, as well as, the RAS pathway. Overexpression of growth factors or deletion of *PTEN* increases the mTOR activation in GBM (14, 36). There are some selective mTOR inhibitors that have been examined in GBM settings. The small molecule sirolimus (Rapamune; Wyeth) was not effective as a single agent. It had limited efficacy in a phase II trial with erlotinib (47, 71). Temsirolimus (Toricel; Wyeth) had some efficacy as a single agent for recurrent GBM. There are now some ongoing trials using it in combination with EGFR/PI3K pathway inhibitors or bevacizumab (72). The derivative of sirolimus, mTOR inhibitor everolimus (Zortress; Novartis) had no clear clinical benefit in combination with gefitinib for recurrent GBM (41).

Other intracellular molecular targets for GBM therapy. One of the recently defined molecular targets now being examined in the treatment of various types of cancer is the family of proteins called polyADP ribose polymerases (PARPs). The PARP protein family acts in DNA repair. Its main role is the detection and signaling of single-strand DNA breaks. PARP inhibitors have been widely examined in clinical trials for therapy of tumors with specific genetic deficits in DNA repair pathways such as *BRCA1* and *BRCA2* (73). In the case of GBM, two new drugs are being examined for the treatment of initial as well as recurrent disease: the specific PARP inhibitors iniparib (BSI 201; Sanofi-Aventis) and veliparib (ABT 888; Abbott).

The mechanisms of epigenetic modifications of genes and their aberrant functions are also very important in cell transformation in the case of malignant glioma. Histone acetylation (by histone acetyltransferases) and deacetylation (by histone deacetylases, HDACs) play fundamental roles in the regulation of gene expression. There are some HDAC inhibitors that were examined for GBM, such as phenylbutyrate, valproic acid, depsipeptide (FK228) and vorinostat (Zolinza; Merck) (74). A recent phase II study of vorinostat as a monotherapy for recurrent GBM showed modest activity with a median OS of 5.7 months (75).

The proteasome complex inhibitors are other prospective anticancer agents. The proteasome complex is involved in important cellular functions, such as protein homeostasis, apoptosis and cell cycle progression, and in resistance to anticancer therapy. The usage of proteasome inhibitors can induce cancer cell apoptosis or growth arrest (76). The proteasome inhibitor bortezomib (Velcade; Millenium) was examined for the treatment of recurrent GBM. It had a low response rate but led to better results in combination with standard therapy for patients with newly diagnosed GBM (77, 78).

Inhibition of angiogenesis in GBM. The role of the tumor microenvironment and angiogenesis has been widely studied in the case of glioblastoma. Extensive microvascular proliferation denotes poor survival and increased risk of

recurrence in GBM (79). The role of vascular growth factors (VEGF, especially VEGF-A) is well established in aberrant angiogenesis. In the case of GBM, the plasma and the tumor level of VEGF has been found to be relatively high and the elevated intracavitary level of these growth factors was discovered in patients with recurrents in comparison to those with non-recurrent GBM (80, 81). VEGF overexpression in tumor histology also correlates with a poor prognosis (82, 83). Therefore, a great effort is being made with the evaluation of antiangiogenic and anti-VEGF agents in GBM settings.

One of the most common used inhibitors of angiogenesis in cancer treatment is bevacizumab (Avastin; Genentech). It is a humanized monoclonal antibody against VEGF-A. Bevacizumab has also been examined in clinical trials for treatment of recurrent, as well as non-recurrent GBM, as a single agent, and in various combinations with chemotherapy and other targeted therapeutics. In combination with irinotecan, the 6-month PFS among 35 patients was 46% and the median OS was 42 weeks, in one of the first prospective phase II trials for patients with recurrent disease. The 4-year OS was reported to be 11% (84, 85). In the phase II BRAIN study, the use of bevacizumab with or without irinotecan was examined in 167 patients with recurrent GBM. In the bevacizumab plus irinotecan arm, 6-month PFS was 50.3% and the median OS was 8.9 months. The 12-, 18-, 24- and 30-month survival rates were 38%, 18%, 17% and 16%, respectively. For the bevacizumab monotherapy arm, the 6-month PFS was 42.6% and the median OS was 9.3 months. The 12-, 18-, 24- and 30-month survival rates were 38%, 24%, 16% and 11%, respectively (86, 87). There are additional phase II studies among patients with recurrent glioblastoma that support the treatment effect of combining bevacizumab with chemotherapy (88, 89, 90, 91, 92). Bevacizumab was approved in 2009 by the US FDA as a monotherapy for treating recurrent GBM due to high response rates and modest survival benefit (86, 93, 94).

There are other antiangiogenic therapies that are being studied as single-agent treatment for recurrent GBM. In one phase II study, the integrin inhibitor cilengitide (Merck) was examined for patients with recurrent disease. In the arm with the higher dose (2000 mg of cilengitide twice weekly), the median OS was 9.9 months and the OS rates were 37%, 23%, 15% and 10% at 12, 24, 36 and 48 months, respectively, cilengitide was also well-tolerated (95, 96). Another antiangiogenic drug, aflibercept (Zaltrap; Sanofi and Regeneron Pharmaceuticals), is a recombinantly prepared fusion protein that can bind VEGF-A, VEGF-B and placental growth factor (PGF). In the ongoing NABTC 0601 phase II study with aflibercept, the preliminary ORR was 30% for recurrent GBM (97). There is also a phase I trial with aflibercept and standard RT/temozolomide therapy for initial GBM (98). The oral inhibitor of MET/VEGFR2 cabozantinib (XL184; Exelixis) was examined in a phase II study in patients with previously treated recurrent GBM. The median PFS of patients without previous antiangiogenic treatment was 16 weeks. Furthermore, 61% of patients on corticosteroids had a more than 50% reduction in corticosteroid dose (99). Another small-molecule kinase inhibitor, cediranib (Recentin; AstraZeneca), led to normalization of tumor vessels and reduction of brain edema among glioblastoma patients (100). On the other hand, cediranib increased tumor infiltration in one phase II study of recurrent GBM (101). One hypothesis is that there is an angiogenesis-independent tumor population, or mechanism, in GBM which can be promoted by antiangiogenic treatment and which limits the efficacy of these new therapeutics (102). The potential for recurrent infiltrative as well as invasive tumor growth after the use of antiangiogenic agents has been reported in some studies (103, 104, 105). Other recent trials reported that there were no significantly changed patterns of relapse of GBM after the antiangiogenic treatments (106-109).

Combinations of antiangiogenic agents and chemoradiaton for newly diagnosed as well as recurrent GBM were also examined. In one study of standard RT/temozolomide treatment in combination with bevacizumab, for patients with newly diagnosed high-grade glioma, the 12-month PFS and OS were 59.3% and 86.7%, respectively (110). The combination of chemotherapy and bevacizumab for patients with newly diagnosed GBM approximately doubled the median PFS compared to standard therapy (14 *vs.* 6.9 months) (111). The combination of cilengitide with RT/temozolomide was examined in a phase I/II trial of newly diagnosed GBM. The median PFS was 8.0 months and the 12- and 24- month OS were 68% and 35%. The median OS was 16.1 months, with no additional toxicities (112). There are two large phase III studies that will evaluate bevacizumab-containing regimes for newly diagnosed GBMs and that have recently begun enrolling patients [AVAglio (NCT00943826) and RTOG-0825 (NCT00884741).

Immunotherapy and vaccines for treatment of GBM. Immunotherapy is a promising new area of multimodal anticancer treatment for many types of human malignancies. The dramatic change in the efficacy of such approaches after decades of relative disappointment was brought about by the recent introduction of vaccine sipuleucel-T and the monoclonal antibody ipilimumab, for the treatment of hormone-refractory prostate cancer and metastatic melanoma, respectively. These two immunotherapeutic agents mean real survival benefit for patients with cancer (113, 114). There has also been great progress in immunotherapy of GBM over the past few years. Although there is no approved anticancer vaccine for GBM at the moment, there is one hot candidate and many others in the pipeline.

Among the immunotherapeutic approaches in GBM research are passive immunotherapy with antibodies, utilization of autologous stimulated lymphocytes and immunotherapy with

cytokines, and active immunotherapy with tumor-based, peptide or dendritic cell (DC) vaccines. Among the peptide vaccines, there is one strong candidate for near future use in GBM treatment, Rindopepimut (CDX-110; Celldex Therapeutics) which is a peptide-based vaccine (13 amino acid sequence) against the antigen EGFRvIII. This specific *EGFR* mutant variant is constitutively activated and expressed in almost 30% of glioblastomas. One phase I/II multicenter study in patients with newly diagnosed GBM who were treated with rindopepimut led to a median PFS of 15.2 months and an OS of 23.6 months (115). Another phase II trial, ACT III, examined rindopepimut in combination with standard RT/temozolomide in 65 patients with newly diagnosed glioblastoma with *EGFR*vIII positivity. The median survival was 21 months from the time of initiating therapy and 24 months from the initial diagnosis. Patients with unmethylated *MGMT* had an OS of 20.9 months from diagnosis, whereas those with methylated *MGMT* had an OS of 40 months from diagnosis (116). The new double-blind, randomized, multicenter phase III study of rindopepimut in patients with newly diagnosed glioblastoma (ACT IV) is now enrolling patients (NCT01480479). In another phase II trial, the HLA-restricted, Wilms tumor 1 (WT1) 9-mer peptide vaccine was examined in patients with recurrent GBM. Partial response was seen in 2 out of 21 patients and the vaccine was well-tolerated (117). The most recent phase II trial with HSPPC-96 (vitespen), an autologous heat-shock protein-peptide vaccine, has shown promise in patients with recurrent GBM. The median OS was 47.6 weeks for vaccine-treated patients, compared to 32.8 weeks for the non-vaccinated group; 6-month OS was 93% for the vaccinated group compared to 68% for the non vaccinated group. There were no grade 3 or 4 side-effects (118).

Vaccines employing dendritic cells are other prospective approaches to GBM treatment. In a trial of relapsed GBM, the use of a vaccine with DCs loaded with autologous tumor lysate was examined in 56 patients. The median PFS was 3 months and the median OS was 9.6 months (119). The same group is investigating the integration of the vaccine in the primary treatment of patients with newly diagnosed GBM (120). Very promising data from a large, double-blind, randomized phase II trial of a DC vaccine in patients with newly diagnosed GBM showed a median survival of 3 years, with 4-year survival reaching 33% of patients and 27% of patients exceeding 6-years survival from initial surgery (121). Another phase I/II trial with DCs pulsed with specific tumor-associated peptides showed a PFS of 6.8 months and median OS of 18.7 months from the time of vaccination in patients with newly diagnosed GBM (122).

New approaches to the treatment of malignant glioma with immunotherapy are emerging and are demonstrating some promise for the near future for significant improvement of GBM therapy.

Conclusion

The prognosis of GBM still remains poor, despite aggressive surgery, RT and chemotherapies. On the other hand, there have been many novel discoveries in basic and translational research made in recent years. Besides the common predictors of the responsiveness to therapy and outcome, such as functional status or simple demographics, there are important underlying molecular characteristics of the tumor which could play a major role in disease evolution and prognosis.

New prognostic biomarkers, such as IDH 1and 2, the CIMP, promoter methylation status of the *MGMT* gene, and others, could be helpful for the determination of prognosis of the disease, as well as for the prediction of outcome of current standard GBM therapy for individual patients. The novel GBM classification according to genetic alterations and gene expression profiles into the Classical, Mesenchymal, Proneural and Neural subtypes could be very useful in the near future for finding important molecular targets within each group, suitable for therapeutic intervention, as well as for the selection of the best targeted therapy for each patient.

The new targeted therapies that are directed against certain tumoral features, such as altered signaling and metabolic pathways, aberrant tumor vessels, angiogenesis and the tumor microenvironment, are being widely examined in clinical trials. Due to the wide molecular diversity and heterogeneity of GBM, there has been a relative lack of success of these new treatment approaches. At the moment, there is only one targeted drug, bevacizumab, approved by the US FDA for the treatment of recurrent GBM, as a single agent. On the other hand, there has been significant progress in immunotherapy for GBM. The most promising agent, currently in phase III clinical trial for newly diagnosed glioblastoma, is the peptide-based vaccine rindopepimut. There are also other promising immunotherapies on the way.

Further progress in GBM treatment will probably be based on the patient's individual tumor analysis and the selection of the best combination of novel targeted agents together with another multimodal therapy for each individual patient, within the actual application of personalized medicine.

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Attachment XIII

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Review

The Role of Cetuximab in the Induction of Anticancer Immune Response in Colorectal Cancer Treatment

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Abstract. *Monoclonal antibodies binding the epidermal growth factor receptor (EGFR), such as cetuximab or panitumumab, are widely used targeted therapeutics for the treatment of patients with colorectal cancer. The clinical significance of these drugs has so far been associated with combined chemotherapy or radiation. It has been shown that these treatment strategies have their clinical limitations and do not fully exploit the immunomodulatory effect of these drugs. In this review, we discuss the mechanisms of immunomodulation together with the anticancer immune response to the monoclonal antibodies targeted to the EGFR. The combination of anti-EGFR monoclonal antibodies with other immunotherapeutic treatment modalities certainly brings new opportunities for targeted therapy in patients with colorectal cancer.*

During the past 10 years, there have been fundamental discoveries in oncogenic transformation which have changed the current view of the diagnosis and treatment of cancer (1, 2). It is receded from reductionist theory, in which cancer is seen as a homogeneous population of tumor cells interacting on the autocrine and paracrine level thereby determining the biological activity of the tumor. More and more scientific articles from basic as well as applied research actually highlight the tumor complex theory in which a tumor is considered to be a complex tissue (3). Whereas the tumor parenchyma consists of cancer cells, the stroma consists partly of non-cancerous cellular components and non-cellular parts. The tumor mass includes a number of non-cancerous cells and structures of the extracellular matrix, affected by local conditions and aberrant signalization of tumor cells, which in turn leads to the feedback influence of tumor cells themselves. The non-cancerous cellular components of the tumor microenvironment include tumor stem cells with unlimited generational potential, cancer-associated fibroblasts (CAF) that form the extracellular matrix, endothelial cells and pericytes involved in pathological angiogenesis, and immune cells affecting the interaction among tumor and immunocompetent cells (3-5). Non-cellular components of the tumor microenvironment are predominantly composed of extracellular matrix containing cytokines, growth and angiogenic factors and other bioactive molecules that are produced and secreted by both cancerous and non-cancerous cells. The communication between tumor and other cells is very intense and actively modifies the biological activity of the tumor itself and its sensitivity to anticancer treatment (1, 6).

The last two decades of intensive research focused on cancer immunology revealed the dual role of the immune system during the process of carcinogenesis. The immune system contributes to the elimination of newly transformed tumor cells and to the eradication of the residual tumor population after treatment. On the other hand, many experiments clearly demonstrated the supportive role of the immune system in survival, growth, and spread of a variety of tumors, including colorectal cancer (7, 8). A better understanding of the importance of the immune system for tumor growth and survival is reflected in the development of new anticancer therapeutic approaches, such as the application of monoclonal antibodies against tumor antigens and growth factor receptors, adoptive transfer of tumor-specific cytotoxic T-lymphocytes, active immunization with tumor vaccines, and

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modulation of the tumor microenvironment *e.g.* monoclonal antibodies against cytotoxic T–lymphocyte-associated antigen 4 (CTLA4) or programmed cell death 1 (PD1) (1, 9).

In this review, we discuss the mechanisms of immunomodulation together with the anticancer immune response to targeting monoclonal antibodies.

Monoclonal Antibodies and Anticancer Immunity

Immunotherapy aims to stimulate the immune system of the patient to reject and destroy the tumor, which is affected indirectly by the patient´s immune cells (10, 11). Immunotherapeutics can be divided into two groups. The first are monoclonal antibodies that bind to specific receptors and cell antigens, thereby blocking transmission of information and cell proliferation through signaling pathways. Monoclonal antibodies also activate the immune system response after binding to the target receptor. The examples of therapeutics in this group are monoclonal antibodies blocking receptors of proliferative signaling pathways *e.g.* epidermal growth factor receptor (EGFR) and those binding to specific cellular antigens *e.g.* cluster of differentiation 20 (CD20) (12-14). Immune effects are also observed using monoclonal antibodies that bind to growth factors *e.g.* vascular endothelial growth factor (VEGF) and other endogenous mediators *e.g.* receptor activator of nuclear factor kappa B ligand (RANKL).

The second group of immunotherapeutics includes substances exclusively affecting the immune system. Their effect depends on the inhibition of immune-response blockage (affecting ligands of CTLA4 or PD1), or conversely, on the specific activation of the immune system (10, 15). The principal biological effects of monoclonal antibody-mediated immune response are the activation of complement and immune cell-mediated cytotoxicity which is antibody-dependent (antibody dependent cellular cytotoxicity, ADCC). In the following text, we focus on monoclonal IgG antibodies that block the proliferative signaling pathways by interference with the EGFR. Specifically, we focus on extracellular inhibitors, whose main advantage is the ability to activate the immune system response against tumor (1, 16).

Mechanism of Action of Antibodies to EGFR in Colorectal Carcinoma

The EGFR is stimulated by transforming growth factor (TGF-α) as well as epidermal growth factor (EGF) (17). Cetuximab and panitumumab are monoclonal antibodies against human EGFR. They act as functional antagonists of the EGF and TGF ligands and are thus inhibitors of the EGFR-dependent signaling pathways EGFR/phosphatidylinositol 3-kinase (PI3K)/protein kinase-B (AKT)/mammalian target of rapamycin (mTOR) and EGFR/retrovirus-associated DNA sequences (RAS)/proto-oncogene serine threonine-protein kinase (RAF)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (12, 18-21). Signal blockage leads to inhibition of cancer cell division in the G1 phase because of the lack of transcription factors, which ultimately leads to cell apoptosis (22). Moreover, both these monoclonal antibodies induce an immune response against cells whose receptors they bind. While cetuximab is a chimeric IgG1 antibody, panitumumab is a fully human IgG2 antibody. This distinction is very important for activation of the immune response. Even though panitumumab binds to EGFR with higher activity than does cetuximab, the IgG2 isotype of monoclonal antibody has a significantly lower immunogenicity for poor binding to fragment crystallizable (Fc) receptor-gamma (FcγR) (23). An immunoglobulin Fc region provides the antibody with the ability to interact with receptors expressed by effector immune cells, or with complement. In contrast to IgG1 antibodies (cetuximab), IgG2 antibodies do not have such a significant ability to induce an immune response by ADCC or by other immune mechanisms.

Cetuximab and its Immune Interactions in Tumor Complex of Colorectal Cancer

Cetuximab is a chimeric antibody with an antigen-binding region of murine origin. Other parts of the heavy and light chains are of human origin (24-26). Typical therapeutic monoclonal antibody consists of two identical fragment antigen-binding (Fab) fragments and one Fc fragment (Figure 1). Fab fragments serve to bind tumor antigen, while the Fc fragment mediates binding and activation of immune cells [macrophages, natural killer (NK) cells, cytotoxic T-lymphocytes, *etc.*]. Monoclonal antibody cetuximab may, therefore, affect the immune response in the tumor complex by various forms of interaction. In the first case, upon binding of the monoclonal antibody to the specific target structure in the tumor cell, binding of the first component of complement (C1q) to Fc fragments of the monoclonal antibody occurs. This results in activation of the classical complement pathway, during which the membrane of the transformed tumor cell is attacked by the complex of complement components C5 to C9, while releasing chemotactic fragments C3a and C5a. Formation of the membrane-lytic complex (membrane attack complex) penetrates the cytoplasmic membrane and this ultimately kills the tumor cell. Simultaneously released chemoattractant lead to accumulation of leukocytes and the initiation of antitumor immune responses. This mechanism is also called complement-dependent cytotoxicity (1, 27).

The second possible mechanism of tumor cell destruction is called antibody-dependent cellular cytotoxicity (ADCC).

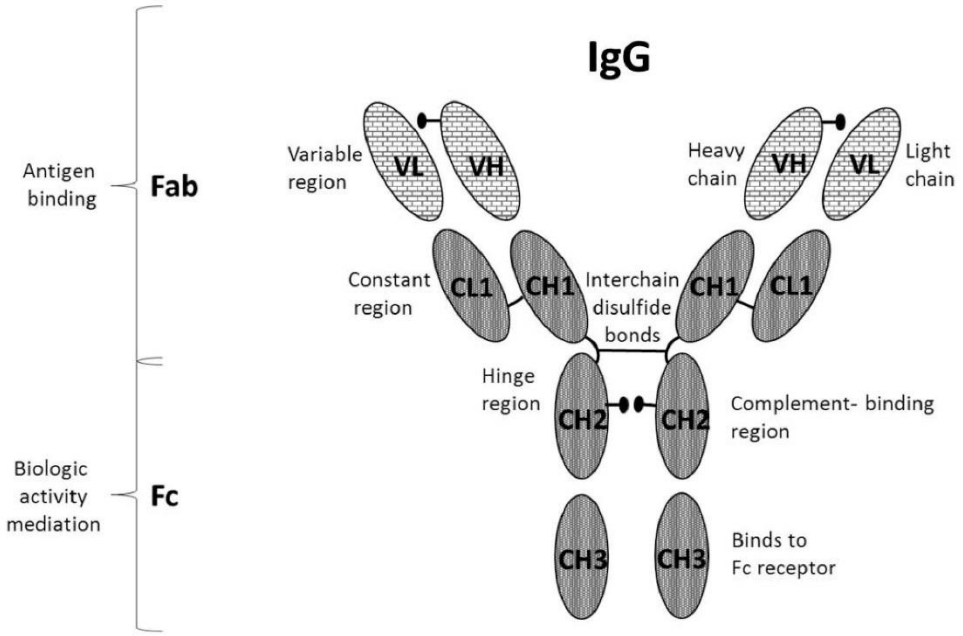

Figure 1. *Immunoglobulin structure: Immunoglobulin G (IgG).*

During this reaction, the tumor cells with bound antibodies (*e.g.* cetuximab) are recognized by NK cells *via* Fcγ receptors (FcγRIIIA=CD16). This leads to activation of NK cells and cytotoxic T-lymphocytes and subsequent effects of cytotoxic agents that damage the membrane of tumor cells (*e.g.* perforin or granzyme B). Moreover, the C3b fragment is produced during the activation of complement which acts as an opsonin for damaged tumor cells and allows phagocytosis by binding to the C3b receptor of macrophages (1, 28-30). This mechanism of immune system activation is called complement-dependent cell-mediated cytotoxicity (Figure 2).

Perspectives on the Use of Cetuximab Immune Response in Colorectal Cancer Treatment

Colorectal carcinoma has generally been regarded as an immunoresistant tumor. In the light of recent research findings, it has become clear that this presumption is not true. The possibility for specific immune system modulation appears to be crucial not only for prognosis, but also for the prediction of response to therapy in patients with colorectal cancer. Tumor-infiltrating lymphocytes ($CD8^+$ and $CD45^+$ T-cells) are increasingly considered to be an independent prognostic factor. Regulatory T-lymphocytes (Tregs) that are responsible for the optimization of the immune response appear to be an optimal predictive factor for monitoring the effect of immunomodulatory therapy (31-33). The determination of the *RAS* mutation status is a powerful predictive factor for the response to the synergistic effect of antibodies to EGFR in combination with chemotherapy. However, *RAS* status is inappropriate for monitoring the activity of the immune response to antibodies EGFR (especially cetuximab) in patients with colorectal cancer. The determination of single nucleotide polymorphisms, variations of individual nucleotides in the DNA sequence (34-36), seems to be more promising. Mutations in Fc fragment domains (especially FcγR2A and FcγR3A) correlate well with an objective response to cetuximab in combination therapy as well as in monotherapy.

