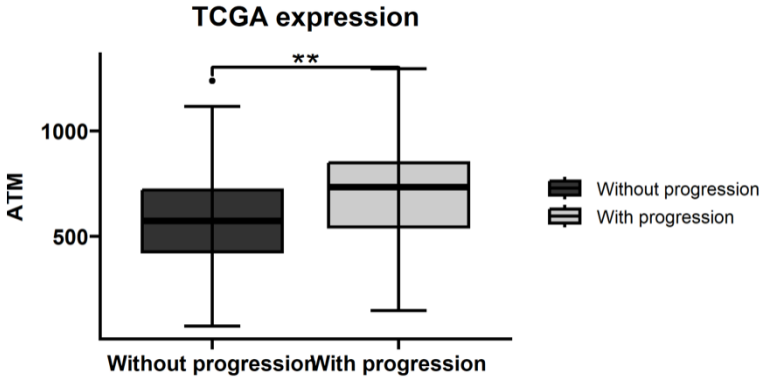
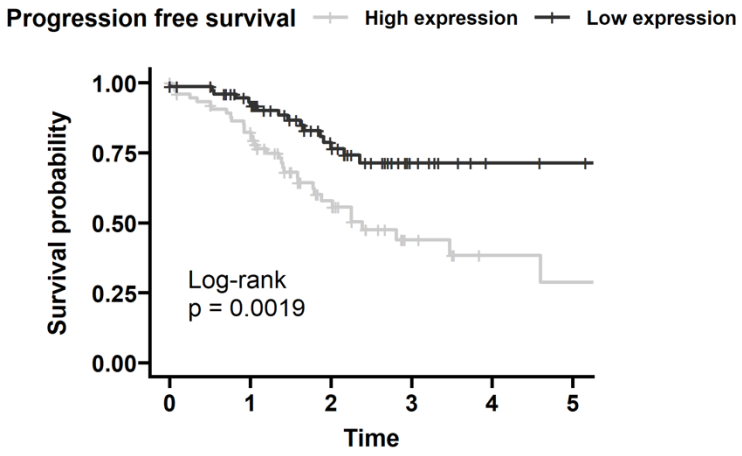
# SUPPLEMENT 1

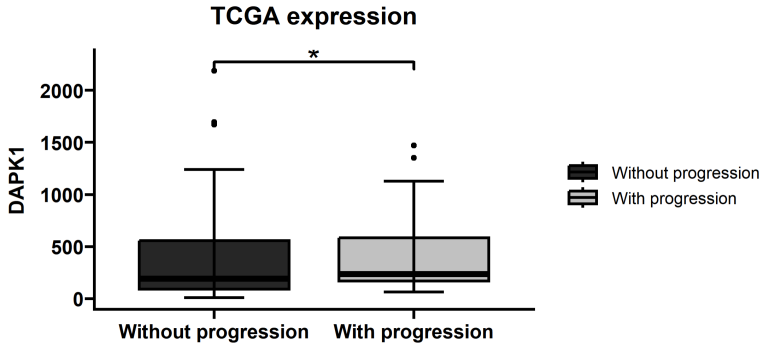
The results from the analysis of TCGA dataset. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001.

## 1. ATM

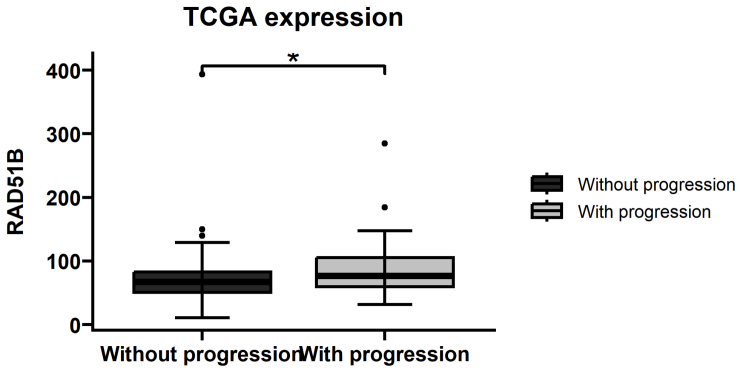


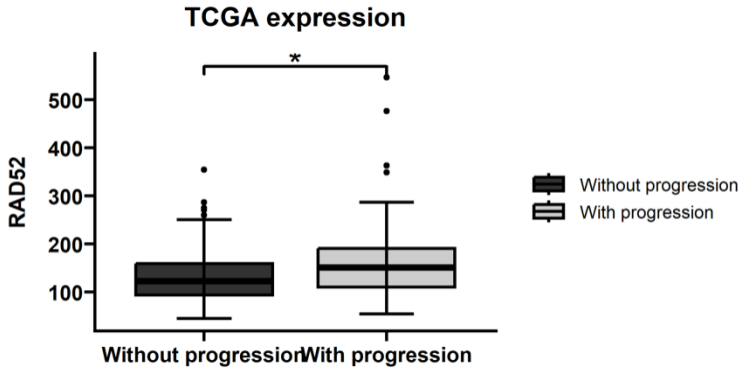

## B. DAPK1

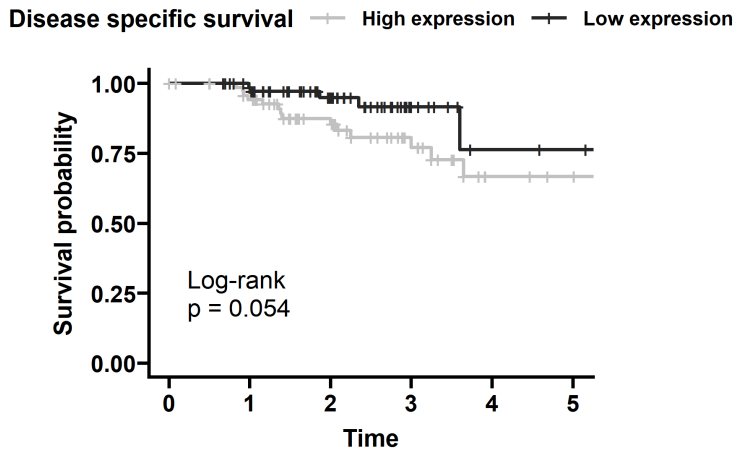


## C. RAD51L1 (RAD51B)

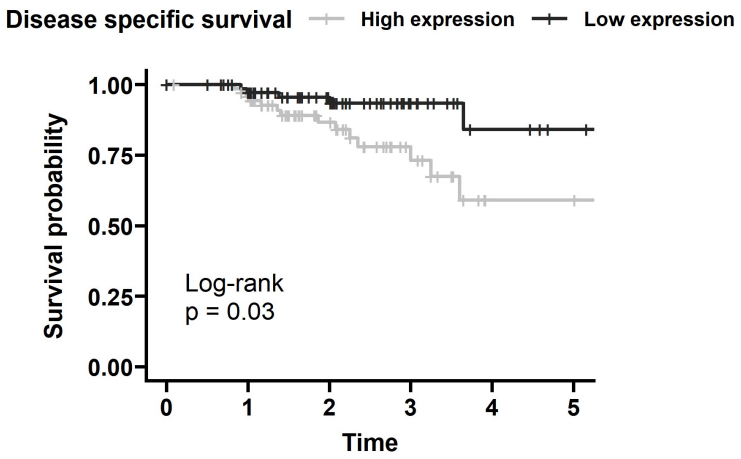



## D. RAD52

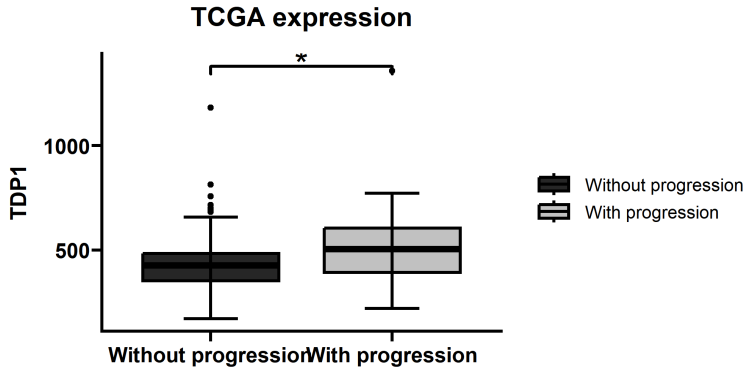


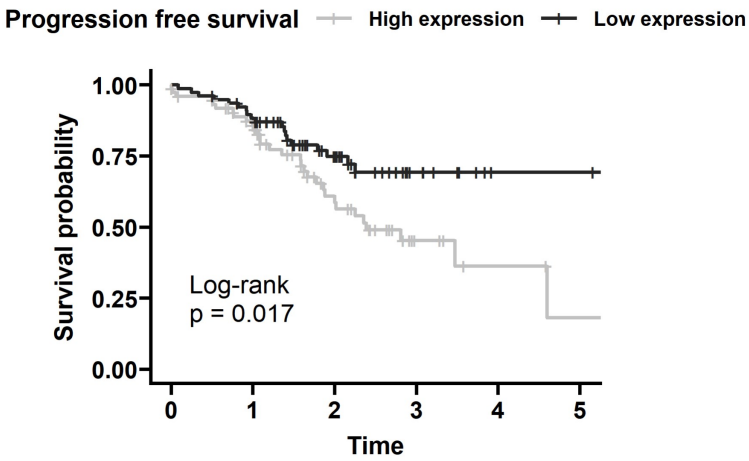



**E. TDG**

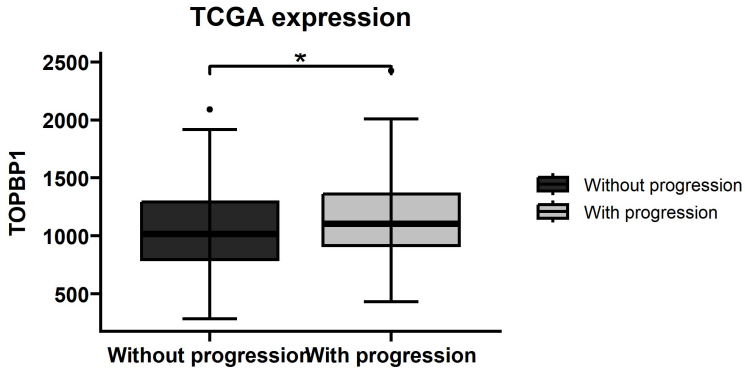

Disease specific survival
High expression
Low expression
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.03
0
1
2
3
4
5
Time

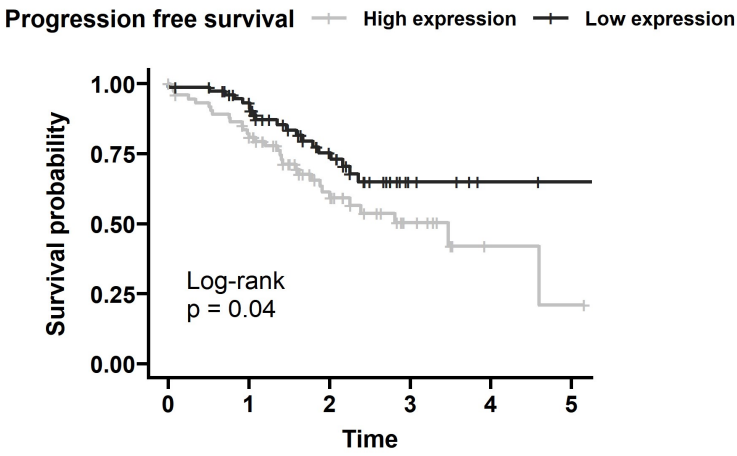
**F. TDP1**


TCGA expression
*
TDP1
1000
500
Without progression
With progression
Without progression
With progression

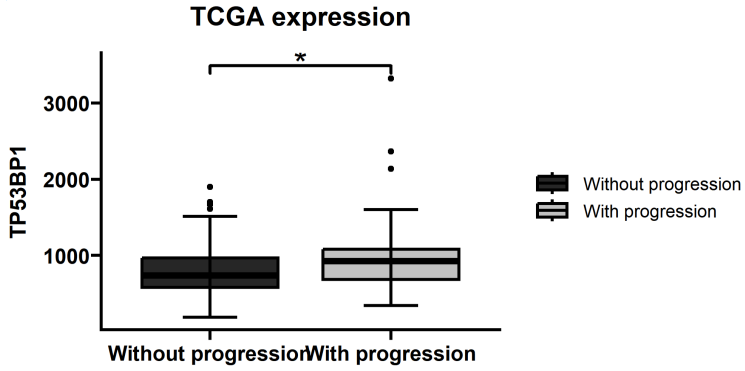

Progression free survival
High expression
Low expression
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.017
0
1
2
3
4
5
Time

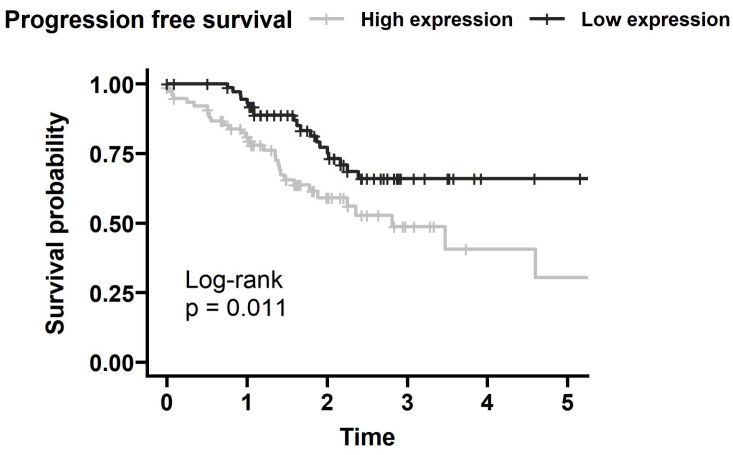
## G. TOPBP1



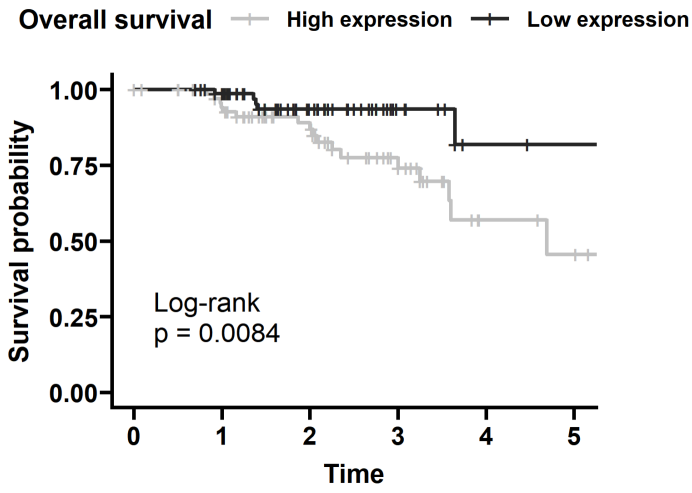



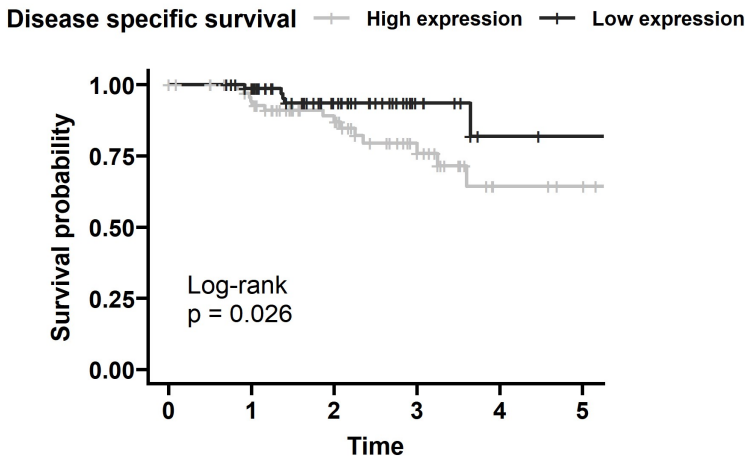

## H. TP53BP1




Progression free survival
High expression
Low expression
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.011
0
1
2
3
4
5
Time

## I. XRCC2


Overall survival
High expression
Low expression
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.0084
0
1
2
3
4
5
Time


Disease specific survival
High expression
Low expression
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank
p = 0.026
0
1
2
3
4
5
Time

# SUPPLEMENT 2

Manuscript 1:

**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.

Manuscript 2:

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.

Manuscript 3:

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.

Manuscript 4:

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

Manuscript 5:

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.

Manuscript 6:

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)

*Mutagenesis*, 2021, 36, 269–279
doi:10.1093/mutage/geab017
Original Manuscript
Advance Access publication 7 June 2021

Original Manuscript

# Genetic variations in 3′UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response

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Received 1 December 2020; Editorial decision 28 May 2021; Accepted 6 June 2021.

## Abstract

Breast cancer (BC) is the most frequent malignancy in women accounting for approximately 2 million new cases worldwide annually. Several genetic, epigenetic and environmental factors are known to be involved in BC development and progression, including alterations in post-transcriptional gene regulation mediated by microRNAs (miRNAs). Single nucleotide polymorphisms (SNPs) located in miRNA binding sites (miRSNPs) in 3′-untranslated regions of target genes may affect miRNA-binding affinity and consequently modulate gene expression. We have previously reported a significant association of miRSNPs in the *SMUG1* and *NEIL2* genes with overall survival in colorectal cancer patients. SMUG1 and NEIL2 are DNA glycosylases involved in base excision DNA repair. Assuming that certain genetic traits are common for solid tumours, we have investigated wherever variations in *SMUG1* and *NEIL2* genes display an association with BC risk, prognosis, and therapy response in a group of 673 BC patients and 675 healthy female controls. Patients with TC genotype of *NEIL2* rs6997097 and receiving only hormonal therapy displayed markedly shorter overall survival (HR = 4.15, 95% CI = 1.7–10.16, $P$ = 0.002) and disease-free survival (HR = 2.56, 95% CI = 1.5–5.7, $P$ = 0.02). Our results suggest that regulation of base excision repair glycosylases operated by miRNAs may modulate the prognosis of hormonally treated BC.

## Introduction

Breast cancer (BC) is the most frequent type of cancer in women and the second most common cancer worldwide (1). The development of BC is caused by the interplay of genetic, epigenetic and environmental factors. High- and medium-penetrance germline mutations in several genes, such as *BRCA1/2*, *PTEN*, *ATM*, *CHEK2*, *TP53*, *PALB2* and *BRIP1* account for about 20% of the familial

BC risk (reviewed by (2)). Recent genome-wide association studies (GWAS) have further identified approximately 200 low-penetrance BC susceptibility loci in sporadic BC (3–13). Altogether, these high-, medium- and low-penetrance variants explain only 50% of the heritable BC risk and much of the remaining genetic susceptibility (so-called missing heritability) for BC is still unknown (14). Most of the known BC-associated genetic variants are in intronic and intergenic regions and their role in BC pathogenesis is unclear (15).

There are four main molecular subtypes of BC. The majority of BC are hormone-receptor positive [oestrogen receptor (ER) and/or progesterone receptor (PR)] divided into (a) luminal A (low Ki-67) and (b) luminal B (high Ki-67). About 20% of BC cases are also positive for the human epidermal growth factor receptor 2 (HER2) while around 10–15% of cases are triple-negative BC (TNBC; ER-negative/PR-negative/HER2-negative). These molecular markers together with tumour stage and grade are influencing BC prognosis and are routinely considered when setting up an individual therapy regimen (16).

Standard BC therapy consists of surgery, radiotherapy and/or preoperative/postoperative systemic treatment (17). For a long time, 5-fluorouracil (5-FU) was used for the treatment of BC with response rates of around 25% when administered as a single agent (18). Currently, BC treatment includes mainly anthracyclines and taxanes (19) both in neo-adjuvant and adjuvant therapies (20). Hormonal therapy (tamoxifen and inhibitors of aromatases) is used in hormone-receptor positive BC, either alone or in combination with chemotherapy. On the other hand, HER2 positive BCs benefit mostly from new targeted therapies (e.g. trastuzumab or pertuzumab). Finally, aggressive TNBC tumours, which have a poor prognosis, do not benefit from either hormonal or HER2-targeted therapies, and treatment with cytotoxic chemotherapy remains their possible option.

More specific targeted BC therapies are represented by PARP inhibitors Olaparib and Talazoparib, approved by the U.S. Food and Drug Administration (FDA) for the use in *BRCA*-mutated BC (21,22) and are an option also in TNBC (23). New immunotherapeutic approaches are nowadays also being applied in BC therapy (24).

MicroRNAs (miRNAs) exert relevant roles in pathogenesis of numerous diseases, including cancer. These small non-coding RNAs modulate the expression of protein-coding genes at post-transcriptional level via degradation or inhibition of translation of specific target mRNA. Deregulated miRNA expression has been observed in several types of cancer (25,26). In BC, miRNA expression levels may be involved in BC initiation, progression, metastasis or resistance to therapy (27–32). Different BC subtypes also exhibit differential patterns of miRNA expression (33,34). Moreover, single-nucleotide polymorphisms (SNPs) in 3′-untranslated regions (UTRs) of miRNA target genes (miRSNPs) may alter miRNA binding and consequently their function. MiRSNPs in 3′UTRs can also alter polyadenylation of target mRNA and protein-mRNA interactions (35,36). Hence miRSNPs can represent promising biomarkers of BC susceptibility and personalized therapy (37,38).

We have previously reported that miRSNPs in the 3′UTR of *SMUG1* (rs2233921 and rs971) and *NEIL2* (rs6997097) modulate colorectal cancer (CRC) prognosis and therapy response. The function of *SMUG1* (rs2233921) was confirmed by dual-luciferase *in vitro* reporter assay (39). Additionally, the common *TP53* haplotype, predictive of CRC and pancreatic cancer risk (40,41), was also associated with increased susceptibility to BC (42).

Here we intended to explore whether a particular genetic background (associated with DNA repair) may exert common features for BC and CRC. Various evidence supports this hypothesis. One of the strongest arguments is 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) (43,44). However, these known mutations cannot explain all the observed familial clustering of BC and CRC.

Defects in the DNA repair and the consequent accumulation of DNA lesions are key hallmarks of cancer (45). *SMUG1* and *NEIL2* genes encode base excision repair (BER) DNA glycosylases, responsible for corrections of small base lesions in DNA. SMUG1 removes mis-incorporated uracil and uracil derivatives that are linked with combination chemotherapy of BC (46). On the other hand, NEIL2 is responsible for removing oxidized derivates of cytosine (e.g. 5-hydroxyuracil). Variations in DNA repair pathway genes, including BER, are often associated with breast carcinogenesis (47).

Considering the important role of DNA repair in breast carcinogenesis, we have investigated the association of miRSNPs in *SMUG1* and *NEIL2* genes with risk, prognosis and therapy response in BC patients.

## Materials and Methods

All methods were carried out in accordance with relevant guidelines and regulations.

### Study population

The study population included 673 BC patients and 675 healthy controls as described in (42). All subjects included in the study provided written informed consent to participate in the study and to use their biological samples for genetic analyses, according to the Helsinki declaration. The design of the study was approved by the Ethics Committee of the Institute of Experimental Medicine, Prague, Czech Republic.

Blood samples of patients were obtained from women with incident BC consecutively diagnosed in three hospitals in Prague (Czech Republic) between February 2002 and December 2010, as described elsewhere (48–50).

The control group consisted of two groups of healthy women, selected from the larger control group composed of both sexes, described previously in 40,51–53. The first group consisted of 332 individuals who were admitted to gastroenterological departments for the 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 centre in Prague. All individuals underwent standard examinations to verify the health status and were cancer-free at the time of the sampling.

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), tumour size, International Union Against Cancer (UICC) tumour-node-metastasis (TNM) classification, histological type and grade of the tumour, expression of ER, PR and HER2; expression of the Ki-67 protein; chemotherapy and hormonal regimen. HER2 status was defined as positive in samples with an immunohistochemical score of 2+ or 3+ (54) and gene amplification confirmed by fluorescence *in situ* hybridization (FISH) or silver *in situ* hybridization (SISH). For all cases, information on radiotherapy, neo-adjuvant and/or adjuvant chemotherapy, and hormonal therapy were collected (Supplementary File, available at *Mutagenesis* Online and Supplementary Table 1, , available at *Mutagenesis*

**Table 1.** Genotype distribution of the investigated polymorphisms in breast cancer patients and controls

| Rs ID | Model | Genotype | Controls[a] | Cases[a] | OR[b] | 95% CI | *P* | Chi-square, P-value HWE[c] |
|---|---|---|---|---|---|---|---|---|
| *SMUG1*_rs2233921 | Co-D | GG | 183 | 203 | ref | | | 0.51, 0.77 |
| | | GT | 342 | 308 | 0.74 | 0.57–0.97 | **0.030** | |
| | | TT | 115 | 143 | 1.12 | 0.79–1.59 | 0.53 | |
| | D | GT+TT | 457 | 451 | 0.86 | 0.64–1.07 | 0.14 | |
| | R | GG+GT | 525 | 511 | ref | | | |
| | | TT | 115 | 143 | 1.36 | 1.01–1.83 | **0.045** | |
| *SMUG1*_rs971 | Co-D | CC | 258 | 241 | ref | | | 1.04, 0.59 |
| | | CT | 306 | 329 | 1.06 | 0.82–1.37 | 0.65 | |
| | | TT | 76 | 94 | 1.37 | 0.96–2.02 | 0.12 | |
| | D | CT+TT | 382 | 423 | 1.12 | 0.88–1.42 | 0.37 | |
| | R | CC+CT | 564 | 570 | ref | | | |
| | | TT | 76 | 94 | 1.30 | 0.92–1.86 | 0.14 | |
| *NEIL2*_rs6997097 | Co-D | TT | 560 | 565 | ref | | | 0.01, 0.99 |
| | | TC | 85 | 83 | 0.91 | 0.64–1.28 | 0.57 | |
| | | CC | 3 | 2 | 0.70 | 0.10–4.82 | 0.72 | |
| | D | TC+CC | 88 | 85 | 0.90 | 0.64–1.27 | 0.54 | |
| | R | TT+TC | 645 | 648 | ref | | | |
| | | CC | 3 | 2 | 0.71 | 0.10 - 4.86 | 0.73 | |

[a]Numbers may not add up to 100% of subjects due to genotyping failure. All samples that did not give a reliable result in the first round of genotyping were resubmitted to up to two additional rounds of genotyping. Data points that were still not filled after this procedure had been left blank.

[b]Logistic regression analysis values are adjusted for age.

[c]Chi-square and *P* values for the deviation from the Hardy–Weinberg equilibrium (HWE) in the controls.

Online). Patients were treated with chemotherapy regimens containing 5-FU, anthracyclines and/or taxanes. Hormonal therapy was based on the administration of inhibitors of aromatases and tamoxifen. Information about distant metastases, relapse and date of death were also collected. The follow-up time was 120 months. The measured outcome variables were overall survival (OS) and disease-free survival (DFS).

### SNP selection and genotyping

*SMUG1* rs2233921 G>T, *SMUG1* rs971 G>A and *NEIL2* rs6997097 T>C polymorphisms were analysed since they showed the significant prognostic value in another solid cancer (39). Freely available software MicroSNiper (55) was used to identify the miRSNPs within the target-binding sites for miRNAs in *SMUG1* and *NEIL2* genes. MiRSNPs were tested for minor allele frequency (MAF; >5% in Caucasian populations) in the SNP database (56) on the basis of HAPMAP CEU population. When this was not possible, other reference populations were considered (i.e. 1000 genomes, Phase I, CEU population). MiRSNPs with the required MAF were further tested for the linkage disequilibrium (LD) with other miRSNPs using HaploReg v. 4.1 (57) using data from the 1000 genomes project.

Genomic DNA was isolated from peripheral blood lymphocytes using standard procedures as previously described in (42). DNA samples from cases and controls were randomly placed on plates where an equal number of cases and controls were run simultaneously. Genotyping was carried out by using the KASP™ chemistry, a competitive allele-specific PCR-based SNP genotyping system (LGC Genomics) as described in (58). Duplicate samples (5%) and non-template controls in each plate were used as quality control tests. The genotype correlation between the duplicate samples was >99%. The genotype call rate ranged between 97.0 and 99.5%.

### Bioinformatic and statistical analyses

**Identification of miRNAs associated with studied miRSNPs.**

Freely available software MicroSNiPer (55), PolymiRTS (59) and Mirsnpscore (60) were used for the prediction of putative miRNAs targeting binding sites within miRSNPs. These online tools offer a computer-based prediction of miRSNPs effects on miRNA-based gene regulation.

**Expression quantitative trait loci (eQTL) analysis of miRSNPs.**

The associations between the miRSNPs analysed in this study and gene expression levels were obtained from the Genotype-Tissue Expression project (GTEx). The GTEx project allows viewing and downloading computed eQTL results and aims to characterise variations in gene expression levels across individuals and diverse tissues of the human body (61).

**Statistical analyses.**

Chi-square test with 1 degree of freedom and a type-I error threshold set at $\alpha = 0.05$ was used to verify the Hardy–Weinberg equilibrium of genotypes in controls. The association between miRSNPs and BC risk was calculated by estimating the odds ratios (ORs) and their 95% confidence intervals (CI) adjusted for age using logistic regression. For all SNPs, the co-dominant, dominant or recessive models were calculated. OS was defined as the time from the surgery to the date of death or of the last follow up. DFS was defined as the time elapsed from surgery to the occurrence of distant metastasis or local recurrence or death, whichever came first. The relative risks of death and recurrence were estimated as hazard ratios (HR) using Cox regression. The survival curves for OS and DFS were derived by the Kaplan–Meier method. Univariate survival analyses were adjusted for therapy regimen, molecular subtypes of BC and TNM stage. Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). The Bonferroni corrected significance threshold for multiple tests was set at 0.017 (for three miRSNPs and $\alpha = 0.05$).

### External validation

Associations of *SMUG1* and *NEIL2* gene expression and DFS and OS were investigated using freely available online tool GEPIA 2 (Gene Expression Profiling Interactive Analysis) (62). This website provides an interactive gene expression profiling based on tumour and normal tissue samples from TCGA and GTEx databases (63).

## Results

### Case–control study

The mean age of patients at the time of diagnosis was 59 years (range 27–92), the mean age of controls at the time of recruitment was 49 (range 22–91) years (Supplementary Table 2, available at *Mutagenesis* Online). The genotype distribution of all the analysed miRSNPs in the control group (consisting of two cohorts as described above) agreed with the Hardy–Weinberg equilibrium.

The GT genotype of *SMUG1* rs2233921 conferred lower risk of BC in a co-dominant model (OR = 0.74, 95% CI = 0.57–0.97, $P = 0.03$). Controversially, the TT genotype of *SMUG1* rs2233921 conferred a higher risk of BC in the recessive model (OR = 1.36, 95% CI = 1.01–1.83, $P < 0.05$). Both associations were not significant after applying the Bonferroni correction ($P > 0.017$). No other significant associations with BC risk were observed in the case–control study (Table 1).

### Clinico-pathological parameters

Sporadic BC represents a complex disease with numerous genetic, environmental and life-style factors in its aetiology. To address the effect of personal and clinico-pathological parameters on survival, age, TNM stage, grade and ER/PR/HER2/Ki76 status were correlated with patient's OS and DFS. Older patients (>70 years), patients with TNM stages 3 and 4, with metastases at the time of diagnosis, and with grade 4 BC had significantly worse OS and DFS. On the other hand, patients with PR, ER or Ki76 positive tumours had significantly better OS and DFS (Supplementary Table 3, available at *Mutagenesis* Online).

### Prognostic significance

The median OS and DFS for the studied population were 86.9 and 85.0 months, respectively. Overall, 115 BC patients died (73 of them due to cancer progression), 558 patients were alive during follow up (96 subjects had recurrence) (Supplementary Table 1, available at *Mutagenesis* Online). None of the studied miRSNPs appeared to be significantly associated with OS or DFS in the overall group of BC patients (Supplementary Table 4, available at *Mutagenesis* Online. After stratification according to therapy regimen, median OS for patients receiving only hormonal therapy was 75.6 months, median DFS 71.9 months; median OS for patients receiving any adjuvant chemotherapy 96.1 months, median DFS 90.9 months; median OS for patients receiving 5-FU based chemotherapy 90.1 months, median DFS 85.1; median OS for patients receiving neoadjuvant

**Table 2.** Association of *SMUG1* and *NEIL2* polymorphisms with OS and DFS in patients with early-stage BC (TNM 1+2) (Cox regression)

| | | | | OS | | | | DFS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rs ID | Model | Genotype | Cases | Events | HR | 95% CI | *P* | Events | HR | 95% CI | P |
| *SMUG1*_rs2233921 | Co-D | GG | 154 | 22 | ref | | | 32 | ref | | |
| | | GT | 226 | 32 | 0.93 | 0.54–1.60 | 0.78 | 47 | 0.94 | 0.60–1.47 | 0.77 |
| | | TT | 109 | 10 | 0.62 | 0.29–1.30 | 0.20 | 23 | 0.98 | 0.58–1.68 | 0.95 |
| | D | GT+TT | 335 | 42 | 0.83 | 0.49–1.39 | 0.47 | 70 | 0.95 | 0.63–1.45 | 0.82 |
| | R | GG+GT | 380 | 54 | ref | | | 79 | ref | | |
| | | TT | 109 | 10 | 0.65 | 0.33–1.27 | 0.21 | 23 | 1.03 | 0.65–1.64 | 0.90 |
| *SMUG1*_rs971 | Co-D | CC | 177 | 19 | ref | | | 39 | ref | | |
| | | CT | 245 | 31 | 1.19 | 0.67–2.10 | 0.56 | 46 | 0.84 | 0.55–1.29 | 0.43 |
| | | TT | 75 | 15 | 2.10 | 1.07–4.14 | **0.03** | 18 | 1.23 | 0.71–2.16 | 0.46 |
| | D | CT+TT | 320 | 46 | 1.39 | 0.81–2.37 | 0.23 | 64 | 0.93 | 0.62–1.38 | 0.71 |
| | R | CC+CT | 422 | 50 | ref | | | 85 | ref | | |
| | | TT | 75 | 15 | 1.90 | 1.07–3.38 | **0.03** | 18 | 1.35 | 0.81–2.25 | 0.25 |
| *NEIL2*_rs6997097 | Co-D | TT | 425 | 53 | ref | | | 83 | ref | | |
| | | TC | 57 | 9 | 1.27 | 0.63–2.58 | 0.50 | 17 | 1.57 | 0.93–2.65 | 0.09 |
| | | CC | 1 | 0 | n/a | | | 0 | n/a | | |
| | D | TC+CC | 58 | 9 | 1.25 | 0.62–2.53 | 0.54 | 17 | 1.54 | 0.91–2.59 | 0.11 |
| | R | TT+TC | 482 | 62 | ref | | | 100 | ref | | |
| | | CC | 1 | 0 | n/a | | | 0 | n/a | | |

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; Co-D, co-dominant; D, dominant; R, recessive model.
Significant results in bold. Bonferroni-corrected *P* value threshold = 0.017

chemotherapy 96.1, median DFS 90.4 and median OS for patients without neoadjuvant therapy 86.7 months, median DFS 84.

