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Molecular mechanisms of sensitivity and resistance towards chemotherapeutics in most frequent solid cancers

Molekulární mechanismy senzitivity a rezistence v chemoterapii nejčastějších solidních nádorů

Dissertation Thesis

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LIST OF ABREVIATIONS

3'UTR	3' untranslated region
5-FU	5-Fluorouracil
ABC	ATP binding cassette
ACTB	Actin beta
APE1	AP endonuclease
ARID1A	AT-Rich interaction domain 1A
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad3-related
BARD1	BRCA1 associated RING domain 1
BC	Breast cancer
BCRP	Breast cancer resistance protein
BER	Base excision repair
BRAF	B-Raf serine/threonine protein kinase
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRIP1	Breast Cancer 1 interacting helicase 1
Cbpt	Carboplatin
CCC	Clear cell carcinoma
CFA	Colony formation assay
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CIS	Chemotherapy-induced senescence
CNU	Chloroethylating nitrosoureas
CRC	Colorectal cancer
CS	Cockayne syndrome
CSA	Cockayne syndrome A protein
CSB	Cockayne syndrome B protein
DAPK1	Death-associated protein kinase 1
DDR	DNA damage response
DFS	Disease-free survival
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
DSBs	Double-strand breaks
DSS	Disease-specific survival
EC	Endometroid carcinoma
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian carcinoma
ER	Estrogen receptor

EXO1	Exonuclease 1
FDA	U.S. Food and Drug Administration
FEN1	Flap structure-specific endonuclease 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEPIA	Gene Expression Profiling Interactive Analysis
GG-NER	Global genome NER
GLC	Ganoderma Lucidum extract
GTEx	Genotype-Tissue expression project
HDI	Human Development Index
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination
HR	Hazard ratio (in statistical analyses)
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
CHT	Chemotherapy
KRAS	Kirsten rat sarcoma virus
LGSOC	Low-grade serous ovarian carcinomas
LIG1	Ligase 1
LTL	Lymphocyte telomere length
MBD4	Methyl-binding domain glycosylase 4
MC	Mucinous carcinoma
MDR	Multidrug resistance
MGMT	Methylguanine methyltransferase
MHL1	MutL homolog 1
miRNA	MicroRNA
miRSNP	SNP polymorphism in miRNA-binding site
MMR	Mismatch repair
MPG	3-methyl-purine glycosylase
MRN	MRE11-RAD50-NBS1 complex
MRP1	Multidrug resistance protein 1
MSH2	MutS homolog 2
MSH3	MutS homolog 3
MSH6	MutS homolog 6
MSI	Microsatellite instability
MUTYH	MutY homolog DNA glycosylase
NACT	Neoadjuvant chemotherapy
NBS1	Nibrin
NEIL1	Endonuclease VIII-like glycosylase 1
NEIL2	Endonuclease VIII-like glycosylase 2
NEIL3	Endonuclease VIII-like glycosylase 3
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining

NSCLC	Non-small cell lung cancer
NTH1	Endonuclease III-like 1
OGG1	8-OxoG DNA glycosylase 1
OS	Overall survival
OvC	Ovarian cancer
PALB2	PALB2 partner and localizer of BRCA2
PARP1	Poly (ADP-ribose) polymerase 1
PARPi	PARP inhibitor
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PMS2	PMS1 homolog 2
POLβ	DNA polymerase beta
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog phosphatase
RAD51C	RAD 51 paralog C
RAD51D	RAD 51 paralog D
RAD51L1	RAD51 like 1
RAD52	RAD52 homolog
ROS	Reactive oxygen species
RPA	Replication protein A
RT	Radiation therapy
SACT	Systemic anti-cancer therapy
SCID	Severe combined immunodeficiency
shRNA	Short hairpin RNA
SMUG1	Single-strand-specific monofunctional uracil DNA glycosylase 1
SNP	Single nucleotide polymorphism
SSBs	Single strand breaks
TCGA	The Cancer Genome Atlas
TC-NER	Transcription-coupled NER
TDG	Thymine DNA glycosylase
TDP1	Tyrosyl-DNA phosphodiesterase 1
TFIIH	Transcription factor II H
TL	Telomere length
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNM	Tumor-node-metastases
TOPBP1	DNA topoisomerase II binding protein 1
TP53BP1	Tumor protein P53 binding protein 1)
TTD	Trichothiodystrophy
UNG1	Uracil-N glycosylase1
UNG2	Uracil-N glycosylase2
UV-DDB	UV-damaged DNA-binding protein
XAB2	XPA binding protein 2

XP	Xeroderma pigmentosum
XPB	Xeroderma pigmentosum group B
XPD	Xeroderma pigmentosum group D
XPG	Xeroderma pigmentosum group G
XRCC1	X-ray repair cross-complementing protein 1
XRCC2	X-ray repair cross-complementing protein 2

ABSTRACT

Despite the great effort, the main obstacle to cancer therapy represents low response towards common chemotherapeutics and/or resistance. Chemoresistance causes cancer relapse and formation of metastases, dramatically challenging the prognosis of patients. It is estimated, that about 90% of cancer mortality can be directly or indirectly attributed to chemoresistance. There are several intrinsic or acquired cellular mechanisms of tumor chemoresistance, with DNA repair being one of the key culprits affecting the response towards chemotherapeutics in cancer cells. This is based on the fundamental principle of their action, as the majority of chemotherapeutics are designed to increase DNA damage and to suppress DNA repair or DNA damage response, ultimately triggering the death of malignant cells. Consequently, understanding the complex mechanisms of DNA repair and its regulation is essential for more targeted and effective treatment of cancer patients.

In this dissertation Thesis, we attempted to elucidate some of the regulatory mechanisms of DNA repair and their effects on response to common chemotherapeutics. We confirmed that single nucleotide polymorphisms in microRNA binding sites of DNA repair genes may influence the patient's survival and response to cancer therapy. We investigated the role of miR-140 in colorectal cancer and proposed that miR-140 ameliorates oxaliplatin response through inhibition of MRE11, an important protein in the repair of DNA double-strand breaks. We also investigated the impact of MRE11 inhibition, using Mirin and observed an increase in the cytotoxic effects of carboplatin on ovarian cancer cells and even re-sensitized resistant cell line to carboplatin. We also established a 5-FU resistant colorectal cancer cell line and demonstrated the crucial role of DNA repair and damage response gene dysregulation in developing 5-FU chemoresistance. Additionally, we were also interested in combination therapies of conventional chemotherapeutics and natural compounds to increase their efficacy. We analyzed the effect of *Ganoderma Lucidum* extract and confirmed its enhancing effect on 5-FU efficacy in colorectal cancer both *in vitro* and *in vivo*.

We believe that our results may add to a better understanding of the molecular mechanisms of resistance and sensitivity to chemotherapeutics in different types of cancer which may ultimately lead to better response and outcome for cancer patients.

ABSTRAKT

Navzdory velkému úsilí je hlavní překážkou léčby rakoviny nízká odpověď na konvenční chemoterapeutika a/nebo rezistence. Chemorezistence způsobuje relaps rakoviny a tvorbu metastáz, což dramaticky ztěžuje prognózu pacientů. Odhaduje se, že přibližně 90 % úmrtí na rakovinu lze přímo či nepřímo přičíst chemorezistenci. Existuje několik vnitřních nebo získaných buněčných mechanismů chemorezistence nádorů, přičemž jedním z klíčových viníků ovlivňujících odpověď nádorových buněk na chemoterapeutika je oprava DNA. To vychází ze základního principu jejich působení, neboť většina chemoterapeutik je navržena tak, aby zvyšovala poškození DNA a inhibovala její opravu nebo odpověď na poškození DNA, což v konečném důsledku vyvolává smrt maligních buněk. Pochopení složitých mechanismů opravy DNA a její regulace je proto nezbytné pro cílenější a účinnější léčbu pacientů s rakovinou.

V této disertační práci jsme se pokusili objasnit některé regulační mechanismy opravy DNA a jejich vliv na odpověď na konvenční chemoterapeutika. Potvrdili jsme, že jednonukleotidové polymorfismy ve vazebných místech mikroRNA v DNA reparačních genech mohou ovlivňovat přežití pacientů a jejich odpověď na protinádorovou léčbu. Zkoumali jsme roli miR-140 u kolorektálního karcinomu a zjistili jsme, že miR-140 zlepšuje odpověď na oxaliplatinu prostřednictvím inhibice MRE11, důležitého proteinu při opravě dvouřetězcových zlomů DNA. Zkoumali jsme také vliv inhibice MRE11 pomocí Mirinu a pozorovali jsme zvýšení cytotoxických účinků karboplatiny na buňky karcinomu vaječníků, a dokonce opětovnou senzibilizaci rezistentní buněčné linie na karboplatinu. Vytvořili jsme také buněčnou linii kolorektálního karcinomu rezistentní na 5-FU a prokázali jsme klíčovou roli dysregulace genů pro opravu DNA a odpověď na poškození při vzniku chemorezistence na 5-FU. Kromě toho jsme se také zajímali o kombinovanou léčbu konvenčními chemoterapeutiky a přírodními látkami s cílem zvýšit jejich účinnost. Analyzovali jsme účinek extraktu *Ganoderma Lucidum* a potvrdili jeho posilující účinek na účinnost 5-FU u kolorektálního karcinomu *in vitro* i *in vivo*.

Věříme, že naše výsledky mohou přispět k lepšímu pochopení molekulárních mechanismů rezistence a citlivosti na chemoterapeutika u různých typů rakoviny, což může v konečném důsledku vést k lepší odpovědi a výsledku léčby onkologických pacientů.

1. INTRODUCTION

1.1. SOLID CANCERS

Cancer comprises an incomprehensible variety of diseases that killed about 10 million people in 2020 and is the second most common cause of death worldwide. Unfortunately, every fourth person is having a lifetime risk of developing cancer (Sung et al. 2021).

Cancers can be divided into 1.) solid cancers, defined as abnormal cellular growths in solid organs such as colon or breast; and 2.) liquid cancers, affecting the blood. Solid tumors consist of many cell types including cancer cells, cancer stem cells, connective tissue cells and immune cells. Two major types of solid tumors are carcinomas (developed from epithelial cells) and sarcomas (developed from mesenchymal cells).

This PhD Thesis discusses DNA damage response and chemoresistance in some of the most common solid cancers: breast cancer (BC), colorectal cancer (CRC), and ovarian cancer (OvC) (Fig 1.).

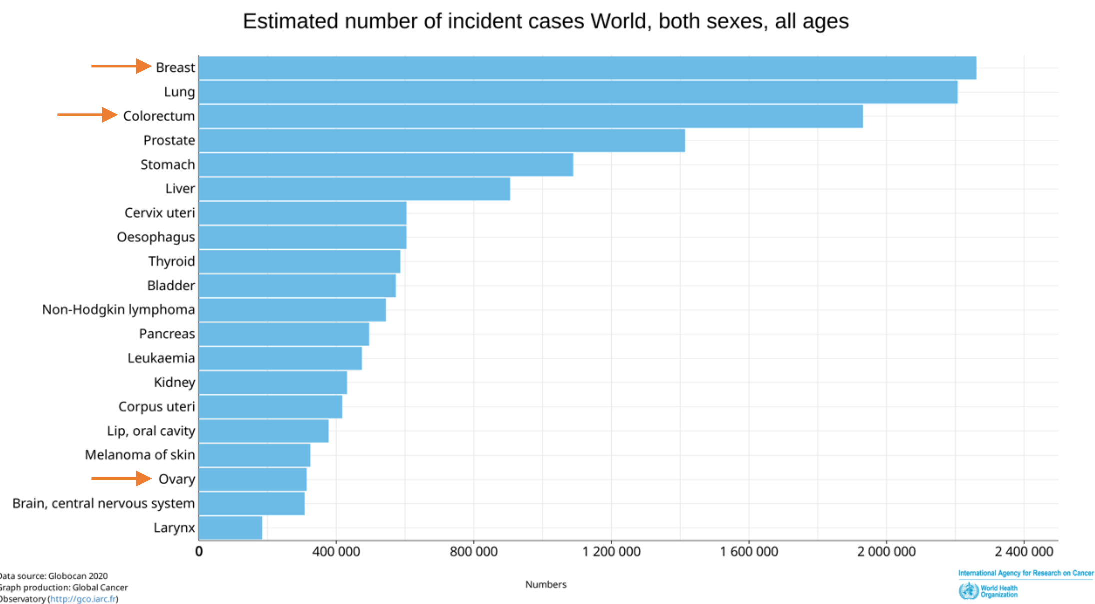


Fig. 1.: Estimated numbers of incident cases in 2020 according to GLOBOCAN 2020 (Sung et al. 2021). Breast cancer is the most diagnosed cancer worldwide while colorectal cancer is in third place in cancer incidence.

1.1.1. Breast cancer

BC is the most frequent cancer worldwide, with an estimated incidence of about 2.3 million new cases and responsible for the estimated number of about 680 thousand deaths

in 2020 (Sung et al. 2021). The incidence is expected to reach 4.4 million in 2070 (Soerjomataram and Bray 2021). However, the incidence and mortality rates vary greatly among the countries. The higher Human Development Index (HDI) levels of countries are associated with higher age-standardized incidence rates and, on the other hand, decreasing HDI levels are associated with increased age-standardized mortality rates (Lei et al. 2021). The overall relative 5-year survival rate for all BC cases is 90%, however, this rate for BC with distant metastases reaches only about 30% and barely 7% in metastatic triple negative BC (Lindman, Wiklund, and Andersen 2022; Young et al. 2001).

Most of the BC cases are sporadic. Epidemiological studies have identified several risk factors for BC as are reproductive and gynecological factors (early menarche, higher age at first birth, low parity, or late menopause), alcohol consumption, smoking, physical inactivity, and obesity, whereas breastfeeding and physical activity are considered as protective factors (Carioli et al. 2017; Macacu et al. 2015; Kashyap et al. 2022). It is estimated that about 5-10% of all BC have a hereditary background (Larsen et al. 2014). However, altogether about 20% of BC cases may be classified as familial. These BC are associated with mutations in genes from several DNA damage response pathways, such as *BRCA1/2*, *PTEN*, *ATM*, *CHEK2*, *TP53*, *PALB2*, *RAD50, RAD51, BRIP1* (Easton et al. 2015).

According to the status and expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 protein, BC may be divided into four main molecular subtypes. The majority of BC are hormone-receptor positive (ER and/or PR) categorized into a) **luminal A** (low Ki-67) and b) **luminal B** (high Ki-67). About 25% of BC cases are **HER2-positive**, while around 10-20% of cases are considered as **triple negative BC** (TNBC; ER-negative/PR-negative/HER2-negative) (Callahan and Hurvitz 2011; Ferrari et al. 2022). Tumor stage, grade and molecular subtypes are influencing BC prognosis and are routinely considered when setting up an individual therapy regimen (Fragomeni, Sciallis, and Jeruss 2018). Luminal A cancers tend to grow more slowly and have the best prognosis. On the other hand, TNBC tumors are associated with the aggressive phenotype and have the worst prognosis (Hennigs et al. 2016).

1.1.2. Colorectal cancer

With almost 2 million new cases, CRC is the second most common cancer worldwide responsible for almost 1 million deaths in 2020 (Sung et al. 2021). Extensive CRC screening and early detection and removal of precancerous adenomas enabled the recent decrease in CRC incidence and mortality (Wolf et al. 2018). The 5-year survival rate of CRC is 65%, but significantly decreases to only 14% with distant metastases of the disease ('Survival Rates for Colorectal Cancer'). Furthermore, a novel medical challenge is represented by the increasing incidence of early onset of sporadic CRC (below 50 years of age) (Archambault et al. 2020).

Most of CRC cases are sporadic and associated with known risk factors such as inflammatory bowel disease, physical inactivity, smoking, obesity, alcohol consumption, and consumption of red meat (Johnson et al. 2013; Murphy et al. 2019). About 5% of CRC cases are linked to known hereditary CRC syndromes, characterized by mutations in mismatch repair genes, or in *APC, POLE, POLD1, PTEN* or *SMAD4*. However, up to 20% of CRC cases may be classified as familial (Dekker et al. 2019).

Three distinct molecular pathways are involved in CRC origin and progression: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) (Singh et al. 2021). Several classifications of CRC have been used, but according to the consensus molecular subtype (CMS) system (Guinney et al. 2015), CRC may be classified into four subtypes: **CMS1** (microsatellite instability immune, 14% of CRC, hypermutated, microsatellite unstable and strong immune activation), **CMS2** (canonical, 37% of CRC, epithelial, WNT and MYC signaling activation), **CMS3** (metabolic, about 13% of CRC, epithelial, evident metabolic dysregulation), and **CMS4** (mesenchymal, 23% of CRC TGF-beta activation, stromal invasion and angiogenesis) (see Fig. 2). About 13% of tumors cannot be assorted into CMS subtypes, possibly because they represent a transition phenotype or due to intra-tumoral heterogeneity (Sawayama et al. 2020).

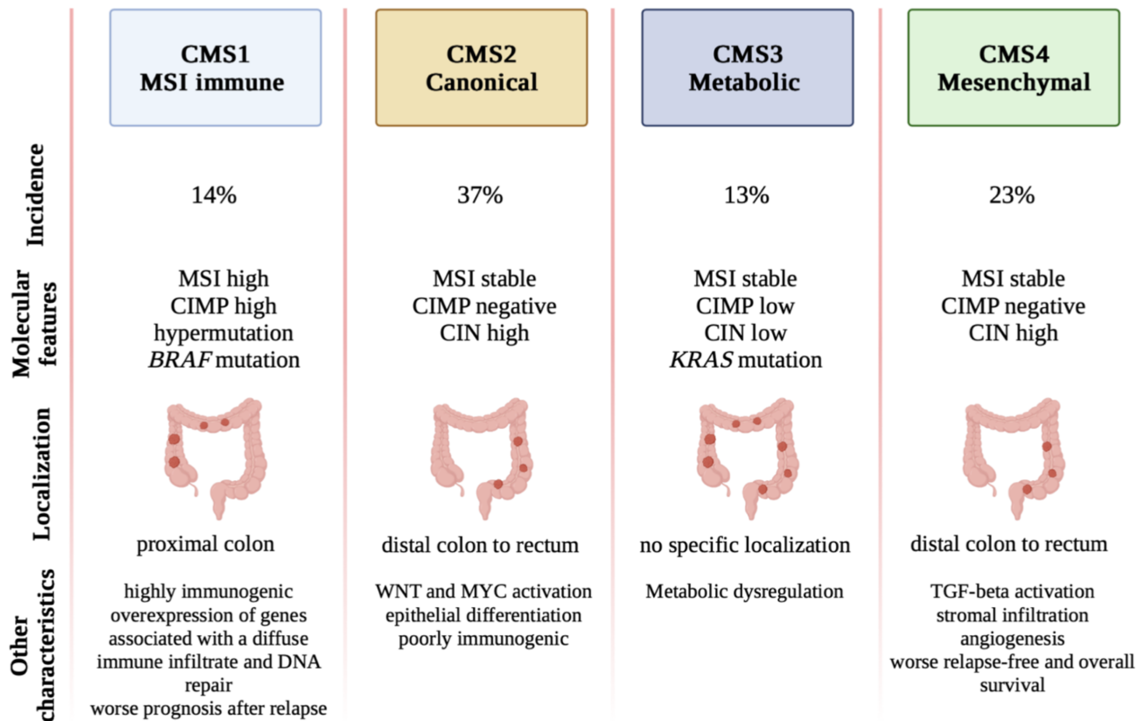


Fig. 2.: Consensus molecular subtype (CMS) system according to (Guinney et al. 2015), adapted from (Cervena et al. 2020) using BioRender.
(MSI: microsatellite instability, CIMP: CpG island methylator phenotype, CIN: chromosomal instability)

1.1.3. Ovarian cancer

Ovarian cancer (OvC) is the 9th most common type of cancer, with more than 300 thousand new cases and more than 200 thousand deaths in 2020 (Sung et al. 2021). It is the leading cause of death among gynecological cancers in most developed countries. For the early stages of OvC (stage I and II) (Prat and Oncology 2014), the overall 5-year survival rates are 85-90% but they rapidly decrease to 10-30% for advanced stages. Unfortunately, due to the difficulties in early detection caused by asymptomatic and inapparent growth of the tumors and lack of proper screening, about 70% of patients are diagnosed in the advanced stages of the disease (Wei et al. 2017).