Another promising predictive factor is the activity of NK cells and cells involved in ADCC. Sophisticated

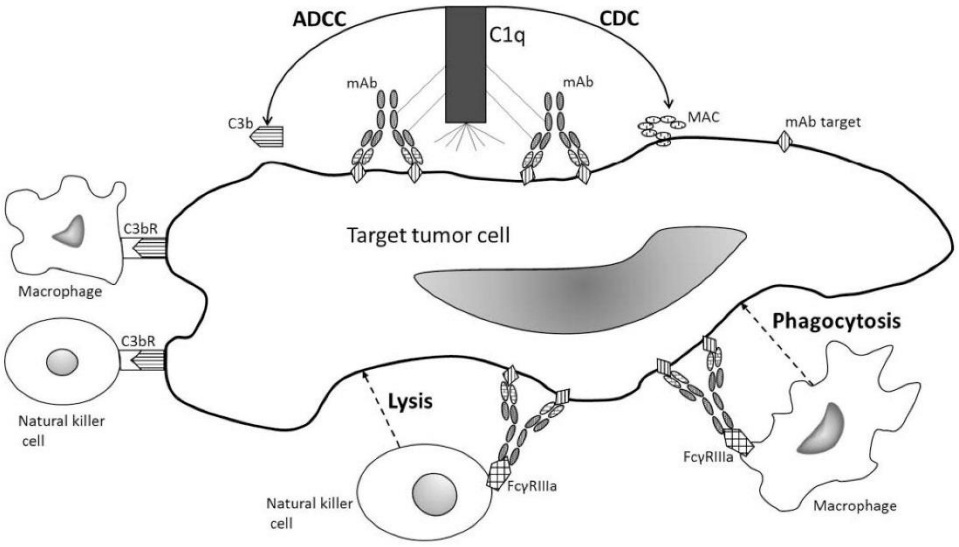

Figure 2. *A schematic model of the effect of monoclonal antibody cetuximab through the immune system. ADCC: Antibody-dependent cellular cytotoxicity, CDC: complement-dependent cytotoxicity, mAb: monoclonal antibody, MAC: membrane attack complex.*

laboratory methods for the determination of NK cell and ADCC activity from peripheral blood samples of individual patients with colorectal cancer are currently being standardized. This activity appears to be an independent prognostic and predictive factor for monitoring of various forms of immunomodulatory treatment, especially the ADCC activity of cetuximab (34, 35, 37-40). These methods are completely independent from the determination of *RAS* mutation and EGFR expression on the surface of tumor cells. Currently there are more than 40 phase I, and III clinical trials evaluating the effectivity of immunomodulatory agents in patients with colorectal cancer in adjuvant as well as palliative treatment settings. The combination of cetuximab with monoclonal antibodies targeted to CTLA4 and PD1 antigens (*in vitro* studies; *in vivo* especially in patients with head and neck tumors and lung cancer) is especially promising. Furthermore, there are numerous studies which are focused on the combination of cetuximab with various vaccines (autologous tumor cells, dendritic cells, adoptive cell therapy *etc.*) or combination with granulocyte-macrophage colony-stimulating factor and several types of interleukin (14, 41-44).

Summary

The clinical significance of cetuximab treatment in patients with colorectal cancer or other cancer types (head and neck tumors, lung tumors) has so far been associated with combined chemotherapy or with radiation. It was shown that these treatment strategies have their clinical limitations and do not fully exploit the immunomodulatory effect of cetuximab, particularly in the induction of ADCC response (45). The combination of cetuximab with other immunotherapeutic treatment modalities certainly opens-up new opportunities for targeted therapy in patients with colorectal cancer.

Conflicts of Interest

The Authors declare that they have no conflict of interests regarding the publication of this article.

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Attachment XIV

Rohan V, Baxa J, Tupy R, Cerna L, Sevcik P, Friesl M, Polivka J Jr, Polivka J, Ferda J. Length of occlusion predicts recanalization and outcome after intravenous thrombolysis in middle cerebral artery stroke. Stroke. 2014; 45(7):2010-7. **(IF = 5.787)**

Length of Occlusion Predicts Recanalization and Outcome After Intravenous Thrombolysis in Middle Cerebral Artery Stroke

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Background and Purpose—The length of large vessel occlusion is considered a major factor for therapy in patients with ischemic stroke. We used 4D-CT angiography evaluation of middle cerebral artery occlusion in prediction of recanalization and favorable clinical outcome and after intravenous thrombolysis (IV-tPA).

Methods—In 80 patients treated with IV-tPA for acute complete middle cerebral artery/M1 occlusion determined using CT angiography and temporal maximum intensity projection, calculated from 4D-CT angiography, the length of middle cerebral artery proximal stump, occlusion in M1 or M1 and M2 segment were measured. Univariate and multivariate analyses were performed to define independent predictors of successful recanalization after 24 hours and favorable outcome after 3 months.

Results—The length of occlusion was measureable in all patients using temporal maximum intensity projection. Recanalization thrombolysis in myocardial infarction 2 to 3 was achieved in 37 individuals (46%). The extension to M2 segment as a category (odds ratio, 4.58; 95% confidence interval, 1.39–15.05; P=0.012) and the length of M1 segment occlusion (odds ratio, 0.82; 95% confidence interval, 0.73–0.92; P=0.0007) with an optimal cutoff value of 12 mm (sensitivity 0.67; specificity 0.71) were significant independent predictors of recanalization. Favorable outcome (modified Rankin scale 0–2) was achieved in 25 patients (31%), baseline National Institutes of Health Stroke Scale (odds ratio, 0.82; 95% confidence interval, 0.72–0.93; P=0.003) and the length of occlusion M1 in segment (odds ratio, 0.79; 95% confidence interval, 0.69–0.91; P=0.0008) with an optimal cutoff value of 11 mm (sensitivity 0.74; specificity 0.76) were significant independent predictors of favorable outcome.

Conclusions—The length of middle cerebral artery occlusion is an independent predictor of successful IV-tPA treatment. **(*Stroke*. 2014;45:2010-2017.)**

Key Words: computed tomography ■ middle cerebral artery ■ outcome ■ stroke ■ thrombolytic therapy

Natural history of acute stroke with middle cerebral artery (MCA) occlusion has a very poor prognosis and localization of occlusion is one of the most important factors.[1] Intravenous thrombolysis with application of recombinant tissue-type plasminogen activator (IV-tPA) is currently the only clinically proven method of acute ischemic stroke treatment[2]; however, its effect is limited in a significant percentage of patients with a large vessel occlusion (LVO). Recanalization of LVO after IV-tPA depends on several factors that have been identified such as location, extent, composition of occlusion, collateral status, and glycemia.[3–8] Endovascular therapy (ET) is an alternative method, more effective in recanalization of occluded vessel, but the clinical superiority of IV-tPA has not yet been proved in large trials.[9–11] In anterior circulation, recanalization rate and clinical outcome after IV-tPA have been worst in patients with terminal intracranial artery occlusion and best in distal MCA occlusion.[12–14] The impact of intravascular thrombus quantity has been evaluated using hyperatenuated arterial sign in noncontrast CT (NCCT) study[15] and using CT angiography (CTA) with semiquantitative scoring as Clot Burden Score.[12,13]

With the advent of multidetector CT scanners allowing volumetric whole brain CT perfusion examinations, time-resolved 4-dimensional CTA (4D-CTA) of cerebral vasculature can be used for noninvasive cerebral hemodynamic assessment.[16–18] The aim of this study was to identify predictors of IV-tPA successful recanalization and outcome in patients with MCA stem (M1) occlusion determined using 4D-CTA temporal maximum intensity projection (tMIP) datasets.

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Materials and Methods

Study Design

We performed a retrospective single-center study of a prospectively acquired data of consecutive patients with final diagnosis ischemic stroke treated with IV-tPA in the period January 2009 to December 2012. Clinical and imaging data, obtained as part of routine stroke care, were reviewed with the approval of The Institutional Review Board.

The standard imaging protocol in patients with suspected acute stroke consists of NCCT followed after exclusion of intracranial hemorrhage with volume perfusion CT examination (VPCT) and CTA of the neck and cerebral arteries for assessment of therapy. The inclusion criteria for our study was the isolated occlusion of the MCA in the M1 and M2 segments, IV-tPA therapy started up to 4.5 hours from symptoms onset, follow-up CTA examination during a time interval of 22 to 26 hours after the IV-tPA administration, and a complete 3-month clinical follow-up. Patients with incomplete M1 occlusion, isolated occlusion of the M2 segment, and occlusion involving internal carotid artery or insufficient technical quality of the VPCT and CTA examination were excluded.

Data on clinical history, demographics, laboratory results, risk factors, prestroke modified Rankin scale (mRS) score were obtained as a part of standard clinical workup by trained staff. The initial neurological deficit and its development 24 hours after IV-tPA administration were assessed using the National Institutes of Health Stroke Scale (NIHSS). Clinical outcome in the 3-month interval was assessed using mRS by a trained neurologist in outpatient clinic or by telephone contact with caregivers and dichotomized into favorable outcome (mRS 0–2) and poor clinical outcome (mRS 3–6). The pathogenesis of stroke was classified according Trial of Org 10172 (TOAST) criteria.

Imaging Protocol

CT examinations consisted of NCCT, VPCT, and CTA on admission and follow-up NCCT and CTA were performed on a dual-source computed tomograph (Somatom Definition, Siemens Healthcare, Erlangen, Germany). NCCT in the range of whole brain was performed in spiral mode with collimation 64×0.6 mm, rotation time 0.5 seconds, tube voltage 120 kV, and tube current 320 mAs. Series in orthogonal projections (6-mm section width) were performed for tissue evaluation. Thin series (1-mm section width) were performed for hyperdense MCA sign (HDMCAS) assessment. VPCT covering the whole brain (136 mm) was performed by using a spiral shuttle mode consisting of 25 repeated scans with a duration of 1.75 seconds (collimation 24×1.2 mm, rotation 0.33 seconds, 100 kV, 150 mAs). The acquisition was started together with the onset of intravenous application of 40 mL (350 mg/mL - Iomeron 350, Bracco, Italy) of iodine contrast agent at a flow-rate of 5 mL/s and a saline flush of 60 mL at the same rate (cubital vein or vein at the hand dorsum). Axial images with a 1.5-mm section width (increment 0.7 mm) were reconstructed (total 4800 images), processed in dedicated VPCT Neuro (Syngo, Siemens Healthcare, Erlangen, Germany) application, and tMIP series were created for further evaluation. The cranio-cervical CTA was performed (collimation 2×64×0.6 mm, 120 kV, 62 mAs, rotation 0.33 seconds, pitch factor 0.7) using 60 mL of iodine contrast agent (350 mg/mL - Iomeron 350, Bracco, Italy) at a flow-rate of 4 mL/s with a saline flush of 50 mL. The examination was started using bolus-tracking technique monitoring the density increase in the ascending aorta (threshold 100 Hounsfield Unit [HU]), the scanning direction being caudo-cranial. Series reconstructed at the section width 0.6 mm (increment 0.4 mm) was performed for further evaluation. The same parameters were used for follow-up examination after IV-tPA.

Image Analysis

Image analysis was performed at the Syngo Leonardo workstation (Siemens Healthcare, Erlangen, Germany). Two readers (radiologists with 8 and 5 years' experience in neuroimaging) blinded to clinical information assessed CT data set of all patients. To determine intraobserver variability, all images were assessed twice in month interval and in randomized order. The presence of HDMCAS was defined by the following criteria[19,20]: spontaneous visibility of the whole or part of horizontal segment of the MCA, density of the MCA higher than that of the surrounding brain, disappearance on bone windows, unilaterality, and absence of subarachnoid bleeding. The M2 dot sign was defined as hyperattenuation of an arterial structure in the Sylvian fissure relative to the contralateral side and was not considered sufficient for diagnosis of HDMCAS when present in isolation. CTA-based measurement of vessel attenuation in HU of occluded M1 and corresponding contralateral site was done by placing a small round region of interest throughout the artery, and the average of the 2 highest HU values was used for further analysis. Absolute HU of symptomatic site was also corrected for hematocrit using the HU ratio (rHU=HU thrombus/HU contralateral MCA).

VPCT data-sets were processed using a dynamic analysis package (VPCT Neuro, Syngo, Siemens Healthcare, Erlangen, Germany) with automatic motion correction and noise reduction technique. To assess initial extent of infarction, cerebral blood volume (CBV) maps were scored using the Alberta Stroke Program Early CT Scale (ASPECTS) score evaluated. Each ASPECTS region was visually evaluated for relatively low CBV compared with the mirror region in the contralateral hemisphere.[21–23] To determine vascular occlusion, initially the differentiation between stenosis and complete occlusion was performed (tMIP and cine 4D-CTA).[24] Subsequently, the occlusion length measurement was performed in the standard viewing software (Syngo 3D, Siemens Healthcare, Erlangen, Germany) using the MIP reconstruction (2-mm section width). According to the thrombus orientation, 2 adapted oblique planes (2D measurement) were performed—in these planes, the occlusion length was measured manually between proximal and distal occlusion end using the freehand curve function with respect of anatomic course. The longer diameter of both planes that corresponded more to the anatomic course of the MCA was used in the statistical analysis. In case of continuous M1 and M2 segment occlusion with substantially different paths of both segments, the lengths were measured separately and were summed. The longest occlusion in the M2 segment was measured using the above-mentioned methods (Figure 1). Finally, the length of M1 proximal stump was measured.

We used 3 angiography parameters of quantitative values in our statistical analysis of recanalization and outcome prediction: (1) the whole length of occlusion including both occlusion in segment M1 and eventually M2, if involved (M1/M2), (2) the occlusion length only in the M1 segment (M1), and (3) the length of nonoccluded proximal part of M1 segment (M1 stump) for localization of clot origin in M1 segment. Moreover (4), we used qualitative parameters for differentiation of pure M1 segment occlusion (M1 only) and occlusion extended to M2 segment (M1+M2).

In addition thick sections tMIP were evaluated for the presence of collaterals in the MCA territory comparing with contralateral hemisphere on a 4-point scale: (0) absent (%), (1) >0 but <50%, (2) ≥50% but <100%, (3) normal (100%).[13,18,25] For statistical analysis, the scale was divided into good (score 2–3) and poor collaterals (score 0–1).

Recanalization Assessment

In all the patients, the follow-up CTA examination was done in a time interval of 22 to 26 hours after the IV-tPA onset. The recanalization effect was evaluated using the thrombolysis in myocardial infarction criteria (Grade 0 – no recanalization of M1, Grade 1 – partial or complete filling of M1 with persistent occlusion a least 1 M2 branch, Grade 2 – partial or complete filling of M1 with complete filling of the distal M2 branches flow, Grade 3 – complete recanalization with filling of all distal branches, including M3 and M4) – grades 2 and 3 were considered to successful recanalization.[26–28]

Statistical Analysis

Data are reported using standard descriptive statistics. Categorical variables were compared with χ^2 and Fisher exact tests and continuous variables with the Mann–Whitney *U* test. Logistic regression was

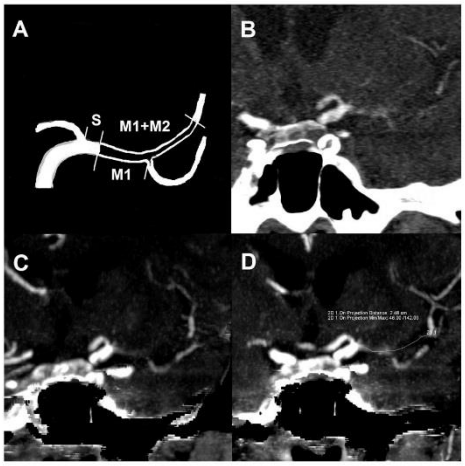

Figure 1. The diagram showing measured parameters: S indicates the length of proximal stump; M1, the occlusion length in M1 segment; M1+M2, the whole length of occlusion, the longest occlusion of M2 segment was measured (**A**). The comparison of conventional maximum intensity projection (MIP) of CT angiography (CTA) with poor filling of peripheral branches of middle cerebral artery behind M1 occlusion without identifiable distal end of occlusion (**B**) and temporal MIP (tMIP) reconstruction of 4-dimensional CTA data with 2D measurement, the longer distance was used in statistical analysis (**C** and **D**).

performed to determine the independent predictors of successful recanalization (thrombolysis in myocardial infarction 2–3), and favorable outcome (mRS 0–2). Variables of interest were age, sex, baseline clinical variables (NIHSS score on admission, atrial fibrillation, arterial hypertension, diabetes mellitus, hypercholesterolemia, previous antithrombotic and statin therapy, blood glucose and cholesterol), pathogenesis, onset-to-treatment time, the total length of M1/M2 occlusion, the length of occlusion in M1 segment, the extension of occlusion from M1 to M2 segment as a categorical variable, the length of M1 stump, presence of HDMCAS, absolute HU, rHU, collateral score, and CBV ASPECTS score. Successful recanalization (thrombolysis in myocardial infarction 2–3) was also included in outcome analysis. Each of these predictors was examined in univariate analysis. Forward stepwise logistic regression including all variables with $P<0.2$ in univariate analysis was performed. The results are expressed as odds ratio (OR) with 95% confidence interval (CI). A probability value of <0.05 was considered statistically significant. Receiver operating characteristic analysis and Matthews correlation coefficient was used to determine the optimal cut-off value of significant continuous variables. Inter-observer (HDMCAS, HU, collateral score, CBV ASPECTS, and CTA) and intraobserver (40 patients, 1 month later in random order, only tMIP) agreements were evaluated using intraclass correlation coefficients and κ statistics.

Results

Baseline Data

Among 411 patients treated with IV-tPA, we identified 142 patients with MCA impairment, 80 of whom with isolated M1-M2 occlusion were included in this study. The reminder were excluded because of significant impairment of ipsilateral common or internal carotid artery (n=27), incomplete M1 occlusion (n=7), occlusion originating beyond M1 (n=25), and insufficient technical quality of CT data (n=10). The main baseline data are summarized in Tables 1 and 2. Mean age was 70.9±12.8 (range, 34–96) years with 41% being male patients. Median baseline NIHSS score was 16 (range, 6–25) and onset to treatment time was 155 minutes (range, 90–275). The distal end of occlusion was not identifiable using CTA datasets in 32 patients (40%). The length of the complete MCA occlusion was measurable in all the patients using the tMIP datasets. In 57 (71%) patients, there was isolated M1 segment occlusion; in 23 patients (29%), there was extension to the M2 segment. The median length of MCA occlusion was 15.4 mm (range, 2.9–26.2), the median length of occlusion in M1 segment was 12.7 mm (range, 2.6–26.2), the median length of M1 stump was 7.8 mm (range, 0–23.0), and HDMCAS was present in 67 (84%) patients. There was no significant relation of HU values and rHU to stroke etiology ($P=0.886$ and $P=0.690$).

Recanalization

Successful recanalization after IV-tPA (thrombolysis in myocardial infarction 2 and 3) was achieved in 37 individuals (46%). Potential predictors of recanalization are summarized in Table 1. In the univariate analysis, baseline NIHSS score ($P=0.015$), the whole length of occlusion (M1/M2) ($P=0.009$), and the length of occlusion in M1 segment ($P=0.003$) were significant negative factors for successful recanalization. Neither presence of HDMCAS nor HU clot density did significantly affect recanalization. In multivariate logistic regression analysis, the length of M1 segment occlusion (OR, 0.82; 95% CI, 0.73–0.92; $P=0.0007$) and the extension to M2 segment as a category (OR, 4.58; 95% CI, 1.39–15.05; $P=0.012$) were significant independent predictors of recanalization with overall model fit $P=0.0004$. Other clinical and CT variables (vascular risk factors, pathogenesis, time to treatment, baseline NIHSS, the whole length of M1/M2 occlusion, the length of M1 stump, HDMCAS, HU, rHU, CVB ASPECTS score) were dropped from final model.

Based on the logistic regression analysis, we performed receiver operating characteristics curve analysis for determination of optimal cutoff value for the length of occlusion in M1 segment. The optimal value was found to be 12 mm, (Matthews correlation coefficient 0.373) with sensitivity 0.67 (95% CI, 0.518–0.833) and specificity 0.71 (95% CI, 0.551–0.844), and odds ratio 4.81 (95% CI, 1.86–12.40; $P=0.0012$) (Figure 2).

Outcome

Intracerebral hemorrhage developed after IV-tPA in 17 patients, and in 3 cases the hemorrhage was symptomatic. The outcome was assessed at a 3-month interval. Follow-up information was obtained in all patients. Favorable outcome (mRS 0–2) was achieved in 25 patients (31%; 6 patients with mRS 0, 10 patients with mRS 1, 9 patients with mRS 2, 8 patients with mRS 3, 10 patients with mRS 4, 22 patients with mRS 5), mortality during a 3-month follow-up was 19% (15 patients). In univariate analysis, lower baseline NIHSS ($P=0.0005$), successful recanalization ($P=0.0007$), and the length of both whole occlusion M1/M2 ($P=0.001$), the length in M1 segment ($P=0.0002$), and CBV ASPECTS score ($P=0.011$) were significant predictive factors for favorable outcome (Table 2).

Table 1. Baseline Characteristics of 80 Patients With Medial Cerebral Artery Occlusion Stroke in Context of Successful Recanalization

Characteristics	Patients With MCA Occlusion (n=80)	Patients With Recanalization TIMI 2–3 (n=37; 46%)	Patients With Recanalization TIMI 0–1 (n=43; 54%)	*P* Value
Age, y; median (range)	73 (23–91)	72 (41–96)	75 (23–91)	0.539
Female, n, %	47 (59)	14 (62)	23 (61)	0.566
Hypertension, n, %	65 (81)	30 (80)	35 (81)	0.971
Diabetes mellitus, n, %	26 (33)	14 (38)	12 (28)	0.346
Baseline glucose, median (range)	7.3 (4.8–27.5)	7.0 (4.8–14.8)	7.5 (5.1–27.5)	0.460
Atrial fibrillation, n, %	40 (50)	21 (57)	19 (44)	0.262
Statin therapy, n, %	13 (16)	8 (22)	5 (12)	0.233
Baseline cholesterol, median (range)	4.7 (2.4–6.8)	4.6 (2.4–6.5)	4.7 (2.4–6.8)	0.519
Antithrombotic therapy, n, %	25 (31)	12 (32)	12 (35)	0.832
Cardioembolic pathogenesis, n, %	46 (58)	22 (59)	24 (56)	0.742
Time-to-treatment, median (range)	155 (90–275)	165 (90–275)	160 (109–250)	0.858
Baseline NIHSS, median (range)	16 (6–25)	14 (6–22)	17 (6–25)	0.015*
Type of MCA occlusion				
M1only, n, %	57 (71)	30 (81)	27 (62)	0.076*
M1+M2, n, %	23 (29)	7 (9)	16 (38)	
Length of MCA occlusion				
M1, median (range)	12.7 (2.9–26.2)	9.3 (2.9–20.5)	13.9 (5.3–26.2)	0.001*
M1/M2, median (range)	15.4 (2.9–44.5)	13.2 (2.9–27.2)	16.9 (6.3–44.5)	0.002*
Length of M1 stump, median (range)	7.8 (0–23.0)	7.1 (0–23.0)	8.0 (0–20.8)	0.317
HDMCAS, n, %	67 (84)	30 (82)	37 (86)	0.549
Absolute HU, median (range)	64 (48–76)	64 (48–72)	65 (49–76)	0.339
rHU, median (range)	1.26 (0.86–1.50)	1.25 (0.89–1.50)	1.26 (0.86–1.43)	0.822
tMIP collateral score, median (range)	2 (1–3)	2 (1–3)	3 (1–3)	0.360
CBV ASPECTS score, median (range)	8 (2–10)	8 (3–10)	7 (2–10)	0.561

ASPECTS indicates Alberta Stroke Program Early CT Scale; CBV, cerebral blood volume; HDMCAS, hyperdense MCA sign; HU, Hounsfield Unit; M1, occlusion length only in M1 segment; M1/M2, complete occlusion length (M1 or M1 and M2 segment); MCA, middle cerebral artery; NIHSS, National Institutes of Health Stroke Scale; and TIMI, thrombolysis in myocardial infarction.