No significant associations were observed after patients' stratification according to the molecular subtype of BC (hormonal-receptor positive luminal subtypes, HER2 positive and TNBC) (Supplementary Tables 5–7, available at *Mutagenesis* Online). After stratification according to the TNM stage (TNM 1+2 vs. 3+4; Supplementary Tables 8 and 9 , available at *Mutagenesis* Online), we found that the TT genotype of *SMUG1* rs971 in patients with early BC (TNM stage 1+2) was 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 (Table 2).

Since different therapies were applied to BC patients according to the diverse molecular subtypes of BC and the stage of the disease (Supplementary Table 1, available at *Mutagenesis* Online), we have further stratified patients into subgroups according to their therapy regimen (outcomes are summarized in Supplementary Tables 10–14, available at *Mutagenesis* Online). 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 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) (Table 3; Figure 1).

### Bioinformatic analyses

Three different freely available online tools (MicroSNiPer, PolymiRTS and Mirsnpscore) were used to identify miRNA binding analysed miRSNPs. Despite each software uses a different algorithm, several miRNAs were predicted by more than one software (see Supplementary Table 15, available at *Mutagenesis* Online: (1) miR-770-5p targeting *SMUG1* rs2233921 when harbouring T allele (by PolymiRTS and Mirsnpscore); (2) miR-455-3p (by PolymiRTS and Mirsnpscore) and miR-655 targeting *SMUG1* rs2233921 when harbouring G allele (by PolymiRTS and Mirsnpscore); (3) miR-541-5p/miR-541* targeting *NEIL2* rs6997097 when harbouring T allele (by MicroSniper and Mirsnpscore); (4) miR-5681a when *NEIL2* rs6997097 when harbouring C allele (by MicroSniper and PolymiRTS).

According to GTEx data, the significant eQTLs were found for *SMUG1* rs2233921 and *SMUG1* rs971 miRSNPs (Supplementary Table 15, available at *Mutagenesis* Online. *SMUG1* rs2233921 regulated *FLJ12825*, *RP11-834C11.10*, *SMUG1* and *RP11-834C11.11* and *SMUG1* rs971 regulated *FLJ12825* and *RP11-834C11.11* genes in breast mammary tissue. No significant eQTLs were found for *NEIL2* rs6997097.

### GEPIA 2 gene expression profiling

GEPIA 2, an interactive online tool, 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. The median gene expression was used for the cut-off of the low/high *SMUG1* or *NEIL2* group of patients. In this set, *NEIL2*-high patients with HER2 positive and non-luminal BC exhibited significantly worse OS than *NEIL2*-low patients (Log rank $P$ = 0.04) (Fig. 2). No other significant associations in other BC groups of patients were observed for *NEIL2* and *SMUG1* genes.

## Discussion

In the present study, we have investigated the potential role of three miRSNPs in the 3′UTRs of genes encoding BER glycosylases SMUG1 and NEIL2 in the susceptibility to BC and clinical outcome in a group of 673 BC patients and 675 healthy controls. We found that *NEIL2* rs6997097 was significantly associated with shorter OS (HR = 4.15, 95% CI = 1.7–10.16, $P$ = 0.002) and DFS (HR = 2.56, 95% CI = 1.5–5.7, $P$ = 0.02) of patients receiving hormonal therapy. On the other hand, *SMUG1* rs2233921 was moderately associated with BC risk and *SMUG1* rs971 conferred shorter OS in patients with early BC (TNM 1+2 stage). However, observed associations of *SMUG1* rs2233921 and rs971 did not pass the Bonferroni's correction for multiple testing. Linkage disequilibrium (LD) analysis of analysed miRSNPs showed, that *SMUG1* rs971 is in LD with 12 other SNPs with $r^2$ from 0.8 to 1. To our best knowledge, none of the SNPs in LD was previously studied for association with BC risk or prognosis.

Our group previously reported that these three miRSNPs significantly modulated CRC prognosis and therapy response (39). Variations in 3′UTR regions may potentially affect the binding of specific miRNAs to miRNA-binding sites resulting in the alteration of expression of target genes. These effects have been documented to affect cancer risk, prognosis or therapy response in many malignancies, including BC (64–72).

Despite the association between *SMUG1* rs2233921 and BC risk, the role of the T allele BC carcinogenesis is not clear as the association was highlighted for the heterozygous carriers. Besides, SMUG1 remains an important DNA glycosylase responsible for the repair of mis-incorporated uracil and this polymorphism may still emerge as a potential BC risk biomarker after future validation in larger and more homogenous case–control studies (73).

Significant associations were observed in the group treated with hormonal therapy, in which the TC genotype of *NEIL2* rs6997097 was significantly associated with shorter OS and DFS. The low frequency of the C allele (only three controls and two patients with the CC genotype) precluded evaluation of the genotype dosage and computing HR for CC bearers as well as the involvement of C allele in the worse prognosis of hormonally treated BC. An explanation for better outcomes in *NEIL2* rs6997097 T allele carriers might be the fact that according to MicroSNiPer and Mirsnpscore miR-541-5p has an increased affinity to the sequence in presence of T allele (60). In this context, several studies identified miR-541 as a tumour-suppressor miRNA in lung cancer (74–76). Additionally, miR-541 directly targets the 3′UTR of *HER2* and inhibits cell growth of HER2-positive breast cancer cell lines(77). However, the role of miR-541-5p in survival and therapy response of BC is still unclear and should be elucidated in the future. Hence, suggested effect of DNA repair gene miRSNP on hormonal therapy response may be explained by the recently reported relationship between DNA repair defects and hormonal therapy resistance (78,79). On the other hand, overexpression of miR-5681a (binding the *NEIL2* rs6997097 when the C allele is present) has been observed in ER-positive breast tumours and downregulation in metastatic pancreatic cancer (80,81).

The binding of several miRNAs was affected by studied miRSNPs for *SMUG1* gene. MiR-770-5p is supposed to target the *SMUG1* 3′UTR sequence when a variant T allele of rs2233921 is

**Table 3.** Associations of *SMUG1* and *NEIL2* polymorphisms with the OS and DFS in patients undergoing hormonal-only therapy (Cox regression)

| | | | OS | | | | | DFS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rs ID | Model | Genotype | Cases | Events | HR | 95% CI | *P* | Events | HR | 95% CI | P |
| *SMUG1*_rs2233921 | Co-D | GG | 60 | 10 | ref | | | 16 | ref | | |
| | | GT | 89 | 18 | 1.10 | 0.51–2.38 | 0.82 | 26 | 1.02 | 0.55–1.90 | 0.95 |
| | | TT | 47 | 3 | 0.34 | 0.09–1.22 | 0.10 | 12 | 0.89 | 0.42–1.89 | 0.77 |
| | D | GT+TT | 136 | 21 | 0.83 | 0.39–1.76 | 0.62 | 38 | 0.98 | 0.54–1.75 | 0.93 |
| | R | GG+GT | 149 | 28 | ref | | | 42 | ref | | |
| | | TT | 47 | 3 | 0.32 | 0.10–1.04 | 0.06 | 12 | 0.87 | 0.46–1.66 | 0.68 |
| *SMUG1*_rs971 | Co-D | CC | 78 | 9 | ref | | | 23 | ref | | |
| | | CT | 90 | 17 | 1.71 | 0.76–3.83 | 0.20 | 24 | 0.89 | 0.50–1.57 | 0.68 |
| | | TT | 32 | 7 | 2.27 | 0.84–6.10 | 0.15 | 9 | 1.10 | 0.51–2.38 | 0.81 |
| | D | CT+TT | 122 | 24 | 1.85 | 0.86–3.98 | 0.12 | 33 | 0.94 | 0.55–1.60 | 0.82 |
| | R | CC+CT | 168 | 26 | ref | | | 47 | ref | | |
| | | TT | 32 | 7 | 1.69 | 0.73–3.90 | 0.22 | 9 | 1.17 | 0.57–2.38 | 0.67 |
| *NEIL2*_rs6997097 | Co-D | TT | 176 | 25 | ref | | | 47 | ref | | |
| | | TC | 16 | 6 | 4.15 | 1.70–10.16 | **0.002** | 7 | 2.56 | 1.15–5.69 | ***0.02** |
| | | CC | 1 | 0 | n/a | | NA | 0 | n/a | | NA |
| | D | TC+CC | 17 | 6 | 3.52 | 1.44–8.61 | **0.006** | 7 | 2.16 | 0.97–4.78 | 0.06 |
| | R | TT+TC | 192 | 31 | ref | | | 54 | ref | | |
| | | CC | 1 | 0 | n/a | | NA | 0 | n/a | | NA |

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; Co-D, co-dominant; D, dominant; R, recessive model.
Significant results in bold. Bonferroni-corrected *P* value threshold = 0.017.

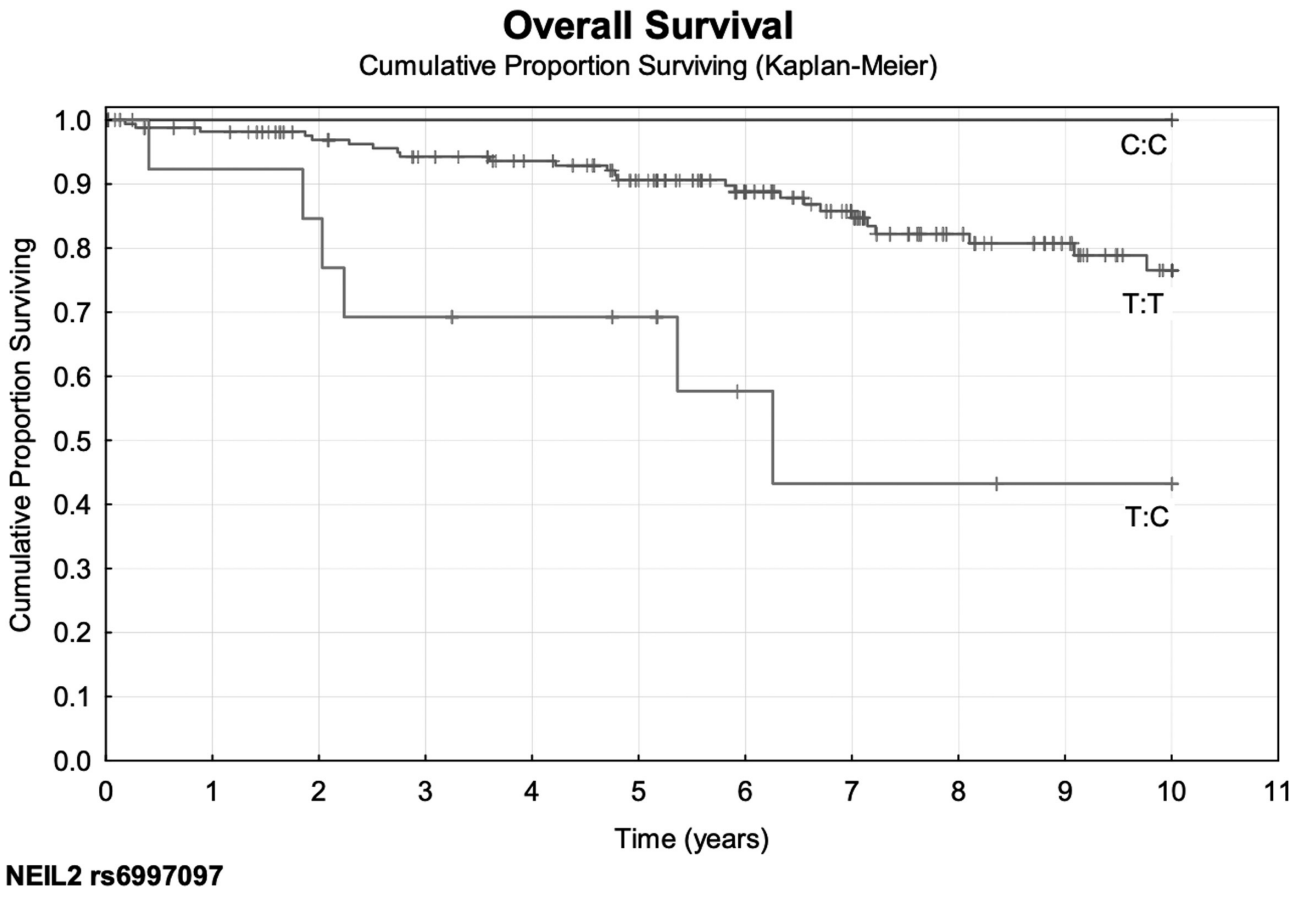


**Figure 1.** Kaplan–Meier overall survival curves of *NEIL* rs6997097 for patients undergoing hormonal-only therapy. TT vs TC vs CC.

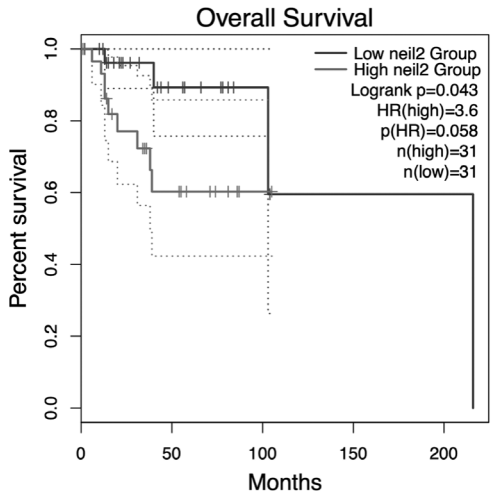


**Figure 2.** Analysis of the overall survival of BC patients with low/high *NEIL2* expression using GEPIA 2. Low vs high *NEIL2*. Image generated by GEPIA2.

present. A recent study showed that miR-770-5p expression was significantly decreased in doxorubicin chemo-resistant TNBC tumours and suppressed the metastasis in TNBC *in vitro* and *in vivo* in mice xenografts (82). Its overexpression also inhibits cell motility and invasiveness in HER2-positive BC cells and potentiates the effect of trastuzumab *in vitro* (83). This miRNA might be thus considered as a potential biomarker of BC invasiveness and therapy response. Overexpression of miR-455-3p that binds to the common G allele of *SMUG1* rs2233921 was associated with G0 cell cycle arrest in CRC cell lines (84) and inhibited cell proliferation, whereas its knockdown promoted cell proliferation in breast cancer cells (85). MiR-665 upregulation has also been recorded in BC and its expression was associated with the metastatic formation and poor prognosis (86). However, the role of miR-665 is ambiguous (being downregulated in other malignancies (87–91)) and it may act both as tumour-suppressor and oncogene, depending on the context.

The importance of *NEIL2* in BC was supported by the analysis with GEPIA 2 (62), a freely available online gene expression profiling tool. HER2-positive patients with *NEIL2*-high displayed significantly worse OS compared to *NEIL2*-low patients. No other significant associations in other BC groups of patients nor the association of *SMUG1* gene expression levels with survival were observed. Because the data on miRNSPs genotypes of BC patients are not available in GEPIA2, we were not able to verify our results in detail. However, results from the GEPIA2 tool highlighted the importance of fine regulation of *NEIL2* levels in BC as its higher expression level was associated with a worse prognosis. Whereas miRNAs have a key role in the regulation of gene expression, the presence of variant allele of certain miRSNPs indeed affects the gene expression levels.

We previously documented that the SNPs investigated in the present study were associated with CRC survival, especially for patients treated with 5-FU based chemotherapy (39). 5-FU is a gold-standard chemotherapeutic used in the treatment of solid tumours (92). Interestingly, we did not find any association of these miRSNPs with the survival of BC patients undergoing 5-FU-based therapy. This

may be attributed to the fact that epigenetic regulation via miRNA regulation and miRNA expression profiles are tissue-specific (93). However, in this study, we searched for germ-line variants and low-penetrance loci in peripheral blood lymphocytes that may not reflect the situation in the target tissue.

After the stratification according to the TNM stage, the TT genotype of *SMUG1* rs971 was associated with shorter OS in patients with TNM stage 1+2. This association did not pass the Bonferroni correction and will, therefore, need additional verification. However, we may anticipate that the association of this miRSNP in BC patients with early stages may also reflect the differences in therapy regimen between early and advanced BC.

Both SMUG1 and NEIL2 have been studied for their role in cancer (73). *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 (94). 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 (95). Regarding NEIL2, the minor allele of *NEIL2* rs1466785 associates with increased BC risk in *BRCA2* mutation carriers (81). 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 (78).

The study by Doherty *et al*. addressed variants *SMUG1* rs971 and *NEIL2* rs6997097 (along with 185 other SNPs in DNA repair genes) in lung cancer risk in 744 patients and 1477 controls. Both miRSNPs failed to be significant risk factors for this cancer (96). With the exception of our previous study on the role of these miRSNPs in CRC, to the best of our knowledge, no other group has studied any of these three miRSNPs and their role in cancer risk and prognosis.

The present study disclosed some promising associations of miRSNPs with BC risk and prognosis. The strongest evidence stands out for *NEIL2* rs6997097 and its association with shorter OS in BC patients treated solely with hormonal therapy.

The above 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 (97). MiRNA-regulated gene expression in general and in particular in DNA repair genes remains largely an unexplored field and further research is inevitable. The variable effect of the same miRSNPs in different types of cancer highlights the extent of inter-tumour heterogeneity in contrast to the universal molecular character of DNA repair in eukaryotic cells.

## Supplementary data

Supplementary data are available at *Mutagenesis* Online.

## Funding

This work was supported by The National Science Foundation of the Czech Republic (GACR 19-10543S); The Grant Agency of Charles University (UNCE/MED/006); The Charles University Research Fund (Progres Q28); The Ministry of Education Youth and Sports of the Czech Republic, the National Sustainability Program I (LO1503) and The Ministry of Health of the Czech Republic (17-28470A to P.S.).

## Acknowledgements

We are thankful to Charles University Research Centre program UNCE/MED/006 'University Centre of Clinical and Experimental Liver Surgery' and INTER-COST project no. LTC19015 provided by the Ministry of Education Youth and Sports of the Czech Republic and by project No. CZ.02.1.01/0.0/0.0/16_019/0000787, Fighting Infectious Diseases, awarded by the MEYS CR, financed from EFRR. The study was also co-sponsored by NU21-03-00145, Ministry of Health of the Czech Republic.
Conflict of interest statement: None declared.

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Mutat Res Gen Tox En 845 (2019) 403065

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

journal homepage: www.elsevier.com/locate/gentox

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

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ARTICLE INFO

*Keywords:*
*Ganoderma Lucidum*
Colorectal cancer
DNA damage
Combination therapy
5-Fluorouracil

ABSTRACT

The first-line chemotherapy of colorectal cancer (CRC), besides surgery, comprises administration of 5-Fluorouracil (5FU). Apart from cytotoxic effect on cancer cells, 5FU may also cause adverse side effects. *Ganoderma Lucidum* (GLC) is a mushroom used in Traditional Eastern Medicine. We propose that natural compounds, particularly GLC extracts, may sensitize cancer cells to conventional chemotherapeutics. This combination therapy could lead to more selective cancer cell death and may improve the response to the therapy and diminish the adverse effects of anticancer drugs.

Here we demonstrate that GLC induced oxidative DNA damage selectively in colorectal cancer cell lines, whereas it protected non-malignant cells from the accumulation of reactive oxygen species. Accumulation of DNA damage caused sensitization of cancer cells to 5FU resulting in improved anticancer effect of 5FU. The results obtained in colorectal cell lines were confirmed in *in vivo* study: GLC co-treatment with 5FU increased the survival of treated mice and reduced the tumor volume in comparison with group treated with 5FU alone.

Combination of conventional chemotherapeutics and natural compounds is a promising approach, which may reduce the effective curative dose of anticancer drugs, suppress their adverse effects and ultimately lead to better quality of life of CRC patients.

## 1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer in the world and the second leading cause of cancer-related deaths in Europe with the highest incidence in Central Europe. With estimated 694 000 deaths per year [1] and with assumed increase by 77% in the number of newly diagnosed CRC cases in 2030, CRC represents a serious health, social and economic problem [2].

Conventional chemotherapeutic treatment of CRC is based on the 5-Fluorouracil (5FU); in the monotherapy or in a combination with irinotecan or platinum derivatives. As the patient's prognosis is primarily determined by the stage of the disease, 5FU based therapy is the standard therapeutic scheme for CRC in stage II and III [3]. Nevertheless, an overall response rate to 5FU monotherapy in more advanced CRC is limited to 10–15% [4]. Combination of 5FU with other cytotoxic agents would not only improve the response to therapy but also reduce the undesirable reaction to these drugs [5,6]. The most common adverse effects of 5FU comprise: nausea, vomiting, diarrhea, mucositis of the

*Abbreviations:* 5FU, 5-Fluorouracil; CRC, colorectal cancer; CTRL, control; DDR, DNA damage response; DMSO, dimethyl sulfoxide; GLC, *Ganoderma Lucidum*; $H_2$DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; PI, Propidium iodide; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulfate

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https://doi.org/10.1016/j.mrgentox.2019.06.001
Received 22 March 2019; Received in revised form 31 May 2019; Accepted 1 June 2019
Available online 03 June 2019
1383-5718/ 

oral cavity (mucosal and submucosal tissue damage), headache, skin pruritus, myelosuppression (suppression of hematopoietic function of the bone marrow, leukopenia, pancytopenia and thrombocytopenia), anemia, cardiotoxicity, agranulocytosis, alopecia (hair loss), photosensitivity, hand-foot syndrome, depression and anxiety [7]. The administration of 5FU in combination with folic acid (leucovorin) enhances patients' survival by approximately 10–15%. Therapy regimen combining 5FU with oxaliplatin and leucovorin (FOLFOX) add additional 7% to 3-year disease-free survival in comparison with the scheme without oxaliplatin [8]. Another possible way to improve conventional therapy may be, among others, targeting DNA damage response (DDR) pathways [9]. DDR plays an essential role in the elimination of DNA damage, thereby preventing cells from genomic instability and malignant transformation. On the other hand, DDR is also involved in patients' response to therapy. DNA damage repair inhibitors can sensitize cancer cells with the main goal to maximize the cytotoxic effect of therapy [10]. Despite disease-free survival improvement, which was achieved by combination therapy, conventional therapy is still accompanied by the significant collateral damage of non-malignant tissues. To achieve better eficiency of conventionally used drugs, we have focused our research on natural compounds with the main aim to promote better eficiency of 5FU therapy leading to a better tolerated treatment.

For many centuries, natural compounds have been used mainly in Eastern medicine. However, many of currently used chemotherapeutics have even their origin in nature, for instance vincristine, irinotecan, etoposide and paclitaxel are plant-derived compounds. Actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs derived from microbial sources, and cytarabine is the first drug originating from a marine source [11]. Natural compounds can target multiple signaling pathways in the cell or organism such as apoptotic and cell cycle pathways [12]. *Ganoderma Lucidum* (GLC), also known as the mushroom of longevity, is a natural compound used in traditional Chinese medicine for more than two thousand years [13]. GLC contains a number of biologically active components, such as triterpenes and polysaccharides [14]. Currently, GLC has been extensively studied from prevention and therapy point of view in many human disorders including cancer (for rev. see [15–17].

We propose that the modulation of DNA damage by natural compounds, particularly GLC, may lead to sensitization of cancer cells to conventional chemotherapeutics and to selective cancer cell death. Potentiation of anticancer effects of conventional chemotherapeutic drugs by well tolerated natural compounds may reduce the effective curative dose of drugs, modify their side effects and lead to better quality of life of CRC patients. Therefore, we have focused on the effect of the GLC on proliferation, migration, cell cycle progression and DNA damage in CRC cell lines as well as in a non–malignant colorectal cell line. To prove our hypothesis, we evaluated the effect of GLC on 5FU treatment both *in vitro* and *in vivo*.

## 2. Material and methods

### 2.1. Ganoderma Lucidum (GLC)

GLC was obtained from Pharmanex (Provo, UT, USA, batch No.: DL12561, Shanghai R&D, Pharmanex). GLC had well defined formulation; it contained 6% of triterpenes, 13.5% of polysaccharides. GLC was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) at the concentration of 50 mg/ml and stored at 4 °C.

### 2.2. Cell treatments

#### 2.2.1. GLC treatment

Fifty mg/ml stock solution of GLC was dissolved in culturing medium to final concentrations 0.25 and 0.5 mg/ml. Medium without GLC was used as a control. Used concentrations were chosen according to results published by Jiang and Sliva [18].

#### 2.2.2. 5FU + GLC co-treatment

5-Fluorouracil (5FU, Sigma Aldrich, St. Louis, MO, USA) was dissolved in DMSO to 500 mM stock solution. For simultaneous co-treatment (5FU + GLC) cells were treated with 5μM 5FU and 0.5 mg/ml GLC. Medium without GLC was used as a control.

### 2.3. Cell cultures

Human adherent colorectal cancer cell lines HCT116, HT29, $HCT116^{p53-/-}$ were a kind gift from Dr. Andera, Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Vestec (Prague, Czech Republic); originally obtained from ATCC (Manassas, USA). Cells were cultured in DMEM medium (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), 1 mM L-glutamine (Biosera, Nuaille, France), 1 mM sodium pyruvate (Biosera, Nuaille, France) and 1 mM penicillin/streptomycin (Biosera, Nuaille, France). Non-cancer human colon mucosal epithelial cell line (adherent) NCM460 cells (originally obtained from INCELL Corporation, San Antonio, TX, USA by Prof. Sliva) were cultured in M3:10™ medium (INCELL, San Antonio, TX, USA) with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) and 1 mM penicillin/streptomycin (Biosera, Nuaille, France). All cells were cultured in a humidified incubator at 37 °C, 5% $CO_2$. Cells were used up to 8 passage.

CT26.WT mouse adherent colon cancer cell line was a kind gift from Prof. B. Rihova, Institute of Microbiology of the Czech Academy of Sciences (Prague, Czech Republic), originally obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin solution (Biosera, Nuaille, France) at 37 °C in 5% $CO_2$ incubator.

### 2.4. Colony forming assay

Cells were plated on 6 well plates (500 cells/well) and treated with different concentrations of GLC (0.25-0.5 mg/ml) for 24 h. Each tested concentration, as well as control, were performed in triplicates. After 24 h, the medium was replaced with fresh medium. After 12 days, colonies were fixed with 3% formaldehyde and stained with 1% crystal violet. Percentages of colonies were measured in ImageJ software [19].

### 2.5. Cell proliferation assay

To measure the proliferation; cells were seeded on 96 well plates ($5 \times 10^4$ cells per well) and treated with GLC (0.25-0.5 mg/ml) in quadruplicates, at different time points (24–72 h). WST1 cell proliferation assay (Roche, Basel, Switzerland) was used according to the manufacturer's protocol (10 μl WST1 reagent per 100 μl of medium, 40 min incubation time). Absorbance was measured using fluorescence reader Biotek ELx808 (Biotek, Vermont, USA), Ex/Em 450/690 nm.

### 2.6. Migration assay

Cells were seeded to 6 well plates ($5 \times 10^5$ cells/ml) and treated with GLC extract (0.5 mg/ml) for 24 h. Cell migration was assayed using Transwell Permeable Supports 8.0 μm (Corning, Sigma Aldrich, St. Louis, MO, USA). Cells were seeded in a density of $1 \times 10^4$ on the top of a transwell support in 24 well plate format and cultured in DMEM medium supplemented with 0.5% FBS. Cells were allowed to migrate for 24 h through the membrane into the lower part of chamber containing DMEM with 20% FBS. The migrated cells were fixed with 3% formaldehyde, stained with 1% crystal violet and counted in four random fields under 200 x magnification.

### 2.7. Reactive oxygen species (ROS) measurement

Cells were cultured in 24 well plates ($5 \times 10^5$ cells/ml). For GLC treatment; cell lines were treated with 0.25 and 0.5 mg/ml concentrations of GLC for 3, 6, 24 h. After incubation, cells were harvested by trypsinization, washed with PBS, and centrifuged (1000 rpm, 10 min). 1 µl of cell-permeant 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (10 µM, Thermo Fisher) was added to cell pellet and incubated for 30 min at 37 °C. The level of relative fluorescence was measured on fluorescent reader Biotek (Vermont, VT, USA) at Ex/Em: 485/538 nm. For the ROS measurement after the 5FU + GLC co-treatment; cells were treated simultaneously with 0.5 mg/ml GLC and 5µM 5FU and then processed in the same way as described above.

### 2.8. Measurement of SBs and oxidative DNA damage using comet assay

DNA damage measurements were performed by alkaline comet assay modified for digestion of nucleoids with DNA repair endonucleases (or single cell gel electrophoresis), fully described in Azqueta et al. [20]. Cells were treated with GLC solutions for 90 min, non-treated cells were used as control. Investigated cells were embedded in duplicates in agarose ($2 \times 10^5$ cells/ml, 0.5% low melting point agarose in PBS, 37 °C) on a microscope slide that was 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 1 h in order to obtain substrate DNA in the form of nucleoids fixed in agarose. Subsequently, slides were washed in washing buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1 M KCl, pH = 8, 3 changes, 5 min each at 4 °C).

Regarding detection of specific oxidative DNA damage, half batch of the nucleoids was incubated with the formamidopyrimidine DNA glycosylase enzyme (Fpg, New England Biolabs, Ipswich, MA, USA), dissolved in reaction buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/ml BSA, 0.1 M KCl, pH = 8, 4 °C) for 30 min at 37 °C. The second part of nucleoids was incubated with reaction buffer only for 30 min at 37 °C to detect SBs. Alkaline incubation followed (freshly prepared - 0.3 M NaOH, 1 mM EDTA, 4 °C) for 30 min in dark, converting alkali-labile sites to SBs. During electrophoresis (1.19 V/cm, 300 mA, 40 min, 4 °C, dark) in the same alkaline buffer, DNA loops containing SBs were drawn towards the anode forming a comet-like image. Slides were then washed in 1xPBS (4 °C) for 10 min, in distilled water (4 °C) for 10 min and dried overnight. Next day, slides were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) diluted 1:10.000 in TE buffer. Comets were visualized with fluorescence microscope Olympus BX63 (Olympus, Tokyo, Japan) and analyzed using semi-automated Lucia Comet Assay™ software (Laboratory Imaging, Prague, Czech Republic). One hundred comets were scored per gel (*i.e.* two hundred comets per slide). Median tail intensity (TI), reflecting the frequency of DNA in tail (% tail DNA), per gel and then the mean TI of replicate gels was used as the parameter to describe the comets. The level of specific oxidative DNA damage was expressed as net values (the level of SBs detected on slides incubated with Fpg minus the level of SBs detected on slides incubated with reaction buffer).

### 2.9. Cell cycle analysis

Cells were seeded on 12 well plates ($5 \times 10^5$ cells/ml) and treated with 0.5 mg/ml GLC for 12–72 h. After the treatment, cells were harvested by trypsinization, washed with PBS and spun down at 1000 rpm for 10 min. Then, 1 ml of Propidium iodide (PI) staining solution (0.02 mg/ml PI, 0.02 mg/ml RNase, 0.05% Triton X-100) was added to the cell pellet and cells were incubated for 30 min at 37 °C in the dark. After incubation, samples were measured using flow cytometer (Apogee A-50 micro, Apogee, Hertfordshire, UK). Measured data were analyzed with Flowlogic software (Inivai Technologies, Mentone, Australia).

### 2.10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Cells after treatment were washed with PBS. Sixty µl of TTL buffer (1 M Tris-HCl, 5 M NaCl, 0.2 M EDTA, 10% Triton X, protease inhibitors) were added to each well and cells were harvested by cell scrappers and transferred into tubes. The tubes were frozen on dry ice. After 15 min cells were gently thawed and incubated for 20 min on ice. After incubation cells were spun down at 20 000xg, 20 min, 4 °C. The supernatants were aspirated into new tubes. The concentrations of proteins were measured by Bradford reagent (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer recommendations. Proteins (20 µg) were loaded and separated in 12% SDS-PAGE gels at 15 mA for 60 min. Then, the separated proteins were transferred to 0.45 µm Amersham Protran Nitrocellulose Blotting Membrane (GE Healthcare, Life science) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline containing Tween 20 (TBST; 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20) for 1 h and incubated with anti-p53 (Cell Signaling, Leiden, The Netherlands) and anti-GAPDH (Abcam, Cambridge, UK) at 4℃ overnight, followed by incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). The membranes were then incubated with SupersignalWest Pico Chemiluminiscent Substrate (Pierce, Thermofisher, Massachusetts, USA) and visualized by Azure c600 (Azure Biosystems, Dublin, CA, USA).

### 2.11. Mice and tumor induction and treatment

Three-month old female BALB/c mice were purchased from Institute of Physiology of the Czech Academy of Sciences (Prague, Czech Republic). All mice were maintained and handled in accordance with the procedures approved by the Institute of Microbiology animal care and use committee (No. 105/2016). Thirty-two mice were inoculated subcutaneously on the right side of their shaved back with a single-cell suspension of CT26.WT cells (200,000 cells in 100 µl) and, after 10 days, divided into four groups with eight mice each. All groups received single or combined therapy when the tumors reached average volume of about 300 $mm^3$ (day 14). The mice were gavaged daily with 100 µl suspension of GLC powder in sterile distilled water (110 mg/ml) according Sliva et al. [21] and injected intraperitoneally three times a week with 200 µl 5FU (Sigma Aldrich, St. Louis, MO, USA) solution in sterile phosphate buffered saline (20 mg/kg). The tumor dimensions were measured twice a week by a caliper and tumor volume was calculated using a formula (length x $width^2$)/2. The mice were sacrificed on day 48. Tumor samples dimensions were measured by caliper and weight out. After measurement tumor tissues were frozen.

### 2.12. Statistical analysis

Statistical analyses were performed using pairwise comparison by Student's *t*-test and Two-way ANOVA (GraphPad Prism5, GraphPad Software, La Jolla California USA, www.graphpad.com). The results represent the mean value of three independent experiments ± SD; the significance level was set at $p \leq 0.05$.