OvC is not a single disease but comprises rather a variety of neoplasms with distinct clinicopathological and molecular features and prognoses (Kossai et al. 2018). The majority of OvC (90%) is designated as epithelial ovarian carcinomas (EOCs) (Matulonis et al. 2016). EOCs may be divided into 2 groups, type I, and type II, which progress along two different tumorigenic pathways (Kurman and Shih Ie 2011). **Type I** includes endometroid (EC), mucinous (MC), clear cell (CCC) and low-grade serous (LGSOC) ovarian carcinomas and **type II** encompasses high-grade serous ovarian

carcinomas (HGSOC, about 70% of all OvC), undifferentiated carcinomas and carcinosarcomas (Kurman and Shih Ie 2016; Rojas et al. 2016).

Type I OvCs exhibit somatic mutations in genes like *KRAS* (Kirsten rat sarcoma virus, coding for K-ras GTPase), *BRAF* (coding for B-Raf serine/threonine protein kinase), *ERBB2* (coding for HER2 receptor), *ARID1A* (coding for AT-Rich Interaction Domain 1A) or *PTEN* (coding for Phosphatase and tensin homolog) and lack mutations in *TP53* (coding for p53 protein) gene. On the other hand, the majority of type II OvCs is predominantly associated with mutations in *TP53* and usually display more aggressive phenotype and worse prognosis.

1.2. CHEMOTHERAPY OF SOLID CANCERS

Management of solid cancers principally implies the combination of surgery, radiation therapy (RT) and systemic anti-cancer therapy (SACT). Several types of SACT are available for treatment, e.g., chemotherapy, endocrine therapy, targeted therapy or immunotherapy that may be used in different settings (such as neo-adjuvant, adjuvant or palliative. An accurate therapy regimen is crucial for a plausible outcome, as many drugs have severe side effects and patients may suffer from fatal toxicities.

1.2.1. Types of chemotherapeutic agents

According to their modes of action and chemical structure, chemotherapeutic drugs may be divided into several categories: 1.) Alkylating and platinum-based agents; 2.) Antimetabolites; 3.) Cytotoxic antibiotics; 4.) Antimicrotubular agents; 5.) Topoisomerase inhibitors.

1.2.1.1. Alkylating and platinum-based agents

Alkylating agents act by attaching an alkyl group to DNA, resulting in the formation of DNA adducts, abnormal base-pairing, DNA cross-linking or fragmentation of DNA, thereby stopping the transcription and resulting in cell death. Alkylating agents have their primary effect on rapidly dividing cells which do not have time for proper DNA repair. Typical alkylating agents used in cancer therapy are nitrogen mustards (e.g., Cyclophosphamide).

Platinum-based chemotherapeutics act in a similar manner by binding to DNA and forming cross-links (as mono-adducts, inter-strand crosslinks, intra-strand crosslinks or DNA protein cross-links). The most commonly used platinum-based chemotherapeutics are Carboplatin, Cisplatin and Oxaliplatin.

1.2.1.2. Antimetabolites

Antimetabolites are the oldest rationally designed and the most widely used anticancer drugs. They are defined as analogs of natural metabolites from cellular metabolism, but in anticancer therapy, they generally refer as analogs of the building block of DNA and RNA or analogs of metabolites essential for DNA and RNA synthesis. Inhibition of DNA and RNA synthesis or their direct misincorporation into DNA or RNA are very effective anticancer treatment strategies (Peters 2014).

According to the chemical structure of antimetabolites, we distinguish 1.) Pyrimidine analogs (e.g., 5-Fluorouracil, Capecitabine); 2.) Purine analogs (structural analogs of guanine, e.g., Cladibrine, Clofarabrine, Nelarabine), 3.) Folate analogs (e.g., inhibit folate cycle, which is essential in the synthesis of purine nucleotides and thymidylate, e.g., Methotrexate, Pemetrexed) and 4.) Cytidine analogs (e.g., Gemcitabine).

1.2.1.3. Cytotoxic antibiotics

Cytotoxic antibiotics are secondary metabolites of microorganisms with anticancer activities. This category encompasses anticancer drugs according to their origin rather than according to their mode of action, as they affect the DNA and RNA in multiple ways.

One of the most commonly used anticancer antibiotics are above mentioned Topoisomerase II inhibitors Anthracyclines produced from the *Streptomyces spp.* Other antibiotics used in cancer treatment may act via DNA breaks formation and induction of oxidative stress (Bleomycin, used also as a radiomimetic agent), via intercalation into DNA (Dactinomycin) or as alkylating agents (Mitomycin) (Gao et al. 2020).

1.2.1.4. Antimicrotubular Agents

Antimicrotubular agents have inhibitory effects on the dynamics of spindle microtubules, causing the arrest of cell cycle progression at mitosis and eventually leading to apoptotic cell death.

They are classified into two groups: 1.) microtubule-destabilizing agents (inhibit microtubule polymerization, e.g., Vinca alkaloids); 2.) microtubule-stabilizing agents (stabilize microtubules and prevent their depolymerization, e.g., Paclitaxel or Docetaxel) (Zhou and Giannakakou 2005; Mukhtar, Adhami, and Mukhtar 2014).

1.2.1.5. Topoisomerase Inhibitors

Topoisomerases are enzymes that catalyze the DNA topology alterations by relaxation of DNA supercoiling, which is essential during transcription, replication, and chromatin remodeling.

Two classes of topoisomerase inhibitors are in use: 1.) Topoisomerase I inhibitors (replication-fork collision is their primary cytotoxic mechanism, e.g., Topotecan and Irinotecan) (Pommier 2006); 2.) Topoisomerase II inhibitors (a. poisons, stabilizing transient intermediates in which DNA Topoisomerase II is linked to DNA leading to strand breaks; Anthracyclines e.g., Doxorubicin, Mitoxantrone, Daunorubicin, Idarubicin; b. catalytic inhibitors that inhibit the catalytic activity of DNA Topoisomerase II, e.g., Aclarubicin, Merbarone, Suramin) (Nitiss 2009).

Several of the above compounds (such as Anthracyclines, Alkylating agents, Platinum-based agents, or Topoisomerase I inhibitors) induce secondary oxidative stress, leading to increased levels of ROS and oxidative DNA damage (Conklin 2004; Vodicka et al. 2019).

1.2.2. Chemotherapy regimens for breast, colorectal and ovarian cancer

Treatment regimens and settings depend largely on the type and stage of the disease. One of the most commonly used chemotherapeutics in both BC, CRC and OvC are antimetabolites (such as 5-FU) and platinum derivatives, of which the chemoresistance and sensitivity are discussed in this Thesis.

1.2.2.1. Chemotherapy of breast cancer

The chemotherapy regimen of BC depends on the molecular subtype and stage of the disease as well as other patient-specific factors such as age or comorbidities (Anampa, Makower, and Sparano 2015). According to the TNM stage (UICC staging, (James D. Brierley 2017)), BC may be classified as: 1.) early-stage (TNM 0,1 and 2; disease is detectable only in the breast and eventually in local lymph nodes); 2.) locally advanced

(TNM 3; advanced breast tumors >5cm, absence of distant metastasis) and 3.) metastatic (TNM 4; cancer is spread beyond the breast and local lymph nodes to other organs such as bones, lungs, liver, or brain).

Neoadjuvant chemotherapy (NACT) is conventionally used prior to surgical removal in both early-stage BC as well as in locally advanced and inoperable BC to downstage the disease and to determine tumor response to therapy. Because luminal A types of BC have an excellent prognosis, they usually do not benefit from neoadjuvant chemotherapy (Collins et al. 2021). On the other hand, the neoadjuvant approach remains a standard approach particularly in high-risk triple-negative and HER2-positive cancers (Korde et al. 2021). NACT regimens are based on combinations of anthracyclines, taxanes, platinum-based chemotherapeutics (Cisplatin) and anti-HER2 targeted therapy (Pertuzumab, Trastuzumab) (Korde et al. 2021).

Adjuvant chemotherapy is used after the surgical removal of tumor. HR-positive cancers benefit from a combination of hormonal therapy and chemotherapy, HER2-positive cancers from a combination of anti-HER2 targeted therapy and chemotherapy, while for TNBC tumors with poor prognosis, cytotoxic chemotherapy remains their only current option (Anampa, Makower, and Sparano 2015; Harbeck et al. 2019). Adjuvant chemotherapy regimens are classified as first, second and third generation and, similarly to NACT, generally consist of the combination of anthracyclines, taxanes, antimetabolites (Methotrexate and 5-FU) and platinum-based chemotherapeutics (Cisplatin) (Fisusi and Akala 2019).

Chemotherapy of metastatic BC employs similar chemotherapeutic agents and regimens are based on the molecular features of the tumors. The addition of one or more chemotherapeutic drugs to the regimen may be contra-productive, as the greater shrinkage of the tumor may go hand in hand with greater toxicity (Butters et al. 2010).

1.2.2.2. Chemotherapy of colorectal cancer

Management of colorectal cancer is mainly influenced by the cancer TNM stage and tumor location. Stage 0 and I CRC tumors are localized only in the mucosa or invade the muscular layer of the colon or rectum. Stage II tumors grow through the wall of the colon or rectum and may infiltrate into nearby structures. Stage III CRC is characterized as when cancer spread to the lymph nodes and stage IV, metastatic CRC, when cancer spread to at least one other distant part of the body, such as liver or lungs (James D. Brierley 2017).

Neoadjuvant chemotherapy is currently not a standard treatment for colon cancer, however, there is increasing evidence to support the use of NACT regimens for advanced CRC (Gosavi et al. 2021; Roth and Eng 2020). On the other hand, the use of neoadjuvant chemotherapy and/or radiation prior to surgery in the management of rectal cancer is well established. NACT are conventionally based on combinations of fluoropyrimidines (5-FU, Capecitabine), Oxaliplatin and Irinotecan (Boland and Fakih 2014).

Adjuvant chemotherapy in early-stage CRC is still a matter of debate, as surgery alone is associated with a high cure rate. It may be recommended in patients with a high risk of recurrence and patients with proficient MMR. Patients with stage III are treated with adjuvant regimens based on fluoropyrimidines (5-FU, Capecitabine) and Oxaliplatin (Rebuzzi et al. 2020; Sargent et al. 2010).

Chemotherapy of metastatic CRC is aimed to prolong the survival of patients, as patients with stage IV CRC have poor outcomes. The addition of biological therapy (e.g., VEGF-A antibody Bevacizumab or EGFR antibodies Cetuximab and Panitumumab) to standard chemotherapeutic agents has improved patients' OS (Ohishi et al. 2023).

1.2.2.3. Chemotherapy of ovarian cancer

Chemotherapy of OvC depends on the histological subtype of the tumor, with about 90% of OvCs being diagnosed as high-grade serous carcinomas (HGSC).

Neoadjuvant chemotherapy and its benefits in OvC are questionable. Some studies showed that platinum-based NACT followed by interval debulking surgery may only be used in patients with advanced OvC (IIIC and IV) who are not eligible for primary debulking surgery (Vergote et al. 2010; Kehoe et al. 2015; Vergote et al. 2018). However, platinum-based NACT failed to show any survival benefits and may potentially induce platinum resistance (Liu, Jiao, and Gao 2020).

Adjuvant chemotherapy of OvC has been based on Carboplatin and Paclitaxel regimens for decades. Because patients with OvC often develop platinum resistance, second-line treatment with non-platinum-based regimens using Docataxel, Paclitaxel, Topotecan and Gemcitabine may be used (Falzone et al. 2021).

Chemotherapy of metastatic OvC is, similarly to non-metastatic OvC, based on Carboplatin and Paclitaxel regimes. If platinum resistance is present, other chemotherapeutic agents combined with anti-angiogenic agents (Bevacizumab), PARP

inhibitors or immune checkpoint inhibitors may be used (Le Saux, Ray-Coquard, and Labidi-Galy 2021).

1.3. CHEMOTHERAPY RESISTANCE AND SENSITIVITY

Cancer as the leading cause of death worldwide is responsible for about 10 million deaths each year (Sung et al. 2021). Resistance towards conventional and new chemotherapeutics remains a main obstacle to successful treatment, causing low response to the therapy, cancer recurrence and eventual patients' death. It is considered that about 90% of cancer mortality can be directly or indirectly attributed to chemoresistance (Housman et al. 2014; Rueff and Rodrigues 2016; Holohan et al. 2013). It occurs when cancer cells became less sensitive or tolerant to a pharmaceutical treatment. Chemoresistance may be restricted only towards a single drug (or a class of drugs with a similar mode of action); or towards multiple drugs with independent modes of action, named multidrug resistance (MDR).

Chemotherapy resistance can be categorized as 1.) intrinsic or 2.) acquired resistance based on the time when it is developed, both based on highly complex and individually variable biological mechanisms (Wang, Zhang, and Chen 2019).

Intrinsic chemoresistance is defined as the innate resistance that exists before the first exposure to chemotherapy. It can be caused by the presence of genetic variations or mutations in tumors that result in decreased responsiveness of cancer cells, or by the high heterogeneity of tumor cell subpopulations, containing resistant subclones, which will be selected upon treatment, leading to relapse in later stages of therapy.

Acquired chemoresistance develops during the course of the drug treatment. It can be established e.g., by the activation of a second proto-oncogene that becomes the newly emerged driver gene, by *de novo* mutations or altered expressions of the drug targets or by dynamic changes in tumor microenvironment (TME).

1.3.1. Mechanisms of chemoresistance

Distinguishing between the intrinsic and the acquired resistance is less clinically significant than understanding the cellular mechanisms of resistance. There are several mechanisms that underlay the patient's resistance towards chemotherapeutic treatment (Fig. 3).

Increased efflux of chemotherapeutic agents leads to their decreased intracellular concentrations. This is the most common cause of so-called multidrug resistance (MDR), a major reason for poor therapy response. Members of the ABC transporter family are the primary ATP-dependent drug efflux proteins, actively pumping drugs out of the tumor cells and protecting them from chemical toxicity. Mutations and overexpression of certain ABC transporters e.g., *ABCB1* (P-glycoprotein, P-gp), *ABCG2* (Breast Cancer Resistance Protein, BCRP) and *ABCC1* (Multidrug Resistance Protein 1, MRP1), directly influence tumor sensitivity and drug efficacy (Fletcher et al. 2016). ABC transporters have a role also in 5-FU response and resistance (Nies et al. 2015). Current strategies to overcome ABC transporter associated chemoresistance include the application of nanoparticles to improve the intracellular drug concentration and development of the ABC transporter inhibitors (Qu et al. 2019; Adamska and Falasca 2018; Xiao et al. 2021; Dong et al. 2019).

Alteration of the drug target during targeted therapy is caused by secondary mutations in the target protein genes or their altered expression, resulting in drug resistance (Dong et al. 2019). For instance, EGFR inhibitors used in the treatment of non-small cell lung cancer (NSCLC) show initially good response rates, however, almost 50% of responsive patients develop a T790M mutation in *EGFR* within one year, resulting in resistance towards 1st and 2nd generation of EGFR inhibitors (Wang, Schmid-Bindert, and Zhou 2012; Gridelli et al. 2011; Tang et al. 2013; Bell et al. 2005; Ma, Wei, and Song 2011). Another example is the development of resistance towards estrogen receptor (ER) inhibitors (Tamoxifen), used in the treatment of ER-positive breast cancers. Mutations in ER gene (*ESR1*) are significantly enriched in endocrine therapy resistant metastatic BC while being rare or non-existent in treatment naïve, primary tumors. The development of novel targeted inhibitors is therefore inevitable (Alluri, Speers, and Chinnaiyan 2014).

Senescence escape is another mechanism of chemoresistance. Chemotherapy-induced senescence (CIS) was initially seen as favorable outcome of chemotherapy, as it leads to arrest of the cell proliferation (Guillon et al. 2019). However, studies have shown that CIS may provide an oncogenic niche, enabling certain populations of tumor cells to perform senescence escape, gain stem-cell properties, restart their proliferation, and become more aggressive and less responsive towards chemotherapeutic treatment (Milanovic et al. 2018). This possesses a great challenge in cancer therapy, as conventional chemotherapeutics are not efficient towards non-proliferating senescent cells. Lately, there is an increasing interest in agents capable to induce apoptosis in

senescent cells, known as senolytic drugs that may be combined with conventional or targeted therapies (Kirkland and Tchkonia 2020; Carpenter, Saleh, and Gewirtz 2021).

Epigenetic alterations contribute to chemoresistance in multiple ways. These include DNA methylations, histone modifications, chromatin remodeling, and non-coding RNA related alterations. DNA demethylation at the promoter region upregulates the expression of a gene (e.g., an oncogene) and *vice versa*, hypermethylation can suppress the gene expression. Hyper- or hypomethylation of a great variety of genes has been associated with resistance towards a number of chemotherapeutics in different cancers (reviewed in (Romero-Garcia, Prado-Garcia, and Carlos-Reyes 2020)). Both miRNAs and lncRNAs may also regulate a variety of processes involved in chemoresistance, including regulation of ABC transporters and decrease in efficacy of chemotherapeutics, inhibition of apoptosis, interaction with DNA repair proteins, alteration of drug targets or involvement in metastatic formation. The involvement of miRNA regulation in therapy response is discussed in Manuscripts 1 and 3 of this dissertation Thesis (Liu et al. 2020; Si et al. 2019).

Tumor heterogeneity provides tumors with significant adaptability and can be present in tumors on several levels: a.) morphologic heterogeneity of tumor cells, where some well-differentiated areas adjacent to poorly or moderately differentiated areas, b.) intra-tumoral genetic heterogeneity caused by the clonal accumulation of somatic mutations and epigenetic alterations, c.) heterogeneity of cell types within the tumor (cancer cells, stromal cells, immune cells, *etc.*), d.) heterogeneity of oxygen and nutrient distribution within the tumor, leading to the expression of stress response genes and activation of compensatory mechanisms favoring more malignant or lethal phenotype (Muz et al. 2015; Saggar et al. 2013). Tumor heterogeneity is one of the major causes of chemoresistance (Crucitta et al. 2022).

Tumor microenvironment (TME) may contribute to intrinsic chemoresistance. More acidic pH in tumor compared to normal tissues (pH 6.5-7.1 vs pH 7.3-7.5) impairs the distribution of weak base chemotherapeutics, such as anthracyclines, anthraquinones, and vinca alkaloids, leading to physiological drug resistance (referred as "ion trapping") (Taylor et al. 2015; Webb et al. 2011; Wojtkowiak et al. 2011). On the other hand, acidic pH may be a potential target for anticancer therapy as proton pump inhibitors have been developed to shrink and sensitize tumors to chemotherapeutic drugs. Besides the acidic pH, fluctuating hypoxia is another characteristic of TME, causing oxidative stress. This

may induce DNA damage, genetic instability and arise of new mutations, contributing to the genetic divergence of tumor cells (Bindra and Glazer 2005).

Epithelial-mesenchymal transition (EMT) is an essential process for metastatic formation but also plays a major role in chemoresistance. During EMT, epithelial cells lose contact with neighboring cells and subjacent matrix and adopt migratory mesenchymal phenotype. This employs the activation of Wnt, Notch and Hedgehog developmental signaling pathways, involved in the regulation of drug efflux, inhibition of apoptosis, cell survival, cell cycle, DNA damage response, TME, etc (Kumar et al. 2021). Additionally, the overexpression of EMT transcriptional factors like Twist, Snail, Slug, ZEB and FOXC2 are known to induce chemoresistance (Deng et al. 2016; Haslehurst et al. 2012; Siebzehnrubl et al. 2013; Lazarova and Bordonaro 2017).