*Variable included in multivariable model ($P<0.2$).

In multivariate logistic regression analysis, baseline NIHSS (OR, 0.82; 95% CI, 0.72–0.93; $P=0.003$) and the length of occlusion M1 in segment (OR, 0.79; 95% CI, 0.69–0.91; $P=0.0008$) were significant independent predictors of favorable outcome with overall model fit $P<0.0001$ (Table 3). Other clinical variables (vascular risk factors, pathogenesis, time to treatment, recanalization status after 24 hours, extension to M2 segment, the whole length of M1/M2 occlusion, the length of M1 stump, HDMCAS, HU, rHU, CVB ASPECTS score) were dropped from final model. The multivariate analysis was repeated with exclusion of recanalization and converged to the same final model.

As in case of recanalization analysis, receiver operating characteristic analysis was used for determination the optimal cut-off value for the M1 occlusion length in favorable outcome prediction. The optimal value was found to be 11 mm, (Matthews correlation coefficient 0.470) with sensitivity 0.74 (95% CI, 0.591–0.887) and specificity 0.76 (95% CI, 0.618–0.892), and odds ratio 8.74 (95% CI, 2.81–27.20; $P=0.0002$) (Figure 3).

There was substantial interobserver agreements for HDMCAS ($\kappa=0.71$) and good agreement for CVB ASPECTS score ($\kappa=0.86$), HU measurement (intraclass correlation coefficient=0.75), and occlusion length (intraclass correlation coefficient=0.93) and intraobserver agreement (intraclass correlation coefficient=0.97).

Discussion

In this study, the length of occlusion in M1 segment in patients with proximal MCA occlusion, measured by tMIP derived from CT perfusion data, was an independent predictor of IV-tPA recanalization after 24 hours and favorable outcome after 3 months. Patients with M1 and continuous M1/M2 segment occlusion were included in our study. Our results confirmed that M2 involvement despite its extent is a significant negative factor for IV-tPA recanalization, but a significant relation with clinical outcome was not proved. The main reason is probably the importance of occluded perforating arteries originating in M1 segment, but probably because of its extreme variability, we did not find any correlation with the length of proximal stump as a localizing parameter of M1 occlusion.[29]

Our results support evidence that IV-tPA radiological and clinical effectiveness in MCA occlusion is limited and highly

Table 2. Baseline Characteristics of 80 Patients With Medial Cerebral Artery Occlusion Stroke in Context of Favorable Outcome

Characteristics	Patients With MCA Occlusion (n=80)	Patients With Favorable Outcome mRS (0–2) (n=25; 31%)	Patients With Poor Outcome mRS (3–6) (n=55; 69%)	*P* Value
Age, y; median (range)	73 (23–91)	69 (41–87)	76 (23–96)	0.062*
Female, n, %	47 (59)	14 (56)	33 (60)	0.736
Hypertension, n, %	65 (81)	20 (80)	45 (81)	0.847
Diabetes mellitus, n, %	26 (33)	7 (28)	19 (35)	0.563
Baseline glucose, median (range)	7.3 (4.8–27.5)	7.3 (5.3–13.2)	7.3 (4.8–27.5)	0.444
Atrial fibrillation, n, %	40 (50)	12 (48)	28 (51)	0.809
Statin therapy, n, %	13 (16)	3 (12)	10 (18)	0.233
Baseline cholesterol, median (range)	4.7 (2.4–6.8)	4.7 (2.4–6.5)	4.6 (3.1–6.8)	0.688
Antithrombotic therapy, n, %	25 (31)	7 (28)	18 (33)	0.673
Cardioembolic pathogenesis, n, %	46 (58)	14 (56)	32 (58)	0.855
Time-to-treatment, median (range)	155 (90–275)	150 (98–270)	160 (90–275)	0.834
Baseline NIHSS, median (range)	16 (6–25)	13 (6–21)	17 (6–25)	0.0005*
Type of MCA occlusion				
M1only, n, %	57 (71)	20 (80)	37 (67)	0.248
M1+M2, n, %	23 (29)	5 (20)	18 (33)	
Length of MCA occlusion				
M1, median (range)	12.7 (2.9–26.2)	8.1 (3.0–18.8)	14.3 (2.9–26.2)	0.0002*
M1/M2, median (range)	15.4 (2.9–44.5)	9.2 (3.0–27.5)	16.5 (2.9–44.5)	0.001*
Length of M1 stump, median (range)	7.1 (0–23.0)	8.5 (0–20.8)	7.1 (0–23)	0.223
HDMCAS, n, %	67 (84)	18 (72)	49 (89)	0.063*
Absolute HU, median (range)	64 (48–76)	63 (48–72)	65 (49–76)	0.060*
rHU, median (range)	1.26 (0.86–1.50)	1.22 (0.89–1.40)	1.28 (0.86–1.50)	0.075*
Collateral score, median (range)	2 (1–3)	2 (1–3)	3 (1–3)	0.488
CBV ASPECTS score, median (range)	8 (2–10)	8 (3–10)	7 (2–9)	0.011*
Recanalization TIMI 2–3, n, %	37 (46)	19 (76)	18 (46)	0.0007*

ASPECTS indicates Alberta Stroke Program Early CT Scale; CBV, cerebral blood volume; HDMCAS, hyperdense MCA sign; HU, Hounsfield Unit; M1, occlusion length only in M1 segment; M1/M2, complete occlusion length (M1 or M1 and M2 segment); MCA, middle cerebral artery; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale; and TIMI, thrombolysis in myocardial infarction.

*Variable included in multivariable model ($P<0.2$).

dependent on age, site of occlusion, amount and character of thromboembolic material, and collateral circulation with its reflection in initial neurological deficit.[12–15,30] Similar predictive factors seem to affect results of ET.[31] So there is a need for selection of reliable parameter which can be used in decision making in selection of more aggressive recanalization therapy.

In CTA the contrast level in an artery depends on scanning protocol and the circulation phase at the time of acquisition, and suboptimal timing of the data acquisition may lead to a significant bias of the examination result.[32] 4D-CTA overcomes this problem because of its dynamic nature and selection of the optimal circulation phase with improvement in image quality especially in case of delayed filling of collateral circulation in LVO and ability of assess completeness of LVO.[16–18] For this reason, we used 4D-CTA for precise evaluation of the presence, extent, and completeness of M1 occlusion.

Recent studies have been published that focused on the possibility of LVO extent determination using the special scoring system. The Clot Burden Score is 10-point scoring system to determine the occlusion extent, and a Clot Burden Score of >6 (corresponding only proximal or distal half of M1 with only 1 M2 occlusion) predicted higher recanalization rate and good clinical outcome.[12,13] However, the assessment was performed using the conventional CTA, a method that has limitations in detection of the distal end of the occlusion, according to our results. Until now, a single study focused on the determination of the absolute length of the LVO and possible prediction of recanalization success—the clot length >8 mm has nearly no potential for recanalization. The authors used the HDMCA sign in 2.5 mm NCCT slices to provide the assessment.[15] This value does not correspond to our results; however, individual methods of the measurement and source datasets were different, and recanalization assessment after 20.7±6.0 hours was done mainly using transcranial Doppler ultrasonography. We did not find such a clear cutoff value because recanalization appeared even in longer occlusions. ROC analysis for the occlusion length in M1 segment as a predictor of successful recanalization with almost linear shape means relatively low specificity and moderate sensitivity of resulting cutoff value.

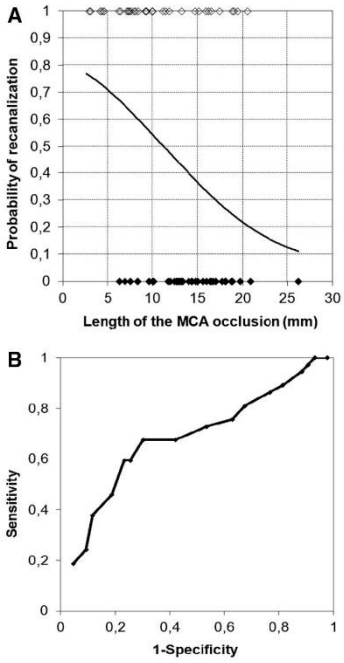

Figure 2. Logistic regression curve representing an estimate of successful recanalization probability by intravenous thrombolysis (IV-tPA). depending on the length of occlusion in M1 segment, odds ratio 0.87 (0.78–0.96) *P*=0.0047 (**A**). Receiver operating characteristic curve for the occlusion length in M1 segment as a predictor of successful recanalization by IV-tPA, the optimal value 12 mm, area under curve of 0.69, *P*=0.0012 (**B**). MCA indicates middle cerebral artery.

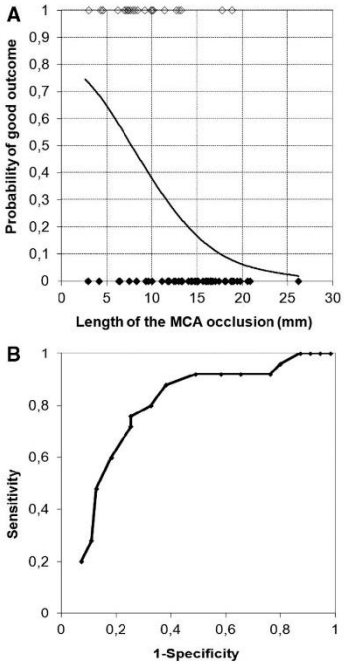

Figure 3. Logistic regression curve representing an estimate of a favorable functional outcome probability after intravenous thrombolysis (IV-tPA). treatment depending on the length of M1 occlusion, odds ratio 0.80 (0.71–0.90), *P*=0.0004 (**A**). Receiver operating characteristic curve for the occlusion length in M1 segment as a predictor of a favorable functional outcome after IV-tPA treatment, the optimal value 11 mm, area under curve 0.78, *P*=0.0001 (**B**). MCA indicates middle cerebral artery.

The main result of our study is identification of crucial impact of the length of M1 segment occlusion for prediction of IV-tPA clinical outcome. Other CT parameters were explored in recent studies. Clot composition assessed by HU density was a predictor of successful IV-tPA early recanalization and stroke subtype.[30] Because of later recanalization assessment, we could not compare this findings. Our results correspond with other study, where neither HDMCAS nor clot HU values were useful for IV-tPA effect and pathogenesis prediction.[19] Moreover, inconsistence in HU quantification methods and lower interobserver agreement limit these findings.

Table 3. Independent Predictors of Recanalization and Favorable Outcome

Independent Predictor	Odds Ratio (95% Confidence Interval)	*P* Value
Favorable outcome		
Baseline NIHSS	0.82 (0.72–0.93)	0.003
Length of M1 occlusion	0.79 (0.69–0.91)	0.0008
Overall model fit		<0.0001
Successful recanalization		
M1 occlusion only	4.58 (1.39–15.05)	0.012
Length of M1 occlusion	0.82 (0.73–0.92)	0.0007
Overall model fit		0.0001

NIHSS indicates National Institutes of Health Stroke Scale; and TIMI, thrombolysis in myocardial infarction.

There is lack of consensus in objective interpretation of CT perfusion parameters. We included the CBV ASPECTS score in our analysis as a predictor of baseline infarct extension, which proved to be a reliable parameter of outcome after IV-tPA and ET.[21,22,33] Insufficient collateral circulation on CTA and 4D-CTA has been shown as a negative predictor in LVO stroke.[13,18,25] We could not replicate this results because most of our patients have a good collateral status CBV ASPECTS score profile. One possible explanation is involvement of deep structures, which can be missed in collateral scoring and their clinical impact could be underestimated in the context of summary ASPECTS score.

Our study has some limitations that are necessary to mention. In a retrospective analysis of prospectively acquired data, there is a potential for bias in selection of subjects. The 24-hour interval from the IV-tPA onset for recanalization assessment, which we used, was considered to be suitable when other recanalization method was not available in our institution. The

main limitation of the occlusion length determination as an independent factor for IV-tPA recanalization is assessment of the effect after ≈24 hours and lack of information about early recanalization. Possibility of later reperfusion in patients with longer occlusion can cause bias in assessment of its effect on outcome. This hypothesis is supported by fact that recanalization status after 24 hours was dropped from final model. The limited number of subjects reduces statistical significance of multivariate analysis. The vessel analysis as gold standard could be questionable, but in our opinion, there is no method more suitable. The digital subtraction angiography is not indicated as the first vascular imaging modality. The comparison of both methods in patient indicated o ET may be limited by bridging IV-tPA therapy and time interval.

The importance of our study is based on detailed assessment of homogenous group of patients with MCA/M1 occlusion with relation to the effect of IV-tPA in the context of searching for a subgroup of patients, who would be optimal candidates for rapid triage for ET, because the position of this method has not been fully established.[9] We identified single independent highly reproducible radiological predictor of IV-tPA clinical outcome. Comparison with results of ET in this subgroup of patients could help in better stratification of stroke patients in recanalization treatment planning.

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Disclosures

None.

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Attachment XV

Polivka J, Rohan V, Sevcik P, Polivka J Jr. Personalized approach to primary and secondary prevention of ischemic stroke. EPMA J. 2014; 5(1):9.

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REVIEW Open Access

Personalized approach to primary and secondary prevention of ischemic stroke

Jiří Polívka[1,2*], Vladimír Rohan[1,2], Petr Ševčík[1,2] and Jiří Polívka Jr[3,4]

Abstract

Primary and secondary prevention of ischemic stroke represents a significant part of stroke management and health care. Although there are official guidelines concerning stroke management, new knowledge are introduced to them with a slight delay. This article provides an overview of current information on primary and secondary prevention of ischemic stroke. It summarizes information especially in the field of cardioembolic stroke, the use of new anticoagulants and the management of carotid stenosis based on the results of recent clinical studies. The optimal approach in stroke management is to follow these recommendations, to know new strategies and to apply an individual personalized approach in our clinical decisions.

Keywords: Ischemic stroke, Primary prevention, Secondary prevention, Anticoagulation therapy, Antiplatelet therapy, Personalized medicine

Review

Introduction

Stroke is the third leading cause of death and the main cause of disability of adults in developed countries. Despite advances in prevention, the prevalence and incidence of ischemic stroke is expected to rise given the aging population [1]. A number of recommendations have been created on the management and prevention of stroke and transient ischemic attack. The guidelines of the European Stroke Organization (ESO) published in 2008 (updated in 2009) [2] cover stroke management in detail. These general recommendations should be transformed to individualized and personalized approach to each patient [3]. As there have been further advances since that time, this review provides an updated look at stroke management especially at stroke prevention.

Primary prevention

Primary prevention aims to reduce the risk of ischemic stroke (IS) in subjects who have been asymptomatic and focuses on influencing and managing known risk factors such as arterial hypertension (AH), diabetes mellitus (DM) and disorders of lipid metabolism. The start and intensity of curative steps depends on an assessment of the total cardiovascular risk (CVR). In asymptomatic individuals, this value is determined by using nomograms from the Systematic Coronary Risk Evaluation (SCORE) [4] project, which evaluates the age, gender, systolic blood pressure (SBP), smoking habits and total cholesterol levels. A value over 5% is considered a high risk (probability of dying of cardiovascular disease in the next 10 years). Among symptomatic individuals with manifested cardiovascular disease, type 2 diabetes or type 1 diabetes with microalbuminuria, or chronic kidney disease, the risk is high (≥5%) or, if there are a combination of factors, very high (≥10%). The primary emphasis is placed on non-drug strategies and lifestyle changes—adopting a healthy diet with a higher proportion of fruits and vegetables and limited salt, increasing regular aerobic physical activity, reducing elevated body weight, limiting alcohol consumption and quitting smoking [2,4].

Arterial hypertension

For AH, which is a proven independent risk factor, the guidelines advocate correcting SBP to under 140 mmHg, except in older patients under 80 years of age, for whom there is a proven benefit of reducing SBP ≥160 to 150 – 140 mmHg. In patients over 80, each case must be assessed individually based on the subject's physical and mental

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condition. Also, the prehypertension (<120/80 mmHg) is associated with higher stroke morbidity [5]. The benefit of correcting SBP values under 140 mmHg has not been proven. A target diastolic value of under 90 mmHg is indicated; for diabetics, the target value is below 85 mmHg. In low-risk patients, non-drug strategies are primarily deployed first; if antihypertensive therapy is started, blood pressure values should be reduced only gradually. The choice of an antihypertensive agent depends on the patient's age and comorbidities, in older patients (over 80 years of age) calcium channel blockers or thiazide diuretics [6]. In women, the screening for arterial hypertension is indicated before prescribing oral contraceptives [7].

Diabetes mellitus

In DM patients, in addition to controlling blood glucose levels, greater emphasis is placed on controlling BP with a target value of under 140/80 mmHg. Angiotensin-conver ting enzyme inhibitors or angiotensin receptor antagonists are preferred for treatment [6]. Concomitant hypercholesterolemia should be corrected at low-density lipoprotein cholesterol (LDL-C) levels exceeding 3.0 mmol/L, primarily through statins [6,8].

Dyslipidemia

As another risk factor, dyslipidemia should be corrected in primary prevention with respect to the overall cardiovascular risk. Strategies should aim at influencing the LDL-C value by making lifestyle changes and, if necessary, through statin therapy [8-10] (Table 1).

Atrial fibrillation

The IS prevention guideline for patients with atrial fibrillation (AF) has undergone the most significant development in connection with the introduction of new oral anticoagulants (NOAs) and the availability of data from patients with implanted devices [11]. In patients with non-valvular AF, the stratification of IS risk has been re-evaluated to reflect the main and secondary clinically relevant risk factors when applying CHA_2DS_2-VASc (Table 2). Antithrombotic therapy is not recommended to AF patients over 65 years without additional risk factors, regardless of gender. As patients with severe renal insufficiency have not only a high risk of IS but also a high risk of death, heart attack and bleeding or hemorrhagic complications, they have been excluded from clinical studies. For this reason, it is difficult to evaluate the benefit of antithrombotic therapy, and it is not included in the score. The benefit of antithrombotic therapy in preventing IS must be higher than the risk of serious hemorrhage, especially intracerebral hemorrhage (ICH), the most feared complication of this type of therapy. It can be stratified using the HAS-BLED score (Table 3), which correlates well with the risk of ICH [12]. It was proven that in patients with the same HAS-BLED score who were treated with acetylsalicylic acid (ASA), the risk of ICH and major hemorrhagic complications is similar [13]. It is recommended to formally establish the risk of hemorrhage in all AF patients. Caution is necessary in case the HAS-BLED score is ≥3; however, this criterion does not exclude the patient from oral anticoagulant (OA) therapy, as the benefit of anticoagulant therapy exceeds the risk of hemorrhage even in patients with a high HAS-BLED score [14]. However, it is necessary to maximally compensate potentially reversible bleeding risk factors, such as uncontrolled arterial hypertension or the concomitant use of ASA or non-steroidal anti-inflammatory drugs (NSAID). In IS prevention among patients with non-valvular AF, ASA should be administered only to patients who reject any form of OA therapy [11].

Other heart diseases

Anticoagulant therapy with warfarin in primary and secondary IS prevention is indicated in patients with a mechanical heart valve replacement (international normalized ratio (INR) 2.5–3.5), the presence of an intraventricular thrombus, mobile thrombus in the ascending aorta, dilated cardiomyopathy, especially in patients under 60 years of age [16], left atrial myxoma and/or mitral stenosis after a previous embolic event (INR 2–3).

New oral anticoagulants in the prevention of ischemic stroke

Until 2012, the only option for OA therapy for AF patients was vitamin K antagonists (VKA), mainly in the form of dose-adjusted warfarin with an INR of 2–3. Based on successful clinical studies which proved non-inferiority in comparison with warfarin in primary and secondary prevention of IS and peripheral embolization in patients with non-valvular AF—RE-LY [17], ROCKET-AF [18], ARISTOTLE [19] and ENGAGE-AF [20] — new oral anticoagulants (NOAs) were approved in 2012: first dabigatran as a direct thrombin inhibitor and later the direct Xa inhibitors rivaroxaban and apixaban, and edoxaban in 2013 in

Table 1 Recommended target treatment levels for LDL-C (adjusted according to Catapano et al. [8])

Cardiovascular risk	LDL-C target value
Very high (manifest cardiovascular disease, type 2 DM, type 1 DM with organ impairment, moderate to severe kidney impairment or cardiovascular score ≥10%)	<1.8 mmol/L and/or ≥50% reduction of LDL-C
High (significantly increased individual risk factor, cardiovascular score 5%–10%)	<2.5 mmol/L
Moderate (cardiovascular score 1%–5%)	<3 mmol/L

Table 2 CHA_2DS_2-VASc score (adjusted according to Lip et al. [15])

Risk factor		Score
C	Congestive heart failure	1
H	Hypertension	1
A_2	Age (≥75 years)	2
D	Diabetes mellitus	1
S_2	Stroke (CS/TIA in history)	2
V	Vascular disease (myocardial infarction/peripheral vascular damage)	1
A	Age (65–74 years)	1
Sc	Sex category (female gender)	1

the USA. Of all NOAs that have been tested, clinical studies proved non-inferiority in comparison with warfarin, with better safety and reduced risk of ICH. This led to an update in the Guidelines for the Management of Atrial Fibrillation by the European Society of Cardiology [11], with NOAs considered more suitable for the majority of patients with non-valvular AF. Because there is only limited experience with NOAs, strict adherence to the approved indications and careful post-marketing monitoring are recommended. Given that there are no direct comparative studies among individual NOAs and indirect comparative analyses do not indicate that there are fundamental differences in efficacy, no conclusions can be drawn regarding preference for individual NOAs [21]. The advantage of NOAs over warfarin is their fixed dosage with no need for regular monitoring of anticoagulant activity. However, when determining the appropriate dosage, it is necessary to consider the patient's age and renal function. Another advantage is the lower quantity of clinically significant drug interactions. The short half-life and rapid onset and decrease in efficacy are important aspects that required careful compliance with treatment, as the anticoagulant effect is insufficient if more than one dose is skipped. Renal values must be monitored, especially for dabigatran. In polymorbid patients, these values may rapidly change in the course of an intercurrent disease [22]. Unlike warfarin, no haemocoagulation test can be used for NOAs that would clearly quantify the anticoagulation effect. Non-specific anticoagulation tests may be used, such as activated partial thromboplastin time (APTT); thrombin time or ecarin clotting time tests are more specific for dabigatran, plus prothrombin time (PT) or determining anti-Xa activity for Xa inhibitors. However, these tests are used more to determine the presence of a medication and cannot be reliably used to estimate the anticoagulant effect of the NOA. As yet, no NOAs have a specific antidote. To rapidly adjust coagulation in case of serious bleeding, in addition to blood derivatives, specific procoagulant reversal agents such as prothrombin complex concentrate (PCC), activated prothrombin complex concentrate (APCC) or recombinant factor VIIa (r-FVIIa) may be administered. Hemodialysis may also be considered for dabigatran, but practical experience is still limited [23].