## 3. Results

### 3.1. Ganoderma Lucidum inhibits growth and invasive behavior of colorectal cancer cell lines

To define the effect of GLC on cell proliferation, HCT116, HT29 and non-malignant NCM460 cells were treated with two different doses of GLC (0.25 mg/ml and 0.5 mg/ml). After 24 h treatment we observed non-significant decrease in cell proliferation (data not shown). However, we recorded significantly decreased cell proliferation in

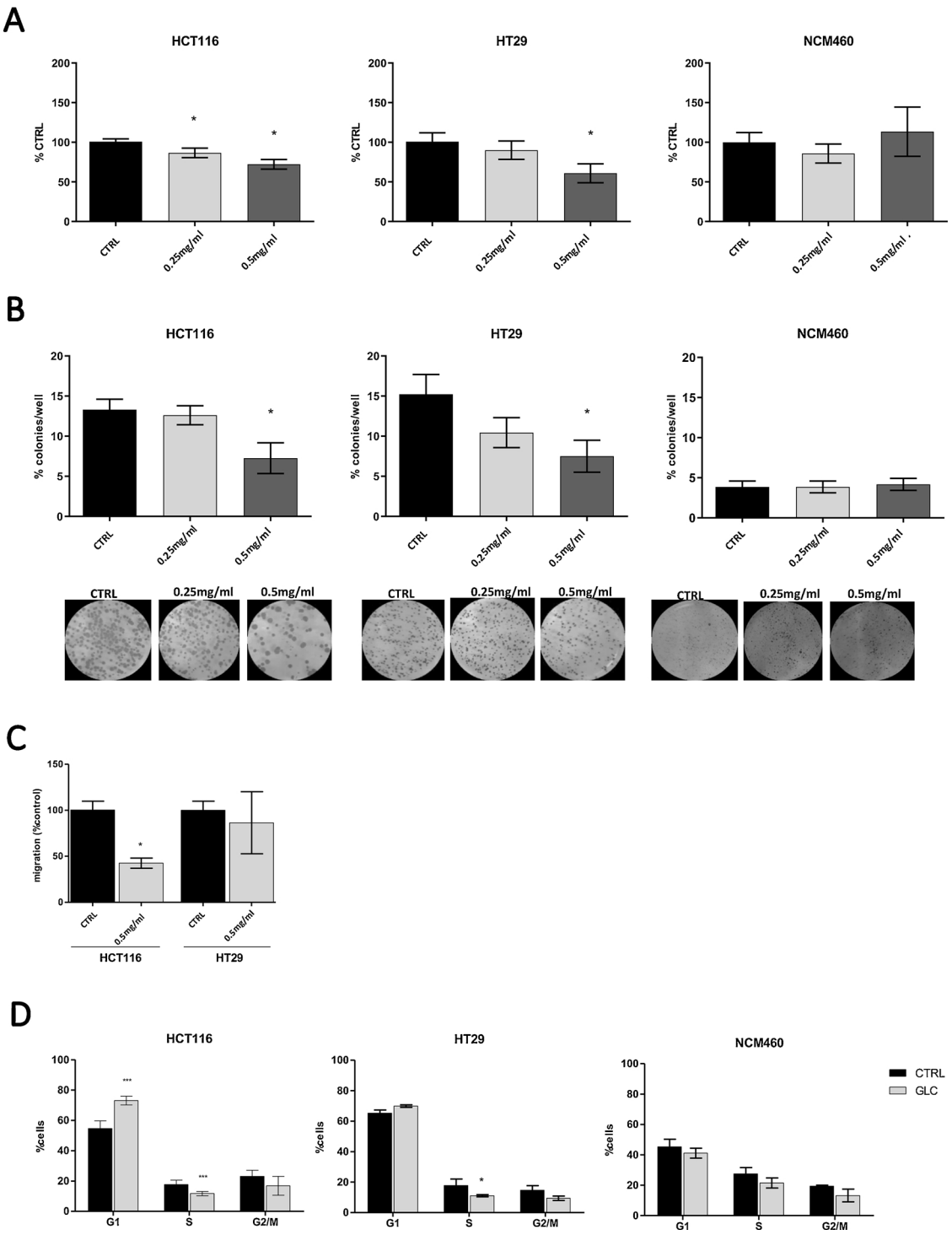


**Fig. 1.** GLC inhibits growth, invasive behavior and cell cycle of colorectal cancer cell lines.
(A) Cell proliferation measured by WST assay after 48 h GLC treatment in HCT116, HT29 and NCM460 cells. (B) Colony forming assay after GLC treatment. (C) Migration of cancer cells after GLC treatment. (D) Propidium iodide analysis to define cell cycle distribution after GLC treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

HCT116 (by 27%, $p < 0.05$) and HT29 (by 39%, $p < 0.05$) cancer cells at 0.5 mg/ml after 48 h treatment. The prolonged GLC treatment for 72 h resulted in persisting lower proliferation in HCT116 cells, whereas no effect on proliferation was recorded in HT29 cells (data not shown). Proliferation of non-malignant cells was not affected (Fig. 1A).

Colony forming assay was performed to verify the anti-proliferative potential of GLC. CRC and non-malignant cells were treated with two different doses of GLC (0.25 mg/ml and 0.5 mg/ml) for 24 h as described in Materials and Methods. After 0.5 mg/ml GLC treatment, the number of colonies significantly decreased by 46% and 45% in HCT116 and HT29 cells, respectively ($p < 0.05$), when compared to non-treated cells (Fig. 1B). GLC treatment did not affect non-malignant NCM460 cells (Fig. 1B). In following experiments, the cells were treated with 0.5 mg/ml GLC, because this concentration showed higher eficacy in our experiments. The effect of GLC on migration of cancer cells was analyzed as well. Significant 57% decrease in migration of HCT116

cells was observed after GLC treatment ($p < 0.05$), however HT29 cells showed only moderate reduction by 14% (Fig. 1C). The cell cycle distribution after GLC treatment was analyzed by flow cytometry after propidium iodide staining. We did not observe any difference in cell cycle distribution in either cell line treated with 0.5 mg/ml GLC for 24 h (data not shown). Fig. 1D is a representative figure depicting effects on cell cycle distribution after 48 h treatment with 0.5 mg/ml GLC. At this time interval significant increase in the amount of HCT116 cells in G1 phase and decrease in the amount of cells in S phase ($p < 0.001$) was pronounced. The same tendency was observed in HT29 cells as well; the reduced proportion of cells in S phase was significant ($p < 0.05$). This indicates the GLC induced G1/S cell cycle arrest. Moreover, in HT29 cells, several cells in G0 phase were observed.

### 3.2. The effect of Ganoderma lucidum treatment on oxidative DNA damage

To asses DNA damage by GLC, the CRC cells were treated with 0.5 mg/ml GLC extract for 90 min. GLC treatment induces significant changes in the amount of DNA strand breaks in HCT116 cell line ($p < 0.05$) but not in HT29. However, the levels of oxidative DNA damage significantly increased in HCT116 and in HT29 cells, respectively (both $p < 0.05$, Fig. 2A and 2B). We found no increase in DNA strand breaks and oxidative DNA damage in non-malignant reference cells – NCM460 (Fig. 2C).

### 3.3. GLC enhances the effect of 5FU in CRC cells

Our results showed that GLC specifically decreased colorectal cancer cell growth and also induced DNA damage. In the next part of our study, we tested the eficacy of GLC in combined treatment with conventionally used chemotherapeutic 5FU.

Cancer cells simultaneously treated with GLC (0.5 mg/ml) and 5FU (5 μM) were analyzed for proliferation, long term survival (colony formation), and DNA damage.

GLC co-treatment with 5FU did not significantly affect cancer cell proliferation in HCT116 (Fig. 3A). However, growth of HCT116 cells was decreased by about 20% compared to the effect of 5FU alone ($p < 0.01$, Fig. 3B). In HT29 cells, GLC enhanced the effect of 5FU by about 15% ($p < 0.05$, Fig. 3B). For further validation, the co-treatment effect was analyzed using Combenefit software [22]. This analysis showed an additive effect of GLC and 5FU treatment (data not shown).

The co-treatment (GLC + 5FU) also increased the level of DNA strand breaks in HT29 cells and oxidative damage in HCT116 cells (Fig. 3C). In HCT116 cells, simultaneous treatment with GLC and 5FU increased the level of oxidative DNA damage ($p < 0.05$) in comparison with 5FU treatment alone. In HT29 cells, co-treatment increased DNA strand breaks ($p = 0.05$). We did not observe any effect of GLC as well as simultaneous treatment of 5FU + GLC on any of the analyzed parameters in non-malignant colonic NCM460 cells (summarized in Fig. 3).

### 3.4. Effect of Ganoderma lucidum on accumulation of reactive oxygen species (ROS) in CRC and non-malignant cell lines

The effect of GLC treatment (0.25 mg/ml and 0.5 mg/ml) on ROS production in CRC and non-malignant cell lines was examined after 3, 6, 24 h incubation. We did not detect any changes in ROS levels after the 3 h treatment (data not shown), whereas 6 h treatment with 0.25 mg/ml GLC induced an increase in ROS levels in HCT116 cells, treatment with 0.5 mg/ml was non significantly increased (Fig. 4A, $p < 0.05$). In HT29 cells, we detected non-significant increase in ROS accumulation. In non-malignant NCM460 cells, the treatment with both doses of GLC extract caused a significant decrease in ROS levels by about 20% after 6 h (0.25 mg/ml, $p < 0.05$; 0.5 mg/ml, $p < 0.01$) and by about 17% after 24 h (0. 25 mg/ml, $p < 0.05$; 0.5 mg/ml, $p < 0.001$, Fig. 4A).

### 3.5. Role of p53 in GLC treatment

The effect of GLC treatment on level of p53 protein was analyzed in all tested cell lines. GLC (0.5 mg/ml) treatment increased the level of p53 in CRC cell lines (Fig. 5A). In NCM460, the level of p53 was not changed in comparison with non-treated control. HCT116$^{p53-/-}$ cells were used to investigate the role of p53 protein in GLC effect. HCT116$^{p53-/-}$ cells were treated with different concentrations of GLC for 48 h. Our results showed a 42% decrease in cell proliferation after treatment with 0.5 mg/ml GLC ($p < 0.05$, Fig. 5B). GLC co-treatment with 5FU also decreased the cell proliferation by 17% ($p < 0.05$, Fig. 5B). This effect was also confirmed by colony forming assay showing significant decrease in cancer cell growth (Fig. 5C). We did not observe any effect on ROS accumulation in HCT116$^{p53-/-}$ cells (Fig. 5D). GLC treatment induced DNA strand breaks and oxidative DNA damage. GLC co-treatment with 5FU enhanced the effect of 5FU on both types of DNA damage ($p < 0.05$, Fig. 5E).

### 3.6. Effect of GLC on 5FU in mice xenograft model in vivo

To confirm the effect of GLC on 5FU treatment *in vivo*, we used mice transplanted with syngeneic CT26 cells. After 14 days of tumor growth, we started to treat the mice with GLC alone or in combination with 5FU. Although non-significant, in GLC + 5FU group, we observed better survival ($p = 0.0628$) and smaller tumor volume in comparison to other groups (Fig. 6A and 6B). These findings were also associated with lower tumor weight measured at the day of experiment termina-tion ($p < 0.05$, Fig. 6C).

## 4. Discussion

*Ganoderma Lucidum* (family *Ganodermataceae)* is basidiomycetous fungi used in traditional Eastern medicine for centuries. This medical mushroom is believed to preserve human vitality and promote longevity. It has been used to treat various human diseases, such as allergy, arthritis, bronchitis, gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia, scleroderma, inflammation, and cancer [23]. In our study, we have

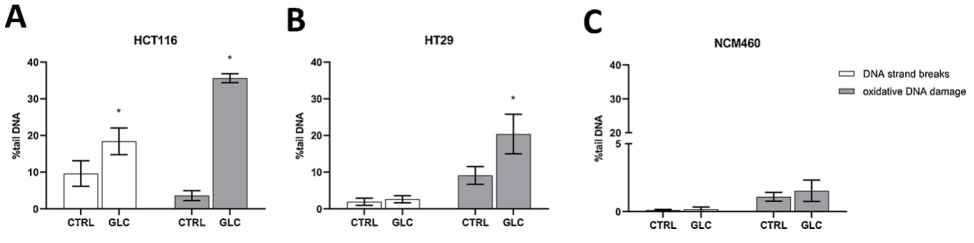


**Fig. 2.** Effect of GLC on DNA damage.
(A) Effect of GLC on DNA strand breaks and oxidative DNA damage measured by comet assay in HCT116. (B) Effect of GLC on DNA strand breaks and oxidative DNA damage in HT29. (C) Effect of GLC on DNA strand breaks and oxidative DNA damage measured in NCM460. Level of oxidative DNA damage is expressed as net values. All data are expressed as means ± SD of triplicates and *$p \leq 0.05$ *versus* control (non-treated cells).

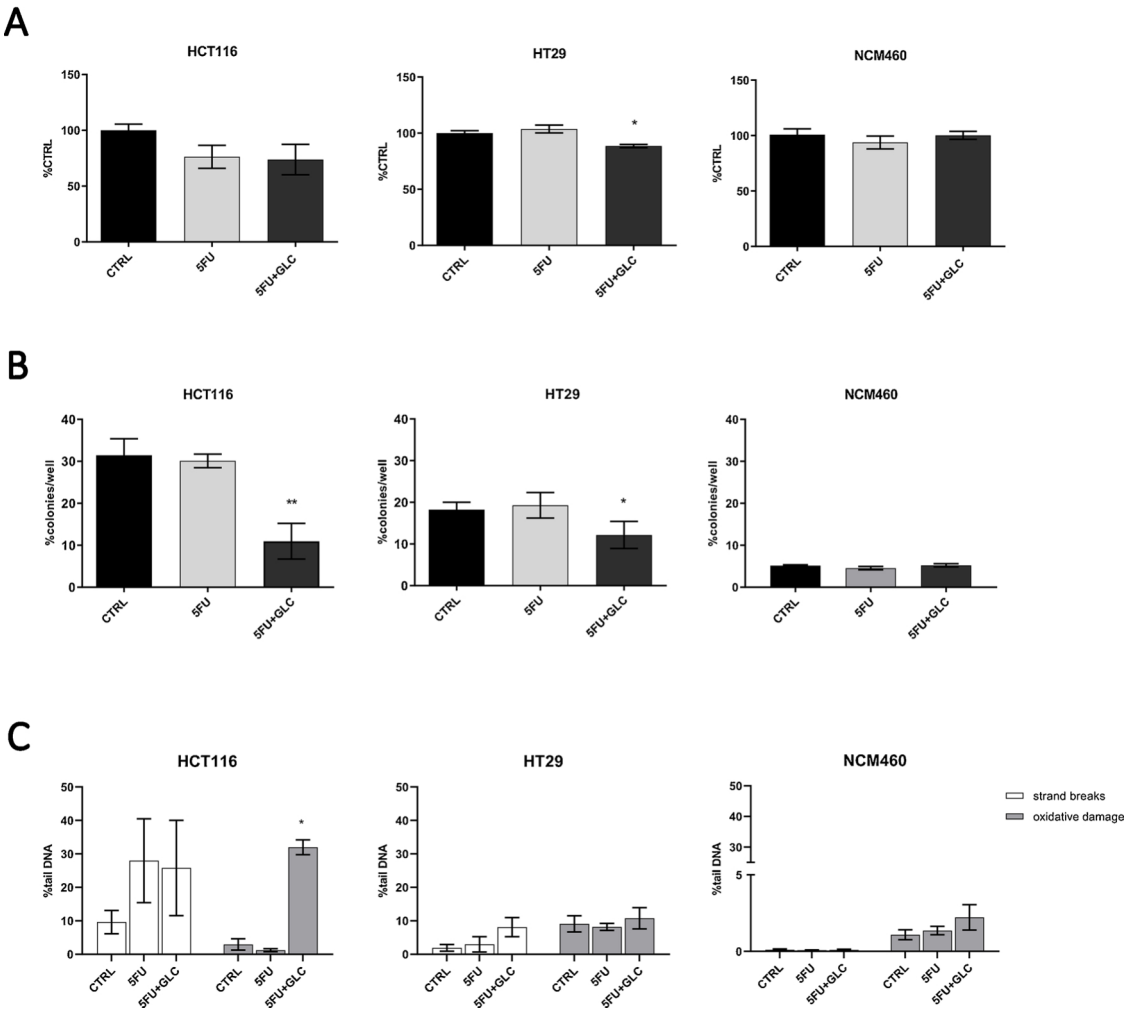


**Fig. 3.** The effect of GLC in combination with 5FU *in vitro*.
(A) Proliferation after GLC and 5FU co-treatment. (B) Effect of GLC and 5FU co-treatment on colony forming assay. (C) Effect of GLC and 5FU co-treatment on DNA damage in HCT116 and HT29 CRC cell lines. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

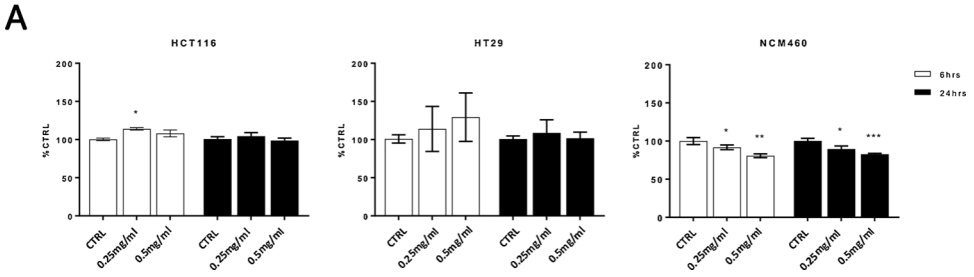


**Fig. 4.** GLC effect on ROS production in colorectal cell.
(A) Intracellular ROS accumulation after GLC treatment detected by 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) in HCT116, HT29 and NCM460 cells after 6 h and 24 h GLC treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

tested the consequence of simultaneous treatment of natural compound GLC together with chemotherapeutic 5FU on colorectal cancer, both *in vitro* and *in vivo.* In our recent review article we hypothesized that potentiating of anti-cancer effects of chemotherapeutics with well tolerated natural compounds may modify the effective drugs dose, diminish their side effects and ultimately lead to a better quality of life for cancer patients [17].

Our original hypothesis that co-treatment of GLC with 5FU enhances its cytotoxic effect in CRC was confirmed in our study. Simultaneous treatment of CRC cells with GLC and 5FU led to increased level of oxidative DNA damage resulting in significantly decreased cancer cell growth. Similar effect on colorectal cancer cells was shown by Jiang et al., who showed that *Ganoderma Lucidum* polysaccharides (GLPs) administered in combination with 5-FU synergistically suppress proliferation of CRC cells [24].

These data suggest that specific DNA damage caused by natural compounds may become a potential tool for improvement of the anticancer treatment. Furthermore, we investigated the effect of GLC on cancer cell proliferation, migration, cell cycle progression, as well as DNA damage in malignant CRC cells and non-malignant colorectal cell line. Additionally, we have tested a well characterized GLC extract containing both polysaccharides and triterpenes and observed that GLC decreased proliferation of HCT116 and HT29 cancer cells. Recently, many authors brought an evidence, that GLC, particularly its component triterpenes, decreases cancer cell proliferation in ovarian [25], breast [26,27] and also in colorectal cancer cells [28,29]. Moreover, to our best knowledge, we have documented for the first time that GLC has no significant effect on non-malignant colorectal cells. To further

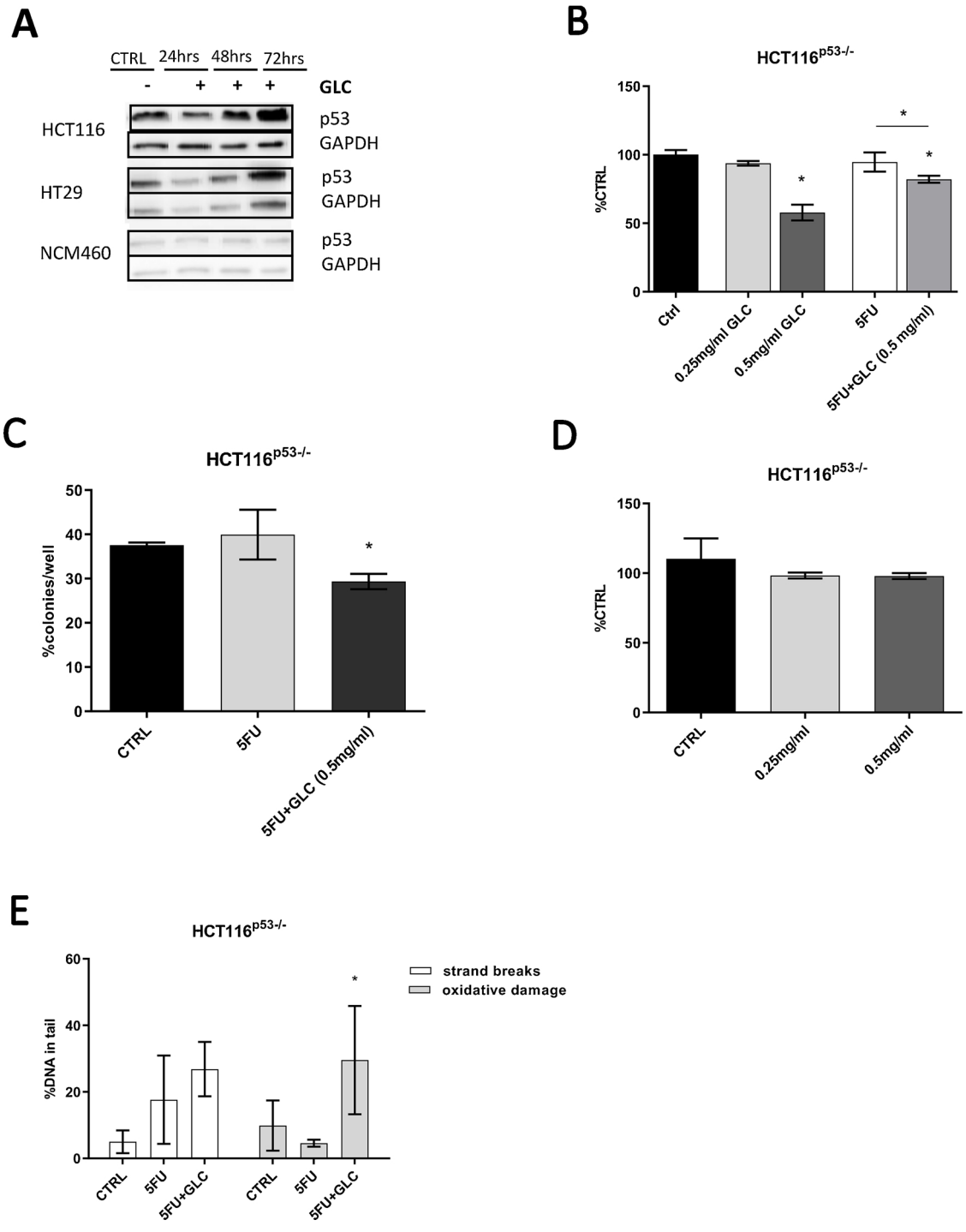


**Fig. 5.** The role of p53 in GLC treatment.
(A) Representative figure of western blot analysis of p53 level after GLC treatment. (B) Proliferation of HCT116$^{p53-/-}$ cells after GLC treatment alone and in 5FU co-treatment. (C) Effect of co-treatment of 5FU with GLC on colony forming assay. (D) ROS accumulation after GLC treatment. (E) Level of DNA damage after 5FU and GLC co-treatment. All data are expressed as means ± SD of triplicates and *p ≤ 0.05 *versus* control (non-treated cells).

investigate the anti-cancer effect of GLC, we also analyzed cells long term survival, which defines the ability of single cell to divide [30]. We detected decreased growth of colorectal cancer cells after GLC treatment, while the growth of non-malignant colorectal cells NCM460 remained unaffected. Another important feature of tumor cells is their invasiveness. Cell migration is a crucial process for normal development and homeostasis, but disturbed cellular migration is also an essential trait for cancer metastasis development. This metastatic spread of the primary tumor accounts for over 90% of patient`s mortality associated with solid tumors [31]. Subsequently, we have observed that GLC significantly reduced also cancer cell migration. Consistently with our results, Li et al. reported that ethanol extract of Ganoderma triterpenes suppressed HCT116 migration through the upregulation of E-cadherin [32]. This effect was also described in breast cancer cells by Martínez-Montemayor et al. [33]. It is well recognized that the inhibition of cancer cells growth and their invasive behavior are important mechanisms for carcinogenesis inhibition. In accordance with this assumption, we proved that GLC showed a pronounced antitumor effect

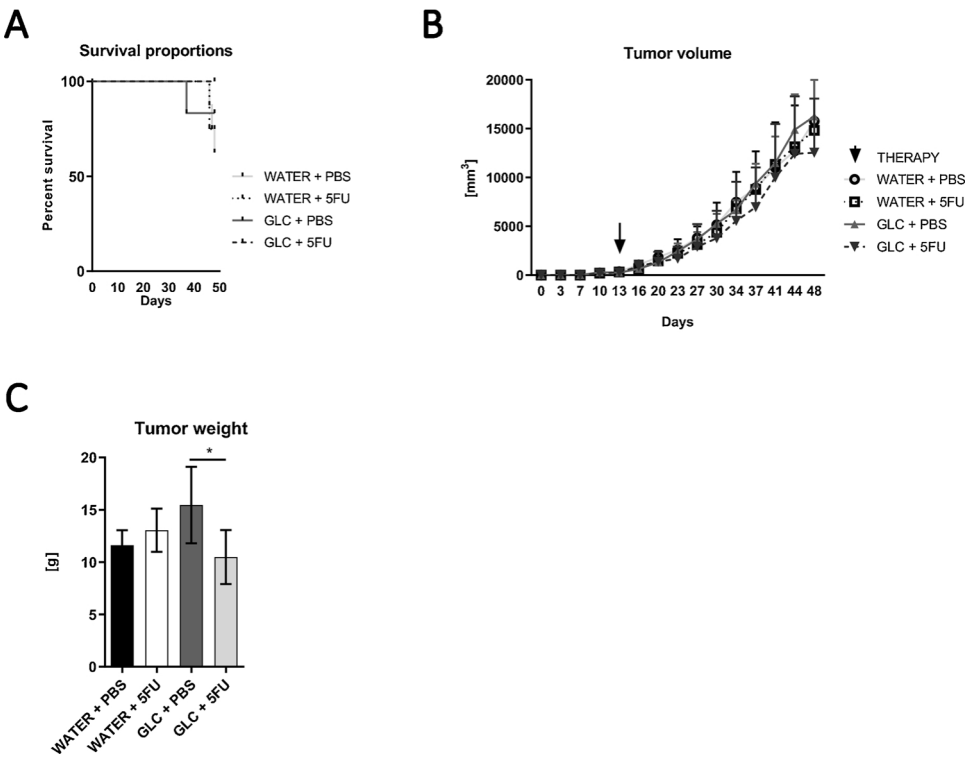


**Fig. 6.** The effect of GLC and 5FU *in vivo*. (A) Kaplan Meier curves representing mice survival after GLC and 5FU administration. (B) Differences in tumor volume after GLC and 5FU administration. (C) Differences in tumor weight after GLC and 5FU administration. All data are expressed as means ± SD and *p ≤ 0.05 *versus* control group.

on CRC cells without affecting non-malignant cells. This may suggest protective effect of natural compounds on non-malignant cells and explain why the natural compounds are well tolerated in humans.

To validate our results from *in vitro* studies, we analyzed the simultaneous effect of GLC with 5FU in a mice xenograft model. The group of mice treated with GLC and 5FU together exhibited better survival. GLC also positively influenced the cytotoxic effect of 5FU on tumor size. Overall fitness of animals is important in evaluation of the toxicity and the side effects of a chemotherapy drug or a natural supplement in animal studies. Groups treated with GLC showed a moderate increase in body weight (data not shown) and had better overall fitness than mice in other groups. Sliva et al. reported that GLC triterpenes could be used as an alternative dietary approach for the prevention of cancer associated colitis [21] and Xu et al. showed that GLC attenuated doxycycline induced cardiotoxicity [34]. Zhao el al described that GLC enhances the sensitivity of ovarian cancer cells to cisplatin [35], and Yue et al. defined synergism between GLC triterpenes and doxorubicine [36]. Li et al. showed that *Ganoderma microsporum* prevented 5FU induced mucositis in mice model [37]. However, studies addressing effect of GLC on 5FU treatment in mice tumor xenograft model are rather scarce. Taking together, we proved that GLC could enhance cytotoxic effect of 5FU, and alongside it protects non-malignant cells from 5FU cytotoxicity.

In our study, we observed important effect of GLC and 5FU on CRC and non-malignant cells. To further understand this phenomenon, we focused on the description of the mechanism involved in the GLC cytotoxicity. Since DNA damage is a complex target for anticancer drugs [38], we focused on the level of DNA strand breaks after GLC treatment. We detected an accumulation of DNA strand breaks (HCT116) in CRC cells after GLC treatment. Furthermore, we documented that GLC induced specific oxidative DNA damage in CRC cells. Some studies reported that triterpenoids isolated from GLC induced oxidative DNA damage due to their structure-activity relationships. Liu et al. suggested that oxidative DNA damage accumulation depends on the degree of acetylation in the structure of GLC triterpenoid [39]. It is well known that many natural compounds are able to cause oxidative DNA damage, such as curcumin [40], resveratrol [41] and *Ginkgo Biloba* extract [42] ultimately resulting in cell cycle arrest and apoptosis of colorectal cancer cells [43]. The importance of studying natural compounds in the cancer therapy is supported by the fact that some of these natural compounds recently underwent clinical trials (curcumin), [44,17].

Under physiological conditions, recognition of DNA damage induces the DNA damage response (DDR) machinery in order to maintain genomic integrity of the cell. Suboptimal activity of DDR may enhance the effect of DNA damaging compounds. Kuo et al. reported that dietary flavonoids can enhance chemotherapeutic effect by inhibiting DDR [46]. We demonstrate that, GLC increased the accumulation of oxidative DNA damage. On the contrary, GLC treatment significantly decreased the level of ROS in non-malignant colorectal cells. Reactive oxygen species can react with different components of DNA and cause DNA lesions. These properties predestine ROS as a potential target for anti-cancer therapy [47]. There is an evidence of protective effect of GLC against ROS formation, mostly in non-cancerous cells. Li et al. has already postulated that GLC polysaccharides exert a protective effect against oxidative stress in the brain cells [48] as well as in cardiomyocytes [34].

Our results showed that HT29 are less sensitive to GLC treatment. The reason for this difference could be due to the fact that HT29 line bears a mutation in *TP53* gene [49]. Mutation in *TP53* is often present in many cancer types, including advanced CRC. Loss of p53 function by mutations leads to uncontrolled cell cycle progression [50]. Jiang et al. published that polysaccharides from GLC restore tumor suppressor function of mutant p53 [24]. Moreover, we observed increased levels of p53 protein after GLC treatment. We hypothesized that restoration of tumor suppressor function of p53 after GLC treatment may lead to restoration of cell cycle regulation and cell cycle arrest and apoptosis. To define the role of p53 protein in GLC treatment, we used HCT116$^{p53-/-}$ cells. p53 in general is not necessary for the induction of the DNA damage, but it is critical for the occurrence of the cell death. In the presence of p53, GLC dramatically enhanced cytotoxicity of 5FU by triggering of the oxidative DNA damage. In case of p53$^{-/-}$ cells, GLC induced the DNA damage, and simultaneously reactivated p53. These changes may subsequently result in cell growth inhibition and apoptosis, as recently shown by Jiang et al. [24].

## 5. Conclusion

In summary, GLC showed a substantial effect on CRC cells by induction of oxidative DNA damage, whereas it protected non-malignant cells from ROS accumulation. Moreover, GLC enhanced the toxic effect of 5FU in CRC cell lines. According to obtained results, we propose natural compounds may represent a promising supplement to conventional cancer therapy, which may finally reduce the effective curative dose of anticancer drugs and improving patients' outcomes.

## Conflict of interests

The authors declare that there are no conflicts of interest.

## Funding

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic [KONTAKT II - LH13061], the Czech Science Foundation [GACR 18-09709S and 19-10543S]; the Czech Health Research Council of the Ministry of Health of the Czech RepublicCzech Health Research Council [AZV 15-27580A]; the Grant Agency of the Charles University [GAUK 800216]; the Research program of Charles University [PROGRES Q28 (Oncology) and "Centrum of clinical and experimental liver surgery", UNCE/MED/006]; the National Sustainability Program I (NPU I) [Nr. LO1503] and EFRR [project No. CZ.02.1.01/0.0/0.0/16_019/0000787 „Fighting INfectious Diseases", awarded by the Ministry of Education, Youth and Sport of the Czech Republic.].

## Acknowledgement

The authors are grateful to the COST Action CA15132, 'hCOMET', for support. The authors also thank to Dr. Andera for a gift of the colorectal cell lines, Prof. Rihova for a gift of the mouse colon cancer cell line and to Dr. Vymetalkova for a consultation of the results.

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 17 October 2022
DOI 10.3389/fonc.2022.959407



OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Gastrointestinal Cancers: Colorectal Cancer, a section of the journal Frontiers in Oncology

RECEIVED 01 June 2022
ACCEPTED 01 September 2022
PUBLISHED 17 October 2022

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



# MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients

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Cancer therapy failure is a fundamental challenge in cancer treatment. One of the most common reasons for therapy failure is the development of acquired resistance of cancer cells. DNA-damaging agents are frequently used in first-line chemotherapy regimens and DNA damage response, and DNA repair pathways are significantly involved in the mechanisms of chemoresistance. MRE11, a part of the MRN complex involved in double-strand break (DSB) repair, is connected to colorectal cancer (CRC) patients′ prognosis. Our previous results showed that single-nucleotide polymorphisms (SNPs) in the 3′ untranslated region (3′UTR) microRNA (miRNA) binding sites of MRE11 gene are associated with decreased cancer risk but with shorter survival of CRC patients, which implies the role of miRNA regulation in CRC. The therapy of colorectal cancer utilizes oxaliplatin (oxalato(trans-l-1,2-diaminocyclohexane) platinum), which is often compromised by chemoresistance development. There is, therefore, a crucial clinical need to understand the cellular processes associated with drug resistance and improve treatment responses by applying efficient combination therapies. The main aim of this study was to investigate the effect of miRNAs on the oxaliplatin therapy response of CRC patients. By the in silico analysis, miR-140 was predicted to target MRE11 and modulate CRC prognosis. The lower expression of miR-140 was associated with the metastatic phenotype ($p < 0.05$) and poor progression-free survival (odds ratio (OR) = 0.4, $p < 0.05$). In the in vitro analysis, we used miRNA mimics to increase the level of miR-140 in the CRC cell line. This resulted in decreased proliferation of CRC cells ($p < 0.05$). Increased levels of miR-140 also led to

increased sensitivity of cancer cells to oxaliplatin ($p < 0.05$) and to the accumulation of DNA damage. Our results, both in vitro and in vivo, suggest that miR-140 may act as a tumor suppressor and plays an important role in DSB DNA repair and, consequently, CRC therapy response.

KEYWORDS

miR-140, colorecal cancer, MRE11, oxaliplatin, therapy response, DNA damage, DNA repair, miRNA

# Introduction

Treatment failure of colorectal cancer (CRC) therapy, represented by the development of drug resistance or outgrowth of metastasis, is a major complication for CRC patients. There is a crucial clinical need for predictive biomarkers that indicate the success or failure of cancer treatment. A better understanding of the cellular processes associated with drug resistance will eventually lead to improved treatment response by applying more effective combination therapies (1).

Cancer cells react toward chemotherapeutics in different modes, such as by modifying DNA repair pathways. DNA repair plays a major role in the cancer therapy response, as chemotherapeutics usually induce various types of DNA damage in cancer cells (2). The overexpression of DNA repair genes in the tumor may confer more efficient repair of induced damage and thus contribute to chemoresistance and impaired therapy response (3). However, downregulation of the DNA repair genes may confer a better therapy response but may also give a basis for the appearance of new mutations and cancer progression (4).

Oxaliplatin (oxalato(trans-l-1,2-diaminocyclohexane) platinum; OX) belongs to the most used chemotherapeutics in CRC treatment. OX is a genotoxic drug that induces the formation of DNA crosslinks, thus directly impairing the structure of DNA, inhibiting DNA replication and RNA synthesis, and inducing apoptosis (5). One of the most crucial repair pathways to deal with DNA crosslinks is homologous recombination (HR), a constituent of double-strand break (DSB) repair (6).