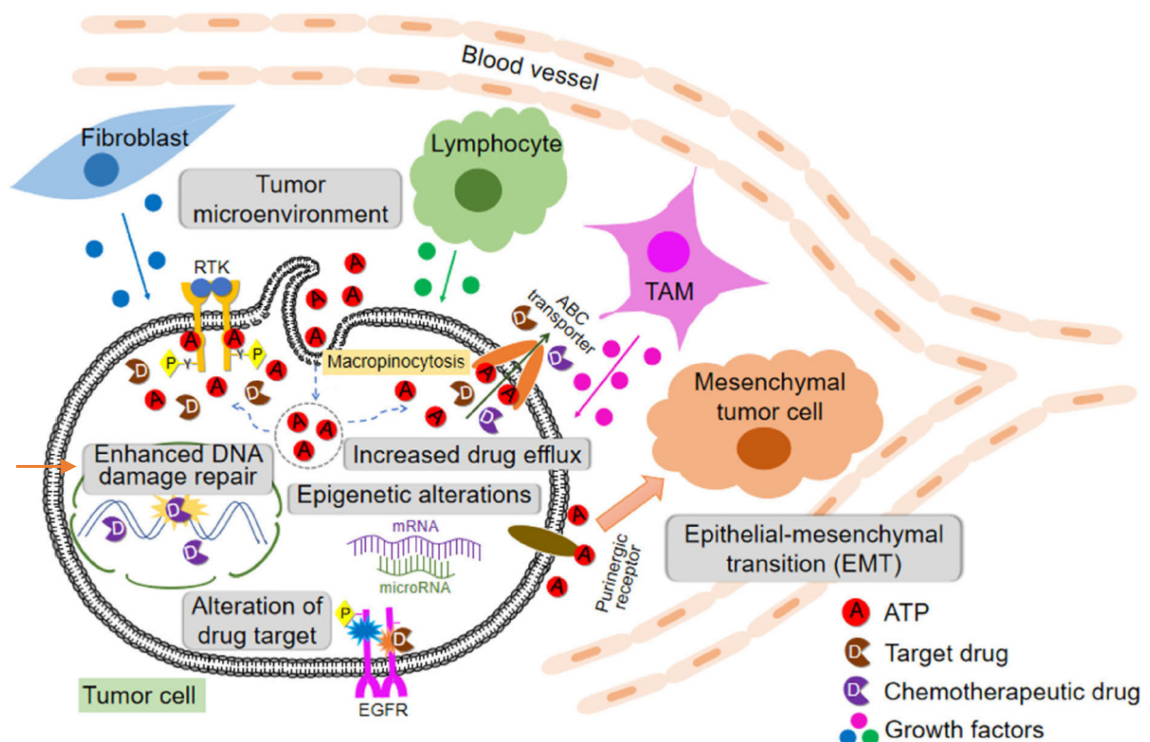


Fig. 3.: Cellular mechanisms of chemoresistance. From (Wang, Zhang, and Chen 2019). Dysregulations of DNA repair are one of the mechanisms of chemoresistance.

1.3.2. Role of DNA repair and damage response in chemoresistance

Besides the cellular mechanisms of chemoresistance discussed above, dysregulated DNA repair and damage response (DDR) is another key mechanism involved in resistance and sensitivity towards chemotherapeutic drugs and it is the main topic of this PhD Thesis.

1.3.2.1. Mechanisms of DNA repair

Because DNA is the repository of genetic information in cells, it is essential to maintain its integrity and stability. However, DNA is constantly exposed to countless damaging agents originating from various endogenous (e.g., byproducts of metabolism, such as free radicals) and exogenous sources (e.g., UV and ionizing radiation, chemotherapeutic drugs, industrial chemicals, or cigarette smoke) (Torgovnick and Schumacher 2015). Therefore, cells have developed robust DNA repairing mechanisms to either remove or tolerate the damage to ensure overall survival (Chatterjee and Walker 2017). Depending on the type of DNA damage, one of the six repair pathways can be utilized: mismatch repair (MMR), base excision repair (BER), homologous recombination (HR), non-homologous end joining (NHEJ), nucleotide excision repair (NER) and direct damage reversal (summarized in Fig. 4)

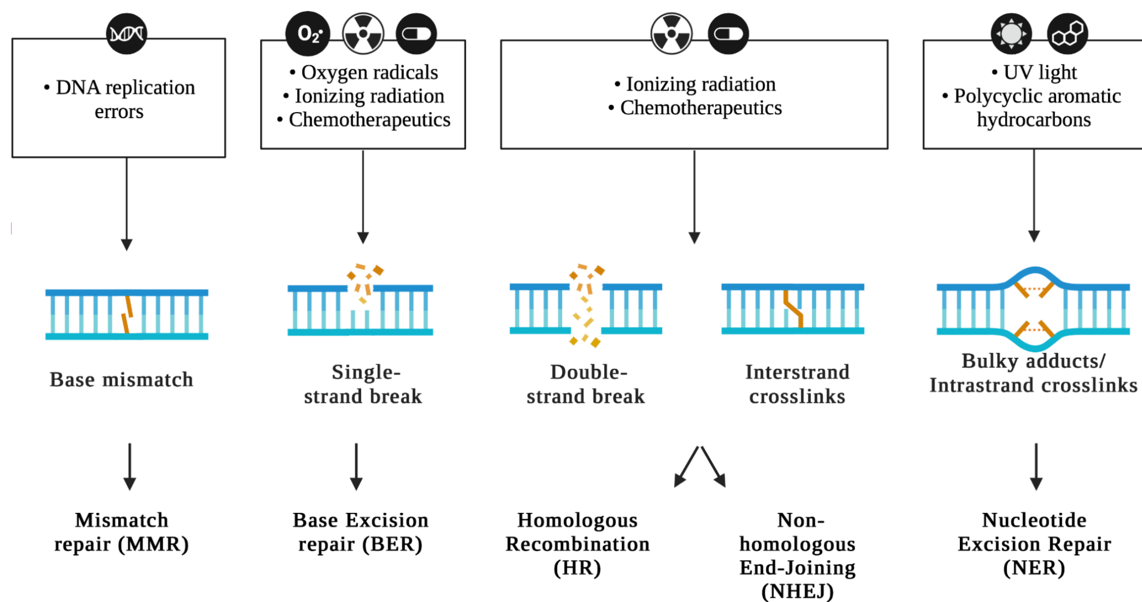


Fig. 4.: DNA damage and repair mechanisms. DNA damaging agents cause different DNA lesions, which are then repaired with relevant DNA repair pathways. Adapted from (Hindi, Elsakrmy, and Ramotar 2021) using BioRender.

1.3.2.1.1. Mismatch repair

The mismatch repair (MMR) pathway corrects spontaneous base-base mispairs and small insertions/deletion loops generated during DNA replication, thereby preventing those mutations from becoming permanent in dividing cells (Pecina-Slaus et al. 2020; Caja et al. 2020). It is estimated that replicative polymerases ε and δ make one error per every 10^4 and 10^5 nucleotides, thus generating 100,000 and more polymerase errors during cell

division (Preston, Albertson, and Herr 2010). The majority is immediately corrected by the proofreading activity of polymerases. Errors that escape proofreading are then corrected by MMR machinery (Baretti and Le 2018).

MMR pathway is initiated by the mismatch recognition by MSH2/MSH6 or MSH3/MSH3 heterodimers. This is followed by the recruitment of MHL1 and its partner PMS2. With PCNA acting as a clamp and EXO1 nuclease, the mismatched base is then excised, DNA re-synthetized with DNA Polymerase δ and the strand sealed with DNA Ligase 1. (see Fig. 5)

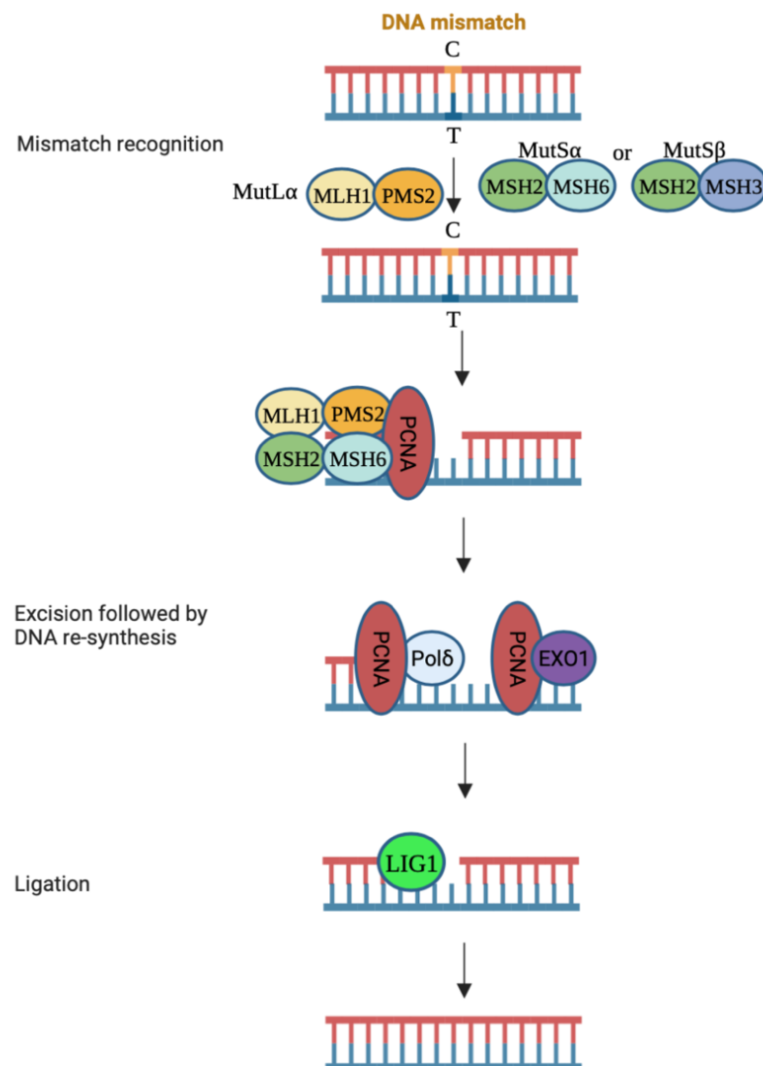


Fig.5.: Mismatch repair pathway. Adapted from (Pecina-Slaus et al. 2020) using BioRender.

MMR deficiency is caused either by a.) germline mutations in MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2* and, rarely, *PMS1*), which are associated with Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC), or b.) epigenetic inactivation of MMR genes, commonly found in sporadic microsatellite instable (MSI)

tumors (Baretti and Le 2018). Microsatellites are a type of repetitive sequences in the genome defined as 1-6 nucleotides repeated 5-50x, prone to DNA replication errors. Most are localized in the non-coding sequences of the genome, but few are localized within exons of tumor suppressor genes. When MMR is deficient, cells fail to repair these errors, leading to alterations of sequence lengths within microsatellites called microsatellite instability (MSI). MSI is one of the typical characteristics of tumor cells (Schmidt and Pearson 2016).

1.3.2.1.2. Base Excision Repair

The base excision repair (BER) is a fundamental and highly conserved part of the DNA repair machinery. BER repairs small base lesions, namely alkylations, oxidations, deaminations, depurinations/depyrimidations or single-strand breaks (SSBs) resulting from endogenous (products of metabolism) as well as exogenous (radiation, chemicals, drugs) sources of DNA damage.

The initial step of the BER pathway is the recognition of the lesion by a DNA glycosylase. Depending on the type of lesion, one of the eleven human glycosylases (summarized in Table 1) is used to excise the damaged base. Glycosylases can be either a) monofunctional - remove the damaged base by cleaving the N-glycosyl bond between the base and the sugar which creates an abasic site (AP-site); recognize uracil, thymine, and alkylated bases; or b) bifunctional - excise not only the damaged base but also cleaving the DNA backbone (AP-lyase activity), recognize oxidative lesions. The aberrant function of DNA glycosylases is linked with various cancers including CRC, esophageal, ovarian, gastric and lung cancer (Wallace 2014; D'Errico et al. 2017; Vodicka et al. 2020; Vodicka et al. 2007; Hans et al. 2020). AP-site is later recognized by APE1 (AP endonuclease), which cleaves the abasic site leaving a sugar attached to the 5′ side of the nick. During the *short patch BER*, DNA polymerase β then removes the sugar and fills the gap in the DNA with DNA ligase 3 sealing this gap. In some cases (e.g., when the sugar is inefficiently removed), this process can be redirected to the *long patch BER*, with different enzymes involved (including replicative Polymerase λ and FEN1) when 2-10 new nucleotides are synthetized. DNA ligase 1 is then used to seal the repaired DNA. Other important players participating in BER are PARP1 (Poly (ADP-ribose) polymerase 1), which recruits additional repair enzymes and XRCC1 (X-ray repair cross-complementing protein 1), which acts as a scaffold protein (as illustrated in Fig. 6).

Gene	Endonuclease	Subcellular localization	Mono/Bi-functional	Major Substrate Specificity
UNG1	Uracil-N glycosylase1	Mitochondria	Mono	U in any context, in ss and dsDNA
UNG2	Uracil-N glycosylase2	Nuclei	Mono	Similar to UNG1
SMUG1	Single-strand-specific monofunctional uracil DNA glycosylase 1	Nucleus	Mono	ssU, U:G, U:A, 5-hydroxymethylU, in ss and dsDNA
TDG	Thymine DNA glycosylase	Nucleus	Mono	U:G, T:G, oxidized/deaminated 5-methylC:G, in dsDNA
MBD4	Methyl-binding domain glycosylase 4	Nucleus	Mono	U:G and T:G, 5-hydroxymethylU in CpG islands, in dsDNA
OGG1	8-OxoG DNA glycosylase 1	Nucleus	Bi	Oxidized purines (8-oxoG:C, FapyG:C), in dsDNA
MUTYH	MutY homolog DNA glycosylase	Nucleus	Mono	A opposite 8-oxo-G/C/G, in dsDNA
NTH1	Endonuclease III-like 1	Nucleus	Bi	Oxidized pyrimidines (Tg, 5-hydroxyC, 5-hydroxyU), FapyG, FapyA, in dsDNA

MPG	3-methyl-purine glycosylase	Nucleus	Mono	3-methylA, 1-methyl, 7-methylG, εA, ethenoA hypoxanthine, in ss and dsDNA
NEIL1	Endonuclease VIII-like glycosylase 1	Nucleus	Bi	Oxidized pyrimidines (Tg, 5-hydroxyU, 5,6-dihydroU, hydantoins Gh and Sp), FapyG, FapyA, in ss and dsDNA
NEIL2	Endonuclease VIII-like glycosylase 2	Nucleus	Bi	Similar to NEIL1 in bubbles and loops
NEIL3	Endonuclease VIII-like glycosylase 3	Nucleus	Bi	Similar to NEIL1 (FapyG, FapyA, Sp and Gh) in ssDNA

Table 1.: List of human glycosylases. Adapted from (Hans et al. 2020; Vodicka et al. 2020).

It is essential for cells to tightly control the BER pathway, as every step generates intermediates (AP sites, 5′-deoxyribose phosphate (5′-dRP) residues and SSBs), which have been shown to be mutagenic and toxic. In fact, sometimes these intermediates are more toxic than the initiating DNA base lesion (Fu, Calvo, and Samson 2012).

The importance of proper BER functioning is illustrated by the fact that mouse knockouts of the core BER proteins (e.g., XRCC1, POLβ, APE1, FEN1 or LIG1) are embryogenic lethal. However, SNPs polymorphisms in BER genes have been widely studied in association with cancer susceptibility. For example, SNPs in *OGG1* have been linked to an increased risk of lung cancer or breast cancer (Rossner et al. 2006); in *XRCC1* to lung cancer (Schneider, Classen, and Helmig 2008), breast cancer (Sanjari Moghaddam et al. 2016), and gastric cancer (Shen et al. 2000); and in *APE1* to lung cancer, breast cancer, colorectal cancer, bladder, or prostate cancer (Zhao et al. 2011; Demple and Harrison 1994; Liu et al. 2021).

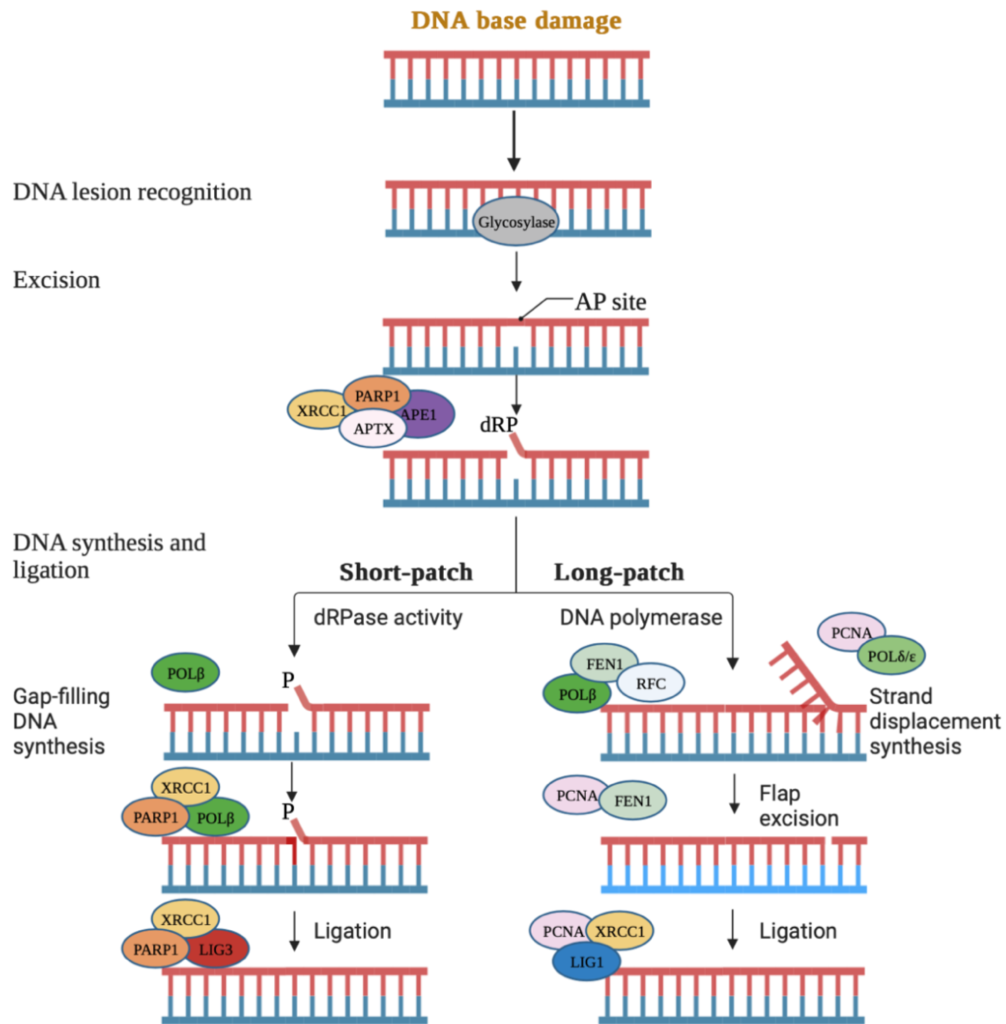


Fig. 6.: A simplified scheme of the short patch and long patch base excision repair (BER) pathways in eukaryotes. Adapted from (Hindi, Elsakrmy, and Ramotar 2021) using BioRender.

1.3.2.1.3. Nucleotide excision repair

The nucleotide excision repair (NER) pathway can repair a great variety of helix-distorting DNA lesions, ranging in size from abasic sites to multi-ring aromatic hydrocarbons and protein-DNA crosslinks without a need for lesion-specific enzymes (as are glycosylases in BER) to initiate the repair pathway (Spivak 2015). Various exogenous factors can cause these lesions, such as UV light, polycyclic aromatic hydrocarbons, benzo(a)pyrene or certain chemotherapeutic drugs like platinum derivatives (Kap, Popanda, and Chang-Claude 2016).