Table 3 HAS-BLED score (modified according to Pisters et al. [12])

Risk factor		Score
H	Hypertension (not controlled, >160 mmHg of systole)	1
A	Abnormal renal function or hepatic function	
	Transplant dialysis, Cr >200 μmol/L	1
	Cirrhosis, bilirubin >2× normal, AST/ALT/AP >3× normal	1
S	Stroke (CS in history, especially lacunar stroke)	1
B	Bleeding (bleeding in history or bleeding diathesis, anemia)	1
L	Labile INR (unstable or high INR)	1
E	Eldery (age ≥65 years)	1
D	Drugs/alcohol (antiplatelet medications, non-steroidal antiphlogistics or excessive use of alcohol)	1 1

Asymptomatic stenosis of the internal carotid artery

Atherosclerotic stenosis of the extracranial part of the internal carotid artery (ICA) is associated with an increased risk of IS [24]. The risk of asymptomatic stenosis progression rises over time, depending on the presence of additional risk factors such as smoking, arterial hypertension, DM, the level of stenosis, the composition of the plaque and contralateral impairment of the ICA [25]. In short-term monitoring, the risk of ipsilateral IS in patients with asymptomatic ICA stenosis ranges between 1% and 3%, depending on the seriousness of the stenosis and the studied population [24]. As there have been significant advances in conservative and invasive treatment, no valid data is available right now that assesses the real risk of asymptomatic ICA stenosis comparing these two different management strategies. In a 10-year study monitoring patients in the ACST trial [26], CEA reduced the risk of stroke, including perioperative stroke, to 13.4%, compared to 17.9% in patients with delayed intervention or conservative treatment. The fact that 80% of the patients in the study were not treated with statins may influence an interpretation of the results. In regard to a comparison of carotid endarterectomy (CEA) and carotid artery stenting (CAS), the last extensive study was CREST [27], which compared these two strategies in symptomatic and asymptomatic patients with significant ICA stenosis. During a mid-term 2.5-year monitoring study, the 4-year risk composite target (IS, myocardial infarction or death) was found to be nearly the same for both CAS and CEA (7.2% and 6.8%), regardless of age or clinical presentation of the stenosis. Surprisingly, patients over 70 years of age profited more from CEA, while patients under 70 profited more from CAS. The risk of CS or death was 6.4% for CAS and 4.7% for CEA, with the difference nearing

significance only among asymptomatic patients. Only differences in perioperative complications were significant, with a higher risk of CD in CAS (4.1% vs. 2.3%) and a higher risk of myocardial infarction in CEA (1.1% vs. 2.3%). The risk was similar in the subsequent period (2.0% vs. 2.4%). The results of this study do not fundamentally change the current guideline that in the case of significant asymptomatic ICA stenosis (60%–90% according to the North American Symptomatic Carotid Endarterectomy Trial—NASCET), intensive drug treatment of the risk factors (DM, arterial hypertension, dyslipidemia) is indicated. CEA is indicated only among patients with a high risk of stroke (men, stenosis >80%, plaque character) and expected survival greater than 5 years, performed in centres where the operative risk is less than 3%. Administration of ASA is indicated before and after CEA. CAS is not indicated for asymptomatic subjects.

Secondary prevention

Secondary prevention aimed at reducing the risk of another IS must be based on the etiology of the past ischemic IS and consider the presence of any additional risk factors. It consists of the optimal compensation of vascular risk factors—arterial hypertension, hyperlipidaemia and diabetes, antiplatelet or anticoagulant therapy and, in indicated cases, the use of invasive surgical or endovascular therapy. The patient's regimen, emphasizing adequate physical activity, elevated body weight loss, sufficient hydration, dietary changes, quitting smoking and a reduction in excessive alcohol consumption, is an integral part of this [2].

Vascular risk factors

As part of optimizing vascular risk factors, after the acute stroke phase subsides, antihypertensive therapy that adjusts BP to normal values is indicated. The BP value must be individualized with regard to possible haemodynamic consequences, however, such as in patients with bilateral stenosis in afferent cerebral arteries or the trunk of the cerebral artery. In contrast, in patients with small artery damage, reducing pressure below 130 mmHg SBP [28] seems suitable. As in primary prevention, individualized DM therapy is indicated. In non-cardiogenic stroke, statin therapy with a target LDL value under 2.5 among high-risk subjects under <1.81 mmol/L is indicated [9]. In patients with sleep-disordered breathing, respiratory therapy with continual positive pressure in the air passages is recommended [29].

Atherothrombotic stroke

In most cases, antiplatelet therapy is indicated for secondary prevention of atherothrombotic (non-cardioembolic) stroke. According to the ESO guidelines, ASA should be administered in combination with dipyridamole (25/200 mg 2× daily) or clopidogrel monotherapy (75 mg/day), alternatively, as an economical alternative ASA monotherapy (75–325 mg/day). The efficacy of higher doses of ASA has not been proven [30]. The combination of ASA + clopidogrel versus clopidogrel monotherapy and the combination of ASA + clopidogrel versus ASA monotherapy have been investigated in MATCH [31] and CHARISMA [32], respectively. In both studies, with a long-term administration of ASA + clopidogrel, the insignificant reduction in the risk of ischemic stroke was accompanied by a significant increase in bleeding complications and mortality. The last SPS3 trial of lacunar stroke came to the same conclusion [33]. The coincidence of stroke and a recent myocardial infarction or status post coronary stenting thus remain the indications for using a combination of ASA + clopidogrel. The new specific indication for the combination of short-term ASA + clopidogrel therapy seems to be significant intracranial symptomatic stenosis in a major artery. The SAMMPRIS study investigated the effect of intracranial stenting and intensive drug therapy (ASA + clopidogrel + statin) versus intensive drug therapy alone [34]. It showed a significantly higher incidence of stroke and death in the stented group during the 30-day (14.7% vs. 5.8%) and the 1-year (20.0% vs. 12.2%) monitoring period. At the same time, the study showed about a 50% drop in the incidence of IS and death in the medicated group compared to historical controls—patients treated with ASA or warfarin in the WASID study [34,35]. In the case of stroke, it is also necessary to consider possible resistance to ASA or clopidogrel when starting antiaggregant therapy. It is necessary to evaluate the compensation of other vascular risk factors, especially the etiology of the stroke with regard to the potential for cardioembolism, especially in the case of paroxysmal AF.

Cardioembolic stroke

According to the ESO guidelines, anticoagulant therapy with warfarin (INR 2–3) is indicated in the secondary prevention of IS in patients with AF both paroxysmal and permanent and also in other cardioembolism or NOAs in the case of AF. Regarding adherence in the management of atrial fibrillation, there are the problems of high differences in the management and compliance [36]. In cryptogenic stroke, paroxysmal atrial fibrillation should always be excluded. Its detection rate is dependent on the intensity of ECG monitoring [37,38]. When deciding on the timing of starting full anticoagulant therapy, one must consider the risk of the hemorrhagic transformation of infarction foci with regard to size and location. The benefit of early anticoagulant therapy versus a delayed start has not been proven [39]. Secondary prevention with ASA alone has little effect, and the risk of major bleeding is not significantly different from OA [13]. ASA + clopidogrel compared to warfarin in patients with AF also shows low

efficacy and does not bring a significant reduction in the risk of bleeding complications—ACTIVE W study [40]. Antiplatelet therapy should be restricted to patients who reject any form of OA therapy. The NOAs discussed above are an alternative to warfarin.

In the case of concurrent acute myocardial infarction, concurrent anticoagulant and antiaggregant therapy for a 3-month period is indicated with regard to the size of the infarction foci and the risk of hemorrhagic transformation. As in primary prevention, anticoagulant therapy with warfarin is also indicated in the case of other cardiac sources of embolism.

Patent foramen ovale

The significance of patent foramen ovale (PFO) is still discussed and studied in patients with cryptogenic stroke. The results of three randomized studies were published in 2012—CLOSURE I [41], PC-Trial [42] and RESPECT [43], comparing the effect of PFO closure with ASA or warfarin drug treatment. REDUCE, a study comparing PFO closure with antiplatelet treatment vs. antiplatelet treatment as monotherapy, is ongoing [44]. Given the low incidence of target events (stroke, death) and relatively short period of monitoring (2 years), none of the studies have shown a statistically significant difference among the monitored groups, despite a certain trend towards mechanical closure of PFO. Subanalyses and meta-analyses of these studies may provide further data. In contrast, data from observational studies with a longer period of monitoring report a statistically significant difference in favor of invasive vs. drug therapy, and in the case of drug therapy, there is a significant benefit of anticoagulant therapy vs. antiplatelet therapy [45]. Although for the reasons described above there is still not enough clear clinical evidence, it is appropriate to consider PFO closure only in patients with embolic-type stroke with a significant shunt in the transesophageal echocardiogram exam and if other risk factors are absent. In other cases, anticoagulant or antiplatelet therapy is indicated.

Thrombophilia

In patients with ischemic stroke of unclear etiology or who are under 40 years of age, testing for thrombophilia is indicated. Anticoagulant therapy is normally indicated in the case of a proven deficiency of antithrombin III, protein C and protein S; resistance to activated protein C (factor V Leiden), especially in the case of deep vein thrombosis, is also detected. Patients with positive antiphospholipid antibodies with no other signs of antiphospholipid syndrome are indicated to only take antiplatelet therapy; anticoagulant therapy is indicated for patients meeting the criteria for antiphospholipid syndrome [46].

Major extracranial arterial stenosis

As is the case for other atherothrombotic IS, intensive drug treatment of the risk factors and antiplatelet therapy are indicated for secondary prevention in patients with major extracranial arterial stenosis [2,46]. In the question of using CEA or CAS in patients with significant ICA stenosis, there have not been any fundamental changes to the ESO guidelines; there is still a lack of data comparing these strategies with current intensive drug therapy. Early CEA within 2 weeks after the stroke is indicated for patients with small-scale infarction who do not have a high risk of hyperperfusion syndrome with potential hemorrhagic transformation of the infarction foci. The benefit of CEA at an interval of 3 months is minimal compared to conservative treatment. In addition to the seriousness of the stenosis, the characteristics of the plaque also plays a role in indicating CEA/CAS, with the presence of ulcers being an indication to operate even lower-level stenosis (50%–69%) while adhering to the principles of low perioperative morbidity and mortality (<3%). Even in the perioperative period, patients should be left on antiplatelet therapy. CAS is recommended only for patients in whom CEA is contraindicated, the location of the stenosis is not surgically accessible, with restenosis following CEA and post-radiation stenosis. After CAS, dual antiplatelet therapy with ASA + clopidogrel for a period of 1 month is indicated.

Intracranial arterial stenosis

Dual antiaggregant therapy with ASA and clopidogrel in combination with optimal compensation of vascular risk factors was compared against the effect of stenting symptomatic intracranial large artery stenosis (50%–99%) in the SAMMPRIS study [34]. Enrolment in the study was halted after 451 patients were included in the study, due to a significantly higher incidence of early stroke/death after stenting compared to conservative treatment (14.7% vs. 5.8%).

Conclusions

Stroke management is a great challenge. Stroke prevention remains the object of intense medical research. Based on new information provided in this review, management strategies of stroke prevention should be personalized, even if general guidelines exist. The management of arterial hypertension, diabetes, dyslipidemia, atrial fibrillation and the other causes of cardioembolic stroke in the primary and secondary stroke prevention, search for thrombophilia in younger patients and the management of extracranial arterial stenosis and the indication and use of new oral anticoagulants are of cardinal importance. An update to the current guidelines can also be expected.

Abbreviations

AF: atrial fibrillation; AH: arterial hypertension; APCC: activated prothrombin complex concentrate; APTT: activated partial thromboplastin time;

ASA: acetylsalicylic acid; CAS: carotid artery stenting; CEA: carotid endarterectomy; DM: diabetes mellitus; ESO: European Stroke Organization; ICH: intracerebral hemorrhage; INR: international normalized ratio; IS: ischemic stroke; NASCET: North American Symptomatic Carotid Endarterectomy Trial; NOAs: new oral anticoagulants; PCC: prothrombin complex concentrate; PFO: patent foramen ovale; PT: prothrombin time; r-FVIIa: recombinant factor VIIa; SBP: systolic blood pressure; TIA: transient ischemic attack..

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PJ and RV conceived the review and coordinated the drafting of the manuscript. SP and PJ Jr. participated in the design of the review, performed literature searches and identified relevant studies. PJ and RV provided content expertise. All authors read and approved the final manuscript.

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Attachment XVI

Polivka J, Polivka J Jr, Peterka M, Rohan V, Sevcik P, Topolcan O. Vitamin D and neurological diseases. Vnitr Lek. 2012; 58(5):393-5.

Vitamin D a neurologická onemocnění

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Předneseno na odborném pracovním setkání Vitamin D (projekt OPVK CZ 1.07/2.3.00/09.0182), konaném dne 22. června 2011 v Plzni a organizovaném LF UK a FN Plzeň ve spolupráci s Českou společností klinické biochemie České lékařské společnosti J. E. Purkyně, sekcí imunoanalýzy České společnosti nukleární medicíny České lékařské společnosti J. E. Purkyně a Endokrinologickým ústavem Praha

Souhrn: Je podán přehled o vztahu vitaminu D a některých neurologických onemocnění, kde je korelace opakovaně popisována. Nejvíce literárních údajů je z oblasti cerebrovaskulárních nemocí, dále u roztroušené sklerózy a kognitivních poruch. Hypovitaminóza D může souviset s nemocemi přímo, může se uplatňovat u rizikových faktorů nemocí (typicky u mozkových cévních příhod). Hypovitaminóza D se může dál uplatnit u osob s reziduálním funkčním postižením v důsledku neurologické nemoci (poruchy hybnosti, nesoběstačnost) a dále zhoršovat funkční stav v důsledku svalové slabosti, instability a pádů.

Klíčová slova: vitamin D – cerebrovaskulární onemocnění – roztroušená skleróza – kognitivní poruchy

Vitamin D and neurological diseases

Summary: We provide an overview of the association between vitamin D and some neurological diseases where the correlation has repeatedly been described. The majority of literature refers to cerebrovascular diseases, followed by multiple sclerosis and cognitive disorders. Vitamin D hypovitaminosis might be associated with the diseases directly or it might contribute to the disease risk factors (typically in cerebrovascular events). Vitamin D hypovitaminosis may also play a role in patients with residual functional involvement due to a neurological disorder (movement disorders, lack of self-sufficiency) and worsen functional status owing to muscle weakness, instability and falls.

Key words: vitamin D – cerebrovascular disorders – multiple sclerosis – cognitive disorders

Úvod

Vitamin D je označován za prohormon a v poslední době mu je věnována značná pozornost. Základní metabolizmus vitaminu D je delší dobu znám stejně jako jeho role v udržování kalciové a fosfátové homeostázy. S rozvojem poznání dějů na buněčné a molekulární úrovni byly objeveny buněčné receptory (VDR) aktivní formy vitaminu D – 1,25-dihydroxyvitaminu D, ve většině tkání: kromě tradičních ledvin, střeva a kostí také v T- a B-lymfocytech, svalech, v buňkách nervového systému i v nádorových buňkách [1,26], v hladkých svalových buňkách cév či v epitelu [12]. Velká pozornost je věnována nedostatku vitaminu D v lidské populaci [15]. Úloha hypovitaminózy D je studována i u řady neurologických onemocnění.

Vitamin D a cerebrovaskulární onemocnění

Cerebrovaskulární onemocnění jsou 3. nejčastější příčinou mortality a nejčastější příčinou dlouhodobé funkční neschopnosti ve vyspělých zemích. V 80 % jde o mozkové ischemie, jejichž nejčastější příčina je aterosklerotická. 20 % iktů je hemoragických. Hypovitaminóza D se podílí na vzniku iktů z několika příčin.

Vztah vitaminu D a arteriální hypertenze byl prokázán několika studiemi týkajícími se geografické variability krevního tlaku [8,35,45] i sezónní variability krevního tlaku dle ročních období u téže populace [17,48]. Jedná se o inverzní vztah mezi hladinou 25(OH)D a hodnotami krevního tlaku. Arteriální hypertenze je jedním z rozhodujících rizikových faktorů ischemických i hemoragických mozkových cévních příhod [42].

Je prokázána souvislost hypovitaminózy D se vznikem diabetu 2. typu experimentálně u zvířat i u lidí [30]. Je popsáno několik cest, které se v této souvislosti uplatňují [1,29,43]. Metaanalýza prokázala inverzní vztah mezi hladinou 25(OH)D a prevalencí diabetu 2. typu [31]. Diabetes mellitus 2. typu je jedním z nejvýznamnějších rizikových faktorů aterosklerózy a mozkových cévních příhod [42].

Role zánětu v rozvoji aterosklerotického procesu je dostatečně známa [9,21]. Bylo prokázáno, že suplementace vitaminu D zvyšuje hladinu protizánětlivého cytokinu IL-10 a snižuje hladinu prozánětlivých cytokinů IL-6, IL-12 a TNF-α [22,36].

Je sledována role vitaminu D v rozvoji aterosklerózy a jejích subklinických projevů, jako je tloušťka intimy-medie karotické cévní stěny [27]. Byl prokázán jednoznačný inverzní vztah

mezi nízkou hladinou vitaminu D a rozvojem subklinické ateroskleró-zy [41]. Deficit vitaminu D je považován za významný nezávislý faktor ve vztahu k ateroskleróze [16]. Reis prokázal souvislost deficitu vitaminu D a zvýšené hladiny parathormonu s rizikem kardiovaskulárních nemocí včetně mozkových cévních příhod a karotické aterosklerózy [34].

Anderson zkoumal prospektivně hladiny vitaminu D v elektronických záznamech více než 41 000 pacientů ve státě Utah [2]. Zjistil normální hladiny (více než 30 ng/ml) u 36,4 % osob, nízké hladiny (16–30 ng/ml) u 46,9 % osob a velmi nízké hladiny (méně než 16 ng/ml) u 16,7 % osob. Nízké a velmi nízké hladiny vitaminu D korelovaly s výskytem mozkových cévních příhod ($p = 0,003$). Obdobně tomu bylo u kardiovaskulárních nemocí a onemocnění periferních tepen. Jejich výskyt byl až dvojnásobně vyšší. Za hlavní mechanizmus autor považuje sekundární hyperparatyroidizmus [20,46], který se uplatňuje 3 způsoby: zvýšenou inzulinovou rezistencí, predisponující metabolickému syndromu a diabetu, aktivací systému renin-angiotenzin, působící zvyšování krevního tlaku, a stimulací systémového zánětu a zánětlivých změn cévní stěny. Zánětlivé změny cévní stěny a rozvoj aterosklerotických změn v cévní stěně a vztahu k vitaminu D sledoval Brewer [4]. Vztah hypovitaminózy D a výskytu mozkových cévních příhod prokázali i jiní autoři [23,25]. Hypovitaminóza D má významnou roli i u osob po mozkové cévní příhodě. Osoby s reziduálním postižením zejména hybnosti po iktu jsou méně mobilní, mají vyšší riziko pádů. Jsou ve srovnání s ostatní populací stejných věkových kategorií méně vystaveni slunečnímu záření, mohou mít problémy s výživou. Častěji mají hypovitaminózu D, sníženou kostní denzitu, osteopenii nebo osteoporózu. Carda to vysvětluje uplatněním endokrinních faktorů (inhibice sekrece parathormonu) a porušením osy vitamin D-parathormon v důsledku iktu, nutričními faktory, případně farmakologickými faktory [7]. Hypovitaminóza D způsobuje svalovou slabost zejména kořenového svalstva, instabilitu a pády [3,11]. Osoby s reziduem po mozkové cévní příhodě tak mají zřetelně zesílené riziko pádů, způsobených horší mobilitou a svalovou slabostí. Pády zhoršují dále kvalitu života, působí bolest a v terénu osteopenie a osteoporózy jsou častou příčinou zlomenin, zejména stehenní kosti.

Vitamin D a roztroušená skleróza

Vitamin D patří mezi vysoce zkoumané látky v problematice roztroušené sklerózy (RS). RS je autoimunitní onemocnění, u kterého se uplatňují komplexní interakce mezi genetickou náchylností a faktory zevního prostředí [10]. Vitamin D je dáván do vztahu k RS z toho důvodu, že RS má typické geografické rozložení incidence a prevalence v závislosti na zeměpisné šířce [18]. Čím větší je vzdálenost od rovníku, tím je výskyt RS vyšší [49]. Rovněž je popsána sezónní fluktuace nemoci [50]. Ačkoli příčin může být více, je menší expozice slunečnímu záření a nižší hodnota vitaminu D jednou z těch možných a nabízí plausibilní vysvětlení, zejména v některých oblastech se specifickými populacemi [13]. Deficience vitaminu D se jeví v korelaci se závažností funkčního deficitu, hodnoceného škálou Expanded Disability Status Scale (EDSS) [19]. U pacientů s častějšími atakami byla popsána hladina vitaminu D nižší ve srovnání s pacienty, kteří měli ataky nemoci méně časté [23,24]. Nižší hladiny vitaminu D byly zjištěny i u pacientů s primárně progresivním průběhem onemocnění [37,38,40]. Úskalí tohoto mechanistického přístupu je však v tom, že pacienti s těžším průběhem nemoci jsou méně hybní a soběstační, mají nižší expozici slunečnímu záření než pacienti méně postižení a nižší hladina vitaminu D je důsledkem tohoto stavu [10,13]. Hypovitaminóza D je jednou z možných příčin i z důvodu ovlivnění imunitního systému [39]. Vitamin D inhibuje proliferaci T-lymfocytů a indukuje jejich apoptózu, působí na ně imunosupresivním účinkem. Byl prokázán vliv vitaminu D na diferenciaci $CD4^+CD25^{+}$ $FoxP3^+$ T buněk, schopných zastavit rozvoj autoimunitní odpovědi. Jakkoli se zdá vliv vitaminu D na vznik a průběh nemoci významný, chybí dostatečně validní data, která by čerpala z validního srovnání homogenních skupin pacientů [44]. Totéž platí i pro dosud proběhlé intervenční studie suplementace vitaminu D. Náhled na úlohu vitaminu D je široký, od velmi rezervovaného přístupu až k charakteru panacea. Ukazuje se, že vitamin D může být jedním z faktorů, podílejících se na vzniku a průběhu nemoci, avšak jeho nižší hladina může být zároveň jejím důsledkem. Zkoumání role vitaminu D je nadále vhodné.

Vitamin D a kognitivní poruchy

Kognitivní poruchy, demence, zejména Alzheimerova nemoc, jsou označovány za epidemii 21. století. Představují mimořádnou zdravotní, sociální i ekonomickou zátěž. Jsou hledány nové strategie včasné diagnostiky a možného ovlivnění progrese degenerativního procesu, vedoucího k nevratné ztrátě neuronů. Receptory vitaminu D jsou zastoupeny v oblastech často postižených Alzheimerovou nemocí, jako je hippocampus a přilehlé struktury. Je popisován pozitivní účinek vitaminu D na kognitivní funkce, způsobený inhibicí syntézy oxidů dusíku, regulací enzymatických dějů v metabolizmu glutationu a neurotrofinu a v regulaci buněčného kalcia. Byly zjištěny nižší hladiny vitaminu D u pacientů s kognitivním deficitem ve srovnání s běžnou populací. Rovněž zde však může být hypovitaminóza D i důsledkem nesoběstačnosti, menší mobility a nižší expozice slunečnímu záření a zhoršení nutrice u pacientů s demencí. Vitamin D může u těchto pacientů pomoci ovlivnit komorbidity, které dále zhoršují průběh základní nemoci, jako jsou kardiovaskulární a cerebrovaskulární nemoci,

záněty, rovněž svalová slabost, pády a zlomeniny [5,6,28,33,47].

Závěr

Působení vitaminu D prolíná celým organizmem, tedy i nervovým systémem. Nedostatek vitaminu D se primárně uplatňuje v rozvoji a průběhu některých typů výše uvedených neurologických onemocnění, častěji je jedním z mnoha faktorů a velmi často je zároveň důsledkem těchto nemocí, především zhoršené soběstačnosti a omezení běžného stylu života. Jsou nutné další výzkumy problematiky.