MRN complex, a protein complex consisting of MRE11-RAD50-NBS1, plays an important role in the initial processing of DSB repair. The impaired function of the MRN complex leads to gene instability and DNA damage accumulation, a prerequisite of malignant transformation (7). Mutations in MRE11 predispose to CRC and are frequent in primary CRC with mismatch repair deficiency (8). Patients with the decreased expression of MRE11 were more sensitive to OX treatment, with more significant tumor mass reduction and more prolonged progression-free survival (9). Moreover, single-nucleotide polymorphisms (SNPs) in the 3′ untranslated region (3′UTR) of MRE11 gene are associated with decreased cancer risk but with shorter survival in CRC patients, which implies the role of microRNA (miRNA) regulation in CRC (10).

MiRNAs are signaling molecules in various cell processes functioning mainly as the suppressors of gene expression through interaction with 3′UTRs of target mRNAs. However, miRNAs have also been shown to interact with other regions of mRNA and can even activate gene expression under certain conditions (11). There are several mechanisms by which the deregulation of miRNAs can influence malignant transformation (for review, see (12)). Regardless of the mechanism, miRNA dysregulation can potentiate CRC development by acquiring one or more hallmarks of cancer (13). Despite some evidence of miRNAs influencing the CRC sensitivity to the therapy, there is a scarcity of miRNAs associated with OX therapy response (14).

The main aim of this study was to investigate the effect of miRNAs on the OX therapy response of CRC patients. Based on our previous published study, where we observed an association of SNPs in the 3′UTR of the MRE11 gene with decreased CRC risk (10), we performed in silico analysis of miRNAs associated with MRE11 and found 187 miRNAs with MRE11 as a predicted target. By additional analysis using The Cancer Genome Atlas (TCGA) database, we have identified miR-140 as the best candidate for further investigation. Our results suggest that the miR-140/MRE11 axis is associated with improved therapeutic response in oxaliplatin-treated CRC patients.

# Materials and methods

## Patient characteristics and samples

Paired tumor and non-malignant adjacent mucosa samples were obtained from 50 patients who underwent surgery between the years 2011 and 2015 and in whom all information was followed and updated in 2021 (patients' characteristics in Table 1 and Supplementary Table 1). All the patients provided signed consent for participation and their medical

TABLE 1 Patients' characteristics.

| | | Number of patients (N = 50) |
|---|---|---|
| Gender, N | Male | 26 |
| | Female | 24 |
| Age of diagnosis | Median | 65 |
| | Range | 37-82 |
| Smoker, N | Smokers | 16 |
| | Non-smokers | 16 |
| | Ex-smokers | 18 |
| TNM stage, N | I | 2 |
| | II | 13 |
| | III | 25 |
| | IV | 10 |
| Metastasis | Yes | 26 |
| | No | 24 |

documentation for research. The design of the study was approved by the Ethical Committee of the Institute of Experimental Medicine, Prague, Czech Republic. RNA was isolated from tissues by miRNeasy® Mini Kit (50) (Qiagen, Hilden, Germany).

## Bioinformatics analysis

Data from TargetScan (15) were extracted by multiMiR R package (16).

All miRNA-Seq transcriptional profiles and detailed clinical information were downloaded from TCGA (https://portal.gdc.cancer.gov) using the TCGAbiolinks R package (17). For the present study, data from the project TCGA-READ (rectal adenocarcinoma, n = 155) and TCGA-COAD (colon adenocarcinoma, n = 476) for every miRNA were separately analyzed and filtered according to the following criteria: 1) analyses were performed on CRC patients who had miRNA expression level data available, and 2) clinical data including survival data were also available. Finally, for miR-140, a total of 570 patients presented expression levels.

## Cell cultures

Human colorectal cancer cell lines HCT116, DLD1, and HT29 were obtained from Merck (Darmstadt, Germany). Cell lines were cultured in Dulbecco′s modified Eagle′s medium (DMEM) (Merck, Germany) with 10% fetal bovine serum (Merck, Germany), 1 mM of l-glutamine (Biosera, Nuaille, France), 1 mM of sodium pyruvate (Biosera, Nuaille, France), and 1 mM of penicillin/streptomycin (Biosera, Nuaille, France). All cells were cultured in a humidified incubator at 37°C, with 5% CO2.

## Transient transfection

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, Austin, TX, USA) or with Negative Control miRNA Mimics (Ambion, USA) with no homology to the human genome using Lipofectamine® RNAiMAX 2000 (Invitrogen™) according to the manufacturer′s protocol. All the experiments in cell lines were performed in three independent repeats. The efficiency of transfection was analyzed by qPCR measuring expression levels of transfected miRNAs as compared to negative controls.

## Isolation and reverse transcription of RNA from cell culture samples

Forty-eight hours after transfection, total RNA (including miRNAs) was extracted from cells using Qiagen miReasy Mini Kit (Qiagen, Germany) according to the manufacturer′s protocol. The concentration of the total RNA was measured by Nanodrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of mRNA (RNA integrity number (RIN)) of each sample was determined by Agilent RNA 6000 Nano Kit by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), according to the manufacturer′s protocol.

## Quantitative PCR of cell culture samples

Expression levels of miR-140 were measured using TaqMan MicroRNA Assays at 7500 Real Time PCR System (Thermo Fisher Scientific, USA). The reaction contained 2 ml of a sample with 40 ng of cDNA, 10 ml of TaqMan™ Universal PCR Master Mix, 1 ml of the assay, and 7 ml of RNAse-free water. The thermal protocol was as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s plus melting curve analysis. MiRNA expression was normalized to RNU6B, and all data were subsequently analyzed by the $2^{-DDCt}$ method.

## Oxaliplatin treatment

Oxaliplatin, obtained from Merck (Germany), was dissolved in dimethyl sulfoxide (DMSO; Merck, Germany) at the concentration of 100 mM and stored at 4°C. To assess the chemosensitivity of CRC cells with overexpressed miR-140 and control cells, both cells were treated with a 6 mM concentration

of oxaliplatin 24 h after miRNA mimics transfection and analyzed for cell viability.

## Viability and proliferation assays

For clonogenicity formation assay (CFA), 48 h after cell transfection with miRNA mimics, 500 cells per well were plated for colony formation assay onto 6-well plates and cultured in DMEM. Twelve days later, colonies were fixed with 3% formaldehyde, stained with 1% crystal violet, and counted.

For proliferation assay, cells were plated onto 96-well plates at a density of 3 × 104 cells per well. The metabolic activity of the cells was measured 24 h after plating by adding WST-1 solution into the media as recommended by the manufacturer (Merck, Germany). Absorbance at 450 and 690 nm was measured on BioTek ELx808 absorbance microplate reader (BioTek, Winooski, VT, USA).

## Cell cycle analysis

Cells were seeded on 12 well plates (5 × 105 cells/ml), harvested, washed with PBS, and centrifuged at 1,000 rpm for 10 min. Then, 1 ml of propidium iodide (PI) staining solution (0.02 µg/µl of PI, 0.02 mg/ml of RNase, and 0.05% Triton X-100) was added to the cell pellet, and cells were incubated for 30 min at 37°C in the dark. After incubation, samples were analyzed using a flow cytometer (Apogee A-50 micro, Apogee, Hertfordshire, UK). Measured data were evaluated with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia).

## Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting analysis

Proteins (20 mg) were loaded and separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels at 15 mA for 60 min. Then, the separated proteins were transferred to 0.45 µm Amersham Protran Nitrocellulose Blotting Membrane (GE Healthcare, Life Sciences, Marlborough, MA, USA) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing Tween 20 (TBST; 20 mM of Tris–HCl, pH 7.4, 0.15 M of NaCl, and 0.1% Tween 20) for 1 h and incubated with anti-MRE11, anti-gH2AX, anti-RAD51 (Cell Signaling, Leiden, the Netherlands) and anti-GAPDH antibodies (Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). The membranes were then incubated with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore Corporation, Billerica, MA, USA) and visualized by Azure c600 (Azure Biosystems, Dublin, CA, USA).

## Preparation and application of recombinant lentiviruses for MRE11 silencing

For the preparation of recombinant lentiviruses expressing MRE11 shRNAs, HEK293FT cells (Thermo Fisher, Waltham, MA, USA) seeded in 6-well plates were co-transfected with pLKO1 mission MRE11 shRNA plasmids and helper plasmids psPax2 and pMD2.g (Addgene, Cambridge, MA, USA) using Lipofectamine 3000 (Thermo Fisher, Massachusetts, USA). Six hours later, the medium was replaced with fresh DMEM without antibiotics. After 48 h, the recombinant lentivirus-containing culture medium was harvested and centrifuged at 15 min, 3,000 rpm, and 4°C to remove any floating cells and cell debris. The cleared media containing lentiviruses were at 1:3 and 1:10 v/v ratios, added to HCT116 cells and plated in a 12-well plate, and after 24 h; the media were replaced with the fresh cultivation medium; cell cultures containing integrated lentiviruses were selected by using 2 mg/ml of puromycin for 4–5 days. Transfected cells were then tested using genomic PCR and Western blotting analysis for the genetic elimination/loss of expression of the MRE11 gene.

## Statistical analysis

Statistical analyses were performed using pairwise comparison by Student's t-test and two-way ANOVA (GraphPad Prism8, GraphPad Software, La Jolla, CA, USA; www.graphpad.com). The results represent the mean value of three independent experiments ± SD; the significance level was set at $p \leq 0.05$. Statistical analysis for TCGA data was performed using the R environment using the dplyr and survival, survminer, and ggplot2 packages. The survival significance was measured by a log-rank test.

# Results

## In silico analysis of miRNAs targeting MRE11

Using TargetScan (15), we found 187 miRNAs with MRE11 as a predicted target (Supplementary Table 2) with 111 miRNAs with data sufficient for progression-free survival (PFS) calculation in the TCGA database. Out of these 111 miRNAs, eight had a statistically significant impact on PFS ($p < 0.05$, Supplementary Table 3). We identified miR-140 as the candidate for further investigation, as it displayed the strongest statistically

significant association with PFS (Figure 1, $p < 0.01$) in the group of analyzed miRNAs supported by data from more than 500 patients.

## MiR-140 is downregulated in colorectal cancer and associated with progression-free survival and with the metastatic phenotype in colorectal cancer patients' samples

We investigated the expression levels of MRE11 and miR-140 in 50 CRC tumor tissues and adjacent non-malignant mucosa samples (Table 1 and Supplementary Table 1). The levels of miR-140 were significantly lower in tumor tissue (Figure 2A, $p < 0.01$) compared to adjacent mucosa. MRE11 levels were moderately, but not significantly, higher in tumor tissues (Figure 2B, $p = 0.11$). A significant decrease in miR-140 in patients' CRC samples led only to a moderate non-significant increase in MRE11, which might be due to broader regulation, mixed phenotype, or complex treatment.

The Kaplan–Meier analysis showed, in concordance with TCGA results, that lower expression of miR-140 in tumor tissue is associated with poor PFS (Figure 2C, $p < 0.05$).

Because metastatic CRC has a higher mortality rate and treatment is much more challenging, we have also investigated the association between miR-140 and metastatic formation. Our data showed that decreased expression of miR-140 is associated with the metastatic phenotype of CRC (Figure 2D, $p < 0.05$).

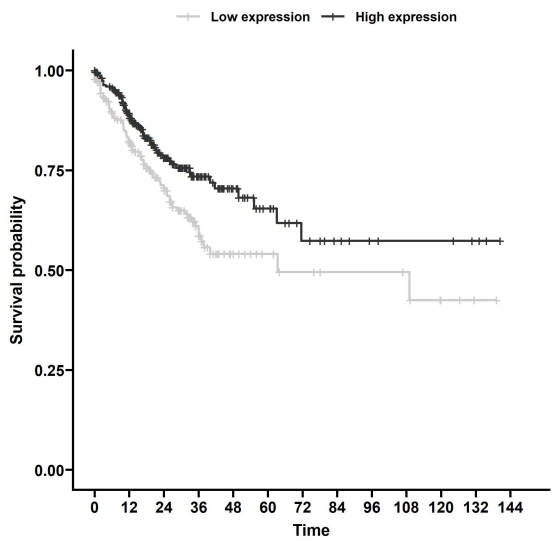


FIGURE 1
Survival analysis of TCGA samples. PFS associated with miR-140 expression of CRC patients from TCGA database. Kaplan–Meier analysis of the TCGA dataset showed that lower miR-140 expression is associated with poor PFS ($p = 0.006$). TCGA, The Cancer Genome Atlas; PFS, progression-free survival; CRC, colorectal cancer.

## MiR-140 represses MRE11 expression

To select the appropriate colorectal cell line for transient transfection, we measured the expression levels of miR-140 in different CRC cell lines (Supplementary Figure 1A), and we decided on DLD1 by transient transfection of miR-140 by miRNA mimics. We have reached a significant increase in miR-140 levels stable up to 72 h (Supplementary Figure 1B). Our data showed that overexpression of miR-140 using miRNA mimics decreased the protein levels of MRE11 (Figure 3A) as well as mRNA levels of MRE11 (Figure 3B).

## Overexpression of miR-140 leads to the accumulation of DNA damage

MRE11 is a crucial component of the MRN complex associated with DSB repair (18). Therefore, we evaluated the effect of miRNA mimic-induced miR-140 overexpression on one of the markers of DSB DNA damage and gH2AX protein accumulation (19). Western blotting analysis showed higher levels of gH2AX after miR-140 miRNA mimics in the CRC cell line (Figure 4).

## Overexpression of miR-140 decreases colorectal cancer cell proliferation

The effect of miR-140 overexpression induced by miRNA mimics on CRC cell proliferation was measured using the WST-1 assay. Figure 5A shows that overexpression of miR-140 leads to decreased cell proliferation, pronounced 24 h after transfection ($p = 0.05$). However, miR-140 overexpression does not affect clonogenic potential (Figure 5B). In addition, flow cytometry analysis of the cell cycle showed that overexpression of miR-140 leads to moderate accumulation of cells in the G1 phase (Figure 5C).

## MiR-140 enhances the chemotherapeutic sensitivity of colorectal cancer cells

Oxaliplatin is a third-generation platinum compound with an important role in CRC treatment. Therefore, we have investigated miR-140 in relation to the oxaliplatin sensitivity of CRC cells. Cell proliferation after oxaliplatin treatment in DLD1 cells overexpressing miR-140 significantly decreased after 48 and 72 h (Figure 6A, $p < 0.05$). The clonogenic potential of the cells (CFA) revealed a significant decrease in colony numbers (Figure 6B, $p <$

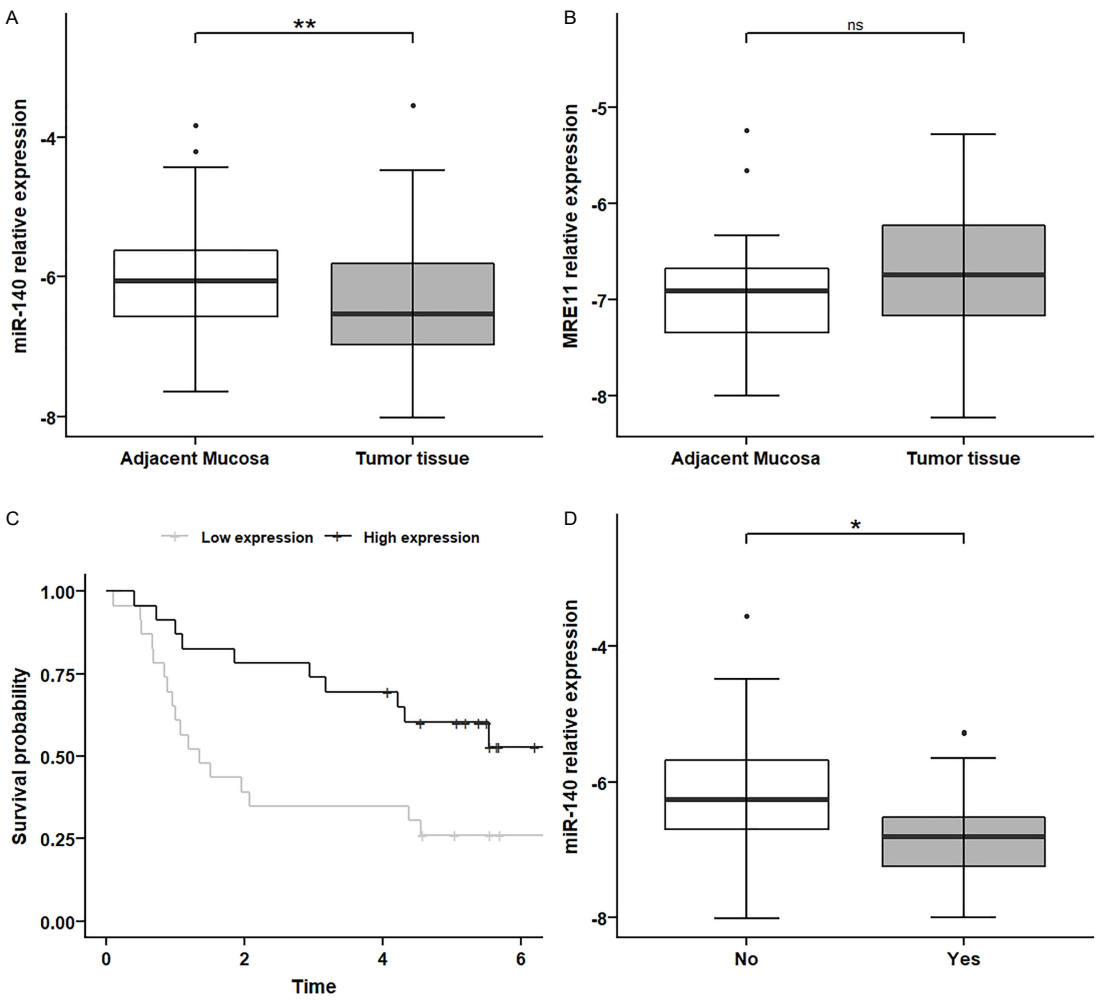


FIGURE 2
MiR-140 downregulation and association with PFS and metastatic CRC phenotype. (A) Relative expression of miR-140 is decreased in tumor tissues compared to non-adjacent mucosa ($p = 0.009$). (B) Relative expression of MRE11 in non-significantly increased in tumor tissue ($p = 0.11$). (C) Kaplan–Meier analysis showed that lower expression of miR-140 in tumor tissue is associated with poor PFS ($p = 0.017$). (D) Decreased relative expression of miR-140 is associated with the metastatic phenotype of CRC ($p = 0.023$). *$p \leq 0.05$, **$p \leq 0.01$. PFS, progression-free survival; CRC, colorectal cancer. ns = $p>0.05$ = non-significant

0.05). Cell cycle analysis of oxaliplatin-treated cells showed that overexpression of miR-140 leads to an increase in cells in the G1 phase and a decrease in those in the S phase (Figure 6C).

## MiR-140 did not affect oxaliplatin sensitivity in shMRE11 cell lines

Our in silico analysis proposed a potential connection between miR-140 and MRE11. To further analyze the effect of miR-140 on oxaliplatin sensitivity through MRE11, we used recombinant lentiviruses expressing MRE11 shRNAs and established CRC cell lines with suppressed levels of MRE11 (Figure 7A). Cellular growth after miR-140 overexpression was not changed in parental and shMRE11 cell lines (Figures 7B, C). The measurement of cellular growth of HCT116 with overexpression of miR-140 and oxaliplatin treatment showed decreased cellular growth ($p = 0.05$) (Figure 7D). However, the analysis of cell growth did not show increased oxaliplatin sensitivity of shMRE11 cells with overexpressed miR-140 (Figure 7E).

# Discussion

Poor therapy response and chemoresistance pose significant complications in CRC treatment, leading to ineffective therapy, tumor progression, metastasis, relapse of disease, and impaired patient survival.

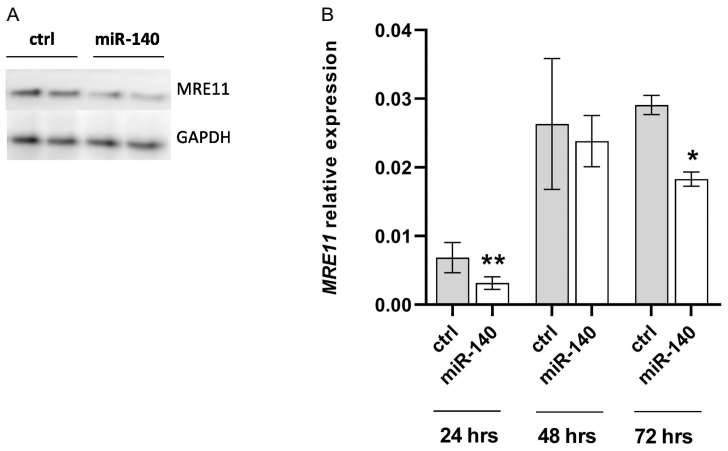


FIGURE 3
Increased levels of miR-140 led to the downregulation of MRE11. (A) Western blotting analysis of cells after transient transfection of miR-140 in the DLD1 cell line showed decreased protein level of MRE11. (B) qPCR analysis of cells showed decreased MRE11 mRNA level. The results represent the mean value of three independent experiments ± SD. *p ≤ 0.05, **p ≤ 0.01.

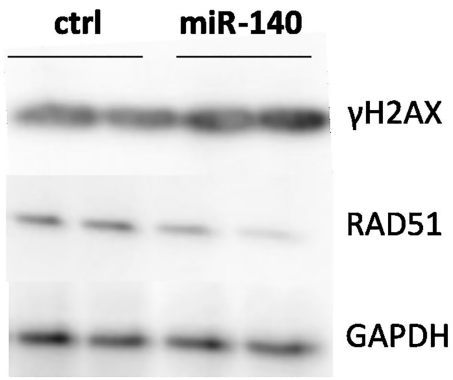


FIGURE 4
Effect of miR-140 on markers of DSBs. Western blotting analysis of cells transiently transfected with miR-140 showed decreased protein levels of RAD51 and increased levels of gH2AX. DSBs, double-strand breaks.

Based on our previous evidence that miRSNPs in the MRE11 gene influence CRC risks and survival (10), in the present study, we investigated the effect of the miRNA/MRE11 axis on the oxaliplatin therapy response of CRC patients.

Despite the multidisciplinary approach and chemotherapy improvement, there is a considerable percentage of patients with inadequate response to treatments and a poor prognosis. Currently, there is a lack of properly validated predictive factors for CRC treatment response, and the emergence of resistant clones is a non-negligible reason for therapeutic failure and potential metastasis development (20). In our study, we defined the association of miR-140 expression with PFS, where lower miR-140 expression is associated with poor survival. Furthermore, our results showed lower levels of miR-140 in tumor tissue. MiR-140 expression has been previously studied mainly in association with cancer development and recurrence. Zheng et al. performed a meta-analysis and found a strong correlation between high expression of miR-140 and better overall survival (OS) in several cancers. Conversely, low expression is associated with advanced stages, worse histologic type, and lymph node metastasis (21). MiR-140 could also remarkably reduce the tumor size in gastric cancer xenograft mice (22). Yuan et al. found that miR-140 is significantly downregulated in non-small lung carcinoma (NSCLC) tissues and cell lines (23). In recent years, there has been increasing evidence of

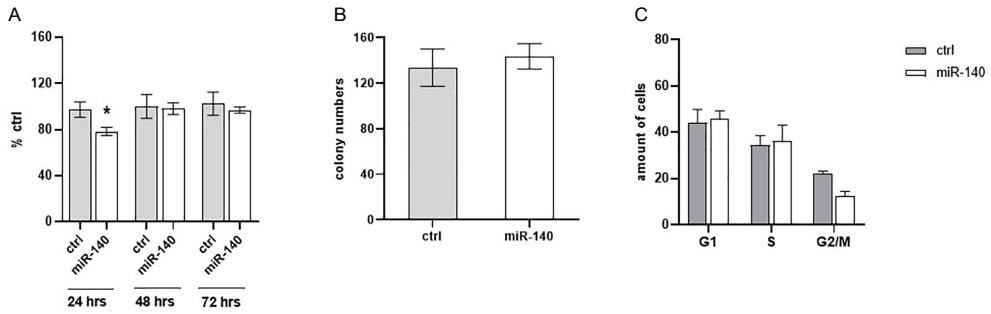


FIGURE 5
Effect of miR-140 on colorectal cancer cells. (A) Proliferation analysis showed a decreased level of proliferation in cells overexpressed miR-140 after 24 h ($p = 0.05$). (B) miR-140 overexpression did not affect the clonogenic potential of the cells. (C) Analysis of cell cycle content showed moderate accumulation of cells in the G1 phase. The results represent the mean value of three independent experiments ± SD. *$p \leq 0.05$.

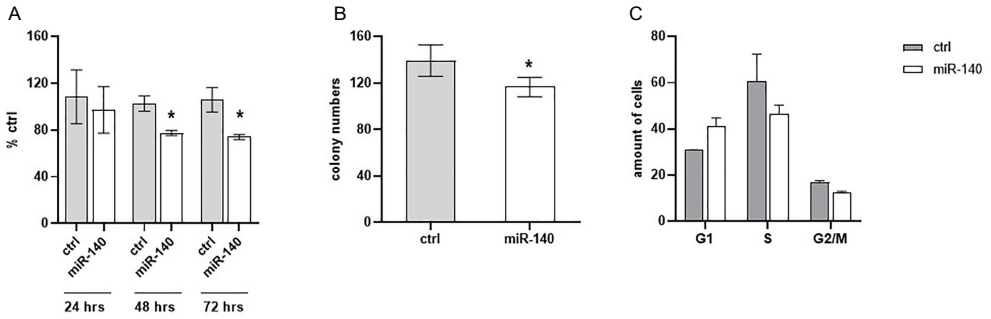


FIGURE 6
MiR-140 enhances the oxaliplatin sensitivity of CRC cells. (A) CRC cell proliferation after miR-140 and oxaliplatin treatment is decreased after 48 h ($p < 0.05$) and 72 h ($p < 0.05$). (B) Analysis of cell clonogenicity potential showed a significant decrease in CFA ($p < 0.05$). (C) Analysis of cell cycles showed an accumulation of cells in the G1 phase and a decrease in the S phase. The results represent the mean value of three independent experiments ± SD. *$p \leq 0.05$. CRC, colorectal cancer; CFA, clonogenicity formation assay.

a miR-140 role in the response to platinum derivative treatment in different cancers. Meng et al. described that miR-140 promoted autophagy mediated by HMGN5 and sensitized osteosarcoma cells to chemotherapy (24). Furthermore, miR-140 acts as a tumor suppressor in breast cancer by inhibiting FEN1 from repressing DNA damage repair. The authors of the published work reveal miR-140 to be a new anti-tumorigenesis factor for adjuvant breast cancer therapy (25). These results suggest a therapeutic potential of miR-140 in cancer treatment. Lui et al. demonstrated that plasma exosomal miR-140 in CRC patients was lower than in healthy controls, and their work supports our findings that miR-140 exerts a tumor suppressor ability (26).

Moreover, we found that decreased expression of miR-140 was associated with metastatic CRC phenotype. Our findings are consistent with a study by Shahabi et al. (2020). The authors showed that low expression of miR-140 is associated with lymph node metastasis in breast cancer (27).

Our in vitro analysis revealed an association of miR-140 overexpression with decreased CRC cell survival and accumulation of DNA damage. Moreover, overexpression of miR-140 enhances the sensitivity of colorectal cells to oxaliplatin. The important role of miRNA in oxaliplatin resistance in CRC was also proven by Wang et al. (28). They published evidence that overexpression of miR-29b re-sensitized OR-SW480 cells to oxaliplatin treatment. MiR-140 also re-sensitizes cisplatin-resistant NSCLC cells to cisplatin treatment through the SIRT1/ROS/JNK pathway (29).

Direct or indirect induction of DNA damage is the main goal of most cancer treatment regimens. Therefore, the process of DNA damage repair plays an important role in therapy response and chemotherapy resistance. Unfortunately, cancer cells can initiate DNA repair, which plays a role in therapy response (3) and chemotherapy resistance (2). The clinical importance of HR for cancer therapy, mainly of MRE11, RAD50, and, NBS, has

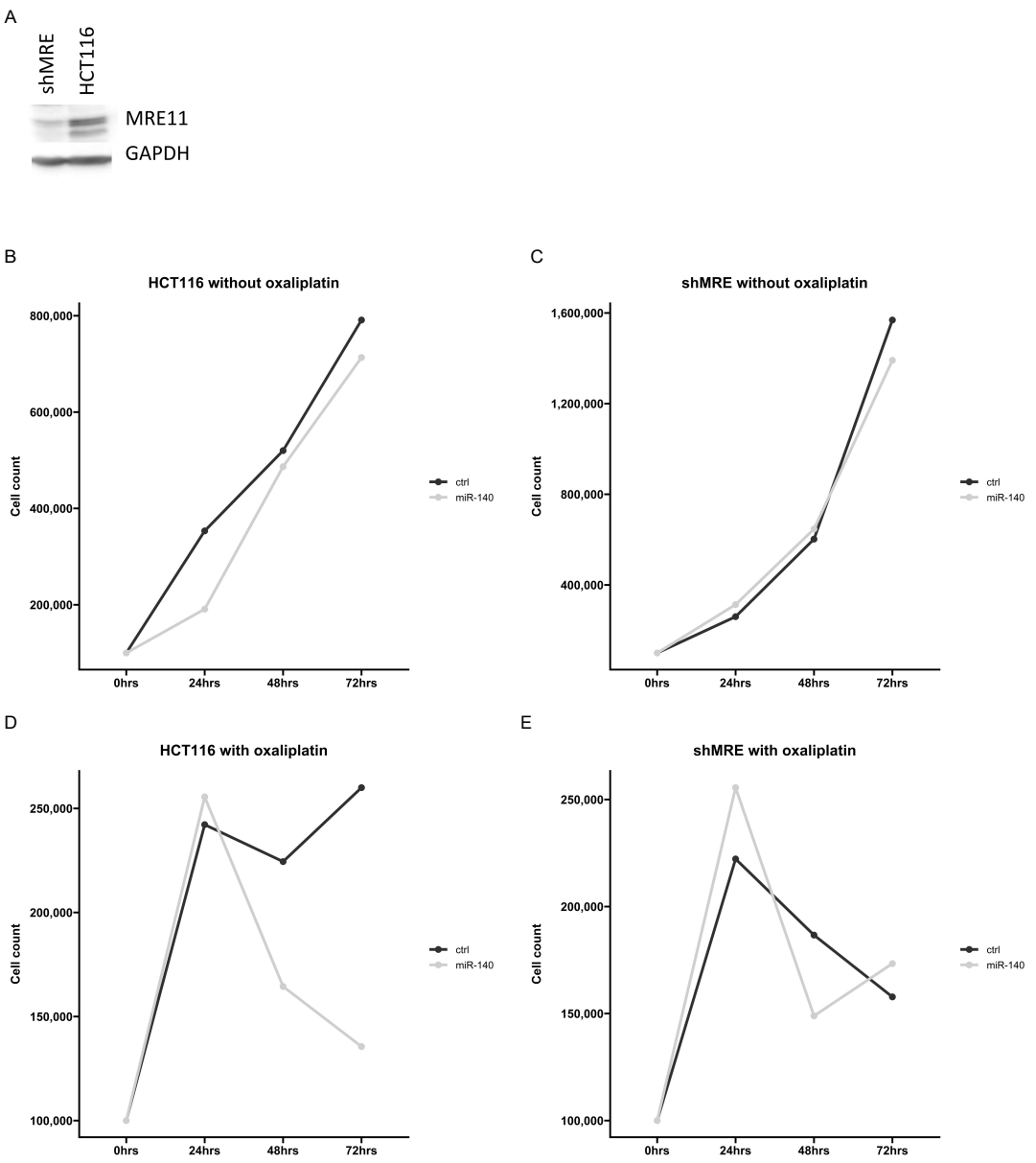


FIGURE 7
MiR-140 did not affect oxaliplatin sensitivity in shMRE11 cell lines. (A) Western blotting analysis of novel established cell line expressing recombinant lentiviruses MRE11 shRNA. (B) Cellular growth of parental cells HCT116 after overexpression of miR-140 was not changed. (C) Cellular growth of shMRE11 cells after overexpression of miR-140 was not changed. (D) Analysis of cellular growth of HCT116 with overexpression of miR-140 and oxaliplatin treatment showed decreased growth ($p = 0.05$). (E) Cellular growth of shMRE11 cells with miR-140 overexpression after oxaliplatin treatment was not changed. The results represent the mean value of three independent experiments.

already been reported (30). According to Pavelitz et al., deficient MRE11 protein is a marker of better prognosis for CRC patients irrespective of treatment in the long term (31). We previously described the significant influence of miRNA binding sites (miRSNPs) in the MRE11 gene on CRC risks and survival (10). The importance of SNPs in miRSNPs of DNA repair genes has been also described in other types of cancer (32). MiR-140 was predicted as a potential interacting partner for MRE11 by TargetScan (15). In vitro overexpression of miR-140 causes the decrease of MRE11 protein levels. We did not observe any effect of miR-140 on cell proliferation and oxaliplatin sensitivity in the cells with inhibited MRE11 (shMRE11). Based on this data, we hypothesize that miR-140 affects oxaliplatin sensitivity in CRC cells via MRE11, or miR-140 may cooperate with MRE11 and may affect oxaliplatin sensitivity in tested cells. MRE11 downregulation may lead to

impairment of MRN complex and thus to inefficient HR and subsequent damage accumulation (33). That is in accordance with our results, as we observed the accumulation of gH2AX, a marker of DNA damage, following overexpression of miR-140.

Despite intensive research, the efficiency of CRC therapy remains low. Searching for novel prognostic and predictive biomarkers may lead to better therapy responses. The presence of miRNAs in blood plasma gives miRNAs a solid potential to be easily accessible biomarkers. However, their use may be compromised by the interindividual variability of cancer patients and large intratumor heterogeneity. Our results indicate miR-140 as a tumor suppressor and potential predictive biomarker for oxaliplatin treatment. We believe that identifying and validating novel biomarkers will ultimately lead to more personalized cancer therapy and improve the quality of a CRC patient's life.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

This study was reviewed and approved by the Ethical Committee of the Institute of Experimental Medicine, Prague, Czech Republic. The patients/participants provided written informed consent to participate in this study.

## Author contributions

JH, AD, AC, OC, and AO performed the experiments. LA coordinated the cell line establishment. ML, LV, and MS were responsible for the collection of patients' samples. PV reviewed the manuscript and discussed the results. AO coordinated the study and wrote a manuscript, JH wrote the manuscript. All authors contributed to the article and approved the submitted version.

## Funding

The study was supported by the Grant Agency of Charles University (GAUK 784120), the Czech Science Foundation (20-03997S, 21-27902S, and 21-04607X), the Czech Health Research Council (grants AZV NV18/03/00199), Charles University grant Unce/Med/006, the Charles University Research Fund (Cooperation No. 43—Surgical Disciplines and the Cooperation Program, research area Oncology and Haematology), EFRR [project No. CZ.02.1.01/0.0/0.0/16_019/0000787 "Fighting INfectious Diseases", awarded by the MEYS CR], and the National Operation Programme: National Institute for Cancer Research LX22NPO05102.