NER can be initiated by two sub-pathways which differ in the initial step of DNA damage recognition (see Fig. 7): anywhere in the genome by global genome NER (GG-NER) or in the transcribed strand of active genes by transcription-coupled NER (TC-

NER) (Gillet and Scharer 2006; Hanawalt and Spivak 2008). GG-NER is initiated by the XPC-RAD53B-CTN2 heterotrimer complex, sometimes with the help of UV-DDB (UV-damaged DNA-binding protein). On the other hand, TC-NER is initiated by RNA-polymerase II which is stalled at the lesion and by other specific factors CSA, CSB and XAB2. After the DNA damage recognition, TFIIH complex together with XPA and RPA unwind the DNA helix around the damage. Two endonucleases, XPG and XPF, excise the oligonucleotide sequence around the damage and DNA polymerase then synthetize new DNA, followed by DNA ligation (Vaughn and Sancar 2020).

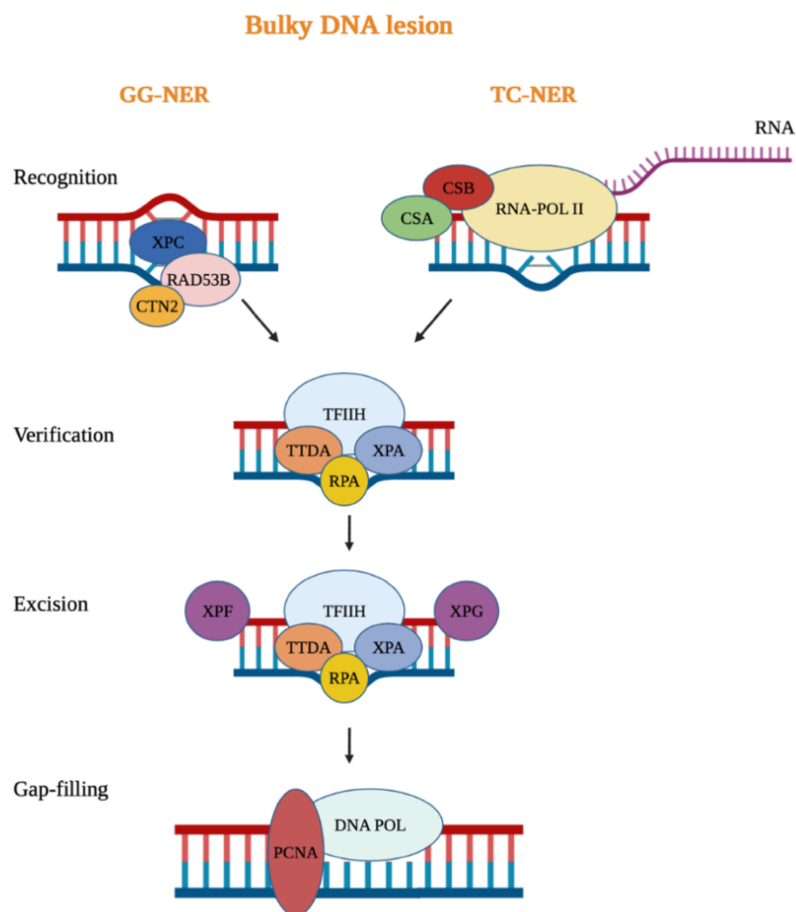


Fig.7.: A simplified scheme of nucleotide excision repair. Adapted (Sassa et al. 2019) using BioRender.

Deficiency of the NER pathway is associated with three distinct photosensitive syndromes: Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and the photosensitive form of trichothiodystrophy (TTD). XP is caused by the different mutations in *XPB*, *XPD* and *XPG* genes and manifests as an extreme sensitivity to UV rays and a 2000-fold risk of developing malignant melanoma compared to the general population. Smokers with XP also have a high risk of developing lung cancer (Lucero and Horowitz 2023).

1.3.2.1.4. Homologous recombination

Homologous recombination (HR) is a high-fidelity DNA repair pathway for providing repair of complex DNA damage including DNA gaps, DNA double-strand breaks (DSBs), and DNA inter-strand crosslinks. These can be generated e.g., by genotoxic chemicals, ionizing radiation, UV-light or during normal cellular processes of DNA replication (replication fork collapse or arrest) and meiosis (process of crossing-over). Unrepaired DSBs are considered the most fatal for the integrity of DNA, as they can lead to aneuploidy, genetic aberrations, and cell death (Reliene, Bishop, and Schiestl 2007). Because the HR pathway is template-dependent, HR is active during the S and G2 phases of the cell cycle when the sister chromatid is available.

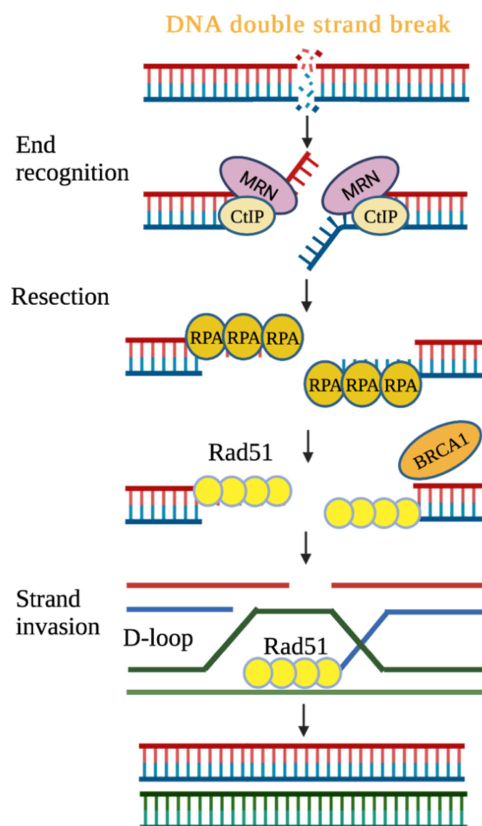


Fig.8.: A simplified scheme of homologous recombination. Adapted from (Brandsma and Gent 2012) using BioRender.

The DSBs (see Fig.8) are firstly sensed by the MRN complex (MRE11-RAD50-NBS1), which activates the ATM (ataxia telangiectasia mutated) serine/threonine kinase. ATM regulates hundreds of substrates and initiates the HR repair response and cell cycle arrest. CtIP (C-terminal binding protein) interacts with the MRN complex and mediates

the short-range DNA resection by MRN, generating a short (~100 nt) 3′-ssDNA overhang (Lamarche, Orazio, and Weitzman 2010). EXO1 (Exonuclease 1) is then recruited to mediate the long-range resection and RPA (Replication Protein A) is responsible for coating of the exposed ssDNA. ATR kinase (Ataxia Telangiectasia and Rad3-related protein) localizes to the ssDNA and facilitate the cell cycle arrest important for the HR to proceed. BRCA2 then helps RAD51 replace RPA and form RAD51-ssDNA nucleofilament, which mediates the homology search, strand invasion and D-loop formation. DNA is then synthetized, ligated and Holliday junctions are resolved (Peng and Lin 2011; Sun et al. 2020).

Germline mutations in genes from HR pathway, such as *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *NBS1*(*NBN*), *PALB2*, *RAD51C* and *RAD51D,* lead to increased susceptibility to different types of cancer, including breast, ovarian, prostate, NSCLC, and pancreas (Yamamoto and Hirasawa 2021).

1.3.2.1.5. Non-homologous end-joining

Non-homologous end-joining (NHEJ) is the main DNA repair pathway for repairing DSBs, responsible for repairing almost all DSBs outside the S and G2 phases and about 80% during the S and G2 phases of the cell cycle. NHEJ differs from HR in fidelity and template requirements. During NHEJ, the broken DNA ends are modified and ligated together, generating deletions and insertion. This is essential during V(D)J recombination in immunity, however, poses a potential mutagenic risk for the cell (Chang et al. 2017).

During NHEJ (see Fig.9), DSBs is first recognized by the Ku70/80 heterodimer which loads other NHEJ protein needed to promote the DSBs repair. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) has a high affinity for Ku and together form the DNA-PK complex. DNA-PKcs undergoes autophosphorylation and activates Artemis endonuclease, which processes the DNA ends. If necessary, ends can be filled in by members of family of polymerases X (Pol μ and/or λ). Finally, the ligation complex (XLF–XRCC4–DNA Ligase IV) ligates the ends of the DNA molecule (Brandsma and Gent 2012).

Mutations in NHEJ genes are associated with severe combined immunodeficiency (SCID) (Chang and Lieber 2016).

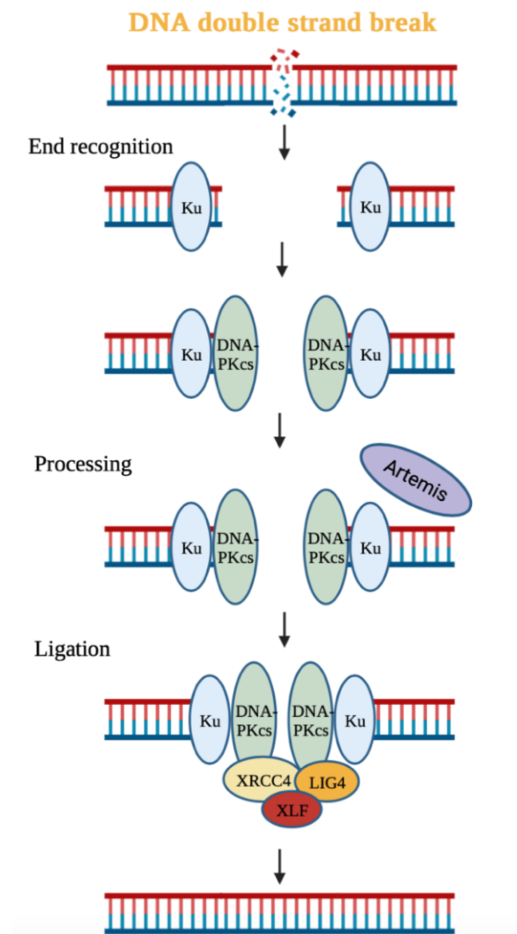


Fig.9.: A simplified scheme of non-homologous end-joining. Adapted from (Brandsma and Gent 2012) using Biorender.

1.3.3. Modulating DNA repair in cancer therapy

Most anticancer therapies (including chemotherapeutics and ionizing radiation) induce cell death by causing direct or indirect DNA damage. Therefore, dysregulation of DNA repair machinery may contribute to resistance or increased sensitivity to chemotherapeutic agents. It is widely acknowledged that enhanced DNA repair is associated with resistance to chemotherapeutics and, on the other hand, defects in DNA repair usually predispose cancer cells to higher sensitivity to DNA-damaging agents (Brandsma and Gent 2012).

Consequently, targeting DDR is one of the key therapeutical strategies to overcome resistance and increase sensitivity to cancer treatment. DNA repair and damage response may be modulated with a variety of molecular mechanisms including 1.) targeting DNA repair via epigenetic regulation; 2.) inhibition of core DNA repair proteins

with inhibitors; 3.) developing new combination therapies with natural compounds that will enhance the effect of conventional chemotherapeutics.

1.3.3.1. Epigenetic regulation of DNA repair

Epigenetic alterations have a significant effect on DNA repair. Examples of epigenetic alterations are DNA hypo/hyper-methylations, histone modifications (e.g., increased/decreased histone acetylation and methylation) and miRNA-mediated regulation.

Hypermethylation of several DNA repair genes (e.g., *MGMT*, *OGG1*, *MHL1*, *BRCA1*) has been observed in a variety of cancers. Hypomethylation of the promoter regions allows the translation machinery to target the gene promoters, while hypermethylation modifies the chromatin and suppresses the gene expression by blocking the access of translation machinery to the promoters. Besides its involvement in tumorigenesis, methylation status is an important biomarker of therapy response. For instance, *MGMT* hypermethylation sensitizes glioma cells to alkylating agents and predisposes to better response to Temozolomid in colorectal cancer patients (Jacinto and Esteller 2007; Inno et al. 2014). CRC patients with hypermethylated *MHL1* do not benefit from 5-FU (Niv 2005), and *MHL1* methylation is also a marker of oxaliplatin resistance in gastric cancer patients (Li et al. 2015). Therefore, hypomethylating agents like 5-azacytidine (Azadine) and 5-aza-2'-deoxycytidine (Decitabine) are used in the treatment of certain cancers, e.g., myelodysplastic syndromes and acute myeloid leukemia (Sato, Issa, and Kropf 2017).

MiRNAs are short (average length 18-23 nucleotides) non-coding RNAs that modulate the expression of protein-coding genes at the post-transcriptional level via degradation or inhibition of translation of specific target mRNA. More than 2600 miRNAs have been predicted to be encoded by the human genome, with the ability to modulate the expression of about 60% of human genes (Chou et al. 2018). Deregulated miRNA expression has been observed in numerous cancers in association with cancer susceptibility, progression, metastatic formation and in therapy sensitivity/resistance (Melo and Esteller 2011). MiRNAs as regulatory elements can modulate the cancer cell sensitivity towards DNA-damaging agents by regulating the expression in DNA repair and damage response genes (Jurkovicova et al. 2022). Therefore, miRNAs represent promising therapeutic tools for improving the therapy response in chemoresistant cancers. Among numerous miRNAs associated with therapy response, we studied the role of miR-

140 in oxaliplatin resistance **Manuscript 3** and also the role of miRSNPs (SNP polymorphisms in miRNA-binding sites in 3'UTRs of protein-coding genes) in therapy response in BC patients in **Manuscript 1**. Elucidating the role of cancer-associated miRNAs in carcinogenesis is also vital for developing new promising strategies of anti-cancer treatment by either restoring their function by applying miRNA mimics or inhibiting them with inhibitors or antagomiRs (Fu et al. 2021). Additionally, their good accessibility and high stability in body fluids is making them suitable as non-invasive predictive and prognostic biomarkers in cancer therapy (He et al. 2020).

1.3.3.2. Novel DNA repair and DNA damage response inhibitors

Over the last years, extensive research has been done on the development of DDR inhibitors and their implementation in cancer therapy. Their therapeutic potential is based on their ability to overcome resistance to conventional cancer therapies and/or produce synergistic anti-cancer effects when combined with conventional therapies such as chemotherapy (Hu and Guo 2020; Tang, Chen, and Xu 2020).

The first DDR inhibitor approved by U.S. Food and Drug Administration (FDA) in cancer therapy was PARP inhibitor (PARPi) olaparib in 2014 (Kim et al. 2015), approved for the treatment of patients with advanced OvC carrying a mutation in *BRCA* genes. Since then, three other PARPi have been developed - rucaparib, talazoparib and niraparib, each indicated in a different type of advanced, metastatic, or recurrent OvC, fallopian tube cancer, primary peritoneal cancer, BC, pancreatic or castration-resistant prostate cancer, predominantly for *BRCA* mutation carriers (Schettini et al. 2021). Their anti-cancer activity is based on the concept of synthetic lethality, where the defect in a DNA repair gene (*BRCA 1* or *BRCA2*) is combined with the inhibition of a DNA repair protein (PARP) that is critical for the survival of cancerous cells but is less important for the survival of normal cells (Hengel, Spies, and Spies 2017).

Subsequently, numerous DDR inhibitors have been discovered, some of them currently investigated in clinical studies with promising results for cancer therapy. These include DNA-PK, ATM, ATR, CHK1 or WEE1 inhibitors (Cheng et al. 2022).

In our **Manuscript 4**, we have investigated the role of another promising MRE11 inhibitor, Mirin. According to our results, Mirin ameliorates carboplatin therapy response in OvC cells.

1.3.3.3. New combination therapies with natural compounds

Several natural compounds have been shown to have anti-cancer activities and many studies have been conducted on the combination of natural compounds with conventional chemotherapeutics to enhance their effect or re-sensitize chemoresistant cancer cells (Rejhova et al. 2018). They are usually well tolerated and do not cause toxic side effects, which makes them an interesting approach for overcoming resistance and increasing sensitivity to conventional drugs.

One of the most studied natural compounds in cancer therapy is curcumin, isolated from *Curcuma longum*. It has been proven to reverse chemoresistance of various agents *in vitro, in vitro,* and even in clinical trials, e.g., cis-platin resistance in OvC cells (Muhanmode et al. 2021), 5-FU resistance in CRC cells (Li et al. 2021), doxorubicin, and etoposide resistance in gastric cancer cells (Yu et al. 2011), paclitaxel resistance in hepatocellular carcinoma (Tian et al. 2019) or oxaliplatin resistance in CRC both *in vitro* and *in vivo* (Ozawa-Umeta et al. 2020). Another extensively studied natural compound is resveratrol, which naturally occurs in about 70 plant species. It has been observed to suppress platin resistance in several cancers or resistance to docetaxel in BC (Muhanmode et al. 2021; Ferraresi et al. 2021; Vinod et al. 2015). In our study (**Manuscript 2**), we investigated the effect of *Ganoderma lucidum* extract (GLC) on 5-FU efficacy. GLC is a popular over-the-counter supplement with numerous anti-proliferative effects on cancer cells (Sohretoglu and Huang 2018; Rejhova et al. 2018). Our study confirmed its enhancing effect on 5-FU in CRC *in vitro* and *in vivo*.

2. AIMS

DNA repair and DDR pathways have, as illustrated above, a crucial role in cancer risk, development, and therapy response. In this dissertation Thesis, we investigated the role of DNA repair and its modulation in therapy response and resistance to most common chemotherapeutics. Our rationales were that A) polymorphisms in repair genes influence the therapy response and survival of cancer patients; that B) combining natural compounds with conventional chemotherapeutics enhance their therapeutic effect; that C) inhibition of vital DNA repair proteins using inhibitors and via epigenetic regulation with miRNAs has an effect on chemoresistance and increases chemosensitivity; and that D) alterations in the expression of DDR and DNA repair genes are involved in chemoresistance,.

Hence, the main **aims** of this dissertation Thesis were:

1. To address the role of polymorphic variations in DNA repair genes in therapy response.
2. To explore the effect of combination therapy with natural compounds on resistance/sensitivity to 5-FU.
3. To address the role of DNA repair and damage response in resistance and sensitivity towards conventional chemotherapeutics.
4. To further explore the role of homologous recombination in resistance/sensitivity to platinum compounds and 5-FU.

3. MATERIAL AND METHODS

Manuscript 1: Genetic variations in 3'UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response.

Manuscript 2: *Ganoderma Lucidum* induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo.*

Manuscript 3: MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients.

Manuscript 4: Inhibition of homologous recombination repair by Mirin in ovarian cancer ameliorates carboplatin therapy response *in vitro*.

Unpublished study: The role of DDR and DNA repair in acquired resistance to 5-FU in CRC *in vitro*.

3.1. POPULATION STUDY - MANUSCRIPT 1

Study population

The study population included 673 incident BC patients consecutively diagnosed in three hospitals in Prague (Czech Republic) between February 2002 and December 2010 and 675 controls. The control group consisted of two groups of healthy women. The first group consisted of 332 individuals who were admitted to gastroenterological departments for colonoscopy examination with negative results and did not have any malignancy at the time of the sampling. The second group consisted of 343 healthy blood donor volunteers from a blood donor center in Prague. The mean age of patients at the time of diagnosis and the mean age of controls were 59 and 49 years, respectively. The genotype distribution of analyzed miRSNPs in the control group agreed with the Hardy-Weinberg equilibrium. All individuals were sampled for peripheral blood. The design of the study was approved by the Ethical Committee of the Institute of Experimental Medicine, Prague, Czech Republic.

Clinical information

The following data on BC patients were retrieved from medical records: date of cancer diagnosis, age, menopausal status, family history of cancer (number of relatives affected by BC, ovarian cancer, or other malignant diseases), tumor size, International Union

Against Cancer (UICC) tumor-node-metastasis (TNM) classification, histological type and grade of the tumor, expression of ER, PR and HER2; expression of the Ki-67 protein; chemotherapy and hormonal regimen.

SNP selection and genotyping

SMUG1 rs2233921 G>T, *SMUG1* rs971 G>A and *NEIL2* rs6997097 T>C polymorphisms were analyzed in this study. These polymorphisms showed a significant prognostic value in CRC (Pardini et al. 2013). Genomic DNA was isolated from peripheral blood lymphocytes. Genotyping was carried out by using the KASP™ chemistry (LGC Genomics, UK), a competitive allele-specific PCR-based SNP genotyping system, described in (Pardini et al. 2008).