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Attachment XVII

Tonar Z, Eberlova L, Polivka J, Daum O, Witter K, Kralickova A, Gregor T, Nedorost L, Kochova P, Rohan E, Kalusova K, Palek R, Skala M, Glanc D, Kralickova M, Liska V. Stereological methods for quantitative assessment of hepatic microcirculation. Current Microscopy Contributions to Advences in Science and Technology. Vol. 1. Microscopy Book Series – 2012 Edition. Formatex Research Center, Badajoz, Spain, pp. 737-748. ISBN 978-84-939843-6-6.

Stereological methods for quantitative assessment of hepatic microcirculation

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This chapter reviews the current knowledge on healthy and pathological hepatic circulation and microcirculation in animal models used in biomedical research and in human histopathology. To demonstrate the clinical importance of this topic, we focus on blood vessels (particularly microvessels) in liver surgery and in hepatic metastases of colorectal carcinoma. Histological techniques of assessment of healthy and tumourous microvessels are reviewed with focus on the immunohistochemistry on the endothelium, basal lamina and lectin immunohistochemistry. The role of angiogenesis and vascularisation in colorectal cancer and liver metastasis is linked to recent knowledge on proliferation, tumour invasion, metastasis formation, prognostic factors, and scoring systems. In addition to histopathological methods, the three-dimensional imaging of hepatic microvessels using corrosion casts is shown using X-ray microtomography. Stereological methods are demonstrated to quantify the volume, surface, length, numerical density and tortuosity of the microvessels.

Keywords liver; quantitative histology; immunohistochemistry; micro-computed tomography; colorectal carcinoma

1. Hepatic circulation

1.1 Hepatic circulation in humans and in laboratory animals

The main animal models for experimental studies on the liver are the pig, dog, rat, and mouse (for reviews see, e.g., [1,2]). Similar to humans, these mammals have a nutritive as well as a functional hepatic circulation. Oxygenated blood enters the liver by the hepatic artery, a branch of the coeliac artery. Nutrient enriched blood is collected from the digestive organs of the abdominal cavity and enters the liver via the portal vein. The main branches of these vessels differ between animal species, as well as inter-individually [3-5]. The primary branches give rise to the lobar and segmental vessels of increasing orders until the interlobular arteries and veins are formed within the so-called portal area between liver lobules [3,6]. The interlobular portal venules comprise smaller branches, which form the axis of the liver acinus [7]. Short inlet venules arising from these smaller branches distribute the blood directly to the sinusoids. In contrast, most of the blood from the interlobular arteries enters a peribiliar capillary plexus within the portal areas. Only a very small portion of the arterial blood reaches the sinusoids directly. From the liver sinusoids, the blood leaves the lobules via the central (terminal) veins of the lobules, sublobular veins and collecting veins of decreasing order, which eventually enter the hepatic vein [6,8,9]. Interestingly, a certain competition exists between the hepatic artery and the portal vein blood flow [10], and the hepatic arterial blood flow can become the primary supply of sinusoids if the blood flow through the portal vein is impaired [11]. Comprehensive reviews on hepatic vascular bed and intrinsic regulation of hepatic blood flow are provided by Lautt and Greenway [12] and Lautt [13].

In general, liver surgery requires a detailed knowledge of the complex vascular anatomy of this organ [14,4,15]. For interventions in human liver pathologies, a number of pre- and perioperative imaging approaches have been developed to visualise individual variations in branching of hepatic vessels, including the portal vein [16,15,17]. Similar studies in experimental animals remain limited [18]. Our understanding of the physiological and pathological implications of microvascular blood flow in the liver as described above is mainly based on the concept of liver acini developed by Rappaport [7]. Hepatic lobules, which are arranged around a central vein and—particularly in the adult pig model—are defined by surrounding connective tissue, which can readily be identified in histological sections. However, they do not represent the functional unit of the liver. Portal lobules—virtual triangular parts of the parenchyma centred proximally to a bile ductule in a portal area—emphasise the exocrine function of the liver [6].

Gradients of metabolic activity, the dissemination of tumours or parasites and the influence of toxins on the liver can be explained by differences in the dynamics of blood flow and pressure in Rappaport's [7] acinus. An acinus is a diamond-shaped area comprised of two adjacent lobules that are supplied by a "backbone" of terminal branches of the interlobular portal veins and hepatic artery as described above [6,8]. The cells around the virtual backbone have the highest metabolic activity, but they are also the first cells to be affected by toxins within the circulation. In contrast, insufficient oxygen supply first affects the cells proximal to the central veins at the ends of the "diamond" [6,8,19,20]. A detailed knowledge of hepatic microcirculation is necessary to explain hepatic ischemia-reperfusion injury [21] and hypothermic hepatic perfusion [22].

1.2 Surgical consequences of hepatic microcirculation

The blood supply of the liver is unique in comparison to the blood supply of other organs in the human body. It is supplied by two vascular systems that are connected immediately before the network of liver sinusoids. The blood from all of the inner organs of the abdominal cavity, including the pancreas, is carried by portal vein that divides into the left and right lobular branches. Arterial blood is delivered by the hepatic artery, which exhibits the same branching pattern as the portal vein. The portal and hepatic arterial blood streams branch many times up until the portal venules and hepatic arterioles, which are within the septa among particular liver lobules. The blood from portal venules and hepatic sinusoids flows into the hepatic sinusoids that lie between the hepatic plates and then into central vein. The central vein empties into the sublobular veins and then into the branches of liver veins and the inferior caval vein [23].

The liver parenchyma is unique in its ability to regenerate after any loss of its functional volume, whether by trauma, liver resection or any type of toxic insult. The remaining liver is able to restore its previous functional capacity by producing more liver cells (hepatocytes, cholangiocytes and undifferentiated stem cells) [24]. The resulting liver remnant volume does not correspond to total liver volume before liver injury, but it is influenced by the status of the hepatic parenchyma (liver cirrhosis, steatohepatitis, steatofibrosis, etc.) [25,26]. Renewal of the functional capacity tends to occur through the establishment of new liver microcirculation. Several studies have demonstrated that the portal vein flow before injury and after restoration is consistent with this notion. Our further understanding of the liver microcirculation is important in light of the development and increasing frequency of more complicated surgical resections of liver parenchyma for liver lesions, whether benign or malignant. The techniques and skills required for current liver surgical procedures are constrained by a resection limit of approximately of 70% of the healthy functional liver parenchyma. As mentioned previously, in cases of diffuse liver parenchymal disease, it is important to reduce the resected volume [27]. We are able to increase the post-resection liver remnant volume by procedures that precede liver resection, such as portal vein embolisation (PVE). However, currently, we are unable to predict the results of liver regeneration after resection or PVE [28]. For these reasons, many patients are precluded from the possibility of radical surgical treatment of their malignancy and are indicated only for palliative oncological treatment. The results of radical and palliative treatment are not comparable. A deeper understanding of the alterations of liver microcirculation during liver regeneration might provide insight into strategies for increasing post-resection liver remnant volume to improve current liver resection procedures.

2. Histological techniques for assessment of healthy and tumourous microvessels

Detection of microvessels in histological sections is still a state-of-the-art technique for generating quantitative data such as vessel density. Independent of the actual quantification method, the first step is detection and visualisation of blood vessels within the sections. It is generally not possible to distinguish the complete vascular tree in routinely stained histological sections because capillaries, as well as tumourous and newly formed vessels, might not exhibit a visible volume or might be otherwise deformed [29,30]. Different strategies can be used to visualise these vessels.

In general, detection of endothelial cells by immunohistochemistry or lectin immunohistochemistry is preferred. In human, rat and mouse material, the pan-endothelial markers CD31/PECAM1 (platelet endothelial cell adhesion molecule) and CD34 are used. Unfortunately, most of the commercially available anti-CD31 and anti-CD34 (Fig. 1A,B) are limited in interspecies immunoreactivity and therefore often cannot be applied in studies using animals other than those for which they were designed. Recently, monoclonal anti-pig and anti-sheep antibodies for use on cryosections have been developed; additionally, single polyclonal antibodies may react with antigens in the tissues of different species [31]. The expression of CD31 and CD34 in endothelial cells has been reported to depend on the vessel and organ type [32].

As an alternative endothelial marker, vWF (factor VIII-related antigen, von Willebrand factor, Fig. 1C) can be used. However, it must be noted that vWF is a functional molecule that is not distributed uniformly in endothelial cells of all vessels [33]. Capillaries of the brain and other organs with limited requirements of coagulation, e.g., lymphatic organs, are not easily detected using anti-vWF antibodies.

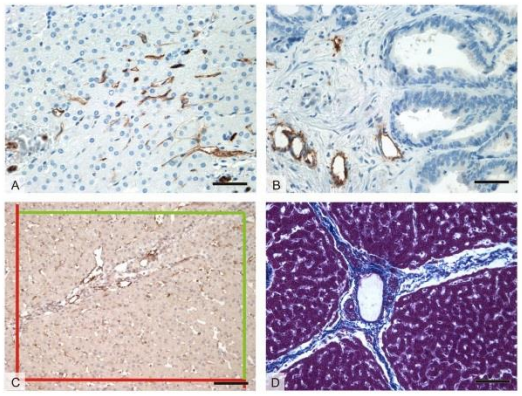

Fig. 1 Microvessels in the human liver can be detected by immunohistochemical staining against the CD34 antigen (A,B). In porcine liver, von Willebrand factor is a usable endothelial marker (C). When counting the microvessel profiles per section area, a projection of unbiased counting frame [34] with admittance (green) and forbidden (red) borders can be used. The filling of hepatic microvessels with the Mercox resin for preparing the corrosion casts can also be confirmed histologically (D). Immunohistochemical detection of CD34 (A,B,) and von Willebrand factor (C), and counterstaining using Gill's haematoxylin (A,B,C). Mallory's trichrome stain (D). The scale bars indicate 50 µm (A,B) and 100 µm (C,D).

The detection of the glycocalyx of endothelial cells in histological sections using appropriate lectins represents an interesting alternative for the visualisation of blood vessels when immunohistochemistry is not a suitable option. The classical lectin for this purpose is UEA (*Ulex europaeus* agglutinin) for human material [e.g., 35]. For animal tissues, it is necessary to test different lectins for the specific species and the organ under study. Good results are often attained using SBA (Soy bean agglutinin), LEA (*Lycopersicon esculentum* agglutinin), GSL-I (*Griffonia simplicifolia* lectin-I) or WGA (Wheat germ agglutinin) [e.g., 36-38, own unpublished results]. The use of lectin histochemistry for vessel detection is limited in organs containing other cells with a prominent glycocalyx (e.g., kidneys) or internal accumulations of carbohydrates (e.g., liver). Organs with large amounts of connective tissue (e.g., skin) often exhibit a prominent "background" staining due to the glycoproteins surrounding their collagen fibres.

In experimental systems, these limitations can be circumvented by injecting solutions of conjugated lectins into the vessels [39-40]. Lectin binding is robust, can survive fixation and embedding and can be detected subsequently using appropriate staining kits, according to the respective conjugate (lectin perfusion-labelling technique; [41,42]. However, the use of this method in large organs, in organs or regions with diffuse afferent vessels (e.g., subcutaneous tumours) and for organs or animals that cannot be injected (e.g., archival material or material that has to be divided for different methods, etc.) can be cost-prohibitive and possibly either non-uniform or insufficient for the perfusion of small vessels. Leakage of the lectin solution from permeable vessels within tumours might produce unwanted artifacts [43].

Detection of the basal lamina of blood vessels rather than endothelial cells is an alternative way to visualise arteries, veins and capillaries. Histochemical methods of detecting glycoproteins from the basement membrane include methenamine silver staining and periodic acid-Schiff-staining (PAS), which is based on a similar mechanism. These methods are still used where glycoproteins and other carbohydrates of the tissue do not interfere (e.g., in the brain or parenchyma of lymphatic organs) [44,38]. These methods are relatively cheap and give good results in the appropriate settings. Whereas thickening of the basement membrane may cause better visibility of the capillaries stained using this method, newly formed vessels appear to lack a prominent glycoprotein component within the basement membrane [45].

Immunohistochemical detection of the basal lamina using anti-laminin and anti-collagen IV antibodies [46-49,37] is a more common approach and represents a robust method for nearly all tissues. The composition of newly formed basal laminae, such as during angiogenesis in organ development, organ remodelling or cancer, differs from that in vessels of homeostatic organs [50-54]. This phenomenon can be used to distinguish young or invasive (tumour) vessels from normal ones. Appropriate anti-laminin antibodies are commercially available. It is necessary to consider that tumour vessels might lack basal laminae [55]. In some organs, basal laminae of other cells (e.g., muscle cells, tubular epithelium of kidneys, or pneumocytes of the lung) might hamper automatic recognition and likely even the segmentation of the vessels by a researcher in immunohistochemically stained sections.

Another classical method used for the detection of microvessels is the injection of the vascular tree with appropriate reagents prior to sectioning [e.g., 56,57]. For this approach, the same limitations apply as listed above for the injection of lectin solutions. Moreover, the artificial filling of vessels might produce artifacts attributed to the interplay of injection pressure and vessel wall elasticity. Quantitative parameters such as vessel luminae should be evaluated with care in these cases. On the other hand, injection techniques allow for the detection of microvascular leakage [58].

Moreover, in some experimental settings, injection of the vascular tree might be advantageous, particularly if other types of examinations (scanning electron microscopy) are planned with parts of the material.

A universal method for the detection and the visualisation of blood vessels does not yet exist. For each specific experimental system (species, organ under study, method of segmentation), an appropriate method has to be developed and tested, particularly when material other than that from human, mouse and rat organs are to be used. If the vessel morphology is expected to be severely perturbed, as is the case in certain tumours, transmission electron microscopy (if necessary, after immunohistochemical staining) can be helpful in the identification of uncertain structures.

3. Prognostic role of vascularisation in colorectal carcinoma and its liver metastases

3.1 Colorectal cancer and liver metastasis

Colorectal cancer (CRC) is one of the most common malignancies, with an annual incidence rate of approximately 1.2 million new cases worldwide [59]. CRC is also the third leading cause of cancer mortality, with a general 5-year survival rate of approximately 65% (and of only 11% if there are distant metastases) [60]. Between 15-25% of patients present with hepatic metastases at the time of initial diagnosis [61,62]. Moreover, 29% of patients develop liver metastases within the 3 years after the diagnosis of primary CRC [63]. The resection of liver metastases provides the only potentially curative treatment with a 5-year survival between 37-58% [64,65]. However, 60% of patients develop recurrent disease after primary hepatectomy, and only one third of these benefit from repeated resection of liver metastases, with a 5-year survival ranging from 26-41% [66]. Some of the novel treatment strategies, such as portal vein embolisation, two-stage hepatectomy and preoperative downsizing chemotherapy, actually increase the number of patients with resectable liver metastases [67,68].

The standard-of-care treatment for the metastatic colorectal cancer (mCRC) is systemic chemotherapy that involves the antimetabolites 5-fluorouracil and its oral pro-drug capecitabine, combined with the DNA-damaging drugs oxalplatin and irinotecan. Despite the therapeutic advances, the overall survival (OS) for mCRC patients remains between 18 and 21 months [69]. Over the past few years, novel targeted therapeutics were introduced into the mCRC therapeutic regimen. Two monoclonal antibodies (moAb) against the epidermal growth factor receptor (EGFR), cetuximab and panatimumab, as well as one moAb against the vascular endothelial growth factor (VEGF), bevacizumab. These agents have demonstrated limited clinical benefit in monotherapy or in combination with chemotherapy [70]. Despite these recent advances, the effects of the mCRC treatment are often transient, and the tumour drug resistance remains the prominent problem. Recent research has focused on the identification of novel biomarkers, which can be used as prognostic factors for the aggressiveness of the disease, the probability of recurrence or the response to the chemotherapy for each patient. The more individualised approach represents significant progress in the cancer management and the new era of truly personalised medicine.

3.2 Prognostic factors and scoring systems for the colorectal cancer liver metastases

Because the resection of liver metastases remains the only potentially curative treatment for patients with mCRC, the efforts of many research groups have focused on the identification of prognostic biomarkers to ensure the maximum treatment benefit after hepatic resection. Some clinicopathological factors were identified to elicit a strong prognostic role in the case of resection of hepatic metastases, such as the staging of the primary tumour, the time to diagnoses of hepatic metastases, the number and size of metastases or the pre-operative carcinoembryonic antigen (CEA) level.

For more than 30 years, simple prognostic factors and more complex scoring systems have been developed and employed to help to stratify patients into risk categories that can improve the selection of candidates for hepatic surgery or to predict tumour recurrence. Fifteen independent prognostic models for the clinical outcome after liver resection for colorectal cancer metastases have been identified using a multistep process by the systemic literature reviews [71,72]. In these prognostic models, 25 different factors were found to influence the prognosis, and 11 were identified by two or more studies. The number of liver metastases was a negative predictive factor in twelve studies. Cancer that spread to the lymph nodes counted for worsening prognosis in ten models. The maximum size of metastases was a predictive factor in six studies, five of which used a cut-off diameter value of 5 cm. The strong independent prognostic factor was the time between the primary CRC surgery and the onset of liver metastases, which was identified by five study groups with a cut-off value of 12 months. In five different studies, the pre-operative CEA levels were identified as another determinant. The positive resection margin and poor differentiation of the CRC were identified as negative prognostic factors in three studies. The serosal invasion of the cancer, hepatic lymph node metastases and bilobar spread of liver metastases were identified by only two models, whereas the other markers were found only by one model. Therefore, the most important prognostic factors were the number of liver metastases, cancer spread to lymph nodes, maximum size of metastases, pre-operative CEA level and non-radical resection [72]. The scoring systems could be used to predict survival and the recurrence risk for patients who undergo hepatic resection or to help clinicians with the selection of patients for resection or for adjuvant therapy. However, there is no consensus over these systems and their application in clinical practise. Moreover, there is a general problem with the validation and applicability of these systems. In one

study, three independent scoring systems were examined for the outcome predictions of the Mayo Clinic patients with mCRC and hepatic resection. The scoring models were only marginally better than chance alone for the overall survival and disease recurrence prediction [73]. These results suggest that the clinical and pathological factors alone are insufficient for accurate individual prognostication and that the scoring systems using only these markers are not widely applicable. Furthermore, patients with identical clinicopathological variables might have significantly different outcomes [74]. It is apparent that increased understanding of the specific aspects of tumour biology, the tumour microenvironment and novel molecular markers will improve the predictive power of such prognostic scoring systems.

3.2 The role of angiogenesis and vascularisation in colorectal cancer and its liver metastases

Angiogenesis is an essential part of the multistep carcinogenesis process of many cancers that facilitates the establishment of new blood supply to the tumour that is established from pre-existing blood vessels [75]. Without angiogenesis, tumours are unable to growth and are limited in size to approximately 1-2 mm. Angiogenesis is crucial for the metastatic spread of the primary tumour, as well as the disease recurrence from residual micrometastatic deposits, such as those found after hepatic resection of mCRC. This complex process depends on the balance between stimulatory and inhibitory factors.

Among many pro-angiogenic factors that initiate the cascade of new microvasculature formation, the VEGF family is considered as the most important group of molecules. The VEGF family comprises seven different molecules classified as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and platelet-induced growth factor (PIGF) [76]. The family of VEGF receptors (VEGFR) includes VEGFR-1, VEGFR-2 and VEGFR-3, which is presented mainly on the lymphatic endothelial cells [77]. The expression of VEGF can be assessed by the immunostaining of postoperative specimens or by the measurement of circulating or urinary levels of this angiogenic growth factor. Similarly, there are the soluble VEGF receptors (VEGFR-1, -2 and -3) that are currently under investigation in various cancers for their utility as biomarkers of angiogenesis, as well as for responsiveness to anti-angiogenic therapies [78,79]. Another approach to the study of tumour vascularisation and angiogenesis and evaluation of the efficacy of anti-angiogenic therapies is based on the measurement of microvascular density (MVD) by immunohistochemical staining of endothelial cells [79]. CD31, CD34, von Willebrand factor, CD105 (endoglin), CD146 and other endothelial cells antigens are suitable for such staining analysis [80]. There are, however, also some drawbacks to this approach, such as invasiveness and the lack of standardisation.

The correlation between MVD, VEGF and clinicopathological factors, together with the clinical applications of these findings, remains controversial. High VEGF-A expression is correlated with increased metastatic spread and poor prognosis of patients with primary CRC in some studies [81,82]. VEGF-C expression at the deepest invasive sites has been correlated with worsening histological grade, depth of invasion, lymphatic invasion and lymph node metastases, venous invasion, Dukes' stage and liver metastasis [83]. One study has also reported that the overexpression of VEGF mRNA was associated with the progression, invasion, metastasis and the poor prognosis of CRC patients [82,84]. Conversely, one recent study found no correlation among MVD, VEGF and clinicopathological factors [85], and another study found that VEGF-D expression did not correlate with the MVD, tumour differentiation or Dukes' stage; instead, it was associated with lymphatic metastases [86]. In two studies of colorectal cancer liver metastasis resections, VEGF expression has failed to show any correlation with survival [87,88].

The role of MVD as a prognostic factor for CRC patients remains unclear. The vessels stained with anti-factor VIII polyclonal antibody showed a significant correlation between MVD and tumour size, depth of invasion and lymph node metastasis [89]. CD34-labelled tumour vascularisation was linked to poor prognosis in overal survival and recurrence of liver metastases [90]. The association of MVD with the so-called hot spots of the primary tumour, with the presence of lymph node infiltration, as well as with distant metastases and shorter survival, was identified in some studies [91-95]. However, lack of correlation of MVD with metastases or with survival has also been reported [96,97]. One study found no correlation of MVD with the Dukes' classification or clinicopathological factors [98]. Interestingly, other studies have observed improved prognosis together with the higher microvessel counts [99-101]. The role of MVD as a prognostic factor for patients after the resection of colorectal liver metastases was examined as well. Lower survival after hepatic resection was correlated with high MVD, which was associated with a 4.9-fold increased risk of death [87,102]. Another study found an association of MVD in hepatic resection with poor clinical outcome [103]. Nonetheless, there are studies that failed to reveal any association between MVD and patient prognosis [104,105]. There are substantial inconsistencies among these studies. The methodological variations in the assessment of MVD might contribute significantly to the lack of any consensus. Standardised methodology, together with larger studies, is needed to confirm the prognostic roles of MVD and of VEGF expression in colorectal cancer and its liver metastases. The preclinical results from a recent and complex study have revealed the different expression profiles of 44 angiogenesis-related cytokines that were assessed by antibody array using primary and metastasis-derived CRC cell lines. The primary CRC cell lines expressed higher levels of interferon gamma, insulin-like growth factor-1, IL-6 and other cytokines in contrast to the metastatic CRC cell lines, which exhibited higher expression of different factors such as angiogenin-2, macrophage chemoattractant proteins-3/4, matrix metalloproteinase-1 and other distinct cytokines. The expression of VEGF exhibited no differences during normoxic conditions, but during hypoxic conditions, the primary CRC cell lines responded with higher up-regulation of expression than the metastatic ones. This study has shown that

there are many more factors other than VEGF that are related to angiogenesis and to metastatic spread and that could be used as prognostic markers or therapeutic targets for mCRC in the future [106].

Another approach to increase our functional understanding of microvasculature and quantifying the angiogenesis of tumours is the usage of novel imaging methods such as perfusion-computed tomography (perfusion CT). This technique could be used in the overall management of CRC, from the diagnosis and prognosis to the monitoring or metastasis and treatment [107]. In the field of angiogenesis, the perfusion CT reflects the tumour physiology and can be used as an indirect imaging biomarker. It might be a more valid method of assessing tumourigenesis in CRC compared to the measurement of MVD [108]. For example, increased hepatic arterial perfusion is an indicator of CRC with liver metastases, whereas progressive disease is correlated with a reduction in the portal perfusion [109]. Perfusion CT could also be used for the monitoring of therapeutic response after chemoradiation, as well as after the antiangiogenic drugs [110,111]. In one clinical trial with rectal cancer patients treated with bevacizumab, the angiogenic changes were monitored by CT perfusion and correlated with the decrease of tumour MVD and other angiogenic markers [112]. Perfusion CT has become the preferred functional imaging technique in the management of CRC and can be used for the monitoring of treatment response as well as for the assessment of disease prognosis.