## Acknowledgments

The results shown in the section "In silico analysis of miRNAs targeting MRE11" are in part based upon data generated by TCGA Research Network: https://www.cancer.gov/tcga.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.959407/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) relative expression of miR-140 in different colorectal cell lines (B) optimization of the transfection with miR-140 mimics. The results represent the mean value of three independent experiments ± SD *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

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# Inhibition of homologous recombination repair by Mirin in ovarian cancer ameliorates carboplatin therapy response *in vitro*

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## Abstract

Chemoresistance poses one of the most significant challenges of cancer therapy. Carboplatin (CbPt) is one of the most used chemotherapeutics in ovarian cancer (OVC) treatment. MRE11 constitutes a part of homologous recombination (HR), which is responsible for the repair of CbPt-induced DNA damage, particularly DNA crosslinks. The study's main aim was to address the role of HR in CbPt chemoresistance in OVC and to evaluate the possibility of overcoming CbPt chemoresistance by Mirin-mediated MRE11 inhibition in an OVC cell line. Lower expression of *MRE11* was associated with in better overall survival in a cohort of OVC patients treated with platinum drugs (TCGA dataset, $p < 0.05$). Using *in vitro* analyses, we showed that the high expression of HR genes drives the CbPt chemoresistance in our CbPt-resistant cell line model. Moreover, the HR inhibition by Mirin not only increased sensitivity to carboplatin ($p < 0.05$) but also rescued the sensitivity in the CbPt-resistant model ($p < 0.05$). Our results suggest that MRE11 inhibition with Mirin may represent a promising way to overcome OVC resistance. More therapy options will ultimately lead to better-personalized cancer therapy and improvement of patients' survival.

**Keywords:** Ovarian cancer; MRE11; DNA repair; chemoresistance; cancer therapy

## 1. Introduction

Cancer therapy is a complex process for eliminating the spread of malignant cells, consisting of surgery and other modalities like radiotherapy, chemotherapy, targeted therapy, or immunotherapy. In fact, cancer cells can survive and adapt to cytotoxic therapy-induced conditions. This process, called chemoresistance, introduces one of the most significant complications of cancer therapy and is the leading cause of cancer-related deaths.

Ovarian cancer (OVC) is the eighth most common cancer in females. In 2020 the incidence was more than 300,000 patients, and the mortality was more than 200,000 worldwide [1]. The most common type of OVC is epithelial ovarian cancer (EOVC), with a 5-year survival rate of only 49%, irrespective of staging. Other subtypes of OVC, stromal or germ cell tumors, are rare but have a much better 5-year survival exceeding 90% [2]. Apart from sporadic OVC, some of the hereditary cancer syndromes with a deficiency in DNA repair pathways are associated with a high risk of OVC. For example, the homologous recombination (HR) deficiency leading to a higher risk of OVC is caused mainly by *BRCA1/2* mutations but also by mutations in other HR genes, such as *RAD50*, *PALB2* [3], *BRIP*, *RAD51C*, *RAD51D* [4]. The higher risk of OVC is also connected with mismatch repair deficiency in Lynch Syndrome [5].

The primary therapy regime in EOVC is cytoreductive surgery and platinum-based chemotherapeutics [6]. Over 80% of OVC patients respond to the first-line therapy, but almost 80% of initially responsive patients will develop resistance [7]. Therefore, the chemoresistance to platinum derivates represents a severe therapeutic complication. The cancer cell can acquire chemoresistance by several mechanisms, including increased efflux of the therapeutics by, for example, modulation of ABCB1 and ATP7A membrane transporters [8,9] or altered expression of pro-survival proteins [10]. As DNA is a main target for platinum-based chemotherapeutics, DDR plays a critical role in the chemoresistance mechanisms [11]. In the cell, platinum-based drugs travel to the nucleus, where they cause intra- and inter-strand DNA crosslinks [12]. DNA intra-strand crosslinks are repaired mainly by nucleotide excision repair (NER). In contrast, inter-strand cross-links are repaired by activation of NER, HR, translational synthesis, and Fanconi anemia (FA) pathways [13]. Therefore, upregulation of these pathways may protect the cell from induced damage, as documented in the example with the upregulation of *ERCC1* from the NER pathway associated with chemoresistance to platinum drugs [14].

HR is an important mechanism for repairing double-strand breaks (DSBs). HR-deficient patients, represented by *BRCA1/2* mutations, are also more sensitive to platinum drugs [15]. Rescuing the efficiency of the HR is one of the critical mechanisms resulting in chemoresistance. The secondary mutations in *BRCA1/2* genes may restore the protein expression and establish a resistance to platinum derivates [16]. Rescuing the protein activity by secondary mutations can be observed in other proteins of HR as well, like RAD51 [17]. HR deficiency also plays a role in targeted therapy. PARP inhibitors are one of the first clinically approved drugs in the concept of synthetic lethality [18]. Inhibition of PARP in HR-deficient tumors leads to cancer cell death. The critical step for recognizing and repairing DSBs is forming the Mre11-Rad50-Nbs1 (MRN) complex. MRE11, with its endo and exonuclease activity, is an essential component of this complex. Impaired expression of MRE11 has been associated with developing colorectal

and gastric cancers and susceptibility to ovarian cancer [19]. Alblihy et al. showed that MRE11 protein overexpression in epithelial ovarian cancers was associated with aggressive phenotype and poor progression-free survival [20].
Mirin [Z-5-(4-hydroxybenzylidene)-2-imino-1,3-thiazolidin-4-one] is a small molecular MRE11 inhibitor. Mirin prevents MRN-dependent activation of ATM without affecting ATM protein kinase activity and inhibits MRE11-associated exonuclease activity. Consistent with its ability to target the MRN complex, Mirin abolishes the G2/M checkpoint and homology-dependent repair in mammalian cells [21].
As stated above, surgery and platinum-based chemotherapy are the first therapy choices in OVC. However, the frequent deficiencies in DNA repair pathways, mainly HR, leave the tumors vulnerable to targeted cancer therapy [22]. Furthermore, acquired resistance to chemotherapy and targeted therapy caused by the rescue of the function of mutated proteins represents immense clinical complications [23]. Therefore, inhibition of HR by small molecular inhibitors in HR-proficient tumors and acquired resistance have been studied with the potential to add new drugs to the concept and diminish the occurrence of acquired chemoresistance.
To study the effect of the HR inhibition by MRE11 inhibitor Mirin on the sensitivity of carboplatin (CbPt) in OVC cell lines, we generated a cell line with the acquired resistance to carboplatin. In our present study, we investigated the role of MRE11, an important player in HR, in chemoresistance development and its overcoming. Describing more possibilities and connections between HR inhibition and the therapy response will offer additional options in personalized medicine and, consequently, may improve the patient's survival and quality of life.

## 2. Material and methods

### 2.1. Cell cultures

Human ovarian cancer cell lines OVCAR3 were kindly gifted from Dr. Vaclavikova from Center of Health Safety of National Institute of Public Health. A resistant cell line to carboplatin was created from maternal OVCAR3 by adding an increasing amount of carboplatin, as described in the Coley protocol [24]. Cell lines were cultured in RPMI medium (Merck, Germany) with 10% fetal bovine serum (Merck, Germany), 1 mM L-glutamine (Biosera, Nuaille, France), 1 mM sodium pyruvate (Biosera, Nuaille, France) and 1mM penicillin/streptomycin (Biosera, Nuaille, France), HEPES (Biosera, Nuaille, France). All cells were cultured in a humidified incubator at 37°C, 5% $CO_2$.

### 2.2. Mirin pretreatment

Mirin, obtained from Sigma-Aldrich® (Germany), was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) at the concentration of 45mM and stored at -20°C.

To assess the MRE11 inhibition by Mirin, we used the protocol by Dupre *et al.* [21]. After one hour of pretreatment with 100 μM solution of Mirin, the cells were washed with PBS and followed by the treatment of carboplatin.

### 2.3. Carboplatin treatment

Carboplatin, obtained from Sigma-Aldrich®(Germany), was dissolved in DMSO (Merck, Germany) at the concentration of 125mM and stored at -20°C.

To assess the chemosensitivity of paternal and carboplatin resistant OVCAR3 cells after inhibition of MRE11, cells were treated with the 10 μM concentration of carboplatin after the Mirin pretreatment. For the assessment of gene expression, viability, proliferation, cell cycle and protein expression the 48hrs Carboplatin treatment was used. For the growth curves the treatment was according to the time point (24hrs, 48hrs, 72hrs).

### 2.4. Isolation and reverse transcription of RNA from cell culture samples

Forty-eight hours after treatment, total RNA was extracted from cells using Qiagen miReasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration of the total RNA was measured by Nanodrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, USA), and the integrity of mRNA (RIN) of each sample was determined by Agilent RNA 6000 Nano Kit by Agilent Bioanalyzer 2100 (Agilent Technologies). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

### 2.5. Quantitative PCR of cell culture samples

Expression levels of DDR and EMT genes were measured using a 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Reaction contained 2 μL of a sample with 40 ng of cDNA, 10 μL SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA), 1 μL of custom-made primers (supplementary table 1), and 7 μL of RNAse-free water. The thermal protocol was: 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15 s, and 62°C for 60 s plus melting curve analysis. mRNAs expression was normalized to ACTB, GAPDH, and RNU19, and all data were subsequently analyzed by the $2^{-\Delta\Delta Ct}$ method.

### 2.6. Viability and proliferation assays

For clonogenicity potential assay (CFA), 500 cells per well were plated for colony formation assay onto 6-well plates treated with appropriate drug concentrations for 24hrs. After 24hrs the medium was changed to a fresh one. Twelve days later, colonies were fixed with 3% formaldehyde, stained with 1% crystal violet, and counted.

For proliferation assay, cells were plated onto 96-well plates in a density of $2x10^4$ cells per well. The metabolic activity of the cells was measured 48 hours after plating by adding WST-1 solution into the media as recommended by the manufacturer (Merck, Germany). Absorbance at 450 and 690 nm was measured on a BioTek ELx808 absorbance microplate reader (BioTek, USA).

### 2.7. Cell cycle analysis

Cells were seeded on 12 well plates ($5x10^5$ cells/ml), harvested, washed with PBS, and centrifuged at 1000 rpm for 10 min. Then, 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 pellet, and cells were incubated for 30 min at 37°C in the dark. After incubation, samples were analyzed using a flow cytometer (Apogee A-50 micro, Apogee, Hertfordshire, UK). Measured data were evaluated with Flowlogic software (Inivai Technologies, Mentone, Australia).

### 2.8. Protein isolation

Forty-eight hours after treatment, the proteins were isolated from cells using RIPA lysis buffer (Sigma-Aldrich®, USA) with added inhibitors of proteases (Roche, Germany). The concentration of the proteins was measured by Bradford reagent (Sigma-Aldrich®, USA) and BSA standards (BioRad Quick Start Set, USA)

### 2.9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis

Proteins (40µg) were loaded and separated in 10% SDS-PAGE gels at 15 mA for 60 min. Then, the separated proteins were transferred to 0.45 µm Amersham Protran Nitrocellulose Blotting Membrane (GE Healthcare, Life science) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline containing Tween 20 (TBST; 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 0.1% Tween 20) for 1 hour and incubated with anti-MRE11, anti-γH2AX, anti-RAD51 (Cell Signalling, Leiden, The Netherlands) and anti-GAPDH antibodies (Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). Next, the membranes were then incubated with Immobilon western Chemiluminescent HRP Substrate (EMD Millipore Corporation, MA, USA) and visualized by Azure c600 (Azure Biosystems, Dublin, CA, USA).

### 2.10. The Cancer Genome Atlas data analysis

The transcriptional profile of MRE11 and clinical information were downloaded from The Cancer Genome Atlas TCGA (https://portal.gdc.cancer.gov) using the cbioportal.org website [25]. For the present study, data from the project TCGA-OV (Ovarian Serous Cystadenocarcinoma) and filtered according to the following criteria: 1) the sample had available expressions levels data of the MRE11 gene, 2) the sample had clinical data of survival available, and 3) the patient was treated by platinum-based drugs. A total number of 251 patients passed the filtering and were included in the survival analyses.

### 2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism8 (GraphPad Prism8, GraphPad Software, La Jolla California USA, www.graphpad.com) and R programming

language and Rstudio with the help of libraries dplyr and ggplot2. The Shapiro test assessed normality, and according to the results, a parametrical test (student t-test) or non-parametrical test (Mann-Whitney test) was applied. The results represent the mean value of three independent experiments ±SD; boxplots show median and interquartile range; the significance level was p≤0.05 (“*”), p<0.01 (“**”) and p<0.001 (“***”). Statistical analysis for TCGA data was performed using the survival and survminer packages, and the survival significance was measured by a log-rank test with the significance level of p≤0.05.

## 3. Results

### 3.1 The expression level of MRE11 impacts patients’ overall survival

Initially we intended to see whether the expression level of MRE11 might impact the survival of the OVC patients who were treated with platinum drugs. For that purpose, we used the data from the TCGA database and stratified the samples by median into groups with higher and lower expressions. The group of patients with the lower expression levels of MRE11 had significantly better overall survival (OS) than the group with the higher expression level (Fig 1, p < 0.05)

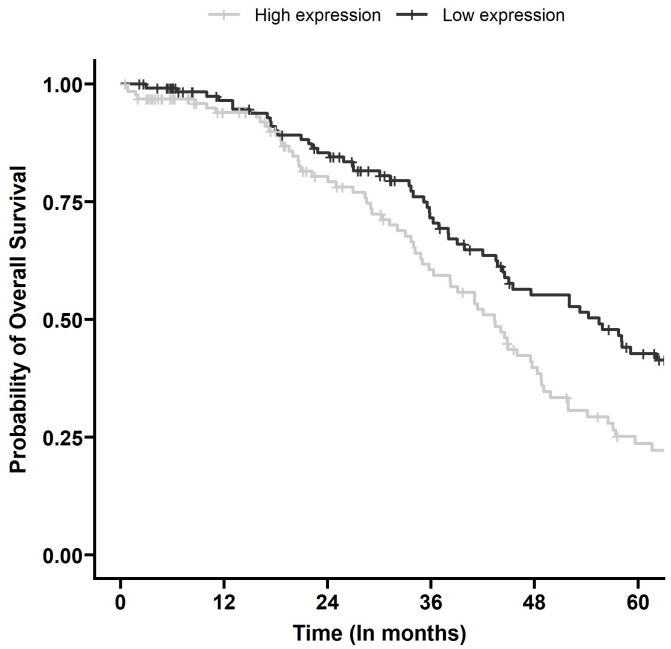


**Fig. 1** Survival analysis of TCGA samples
OS associated with MRE11 expression of OVC patients from TCGA database. Kaplan Meier analysis of TCGA dataset showed that lower MRE11 expression is associated with better OS (p<0.05).

### 3.2. Effect of Mirin on OVCAR3 cell line

As the MRN complex is essential for HR and may significantly impact cell line survival, we first evaluated the effect of Mirin treatment on the accumulation of DNA damage and the level of MRE11 expression. We adopted the protocol of Dupre et al. [21]. For the combinatory effect of HR inhibition and carboplatin treatment, we performed one-hour Mirin pretreatment. Mirin pretreatment does not significantly affect cell proliferation (Fig 2A), clonogenic potential (Fig 2B) or cell growth (Fig 2C). As expected, Mirin pretreatment leads to a decreased level of MRE11 (Fig 2D and E, $p<0.05$), mild accumulation of DNA damage (Fig 2F) and mild S phase arrest on the cell cycle (Fig 2G).

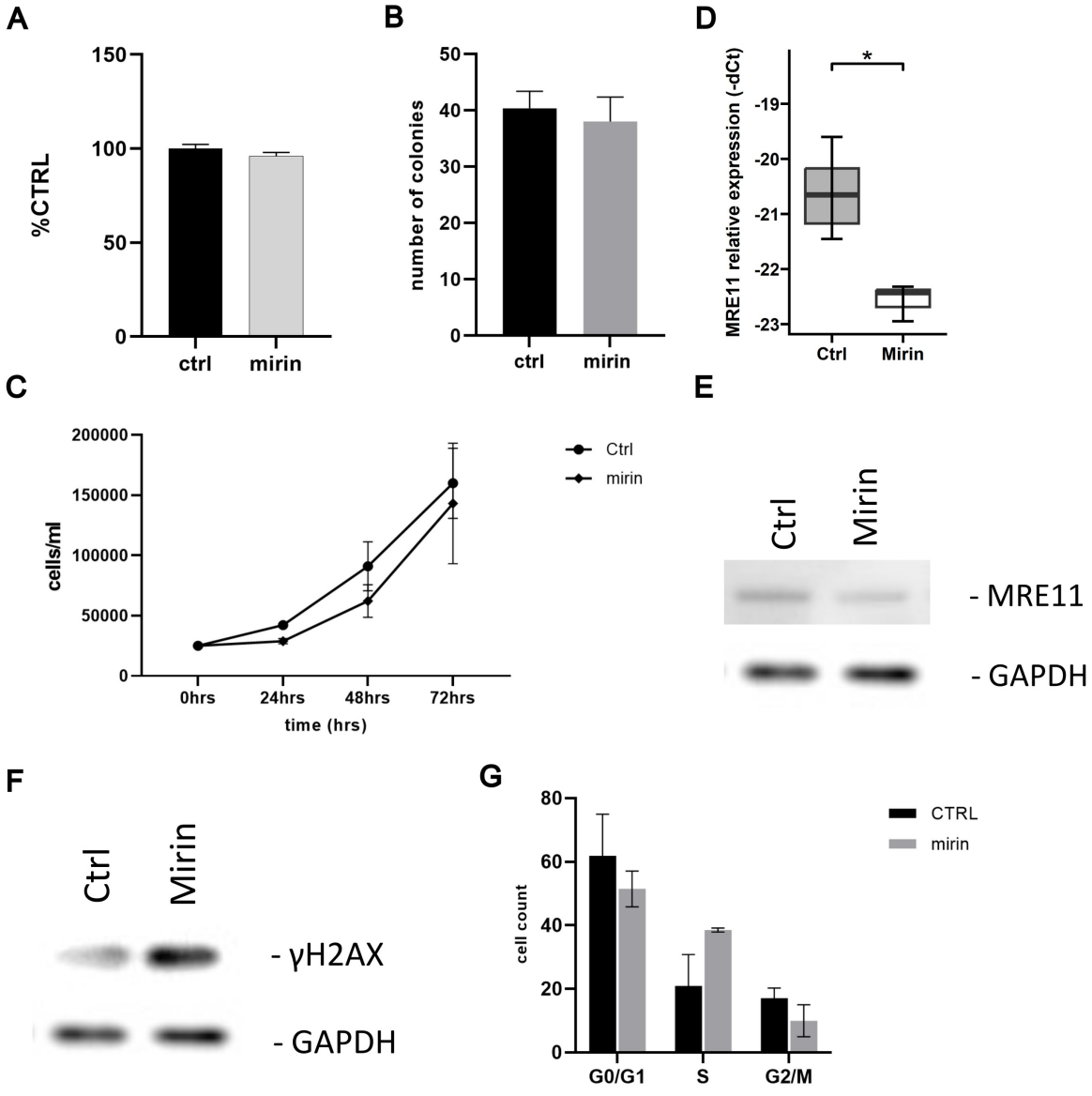


**Fig. 2** Effect of Mirin pretreatment
Mirin pretreatment (1hr) does not significantly change A) cell proliferation measured by WST1 assay,

B) Clonogenic potential measured by colony-forming assay and C) cell growth. Mirin pretreatment downregulates MRE11 D) on RNA level ($p<0.05$) and E) on protein level. It also causes F) mild accumulation of DNA damage and G) mild S-phase arrest in cell cycle. Significance level "*" is $p < 0.05$, "**" is $p<0.01$, "***" is $p<0.001$. Bars indicate mean ±SD from three individual experiments. Boxplots show median and interquartile range.

### 3.3. HR inhibition increased the sensitivity to carboplatin

We used proliferation and colony-forming tests to assess the impact of HR inhibition on carboplatin sensitivity. Carboplatin itself moderately decreases the proliferation of the OVCAR3 cells (Fig 3A, $p < 0.05$). However, after the Mirin pretreatment, the decrease in viability of cancer cells is significantly pronounced (Fig 3A, $p < 0.05$). Moreover, supporting the proliferation, we observe a similar effect on clonogenic potential (Fig 3B, $p < 0.05$) and cell growth (Fig 3C, $p < 0.05$).

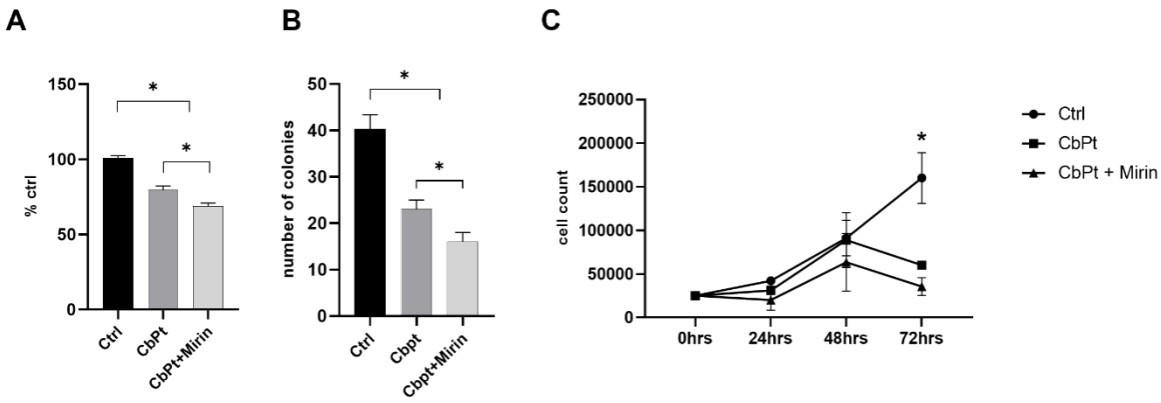


**Fig. 3** Effect of Mirin pretreatment on OVCAR3 sensitivity to carboplatin
A) OVCAR3 proliferation is impaired by the CbPt treatment (48hrs) ($p < 0.05$), and Mirin pretreatment
(1hr) led to the increase of the carboplatin effect ($p < 0.05$), measured by the WST1 assay. B) CbPt treatment (48hrs) impaired the clonogenic potential measured by colony-forming assay ($p < 0.05$), and Mirin pretreatment (1hr) further pronounced that effect ($p < 0.05$). C) Also, the cell growth, measured by counting cells, was decreased by CbPt treatment (48hrs) ($p < 0.05$) and also by a combination of Mirin pretreatment (1hr) and CbPt treatment (48hrs) ($p < 0.05$). Significance level "*" is $p<0.05$, "**" is $p<0.01$, "***" is $p<0.001$. Bars indicate mean ±SD from three individual experiments.

### 3.4. Effect of HR inhibition and carboplatin treatment on DNA damage accumulation and cell cycle

Carboplatin induces DNA damage, whereas Mirin inhibits DNA repair (MRE11 and NBS1 are downregulated after the Mirin pretreatment, supplementary Figure 1). Therefore, we explored the option of DNA damage accumulation as a consequence of the Mirin

treatment. We can see the increase in γH2AX expression as a marker of DNA damage (Fig 4A). Accumulation of DNA damage is associated with changes in the cell cycle. It leads to the arrest in the S phase, where the replication machinery proceeds to repair the damage. We have recorded the S phase arrest after the combinatory effect of carboplatin treatment and Mirin pretreatment (Fig 4B).

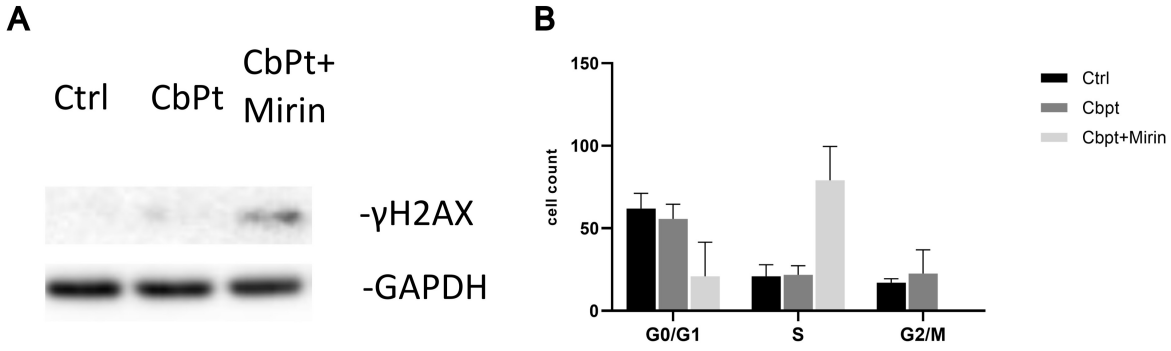


**Fig. 4** Effect of combination treatment of Mirin and CbPt to DNA damage accumulation and cell cycle
of OVCAR3 cell line A) Western Blot of accumulation of γH2AX level after Mirin pretreatment and CbPt treatment. B) Cell cycle distribution of OVCAR3 cell line without treatment (black), with carboplatin treatment (48hrs, dark grey) and Mirin pretreatment (1hrs) followed by carboplatin treatment (48hrs, light grey). Bars indicate mean ±SD from three individual experiments.

### 3.5. Differences between OVCAR3 and Carboplatin resistant cell line

To assess the potential of HR inhibition for overcoming the CbPt resistance, we have established the carboplatin-resistant (CbPtR) cell line. The CbPtR cell line has approximately 3.5x higher IC50, and it exhibits a changed morphology (Fig. 5B) than the paternal OVCAR3 cell line (Fig. 5A).

CbPtR cell line also has extensive changes in the expression profile of many genes involved in the repair of carboplatin-induced damage. Our analysis by qPCR showed significant increases in HR-genes expression, including all members of the MRN complex (*MRE11*, *NBS1*, and *RAD50*), its activation target ATM, and most of the HR effectors genes. The resistant cell line also activated the error-prone but still very accurate NHEJ. However, the fork to very inaccurate single-strand annealing is not activated (Fig. 5C). Several other genes involved in autophagy and apoptosis, or the efflux transporter MDR1, are also dysregulated (Supplementary table 1).

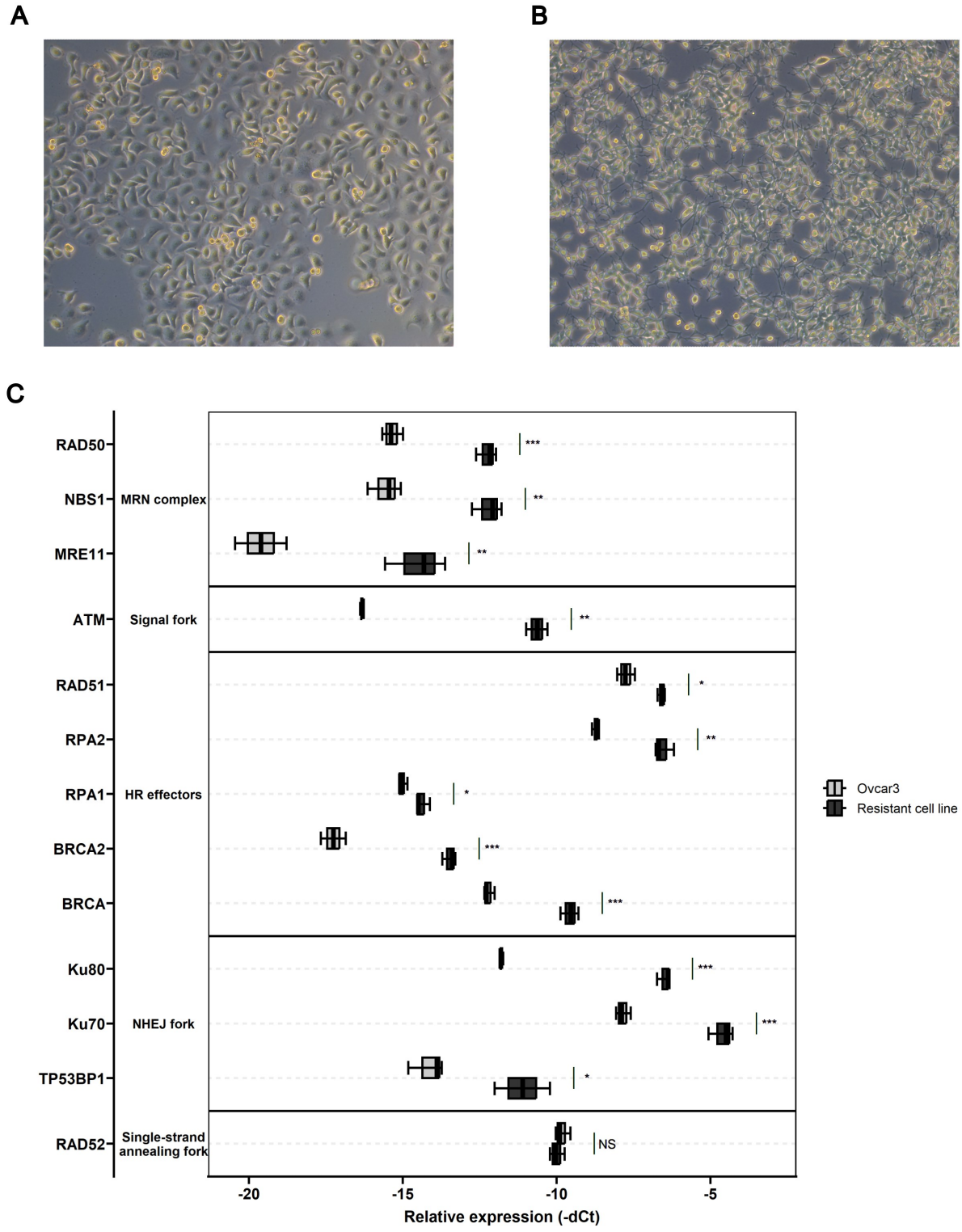


**Fig. 5** Characteristics of carboplatin-resistant cell line

A) Morphology of maternal OVCAR3 cell line. B) Morphology of resistant cell line to carboplatin. C) Difference of expression profiles of double-strand break repair genes between maternal (dark grey) and resistant (light grey) cell lines. Overexpressed genes of MRN complex (MRE11, NBS1, and RAD50) and their signaling target ATM (second row from the top). Overexpressed HR downstream effectors (BRCA1/2, RPA1/2, RAD51) are in the third row, and NHEJ (TP53BP1,

Ku70, and Ku80) is in the fourth row from the top. RAD52, responsible for Single-strand annealing, was unchanged (bottom row). Significance levels, "*" is p<0.05, "**" is p<0.01, "***" is p<0.001. Boxplots show median and interquartile range.

### 3.6. Rescuing the sensitivity to carboplatin in a resistant cell line by Mirin

To explore the possible rescuing of the carboplatin sensitivity by MRE11 inhibition by Mirin pretreatment, we performed the analysis of viability, cellular growth, and clonogenic potential on the CbPtR cell line after Mirin pretreatment. Carboplatin-resistant cell line proliferation was not impaired by the CbPt treatment. CbPtR proliferation is significantly decreased after combining carboplatin treatment and Mirin pretreatment (Fig 6A, $p < 0.05$), as are clonogenic potential (Fig 6B, $p < 0.05$) and growth curves (Fig 6C, $p < 0.05$). Mirin also decreased the expression of HR genes ATM and RAD51 (Fig 6D, $p < 0.05$). Our results showed that HR inhibition leads to an increase in the sensitivity to carboplatin.

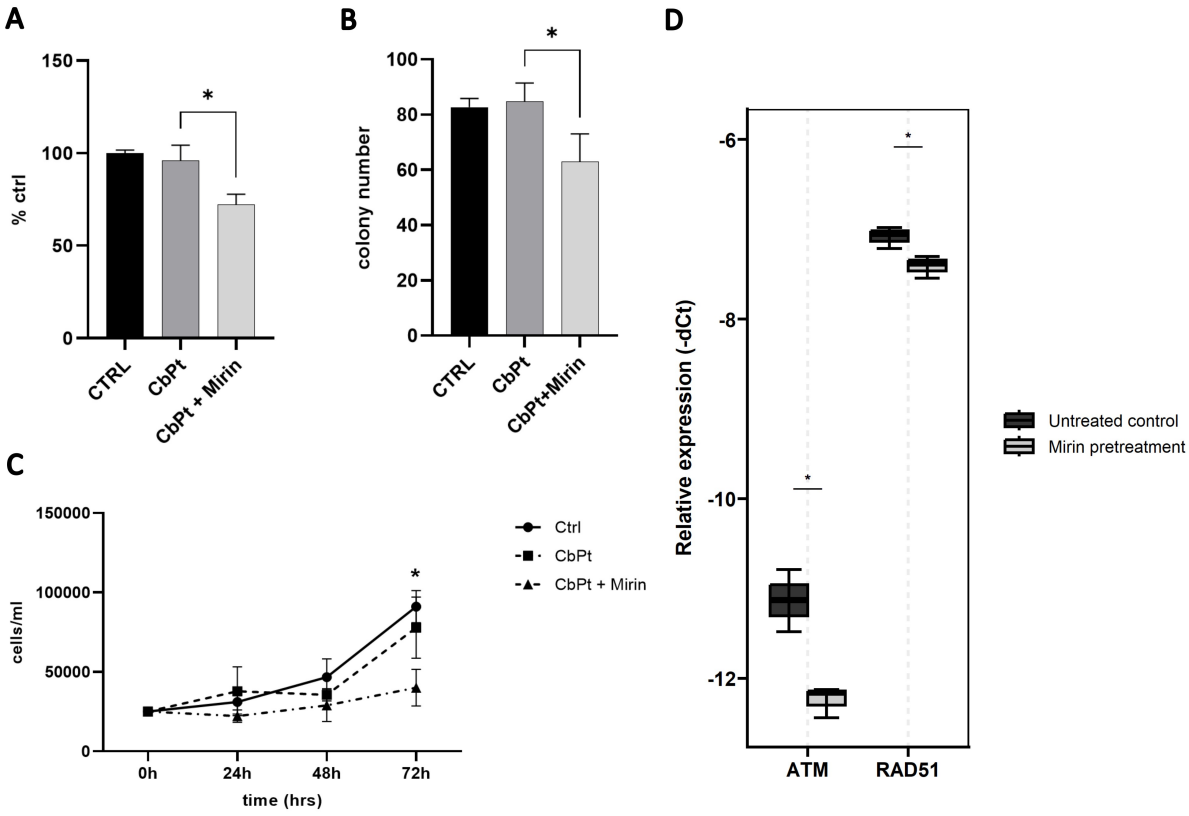


**Fig. 6** Mirin rescued the resistant cell line sensitivity
A) Carboplatin-resistant cell line proliferation was not impaired by the CbPt treatment (48hrs). Still,
Mirin (1hr) pretreatment led to rescuing of the sensitivity of the carboplatin ($p < 0.05$), measured by the WST1 assay. B) CbPt treatment (48hrs) did not impair the clonogenic potential measured by colonyforming assay, but Mirin pretreatment (1hr) rescued that effect ($p < 0.05$). C) Also, the cell growth, measured by counting cells, was not decreased by CbPt treatment (48hrs), but the combination of Mirin pretreatment (1hrs) and CbPt treatment (48hrs) decreased it ($p < 0.05$) D) Mirin pretreatment (1hr) led to the decreased expression of ATM ($p < 0.05$) and RAD51 ($p < 0.05$)

genes. Significance level "*" is $p < 0.05$, "**" is $p<0.01$, "***" is $p<0.001$. Bars indicate mean ±SD from three individual experiments. Boxplots show median and interquartile range.