Bioinformatic and statistical analyses

Freely available software MicroSNiPer, PolymiRTS and Mirsnpscore were used for the prediction of putative miRNAs targeting binding sites within miRSNPs. The associations between the miRSNPs analyzed in this study and gene expression levels were obtained from the Genotype-Tissue Expression project (GTEx). All statistical analyses were performed using SAS software (SAS Institute, USA). The Bonferroni corrected significance threshold for multiple tests was set at 0.017 (for 3 miRSNPs and α= 0.05). External validation was performed using the freely available online tool GEPIA 2 (Gene Expression Profiling Interactive Analysis). This website provides interactive gene expression profiling based on tumor and normal tissue samples from TCGA and GTEx databases.

3.2. *IN VITRO* STUDIES - MANUSCRIPTS 2,3,4, UNPUBLISHED STUDY

Cell Cultures

Manuscript 2

The study was performed using human colorectal cancer cell lines HCT116, HT29, HCT116$^{p53-/-}$ (obtained from ATCC, USA) and non-cancer human colon mucosal epithelial cell line NCM460 cells (originally obtained from INCELL Corporation, USA by Prof. Sliva). Cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium (Sigma Aldrich, USA) with 10% fetal bovine serum (Sigma Aldrich, USA), 1 mM L-glutamine (Biosera, France), 1mM sodium pyruvate (Biosera, France)

and 1 mM penicillin/streptomycin (Biosera, France). NCM460 cells were cultured in M3:10TM medium (INCELL, USA) with 10% fetal bovine serum (Sigma Aldrich, USA) and 1 mM penicillin/streptomycin (Biosera, France). All cells were cultured in a humidified incubator at 37°C, 5% CO2.

Manuscript 3

The study was performed using human CRC cell lines HCT116, HT29 and DLD1 (Merck, Germany). Cell lines were cultured in DMEM medium (Merck, Germany) with 10% fetal bovine serum (Merck, Germany), 1 mM L-glutamine (Biosera, France), 1 mM sodium pyruvate (Biosera, France) and 1 mM penicillin/streptomycin (Biosera, France). All cells were cultured in a humidified incubator at 37°C, 5% CO_2.

Manuscript 4

The study was performed using the human ovarian carcinoma cell line OVCAR3 (Merck, Germany). Cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Merck, Germany) with 10% fetal bovine serum (Merck, Germany), 1 mM L-glutamine (Biosera, France), 1 mM sodium pyruvate (Biosera, France) and 1 mM penicillin/streptomycin (Biosera, France). All cells were cultured in a humidified incubator at 37°C, 5% CO_2.

Unpublished Study

The study was performed using human CRC cell line DLD1 (Merck, Germany). Cell lines were cultured in DMEM medium (Merck, Germany) with 10% fetal bovine serum (Merck, Germany), 1 mM L-glutamine (Biosera, France), 1 mM sodium pyruvate (Biosera, France) and 1mM penicillin/streptomycin (Biosera, France). Cells were cultured in a humidified incubator at 37°C, 5% CO_2.

Cell treatments

Manuscript 2

Cells were treated with *Ganoderma lucidum* extract (GLC), 5-FU and their combination. GLC was obtained from Pharmanex (USA, batch No.: DL12561) and contained well-defined formulation of 6% of triterpenes and 13.5% of polysaccharides. GLC was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) at the concentration of 50 mg/ml and stored at 4°C. 5-Fluorouracil (5-FU, Sigma Aldrich, USA) was dissolved in DMSO to 500 mM stock solution.

Manuscript 3

Cells were treated with the 6 µM concentration of oxaliplatin (Merck, Germany) 24 hrs after miRNA mimics transfection. Oxaliplatin was dissolved in DMSO (Merck, Germany) at the stock concentration of 100 mM and stored at 4°C.

Manuscript 4

Cells were pretreated for 1 hour with a MRE11 inhibitor Mirin (Sigma-Aldrich, Germany) at the concentration of 100 µM as described in (Dupre et al. 2008). Mirin was dissolved in DMSO at the stock concentration of 45 mM and stored at -20°C. After the pretreatment, cells were treated with a 6 µM concentration of carboplatin (Sigma-Aldrich, Germany). Carboplatin was dissolved in DMSO (Merck, Germany) at the stock concentration of 45 mM and stored at -20°C.

Unpublished Study

Cells were treated with an increasing concentration of 5-FU (Sigma-Aldrich, USA) following protocol from Coley (Coley 2004). Two 5-FU resistant cell lines were established, stably proliferating in 40 µM and 160 µM concentrations of 5-FU.

Viability, proliferation and growth assays

Manuscript 2,3,4, Unpublished Study

Colony formation assay (CFA) was used to determine the clonogenicity potential and viability of cells in association with studied agents. Briefly, 500 cells/well were plated on 6 well plates. After the corresponding treatment, cells were grown in the fresh medium. After 10-12 days, colonies of at least 50 cells were fixed using 3% formaldehyde, stained with 1% crystal violet, and manually counted.

Manuscript 2,3,4, Unpublished Study

WST-1 cell proliferation assay (Roche, Switzerland) was used to measure cell proliferation after exposure to studied agents. WST-1 method is a colorimetric nonradioactive method to assess the number of metabolically active cells. $5x10^4$ cells per well were seeded on 96 well plates and treated with analyzed agents in quadruplicates. After the corresponding treatment, WST-1 reagent was added according to the manufacturer's manual (10 µl WST-1 reagent per 100 µl of medium). Absorbance was measured at 450nm, with 690nm as reference using fluorescence reader Biotek ELx808 (Biotek, USA).

Manuscript 4, Unpublished Study

Cell growth was analyzed by seeding cells on 12 well plates ($2.5x10^4$ of OVCAR3 and 10^5 of DLD1 cells) and after corresponding treatment, viable cells were manually counted after 24, 48 and 72hrs using Trypan blue.

Migration assay

Manuscript 2

Cell migration was measured using Corning Transwell Permeable Supports 8.0 µm (Sigma Aldrich, USA) according to the manufacturer's manual. Briefly, cells were seeded on 6 well plates ($5x10^5$ cells/ml) and treated with 0.5 mg/ml GLC for 24h. Subsequently, $1x10^4$ of the treated cells were seeded on the top of the transwell inserts in 24 well plate format and cultured in DMEM medium containing 0.5% FBS. Cells were allowed to migrate for 24h through the membrane into the lower part of the chamber with medium containing 20% FBS. Migrated cells were fixed with 3% formaldehyde, stained with 1% crystal violet and counted in four random fields under 200x magnification.

Cell cycle analysis

Manuscript 2,3,4, Unpublished Study

Cells were seeded on 12 well plates ($5x10^5$ cells/ml). After the corresponding treatment, cells were harvested, washed with PBS, and spun down at 1000 rpm for 10min. 1 ml of Propidium iodide (PI) staining solution (0.02 µg/µl PI, 0.02 mg/ml RNase, 0.05% Triton X-100) was added to the cell pellets and cells were incubated for 30min at 37°C in the dark. After incubation, samples were analyzed using a flow cytometer Apogee A-50 micro (Apogee, UK). Obtained data were analyzed with FlowLogic™ software (Inivai Technologies, Australia).

Tumor samples

Manuscript 3

Tumor and non-malignant adjacent mucosa paired samples from 50 CRC patients (26 males and 24 females), who underwent surgical tumor resection, were obtained between 2011 and 2015 and stored at -80°C. The median age at diagnosis was 65 years (range 37-82). Information about patients' survival or disease progression were followed until 2021. All patients provided signed consent for participation in this study. The design of the

study was approved by the Ethical Committee of the Institute of Experimental Medicine, Prague, Czech Republic.

Reverse transcription and quantitative PCR (qPCR)

Manuscript 3,4, Unpublished Study

After treatment with a studied agent, total RNA from cells or tumor samples was isolated using Qiagen miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration of the isolated RNA in samples was measured using Nanodrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, USA), and integrity of mRNA (RIN) was determined using Agilent RNA 6000 Nano Kit by Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Reverse transcription to cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

The expression of studied protein-coding genes was measured using qPCR SybrMaster (Jena Bioscience, Germany) according to manufacturer's protocol. All PCR primers (Sigma-Aldrich, USA) were in-house designed using OligoArchitect™ Primer and Probe Design (Sigma-Aldrich, USA). The thermal protocol was 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15s, and 62°C for 60s and followed by melting curve analysis. MRNA expression was normalized to ACTB, GAPDH, and RNU19. Expression of miRNA coding gene was measured using TaqMan MicroRNA Assay according to manufacturer's protocol. The thermal protocol was 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15s, and 60°C for 60s. MiRNAs expression was normalized to RNU6B. QPCR analyses were run on 7500 Real Time PCR System (Thermo Fisher Scientific, USA). Data were subsequently analyzed by the $2^{-\Delta\Delta Ct}$ method.

Protein isolation, SDS-PAGE and Western blot analysis

Manuscript 2,3,4

Proteins were isolated using RIPA lysis buffer (Sigma-Aldrich, USA) with added cOmplete™ Protease Inhibitor Cocktail (Roche, Germany) according to the manufacturer’s manual. Protein concentration was measured using Quick Start™ Bradford Protein Assay Kit (Bio-Rad Laboratories, USA).

After protein isolation, 20 µg of proteins per sample were loaded and separated in 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels at 15 mA for 60min. Separated proteins were then transferred to 0.45 µm Amersham ™ Protran ®

Western blotting membranes (Cytiva Life sciences, UK) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, USA). Membranes were blocked with 5% BSA in TBST (Tris-buffered saline containing Tween 20; 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20) for 1 hour. Then, membranes were incubated with the corresponding primary antibodies from Cell Signaling Technology (USA) or Abcam (UK) overnight at 4°C and incubated with a secondary antibody conjugated with horseradish peroxidase (Abcam, UK). Membranes were then incubated with Immobilon western Chemiluminescent HRP Substrate (EMD Millipore Corporation, USA) and visualized by Azure c600 (Azure Biosystems, USA).

Transient transfection

Manuscript 3

To study the effect of miRNA overexpression, MISSION miRNA mimics technique was used (Ambion, USA). Cells were transfected in 6 well plates at 60-80% confluency with 2.5 pmol of MISSION miRNA hsa-miR-140-3p miRNA Mimics (Ambion, USA) or with Negative Control miRNA Mimics (Ambion, USA) with no homology to the human genome using Lipofectamine® RNAiMAX 2000 (Invitrogen™, USA) according to the manufacturer's protocol.

MRE11 silencing

Manuscript 3

For creating HCT116 cells with silenced MRE11, shRNAs technique was employed. Briefly, HEK293FT cells (Thermo Fisher Scientific, USA) were seeded on 6 well plates and co-transfected with pLKO1 mission MRE11 shRNA plasmids and helper plasmids psPax2 and pMD2.g (Addgene, USA) using Lipofectamine 3000 (Thermo Fisher Scientific, USA). After 6h, media were replaced with fresh DMEM without antibiotics. After 48h, culture media containing recombinant lentiviruses were harvested and spun down at 3000 rpm, at 4°C to for 15min to remove any floating cells and cell debris. The cleared media were added to HCT116 cells at ratios 1:3 and 1:10 v/v and seeded on 12 well plates. After 24h, media were replaced with fresh DMEM and colonies containing integrated lentiviruses were selected by cultivating cells with 2 μg/ml of Puromycin (Sigma Aldrich, USA) for 4–5 days. Successful MRE11 silencing was confirmed using PCR and Western blot.

Reactive oxygen species (ROS) measurement

Manuscript 2

Cells ($5x10^5$ cells/ml) were seeded on 24 well plates. After the treatment as described in the manuscript, cells were harvested and spun down at 1000 rpm for 10min. One µl of 10 µM solution of cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Thermo Fisher Scientific, USA) was added to cell pellet followed by incubation for 30min at 37°C. The levels of relative fluorescence were measured using fluorescent reader Biotek (Vermont, VT, USA) at excitation/emission wavelengths Ex/Em: 485nm/538nm.

Comet assay

Manuscript 2

DNA damage associated with studied agents was measured using Comet assay. Briefly: after the treatment, cells were embedded in duplicates in agarose cells ($2x10^5$ cells/ml, 0.5% low melting point agarose in PBS, 37°C) on a microscope slide pre-coated with 1% normal melting point agarose dissolved in distilled water. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, 1% Triton X-100, pH = 10, 4°C) for 1h to obtain substrate DNA that is fixed in agarose in the form of nucleoids. Subsequently, slides were washed at 4°C 3 times in the washing buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1 M KCl, pH = 8) for 3x5 min.

To investigate the overall DNA damage, slides were incubated with reaction buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1M KCl, pH=8, stored at 4°C) for 30min at 37°C. Subsequent alkaline incubation (freshly prepared 0.3M NaOH, 1 mM EDTA, stored at 4 °C) for 30min in the dark converted alkali-labile sites to strand breaks (SBs). During electrophoresis (1.19 V/cm, 300 mA, 40 min, at 4°C, in the dark) in the same alkaline buffer, DNA loops containing SBs were drawn towards the anode forming a comet-like image. To investigate the oxidative DNA damage, at the beginning of the experiment, nucleoids were incubated in the above-described reaction buffer with added formamidopyrimidine-DNA-glycosylase enzyme (Fpg; New England Biolabs, USA) for 30min at 37°C. After the electrophoresis, slides were washed in 1xPBS (4°C) for 10min, in distilled water (4°C) for 10min, dried overnight and stained in SYBR Gold (Invitrogen, USA) diluted 1:10.000 in Tris-EDTA (TE) buffer (10 mL of 1 M Trizma® base, 2 mL of 0.5 M EDTA-Na_2 in 988 mL of distilled water). Comets were visualized using a fluorescence microscope Olympus BX63 (Olympus, Japan) and scored using semi-automated Lucia Comet Assay™ software (Laboratory Imaging, Czech Republic).

In vivo experiment

Manuscript 2

Thirty-two 3 months old female BALB/c mice were inoculated with a suspension of CT26.WT cells (mouse colon carcinoma cell line). They were divided into 4 groups and treated with single or combined therapy when the tumors reached an average volume of about 300 mm^3 (day 14). GLC was administered daily via an oral gavage as a 100 µl GLC powder suspension in sterile distilled water (110 mg/ml) and 5-FU injected intraperitoneally 3 times a week with 200 µl suspension of 5-FU in PBS (20 mg/kg). The mice were sacrificed on day 48, tumors were measured, weighted and frozen.

Bioinformatical analyses

Manuscript 4, Unpublished Study

Data from patients with OVC or CRC respectively, who were treated with platinum or 5-FU based chemotherapy, with available data on expression profiles of analyzed genes and clinical information on survival, were downloaded from The Cancer Genome Atlas TCGA project using cBioPortal website ('cBioPortal').

Statistical analyses

Manuscript 2,3,4, Unpublished Study

The appropriate statistical tests (two-way ANOVA, multiple unpaired t-tests, Mann-Whitney test) were performed using GraphPad Prism8 (GraphPad Software, USA) or RStudio (Posit, USA). Results represent the mean value ± SD of at least three independent experiments. The level of significance was set at $p \leq 0.05$. The survival analysis for TCGA data was performed using survminer package in RStudio, significance was measured with a log-rank test.

4. RESULTS AND DISCUSSION

This Thesis, based on the related research articles and yet an unpublished study, attempts to elucidate some of the molecular mechanisms involved in response towards most common chemotherapeutics, especially how to achieve better response, overcome resistance and increase sensitivity. We investigated these mechanisms in three solid cancers: breast cancer and colorectal cancer as one of the most common cancers in the world, including the Czech Republic, and ovarian cancer, as a cancer with a particularly high occurrence of chemoresistance and high mortality.

4.1. MANUSCRIPT 1: GENETIC VARIATIONS IN 3'UTRS OF *SMUG1* AND *NEIL2* GENES MODULATE BREAST CANCER RISK, SURVIVAL AND THERAPY RESPONSE.

Contribution: preparation of the analyzed databases with clinicopathological information on patients and controls (such as complementing information about patients' survival or TNM staging), analysis of results, writing and revising the manuscript

In this study, we have investigated the potential role of three miRSNPs (*SMUG1* rs2233921 G>T, *SMUG1* rs971 G>A and *NEIL2* rs6997097 T>C) in the 3'UTRs of BER glycosylases SMUG1 and NEIL2 in the susceptibility to BC and clinical outcome in a group of 673 BC patients and 675 healthy controls. SMUG1 and NEIL2 are BER-associated endonucleases (Table 1) responsible for the recognition and excision of modified bases and small DNA lesions. Our group previously reported that these miRSNPs are involved in the CRC prognosis and therapy response (Pardini et al. 2013). The strongest association was found between *SMUG1* rs2233921 and survival in patients undergoing 5-FU-based chemotherapy. This provided the first evidence that variations in miRNA-binding sites may modulate the response to chemotherapy. Based on these findings, we strove to explore, whether a particular genetic background associated with BER may exert common features for BC and CRC. One of the strongest arguments for that would be the observed clustering of BC and CRC cases in some families, partly caused by mutations in high-penetrance genes e.g., *BRCA1*; *BRCA2*; *CHEK2*; *MLH1* or *MSH2* (Lynch syndrome); and *LKB1/STK11* (Peutz-Jeghers syndrome). However, these known mutations cannot explain all the observed familial clustering of BC and CRC.

In our study, we did not find a significant association (lower than p<0.017, see Bonferroni correction) between these miRSNPs and BC risk. None of these miRSNPs were either associated with the survival of the BC patients. However, because BC patients had different molecular subtypes of the disease, have different stage of the disease, and received different therapy regimens, we divided patients in our subsequent statistical analyses into several groups. No significant associations were observed after patients' stratification according to the molecular subtype of BC (HR-positive luminal subtypes, HER2-positive and TNBC). After stratification according to the TNM stage (TNM 1+2 vs. 3+4), we found that the TT genotype of *SMUG1* rs971 in patients with early BC (TNM stage 1+2) was moderately associated with shorter OS both in the co-dominant and recessive models (HR=2.1, 95% CI=1.07-4.14, p=0.03 and HR=1.9, 95% CI=1.07-3.38, p=0.03, respectively**)**; however, the association did not pass Bonferroni's correction. After stratification according to the therapy regimen (patients with neo-adjuvant chemotherapy, without neo-adjuvant chemotherapy, with any adjuvant chemotherapy, with 5-FU-based chemotherapy and with hormonal-based therapy), we observed that the TC genotype of *NEIL2* rs6997097 in patients receiving only hormonal-based therapy was associated with shorter OS both in the co-dominant and the dominant model (HR=4.15, 95% CI=1.7-10.2, p=0.002; HR= 3.52, 95% CI=1.4-8.6, p=0.006, respectively). The same group of patients showed also a shorter DFS in the co-dominant model, moderately exceeding the Bonferroni-adjusted threshold of significance (HR=2.56, 95% CI=1.5-5.7, p=0.02).

According to *in silico* analysis with three different online tools (MicroSNiPer, PolymiRTS and Mirsnpscore), several miRNAs were predicted to bind to analyzed miRSNPs. Despite these programs using different algorithms, following miRNAs were predicted by more than one software: 1) miR-770-5p targeting *SMUG1* rs2233921 when harboring T allele; 2) miR-455-3p and miR-655 targeting *SMUG1* rs2233921 when harboring G allele; 3) miR-541-5p/miR-541* targeting *NEIL2* rs6997097 when harboring T allele and 4) miR-5681a when *NEIL2* rs6997097 when harboring C allele.

Another online tool, GEPIA 2, was used to perform the survival analysis based on the expression levels of *SMUG1* and *NEIL2* genes. An analysis of a total of 808 BC expression profiles (n luminal A=415, n luminal B=192, n TNBC=135, HER2 positive non-luminal=66) was performed. *NEIL2*-high patients with HER2 positive and non-luminal BC exhibited significantly worse OS than *NEIL2*-low patients (Logrank p=0.04).