A number of other factors influence the complex process of tumour angiogenesis that might represent prognostic biomarkers in CRC. For example the endothelial growth factors angiopoietins were examined in colorectal adenocarcinoma, where the increased expression of angiopoietin-2 and decreased expression of angiopoietin-1 might be responsible for blood vessel formation. The overexpression of VEGF and angiopoietin-2 was correlated with adenocarcinomas with a diameter of more than 5 cm and with lymph node metastases [113]. Another angiogenic factor, trombospondin-1, was analysed in both primary CRC [114] and in mCRC with the hepatic resection, where thrombospondin-1 expression correlated with the poor survival [115].

There is a strong evidence to support the notion that not only do basic clinicopathological factors influence the prognosis of primary and metastatic colorectal cancer, but that many recently identified novel biomarkers might help in the future of personalised management of this type of cancer. As angiogenesis is one of the most important aspects of tumour progression and metastasis, the identification of novel pro-angiogenic and anti-angiogenic biomarkers will undoubtedly guide the future prognostic models of many human cancers and their therapies.

4. Three-dimensional reconstruction of hepatic microcirculation

4.1 Vascular corrosion casts

In addition to histological methods, three-dimensional imaging of hepatic microvessels can be performed using microvascular corrosion casts. The recent advances in X-ray microtomography (micro-CT) has introduced resolution similar to that of routine histopathology, now allowing for the application of *ex-vivo* micro-CT to bridge the gap between the macroscopic imaging of liver vasculature and the histopathology of hepatic microcirculation. Tomographic modelling of vascular corrosion casts provides quantifiable three dimensional (3D) data on the vascular bed [10,116], whereas micro-CT is currently the only structural modality that can reflect angiogenesis [117].

Table 1 Recently published studies on hepatic corrosion casting.

Material and vasculature described	Vessel diameter differentiated	Casting media used
dissected porcine liver, resin injected into the portal vein, hepatic artery and inferior vena cava [118]	segmental branches	Epoxy Resin R180 (Fibreglass International, Adelaide, Australia)
dissected bovine liver, resin injected into portal system, hepatic arteries and hepatic veins [119]	sub/segmental branches	Technovit (Heraeus Kluzer, Hanau, Germany)
porcine liver, in situ vascular cast of portal vein [18]	0.5 – 12 mm	Technovit 7143 (Heraeus Kluzer, Hanau, Germany)
human liver, resin injected into hepatic artery and portal vein [116]	liver sinusoids	Batson's™ #17 Corrosion Kit (Polysciences, Warrington, USA), 25% $BaSO_4$ added

Vascular corrosion casts offer 3D replicas of vascular trees. The procedure in general includes the following: initial exsanguination to prevent intravascular blood clotting, injection of the casting media, its polymerisation in the fully filled vascular bed and the subsequent maceration of the surrounding tissues by a highly aggressive corrosive solution. Every step of this procedure, particularly the choice of resin, the preparation of the organ and the manner of injection may dramatically influence the final quality of the cast. As for the casting media, it must be of adequate viscosity to pass through the vessel but not to penetrate the wall. It must also be capable of even and fast polymerisation with

minimal shrinkage, and it must exhibit chemical resistance to the subsequent corrosion procedures. Methylmethacrylate (MMT) resin embodies all of these properties. Table 1 shows the casting media, methods and the results of several recent publications on liver vascular corrosion casts. Mercox is another widely used, low-viscosity acrylic resin [120,121]. Mercox allows for optimal permeability through the entire vascular bed, optimal infiltration properties, minimal shrinkage, high chemical resistance, and short preparation [122]. For the high volume of the liver vascular bed, quick polymerisation (cure time is approximately 5 minutes for the lowest concentration) appeared to be a disadvantage of using Mercox. Another critical factor for the microvascular hepatic corrosion cast is the air embolism, which must be prevented during any handling, both during the surgery and while injecting the corrosion media.

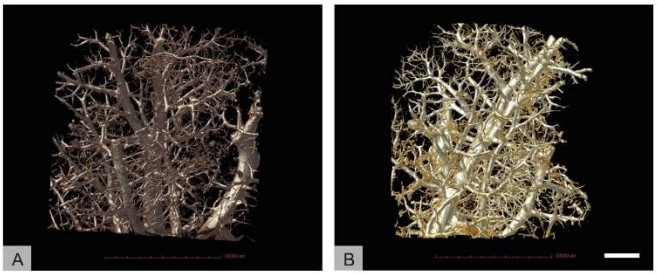

Fig. 2 Snapshots from a micro-CT-based 3-D reconstruction (A,B) of a vascular corrosion Mercox cast of porcine hepatic microcirculation. Due to the resolution of 36.26 µm, minor blood vessels (some of the interlobular blood vessels, sinusoids and central veins) are not distinguished. The scale bar indicates 600 µm.

For our corrosion casts, we used Mercox II (Ladd Research, Williston, Vermont, USA), which is commercially offered in two colours (blue and red). The kit contains the MMT resin and catalyst (benzoyl peroxide). To obtain the corrosion cast of the vasculature in a porcine liver, we first rinsed the liver with 5 l of saline solution containing 50,000 IU of heparin before sacrificing the animal. Before the hepatectomy, the portal vein, hepatic artery proper and inferior vena cava were clamped. During the entire casting procedure, the liver was immersed in the lukewarm water. To extend the time necessary to cast a larger amount of Mercox (Fig. 1D), we used the highest possible dilution of 0.4 g of catalyst per 20 ml resin with a stirring time of 4 minutes. As the hepatic artery was too small to be cannulated, Mercox was injected only into the portal vein. The injected fluid polymerised at room temperature for 12 h. Afterwards, the liver was macerated in 10 % KOH for 48 h. MicroCT scans showed low Mercox opacity for differentiation of vessels with diameters below 40 µm (Fig. 2-3). Because the smallest capillaries have lumens that are approximately 6 µm in diameter, higher resolution with a sufficient radiopacity is required. The choice of the contrast agents is also a critical factor for the micro-computed tomography imaging because it should not change the rheological properties of resin. Debbaut et al. [10] successfully added 25% barium sulphate into the resin to preserve its permeability through the microvascular bed. Another contrast agent used to study microvasculature ex-vivo is a radio-opaque silicone rubber Microfil (Microfil, Flow Tech, Carver, MA) [122]. However, Microfil is not suitable for subsequent corrosion casting.

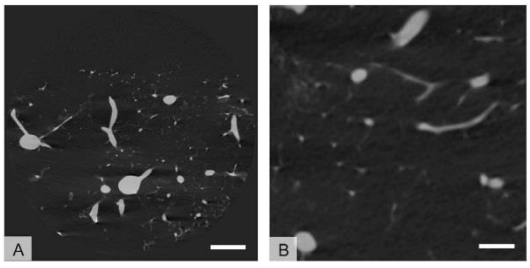

Fig. 3 Two-dimensional micro-CT section (A) of a vascular corrosion Mercox cast of porcine hepatic microcirculation. A large piece of the cast is visualised, however, the limits of the resolution (pixel size 36.26 µm) are obvious upon zooming in to assess image in detail (B). The scale bars indicate 5 mm (A) and 1 mm (B).

4.2 Quantitative stereological techniques

When deriving large three-dimensional data sets from the micro-CT, several unbiased stereological methods can be used that are already acknowledged in quantitative light or confocal microscopy.

The volume of the microvessels can be expressed as their volume fraction V_V within a spatial region of interest. This fraction can be determined using thresholding, which requires high-contrast images, without artifacts and shadows that might result in false positivity of the thresholded microvessels. Optionally, the volume occupied by the microvascular corrosion cast can be easily estimated using the stereological Cavalieri principle [124]. This estimate is achieved by counting points of a stereological grid superimposed on the profiles of the microvessels while the area corresponding to each point and the distance between the sections are known constants.

When estimating the surface of microvascular casts (often expressed as their surface density S_V within a spatial region of interest), either thresholding or stereological methods can be used. Whenever the methods based on automatic thresholding might introduce bias (e.g., due to a high sensitivity of the threshold settings, due to non-homogeneous and unbalanced images), we can use virtual isotropic spatial grids of orthogonal lines with a random initial orientation for the same purpose. The latter method is more laborious because it relies on counting intersections between three sets of test lines (the fakir probe [125,126]) and the profiles of microvessels on a series of sections.

When estimating the length of the microvessels (often expressed as their length density L_V within a spatial region of interest), the profiles of microvessels are to be either connected and skeletonised automatically or, traced manually to provide a unidimensional representation of the vascular tree. With a known length, the tortuosity can be calculated as a ratio between the true length and the shortest distance between the endpoints of each microvessel. When detecting the branching nodes, the valence of each node n is defined as the number of vessel segments joined at the node. Provided that a capillary is defined as a loop between two nodes of the vascular network, the numerical density of capillaries within a reference volume $N_V(cap/ref)$ can be estimated with the optical disector [44], see Equation 1:

$$N_V(cap/ref) = \frac{N(cap)}{V(ref)} = \frac{\sum(\frac{n-2}{2} \cdot P_n)}{\sum v(dis)} + 1 = \frac{\sum(\frac{n-2}{2} \cdot P_n)}{\sum h \cdot a(fra)} + 1, \quad (1)$$

where $N_V(cap/ref)$ is the number of capillaries $N(cap)$ per reference volume $V(ref)$; P_n is the number of nodes of valence n (number of vessel segments joined at the node); the $v(dis)$ is the volume of the disectors; h is the height of the disector; and $a(fra)$ is the area of the counting frame. Once the microvessels are represented as oriented lines within a reference space, it is also possible to assess their orientation using a spherical coordinate system. Each microvessel can be represented by a vector with a known length and a combination of azimuth (longitude) and elevation (latitude) when connecting the centre of the coordinate system with the surface of a virtual sphere. Moreover, the preferential orientation and degree of anisotropy of the microvessels can be calculated as well [127]. Such a description of the vascular tree can be useful, for instance, when devising advanced models of hepatic circulation [128,129]. Computer simulations of liver tissue perfusion should be at least statistically similar to real microvessel networks.

5. Conclusion

Our current knowledge of healthy and pathological hepatic microcirculation in animal models used in biomedical research and in human histopathology is of great clinical importance because it is linked to the role of vascularisation and angiogenesis after liver surgery and in hepatic metastases of colorectal carcinoma. Histological techniques of the assessment of healthy and tumourous microvessels rely mostly on endothelial markers, such as CD34 in humans and von Willebrand factors in some animals. Quantitative assessment of microvessels in colorectal cancer liver metastases is linked to our recent knowledge on proliferation, tumour invasion, metastasis formation, prognostic factors and scoring systems. In addition to histopathological methods, three-dimensional imaging of hepatic microvessels has been achieved using corrosion casts visualised by X-ray microtomography. Stereological methods represent a favourable option when quantifying volume, surface, length, numerical density and tortuosity of the microvessels.

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Attachment XVIII

Rohan V, Polivka J, Sevcik P, Polivka J Jr. Current approach to the options of primary and secondary prevention of ischemic cerebrovascular accident. Kardiol Rev. 2013; 15(4):218 223.

AKTUÁLNÍ POHLED NA MOŽNOSTI PRIMÁRNÍ A SEKUNDÁRNÍ PREVENCE ISCHEMICKÝCH CÉVNÍCH MOZKOVÝCH PŘÍHOD

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Souhrn

Léčba a prevence ischemických cévních mozkových příhod jsou předmětem intenzivního medicínského výzkumu, na jehož základě jsou s určitým zpožděním modifikovány i doporučené léčebné postupy sloužící jako opora pro klinickou praxi. Toto sdělení přináší přehled aktuálních informací na poli prevence ischemických cévních mozkových příhod, které by měly být zohledněny v klinickém rozhodování ještě před začleněním do oficiálních doporučených postupů. Shrnuje aktuální informace především v oblasti kardioembolických iktů, užití nových antikoagulancií a přístupu ke karotickým stenózám na podkladě výsledků klinických studií v primární i sekundární prevenci cévních mozkových příhod.

Klíčová slova

ischemické cévní mozkové příhody – primární prevence – sekundární prevence – antikoagulační léčba – protidestičková léčba

Abstract

Current approach to the options of primary and secondary prevention of ischemic cerebrovascular accident. The treatment and prevention of ischemic cerebrovascular accidents are subject to intensive medical research based on which the recommended treatment procedures used as a support in clinical practice are modified with a certain delay. This paper provides an overview of current information about the prevention of cerebrovascular accidents, which should have been reflected in the clinical decision-making before incorporation into the official recommended procedures. It summarizes the latest information primarily regarding cardioembolic strokes, use of new anticoagulants, and an approach to carotid stenoses based on the results of clinical trials in primary and secondary prevention of cerebrovascular accidents..

Keywords

ischemic stroke – primary prevention – secondary prevention – anticoagulation therapy – antiplatelet therapy

Cévní mozkové příhody (CMP) jsou jednou z hlavních příčin morbidity a mortality zejména v rozvinutých zemích. Ischemické CMP představují 85–90 % všech CMP. I přes pokroky v prevenci lze předpokládat, že v souvislosti se stárnutím populace bude trend v prevalenci a incidenci CMP vzrůstající [1]. Byla vypracována řada doporučení pro léčbu a prevenci ischemických CMP a tranzitorních ischemických atak (TIA). Zatím poslední dosud platná doporučení České neurologické společnosti pocházejí z roku 2008 [2]. Doporuční European Stroke Organization (ESO) rovněž z roku 2008 (doplněná 2009) [3] podrobně rozebírají danou problematiku. Jejich zkrácená verze byla publikována i v časopise Neurologie pro praxi [4]. Vzhledem k tomu, že od té doby došlo na tomto poli k dalšímu vývoji, přináší toto sdělení aktualizovaný pohled na problematiku.

Primární prevence

Cílem primární prevence je snížení rizika CMP u dosud asymptomatických osob. Je zaměřena na ovlivnění a léčbu známých rizikových faktorů, jako je arteriální hypertenze (AH), diabetes mellitus (DM) či porucha metabolizmu tuků. Zahájení a intenzita léčebných opatření závisí na stanovení celkového kardiovaskulárního rizika (CVR). U asymptomatických jedinců se k jeho stanovení používá nomogramů vycházejících z projektu The Systematic Coronary Risk Evaluation (SCORE) [5] hodnotících věk, pohlaví, hodnotu systolického krevního tlaku (STK), kuřácké návyky a hodnotu celkového cholesterolu. Hodnota >5 % je považována za vysoké riziko (pravděpodobnost úmrtí na kardiovaskulární onemocnění v následujících 10 letech). U symptomatických jedinců s již manifestním onemocněním kardiovaskulárním, diabetem 2. typu, případně 1. typu s mikroalbuminurií nebo chronickým onemocnění ledvin se jedná o riziko vysoké (≥ 5 %) nebo při kombinaci faktorů riziko velmi vysoké (≥ 10). Důraz je kladen primárně na nefarmakologické postupy a úpravu životního stylu ve smyslu zdravé výživy se zvýšením podílu zeleniny a ovoce, omezením soli, dále na zvýšení pravidelné aerobní fyzické aktivity, redukci zvýšené tělesné hmotnosti, omezení konzumace alkoholu a zanechání kouření [2–5].

Arteriální hypertenze

V případě AH, která je prokázaným nezávislým rizikovým faktorem, trvá doporučení korekce STK na hodnoty systoly < 140 mm Hg s výjimkou starších pacientů do 80 let věku, kde byl prokázán prospěch při snížení STK ≥ 160 na hodnoty 150–140 mm Hg. U pacientů starších 80 let nutno postupovat individuálně v závislosti na fyzickém a psychickém stavu. Prospěch korekce hodnot STK < 140 mm Hg nebyl prokázán. Je indikována cílová hodnota

diastoly <90 torr, u diabetiků < 85 torr. U pacientů s nízkým rizikem začínáme primárně nefarmakologickými postupy, v případě nasazení antihypertenzní terapie nutno hodnoty krevního tlaku snižovat postupně. Volba antihypertenziv závisí na věku a komorbiditách pacienta, u pacientů vyššího věku (> 80 let) se volí blokátory kalciových kanálů nebo thiazidová diuretika [6,7].

Diabetes mellitus

U pacientů s DM je kromě kontroly glykemie kladen vyšší důraz na kontrolu TK s cílovou hodnotou < 140/80 mm torr, v léčbě jsou preferovány inhibitory angiotenzin-konvertujícího enzymu nebo antagonisté angiotenzinových receptorů [6,7]. Současná hypercholesterolemie by měla být korigována již při hodnotách LDL-cholesterolu (LDL-C) > 3,0 mmol/l primárně pomocí statinů [7,9].

Dyslipidemie

Dyslipidemie jako další rizkový faktor by měla být korigována v primární prevenci s ohledem na celkové kardiovaskulární riziko. Léčebným cílem je ovlivnění hodnoty LDL-C úpravou životního stylu, případně léčbou statiny (tab. 1) [7].

Fibrilace síní

Doporučení pro prevenci CMP u pacientů s fibrilací síní (FS) doznala v souvislosti se zavedením nových perorálních antikoagulancií (NPA) a dostupností dat od pacientů s implantovanými přístroji nejvýznamnější vývoj [11]. U pacientů s nevalvulární FS byla přehodnocena stratifikace rizika CMP reflektující hlavní a vedlejší klinicky relevantní rizikové faktory za použití CHA_2DS_2-VASc (tab. 1). Antitrombotická léčba není doporučována pacientům s FS bez dalších rizikových faktorů ve věku < 65 let bez ohledu na pohlaví. Pacienti s těžkou renální insuficiencí mají rovněž vysoké riziko CMP, ale zároveň vysoké riziko smrti, koronárních příhod a krvácení, proto byli vylučováni z klinických studií. Hodnocení prospěchu antitrombotické léčby je zde proto obtížné a není v uvedeném skóre zařazeno. Přínos antitrombotické léčby v prevenci CMP musí převýšit riziko závažného krvácení, zejména intracerebrálního krvácení (ICH), nejobávanější komplikace této léčby. Jeho stratifikace je možná pomocí HAS-BLED skóre (tab. 2), které dobře koreluje s rizikem ICH [12]. Zajímavým poznatkem je to, že u pacientů léčených ASA je riziko ICH a velkých krvácení při stejném HAS-BLED skóre podobné [13]. U všech pacientů s FS je tedy doporučováno formální stanovení rizika krvácení. Při HAS-BLED ≥ 3 je nutná opatrnost, nejedná se ale o vylučující kritérium z léčby PA, neboť i u pacientů s vysokým HAS-BLED skóre převyšuje benefit antikoagulační léčby nad rizikem krvácení [14]. Je však nutná maximální kompenzace potenciálně reverzibilních rizikových faktorů krvácení jako nekontrolovaná arteriální hypertenze, současné užívání ASA nebo nesteroidních antiflogistik. V prevenci CMP u pacientů s nevalvulární fibrilací síní by měla být ASA podávána pouze těm pacientům, kteří jakoukoli formu léčby PA odmítají [11].

Jiná srdeční onemocnění

Antikoagulační léčba warfarinem v primární i sekundární prevenci CMP je indikována při mechanické náhradě srdeční chlopně (INR 2,5–3,5), při přítomnosti intraventrikulárního trombu, mobilního trombu v ascendentní aortě, při dilatační kardiomyopatii zvláště u pacientů do 60 let věku [15], myxomu levé síně a u mitrální stenózy po jakékoli předchozí embolizační příhodě (INR 2–3).

Nová perorální antikoagulancia v sekundární prevenci

Do roku 2012 byly jedinou možností PA léčby pacientů s FS antagonisté vitaminu K (VKA) převážně v podobě warfarinu s úpravou dávkování v rozmezí INR 2–3. V roce 2012 na základě úspěšných klinických studií prokazujících noninferioritu ve srovnání s warfarinem v primární a sekundární prevenci CMP a periferních embolizací u pacientů s nevalvulární FS – RE-LY [16], ROCKET-AF [17], ARISTOTLE [18] byla schválena nová perorální antikoagulancia (NPA) – nejdříve dabigatran jakožto přímý inhibitor trombinu, z přímých inhibitorů faktoru Xa pak rivaroxaban a apixaban, ve stadiu klinických zkoušek je edoxaban [19]. U všech dosud zkoušených NPA byla v klinických studiích prokázána noninferiorita ve srovnání s warfarinem s lepší bezpečností a snížením rizika ICH. To vedlo i k aktualizaci Doporučení pro management FS Evropské kardiologické společnosti [11] a recentně i České kardiologické společnosti JEP [20], kdy jsou NPA u většiny pacientů s nevalvulární FS považována za vhodnější. Protože jsou zkušenosti s NPA zatím omezené, doporučuje se striktní dodržování schválených indikací a pečlivý postmarketingový dohled. Vzhledem k tomu, že není přímá srovnávací studie mezi jednotlivými NPA a nepřímé srovnávací analýzy nesvědčí pro zásadní rozdíly v účinnosti, nelze učinit závěr o preferenci jednotlivého NPA [21]. Výhodou NPA oproti warfarinu je fixní dávkování bez nutnosti pravidelné monitorace antikoagulační aktivity. Je však nutno zohlednit při volbě dávky věk a renální funkce pacienta. Další výhodou je nižší množství klinicky významných lékových interakcí. Důležitým aspektem je krátký poločas s rychlým nástupem a poklesem účinku vyžadující pečlivé dodržování léčby, neboť při vynechání více než jedné dávky léku je antikoagulační efekt nedostatečný. Zvláště u dabigatranu je nutné monitorování renálních parametrů, které se mohou u polymorbidních pacientů rychle změnit např. v průběhu interkurentního onemocnění [22]. Na rozdíl od warfarinu není pro NPA použitelný žádný hemokoagulační test, který by jednoznačně kvantifikoval antikoagulační účinek. Lze použít nespecifické antikoagulační testy jako aktivovaný parciální tromboplastinový čas (APTT), specifičtější je trombinový čas nebo ekarinový test u dabigatranu, dále protrombinový čas (PT), případně stanovení anti Xa u inhibitorů Xa. Tyto testy však slouží spíše ke zjištění přítomnosti medikace, nelze je spolehlivě použít k odhadu antikoagulačního účinku NPA. Žádné z NPA nemá zatím specifické antidotum, k rychlé úpravě koagulace v případě závažného krvácení lze použít kromě krevních derivátů specifické prokoagulační reverzní látky, jako je kon-

Tab. 1. Doporučené cílové léčebné hodnoty LDL-cholesterolu (LDL-C).

Kadiovaskulární riziko	Cílová hodnota LDL-C
velmi vysoké (manifestní kardiovaskulární onemocnění, DM 2. typu, DM 1 typu s orgánovým postižením, střední až těžké postižení ledvin nebo kardiovaskulární skóre ≥ 10 %)	< 1,8 mmol/l a/nebo ≥ 50 % redukce LDL-C
vysoké (výrazně zvýšený jednotlivý rizikový faktor, kardiovaskulární skóre 5–10 %)	< 2,5 mmol/l
střední (kardiovaskulární skóre 1–5 %)	< 3 mmol/l

centrát protrombinového komplexu (PCC), aktivovaný koncentrát protrombinového komplexu (APCC) nebo rekombinantní faktor VIIa (r-FVIIa). V případě dabigatranu lze ještě zvážit použití hemodialýzy, praktické zkušenosti jsou ale dosud omezené [23].