## 4. Discussion

Chemoresistance is one of the major complications of OVC therapy, leading to treatment failure, metastases, relapses, and consequently to patients' death. Therefore, discovering new targets and therapy combinations is necessary to improve treatment options in personalized medicine and increase the chance of better patient survival. Consequently, we focused on the chemoresistance of OVC cancer to carboplatin and investigated the possibility of the tentative use of HR inhibition and a possible source of at least partial rescue of the CbPt sensitivity in tumor cells. Alblihy et al. similarly explored the MRE11 inhibition to increase cisplatin sensitivity and discovered that the combination of XRCC1 deficiency and MRE11 inhibition is lethal in ovarian cancer cells [20].

Our research demonstrated that inhibition of MRE11 by Mirin led to increased sensitivity to carboplatin, DNA damage accumulation, and cell cycle arrest in the S phase. Moreover, we demonstrated that MRE11 inhibition by Mirin successfully rescued the sensitivity to carboplatin in carboplatin-resistant cell lines. Impairment of HR in OVC treatment response has been previously studied, but it was mainly focused on impairment via mutations in *BRCA1* and *BRCA2* genes. *BRCA1* and *BRCA2* mutations not only lead to the possibility of using PARP inhibitors in the treatment but are also connected to increased sensitivity to platinum-based drugs [26]. However, other genes of the HR pathway are also related to the sensitivity to platinum-based drugs [27], like RAD50 [28] and RAD51 [29]. We also documented on the dataset from the TCGA that the lower MRE11 expression led to better survival of OVC patients who were treated with platinum-based drugs. Mirin as an inhibitor was also previously shown to induce cell death in multiple myeloma [30] and neuroblastoma through increasing replication stress [31], otherwise Mirin's potential anticancer effects are not much explored.

Overcoming resistance is a difficult task as chemoresistance is a complex process in chemotherapy in general and in platinum-based chemotherapy in particular [32]. Changes during the chemoresistance development often lead to morphology changes [33]. Our carboplatin-resistant cell model also exhibited morphology changes and showed a distinct expression profile in DNA damage repair genes and known multidrug resistance genes, such as the MDR1 transporter. However, ATP7A and ATP7B transporters are responsible for transporting the carboplatin inside the cancer cells [8,9]. Therefore, the upregulation of MDR1 might be reactive, but the main resistance mechanism is the upregulation of DNA repair genes. We showed the upregulation of most HR genes from sensing DNA damage by MRN complex through ATM signaling and repair effectors like RAD51. Also, another pathway from the double-strand break repair NHEJ was activated. However, the fork to the most error-prone single-strand annealing is not activated. Inhibition of NHEJ was studied for synthetic lethality in p53 mutated tumors [34]. It also increased the sensitivity of leukemic KRAS mutated cells to chemotherapy [35]. Inhibition of HR by targeting MRE11 was previously explored to increase the sensitivity to radiotherapy in several types of cancer (oral, breast, bladder, rectal, or anal cancer) [36]. Moreover, Mirin itself was used in glioblastoma for that

purpose [37]. High expressions of MRE11 also correlated with worse survival in gastric cancer [38], and it was a prognostic marker in left-sided colorectal cancer [39] or breast cancer [40], where the high expression is also connected to more malignant behavior and in forming metastases. Besides a role in DDR, MRE11 is essential for countering oncogene-driven replication stress. The MRE11-ATM axis is also involved in pro-survival signaling, epithelial-mesenchymal transition (EMT), invasion, and migration [41]. We showed that in our carboplatin-resistant model, among others, MRE11 was upregulated, and its inhibition by Mirin led to decreased expression of *ATM* and *RAD51*, consequently sensitizing the cells to carboplatin.

Despite the successes of PARP inhibitors and other therapeutics of targeted therapy, current treatment combinations are not universal for all cancers. In addition, not all patients are eligible for treatment with currently used targeted inhibitors. Our results suggest that MRE11 inhibition with Mirin represents a promising therapeutic approach. Searching for novel therapeutic targets may lead to a better understanding of chemoresistance mechanisms and bring new possibilities in cancer therapy to the table. More therapy options will ultimately lead to better-personalized cancer therapy and improvement of patients' survival.

**Acknowledgment**

JH and PH acknowledge the support of the project Grant Schemes at Charles University (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935). PV and his team acknowledge the support from the grants awarded by the National Science Foundation 19-10543S and 21-27902S. We would like to thank Dr. Vaclavikova from Center of Health Safety of National Institute of Public Health in Czech Republic for kindly gift of OVCAR3 cell line.

**Data Availability Statement**

The original data presented in this study are available in the study and supplementary material. The transcriptional profile of MRE11 and clinical information were downloaded from The Cancer Genome Atlas TCGA (https://portal.gdc.cancer.gov)

**Conflicts of Interest**

The authors declare no conflict of interest.

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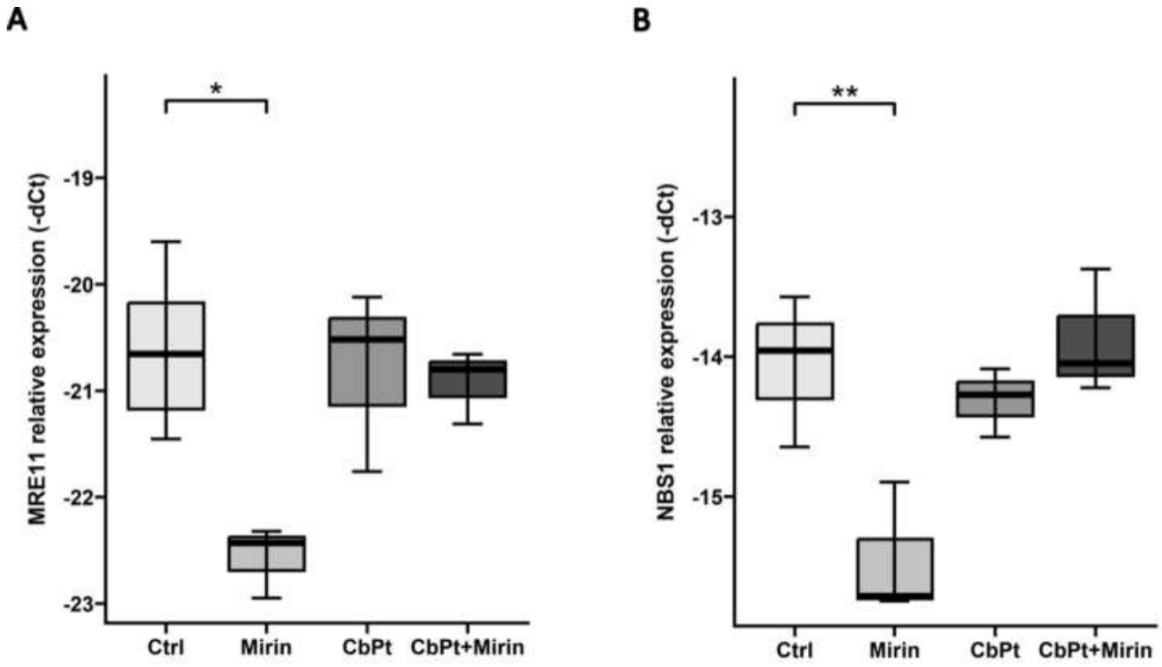


**Supplementary figure 1**

Effect of Mirin pretreatment and carboplatin treatment to DNA repair genes expression

Mirin pretreatment (1hrs) led to decrease in A) MRE11 gene expression and B) NBS gene expression. Significance levels, "*" is p<0.05, "**" is p<0.01, "***" is p<0.001. Boxplots show median and interquartile range.

| Gene | p_value | fold_change |
|---|---|---|
| AKT | 0,521048 | 1,151177261 |
| APEX1 | 0,03093 | 2,403736908 |
| ATG5 | 0,000242 | 28,74995913 |
| ATG7 | 0,079901 | 3,305835644 |
| ATM | 0,001049 | 51,89725523 |
| ATR | 2,22E-05 | 12,88980619 |
| BAD | 0,179343 | 1,637198973 |
| BAK1 | 0,001742 | 0,328373133 |
| BCL10 | 0,000235 | 47,82489845 |
| BECLIN1 | 0,248177 | 1,396314573 |
| BRCA | 0,000532 | 6,252466113 |
| BRCA2 | 0,000641 | 13,79893163 |
| CASP9 | 0,079141 | 1,233128945 |
| CMYC | 0,014686 | 52,7253916 |
| DAPK1 | 0,017971 | 1,888539117 |
| DCLRE1C (ARTEMIS) | 0,001269 | 32,14814673 |
| DDB1 | 0,703861 | 1,046251241 |
| DDB2 | 0,004484 | 0,296023647 |
| ERCC2/XPD | 0,056873 | 2,893026089 |
| ERCC3/XPB | 0,506897 | 0,861446238 |

| | | |
|---|---|---|
| ERCC4/XPF | 0,001766 | 17,55684044 |
| ERCC6/CSB | 0,260852 | 1,826216742 |
| ERCC8/CSA | 2,83E-06 | 60,98462521 |
| H2AFX | 0,001618 | 1,734386782 |
| HIF1 | 0,000639 | 16,35505078 |
| CHEK1 | 0,000586 | 62,64332998 |
| CHEK2 | 0,029197 | 1,811692785 |
| LC3 | 0,033266 | 8,998584003 |
| LIG1 | 0,00554 | 2,430489104 |
| LIG3 | 0,726534 | 1,103691267 |
| MDB4 | 0,302322 | 0,780455168 |
| MDR1 | 0,02352 | 50,44553471 |
| MRE11 | 0,002651 | 34,47813103 |
| MSH2 | 4,86E-06 | 44,68432729 |
| MSH3 | 0,000976 | 30,42138645 |
| MSH6 | 0,002238 | 1,557038913 |
| MUTYH | 0,01675 | 0,426211768 |
| NBS1 | 0,001452 | 10,13493783 |
| NEIL1 | 0,00057 | 0,491244899 |
| NEIL2 | 0,325191 | 1,403573226 |
| NEIL3 | 4,99E-06 | 85,06173244 |
| NTH1 | 7,19E-06 | 0,136240355 |
| PARP1 | 0,005125 | 5,064240782 |
| PMS1 | 0,052608 | 7,85147296 |
| PMS2 | 0,00175 | 16,87369472 |
| POLB | 4,98E-05 | 21,22483843 |
| POLD1 | 0,581299 | 1,157093408 |
| POLD2 | 0,088729 | 1,599343321 |
| POLD3 | 0,001317 | 62,31756684 |
| POLD4 | 0,82378 | 0,902184455 |
| POLE1 | 0,001685 | 0,428267667 |
| POLE2 | 0,000287 | 5,980451198 |
| POLE3 | 9,04E-05 | 9,83822466 |
| POLE4 | 0,004456 | 3,286153033 |
| RAD21 | 0,002997 | 7,215626519 |
| RAD23B | 0,000921 | 3,760530853 |
| RAD50 | 0,000359 | 8,513942421 |
| RAD51 | 0,010453 | 2,238837265 |
| RAD51C | 0,000112 | 9,662668496 |
| RAD51D | 0,00036 | 4,612044181 |
| RAD51L1 | 6,22E-05 | 4,710706126 |
| RAD52 | 0,446686 | 0,890606883 |
| RAD54L | 0,00061 | 4,889409818 |
| RPA1 | 0,021169 | 1,54877403 |
| RPA2 | 0,003929 | 4,5134981 |

| | | |
|---|---|---|
| SMUG1 | 0,40636 | 0,829427431 |
| SOD2 | 0,011322 | 1,32629221 |
| TDG | 0,000144 | 15,25385867 |
| TDP1 | 0,884332 | 1,024843298 |
| TOPBP1 | 9,2E-05 | 40,73745646 |
| TP53 | 0,001242 | 0,283018252 |
| TP53BP1 | 0,011785 | 8,209729379 |
| UNG | 0,916147 | 1,017847201 |
| WIP1 | 2,98E-05 | 8,742785478 |
| XPA | 0,009901 | 45,17496777 |
| XPC | 0,00025 | 6,320041361 |
| XRCC1 | 0,322148 | 0,916498654 |
| XRCC2 | 0,133548 | 1,438501441 |
| XRCC3 | 0,161924 | 2,238606053 |
| XRCC4 | 4,27E-05 | 26,05575221 |
| XRCC5 (KU80) | 0,000299 | 39,30918825 |
| XRCC6 (KU70) | 0,000861 | 9,382495328 |

**Supplementary table 1** Expression profile of DNA repair genes in Carboplatin resistant cell line in comparison to maternal OVCAR3

European Journal of Medicinal Chemistry 144 (2018) 582e594

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Mini-review

# Natural compounds and combination therapy in colorectal cancer treatment

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a r t i c l e i n f o

Article history:
Received 22 September 2017
Received in revised form
11 December 2017
Accepted 12 December 2017
Available online 16 December 2017

Keywords:
5-Fluorouracil
Apoptosis
Adverse effect
Cell proliferation
Chemotherapy
Colorectal cancer
Combination therapy
Drug resistance
Natural compound
Oxaliplatin

a b s t r a c t

Colorectal cancer (CRC) therapy using conventional chemotherapeutics represents a considerable burden for the patient's organism because of high toxicity while the response is relatively low. Our review summarizes the findings about natural compounds as chemoprotective agents for decreasing risk of CRC. It also identifies natural compounds which possess anti-tumor effects of various characteristics, mainly in vitro on colorectal cell lines or in vivo studies on experimental models, but also in a few clinical trials. Many of natural compounds suppress proliferation by inducing cell cycle arrest or induce apoptosis of CRC cells resulting in the inhibition of tumor growth. A novel employment of natural substances is a so-called combination therapy - administration of two or more substances - conventional chemothera-peutics and a natural compound or more natural compounds at a time. Some natural compounds may sensitize to conventional cytotoxic therapy, reinforce the drug effective concentration, intensify the combined effect of both administered therapeutics or exert cytotoxic effects specifically on tumor cells. Moreover, combined therapy by targeting multiple signaling pathways, uses various mechanisms to reduce the development of resistance to antitumor drugs. The desired effect could be to diminish burden on the patient's organism by replacing part of the dose of a conventional chemotherapeutic with a natural substance with a defined effect. Many natural compounds are well tolerated by the patients and do not cause toxic effects even at high doses. Interaction of conventional chemotherapeutics with natural compounds introduces a new aspect in the research and therapy of cancer. It could be a promising approach to potentially achieve improvements, while minimizing of adverse effects associated with conventional chemotherapy.



## 1. Introduction

### 1.1. Colorectal cancer, prevalence and risk

Colorectal carcinoma (CRC) is a 3rd most common type of cancer (after lung and breast cancer) and the fourth leading cause of cancer death worldwide [1]. CRC occurs predominantly in persons over 50 years of age, about 690,000 people annually die for this disease. The Czech Republic, together with Slovakia (first in rank) and Hungary (second in rank), is one of the worst affected regions in the world. According to a 2012 comparison of GLOBOCAN data, the Czech Republic occupies the 3rd place. With the early detection of the disease is a five-year survival of 80%, while at the diagnosis of more advanced forms the prognosis is poor, the average survival time is 11 months. Data from the National Oncological Registry for 2013 show the incidence of CRC in the Czech Republic of 52/100 thousand in men and 39/100 thousand for women [2]. Mortality for 2013 in men reached 23/100 thousand and 18/100 thousand in women. From the long-term point of view, CRC mortality decreases slightly due to early detection of the disease.

CRC predisposition is an inherited susceptibility with an estimated risk 12e35% attributed to genetic factors [3] [4]. The genetic risk factors vary between two extreme situations: rare high-penetrance mutations which have significant increment of risk for hereditary syndromes and common variants (polymorphisms)

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https://doi.org/10.1016/j.ejmech.2017.12.039
0223-5234/

which have weak effects on sporadic risk in individuals without family history in CRC [5]. Risk of CRC can be reduced with dietary habits and lifestyle [6]. Diet appears to be one of the ways by which various carcinogens challenge the DNA repair daily [7]. Dietary habits also modulate the intensity of early colon carcinogenic events [6]. Since genetic polymorphisms modulate the cancer risk by the activity of enzymes metabolizing xenobiotics and DNA repair proteins, it might be a critical step in the mutational process. Even at high-risk population the risk of tumor development can be modified by natural compounds. For example, in Chinese women that are at a higher risk of breast cancer due to a genetic predisposition, there has been reported that regular green tea consumption is associated with reduced risk of breast cancer [8]. A recent clinical trial phase II reported that in younger postmenopausal women (50e55 years) at high risk of breast cancer, a percent of mammographic density was significantly reduced by 4.40% with decaffeinated green tea extract supplementation as compared with the placebo group [9].

### 1.2. Conventional treatment

Current treatment of CRC is based on surgery and chemotherapy. Chemotherapeutics interfere with the cell cycle, the most sensitive are rapidly dividing cells and cells with reduced DNA repair capacity [10]. The chemotherapeutic agents are classified according to their mechanism of action on mitosis inhibitors, DNA replication inhibitors, and other cytotoxic agents. Adjuvant chemotherapy (therapy given in addition to the primary therapy to maximize its effectiveness) is not recommended for rectal cancer stage II or III [11]. The most commonly used chemotherapeutic agents are platinum derivatives (oxaliplatin), antimetabolites (capecitabine, 5-fluorouracil), topoisomerase inhibitors (irinotecan) and Tegafur/uracil (UFT), which inhibit the catabolism of 5-fluorouracil. First line chemotherapy in advanced CRC is based on 5-fluorouracil (5-FU) with leucovorin (folinic acid), alone or in combination with oxaliplatin [12]. In metastatic CRC the treatment consists of 5-FU or its combination with irinotecan [13]. The overall response rate to 5-FU monotherapy in advanced CRC is limited to 10e15% [14]. A potential way to improve therapy and postpone adverse effects is combination or multicomponent therapy [15]. 5-FU combined with other cytotoxic drugs could not only improve the response rates, but also reduce the undesirable reaction of these drugs [15]. In many cases, as described further, the combination therapy shows a better outcome eenhancement of the cytotoxic effect of the conventional therapy to cancer cells (for example curcumin [16]). The other problem with chemotherapy is toxicity and drug resistance.

The non-specific action of chemotherapeutics is connected with a number of side effects. Chemotherapeutics affect not only the tumor cells but also non-malignant cells. Chemotherapy destroys rapidly dividing cells - tumor cells, but also hair follicle cells, mucous membranes of the oral cavity and gastrointestinal tract, erythrocytes and leukocytes [17]. The adverse effects of the treatment differ among patients, depending on the type of used chemotherapy. As mentioned above, the first line chemotherapeutics in CRC therapy is 5-FU [12]. The most common adverse effects of 5-FU are: nausea, vomiting, diarrhea, mucositis of the oral cavity (mucosal and submucosal tissue damage), headaches, skin pruritus, myelosuppression (suppression of hematopoietic function of the bone marrow, leukopenia, pancytopenia and thrombocytopenia), anemia, cardiotoxicity, agranulocytosis, alopecia (hair loss), photosensitivity, hand-foot syndrome, depression and anxiety [18] [19].

For the capecitabine, a 5-FU precursor, which is enzymatically metabolized in the body to effective 5-FU, some of the serious adverse effects are occurring quite commonly (in more than 10% of patients) [20]. Common side effects of oxaliplatin therapy are neuropathy (peripheral nerve disorder resulting from drug neurotoxicity, starting with limb tingle), proprioceptive disorders (body coordination), ototoxicity (loss of hearing), nausea, vomiting, diarrhea, neutropenia, hypokalemia (low concentration of potassium in blood), rhabdomyolysis (acute skeletal muscle breakdown, followed by renal failure) [21]. Adverse effects of cytotoxic therapy impair the quality of patient's life and may adversely affect the course of the treatment, the treatment outcome, and treatment costs. We may hypothesize, that combination of conventional chemotherapeutics with well tolerated natural non-toxic compounds may contribute to better response to therapy and better quality of patient's life.

### 1.3. Chemopreventive approaches, natural compounds in food, antioxidant supplements, prebiotics and probiotics, healthy dietary habits

Natural compounds (sometimes called phytochemicals or phytonutrients) are biologically active substances present in plants (pigments), such as carotenoids, flavonoids, anthocyanins or terpenoids. Some of these beneficial substances are not found only in plants, but also in mushrooms, bacteria or marine organisms. Plants are protected against external influences or against predators by phytochemicals, mostly by antioxidant mechanisms. On the other hand, some other plant-derived substances may be toxic to the human body (phytotoxins), e. g. aristolochic acid [22]. For many natural compounds a precise mechanism of their action is not known and is being investigated. Many natural compounds that show the chemopreventive effect on DNA we uptake with food, but the ingested dose may not represent an effective amount. Food is both a source of macronutrients (proteins, fats, carbohydrates) and micronutrients (vitamins, minerals), but it also contain a large number of natural compounds e a complex mixture of bioactive compounds - of that our organism can benefit of [23].

The concept of so-called chemoprevention can be defined as the use of natural substances capable of preventing or interacting with the development of processes leading to the onset of neoplasia, during its initiation, promotion and progression, or the capability to discontinue its development [24]. Primary chemoprevention is an approach focused on cancer risk in the high-risk population, for example the prevention of lung tumors in smokers or the prevention of breast cancer development in women with positive biomarkers BRCA1 and BRCA2. Secondary chemoprevention focuses on patients diagnosed with a pre-malignant lesion that could grow into an invasive tumor. Tertiary chemoprevention is focused on prevention of recurrence after experienced cancer [25] [26].

One of the studied chemopreventive approaches is based on supplementation with compounds exhibiting antioxidant properties, as it is based on the assumption that oxidative stress and free radical formation in normal cells are one of the causes of cancer [27]. However, some clinical studies testing chemopreventive strategies have provided controversial outcomes. The SELECT 2011 study (Selenium and Vitamin E Cancer Prevention Trial), in which 35.5 thousand of patients with positive prostate cancer markers were administered with selenium, alpha-tocopherol (vitamin E), both agents together, or placebo, had to be prematurely stopped because it came out that vitamin E significantly increases the risk of prostate cancer [28]. From the 2004 meta-analysis of beta-carotene, vitamins A, C, E and selenium in cancer of the esophagus, stomach, colon and rectum, pancreas and liver appears that antioxidant supplementation has either no effect, or even increases mortality [29]. A study from 2011 says that green tea polyphenols which possess anti-cancer activity exhibit both antioxidant (radical

scavenging) and pro-oxidant reactions (inhibition of cancer cell viability and induction of apoptosis) [30].

Another chemopreventive approach presumes that dietary modulation of intestinal microflora by prebiotics can help to prevent the development of CRC. Prebiotics, as dietary natural compounds that improve intestinal function, can modulate microbial environment of the colon and thereby ensure a healthy gastrointestinal tract [31]. A study from 2013 was focused on effect of natural prebiotic compound inulin on rats treated with dimethylhydrazine to induce CRC development. Inulin significantly decreased coliform counts, increased lactobacilli counts and decreased the activity of b-glucuronidase. Inulin suppressed the numbers of COX-2- and NF-kB-positive cells. The expression of IL-2, TNFa, and IL-10 was also reduced [31]. This effect of inulin could prevent pre-neoplastic changes and inflammation. A clinical study from 2015 on CRC patients treated with combination of probiotics (Bifidobacterium longum, Lactobacillus acidophilus and Enterococcus faecalis) suggests that probiotics significantly reduce abundance of mucosa-associated pathogens [32]. Prebiotics and probiotics intake seems to be a very promising chemopreventive approach in CRC incidence and development.

Studies concerning dietary effects on CRC risk and progression agree on the increased risk of CRC when eating red and processed meat and, on the contrary, the reduced risk associated with regular physical activity [33] [24]. Calcium, fiber and whole grain have been associated with a lower risk of CRC, saturated fats with its increased risk (for rev see Ref. [34]). A recent epidemiological research suggests that obesity has stronger associations with CRC in men than in women [35]. The effect of cholesterol on CRC metastasis has also been studied. The level of low-density lipoprotein cholesterol (LDL) was positively correlated with liver metastases, and a higher level of LDL receptor (LDLR) expression was associated with advanced stages (Nodes and Metastases e N and M stages) of CRC [36]. Another study describes the various types of fruit and vegetables on proximal and distal colon cancer risk. Risk of proximal colon cancer and rectal cancer does not seem to be associated with intakes of total fruit and vegetable, except for brassica vegetable. Distal colon cancer risk was significantly decreased by intake of carrots, pumpkin and apples [37].

Modulation of gut microbiota by probiotics and prebiotics, either alone or in combination seems to be an effective chemopreventive approach, which could positively influence inflammation and CRC. The solely employed antioxidant supplements may not the best curative option, the better approach seems to be a conventional treatment in combination with natural compounds that affect more signal pathways, may reinforce the effect of the drug effective concentration and minimize adverse effects. For example, Ganoderma lucidum extract polysaccharides can immunity response, and Ganoderma lucidum triterpenes are capable to enhance reactive oxygen species (ROS)-producing effect of chemotherapeutic agent doxorubicine [38] [39].

At last but not least, the suitable dietary habits and moderate physical activity also contribute to reduce the CRC risk.

## 2. Natural compounds in colorectal cancer treatment

Natural compounds have historically occurred in the treatment of cancer, especially in traditional Chinese medicine or Indian Ayurveda. The term natural compound means chemical substances found in plants, fungi, marine animals or those produced by bacteria, with significant pharmacological effects. Many current anticancer drugs also originate from natural sources - irinotecan, vincristine, etoposide and paclitaxel are classic examples of plant-derived compounds. Actinomycin D and mitomycin C are from Streptomyces and bleomycin is the first marine compound [23]. On the other hand, many natural extracts are rather impure or may contain additional compounds/contaminants, some of them toxic (for example aflatoxin [40]). Consequently, not every so-called natural compound would be beneficial to human health, unless properly stated composition is provided. Some of the compounds are already stated as safe to human, as they are enlisted in the WHO list of essential medicines (artesunate an antimalarial medicine). The future research in this field is still needed.

Some natural compounds have the ability to modulate signaling pathways and regulate the expression of genes involved in cell cycle regulation, cell differentiation and apoptosis [40]. At present, the so-called combined therapy, the simultaneous therapy with two substances, a conventional chemotherapeutics and a natural compound, or two or more natural compounds together, is coming to the forefront of interest. In addition, combined therapy, due to the fact that it targets multiple metabolic pathways, uses various mechanisms to reduce the development of resistance to anticancer drugs, thereby also increasing the sensitivity to the effect of the chemotherapeutics [41]. Some recent studies have highlighted the importance of a combined approach and consider this therapy more effective than using conventional chemotherapeutic agents [42].

The effect of the combined approach in the treatment of tumors can be divided into synergistic (increased), antagonistic (reduced), or additive (identical) to their effect on separate administration and can be mathematically determined by the Chou-Talay combination index [43].

The resulting effect could be a reduction of the toxic burden for the patient's organism by replacing part of the dose of a classical chemotherapeutic agent with-well tolerated natural substance with a defined effect [44].

Natural compounds can be classified according to their chemical structure, function, or signaling pathways through which they act. According to the chemical structure we can classify natural compounds as follows: terpenes (artesunate), carotenoids (lycopene, alpha and beta carotene), phenolic compounds - phenolic acids, flavonoids, stilbenes (resveratrol), coumarins, tannins; alkaloids, nitrogen compounds; organosulphates - isothiocyanates and indoles, allylsulphates (contained, for example, in garlic). Flavonoids are further divided into chalcones (isoliquiritigenin), flavanones (naringenin), flavones (apigenin, luteolin), flavonols (quercetin, kaempferol), flavanols (epigallokatechin), isoflavones (genistein) and anthocyans. In some cases, extracts from whole plants that contain a complex of natural compounds demonstrated activity in in vivo or in vitro studies.

In the following paragraphs we summarized the most investigated natural compounds related to CRC.

### 2.1. Curcumin

At present, one of the most studied natural compounds is the phenolic acid derivative of turmeric (Curcuma longum), curcumin. This compound is known for its chemopreventive effects [45]. Preclinical studies are consistent with its antitumor activity in vitro in tumor cells of the breast, cervix, liver, leukemia, pancreas, colon, epithelium of the mouth, ovary and prostate, and in vivo in animal model organisms [45]. Curcumin possesses potent anti-inflammatory, antioxidant ant anti-cancer properties [46]. It is likely to have a great therapeutic potential due to the modulation of tumor growth progression [47]. Curcumin probably acts on multiple pathways. It suppresses colon cancer cell invasion via AMPK-induced inhibition of NF-kB, uPA and MMP9 in human CRC cells SW480 and LoVo cells [46]. Consequently, curcumin reduces the production of TNF-a, COX-2 and IL-6, demonstrating anti-inflammatory activity [48]. This compound also inhibits cell

proliferation by increasing the activity of biotransformation enzymes and cell-cycle proteins (glutathione-S-transferases and p21) [49]. It may also induce the expression of pro-apoptotic proteins (Bax, Bim, Bak, Noxa) and the inhibition of anti-apoptotic proteins (Bcl-2, Bcl-xL) [50]. Finally, curcumin also reduces the expression of VEGF and matrix metalloproteases to prevent the development of metastases [51].

Curcumin enhances effect of conventionally used chemoterapeutics. Curcumin showed synergistic effect with dasatinib (inhibitor of Src and Abl kinases) in HCT116 and HT-29 cell lines under FOLFOX (5-FU, leucovorin, oxaliplatin) treatment [16]. In a study from 2008, curcumin has been shown to potentiate the proapoptotic and antimetastatic effects of capecitabine in HCT116 and HT-29 cells [52]. A turmeric ethanol extract (including curcumin) in a combinational therapy with bevacizumab (monoclonal antibody which targets VEGF, vascular endothelial growth factor inhibited tumor growth of HT-29 human xenografts in mice, which inhibited tumor growth [53]. In a Phase I clinical trial, the combination of curcumin and FOLFOX demonstrated an antiproliferative effect of tumor explants from CRC patients by reducing the overall survival of CRC cells [54].

One of the obstacles on the way to the clinical use of curcumin is its bioavailability. To improve its absorption by intestinal epithelial cells, chitosan nanoparticle coating can be used. This approach has ensured a sustained release profile of curcumin in combination with 5-FU in blood for 4 days in mice [55].

### 2.2. Resveratrol

Another chemopreventive and chemotherapeutic natural compound is resveratrol. From a point of view of structural chemistry, it is a stilben, which naturally occurs in about 70 plant species, for example in blueberries, raspberries, grapes, peanuts, or in a plant Reynoutria japonica. Resveratrol is a natural inhibitor of cell proliferation, synthesized by plants in response to a pathogenic invasion [42]. In 1997 its chemopreventive effect was tested in mouse skin tumor for a first time [56]. Resveratrol interacts with cytochrome P450 isoenzymes, inhibits and downregulates cyclooxygenase inflammation mediator enzymes, and reduces NF-kB transcription factor binding activity to DNA which is usually increased in cancer [57].

In addition to its chemopreventive effects, resveratrol can also possess synergistic effects in combination with other chemotherapeutics such as 5-FU, etoposide, mitomycin, oxaliplatin or another natural compound, curcumin (as investigated on the colorectal line HCT116 and xenograft models) [44]. Resveratrol sensitizes tumor cell line HCT116 and p53-deficient HCT116 colorectal carcinoma lines to the effect of 5-FU and induces apoptosis in both lines [58]. It also sensitizes the SW620 and HT-29 lines to cytotoxic oxidative stress caused by 5-FU by inhibiting endogenous antioxidant capacity [59]. Resveratrol has already been tested several times in clinical studies on patients with CRC [60]. Approximately 70e80% of the orally received resveratrol is rapidly absorbed by passive diffusion into the enterocytes, rapidly producing conjugated derivatives (glucuronides and sulfates). The limiting factor for its use in clinical practice is the fact that only 2% of unmodified trans-resveratrol can be found in the blood at maximum 30e60 min after ingestion [61]. When administered orally 25 mg of resveratrol, only trace amounts of unmodified resveratrol (less than 5 ng/ml) are found in serum [62]. Additionally, after ingestion of a higher dose than 1 g daily, volunteers experienced gastrointestinal adverse effects [63]. However, the amount of resveratrol and its metabolites found in colorectal tissue samples is higher than in the serum, so resveratrol is a suitable chemopreventive agent in combination with other chemotherapeutic agents at a dose of up to 1 g [64]. The clinical trial proved that resveratrol was well tolerated and it reduced tumor cell proliferation by 5% [64]. Another study from 2013 states that resveratrol metabolites inhibit human metastatic colon cancer cells progression and synergize with chemotherapeutic drugs to induce cell death [65].

### 2.3. Ganoderma lucidum

One of the mushrooms used in China, Japan and other Asian regions for its anti-inflammatory and immunomodulating effects and longevity is Ganoderma lucidum (GLC) or Reishi. GLC has also been used as preventive agent and cancer therapeutics. The main bioactive components of Ganoderma lucidum are polysaccharides and triterpenoids.

The GLC extract shows anti-tumor and immunotherapeutic properties. GLC has been shown to stimulate NK cell cytotoxicity by increased expression of NKG2D and natural cytotoxicity receptors (NCR), increased phosphorylation of intracellular MAPK, and perforin and granulysin secretion, which could explain the possible molecular mechanism of antitumoral effects of GLC extract in humans [66]. Ganoderma lucidum induces cell cycle arrest and apoptosis in various human tumor cell lines. One of the characteristics of highly metastatic tumor cells is the constitutive activation of transcription factors AP-1 and nuclear factor kappa B (NF-kB). GLC has been shown to inhibit these factors, resulting in inhibition of expression of the urokinase plasminogen activator (uPA) and its uPAR receptor [67].

Polysaccharides extracted from Ganoderma lucidum (GLP) show anti-inflammatory, hypoglycemic, anti-tumor and immunostimulatory effects and inhibit the formation of reactive oxygen species (ROS) and reduce oxidative DNA damage [68] [69] [66] [67]. Polysaccharides induced apoptosis in HCT116 by increasing caspase-8, caspase-3 and Fas [70]. GLP also reactivated mutant p53 in HT29 and SW480 colorectal line alone in separate administration or together with 5-FU as well [71].

More than 200 pharmacologically active triterpenoids have been isolated from GLC, some of which are called ganoderic acids. Ganoderic acids A and C inhibit farnesyl protein transferases, key enzymes for activating the Ras oncoprotein responsible for cell transformation [69]. The triterpene extract (GLT) suppresses the proliferation of human HT-29 colon carcinoma cells and inhibits tumor growth in the colon carcinoma xenograft model. This activity is associated with cell cycle arrest in G0/G1 phase transition and induction of apoptosis. GLT induces the formation of autophagous vacuoles and increases the expression of Beclin-1 and LC-3 proteins in cell lines and in the xenograft model. Autophagy is mediated by inhibition of p38 MAPK (mitogen-activated p38 protein kinase) [72].