Our results showed a strong association between the *NEIL2* rs6997097 TC genotype and shorter survival in patients receiving hormonal therapy (inhibitors of

aromatases and tamoxifen) (Fig. 10). However, the low frequency of the C allele (only 3 controls and 2 patients with the CC genotype in our studied population) precluded evaluation of the genotype dosage and computing HR for CC bearers. Unfortunately, no significant associations were found between analyzed miRSNPs and the survival of patients receiving 5-FU-based chemotherapy or other types of chemotherapy.

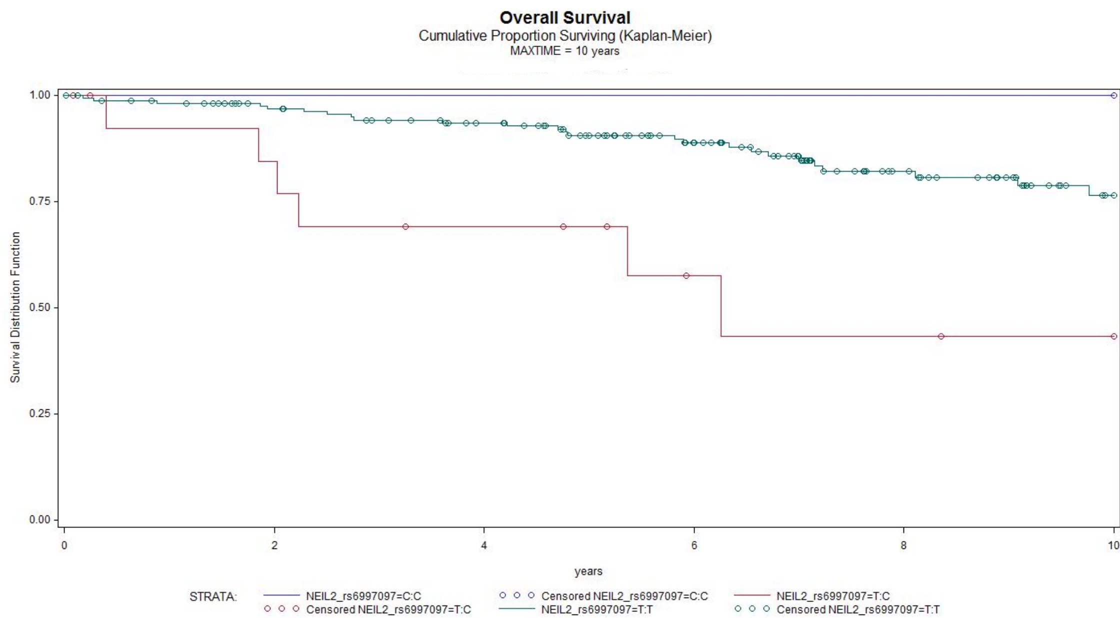


Figure 10. Kaplan-Meier overall survival curves of *NEIL* rs6997097 for patients undergoing hormonal-only therapy.

TT (green) vs TC (red) vs CC (blue), C = variant allele

According to the *in silico* analysis, miR-5681a targets the *NEIL2* rs6997097 when the C allele is present. This miRNA is overexpressed in ER-positive breast tumors (Sidorova et al. 2023). On the other hand, a tumor-suppressor miRNA miR-541-5p/miR-541* has a high affinity to this sequence, when the T allele is present (Shen et al. 2020; Leivonen et al. 2014; Xu et al. 2018; Lu et al. 2016).

Both SMUG1 and NEIL2 have been studied for their role in cancer (Vodicka et al. 2020). *In vivo* experiments showed that SMUG1 effectively collaborate with UNG to eliminate incorporated uracil in the genome and is important for preventing the accumulation of spontaneous mutations in DNA (Alsoe et al. 2017). Moreover, low *SMUG1* expression is linked to aggressive clinicopathological phenotypic features of BC (like the absence of hormonal receptors, EGFR overexpression, the presence of basal-like phenotype and triple-negative phenotype) and poor prognosis. Low *SMUG1* expression

was associated also with aberrant expression of several other DNA repair, cell-cycle control and apoptosis genes and overall genomic instability in SMUG1-low tumours. This endorses the important role of SMUG1 in breast carcinogenesis (Abdel-Fatah et al. 2013). Regarding NEIL2, the minor allele of *NEIL2* rs1466785 associates with increased BC risk in *BRCA2* mutation carriers (Osorio et al. 2014). Loss of *NEIL2* expression, simultaneously with alterations of nucleotide excision repair genes *CETN2* and *ERCC1,* was associated with resistance to endocrine treatment for ER+ breast tumours (Anurag et al. 2018).

Our findings support the assumption that DNA repair is one of the most crucial processes in the cell and defects in its fine regulation may have large consequences on human health. MiRNA-regulated gene expression in general and in particular of DNA repair genes remains a largely unexplored field. It is involved in BC initiation, progression, metastasis, or resistance to therapy (Graveel et al. 2015; Le Quesne and Caldas 2010; Mulrane et al. 2013; Serpico, Molino, and Di Cosimo 2014; Takahashi, Miyazaki, and Ochiya 2015; Kayani et al. 2011). Although we did not find an association of these miRNA-binding sites polymorphisms with chemotherapy response, our results suggest that individual genetic variations in miRSNPs may influence the patient's prognosis and response to hormonal anticancer therapeutics. The exact molecular mechanisms underlying the association of miRNAs and therapy efficacy have yet to be elucidated. That would help to tailor the treatment regimen to the individual's genetic background and indeed improve the patient's survival.

Additionally, we addressed the relationship between telomere length (TL) in lymphocytes (LTL), prognosis and clinicopathological features in the same set of BC patients as analyzed in our Manuscript 1 (see Manuscript 7 (Kroupa et al. 2020)). BC patients had significantly longer LTL than healthy controls. Moreover, patients were genotyped for nine TL-associated polymorphisms and CC genotype of *hTERC* (coding for human telomerase RNA component) rs16847897 was associated with longer LTL as well. Telomere maintenance, besides DNA repair, is another complex component for maintaining the genome integrity of cells and hence represents another key mechanism of carcinogenesis. The interplay between telomere maintenance and DNA repair possesses an interesting research topic with telomerase and telomeres being a possible target of anticancer therapy (Tomasova, Kroupa, et al. 2020).

4.2. MANUSCRIPT 2: *GANODERMA LUCIDUM* INDUCES OXIDATIVE DNA DAMAGE AND ENHANCES THE EFFECT OF 5-FLUOROURACIL IN COLORECTAL CANCER *IN VITRO* AND *IN VIVO*.

Contribution: *in vitro* experiments (CFA, proliferation, migration assays), manuscript revision

In this study, we have investigated the effect of *Ganoderma Lucidum* extract (GLC), as a possible modulator of DNA damage that may be used in combination with conventional chemotherapeutics to enhance the response and minimize their side effects in colorectal cancer. The study comprises both *in vitro* and *in vivo* experiments.

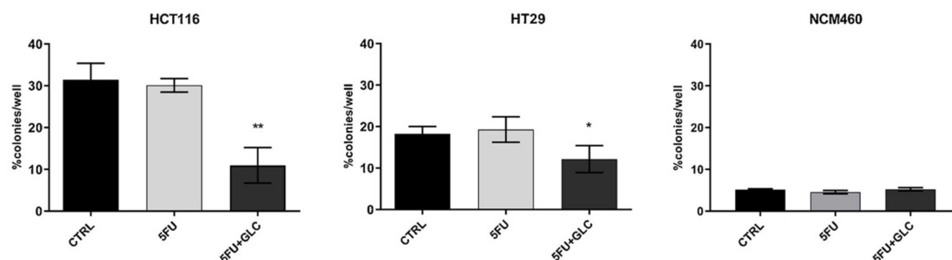
Ganoderma Lucidum is basidiomycetous fungi used in traditional Eastern medicine for centuries to treat various diseases including cancer (Sliva 2003). Our hypothesis that the efficacy of conventional chemotherapeutic 5-FU may be modulated by GLC extract was confirmed in the present study. Our results show that GLC exerts its anticancer effect by increasing the oxidative DNA damage in cancer cells while protecting non-malignant cells against ROS formation. It also decreased the migratory properties of cancer cells that are essential for metastatic spread. Metastases are responsible for about 90% of cancer deaths (Jiang et al. 2015).

GLC treatment alone showed a number of anticancer effects. To study its inhibitory effect, we measured the cell proliferation after GLC treatment for 24, 48 and 72 hours. The most significant decrease in proliferation was observed after 48 hours of treatment with 0.5 mg/ml concentration of GLC. The proliferation of HCT116 decreased by 27% ($p<0.05$) and HT29 by 39% ($p<0.05$). The proliferation of non-malignant NCM460 cells was not affected. Results from CFA confirmed the inhibitory effect of GLC, as the number of colonies after 0.5 mg/ml GLC treatment significantly decreased by 46% and 45% in HCT116 and HT29 cells, respectively ($p<0.05$). CFA did not show any effect of GLC on non-malignant cells. GLC also showed an inhibitory effect on invasive cancer behavior. Migratory properties of HCT116 decreased by 57% ($p<0.05$) and of HT29 by 14% (not significant). Cell cycle analysis indicates that GLC induced G1/S cycle arrest both in HCT116 ($p<0.001$) and HT29 cells ($p<0.05$). GLC treatment increased the amount of oxidative DNA damage in both cancer cell lines ($p<0.05$), however, we did not find any decrease in DNA strand breaks and oxidative DNA damage in non-malignant NCM460 cells. GLC also caused a significant decrease in ROS

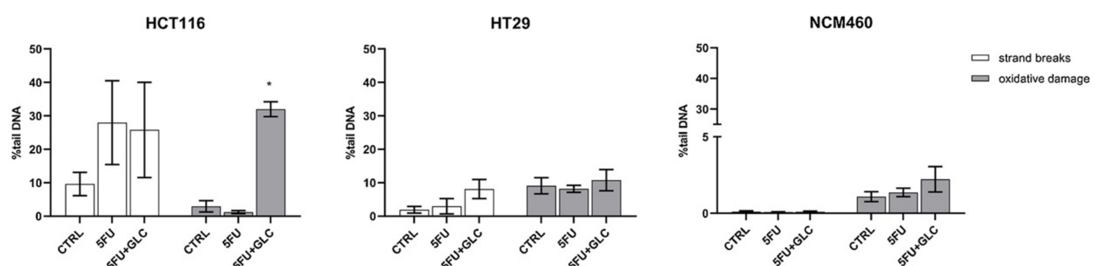
accumulation in non-malignant cells by about 20% after 6h (0.25 mg/ml, $p<0.05$; 0.5 mg/ml, $p<0.01$) and by about 17% after 24h (0. 25 mg/ml, $p<0.05$; 0.5 mg/ml, $p<0.001$).

To study the effect of combing GLC with conventional chemotherapeutic 5-FU on cancer and non-malignant colorectal cells, we performed the co-treatment of 0.5 mg/ml GLC and 5 μM 5-FU both on cancer and non-malignant colorectal cells (Fig. 11). The growth of HCT116 cells was decreased (Fig.11A) by about 20% compared to the effect of 5-FU alone ($p<0.01$) and by about 15% in HT29 cells ($p<0.05$). Furthermore, co-treatment of GLC+5-FU caused a significant ($p<0.05$) increase in strand breaks in HT29 and oxidative DNA damage in HCT116 compared to 5-FU alone (Fig.11B). We did not observe any effect of 5-FU+GLC on any of the analyzed parameters in non-malignant NCM460 cells. To analyze the effect of GLC on 5-FU efficacy, we performed *in vivo* experiments on BALB/c mice with transplanted syngeneic CT26 cells. After 14 days of tumor formation, mice were treated with GLC+5-FU and with GLC or 5-FU alone. Group treated with GLC+5-FU displayed only non-significantly better survival and smaller tumor volume compared to other groups. However, we observed significantly lower ($p<0.05$) tumor weight in GLC+5-FU group (Fig.11C). Further analysis with Combenefit software (Di Veroli et al. 2016) revealed an additive effect of GLC on 5-FU treatment.

A.



B.



C.

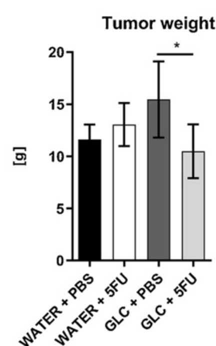


Fig. 11.: ***In vitro*** **and** ***in vivo*** **analysis of the GLC+5-FU co-treatment. A.)** CFA analysis of the cell growth. The number of colonies in HCT116 was decreased by about 20% compared to the effect of 5-FU alone ($p<0.01$) and by about 15% in HT29 cells ($p<0.05$). NCM460 were not affected. **B.)** Significant increase in oxidative DNA damage in HCT116 ($p<0.05$) and of strand breaks in HT29 ($p=0.05$) compared to 5-FU treatment alone. **C.)** Tumor volume comparison in BALB/c mice after treatment with 5-FU/GLC/GLC+5-FU. Tumors were significantly smaller in the group treated with combination therapy. *p ≤ 0.05, **p ≤ 0.01.

Natural compounds are usually well tolerated by patients and alone possess various anticancer effects. They suppress cell proliferation, induce cell cycle arrest, or induce apoptosis (Rejhova et al. 2018). Many of currently used anticancer drugs originate from natural sources as plants (e.g., Irinotecan, Paclitaxel) or microorganisms (e.g., Actinomycin D, Mitomycin C). However, the administration of natural compounds in cancer treatment is limited by their not well-defined or stable composition or the possible presence of contaminants. Therefore, current research goes towards the combined approach, where natural compounds with defined composition and known action mechanisms would be administered with conventional chemotherapeutics to lower their necessary dose and reduce the toxic burden for patients.

Co-treatment of GLC with 5-FU increased the anticancer effect of 5-FU against both cancer cell lines and *in vivo* as well. Current studies confirm the great potential of GLC in combination therapy. Qiu *et al.* recently discovered that co-treatment of WSG, a polysaccharide from GLC, and cisplatin synergistically inhibit lung cancer *in vitro* and *in vivo* while decreasing its cytotoxic effect in macrophages and normal lung fibroblasts (Qiu et al. 2021). Results from an *in vivo* study by Pan *et al.* show that *Ganoderma* spore lipid protects bone marrow against cytotoxic effects of Cyclophosphamide (Pan et al. 2019).

However, the particular cellular mechanism underlying the anticancer effects of GLC must be elucidated. Results from Jiang *et al.* suggest that GLC may restore the p53 function in p53-mutated cancer cells (Jiang et al. 2017). Other results indicate that spore oil from *Ganoderma Lucidum* induces apoptosis by activating caspase-3 and caspase-9. Li *et al.* reported that ethanol extract of *Ganoderma* triterpenes upregulates E-cadherin and suppresses HCT116 migration (Li et al. 2017).

After further research, GLC or its specific chemical components may be promising additives to conventional cancer chemotherapy, increasing its efficacy and lowering its adverse effects.

4.3. MANUSCRIPT 3: MIR-140 LEADS TO MRE11 DOWNREGULATION AND AMELIORATES OXALIPLATIN TREATMENT AND THERAPY RESPONSE IN COLORECTAL CANCER PATIENTS.

Contribution: qPCR standardization and analysis, manuscript revision

In this study, we investigated the role of miRNA miR-140 and its target protein MRE11 in the response to conventional chemotherapeutic oxaliplatin. Our previous study confirmed the important role of miRNA regulation in the response towards anticancer agents (see Manuscript 1).

Oxaliplatin is commonly used in the treatment of CRC, but the efficacy of the therapy is often compromised by the development of chemoresistance. Its genotoxic effect is based on the formation of DNA crosslinks. One of the most crucial pathways for repairing such DNA damage is homologous recombination. MRE11 is a part of the MRN complex involved in the HR repair of the DSBs (Hashimoto, Anai, and Hanada 2016).

Our results show that overexpression of miR-140 leads to decreased proliferation of CRC cells and increased sensitivity to oxaliplatin.

Firstly, we identified miR-140 as the best candidate for our study using TargetScan (McGeary et al. 2019) and TCGA database analysis ('The Cancer Genome Atlas Program (TCGA)'). MiR-140 showed the strongest statistically significant association ($p<0.01$) with PFS out of 187 predicted miRNAs targeting MRE11, where miR-140 overexpression was associated with better survival. Our results on 50 CRC patient's samples (tumor vs. adjacent non-malignant mucosa) were in concordance with TCGA analysis. Higher

expression of miR-140 in tumors was associated with better PFS (p=0.017). Comparing tumors vs. non-malignant mucosa, levels of miR-140 were significantly lower in tumor tissues (p<0.01). Lower levels of miR-140 were also associated with the metastatic phenotype (p<0.05).

To confirm the MRE11 as a target of miR-140, we used miRNA mimics to increase the levels of miR-140 v DLD1 cells. After transfection, mRNA and protein levels of MRE11 were decreased (Fig. 12). To evaluate the effect of miR-140 overexpression on DSBs, we measured the expression of gH2AX protein. Western blot analysis showed increased levels of gH2AX, a marker of DSBs damage, in DLD1 cells. Overexpression of miR-140 also resulted in decreased proliferation, measured with WST-1 assay.
For detailed figures on miR-140 overexpression effect on CRC cells see Manuscript 3. (Horak et al. 2022).

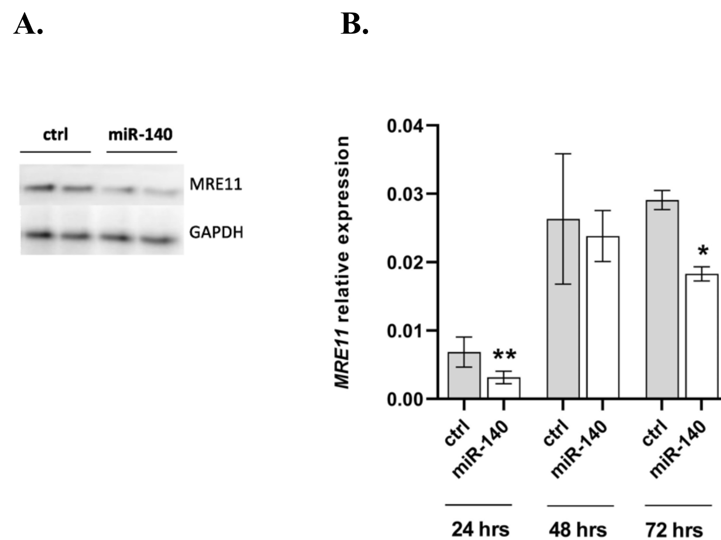


Fig. 12.: Protein and mRNA expression of MRE11 after miR-140 mimics. Increased levels of miR-140 downregulate MRE11. **A.)** WB analysis and **B.)** qPCR analysis of MRE11 expression. *p ≤ 0.05, **p ≤ 0.01.

As oxaliplatin is an important part of CRC treatment regimes, we examined the effect of miR-140 overexpression on DLD1 cells sensitivity to oxaliplatin (Fig. 13). Overexpression of miR-140 significantly decreased cell proliferation after oxaliplatin treatment, significantly decreased cells clonogenic potential (CFA) and increased the number of cells in G1 phase and decreased of those in S phase.

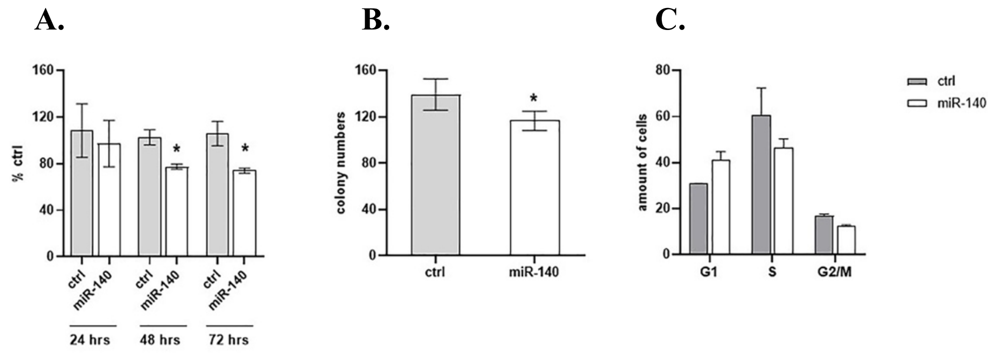


Fig. 13.: Effect of miR-140 overexpression on oxaliplatin sensitivity. A.) cell proliferation after 6 μM treatment with oxaliplatin was significantly decreased after 48 and 72 hours in miR-140 overexpressing cells ($p<0.05$). **B.)** CFA analysis revealed a significant decrease in colony number after 6 μM treatment with oxaliplatin in miR-140 overexpressing cells ($p<0.05$). **C.)** cell-cycle analysis revealed a increase in cells in G1 phase and decrease in the S phase (not significant) $*p \leq 0.05$, $**p \leq 0.01$.