Asymptomatická stenóza vnitřní krkavice

Aterosklerotická stenóza extrakraniální části vnitřní krkavice (ICA) je spojena se zvýšeným rizikem CMP [24]. Riziko progrese asymptomatické stenózy stoupá s časem v závislosti na přítomnosti dalších rizikových faktorů jako kouření, arteriální hypertenze, DM, stupeň stenózy, složení plátu a kontralaterální postižení ICA [25]. Riziko ipsilaterální CMP pacientů s asymptomatickou stenózou ICA se v závislosti na tíži stenózy a studované populaci při krátkodobém sledování pohybuje mezi 1 a 3 % [24]. Vzhledem k tomu, že došlo k výraznému pokroku v konzervativní i intervenční léčbě, nejsou zatím k dispozici validní data posuzující přesněji reálné riziko asymptomatické stenózy ICA srovnávající tyto odlišné léčebné postupy. V desetiletém sledování pacientů ze studie ACST [26] CEA redukovala riziko iktu včetně peroperačních iktů na 13,4 % oproti 17,9 % u pacientů s odloženým výkonem nebo léčených konzervativně. Interpretaci výsledků však může ovlivnit fakt, že 80 % pacientů studie nebylo léčeno statiny. Pokud jde o srovnání karotické endarterektomie (CEA) a stentingu (CAS), je zatím poslední rozsáhlá studie CREST [27] srovnávající tyto dva postupy u symptomatických i asymptomatických pacientů s významnou stenózou ICA. Při střední době sledování 2,5 roku bylo shledáno čtyřleté riziko kompozitního cíle (CMP, infrakt myokardu nebo smrt) téměř stejné u CAS i CEA (7,2 % a 6,8 %) bez ohledu na věk nebo klinickou manifestaci stenózy. Překvapivě více profitovali z CEA pacienti starší 70 let, naopak pacienti mladší 70 let více profitovali z CAS. Riziko CMP nebo smrti bylo 6,4 % u CAS a 4,7 % u CEA, kdy se rozdíl blížil významnosti pouze u asymptomatických pacientů. Významné byly pouze rozdíly v periprocedurálních komplikacích, kdy u CAS bylo vyšší riziko CMP (4,1 vs 2,3 %), u CEA naopak převyšovalo riziko infarktu myokardu (1,1 vs 2,3 %). V dalším období bylo riziko podobné (2,0 vs 2,4 %). Výsledky této studie tedy nijak zásadně nemění stávající doporučení, že v případě významné asymptomatické stenózy ICA (60–90 %) dle North American Symptomatic Carotid Endarterectomy Trial (NASCET) je indikována intenzivní medikamentózní léčba rizikových faktorů (DM, arteriální hypertenze, dyslipidemie). CEA je indikována pouze u pacientů s vysokým rizikem iktu (muži, stenóza > 80 %, charakter plátu), s předpokládaným přežitím > 5 let, provedení výkonu v centrech, kde je operační riziko < 3 %. Je indikováno podávání ASA před a po CEA. U asymptomatických osob není provedení CAS indikováno.

Tab. 2. CHA_2DS_2-VASc skóre (upraveno podle Lip et al, 2010).

Rizikový faktor		Skóre
C	congestive heart failure – městnavé srdeční selhání	1
H	hypertension – arteriální hypertenze	1
A2	age – věk ≥ 75 let	2
D	diabetes mellitus	1
S2	stroke – CMP/TIA v anamnéze	2
V	vascular disease – infarkt myokardu/periferní onemocnění cév	1
A	age – věk 65–74 let	1
Sc	sex category – ženské pohlaví	1

Sekundární prevence

Sekundární prevence s cílem snížení rizika výskytu další ischemické CMP musí vycházet z etiologie proběhlé ischemické CMP a zohledňovat i přítomnost případných dalších rizikových faktorů. Skládá se z optimální kompenzace vaskulárních rizikových faktorů – arteriální hypertenze, hyperlipidemie a diabetu, protidestičkové nebo antikoagulační léčby, případně v indikovaných případech z použití intervenční léčby operační nebo endovaskulární. Nedílnou součástí jsou režimová opatření s důrazem na přiměřenou fyzickou aktivitu, redukci zvýšené tělesné hmotnosti, dostatečnou hydrataci, úpravu stravovacích návyků, abstinenci kouření a redukci nadměrné konzumace alkoholu [2,3].

Cévní rizikové faktory

V rámci optimalizace cévních rizikových faktorů je indikována po odeznění akutní fáze iktu antihypertenzní léčba s korekcí TK do normální hodnoty. Její výše však musí být individualizována s ohledem na možné hemodynamické konsekvence např. u pacientů s bilaterální stenózou přívodných mozkových tepen nebo kmene mozkové cévy. Naopak u pacientů s postižením malých tepen se zdá výhodné snižování hodnot tlaku i pod hranici 130 mmHg STK [28]. Je indikována individualizovaná léčba DM stejně jako v primární prevenci. U nekardiogeních iktů též léčba statiny s cílovou hodnotou LDL < 2,5, u velmi rizikových < 1,81 mmol/l [9]. U pacientů s poruchami dýchání ve spánku je doporučována léčba respirátory s kontinuálním pozitivním tlakem v dýchacích cestách [29].

Aterotrombotické CMP

V případě sekundární prevence aterotrombotického (nekardioembolického) iktu je ve většině případů indikována protidestičková léčba. Dle doporučení ESO by měla být podávána ASA v kombinaci s dipyridamolem (25/200 mg 2× denně) nebo samotný klopidogrel (75 mg/den), alternativně jako ekonomická varianta ASA samostatně

Tab. 3. HAS-BLED skóre (modifikováno podle Pisters et al, 2010).

Rizikový faktor		Skóre
H	hypertension – hypertenze (nekontrolovaná, >160 mmHg systoly)	1
A	abnormal renal function – abnormální renální funkce (dialýza transplantace, Cr >200 μmol/L) nebo jaterní funkce (cirhóza, bilirubin >2× normy, AST/ALT/AP >3× normy)	1
S	stroke – CMP v anamnéze, zvláště lakunární	1
B	bleeding – krvácení v anamnéze nebo hemoragická diatéza, anemie	1
L	labilní INR – nestabilní nebo vysoké INR	1
E	eldery – věk ≥ 65 let	1
D	drugs/alcohol – protidestičkové léky, nesteroidní antiflogistika nebo nadužívání alkoholu	1

(75–325 mg/den). ASA je z důvodu současných indikačních omezení používána nejčastěji, v našich podmínkách v dávce 100 mg/den. Efektivita vyššího dávkování ASA nebyla prokázána [30]. Opakovaně byla zkoumána kombinace ASA + klopidogrel oproti monoterapii klopidogrelem – studie MATCH [31] i kombinace ASA + klopidogrel oproti monoterapii ASA – studie CHARISMA [32]. V obou studiích při dlouhodobém podávání ASA + klopidogrel bylo nesignifikantní snížení rizika ischemického iktu provázeno významným zvýšením krvácivých komplikací a mortality. Poslední studie SPSS3 u lakunárních iktů došla ke stejnému závěru [33]. Indikací užití kombinace ASA + klopidogrel tedy zůstává koincidence iktu a recentního akutního infarktu myokardu nebo stav po koronárním stentingu. Novou specifickou indikací pro kombinaci ASA + klopidogrel v krátkodobém podávání se zdá být významná intrakraniální symptomatická stenóza velké tepny. Studie SAMMPRIS zkoumala efekt intrakraniálního stentingu a intenzivní medikamentózní léčby (ASA + klopidogrel + statin) oproti intenzivní medikamentózní léčbě samotné [34]. Prokázala významně vyšší výskyt iktu a smrti ve stentované skupině při 30denním sledování (14,7 vs 5,8 %) i ročním sledování (20,0 vs 12,2 %), přičemž byla prokázána zhruba poloviční redukce výskytu CMP a smrti v medikamentózní skupině oproti historickým kontrolám – pacientům léčeným ASA nebo wafarinem ze studie WASID [34,35]. V případě iktu při zavedené antiagregační léčbě je nutno pomýšlet také na možnost rezistence na ASA nebo klopidogrel. Nutné je zhodnocení kompenzace dalších cévních rizikových faktorů, ale především etiologie iktu s ohledem na možnost kardioembolizace zejména při paroxysmální FS.

Kardioembolizační CMP

V sekundární prevenci ischemické CMP při FS, paroxysmální i permanentní, i většině ostatních kardioembolizací je dle doporučení ESO indikována antikoagulační léčba warfarinem (INR 2–3) nebo NPA v případě FS. Při rozhodování o načasování zahájení plné antikoagulační léčby je nutné zvážit riziko hemoragické transformace infarktového ložiska s ohledem na jeho velikost a lokalizaci. Zatím nebyl prokázán benefit časné antikoagulační léčby oproti odloženému zahájení [36]. Sekundární prevence samotnou ASA je málo účinná a riziko velkého krvácení se významně neliší od PA [13]. ASA + klopidogrel ve srovnání s warfarinem u pacientů s FS je rovněž méně účinná a nepřináší významné snížení rizika krvácivých komplikací – studie ACTIVE W [37]. Protidestičková léčba by měla být omezena na pacienty, kteří jakoukoli formu PA odmítají. Alternativou warfarinu jsou NPA diskutovaná výše.

Při současném akutním infarktu myokardu je indikován tříměsíční souběh antikoagulační a antiagregační léčby s ohledem na velikost infarktového ložiska a rizika hemoragické transformace. Antikoagulační léčba warfarinem je též indikována v případě ostatních kardiálních zdrojů embolizace stejně jako v primární prevenci.

Foramen ovale patens (PFO)

U pacientů s kryptogenním iktem je stále diskutován a studován význam PFO. V roce 2012 byly publikovány výsledky tří randomizovaných studií – CLOSURE I [38], PC-Trial [39] a RESPECT [40] – porovnávající efekt okluze PFO s medikamentózní léčbou ASA nebo warfarinem. Probíhá studie REDUCE porovnávající uzávěr PFO s protidestičkovou léčbou oproti protidestičkové léčbě samotné [41]. Žádná ze studií neprokázala vhledem k nízkému výskytu cílových událostí (iktus, smrt) a relativně krátkému sledování (dva roky) statisticky významný rozdíl mezi sledovanými skupinami i přes určitý trend ve prospěch mechanického uzávěru PFO. Další data by mohly poskytnout subanalýzy a metaanalýzy těchto studií. Data z observačních studií s delším sledováním oproti tomu přinášejí statisticky významný rozdíl ve prospěch intervenční léčby oproti medikamentózní a v případě medikamentózní léčby vychází významný přínos antikoagulační léčby oproti protidestičkové [42]. I když zatím chybí z výše uvedených důvodů jasná klinická evidence, uzávěr PFO je vhodné zvážit pouze u pacientů s iktem embolizačního typu s významným zkratem při transezofageálním echokardiografickém vyšetření a při absenci jiných rizikových faktorů. V ostatních případech je indikována léčba antikoagulační, případně protidestičková.

Trombofilní stavy

U pacientů s ischemickým iktem nejasné etiologie nebo mladších 40 let je indikováno vyšetření trombofilních stavů. Antikoagulační léčba je obvykle indikována při prokázaném deficitu antitrombinu III, proteinu C a proteinu S, při rezistenci k aktivovanému proteinu C (Faktor V Leiden), zvláště při současném průkazu hluboké žilní trombózy. Pacienti s pozitivními antifosfolipidovými protilátkami bez jiných známek antifosfolipidového syndromu jsou indikováni pouze k protidestičkové léčbě, pacienti splňující kritéria antifosfolipidového syndromu pak k léčbě antikoagulační [43].

Významná stenóza extrakraniálních tepen

V sekundární prevenci u pacientů s významnou stenózou extrakraniálních tepen je stejně jako v případně ostatních aterotrombotických CMP indikována intenzivní medikamentózní léčba cévních rizikových faktorů a léčba protidestičková [2,3,43]. V otázce užití CEA nebo CAS u pacientů s významnou stenózou ICA zatím nedochází k zásadním změnám oproti doporučením ESO, stále chybí data porovnávající tyto postupy se současnou intenzivní medikamentózní léčbou. Časná CEA do dvou týdnů po iktu je indikována u pacientů s infarktem menšího rozsahu, u kterých není velké riziko hyperperfuzního syndromu s eventuální hemohagickou transformací infarktového ložiska. Přínos CEA v odstupu tří měsíců je již oproti konzervativní léčbě minimální. V indikaci CEA/CAS hrají roli kromě tíže stenózy i charakteristika plátu, kdy přítomnost ulcerace je indikací k operaci stenózy i nižšího stupně (50–69 %) při dodržení zásad nízké perioperační morbidity a mortality (<3 %). Pacienti by měli být i perioperačně ponecháni na protidestičkové léčbě. CAS je doporučen pouze u pacientů s kontraindikací CEA, chirurgicky nepřístupnou lokalizací stenózy, restenózou po CEA a poradiační stenózou. Po CAS je indikována duální protidestičková léčba ASA + klopidogrel minimálně po dobu jednoho měsíce.

Stenóza intrakraniální tepny

Efekt stentingu významné symptomatické stenózy intrakraniální tepny (50–99 %) byl srovnáván s duální antiagregační léčbou ASA + klopidogrel v kombinaci s optimální kompenzací vaskulárních rizikových faktorů ve studii SAMMPRIS [34]. Nábor byl zastaven po zařazení 451 pacientů z důvodu podstatně vyššího výskytu časného iktu/úmrtí po stentingu ve srovnání s konzervativní léčbou. (14,7 vs 5,8 %).

Závěr

Léčba a prevence CMP jsou stále objektem intenzivního medicínského výzkumu. Na základě nových informací, jejichž přehled uvádí

toto sdělení, lze individualizovat léčebný postup. Lze také očekávat aktualizaci současně platných doporučených postupů.

Použité zkratky

AH arteriální hypertenzeAPTT – aktivovaný parciální tromboplastinový čas
APPC aktivovaný koncentrát protrombinového komplexu
ASA kyselina acetylosalicylová
CAS karotický stenting
CEA karotická endarterektomie
CMP cévní mozková příhoda
DM diabetes mellitus
ESO European Stroke Organization
FS fibrilace síní
ICH intracerebrální krvácení
INR international normalized ratio
NPA nová perorální antikoagulancia
NASCET North American Symptomatic Carotid Endarterectomy Trial
PA perorální antikoagulancia
PCC koncentrát protrombinového komplexu
PFO patentní foramen ovale
PT protrombinový čas
r-VIIa rekombinantní faktor VIIa
STK systolický krevní tlak
TIA tranzitorní ischemická ataka

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Attachment XIX

Karas M, Steinerova K, Lysak D, Hrabetova M, Jungova A, Sramek J, Jindra P, Polivka J, Holubec L. Pre-transplant quantitative determination of NPM1 mutation significantly predicts outcome of aIlogeneic hematopoietic stem cell transplantation in patients with normal karyotype AML in complete remission. Anticancer Res 2016; 36. ***Article in press.*** **(IF = 1.895)**

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Pre-transplant Quantitative Determination of *NPM1* Mutation Significantly Predicts Outcome of AIlogeneic Hematopoietic Stem Cell Transplantation in Patients with Normal Karyotype AML in Complete Remission

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Abstract. *Background: Minimal residual disease (MRD) in patients with acute myeloid leukemia (AML) before allogeneic hematopoietic stem cell transplantation (alloHSCT) can influence the results. With the aim of evaluating the potential role of pre-transplant MRD, we studied the impact of pre-transplant MRD level on the outcome of alloHSCT in patients with AML in complete remission (CR). Patients and Methods: From 2/2005 to 9/2014, 60 patients with a median age of 54 years (range=30-66 years) with normal karyotype-AML harboring nucleophosmin 1 (NPM1) mutation [53% Fms-related tyrosine kinase receptor 3 internal tandem duplication (FLT3/ITD)-positive] in first (n=45) or second (n=15) CR underwent myeloablative (n=16) or reduced-intensity (n=44) alloHSCT (27% related, 73% unrelated). The MRD level was determined from bone marrow samples using real-time polymerase chain reaction for detection of NPM1 mutations before starting the conditioning regimen. Results: The estimated probabilities of 3-year relapse, event-free survival (EFS) and overall survival (OS) for the whole cohort were 28%, 54%, and 59%, respectively. Statistical analysis showed that only age over 63 years and high MRD level affected alloHSCT outcome. Pre-transplant MRD level of 10 mutant copies of NPM1 per 10,000 Abelson murine leukemia viral oncogene homolog 1 (ABL) copies had the strongest statistical significance, and detection of higher MRD level (>10 NPM1-mutant copies) before alloHSCT was associated with increased overall mortality (hazard ratio=3.71; 95% confidence interval=1.55-9.06; p=0.004). The estimated probabilities of 3-year relapse, EFS, and OS were 6%, 72%, and 75% for patients with a low level of MRD and 48%, 35%, and 40% for patients with a higher level. Conclusion: Our data show that the pre-transplant level of MRD in patients with normal karyotype AML harboring NPM1 mutation in CR provides important prognostic information, which as an independent prognostic factor predicts transplant results.*

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Acute myeloid leukemia (AML) is currently curatively treated using an intensive induction chemotherapy treatment, which generally achieves complete remission (CR) in about 60-80% of cases. However, without further consolidation treatment, relapse would occur in most patients. Consolidation treatment options essentially consist of either further chemotherapy or allogenic hematopoietic stem cell transplantation (alloHSCT), and a range of prognostic factors are important for the choice of consolidation therapy (1-5). While the strategies and methods of curative treatment of AML have undergone no major changes over the last 20 years, the past decade has seen an improvement in treatment results owing to more detailed insight into AML biology and improvements in supportive treatment, which make it possible to manage once-fatal complications of intensive chemotherapy or alloHSCT. AlloHSCT is currently the most effective treatment for AML. Mainly due to improvements in supportive treatment and transplant procedures, mortality from transplant (TRM) has fallen significantly, and a wider spectrum of patients can now undergo alloHSCT (6, 7). At

the same time, a number of studies have investigated the role of alloHSCT in AML treatment (8-12). With TRM decreasing, AML relapse after alloHSCT remains the principal limitation to transplant outcomes, especially because the prognosis of AML relapse after alloHSCT is highly unfavorable (13). For this reason, efforts are underway to identify additional prognostic factors that may help to determine which patients are at increased risk of AML relapse after alloHSCT.

In recent years, the importance of the detection of minimal residual disease (MRD) for the prediction of AML treatment outcomes has been rising. With regard to chemotherapy of AML, the published data seems to indicate that treatment response evaluation based on MRD detection is among the independent prognostic factors relevant to risk of relapse and therefore to AML treatment results (14-26). In alloHSCT, where the efficacy of treatment is significantly influenced by the graft-*versus*-host disease (GVHD) effect and which has proven effective even in the case of chemoresistant AML, with reported long-term survival of 20-30%, it is important to ask whether a finding of MRD before alloHSCT has an impact on transplant outcome (27, 28). Moreover, most patients with AML undergo alloHSCT in CR, *i.e.* at a time when any potential residual disease is generally lower than in resistant AML. Considering the importance of the GvL effect, it is a key question whether determining MRD in AML in CR before alloHSCT can offer prognostic information about the treatment outcome, as it does with intensive chemotherapy. The importance of determining MRD before alloHSCT is thus the subject of intensive research; findings published so far, which mostly used multiparametric flow cytometry to detect MRD, indicate that determining MRD before alloHSCT may carry prognostic information about the transplant outcome. However, the existing research is frequently limited by a small number of studied patients, inclusion of heterogeneous AML types, different diagnostic methods for MRD determination, as well as the fact that support for MRD importance is not universal among these studies (29-35).

With the aim of evaluating the importance of determining the level of MRD levels before alloHSCT in patients with AML in CR, we analyzed alloHSCT outcomes in patients in first or second CR of normal karyotype AML (NK-AML) with *NPM1* gene mutation. AML with normal karyotype represents the largest group of patients with AML and most common molecular lesion in this group is a mutation in the gene encoding nucleophosmin (NPM1). *NPM1* mutation was also previously found to be a suitable and stable marker of MRD in a number of published studies (16-19). We used the relative expression of the mutated *NPM1* gene, evaluated using real-time polymerase chain reaction (RT-PCR), as a marker for MRD monitoring immediately prior to the start of the conditioning regimen.

Patients and Methods

Study group. Our study group was made up of all patients aged 18 and above diagnosed with NK-AML with an *NPM1* gene mutation (types A, B, and D) at the Department of Hematology and Oncology of the University Hospital in Pilsen and who underwent alloHSCT in first or second CR between January 2005 and September 2014. AML was diagnosed according to the World Health Organization (WHO) classification (36).

Cytogenetic examination by G-banding was performed on all AML samples at the time of diagnosis. *NPM1* gene mutation presence and type were determined by DNA sequencing and quantitative examination of initial relative expression of mutated NPM1 was also performed. As part of the molecular genetic marker assay, presence of Fms-related tyrosine kinase *receptor* 3 internal tandem duplication (*FLT3/ITD*) was also determined for all patients at the time of diagnosis. Complete remission of AML was evaluated according to standard recommendations (37).

Human leukocyte antigen (HLA) typing of donors and recipients was performed according the European Federation for Immunogenetics/European Society for Blood and Marrow Transplantation (EFI/EBMT) recommendations (accessible at www.efiweb.eu/efi-committees/standards-committee.htlm) in an EFI-accredited laboratory. Acute and chronic GVHD was diagnosed according to published criteria (38, 39).

All patients were evaluated post-transplant for overall survival (OS), event-free survival (EFS), TRM, and incidence of relapse. All patients were treated according to protocols approved by the Quality Control Boards of a Joint Accreditation Committee-ISCT and EBMT accredited facility and all patients provided consent for monitoring and data processing in accordance with the Declaration of Helsinki.

Detection of MRD. Bone marrow sample collection for MRD detection was performed no later than 1 week before the start of the pre-transplant conditioning regimen. RNA was isolated from bone marrow samples using the commercial QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng of RNA using SuperScript III First Strand Synthesis SuperMix commercial kit (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR was performed on the samples using the NPM1Quant kit (Ipsogen SA, Marseille, France). The calibration curves for the quantitative assessment of the number of copies of the mutated *NPM1* gene and the control gene *ABL* were determined based on the standards supplied with the kit for all analyses. All samples were analyzed in duplicate, using the average of the two analyses in further calculations. If the threshold cycle (Ct) discrepancy between the two samples exceeded 0.6 cycles, the analysis was repeated. The minimum required expression for the *ABL* gene control was set at 3,000 copies. The results of the expression analysis of the mutated *NPM1* gene are given as the number of copies of mutated *NPM1* per 10,000 copies of *ABL*. Amplification and data analysis were performed on Light Cycler version 1.5 or version 2.0 devices (Roche Applied Science, Mannheim, Germany). The assays were performed at the accredited Molecular Genetics Laboratory of Department of Hematology and Oncology, Faculty Hospital, Plzen, Czech Repblic and the method of relative quantification of mutated *NPM1* was regularly verified, validated and monitored as part of external inter-laboratory quality control processes.

Table I. *Characteristics of the study group of patients with acute myeloid leukemia (AML).*

Characteristic	Value
Median of age (range), years	54 (30-66)
Gender, n (%)	
Male	32 (53%)
Female	28 (47%)
AML status, n (%)	
CR1	45 (75%)
CR2	15 (25%)
FLT3/ITD positivity, n (%)	32 (53%)
Conditioning protocol, n (%)	
Myeloablative	16 (27%)
Reduced-intensity	44 (73%)
Type of donor, n (%)	
Related	16 (27%)
Unrelated	44 (73%)
Gender recipient/donor, n (%)	
Male/female	12 (20%)
Other	48 (80%)
CMV status recipient/donor,n (%)	
Negative/negative	6 (10%)
Other	54 (90%)
Source of stem cells, n (%)	
Bone marrow	12 (20%)
PBSC	48 (80%)
WHO status, n (%)	
0-1	60 (100%)
≥2	0 (0%)

CR: First complete remission; CR2: second complete remission; *FLT3/ITD*: Fms-related tyrosine kinase receptor 3 internal tandem duplication; CMV: cytomegalovirus; PBSC: peripheral blood stem cells.