### 2.4. Cannabinoids

Chinese medicine uses the effects of cannabis (Cannabis sativa) to relieve pain and hallucinations for many centuries. This plant contains three main classes of bioactive molecules - flavonoids, terpenoids and more than 60 types of cannabinoids [73]. Cannabinoids are commonly used in palliative treatment of chemotherapy in cancer patients [74]. However, several studies suggest the use of cannabinoids in anti-cancer therapy as well [75]. Cannabinoids possess antitumor effects in vitro or in vivo in animal models by various mechanisms - induction of apoptosis in tumor cells, inhibition of proliferation and angiogenesis or anti-metastatic effects by inhibition of tumor cell migration [76] [77] [78] [79] [80]. The most bioactive compound of this plant is the psychotropic $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), which exhibits inhibition of tumor growth, as well as several other cannabinoids. Several preclinical

studies indicate that $D^9$-THC and other naturally occurring cannabinoids, synthetic cannabinoid agonists, and endocannabinoids have in vitro anticancer effects in lung, glioma, thyroid epithelioma, lymphoma, skin cancer, uterine carcinoma, breast cancer, prostate carcinoma, pancreatic carcinoma and neuroblastoma [81].

#### 2.4.1. Cannabidiol

Cannabidiol is the most represented ingredient in Cannabis sativa extract. Cannabis sativa extract with high content of non-psychotropic cannabidiol (CBD BDS, Cannabidiol botanical drug substance) containing other components including THC, and even CBD alone, showed the same antiproliferative effect in HCT116 and DLD-1, while the proliferation of healthy cells was not affected [82]. In vivo CBD BDS extract reduced pre-neoplastic lesions and azoxymethane induced polyps as well as tumor growth in the colorectal carcinoma xenograft model [82]. In HCT116 and Caco-2 cell lines CBD showed the chemopreventive effect from oxidative damage and decreased cell proliferation through CB1, TRPV1 and PPARg [79]. Cannabidiol is already being administered as a Sativex drug (or in the US Nabiximol) to patients with multiple sclerosis [82].

### 2.5. Flavonoids

Flavonoids are the most abundant group of bioactive compounds in plants that are essential for their morphology and physiology. Flavonoids occur in plant stems, leaves, flowers and seeds and they are constituents of their pigments. They participate in growth and reproduction of plants and protect the plant from UV rays and microbial infections. Flavonoids are powerful antioxidants that are potent in protection of cells against the development of cancer. Additionally, they have phytoestrogenic, antidiabetic, anti-inflammatory, antibacterial and antiviral effects [83].

Individual flavonoid compounds are adressed below.

#### 2.5.1. Epigallocatechin

Epigallocatechin-3-gallate (EGCG) is the main representative of polyphenols that are contained in green tea, which antitumor effects are supported by many in vitro, in vivo and several clinical trials [84]. EGCG inhibits several significant signaling pathways. It blocks the proliferation and migration of CRC cells by inhibiting the TF/VIIa/PAR2 signaling pathway (Protease-Activated Receptor 2), which mediates ERK1/2 phosphorylation and final activation of proinflammatory NF-kB. Reduced NF-kB transcription factor activity induces increased expression of caspase-7 and decreased expression of MMP-9 (matrix metalloproteinase 9) [85]. EGCG is also a regulator of epigenetic processes - it contributes to the degradation of DNMT3A (DNA methyltransferase 3A) and HDAC (histone deacetylases) through the ubiquitination process in colorectal cells sensitive to methylation [84] [86]. Furthermore, the effect of EGCG was tested in combination with 5-FU, which demonstrated the synergistic growth inhibition of DLD1, SW480 and COLO201 [87]. EGCG also potentiates the 5-FU effect by downregulation of ABC transporter expression, resulting in a higher intracellular concentration of 5-FU [88].

#### 2.5.2. Genistein

Genistein is an isoflavone found in soy, beans, lentils, and chickpeas. Soy and its products, such as tofu (fermented soy), contain also other isoflavones - daidzein and glycine. Many epidemiological studies confirm a negative correlation between the incidence of CRC and a diet rich in soybeans. Genistein and other soy isoflavones have a similar chemical structure as endogenous estrogen, they are competitive estrogen receptor agonists. They have anti-angiogenic and antiproliferative effects and inhibit tyrosine kinases [89]. Genistein acts proapoptotically specifically on tumor cells by following mechanisms: by increasing the expression of Bax or p21 proteins in the HT-29 line, inhibiting NF-kB and topoisomerase II, increasing expression of the antioxidant enzyme glutathione peroxidase and by preventing metastases in CRC patients by inhibiting the metalloproteinase MMP2 [90] [91] [92] [93] [94]. Genistein was tested on a 5-FU-resistant HT-29 colorectal cell line, where in combination with 5-FU it decreased significantly viability, inhibited cell growth inhibition by increased expression of the pro-apoptotic p53 and p21 genes, and further inhibited the expression of COX-2 [95]. In combination with cisplatin, genistein inhibits synergistically cell growth and induces apoptosis [96]. However, a clinical trial from 2005 performed of 125 patients concluded that supplementation with soy protein containing isoflavones does not reduce colorectal epithelial tumor cell proliferation [97].

#### 2.5.3. Apigenin

Apigenin ($4^0$,5,7-trihydroxyflavone) is one of the most widespread flavones, found in Chinese cabbage, parsley, paprika, garlic and celery. It exhibits chemopreventive and cytostatic properties, reduces angiogenesis, induces inhibition of colorectal cell growth, cell arrest, and apoptosis in vitro [98]. Apigenin increases the expression of proapoptotic proteins NAG-1 and p53 and cell cycle inhibitor p21 in colorectal cells in vivo and in vitro, reducing intestinal tumor load and number [99]. Apigenin is an inhibitor of ABC receptors that increase the efflux of a chemotherapeutic agent in colorectal epithelial cells, and therefore it increases their bioavailability [100].

CD26 is a membrane protein that suppresses pathways responsible for growth and metastasis and is usually down-regulated in many types of cancer, including CRC. Apigenin increases the number and activity of CD26 in the colorectal lines HT-29 and HRT-18. Apigenin was found to potentiate activity of CD26 with the co-administration of irinotecan [101].

#### 2.5.4. Chrysin

Another polyphenolic flavone is chrysin (5,7-dihydroxyflavone), which is found in honey, propolis, chamomile and martyrs (Passiflora caerulea). Chrysin shows anti-tumor effects in various tumor lines and seems to be a natural chemopreventive agent. Chrysin induces cell apoptosis in HCT116, DLD-1 and SW837 cells. This compound also up-regulates Tumor necrosis factor (Tnf) a and b genes and activates TNF and AHR signaling pathways [102]. A study on the colorectal line SW480 has demonstrated that chrysin induces a cell cycle arrest at the G2/M transition, whereas the combination with apigenin doubled the effect and restrained the progression of the tumor [103]. Chrysin also protects cells against induced cisplatin toxicity in vivo in a mouse model, which is related to oxidative stress suppression by activation of p38MAPK, p53 and apoptosis [104].

#### 2.5.5. Isoliquiritigenin

Isoliquiritigenin is a chalk originating in liquorice (Glycyrrhiza glabra), which has antioxidant and anti-tumor effects [105]. In a study from 2010 there was tested isoliquiritigenin and another flavonoid, formononetin. They inhibited the growth of HCT116 CRC cells and promoted apoptosis, which was accompanied by caspase activation and downregulation of the antiapoptotic proteins Bcl-2 and Bcl-x(L) [106]. In another study the synergistic effect of isoliquiritigenin and cisplatin was demonstrated in mouse CT-26 colorectal line xenografts, with a 79% tumor reduction. This combination achieved a reduction of oxidative damage levels of nitric oxide, lipid peroxidase and GSH in serum, in comparison to cisplatin treatment alone [107].

#### 2.5.6. Kaempferol

Kaempferol is a flavonol, found in propolis, grapefruit, black tea or broccoli. In the colorectal tumor lines, kaempferol demonstrated antitumor activity. In the HT-29 line kaempferol induces apoptosis and activity of CDK2, CDK4 and Cdc2, thereby causing cell cycle arrest in the G1 and G2/M phase [108] [109]. Kaempferol increased chromatin condensation and DNA fragmentation, and the number of early apoptotic cells in HT-29 cell line. It also increased the levels of cleaved caspase-9, caspase-3 and caspase-7. These mechanisms indicate, that kaempferol induces the apoptosis of HT-29 cells via events associated with the activation of cell surface death receptors and the mitochondrial pathway [110]. It also induces apoptosis in SW480 and DLD-1 colorectal cell lines, the higher effect is achieved by TRAIL co-treatment (TNF-associated apoptosis-inducing ligand, apoptosis-inducing ligand), kaempferol sensitizes cells to TRAIL treatment [111].

#### 2.5.7. Quercetin

Quercetin is a widespread flavonol, found in apples, onions, and many other types of vegetables and fruits. Quercetin has antiproliferative effects - it inhibits the expression of RASA1 (RAS p21 protein activator) and thus prevents the activation of RAS in human colon cancer cells Colo-205, Colo-320HSR, Colo-201, LS-174 and WiDr [112]. Likewise, quercetin, as well as kaempferol, sensitizes SW-620, HT-29 (both synergistic effects) and Caco-2 (additive effect) cells against TRAIL, causing their apoptosis [113]. In quercetin treated HT29 cells, over expression of COX-2 and increased generation of reactive oxygen species (ROS) was observed [114]. Quercetin also markedly decreased lung metastasis of colon cancer CT26 cells in an experimental in vivo metastasis model. Migration and invasion of CT26 cells were inhibited by quercetin through expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) regulation. Therefore, quercetin may be a potent therapeutic agent for the treatment of metastatic CRC [115].

Other flavonoids with significant effects tested in vitro on colorectal lines or in vivo are silymarin (Sylibum marianum), scutellarin (Scutellaria barbata) and oroxylin (Scutellariae radix).

### 2.6. Terpenes

Terpenes include a large number of organic compounds, which are constituents of essential oils. Terpenes are volatile fragrances contained in leaves, fruits, flowers, rhizomes and plant roots. Oxidized terpenes are called terpenoids, including limonene, vitamin A, b-carotene and steroids.

Individual terpenes are adressed below.

#### 2.6.1. Artesunate

Artesunate is a derivative of the sesquiterpen artemisinin, found in the traditional Chinese wormwood annual herb (Artemisia annua), used for treatment of fever and rheumatism [116]. The major active ingredient artemisinin was identified and isolated in the 1970s and traditionally it was used to treat malaria because it increases the reactive oxygen species (ROS) in the Plasmodium parasite [117]. The other compounds are not included on the WHO list. There is still lack of clinical research data.

Artesunate also possesses antiproliferative activity, which makes it potential anticancer agent. Artesunate is cytotoxic to HCT116 colorectal carcinoma cells, inducing cell cycle arrest in the G1 phase by reducing the expression of cyclin D1 and increasing p21 expression. Artesunate creates more ROS in co-treatment with oxaliplatin [116]. Another study was aimed to investigate the effect of artesunate on tumor immunosuppression, which is the main source of ineffective treatment on tumor. Artesunate, reverses the immunosuppression from murine colorectal cancer cells Colon26 and human colorectal cancer cell RKO colorectal cancer cells by decreasing TGF-b1 and IL-10 [118]. This is probably one of the antitumor mechanisms of artesunate.

#### 2.6.2. Geraniol

Geraniol is known primarily as a component of essential oils, it is contained in the rose oil, palmarosa oil (Cymbopogon martinii, palmarosa) or citronella oil, and is also found in geraniums (Pelargonium). Geraniol represents a promising dietary chemopreventive agent. Its chemopreventive effect was evaluated in colon tumor induced by dimethylhydrazine in vivo in male Wistar rats. Number of total aberrant crypt foci in the distal colon significantly decreased and apoptosis level in the distal colon was significantly higher [119]. In combination with 5-FU geraniol increased the apoptosis of the Caco-2 cell line in vitro. In xenografts of the TC118 cell line, the combination with 5-FU caused a tumor reduction of up to 83% [120].

#### 2.6.3. Ginsenosides

Ginsenosides are a subset of panaxosides, which are glycosides with triterpenoid (steroidal) aglycone and one or more sugar residues. Panaxosides are effective saponins typical for ginseng (Panax ginseng, Panax notoginseng). Ginseng has been used in chinese traditional medicine over centuries. Panaxosides show antitumor effects and target multiple signaling pathways. A notoginsenoside R1 compound was tested in CRC line HCT-116 when metalloproteinase (MMP)-9 expression was reduced in comparison to the control group. In the adhesion reaction assay, treatment with R1 significantly decreased adhesion of the HCT-116 cells to endothelial cells [121]. An another compound, Ginsenoside Rg3, inhibits CRC cell migration by suppressing NF-kB activity in SW480 cells [122]. Panaxadiol causes apoptosis in HCT116 cells [123] and protopanaxadiol significantly enhances the effect of 5-FU treatment on HCT116 cells and causes cell cycle arrest in the G1 phase, and this data was confirmed in the model of murine xenograft [124].

#### 2.6.4. Betulinic acid

Betulinic acid, together with betulin, belongs to the triterpene class and both substances are found in the birch (Betula pubescens), rosemary, Pseudocydonia sinensis, or Prunella vulgaris. Betulin is found in parasitic mushrooms Piteroporus betulinus or Chaga (Innonotus obliquus), which parasite on birch and other deciduous trees. Despite the fact that betulin has been known for over 200 years, the crystal structures of this compound have been described for the first time in 2010 [125]. Betulinic acid targets the mitochondrial pathway of apoptosis which makes it a promising potential anticancer drug [126]. Betulin has a cytotoxic effect on the SW707 human colorectal adenocarcinoma cell line, but higher cytotoxic effects exert acetylene derivatives of betulin [61]. Betulinic acid has anti-inflammatory, antimalarial, antiretroviral and anti-tumor effects by inhibiting topoisomerases and triggers apoptosis in the colorectal line SNU-C5 by induction of caspase 3 [127]. There were generated resistance variants of this line against 5-FU, irinotecan and oxaliplatin, which were more sensitive to betulinic acid than the original line. In addition, the combination of 5-FU and betulinic acid has been shown to induce apoptosis in 5-FU resistant cells and this occurred similarly with a co-treatment of betulinic acid and oxaliplatin in the oxaliplatin-resistant line. These results imply that combination therapy has succeeded in bypassing the obtained chemoresistance [128].

#### 2.6.5. Carotenoids a xanthophylls

Terpenoid compounds with antitumor effects include a large number of carotenoids such as lycopene (e.g., tomatoes, rose hips,

grapefruits), alpha and beta carotene (carrots, apricots, mangoes) or xanthophyll fucoxanthin. In plants, xanthophylls occur in green plants and photosynthetic bacteria, we call them pigments.

Lycopene exhibits anti-inflammatory effects in vitro in SW480 CRC line. It inhibits the activation of NF-kB and JNK which cause inflammation, and suppresses the expression of TNF-a (tumor necrosis factor) and interleukins IL-1, IL-6, iNOS and COX-2 [129]. Lycopene also suppresses growth and progression of colorectal carcinoma in vivo, as demonstrated in mouse xenografts. Lycopene suppresses the expression of PCNA and b-catechins in tumor tissue, plasma levels of matrix metalloproteinase 9 (MMP-9) in murine xenografts negatively correlated with the inhibitory effect [130].

Fucoxanthin is a carotenoid, in terms of the chemical structure a tetraterpenoid, which is found in the chloroplasts of an edible seaweed Wakame (Undaria pinnatifida) and other seaweeds. Fucoxanthin and its metabolite, fucoxanthinol, possess anticancer effect which was demonstrated in studies on Caco-2, WiDr, HCT116, and DLD-1 cell lines [131]. Studies on the Caco-2 cell line demonstrated the induction of apoptosis by fucoxanthin due to the reduction of Bcl-2 protein expression [132].

While alpha and beta carotene exhibit antiproliferative and antioxidant effects in relation to breast and prostate tumors, data regarding their effect on colorectal carcinomas are inconsistent. The case-control study from 2015 among adult Chinese reports that consumption of alpha-carotene and beta-carotene is indirectly associated with a risk of colon cancer [133]. On the contrary, authors of the meta-analysis of clinical studies from 2017 did not find a significant association between the intake of carotenoids from food sources and the overall risk of colorectal carcinoma [134]. In vitro, beta-carotene has been shown to reduce the expression of COX-2 and PGE2 (prostaglandin E (2)) in adenocarcinoma cell line LS174 and colon carcinoma cell line HCT116, which is a possible mechanism of chemopreventive and antiproliferative effects of beta carotene in tumor cells [135].

Other bioactive compounds with chemoprotective properties from the terpenes family associated with colorectal carcinoma are celastrol, triptolide (both from Tripterygium wilfordii), ursolic acid (basil, rosemary) or irofulven (from the sponge Omphalotus illudens) [44].

### 2.7. Gossypol

Gossypol is a natural phenolic aldehyde, and is found in cotton plants (Gossypium). Gossypol toxicity has limited cottonseed use in animal feed and the most common toxic effect was found to be the impairment of male and female reproduction [136]. Therefore, originally it has been tested as a male oral contraceptive in China in clinical trials in 1972 [137]. Its antitumor effects have been studied since 1980s. Gossypol inhibits the proliferation of tumor cells and induces apoptosis in various colorectal lines [138]. In mouse HCT116 xenografts gossypol inhibited cell growth [139]. Gossypol also exhibited a synergistic effect with 5-FU in the HCT116, HT-29 and RKO cell lines [140]. Gossypol down-regulates thymidylate synthase and cyclin D1 expression was decreased after the treatment, suggesting an additional mechanism of the observed antiproliferative synergistic interactions [141]. In a study from 2010 the authors found that gossypol treatment induced DNA damage and activated p53 in prostate cancer cell lines [142]. Gossypol has completed Phase IIb multi-center clinical trials for treating prostate cancer and small lung cancer [60]. Both gossypol and AT-101 molecule, derived from gossypol, have been found to be well tolerated by humans with a low incidence of serious adverse effects [143] [144].

### 2.8. Isothiocyanates and indoles

For a group of cruciferous vegetables (broccoli, cauliflower, cabbage, brussels sprout, etc.), some meta-analyzes documented a decrease in the incidence of colorectal carcinoma by 18% due to the high concentration of phytochemicals [145]. These substances are isothiocyanates (sulforaphane, phenethyl isothiocyanate, benzyl isothiocyanate) and indole-3-carbinol, both belonging to the group of organosulfate compounds. Above compounds modulate PI3K/Akt/mTOR, two key signaling pathways that affect many physiological aspects of cell growth and survival, but also pathological conditions including carcinogenesis. Their antitumor effect consists of blocking activation of carcinogen in phase I metabolic transformation e they block cytochromes P450 and, on the contrary, induce phase II detoxification enzymes, glutathione S-transferases, to accelerate the elimination of carcinogen from the organism [146]. One compound from the isothiocyanate group, sulforaphane, induced cell death through G2/M phase arrest and induced apoptosis in HCT 116 CRC cell line [147]. Another compound, allyl isothiocyanate, inhibits cell metastasis by inhibiting invasive and migratory ability of HT29 cells and downregulating the protein levels of matrix metalloproteinase-2 (MMP-2), MMP-9 and mitogen-activated protein kinases (MAPKs) [148].

### 2.9. Allylsulfates

The second group of substances belonging to the group of organosulfates are allylsulfates, which are included in garlic, onion, shallot, but also in chive or leek (Alliaceae). One of the most important bioactive compounds in this group of vegetables is diallyltrisulfide (DTS), which suppresses proliferation and induces apoptosis of human colon carcinoma cells (HCT-15 and DLD-1) via oxidative modification of beta-tubulin [149]. Diallyl trisulfide also inhibits growth of mouse colon tumor in a mouse CT-26 cell alloimplant model in vivo [150]. It also increases the production of reactive oxygen species (ROS) in human primary CRC cells and induces apoptosis [151].

### 2.10. Ginkgo biloba

The Ginkgo biloba extract contains over 60 biologically active substances, the most important of which are terpentins, flavonoids, carboxylic acids and L-ascorbic acid. Ginkgo biloba EGb 761 extract inhibits the progression of the HT-29 cell line, and its effect may be related to increased caspase-3 activity, elevated p53 expression, and decreased expression of bcl-2 [152]. EGb was also tested in Phase II clinical research in combination with 5-FU, the authors of the study would recommend this approach as second-line treatment in patients with metastatic CRC [153]. On the contrary, in 2013 National Toxicology Program conducted a 2- year gavage studies on one Ginkgo biloba leaf extract and concluded there was a strong evidence of carcinogenic activity of this extract in mice based on increased incidence of hepatocellular carcinoma and hepatoblastoma [154]. According to International Agency of Research on Cancer [1], Ginkgo biloba extract (EGb) has been classified as a possible carcinogen since 2016 (Group 2B) [155].

## 3. Bioavailability

Some natural compounds have already been tested in clinical trials. Unfortunately, these trials revealed several limitations related to that sometimes administration of these substances result in low levels in blood (curcumin, resveratrol) [156] [64]. Experimental cell cultures or laboratory animals are generally controlled and homogenous, while in clinical trials the patients form a

Table 1
A brief summary of natural compounds and their in vitro and in vivo effects on CRC.

| Compound | Experimental model | Main effect | Reference |
|---|---|---|---|
| Curcumin | HCT116, HT-29 | Potentiates effect of dasatinib&FOLFOX, capecitabine | [16] [52] |
| | HT-29 mice xenograft | in comb. with bevacizumab inhibits tumor growth | [53] |
| Resveratrol | HCT116, HCT116 p53/ | Sensitizes to effect of 5-FU | [58] |
| | SW620, HT-29 | Sensitizes to oxidative stress caused by 5-FU | [59] |
| Ganoderma lucidum | HCT116 | induced apoptosis in HCT116 and increased caspase-8, caspase-3 and Fas | [70] |
| -polysacharides | HT-29, SW480 | Reactivated mutant p53 alone in separate administration or together with 5-FU | [71] |
| -triterpenes | HT-29 | Suppresses the proliferation, inhibits tumor growth in the colon carcinoma xenograft model | [72] |
| Cannabidiol | HCT116, DLD-1 | Antiproliferative effect | [82] |
| | HCT116, Caco-2 | chemopreventive effect from oxidative damage | [82] |
| | HCT116 mice xenograft | reduced pre-neoplastic lesions and azoxymethane induced polyps | [82] |
| Genistein | HT-29 | Pro-apoptotic effect - increases expression of Bax or p21 proteins; inhibits NF-kB and topoisomerase II, in comb. with cisplatin inhibits cell growth and induces apoptosis | [90] [91] |
| | 5-FU-resistant HT-29 | Decrease in viability in comb. with 5-FU, cell growth inhibition | [95] [96] |
| Apigenin | HT-29 and HRT-18 | Increases activity of CD26, more in comb. with irinotecan | [101] |
| | HT-29, HCT116 | Increases expression of NAG-1, p53, p21 | [99] |
| Chrysin | SW480 | Cell cycle arrest at C2/M, co-treatment with apigenin doubled the effect | [103] |
| | HCT116, DLD-1 and SW837 | Induces cell apoptosis, upregulates Tnf a and b | [102] |
| Isoliquiritigenin | HCT116 | Inhibits growth, induces apoptosis | [106] |
| | CT-26 mice xenograft | Synergistic effect with cisplatin | [107] |
| Kaempferol | HT-29 | Induces cell cycle arrest in G1 and G2/M, induces apoptosis | [108] [109] [110] |
| | SW480, DLD-1 | Induces apoptosis, higher effect by TRAIL co-treatment | [111] |
| Quercetin | SW-620, HT-29, Caco-2 | Sensitizes cells against TRAIL, causing apoptosis, generating of ROS | [113] [114] |
| | Colo-205, Colo-320HSR, Colo-201, LS-174 and WiDr | Inhibits expression of RASA1 | [112] |
| | in vivo CT26 model | decreased lung metastasis, migration and invasion | [115] |
| Artesunate | HCT116 | induces cell cycle arrest in the G1 phase, in comb. With cisplatin creates ROS | [116] |
| Geraniol | Caco-2 | Increased apoptosis comb. with 5-FU | [120] |
| | TC-118 mice xenografts | In comb. with 5-FU tumor volume reduction | [120] |
| | in vivo Wistar rats CRC | Increased apoptosis of tumor cells, decreased no. of aberrant crypt foci | [119] |
| Panaxadiol | HCT116 | Apoptosis induction | [123] |
| Protopanaxadiol | HCT116 mice xenograft | Enhances effect of 5-FU, cell cycle arrest in the G1 phase | [124] |
| notoginsenoside R1 | HCT116 | Induced apoptosis | [121] |
| Ginsenoside Rg3 | SW480 | Inhibits migration by suppressing NF-kB activity | [122] |
| Betulinic acid | SNU-C5 | Triggers apoptosis by induction of caspase 3 | [127] |
| | 5-FU/oxaliplatin resistant SNU-C5 | Induces apoptosis in 5-FU and oxaliplatin resistants | [128] |
| Lycopene | SW480 | Acts anti-inflammatory suppresses the expression of PCNA and b-catechins | [129] |
| | Mouse xenograft | Suppresses growth and progression of CRC | [130] |
| Fucoxanthine | Caco-2 | Induction of apoptosis by reduction of Bcl-2 protein expression | [132] |
| Beta-karotene | LS-174, HCT116 | Chemopreventive and antiproliferative effect by reduction the expression of COX-2 and PGE2 | [135] |
| Gossypol | HCT116 mice xenograft | Inhibition of cell growth | [139] |
| | HCT116, HT-29 and RKO | Synergistic effect with 5-FU | [140] |
| Sulforaphane | HCT116 | Induces cell death through G2/M phase arrest, induces apoptosis, induces generation of ROS | [147] |
| Allyl isothiocyanate | HT29 | Inhibits cell metastasis | [148] |
| Diallyltrisulfide | HCT-15, DLD-1 | Suppresses proliferation and induces apoptosis | [149] |
| | CT-26 cell mouse alloimplant | Inhibits mouse colon tumor, increases production of ROS | [150] |
| | Human primary CRC cells | Induces apoptosis, increased ROS | [151] |
| Ginkgo biloba | HT29 | Inhibits progression of the tumor, increases caspase-3 activity, elevates p53 expression, and decreases expression of bcl-2 | [152] |

Table 2
An overview of clinical trials with natural compounds.

| Compound | Phase | Dosing | No. Of patients | Main effect | Reference |
|---|---|---|---|---|---|
| Curcumin | Phase II a | 2 g or 4 g per day for 30 days | 41 | significant 40% reduction in aberrant crypt foci number occurred with the 4 g dose | [161] |
| | Phase I | 0.45 and 3.6 g per day/4 months | 15 | 3,6 g decreases in inducible $PGE_2$ production in blood | [162] |
| Resveratrol | Phase I | 0,5-1 g/8 times per day for 8 days | 20 | resveratrol reduced tumor cell proliferation by 5% | [64] |
| | Phase I | 5 g per day 10e21 days preoperatively | 9 | caspase-3, a marker of apoptosis, was significantly increased by 39% in malignant hepatic tissue | [157] |
| Artesunate | Clinical Pilot study | 200 mg per day/14 days preoperatively | 20 | Apoptosis was of 13% higher than in control group. Artesunate has anti-proliferative properties in CRC | [163] |
| Ginkgo biloba | Phase II | 350 mg GBE 761 ONC on days 1e6 followed by 500 mg 5-FU on days 2e6 | 44 | progression of disease in 22 patients, no change in 8 patients and a partial response in 2 patients. Second line treatment in metastatic colorectal cancer | [164] |

heterogeneous group with various genetic background, lifestyle and dietary habits. Oral bioavailability is known only for a smaller group of natural compounds. For some compounds there is also possible to use subcutaneous administration, but the data for natural compounds are rare. To increase the oral bioavailability there are several obstacles to be considered: poor solubility in water (lipophilic nature), poor absorption or conversion to conjugates (metabolites). To enhance the bioavailability, there is possibility to consider the use of nano-carriers, for example incorporation into polymers (proteins, phospholipids, liposomes) or to create less polar derivates [24]. In a clinical trial with resveratrol, the bioavailability was increased by micronization, which allowed to increase drug absorption [157].

## 4. Discussion

Dietary habits modulate the intensity of early colon carcinogenic events [6]. Since genetic polymorphisms modulate the cancer risk by the activity of enzymes metabolizing xenobiotics, it might be a critical step in the mutational process. This review identifies natural compounds found in plants, fruits, vegetables or other natural sources, their groups or extracts of natural substances in which chemopreventive and also anti-tumor effects of various types have been observed. Antiproliferative, antimetastatic effects of natural compounds, in vitro or in vivo involving colorectal tumors are summarized in Table 1. Effects of natural compounds are mainly reported in vitro or in vivo using animal models. There is still a scarcity of clinical trials. The overview of natural compounds a their effects in clinical trials is summarized in Table 2.

Natural compounds and their potential use in clinical practice have undoubtedly many advantages. With regard to anticancer properties, they possess antioxidant (chemoprotective) and even prooxidant (pro-apoptotic) properties that affect proliferation, growth, angiogenesis, tumor metastasis or induce tumor cell apoptosis [158]. Moreover, a lot of natural compounds are well tolerated by humans. Although natural compounds may be very helpful in tackling chronic diseases, a priori contentwise and effectwise characterization is inevitable.

Conventional chemotherapy therapy represents a considerable burden for the patient's body. A number of studies has been investigating the use of natural substances to reduce the toxic burden of a patient's organism by a dose-substitution of a classical chemotherapeutic agent such as 5-FU or oxaliplatin with a natural compound with a well-defined effect. Some natural compounds are well tolerated and do not exert toxic effects even at high doses e e. g. 8 g of curcumin by oral administration [159]. Moreover, some natural compounds may increase susceptibility to conventional therapy via cytotoxicity, mutually reinforce the combined effect of both administered therapeutics (e. g. EGCG and sunitinib [160]) or act cytotoxically on tumor cells only.

Combined therapy, due to the fact that it targets multiple signaling pathways, uses various mechanisms to reduce the development of resistance to antitumor drugs. In some cases, the addition of natural compound to conventional therapy may overcome altered regulatory cell pathways which may be responsible for drug resistance mechanism. Interaction of conventional chemotherapeutics with natural substances introduces a new perspective in the research and therapy of cancer. It could be a promising approach to potentially achieve improvements in minimizing of adverse effects associated with conventional chemotherapy.

## 5. Conclusion

Our review presents a summary of previously published works of natural compounds in CRC therapy, mainly in vitro on colorectal cell lines or in vivo studies on experimental models. While there is still a long way to go, natural compounds are promising chemoprotective agents and supplements to conventional cancer therapy. Many of them are multitargeted agents, which induce apoptosis of CRC cells, inhibit cell or tumor growth, suppress proliferation or induce cell cycle arrest. The most interesting approach is to combine natural compounds with another compound, mainly 5-fluorouracil or platinum derivatives, to modify chemotherapy burden. Some of the natural compounds have been tested in clinical trials, but there is still lack of evidence and further of them are needed, after overcoming the problems like low solubility and a connected problem with bioavailability. Therefore, the deep investigation of targeted signaling pathways is still needed.

## Funding

This review was supported by Czech-American scientific and technological cooperation (AMVIS) [grant number LH13061] and Grant agency of the Czech Republic [grant number P303/15/14789S].

## Conflicts of interest

The authors declare no conflict of interest.

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cancers

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Review

# DNA Repair and Ovarian Carcinogenesis: Impact on Risk, Prognosis and Therapy Outcome

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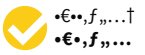
Received: 30 May 2020; Accepted: 24 June 2020; Published: 28 June 2020



**Abstract:** There is ample evidence for the essential involvement of DNA repair and DNA damage response in the onset of solid malignancies, including ovarian cancer. Indeed, high-penetrance germline mutations in DNA repair genes are important players in familial cancers: BRCA1, BRCA2 mutations or mismatch repair, and polymerase deficiency in colorectal, breast, and ovarian cancers. Recently, some molecular hallmarks (e.g., TP53, KRAS, BRAF, RAD51C/D or PTEN mutations) of ovarian carcinomas were identified. The manuscript overviews the role of DNA repair machinery in ovarian cancer, its risk, prognosis, and therapy outcome. We have attempted to expose molecular hallmarks of ovarian cancer with a focus on DNA repair system and scrutinized genetic, epigenetic, functional, and protein alterations in individual DNA repair pathways (homologous recombination, non-homologous end-joining, DNA mismatch repair, base- and nucleotide-excision repair, and direct repair). We suggest that lack of knowledge particularly in non-homologous end joining repair pathway and the interplay between DNA repair pathways needs to be confronted. The most important genes of the DNA repair system are emphasized and their targeting in ovarian cancer will deserve further attention. The function of those genes, as well as the functional status of the entire DNA repair pathways, should be investigated in detail in the near future.

**Keywords:** ovarian cancer; DNA repair; carcinogenesis; prognosis; therapy response

## 1. Introduction

Recent reports highlight the importance of DNA repair and DNA damage response (DDR), involved in the genomic instability that accompanies tumorigenesis and cancer progression [1–3]. Pearl et al. [4] found that every DDR process was functionally impaired to some extent in one or more cancer types. Among eector pathways of DDR, genomic alterations in DNA repair genes represent substantial changes underlying the genetics of many solid cancers e.g., breast, colorectal,

and ovarian cancer (OvC) [5,6]. This paradigm is particularly pronounced in familial cancers with known germline mutations of high penetrance in DNA repair genes, e.g., breast cancer 1 and 2 (BRCA1 and 2) mutations in breast cancer; MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 6 (MSH6), PMS1 homolog 2 (PMS2), and DNA polymerase epsilon (POLE) mutations linked to mismatch repair or polymerase deficiency in colorectal and ovarian cancers; RAD51 paralog C and paralog D (RAD51C and D) deleterious mutations and BRCA1 mutation in OvC [6–9]. The present review article addresses the role of DNA repair machinery in OvC.

OvC is the 9th most common type of cancer and the 8th leading cause of death among female malignant diseases with an estimated annual incidence of 295,400 new cases and 184,800 deaths worldwide [10]. The majority (90%) of OvC is designated as epithelial ovarian carcinomas (EOCs) [11], divided into two major subtypes; (i) type I is composed of endometrioid, mucinous, clear cell and low grade serous ovarian carcinomas and (ii) type II includes high-grade serous ovarian carcinomas (HGSOCs) as histological dominant subtype [12]. It exhibits aggressive behavior and accounts for 70–80% of OvC deaths [13–15]. The other type II ovarian carcinomas present carcinosarcomas and undierentiated carcinomas [14,16]. The present standard of care for EOC consists of optimal cytoreductive surgery and chemotherapy that includes platinum-based chemotherapy usually in combination with taxanes [17,18]. In most cases, new therapeutic approaches are tested directly against molecular targets and pathways, e.g., poly(ADP-ribose) polymerase inhibitors (PARPi) such as olaparib, rucaparib or niraparib; anti-angiogenic agents such as bevacizumab or pazopanib; inhibitors of growth factor signaling or folate pathway inhibitors; protein kinase B (AKT) signaling inhibitors; and many immunotherapeutic approaches [19,20]. Despite the advent of new treatments, long term outcomes have not significantly improved in the past 30 years with the latest five-year survival rates largely falling between 30% and 50% across the globe [21,22]. At present, the main attention is dedicated to the improvement of the overall survival (OS) of OvC patients. As stated above, the functional status of DNA repair along with DDR determines cancer onset and impacts prognosis and ecacy of chemotherapy (often acting via DNA damage generation).