To further analyze the effect of miR-140 on oxaliplatin sensitivity, we established shMRE11 HCT116 cell line with suppressed levels of MRE11. However, we did not observe increased oxaliplatin sensitivity after miR-140 overexpression in this cell line.

In this study, we confirmed our original hypothesis that decreased expression of MRE11 via miR-140 inhibition increases the oxaliplatin sensitivity of CRC cells. MiR-140 was previously widely studied in association with different cancers. A meta-analysis from Zheng *et al.* found a strong correlation between miR-140 overexpression and better OS in several cancers and vice versa, low expression is associated with advanced stages, worse histologic type, and lymph node metastases (Zheng et al. 2021). Other studies described the important role of miR-140 in response to therapy. MiR-140 regulates, besides MRE11, a HMGN5 nucleosome-binding protein, promotes autophagy and sensitize osteosarcoma cells to chemotherapy (Meng et al. 2017). It was described also to re-sensitize cisplatin resistant NSCLC cells to cisplatin through the SIRT1/ROS/JNK pathway (Lin et al. 2020). It also sensitizes lung adenocarcinoma cells towards several chemotherapeutics and targeted agents by targeting ADAM10/Notch pathway (Meng et al. 2022). Wu *et al.* similarly demonstrate the ability of miR-140 to enhance the cisplatin sensitivity in lung adenocarcinoma cells (Wu et al. 2020). MiR-140 also enhances the sensitivity to doxorubicin in hepatocellular carcinoma cells (Gao, Jiang, and Li 2021). Our results are therefore in concordance with previous studies, where miR-140 was associated with higher sensitivity towards different chemotherapeutics even in chemoresistant cell lines.

Our results confirmed that MRE11 is one of the targets of miR-140. MiRNAs, including miR-140, are able to target and regulate the expression of several genes, affecting several cellular pathways. Its inhibition may be a potential tool for overcoming the resistance to platin derivatives. Similar results were obtained from Alblihy *et al.*, who observed an overcome of cisplatin resistance and induced synthetic lethality in XRCC1-deficient epithelial OvC (Alblihy et al. 2022).

Despite intensive research, the response to CRC therapy remains low. An in-depth understanding of miRNAs role in carcinogenesis and chemoresistance and their potential use as novel therapeutic tools or novel prognostic and predictive biomarkers may indeed lead to better efficacy of cancer therapy, especially in chemoresistant tumors.

4.4. MANUSCRIPT 4: INHIBITION OF HOMOLOGOUS RECOMBINATION REPAIR BY MIRIN IN OVARIAN CANCER AMELIORATES CARBOPLATIN THERAPY RESPONSE *IN VITRO*.

Contribution: designing primers for qPCR analysis, qPCR standardizations and analysis, manuscript revision

To further understand the role of MRE11 and HR in chemoresistance to platin derivatives, we investigated to effect of MRE11 inhibitor Mirin on a model of ovarian cancer cells OVCAR3 treated with carboplatin. In our review (Manuscript 6, Supplement 2) on DNA repair and ovarian cancer risk, prognosis, and therapy outcome we emphasize the vital role of HR and its deficiency in OvC carcinogenesis (Tomasova, Cumova, et al. 2020).

To inhibit the MRE11 and evaluated its effect, we performed 1 hour treatment with 100 μM Mirin before the experiments according to Dupré *et al* (Dupre et al. 2008). Mirin alone does not affect the cell proliferation measured by WST-1 assay. However, the protein expression of MRE11 was, as expected, decreased while expression of γH2AX was increased, signifying an accumulation of DNA damage.

To assess the effect of Mirin on carboplatin (Cbpt) sensitivity in OVCAR3, cells with and without 1 h pre-treatment with 100 μM Mirin were treated with 6 μM concentration of carboplatin. OVCAR3 responded only moderately to carboplatin alone. However, Mirin pre-treatment caused a significant decrease in cell viability and

clonogenic potential (Fig. 14, $p<0.05$). We also observed increased expression of γH2AX and S phase arrest after Mirin+Cbpt treatment, compared to Cbpt alone.

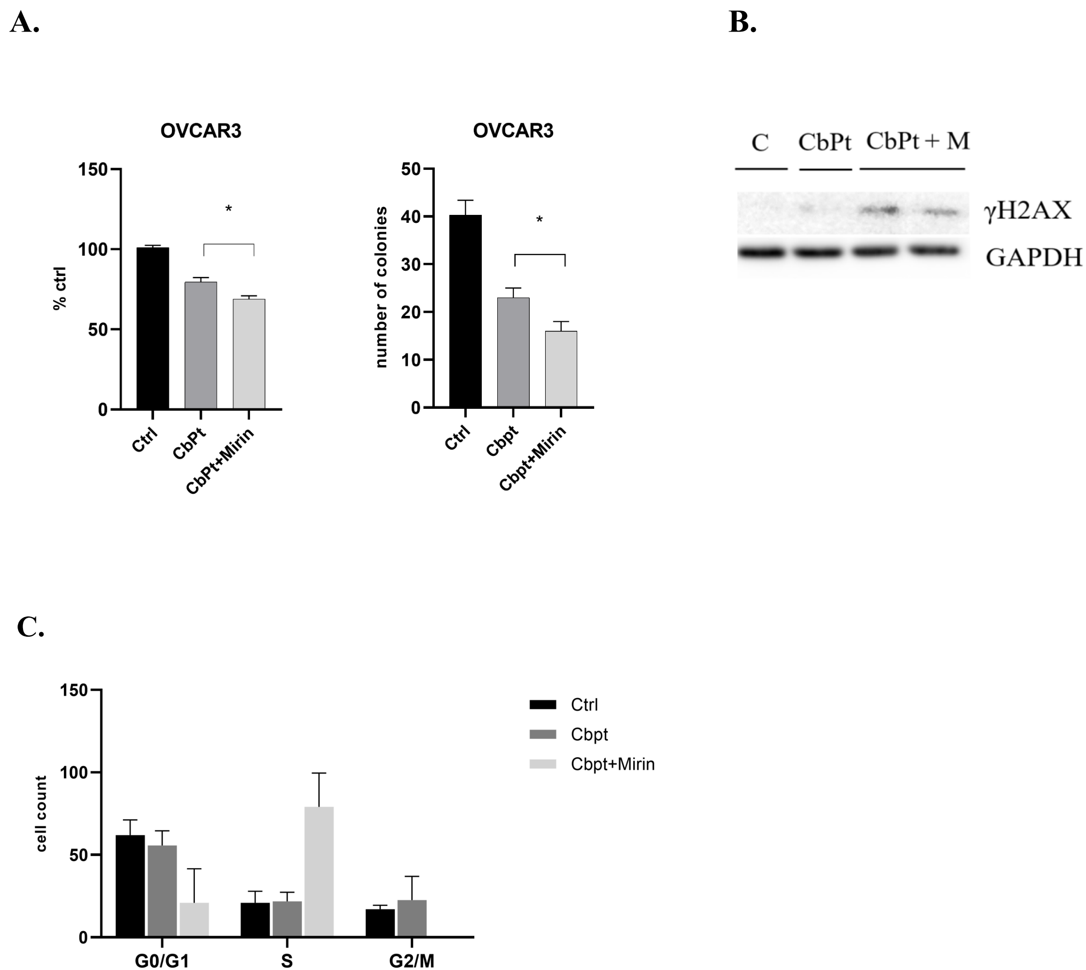


Fig. 14.: Effect of Mirin pretreatment on OVCAR3 sensitivity to carboplatin. Mirin pre-treatment caused **A.)** a significant decrease in cell viability (WST1-assay) and clonogenic potential (CFA), **B.)** an increased expression of γH2AX and **C.) the** S phase arrest after Mirin+Cbpt treatment compared to Cbpt alone. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

To further study the effect of Mirin on overcoming carboplatin resistance, we have established carboplatin resistant OVCAR3 cell line (carboplatin IC50 3.5x higher than in the paternal cell line). This cell line exhibited different morphology and dysregulation of various genes involved in DNA repair, DDR, apoptosis, autophagy, or drug efflux. We observed a significant increase in the expression of genes involved in different phases of the HR pathway as well as the increase in the expression of genes involved in the error-prone NHEJ pathway (see Manuscript 4). After 1 hour of Mirin pre-treatment,

carboplatin-resistant OVCAR3 retrieved the sensitivity to carboplatin and exhibited significantly decreased growth and clonogenic potential after Mirin+Cbpt (Fig. 15).

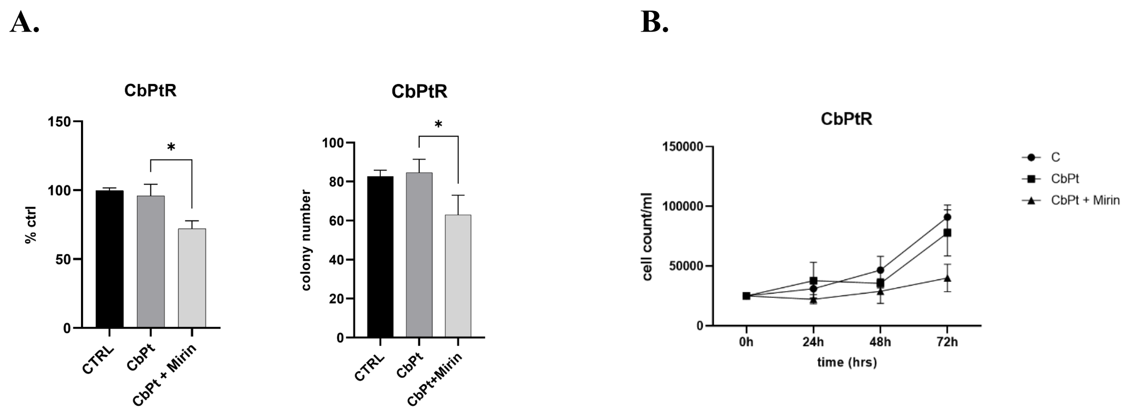


Fig. 15.: Retrieved carboplatin sensitivity after Mirin pre-treatment in OVCAR3 Cbpt-resistant cells. A.) WST1-assay and CFA assay, **B.)** growth analysis. $*p \leq 0.05$, $**p \leq 0.01$.

These results confirm our hypothesis that MRE11 inhibition may be a potent therapeutic approach to overcome carboplatin resistance or enhance the cancer cell sensitivity to its treatment. Chemoresistance is a major obstacle to ovarian cancer therapy, with the majority of initially responsive patients eventually developing platinum resistance (van Zyl, Tang, and Bowden 2018). Our results show that inhibition of MRE11 with Mirin leads to increased sensitivity of OVCAR3 cells to carboplatin, causes DNA damage accumulation and S phase arrest. MRE11 inhibition was previously explored in association with radiotherapy in several types of cancer. Wang *et al.* in their review concluded that increased MRE11 expression is associated with worse patient outcomes following radiotherapy and its inhibition with small or large molecule inhibitors may be used for enhancing radiosensitivity of tumors (Wang et al. 2021). Mirin in combination with CHEK1 inhibitor Prexasertib also showed a potent anticancer effect against colorectal cancer stem cells (CSCs) (Mattiello et al. 2021). Berte *et al.* proposed the use of Mirin or other DSBs inhibitor for increasing the sensitivity against alkylating agents (chloroethylating nitrosoureas, CNU) in the treatment of glioblastoma (Berte et al. 2016).

In our study, we proposed that Mirin not only sensitizes cells against carboplatin but also can re-sensitize platinum resistant OVCAR3 cells. These results are in concordance with our previous study on CRC and oxaliplatin resistance (Manuscript 3), where we used miR-140 mimics to downregulate the MRE11 expression.

Several inhibitors of DNA repair pathway members were successfully implemented into clinical practice of cancer therapy over the last years, such as Olaparib, Rucaparib, Niraparib or Talazoparib. Additionally, many clinical trials are currently evaluating the use of novel inhibitors or therapy regimens. According to our results, MRE11 inhibition may be a powerful strategy for increasing the cancer cell sensitivity towards conventional chemotherapeutics and/or overcoming chemoresistance.

4.5. UNPUBLISHED STUDY ON THE ROLE OF DNA REPAIR IN ACQUIRED RESISTANCE TO 5-FU IN CRC *IN VITRO.*

5-FU is used as a backbone of most conventional CRC chemotherapy regimens (mostly with platinum-based drugs and/or Irinotecan). It is used also in novel cancer therapy regimens, mainly in combination with targeted therapeutics (e.g., endothelial growth factor (VEGF) inhibitors or anti-epidermal growth factor receptor (EGFR) inhibitors) (Ghafouri-Fard et al. 2021). However, acquired chemoresistance greatly affects the clinical use of 5-FU and it is a predominant factor for therapy failure, leading to cancer progression and death (Azwar et al. 2021).

In this study (Manuscript in preparation, data are yet unpublished), we aimed to elucidate the role of DDR and DNA repair pathways in the development of acquired resistance to 5-FU. As DNA repair is one of the crucial factors involved in carcinogenesis and chemoresistance, we were interested in how gene expression of DDR and DNA repair genes would be altered during the process of establishing the resistance. As a model, we used parental DLD1 adenocarcinoma cell line and established two 5-FU resistant (5FUR) cell lines, stably proliferating in 40 (5FUR40) and 160μM (5FUR160) 5-FU in medium according to the protocol from Coley (Coley 2004).

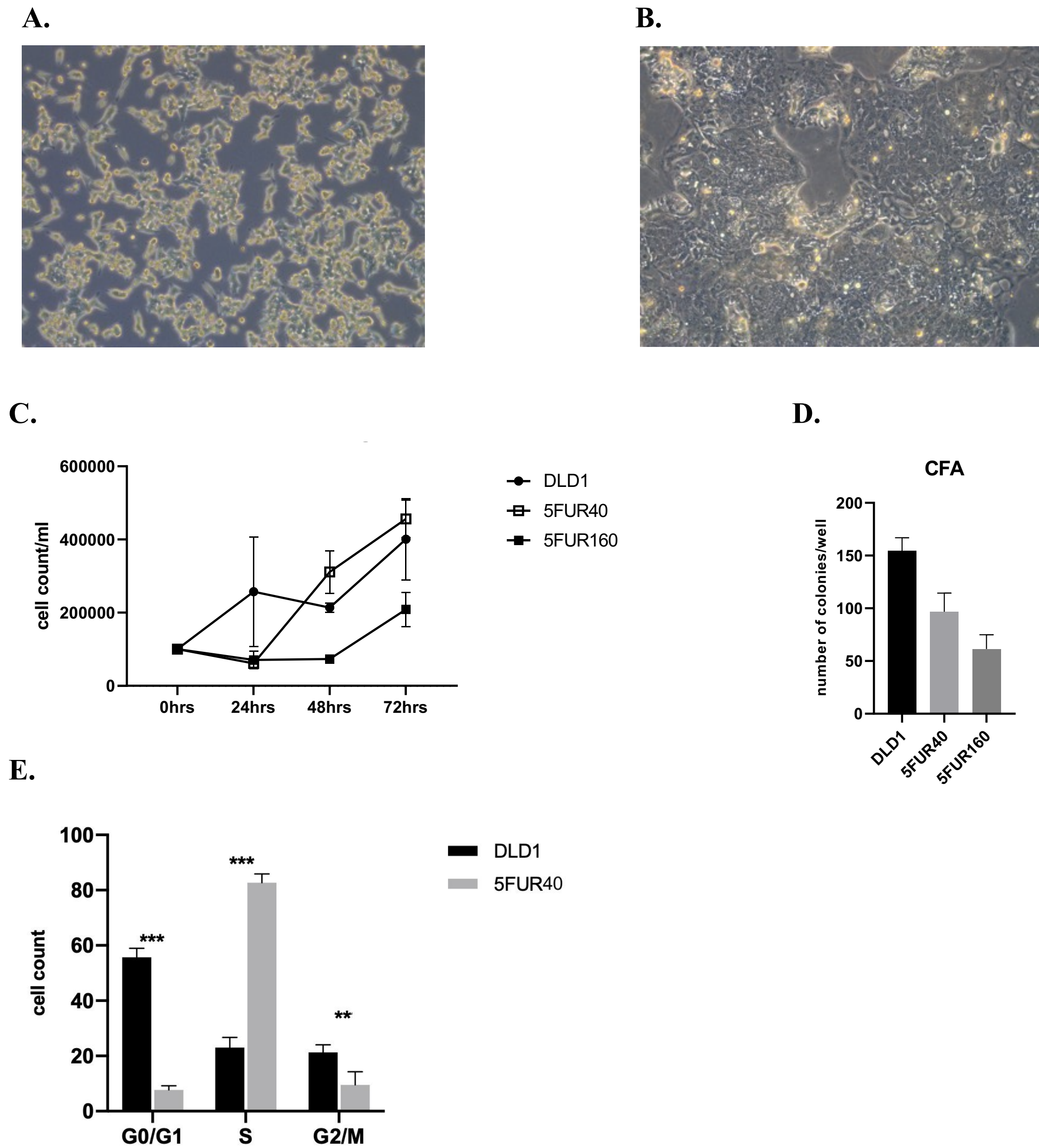


Fig. 16.: **Characterization of novel resistant cell lines**. Resistant cells displayed altered morphology. Parental (**A.**) vs DLD 5FUR40 cell line (**B.**). We observed differences in cell growth (**C.**, **D.**) and a prominent accumulation of 5FUR40 in the S phase (**E.**). *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001.

Resistant cell lines displayed altered morphology. Overall, the proliferation rates of DLD1 chemoresistant cells were slower compared to the paternal cell line. We observed slower cell growth in 5FUR160 compared to 5FUR40 and parental cells (not significant). Both resistant cell lines displayed a lower ability to form colonies (CFA; not significant). The cell cycle analysis revealed a significant decrease in the cell population in the G0/G1 and G2/M phases ($p<0.000001$ and $p<0.001$, respectively) and an accumulation of cells in the S phase in 5FUR40 cells ($p<0.000001$) (Fig.16). That

indicates that the resistant cells likely overcome the G0/G1 cell cycle arrest and proceed to the synthetic phase of the cell cycle when DNA replication and most of the DNA repair occurs.

Because we were focused on the role of DNA repair, we subsequently measured the expression of 88 genes involved in the DNA damage response and DNA repair in all main DNA repair pathways (Tab. 2). These genes were previously studied and associated with different cancers.

List of analyzed DDR and DNA repair genes			
AKT	*ERCC3*	*NEIL3*	*RAD51L1*
APEX1	*ERCC4*	*NHEJ1*	*RAD52*
ATG12	*ERCC6*	*NTH1*	*RAD54L*
ATG5	*ERCC8*	*OGG1*	*RPA1*
ATG7	*H2AFX*	*PARP1*	*RPA2*
ATM	*HIF1*	*POLE2*	*SMUG1*
ATR	*CHEK1*	*PMS1*	*SOD2*
BAD	*CHEK2*	*PMS2*	*TDG*
BAK1	*LC3*	*POLB*	*TDP1*
BCL10	*LIG1*	*POLD1*	*TOPBP1*
BCL2	*LIG3*	*POLD2*	*TP53*
BECLIN1	*LIG4*	*POLD3*	*TP53BP1*
BRCA	*MDB4*	*POLD4*	*UNG*
BRCA2	*MDR1*	*POLE1*	*WIP1*
CASP3	*MRE11*	*POLE3*	*XPA*
CASP9	*MSH2*	*POLE4*	*XPC*
MYC	*MSH3*	*RAD21*	*XRCC1*
DAPK1	*MSH6*	*RAD23B*	*XRCC2*
DCLRE1C	*MUTYH*	*RAD50*	*XRCC3*
DDB1	*NBS1*	*RAD51*	*XRCC4*
DDB2	*NEIL1*	*RAD51C*	*XRCC5*
ERCC2	*NEIL2*	*RAD51D*	*XRCC6*

Tab. 2.: List of analyzed DDR and DNA repair genes. Expression of the relevant genes was measured using qPCR.