Statistical analysis. Patient characteristics were summarized using frequency tables and standard descriptive statistics, Pearson chi-square test, Fisher's exact test, and *t*-test.

OS was calculated from the date of alloHSCT until death from any cause, and surviving patients were censored at the last follow-up. The EFS was calculated from the date of alloHSCT until death or relapse, and patients who were alive and disease-free were censored at the last follow-up. Probabilities of OS and EFS were estimated using the Kaplan–Meier method. TRM was defined as death due to any cause unrelated to disease. Probabilities of TRM and relapse were summarized using cumulative incidence estimates. Cumulative incidence of TRM and relapse were adjusting for competing risk. TRM was a competing risk for relapse, while relapse was a competing risk for TRM. Univariate analyses to evaluate differences in survival between groups of patients were performed using the log-rank and Wilcoxon tests. The Cox proportional hazards model was considered for the survival modeling to specify the role of individual prognostic factors in assessing the OS and EFS. The multivariable Cox proportional hazards model (stepwise regression) was used for identification of the significant prognostic factors in OS and EFS. The level of statistical significance of α=0.05 was used in all analyses. All computations were performed using SAS software (SAS Institute Inc., Cary, NC, USA) and STATISTICA software (StatSoft, Inc., Tulsa, OK, USA).

Table II. *Transplant results of the entire study group.*

Characteristic	n (%)
Acute GVHD	36 (60%)
Acute GVHD III-IV	8 (13%)
Chronic GVHD	24 (40%)
Mild chronic GVHD	12 (20%)
Moderate chronic GVHD	8 (13%)
Severe chronic GVHD	4 (7%)
3-Year cumulative relapse	28%
3-Year cumulative TRM, %	21%
3-Year EFS	54%
3-Year OS	59%

GVHD: Graft-*versus*-host disease; TRM: transplant-related mortality; EFS: event-free survival; OS: overall survival.

Results

Patient characteristics. The group of patients consisted of 60 individuals (32 women, 28 men), with a median age of 54 years (range: 30-66) with *NK-AML* and NPM1 mutation. *FLT3/ITD* was found in 32 patients (53%) at the time of diagnosis. All patients underwent alloHSCT, most (73%) from an unrelated donor. More patients underwent alloHSCT after reduced-intensity conditioning (RIC) (73%) than after myeloablative conditioning (MAC). The RIC consisted of a combination of fludarabine (30 mg/m^2/day for 4 days) and melphalan (140 mg/m^2/day for 1 day); the MAC consisted of a combination of busulfan (3.2 mg/kg/day *i.v.* for 4 days) and cyclophosphamide (60 mg/kg/day for 2 days). In unrelated alloHSCT, ATG Fresenius S (15 mg/kg dose) was used as part of the conditioning regimen. The source of the hematopoietic stem cells was mainly peripheral blood stem cells (80%).

At the time of transplant, the WHO performance status of patients was between 0 and 1. The median follow-up of surviving patients was 55 months (range=6-101 months). Cyclosporine A and methotrexate were administered to all patients as GVHD prophylaxis. Patient characteristics are summarized in Table I.

Transplant outcomes of entire study group. All patients underwent transplant and were in CR at day 30 post-transplant. Although many patients developed acute GVHD (60%), only eight developed acute GVHD grade III-IV.

With a median follow-up of 55 months (range=6-101 months), 36 patients (60%) remained alive. Out of the entire

Table III. *Univariate analysis of factor affecting event-free survival (EFS) and overall survival (OS).*

	EFS			OS		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age >63 years	3.40	1.24-9.62	0.0341	5.40	1.82-16.02	0.0071
High *vs.* low MRD*	3.69	1.60-8.51	0.0021	3.50	1.40-8.47	0.0034
FLT3/ITD positivity	1.29	0.60-2.78	0.51	1.05	0.47-2.34	0.90
CR2 *vs.* CR1	1.72	0.77-3.87	0.19	1.92	0.82-4.49	0.13
RIT *vs.* MAT	1.14	0.48-2.71	0.76	1.71	0.63-4.58	0.29
Unrelated *vs.* related donor	0.98	0.42- 2.32	0.97	1.22	0.45- 2.83	0.81
BM *vs.* PBSCs	1.18	0.50-2.81	0.70	1.09	0.43-2.74	0.86
Recipent M/donor F *vs.* other	1.00	0.38-2.66	0.99	1.77	0.53-5.95	0.35
CMV donor/recipient positive *vs.* other	2.16	0.97-4.83	0.06	1.75	0.76-3.99	0.18

HR: Hazard ratio; CI: confidence interval; CR1: first complete remission; CR2: second complete remission; RIT: reduced-intesity transplantation; MAT: myeloablative transplantation; BM: bone marrow; PBSC: peripheral blood stem cells; M: male; F: female. *High level of minimal residual disease (MRD): nucleophosmin 1 (*NPM1*) >10 copies/10000 copies Abelson murine leukemia viral oncogene homolog 1 (*ABL*).

Table IV. *Multivariate analysis of factors affecting event-free survival (EFS) and overall survival (OS).*

	EFS			OS		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age >63 years	3.40	1.24-9.62	0.0341	6.23	1.99-19.48	0.0017
High *vs.* low MRD*	3.69	1.60-8.51	0.0021	3.71	1.52-9.06	0.0040

*High level of minimal residual disease (MRD): nucleophosmin 1 (*NPM1*) >10 copies/10000 copies Abelson murine leukemia viral oncogene homolog 1 (*ABL*).

group, 16 patients experienced relapsed. The median time from transplant to relapse was 4 months (range=3-13 months). Thirteen patients died as a result of relapse, two patients achieved a subsequent CR lasting 51 and 61 months, respectively, and one patient was alive in relapse.

Eleven patients (18%) had died due to TRM. The most common cause of death (64% of TRM cases) was infectious complications related to acute or chronic GVHD. One 1-year TRM was 13%. Estimated 3-year EFS, OS, cumulative incidence of TRM, and cumulative incidence of relapse were, for the whole group, 54%, 59%, 18%, and 28%, respectively. Transplant outcomes for the entire study group are summarized in Table II and Figure 1.

Importance of pre-transplant prognostic markers for transplant outcome. In univariate analysis of the listed pre-transplant prognostic factors, only age over 63 years and the pre-transplant MRD level (most significant for those above 10 mutated *NPM1* copies per 10,000 *ABL* copies) had a statistically significant negative impact on EFS and OS. A negative trend in EFS and OS was found for alloHSCT having been performed in the second (as opposed to first) CR and for positive serological cytomegalovirus (CMV) status of donor and recipient, but these trends were not statistically significant. Univariate analysis of factors affecting EFS and OS is summarized in Table III.

Multivariate analysis of the listed pre-transplant factors confirmed a statistically significant negative prognostic impact on EFS and OS for age over 63 years and the level of pre-transplant MRD (most significant for levels above 10 mutated *NPM1* copies per 10,000 *ABL* copies). Multivariate analysis results are summarized in Table IV.

Importance of pre-transplant residual disease level for transplant outcome. As the relative expression of mutated *NPM1* was known at the time of *AML* diagnosis [median=38,245 (range=14,350-144,707) mutated *NPM1* copies per 10,000 *ABL* copies], it was possible to evaluate pre-transplant MRD with two different methods: (i) as solely the pre-transplant relative expression of mutated *NPM1*, and (ii) as the decrease in relative expression of mutated *NPM1* between initial AML diagnosis and immediately before the

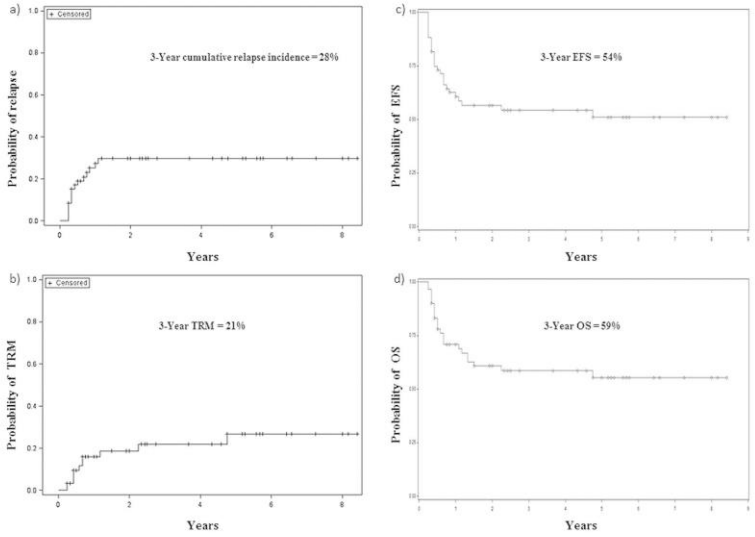

Figure 1. *Probability of relapse (a), transplant-related mortality (b), event-free survival (EFS) (c) and overall survival (OS) (d) for the entire group of patients.*

transplant procedure. Higher levels of statistical significance were achieved using the first method, *i.e.* measuring only the pre-transplant level of relative expression of mutated *NPM1*. We set the cut-off point at 10 mutated *NPM1* copies per 10,000 ABL copies, as this division of the patient group produced the most statistically significant difference in EFS and OS, although statistically significant differences were also found for several other cut-off values (see Figure 2).

The division of the patient cohort by pre-transplant MRD level (more or less than 10 mutated *NPM1* copies per 10,000 *ABL* copies) reveals a marked difference in the results of the alloHSCT. Considering the entire patient group, 16 patients (28%) experienced relapse. However, in the low-MRD group there were only two patients with relapse (6% of the low-MRD group), while in the high-MRD group there were 14 (48% of the high-MRD group). A total of 11 patients (18%) died due to TRM, five (17%) in the high-MRD group and six (19%) in the low-MRD group. Estimated 3-year EFS and OS for the entire study group were 54% and 59%, respectively. Risk of relapse or death was 3.69 times higher in the high-MRD group than in the low-MRD group [hazard ratio (HR)=3.69; 95% confidence interval (CI)=1.60-8.51, p=0.0021). Estimated 3-year EFS was 35% in the high-MRD group – significantly lower than the 72% found in the low-MRD group (p=0.0021). This is presented in Figure 3a.

Table V. *Outcome probalities stratified by minimal residual disease (MRD) status whereby a high level of MRD was defined as nucleophosmin 1 (NPM1) >10 copies/10000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL).*

3-Year endpoint	Low MRD	High MRD
CIR	6%	48%
EFS	72%	35%
OS	75%	40%

CIR: Cumulative incidence of relapse; EFS: event-free survival; OS: overall survival.

Risk of death was 3.71 times higher in the high-MRD group than in the low-MRD group (HR=3.71; 95% CI=1.52-9.06, p=0.0040). The estimated 3-year OS rate was 40% in the high-MRD group – significantly lower than the 75% found in the low-MRD group (p=0.004). This may be seen in Figure 3b.

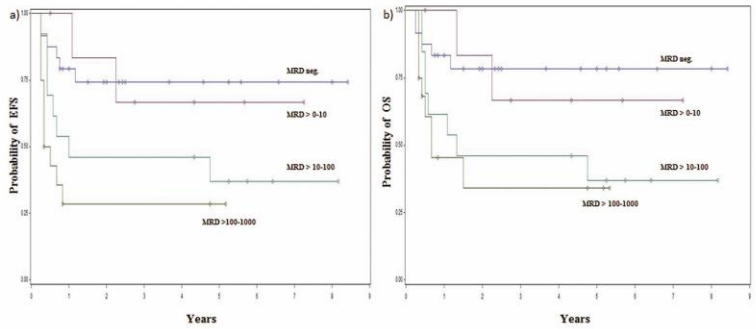

Figure 2. *Probability of event-free (EFS) (a) and overall (OS) (b) survival for transplanted patients stratified by pre-transplant minimal residual disease (MRD) level [mutated nucleophosmin 1 (NPM1) copies/10000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL)].*

The transplant outcomes for the patients according to pre-transplant MRD level are summarized in Table V.

Discussion

We investigated the importance of pre-transplant MRD level for alloHSCT outcome in patients with NK-AML with a mutation of the *NPM1* gene in first or second CR of AML. While our sample is somewhat small, it has the virtue of being homogeneous in both diagnosis and treatment. We determined MRD according to the relative expression of mutated *NPM1*, evaluated using standardized RT-PCR. *NPM1* mutation was previously found to be a suitable and stable marker of MRD in a number of published studies (16-19, 40-42). Our sample is among the largest of studies of patients with AML examining the impact of pre-transplant MRD quantified by the expression of mutated *NPM1* on transplant outcome. Thanks to a distinct molecular genetic marker, the use of RT-PCR eliminates certain limitations of multiparametric flow cytometry – in particular, its lower sensitivity and the risk of antigenic shifts in the leukemia cells (33, 43-45). MRD was determined immediately (less than 1 week) prior to the start of conditioning regimen. This approach reduces the risk of error in MRD measurement arising from potentially fast changes in MRD in patients with AML (46, 47). Other published studies either do not disclose the time interval between MRD assessment and the transplant procedure, or this interval was longer than in our study (34, 48).

The results of our analysis show that in our sample population, the pre-transplant MRD level in patients with NK-AML and *NPM1* gene mutation in CR was an independent prognostic factor for alloHSCT outcome. In our study group, determining the pre-alloHSCT relative expression of mutated *NPM1* proved to be a superior method of predicting alloHSCT outcome compared to the evaluation of the decrease of mutated *NPM1* expression between diagnosis and the transplant procedure. We assume this is due to a large range of expression levels of mutated *NPM1* in the AML diagnostic samples. In our study group, these results were statistically significant for several levels of pre-transplant mutated *NPM1* expression (negative *vs.* positive; more *vs.* less than 1; more *vs.* less than 10; more *vs.* less than 100 mutated *NPM1* copies per 10,000 *ABL* copies), which suggests that alloHSCT outcomes (EFS, OS) deteriorate with increasing pre-transplant MRD. This finding is not completely in line with some prior published studies, where a difference in alloHSCT outcomes had been documented only between MRD-positive and MRD-negative patients, and the impact on transplant outcome of different levels of positivity among MRD-positive patients was not further documented (29, 31). This might be explained by the fact that these studies used a less sensitive method of MRD detection (multiparametric flow cytometry), as well as by their lower number of enrolled MRD-positive patients which might have caused further sample divisions to fall short of statistical significance.

In our study, we chose a cut-off point of 10 mutated NPM1 copies per 10,000 ABL copies (0.1% mutated NPM1 to ABL ratio); this cut-off value divided the entire patient cohort into two groups between which the statistical significance of the difference in EFS and OS was highest, in univariate as well as multivariate analysis. Patients with an *NPM1* mutation in CR and higher pre-transplant MRD level (>10 mutated *NPM1* copies per 10,000 *ABL* copies) exhibited higher incidence of relapse and lower EFS and

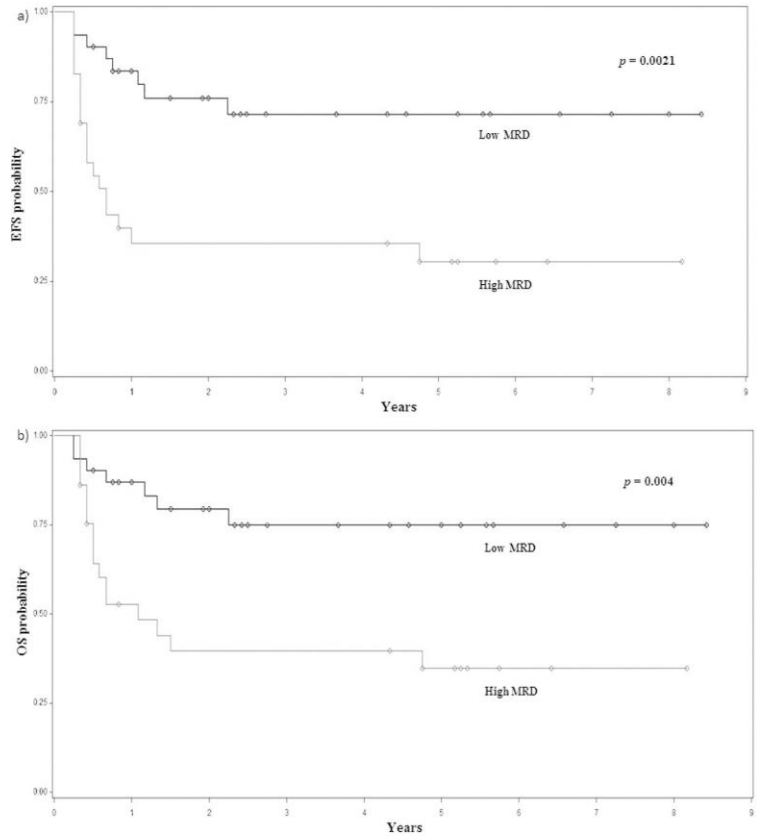

Figure 3. *Probability of event-free (EFS) (a) and overall (OS) (b) survival for patients with acute myeloid leukemia (AML) with negative/low level vs. high level pre-transplant minimal residual disease (MRD) status [defined by cutoff of 10 mutated nucleophosmin 1 (NPM1) copies/10000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL)].*

overall survival, while TRM was not significantly different between the two groups. Our results thus provide further support for the importance of MRD determination in alloHSCT prognosis among patients with AML, in line with the majority of previously published research (29-34, 48, 49).

An interesting finding is that *FLT3/ITD* positivity had no adverse effect on the transplant outcome in our group of patients with NK-AML with an *NPM1* mutation in CR. In other published research, diagnostic *FLT3/ITD* positivity had a negative impact on the prognosis of such patients (19, 46, 50, 51). From our results it would seem that as long as the MRD level is taken into account, diagnostic *FLT3/ITD* positivity or negativity among patients with AML harboring *NPM1* mutations in CR has only minimal impact on alloHSCT outcome. Certain other researchers' results also support this conclusion (42). The status of CR of AML also had no impact on transplant outcomes in our population when pre-transplant MRD levels were taken into account, outcomes were not significantly different between patients

in first and second CR. Similar results are also reported by other researchers who took pre-transplant MRD levels into account for transplant outcome evaluation (29, 31, 33). However, we cannot rule out the influence of sample size on our results. We noticed a trend towards poorer transplant outcomes in patients in second CR, but this trend was not statistically significant. This ambiguity is also in line with the results of a recent study of alloHSCT outcomes in patients with AML with mutated *NPM1* which also found worse outcomes in patients in second CR compared with those in the first; however, it did not evaluate pre-transplant MRD level (52).

It is also important to mention the role of the intensity of pre-transplant conditioning regimen in alloHSCT outcomes – especially since, unlike other potential pre-transplant factors (donor type and gender, *etc.*), we are able to influence this. In our study group, we did not find a statistically significant difference between patients transplanted after MAC and those after RIC. This is supported by the results of several other published studies, where alloHSCT outcomes in AML in CR were also not influenced by the conditioning regimen, but rather were only influenced by pre-transplant MRD positivity (29-32). In general, however, the published data suggests that reduced-intensity pre-transplant conditioning is associated with a higher risk of post-transplant AML relapse when compared to a MIC; this includes several studies which also evaluated pre-transplant MRD (35, 53-55). In our study group, other potentially prognostic factors (donor type and gender, graft type, donor CMV status) did not significantly influence alloHSCT outcomes, with the exception of age over 63 years, where the 3-year EFS and OS were 38%. However, this last result was impacted by higher TRM (38%) among these older patients.

From a practical standpoint, our sample includes a group of patients with low MRD whose 3-year OS was 75%, especially due to low incidence of relapse (only 6%). Similar results may be found in other published studies – pre-transplant MRD-negative patients were reported across several studies to have 3-year OS of 62-77% and relapse incidence rates of 0-21% (29-31, 34). Thus, this patient group has an overall low risk of AML relapse, leaving TRM, morbidity and quality of life as the key factors for alloHSCT outcome. Therefore, for these patients we can preferentially choose an RIC regimen to reduce transplant toxicity. We can also use more potent GVHD prophylaxis and slower tapering of immunosuppression to reduce the risk of GVHD, which is the principal cause of morbidity and mortality after alloHSCT. However, such approaches should be verified in further research. A bolder question is whether alloHSCT is necessary at all in the treatment of these patients, with regard to some recently published data (42).

On the other hand, our study group also included the high pre-transplant MRD group, whose prognosis was notably worse. In our study, these patients had a 3-year OS of 40% and a relapse incidence rate of 48%. Similar results are again found in other published studies, where 3-year OS in pre-transplant MRD-positive patients were in the range of 18-47% and relapse incidence rates were 41-70% (29-31, 34). The outcomes for pre-transplant MRD-positive patients are thus worse than for MRD-negative patients at a statistically significant level. MRD positivity has been shown to be an independent negative prognostic factor for AML treatment outcomes of patients treated with only standard chemotherapy in a fairly large body of research (15-18, 20-22, 26, 42, 56). Therefore, MRD positivity can be considered an independent negative biological characteristic of the disease in the context of standard chemotherapy of AML. Outcomes in MRD-positive patients where chemotherapy treatment is used alone are highly unfavorable. One of the most recent larger published studies on patients with AML with *NPM1* mutation gives 3-year OS of only 24% for patients with persistent positive NPM1 expression after two treatment cycles (42). Combining our results with other published research, pre-transplant MRD-positive patients with AML in CR achieve 3-year OS of around 40%; we can thus infer that alloHSCT partially improves their unfavorable prognosis compared to chemotherapy alone. At present, it remains unclear whether the outcome of alloHSCT in patients with MRD-positive AML in CR can be improved. In this potentially high-risk group, several ways to influence alloHSCT outcomes can be contemplated. In cases where MRD positivity persists during chemotherapy, there is the possibility of attempting to achieve MRD negativity through further cytostatic treatment; however, based on published data, the efficacy of this approach is debatable (26, 57). Another option is to try to influence MRD positivity with a more intensive pre-alloHSCT regimen, but based on our results as well as the results of several other published studies, this approach also does not guarantee an improvement in transplant outcomes (21, 35, 53-55). Furthermore, more intensive pre-transplant conditioning can increase the risk of TRM; this may offset any potential decrease in the risk of relapse, so that OS among alloHSCT patients with an MAC regimen might not change significantly (58). Other options for influencing alloHSCT outcomes in patients with MRD-positive AML in CR include an attempt to increase the graft *versus* leukemia effect by early post-transplant tapering of immunosuppression or by a pre-emptive infusion of donor lymphocytes (59, 60). In recent years, it has also become attractive to combine the abovementioned graft *versus* leukemia potentiation with other treatments, either standard cytostatics or newer targeted therapy (hypomethylating agents, antibodies, tyrosine kinase inhibitors, *etc.*). Certain recent publications have shown azacytidine and deoxyazacytidine to be effective in post-transplant pre-emptive relapse treatment (61-63). However,

the studies published so far are not large and no standard post-transplant pre-emptive treatment is currently being generally recommended for AML.

The results of our study show that any potential post-transplant therapeutic intervention should be initiated in a timely manner, as our patients with AML in CR and high MRD levels experienced relapse after a fairly short median period of 4 months. In general, we can say that the inferior alloHSCT outcomes found in patients with MRD-positive AML in CR open the field for further research that would identify additional negative prognostic factors for this patient group, as well as for prospective intervention studies which would attempt to further investigate the benefits of the discussed therapeutic options with regard to improving the prognosis of these patients.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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