The expression of 41 genes was significantly changed in both resistant cell lines. Thirteen genes were significantly changed only in 5FUR40 cell line suggesting the importance in early 5FU resistance and 15 were significantly changed in 5FUR160 cell line, suggesting the importance in later stages of adaptation to 5-FU (Fig.17, Fig.18).

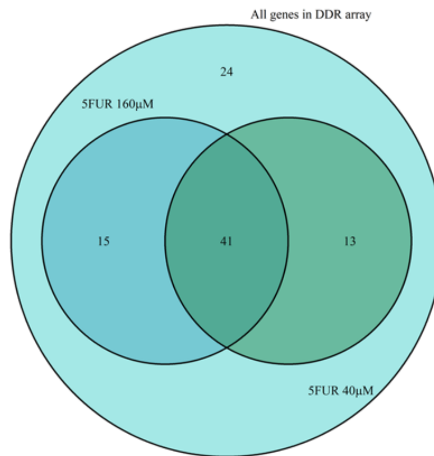


Fig.17.: Venn diagram. Out of 88 analyzed genes, 41 were significantly changed in both resistant cell lines, 13 only in 5FUR40 and 15 only in 5FUR160.

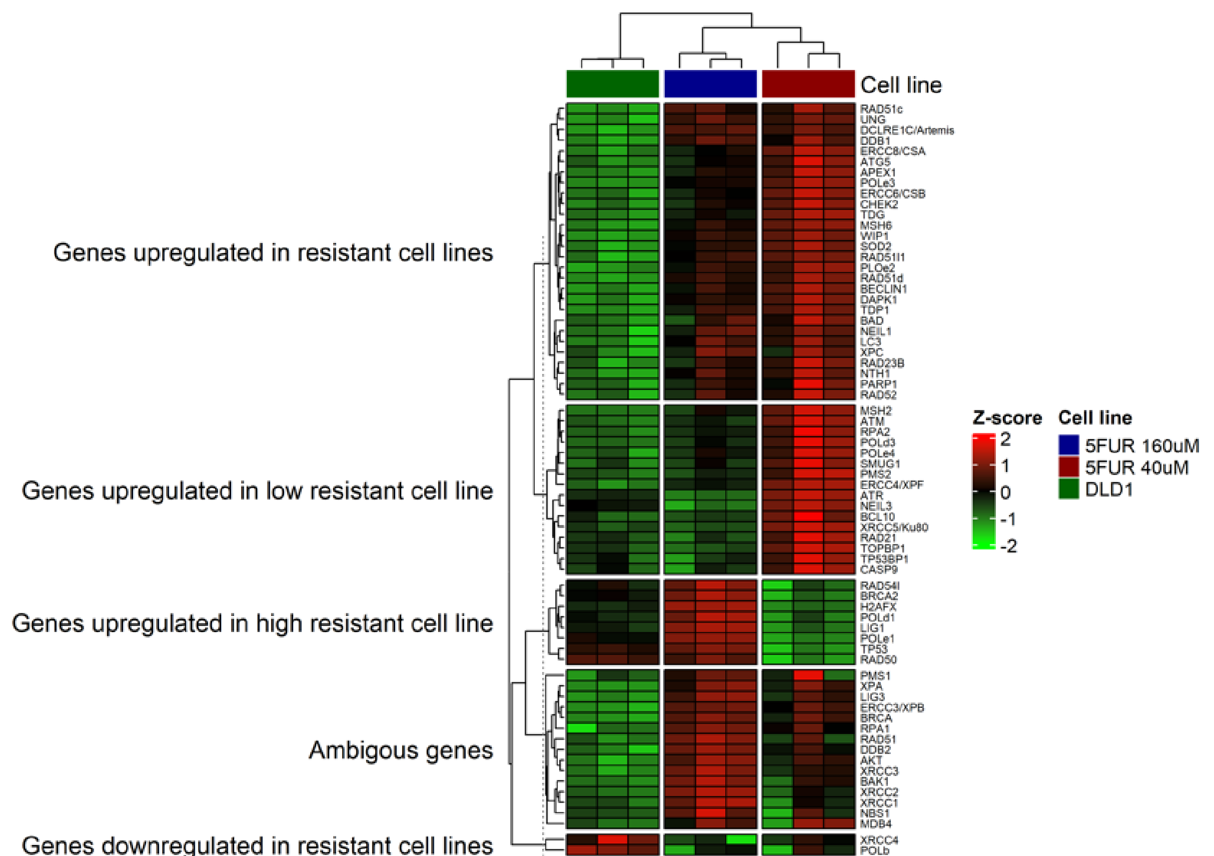


Fig.18.: DDR and DNA repair genes heatmap. The green color represents lower expression, the red color higher expression.

Altogether, the expression of 69 out of 88 analyzed genes was significantly altered at least in one resistant cell line, making it almost 80% of DDR and DNA repair genes being dysregulated in the development of 5-FU resistance. However, comparing our data with data from TCGA database (Supplement 1) and analyzing data on gene expression of 155 CRC patients treated with 5-FU-based therapy, 9 genes were significantly associated with therapy response and at the same time, significantly dysregulated in our resistant cell lines: *ATM*, *DAPK1*, *RAD51L1*, *RAD52*, *TDG*, *TDP1*, *TOPBP1*, *TP53BP1* and *XRCC2*. These genes are involved in HR (*RAD51L1*, *RAD52*, *XRCC2*), BER (*TDG*, *TDP1*), cell death (*DAPK1*) and DDR (*ATM*, *TP53BP1*, *TOPBP1*) (Fig.19).

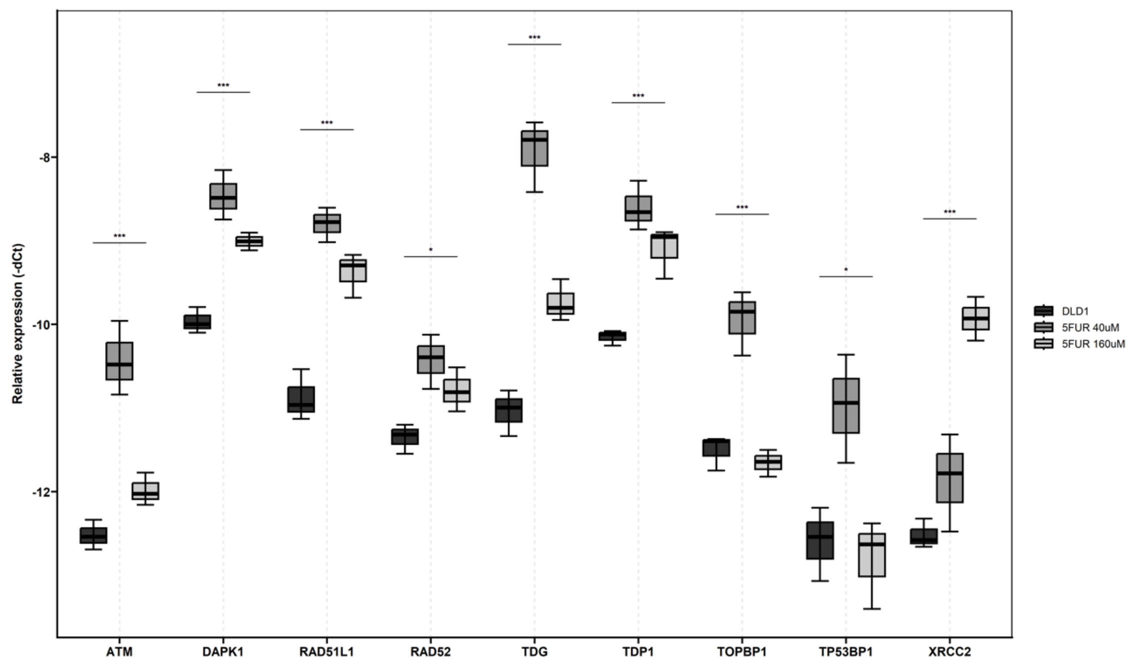


Fig.19.: Gene expression boxplot. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001.

Because the fundamental role of 5-FU and other chemotherapeutics is to cause DNA damage, it is evident, that the associations of DNA repair and DDR pathways with 5-FU resistance are of great interest. The involvement of BER and MMR in 5-FU therapy response is already well acknowledged (Vodenkova et al. 2020). However, the role of HR in 5-FU sensitivity, resistance and consequently therapy efficacy is still not well elucidated.

All 9 DDR and DNA repair genes, which expression was significantly changed and were associated with survival of CRC patients treated with 5-FU, were already studied in relationship with different cancers. Germline mutations in *RAD51L1* are rare

but have been linked with breast and ovarian cancer risk (Buys et al. 2017; Song et al. 2015). RAD51L1 (also called RAD51B) is a paralog of RAD51 and a part of a multi-protein complex (BCDX2). Its inhibition leads to HR deficiency and higher sensitivity to DNA-damaging agents (Lee et al. 2014). RAD52 is another important protein in the repair of DSBs, responsible for promoting complementary ssDNA annealing (Nogueira et al. 2019). RAD52 has been proposed to be a new interesting target for synthetic-lethality-based therapies when resistance to PARP inhibitors occurs (Malacaria et al. 2020). XRCC2 is another paralog of RAD51, and its downregulation has already been linked to higher sensitivity to 5-FU in CRC (Zhang et al. 2017). TDG is a DNA-glycosylase involved in BER. Controversially, Miao *et al.* recently associated TDG overexpression with the better OS of CRC patients and suppression of invasive behavior. However, we observed its overexpression in both 5-FU resistant cell lines and, according to TCGA database, its overexpression is associated with worse disease-specific survival (DSS). TDP1 is a phosphodiesterase with a role not only in BER but also in NHEJ, which is another pathway for repairing of DSBs (Heo et al. 2015). DAPK1 protein, a member of the DAPK family, is involved in apoptosis, necrosis, and autophagy. It is considered a tumor-suppressor, which could indicate that its overexpression is not associated with chemoresistance, but rather with the cellular response to high doses of cytotoxic 5-FU (Bialik and Kimchi 2006). ATM kinase is an essential part of HR, responsible for sensing of DSBs and downstream activation of DDR pathways. ATM inhibitors are already studied for their utilization as chemo-/radio-sensing agents in cancer therapy, e.g., in combination therapy with Irinotecan and 5-FU in CRC (Davis et al. 2022). The loss of 53BP1 encoded in the *TP53BP1* gene is associated with resistance to 5-FU in CRC (Li et al. 2013). Interestingly, we observed the overexpression of *TP53BP1* in the 5FUR40 resistant cell line and its downregulation in the 5FUR160 cell line. *TOPBP1* encodes Topoisomerase IIβ binding protein 1 which interacts with Topoisomerase IIβ and is involved in processes of DDR, checkpoint activation, replication, and transcription (Wu et al. 2017; Lv et al. 2016). Its downregulation sensitizes cancer cells to a variety of genotoxic agents such as doxorubicin, cisplatin, or Mitomycin C. This protein interacts with NBS1 and is directly involved in HR (Morishima et al. 2007).

In this study, we showed that dysregulated expression of genes involved in DDR and DNA repair is crucial for the development of acquired resistance to 5-FU. It is noteworthy, that most of the relevant genes, that were significantly overexpressed in our resistant cell lines and simultaneously their expression was significantly associated with

survival and/or progression of 5-FU-treated CRC patients (TCGA database, Supplement 1), were mostly related to HR repair pathway, either in sensing of DSBs, promoting the downstream reaction or as the core repair proteins. That underlies the fundamental importance of HR, not only in response to chemotherapeutics designed to cause DSBs (such as platinum compounds) but also to other cytotoxic agents like 5-FU. 5-FU, when incorporated into DNA, is recognized with the BER pathway. These lesions, when unrepaired, give arise to DSBs, which are then repaired by HR.

We previously (Manuscript 4) studied Mirin, a HR inhibitor, and described its ability to sensitize OVC cells to carboplatin. Therefore, we focused on the effect of Mirin on 5-FU sensitivity in parental CRC DLD1 cells. Cells were treated with 5 µL 5-FU with an eventual 1hr of 100 µM Mirin pre-treatment. We observed a similar decrease in cell proliferation (Fig. 21), compared to 5-FU treatment alone.

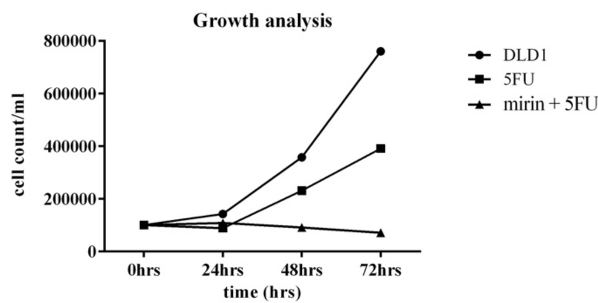


Fig. 21.: Growth analysis of parental DLD1 cells. Untreated cells vs. 5 µL 5-FU vs 5 µL 5-FU with 1hr 100 µM Mirin pre-treatment

These results point out the key role of HR in response to 5-FU. The addition of HR inhibitors, such as Mirin, is a promising tool for enhancing sensitivity to conventional chemotherapeutics. To evaluate the effect of Mirin on 5-FU response in CRC, further research is necessary, such as studying its effect on different parental and 5-FU resistant CRC cell lines or *in vivo* experiments on mice. HR inhibitors (such as RAD51, RAD52, MRE11, ATM, ATR inhibitors) and their possible utilization in a great variety of cancers and therapy regimes, are currently of great interest to researchers.

5. CONCLUSIONS

This dissertation Thesis aimed to elucidate some of the molecular mechanisms involved in resistance and sensitivity towards commonly used chemotherapeutics.
The following paragraphs summarize the results of the present Thesis:

1. We addressed the role of three miRSNPs variations in DNA repair genes *SMUG1* and *NEIL2* in the therapy response of BC patients. We found an association of *NEIL2* rs6997097 C allele with worse OS and DFS in a group of patients receiving only hormonal therapy. No associations of these miRSNPs were found in patients receiving conventional chemotherapeutics, as our group previously reported in CRC patients. However, these results confirm the importance of fine regulation of DNA repair genes expression via miRNA in cancer therapy response.

2. We investigated the possibility of combining natural compounds, such as *Ganoderma lucidum* extract (GLC), with conventional chemotherapeutics like 5-FU, to improve the cell chemosensitivity and therapy response. We confirmed that GLC has anticancer activity, causes oxidative DNA damage, and enhances the effect of 5-FU both *in vitro* and *in vivo*. Interestingly, GLC has rather a protective effect on non-malignant cells, making it a promising addition to conventional therapy regimes for decreasing a necessary dose of cytotoxic chemotherapeutics and minimizing their side effects.

3. We investigated the role of miRNA regulation of MRE11, an important DNA repair protein involved in homologous repair, in sensitivity to oxaliplatin. We confirmed that miR-140 presumably targets MRE11, suppresses its expression and sensitize CRC cells to oxaliplatin. Our results suggest that miR-140 act as a tumor suppressor and plays an important role in HR and CRC therapy response.

4. We explored the effect of MRE11 inhibitor, Mirin, on chemoresistant OvC cells. Mirin significantly increased cell sensitivity to carboplatin and was able to retrieve the carboplatin sensitivity also in carboplatin-resistant OvC cells. These results confirmed the importance of HR in response to platin derivatives.

5. We addressed the role of dysregulated expression of DDR and DNA repair genes in the process of establishing the acquired resistance to 5-FU in a CRC *in vitro* model. These results suggest the fundamental role of HR gene overexpression in 5-FU chemoresistance.

Our findings highlight the important role of DNA repair and damage response in therapy response, sensitivity, and resistance. We believe that elucidating the molecular mechanisms of resistance and sensitivity to chemotherapeutics would ultimately lead to more effective and targeted cancer treatment.

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7. PUBLICATION ACTIVITY

7.1. MANUSCRIPTS RELATED TO THE THESIS

7.1.1. Original Research Articles: Manuscripts 1-4 (Supplement 2)

Manuscript 1: Genetic variations in 3'UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response.

Cumova, A.; Vymetalkova, V.; Opattova, A.; Bouskova, V.; Pardini, B.; Kopeckova, K.; Kozevnikovova, R.; Lickova, K.; Ambrus, M.; Vodickova, L.; Naccarati A.; Soucek P.; Vodicka P. Genetic variations in 3'UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response. *Mutagenesis* 2021, *36*, 269-279, doi:10.1093/mutage/geab017.

IF (2021) = 3.000

Manuscript 2: *Ganoderma Lucidum* induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo.*

Opattova, A.; Horak, J.; Vodenkova, S.; Kostovcikova, K.; **Cumova, A.**; Macinga, P.; Galanova, N.; Rejhova, A.; Vodickova, L.; Kozics, K., Turnovcova K.; Hucl T.; Sliva D.; Vodicka P. Ganoderma Lucidum induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo*. *Mutat Res Genet Toxicol Environ Mutagen* 2019, *845*, 403065, doi:10.1016/j.mrgentox.2019.06.001.

IF (2019) = 2.506

Manuscript 3: MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients.

Horak, J.; Dolnikova, A.; Cumaogullari, O.; **Cumova, A.**; Navvabi, N.; Vodickova, L.; Levy, M.; Schneiderova, M.; Liska, V.; Andera, L.; Vodicka P.; Opattova A. MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients. *Front Oncol* 2022, *12*, 959407, doi:10.3389/fonc.2022.959407.

IF (2022) = 5.738

Manuscript 4: Inhibition of homologous recombination repair by Mirin in ovarian cancer ameliorates carboplatin therapy response *in vitro.*
Horak. J.; Vallusova D.; **Cumova, A.**; Holy, P.; Vodicka, P.; Opattova A.
Submitted to *Mutation Research: Genetic Toxicology and Environmental Mutagenesis*
IF (2023) = 3.189

7.1.2. Review Articles: Manuscripts 5 and 6 (Supplement 2)

Manuscript 5: Natural compounds and combination therapy in colorectal cancer treatment.
Rejhova, A.; Opattova, A.; **Cumova, A.**; Sliva, D.; Vodicka, P. Natural compounds and combination therapy in colorectal cancer treatment. *Eur J Med Chem* 2018, *144*, 582-594, doi:10.1016/j.ejmech.2017.12.039.
IF (2018) = 4.833

Manuscript 6: DNA Repair and Ovarian Carcinogenesis: Impact on Risk, Prognosis and Therapy Outcome.
Tomasova, K.∗; **Cumova, A.∗**; Seborova, K.; Horak, J.; Koucka, K.; Vodickova, L.; Vaclavikova, R.; Vodicka, P. DNA Repair and Ovarian Carcinogenesis: Impact on Risk, Prognosis and Therapy Outcome. *Cancers (Basel)* 2020, *12*, doi:10.3390/cancers12071713.
(∗ shared first authorship)
IF (2020) = 6.639

7.2. MANUSCRIPTS NOT DIRECTLY RELATED TO THE THESIS

7.2.1. Original Research Articles: Manuscript 7

Manuscript 7: Telomere length in peripheral blood lymphocytes related to genetic variation in telomerase, prognosis and clinicopathological features in breast cancer patients.
Kroupa, M.; Rachakonda, S.; Vymetalkova, V.; Tomasova, K.; Liska, V.; Vodenkova, S.; **Cumova, A.**; Rossnerova, A.; Vodickova, L.; Hemminki, K., *et al.* Telomere length in peripheral blood lymphocytes related to genetic variation in telomerase, prognosis and clinicopathological features in breast cancer patients. *Mutagenesis* 2020, *35*, 491-497, doi:10.1093/mutage/geaa030
IF (2020) = 3.379