## 2. Main Molecular Hallmarks of Ovarian Cancer and Association with DNA Repair System

The whole system of DNA repair system is encoded by more than 150 genes and well-characterized [23]. Among existing DNA repair pathways, six pathways are implicated in OvC. In general, defective homologous recombination repair (HR), non-homologous end-joining (NHEJ), mismatch repair (MMR), base excision repair (BER), and disorders in nucleotide excision repair (NER) are typically reflected in OvC origin, pathogenesis and response to chemotherapy [20,24], whereas direct reversal of lesions is in connection with OvC addressed scarcely. Interestingly, there is sucient evidence on the participation of all DNA repair pathways in ovarian tumorigenesis due to complex exposures from environment [25,26]. Main DNA repair pathways relevant in ovarian carcinogenesis and their role in cellular biology are illustrated in Figure 1.

In general terms of genetic profiles, tumor protein p53 (TP53) somatic mutations, chromosomal instability, and frequently defective HR are typical for the most usual and aggressive type II category of ovarian carcinomas largely composed of HGSOC [14]. TP53 is a tumor suppressor which, in response to various cellular stresses (such as DNA damage, oxidative stress or hypoxia), binds to the promoter region of many genes controlling cell proliferation, apoptosis, DNA repair, etc., hereby regulates their expression [27].

Somatic mutations of TP53 occur in more than half of human tumors, making it the most frequent cancer-related gene [28]. HGSOC bears TP53 mutations in 96% of cases and about 50% of these tumors displayed defective HR due to germline and somatic BRCA mutations, epigenetic inactivation of BRCA, and abnormalities of DNA repair genes [15].

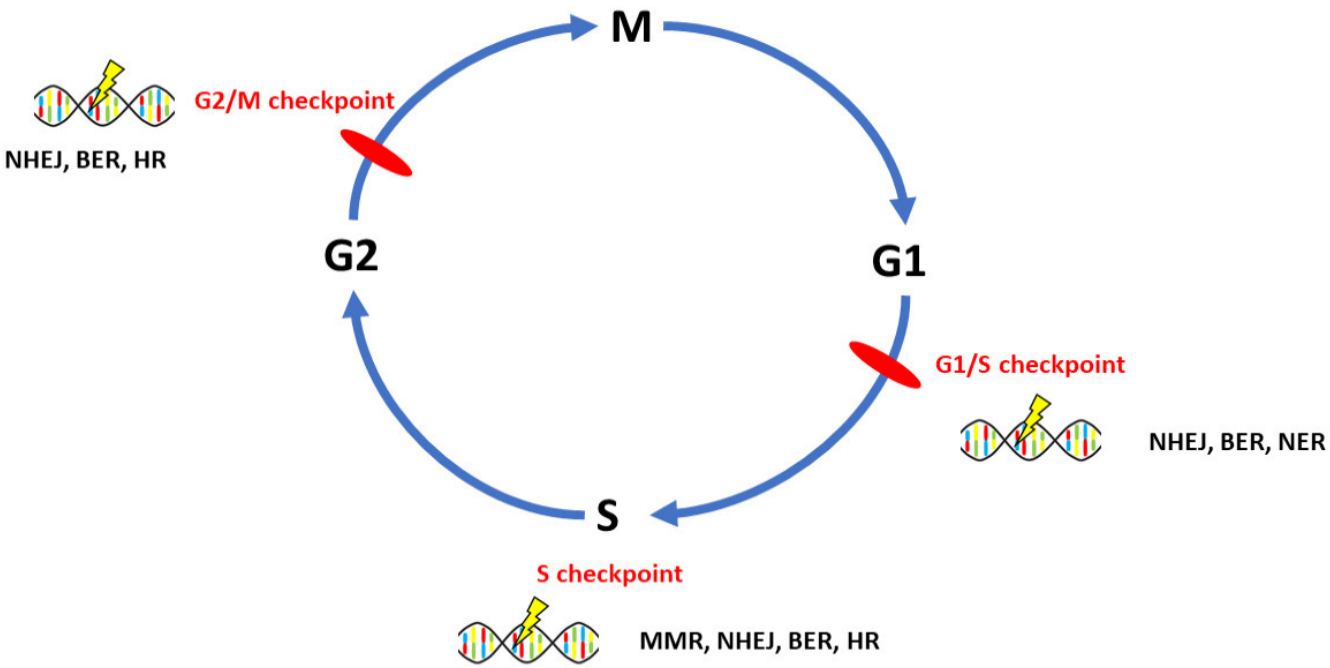



**Figure 1.** DNA repair pathways and implications in cell biology. DNA damage in the G1/S checkpoint is repaired by non-homologous end-joining repair (NHEJ), base excision repair (BER) and nucleotide excision repair (NER). In the S phase checkpoint, DNA damage is repaired by mismatch repair (MMR), homologous recombination (HR), NHEJ, BER. G2/M checkpoint DNA damage repair pathways are NHEJ, BER, HR. [29–32].

The deficiencies in MMR and BRCA1 mutations are important hallmarks for OvC [7,8,33]. BRCA1/2 germline mutations are estimated as risk factors of 10–20% of EOC [15]. Type I EOCs including low grade serous and mucinous carcinomas are typically Kirsten rat sarcoma viral oncogene homolog (KRAS)- and v-Raf murine sarcoma viral oncogene homolog B (BRAF)-mutated. Frequent mutations were also found in AT-rich interactive domain A1 (ARID1A), catenin beta 1 (CTNNB1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit (PIK3CA), phosphatase and tensin homolog (PTEN) genes [14]. Other recently found genes in women diagnosed for EOC and associated with the risk of EOC onset are BRCA1-interacting protein C-terminal helicase (BRIP1), RAD50 homolog (RAD50), RAD51C, RAD51D, BRCA1-associated RING domain 1 (BARD1), checkpoint kinase 2 (CHEK2), meiotic recombination 11 homolog A (MRE11A), partner and localizer of BRCA2 (PALB2) and ataxia telangiectasia mutated (ATM) gene (as summarized in [20]).

Particularly, deleterious mutations in RAD51C and RAD51D (genes involved in HR) have been shown to confer the risk of EOC implicating their use alongside BRCA1 and BRCA2 in routine clinical genetic testing [9]. Except for the association of DNA repair genes variations with modulating EOC risk, some recent studies overviewed the involvement of DNA damage repair pathways in EOC progression and therapeutic response. For instance, deficiency in HR, often occurring in OvC, was associated with worse outcomes in other solid cancers [34]. Nevertheless, except for the U.S. Food and Drug Administration (FDA) agency-approved treatment of germline BRCA-mutated OvC or maintenance treatment of platinum-sensitive relapsed BRCA-mutated EOC patients by PARPi [35], other DNA repair genes and pathways are not used as therapeutic targets in clinical practice at present. Recent period witnessed approaches with utilization of dierent kinds of DNA damage (repaired by dierent DNA repair pathways) induced simultaneously in frame of combinational chemotherapy (e.g., radiation and chemotherapy, the use of natural compounds in parallel with cytostatics [36]). These concepts are believed to diminish adverse eects of chemotherapeutics and postpone the advent of resistance. The role of genes and pathways of DNA repair system in ovarian carcinogenesis, prognosis, therapy response, and their potential as possible therapeutic targets are the main focuses of this review.

## 3. DNA Repair Pathways Involved in the Onset, Progression and Prognosis of Ovarian Cancer

### 3.1. Homologous Recombination Repair

HR is an essential high-fidelity DNA repair pathway, which provides template-dependent repair of complex DNA damage including DNA gaps, DNA double-strand breaks (DSBs), and DNA inter-strand crosslinks (repair mechanism is illustrated in Figure 2). It has also a prominent role in DNA replication and telomere maintenance. HR is active during S and G2 phases of the cell cycle when the sister chromatid is available and serves as a template. Normal cellular processes during DNA replication (due to replication fork collapse or arrest) and meiosis (during the process of crossing-over) may also produce DNA damage, taken care of HR. However, a variety of exogenous agents can induce DNA damage employing HR such as radiation, UV light, and crosslinking agents (e.g., platinum derivatives) [37,38].

Unrepaired DSBs are considered to be the most deleterious and fatal for DNA integrity. Defects in HR may result in deletions, translocations, duplications, loss of heterozygosity or aneuploidy [39]. Consequently, defective HR is linked to various types of cancers, especially OvC and breast cancer.

The defective HR pathway is found in about 50% of HGSOCs. However, non-serous histological types including clear cell, endometrioid, and carcinosarcomas have also been shown to harbor alterations in HR [40]. In OvC, HR deregulation is driven mostly by somatic and germline mutations in high-penetrance susceptibility genes BRCA1/2 [41,42].

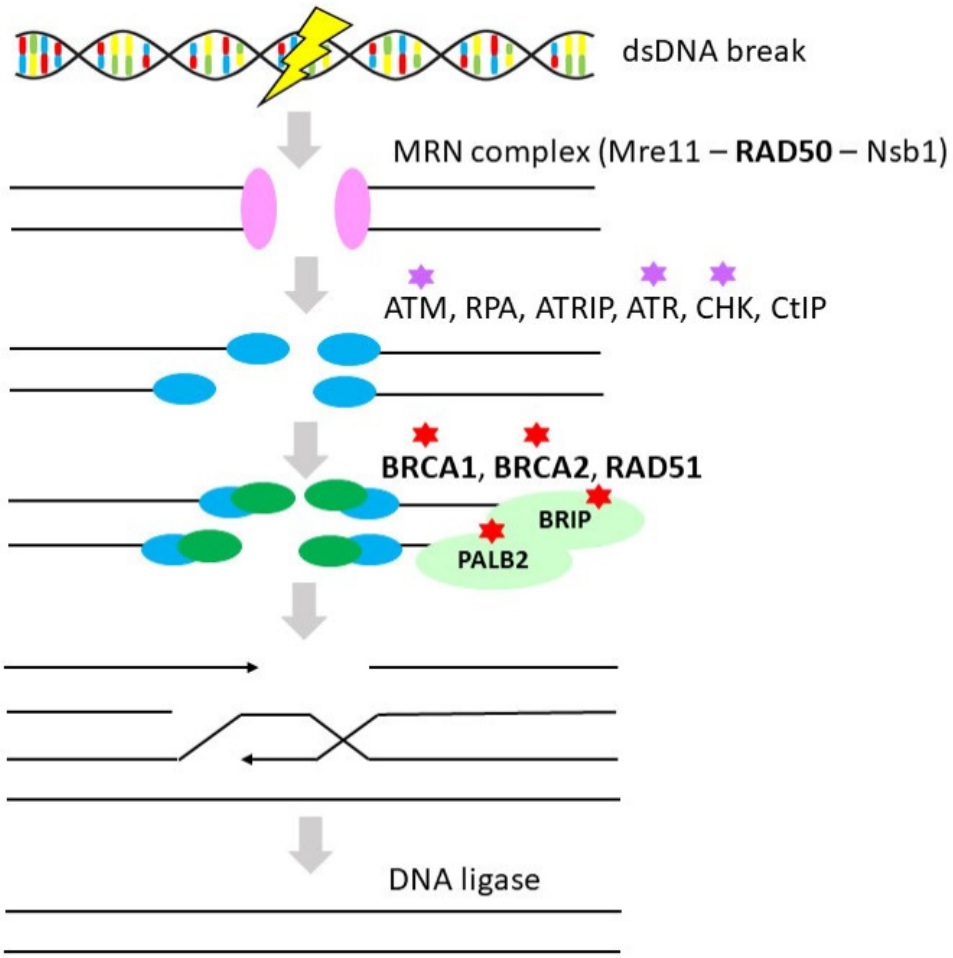



**Figure 2.** Homologous recombination. Simplified scheme of homologous recombination in double-strand DNA breaks and DNA inter-strand crosslinks (gene alternations in OvC in **bold**, therapeutic interventions considered in OvC therapy marked by a red star (PARP inhibitors), purple star (check-point inhibitors)) [43]. Protein names: meiotic recombination 11 (MRE11), RAD50 homolog (RAD50), Nijmegen breakage syndrome 1 (NBS1), Ataxia telangiectasia mutated (ATM), replication protein A (RPA), Ataxia telangiectasia and RAD3 related-interacting protein (ATRIP), Ataxia telangiectasia and RAD3 related (ATR), checkpoint kinases (CHEK), retinoblastoma binding protein 8 (CtIP), breast cancer 1 and 2 (BRCA1 and 2), RAD51 homolog 1 (RAD51), BRCA1-interacting protein C-terminal helicase (BRIP1), partner and localizer of BRCA2 (PALB2) [44].

BRCA1 and BRCA2 proteins play crucial roles in repairing DBSs. The deficiency of BRCA1 or 2 is caused by germline or somatic loss of function mutations (mainly deletions) in BRCA1/2 genes or by hypermethylation of the BRCA1 promoter. BRCA1 is active in the early phases of HR and binding sites for multiple proteins acts as a scaold that organizes other repair proteins to the site of the repair. BRCA2 acts later and is responsible for the loading of RAD51 onto replication protein A (RPA)-coated DNA.

Mutations in BRCA1 and BRCA2 genes are associated with a high risk of hereditary breast cancer and OvC. From a current prospective study of 9856 BRCA mutation carriers, the cumulative risk for OvC to age 80 was 44% for BRCA1 mutation carriers and 17% for BRCA2 mutation carriers [45]. BRCA1 mutation carriers develop OvC earlier compared to BRCA2 mutation carriers (mean age at the diagnosis for BRCA1-mutation carriers is 51.3y, for BRCA2-mutation carriers 61.4y) [46], while typical age at the diagnosis for the general population is about 63 years [47,48]. The risk for OvC varies also with the type and the location of BRCA gene mutations. Results suggest that there are “ovarian cancer cluster regions” (OCCRs) that lie in or near exons 11 of both genes and mutations in these regions are

associated with OvC rather than with breast cancer [49]. Additionally, "breast cancer cluster regions" (BCCRs) were identified in both genes as well, predisposing mainly to breast cancer and suggesting dierent mutation spectrum for ovarian and breast cancer [49].

Pooled analysis of several OvC studies revealed that BRCA1/2 mutation carriers exhibit significantly improved survival compared to non-carriers. This eect is pronounced in BRCA2-mutation carriers. The five-year survival rate in non-carriers was 36%, 44% for BRCA1-mutation carriers, and 52% for BRCA2-mutation carriers [50]. The survival advantage may be partly related to their enhanced sensitivity to platinum-based chemotherapy, which is conventionally used as a first-line OvC chemotherapy. Interestingly, epigenetic silencing of BRCA1 through promoter hypermethylation was not associated with better response to platinum-based chemotherapy and with improved survival in HGSOC patients [51]. However, BRCA1/2-mutated tumors are more likely to develop distant metastases. This may be partly related to the high degree of genomic instability present in these tumors [52].

Patients with HR-deficient OvC exhibit significantly higher response rates and prolonged progression-free survival (PFS) following platinum-based chemotherapy [50,53]. Even after disease recurrence, HR-deficient OvCs exhibit good response for other lines of platinum chemotherapy, while other OvCs often acquire chemo-resistance [54]. Nowadays, several PARPi are used in the treatment of BRCA-mutated OvC. Their cytotoxic eect is based on the synthetic lethality principle, where PARPi kill cancer cells with defective HR. The response to olaparib, the first FDA-approved PARPi, is the best in germline BRCA-mutated platinum-sensitive OvC and the worst in wild-type (wt) BRCA platinum-resistant OvC [55]. However, patients with BRCA-mutated OvC may develop resistance towards PARPi through multiple mechanisms including somatic reversion mutations of BRCA genes, reversion of BRCA-promoter methylation, overexpression of hypomorphic BRCA, decreased poly(ADP-ribose) polymerase 1 (PARP1) expression due to de novo mutations, drug eux, acquisition of new mutations in/silencing of other DNA repair genes. These mechanisms lead to either restoration of HR or protection of replication fork [56].

Other HR pathway alterations include medium penetrance mutations in several Fanconi anemia genes (mainly PALB2 and Fanconi anemia complementation group A, C, I, and L (FANCA, -C, -I, and -L), in RAD genes (such as RAD50, RAD51 homolog 1 (RAD51), RAD51C, RAD51D and RAD54-like (RAD54L)), and in DDR genes involved in HR (ATM, Ataxia telangiectasia and RAD3 related (ATR), checkpoint kinase 1 (CHEK1), and CHEK2) [43].

In particular, mutations in RAD51C and RAD51D have been associated with the risk of EOC, having potential use in routine clinical genetic testing [9]. RAD51 homolog genes are considered to be moderate penetrance OvC susceptibility genes, responsible for about 1% of OvC cases. Both proteins are important parts of the complex named BCDX2 (together with RAD51 paralog B (RAD51B) and X-ray repair cross-complementing 2 (XRCC2)) which is required for the formation of RAD51 foci in response to DNA damage. Biallelic mutations in RAD51C gene are present in Fanconi anemia-like syndrome [57]. Mutations in RAD51 genes are usually of deleterious type or hypermethylation of the RAD51C promoter [58].

Various studies disclosed strikingly elevated risk for OvC, reflected by odds ratio for RAD51C mutations ranging from 5 to 12 [59,60]. Similar odds ratios (5 to 12) have been assessed for mutations in RAD51D [9,61–63]. The lifetime risk for developing OvC for RAD51D mutation carriers is estimated to be 10–15% [62].

In the recent study, the median age at diagnosis in RAD51C and RAD51D mutation carriers was 39 and 32.5 years respectively, suggesting the involvement of RAD51 genes mutations in earlier onset of OvC [60].

Available results demonstrate that RAD51C and RAD51D are OvC predisposition genes, but further studies should evaluate their exact contribution to the OvC risk and onset.

Current studies suggest that mutations in RAD51 paralogs predispose ovarian tumors to be sensitive to PARPi. In vivo study on the patient-derived xenograft mice model revealed that RAD51C

promoter methylation predisposes to the sensitivity of ovarian tumors to niraparib (PARPi) [64]. Primary mutations in RAD51C and RAD51D confer to PARPi rucaparib sensitivity and, on the other hand, reverse secondary mutations in these genes contribute to acquired PARPi resistance [65].

RAD50 is a part of the so-called MRN complex (consisting of meiotic recombination 11 (MRE11), RAD50, and Nijmegen breakage syndrome 1 (NBS1)) which is essential for response to DSB damage and HR initiation. Heeke et al. identified mutations in HR genes in several types of solid tumors including OvC and found that RAD50 is mutated in about 0.12% of tumors [66]. Interestingly, immunohistochemical detection of MRN complex revealed that 41% of epithelial low-grade OvC lacked MRN complex and 10.3% of tumors lacked RAD50 specifically. The role of RAD50 mutation on OvC risk and onset must, therefore, be further evaluated [67].

Kessous et al. correlated the survival of OvC patients with expression profiles of dierent HR genes and found that expression of RAD50 correlates with better PFS [68]. In BRCA-wt OvC patients, 18% of patients exhibit RAD50 copy number deletion which was associated with significantly better OS and PFS [69].

According to an in vitro study from Zhang et al. [69], knockdown of RAD50 gene expression in OvC cell lines was associated with better response to PARPi (olaparib and rucaparib). Further research may help to better define the group of patients who may profit from PARPi, even if they are BRCA-wt but simultaneously have deficient other steps of HR pathway.

PALB2 is another important member of HR, interacting with BRCA2 as well as with BRCA1 and several members of the DDR family [70]. Its mutations are associated with an elevated risk of developing several cancers including breast cancer [71]. A polish study on 460 BRCA-wt OvC patients revealed that 1.5% of patients had germline deletion in the PALB2 gene [72]. A recent study on 524 families from 21 countries harboring pathogenic variants of the PALB2 gene estimated the relative risk of OvC to be nearly 3. The estimated risk of developing OvC to age 80 is almost 5% [73].

Studies of therapy outcome suggest that PALB2-deficient ovarian tumors, similarly to other HR deficient OvC, may benefit from PARPi therapy [74]. In vivo study on pediatric cancers suggests that PALB2 mutations are associated with exceptional response to talazoparib in mouse xenografts [75].

BRIP1 is another member of HR pathway with ATPase and helicase activity known for its role in OvC predisposition. It was previously associated with breast cancer risk [76–79] however, results from these studies are inconsistent and several other studies found no association of BRIP1 mutations and breast cancer risk [80,81]. It is one of the most common OvC susceptibility genes with 0.9–2.5% frequency in all patients carrying a mutation in this gene [62,82–84]. A study from Weber-Lassalle et al. on the loss of function BRIP1 mutations found that these mutations confer a high OvC risk in familial OvC patients as well as in late-onset OvC patients (OR = 20.97 and 29.91 respectively) [83]. Another study assessed the relative risk (RR) of EOC being 11.22 (95% confidence interval [CI] = 3.22 to 34.10, P = 1 10 $^{4}$) and cumulative risk of developing EOC by age 80 years to 5.8% (95% CI = 3.6% to 9.1%) making it a moderate risk factor for OvC [82].

Similarly, as in other members of HR pathway, mutations in BRIP1 are believed to predispose OvC tumors to better respond to both PARPi and platinum [55].

The overall DNA repair system is tightly coordinated with cell cycle checkpoints as an essential part of DDR. A large recent genome-wide association study (GWAS) identified an association of CHEK2 gene variants with EOC risk. CHEK2 is a serine-threonine kinase which, in response to DSB, phosphorylates serine 988 in BRCA1 [85]. This phosphorylation is required for the formation of BRCA1–PALB2–BRCA2 eector complex critical in RAD51-mediated HR [86,87]. According to the GWAS, the strongest association showed CHEK2 single-nucleotide polymorphism (SNP) rs17507066 with serous EOC. The authors reported an additional association of CHEK2 rs6005807 with HGSOC. Both SNPs, i.e., rs17507066 and rs6005807 showed linkage disequilibrium $r^2$ = 0.84 [88]. Additionally, CHEK2 gene variant rs6005807 was associated with EOC risk (irrespective of BRCA1/BRCA2 mutations) in an independent, large GWAS study of Phelan et al. (for detailed description of SNPs discussed in our review see Table 1) [89].

**Table 1.** List of OvC-associated SNPs. Symbols: " means higher, or better; # means lower. Gene names: checkpoint kinase 2 (CHEK2), 8-oxoguanine DNA glycosylase 1 (OGG1), apurinic/apyrimidinic endonuclease 1 (APE1), X-ray repair cross-complementing 1 (XRCC1).

| Gene | SNP | Functionality | Eect | Odds Ratio (OR), Hazard Ratio (HR), Confidence Interval (CI) | Population | Reference |
|---|---|---|---|---|---|---|
| CHEK2 | rs17507066 | Intron variant | " risk of serous EOC | OR: 0.86; 95% CI: 0.81–0.91 | 15,397 patients, 30,816 controls | [88] |
| | rs6005807 | Intron variant | " risk of EOC | OR: 1.12, 95% CI: 1.07–1.18 | 15,397 patients, 30,816 controls | [88] |
| | | | " risk of serous EOC | OR: 1.17, 95% CI: 1.11–1.23 | 25,509 patients, 40,941 controls | [89] |
| OGG1 | rs1052133 | Missense variant, Ser326Cys | " risk | OR: 2.89; 95% CI: 2.47–3.38 | 720 patients, 720 controls | [90] |
| | | | " risk type II EOC | OR: 1.66; 95% CI: 1.26–2.17 | 420 patients, 840 controls | [91] |
| | rs2304277 | Intron variant | " risk for BRCA1/2 carriers | HR: 1.12, 95% CI: 1.03–1.21 | Stage I 1782 mutations carriers<br>Stage II 23,463 mutations carriers | [92] |
| APE1 | rs1130409 | Missense variant, Asp148Glu | # risk | OR: 0.486; 95% CI: 0.344–0.688 | 124 patients, 141 controls | [93] |
| XRCC1 | rs25487 | Missense variant, Arg399Gln | " risk | OR: 2.54; 95% CI: 1.22–5.29 | 50 patients, 78 controls | [94] |
| | | | " risk of death | HR: 1.98; 95% CI: 1.09–3.93 | 195 patients | [95] |
| | rs1799782 | Missense variant, Arg194Trp | " OS | HR: 0.61, 95% CI: 0.34–0.96 | 229 patients | [96] |

### 3.2. Non-Homologous End-Joining

NHEJ is a most robust pathway which repairs DSBs in DNA. Unlike HR, DNA lesions are directly ligated without a need of a homologous template. Since it doesn't require sister chromatid (available during S and G2 phases), it may be executed throughout the entire cell cycle (repair mechanism is illustrated in Figure 3). In fact, NHEJ appears to repair almost all DBSs outside the S and G2 phases of the cell cycle and about 80% during the S and G2 phases [97]. However, since the process involves losses of sequences during the junction formation, NHEJ is a potentially a mutagenic process. Apart from its key role in repairing DSBs, NHEJ is an essential part of adaptive immunity during V(D)J recombination, giving rise to a highly diverse repertoire of immunoglobulins and T cell receptors.

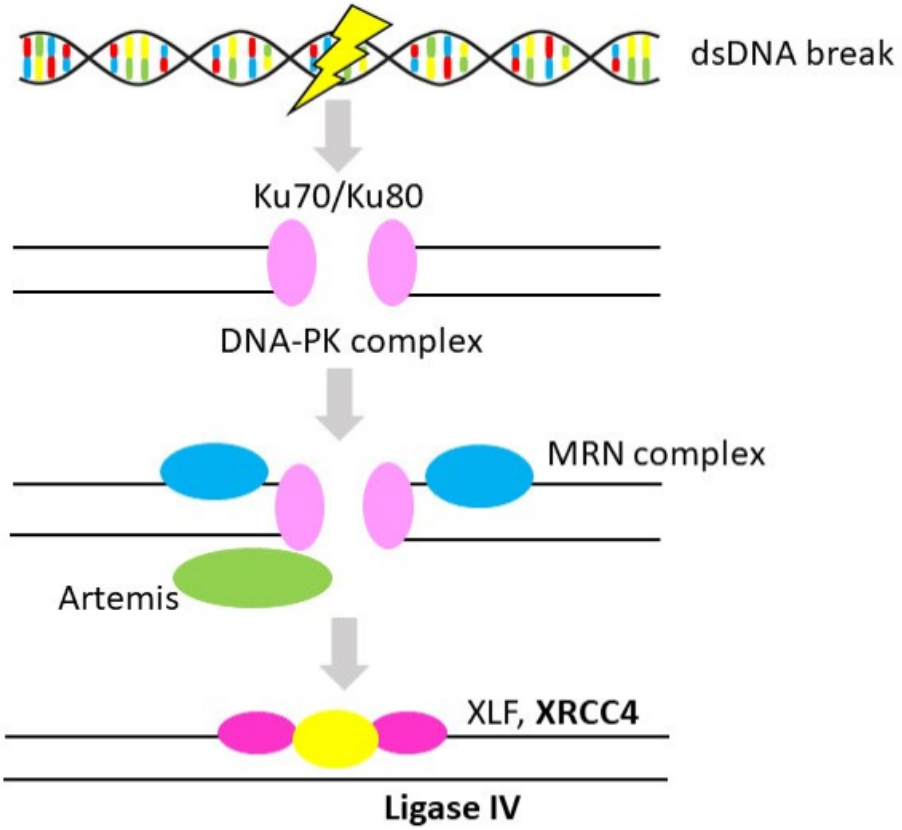



**Figure 3.** Non-homologous end-joining repair. Simplified scheme of non-homologous end-joining repair of double strand DNA breaks (gene alterations in OvC in **bold**) [32]. Protein names: DNA end-binding proteins Ku70/Ku80 (Ku70/Ku80), DNA-dependent protein kinase (DNA-PK), MRE11-RAD50-NBS1 complex (MRN complex), artemis (DCLRE1C), X-ray repair cross complementing-like factor. (XLF), X-ray repair cross complementing 4 (XRCC4), DNA ligase 4 (LIG4) [44].

Germline mutations in genes involved in NHEJ are associated with severe immunodeficiency and developmental abnormalities [98,99], genomic instability as well as with dierent cancers, such as leukemias or bladder cancer [100–103]. Whereas excessive research has been done on HR and OvC, less is known about the relationship between mutations in NHEJ genes and OvC. McCormick et al. assessed NHEJ in a panel of OvC cell lines and 47 primary OvC cell cultures. This study shows that about 40% of OvC cell lines and primary cultures were defective in NHEJ, independently of HR [104]. Interestingly, NHEJ-deficient cell lines and cell cultures were resistant to rucaparib (PARPi). Sensitivity to this PARPi was observed only in NHEJ-competent/HR-deficient cultures, potentially explaining why some HR-deficient tumors are resistant to PARPi.

Probably because of its predominant role in DSBs repair, mutations in NHEJ genes are less common and only a minor part of human cancers are associated with their loss or alterations. X-ray repair

cross-complementing 4 (XRCC4) and DNA ligase 4 (LIG4) were two members of the NHEJ pathway studied in association with OvC.

XRCC4 protein is involved in the ligation phase of NHEJ pathway. Mutations in XRCC4 have been linked mainly to developmental disorders as microcephaly and dwarfism [105]. However, high expression of XRCC4 has been linked also to the poor outcome of OvC patients, making it one of the candidate biomarkers for OvC [106].

LIG4 is an essential protein in NHEJ, making a complex with XRCC4. Mutations in LIG4 are a cause of rare autosomal recessive LIG4 syndrome. Polymorphisms in the LIG4 gene have been associated with increased risk for several cancers [107]. Currently, there is insucient evidence that LIG4 gene variants are involved in OvC risk or prognosis. A SNP rs1805386 in LIG4 was believed to be associated with OvC risk, but this association was later dismissed [108].

### 3.3. Mismatch Repair

Besides the BRCA1 and BRCA2 mutations, MMR deficiency is the most common cause of hereditary OvC [109]. MMR system corrects DNA base mismatches in newly replicated DNA which were not recognized by DNA replication machine, or insertion/deletion mispairs as is illustrated in Figure 4. MMR acts mainly in the S phase of the cell cycle [110].

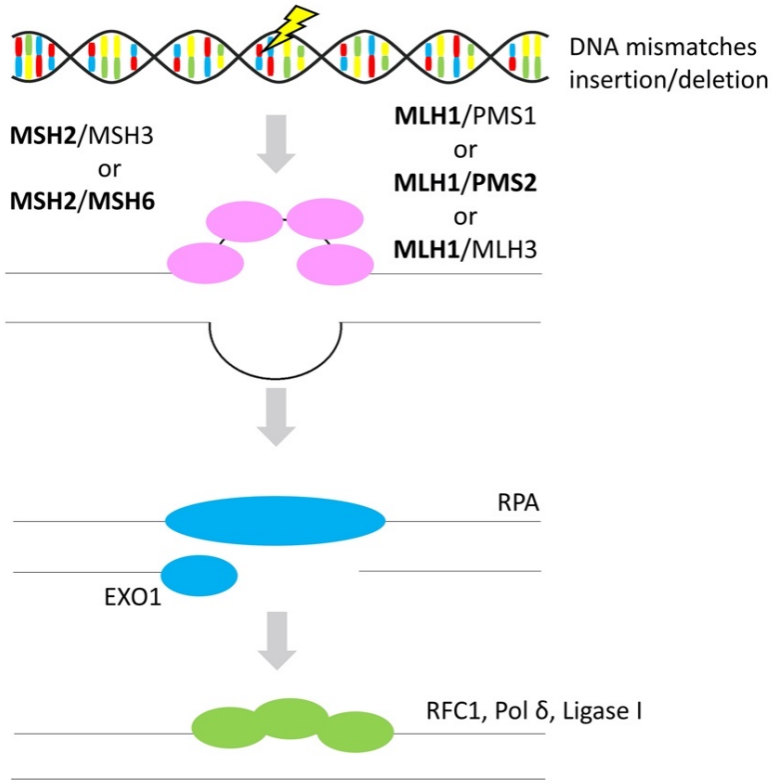



**Figure 4.** Mismatch repair. Simplified scheme of mismatch repair of DNA mismatches or insertion/deletions mispairs (gene alternations in OvC in **bold**) [111]. Protein names: MutS homolog 2 (MSH2), MutS homolog 3 (MSH3), MutS homolog 6 (MSH6), MutL homolog 1 (MLH1), PMS1 homolog 1 (PMS1), PMS1 homolog 2 (PMS2), MutL homolog 3 (MLH3), replication protein A (RPA), exonuclease 1 (EXO1), replication factor C subunit 1 (RFC1), DNA polymerase delta (POLD), DNA ligase 1 (LIG1) [44].

In humans, seven genes are implicated in the MMR system. Mismatch recognition is mediated by a heterodimer, composed of MSH2 and MutS homolog 3 (MSH3), or MSH2 and MSH6. This heterodimer then interacts with another heterodimer, composed of MutL homologs MLH1 and PMS1 homolog 1 (PMS1), MLH1 and PMS2, or MLH1 and MutL homolog 3 (MLH3), which forms single-stranded nicks on either side of the mismatch [112].

Germline mutations in MMR genes MLH1, MSH2, MSH6, and PMS2 or loss of expression of MSH2 cause Lynch syndrome, also known as hereditary non-polyposis colorectal cancer [113]. Depending on the particular MMR gene, this multi-cancer syndrome increases the cumulative lifetime risk of OvC from 6% to 12% [114].

Characteristic molecular signature occurring as a result of inactivation of the DNA MMR is called microsatellite instability (MSI) [115]. It is a hypermutable phenotype manifested through alterations in the size of repetitive DNA sequences. Tumor profiling for MSI serves as a measure for the personalized management of several cancers [116,117]. Regarding OvC, MSI occurs in a limited percentage of the tumors (2–20%) and aects predominantly endometrioid (19.2%), mucinous (16.9%), clear cell (11.2%), and serous (7.9%) subtypes [118,119]. Both endometrioid and clear cell subtypes with MSI show increased levels of tumor-infiltrating lymphocytes and thus may be susceptible to immune checkpoint inhibitor monotherapy [120]. In a very recent study of 478 OvCs by Fraune et al., MMR deficiency occurred almost exclusively in endometrioid subtype (8 of 32) and also in one of 358 serous carcinomas. MMR of other subtypes (mucinous, clear cell, carcinosarcomas of Mullerian origin, and mixed carcinosarcomas) was functional [121]. Whereas all MMR-deficient endometrioid cancers were MSI and showed loss of MLH1/PMS2 proteins in five of 32 cases, MSH2/MSH6 in two of 32 cases, and isolated MSH6 in one of 32 cases; the MMR-deficient serous carcinoma was microsatellite stable and showed PMS2 protein loss and an altered pattern of MLH1 with putative partial MLH1 protein loss [121].

Earlier studies suggested the role of MMR in signaling that triggers apoptotic activity [122]. This was further confirmed by the proof that MMR-deficient cells can continue to proliferate despite DNA damage [123]. The chemical nature of platinum derivatives may explain the resistance as well. They induce, by attacking -SH, -NH and -OH nucleophilic centers of DNA bases, either monofunctional N7-guanine adducts (minor product) or the bifunctional adducts resulting in guanine-guanine intrastrand crosslinks, guanine-adenine intrastrand crosslinks (both representing a majority of lesions) and guanine-guanine interstrand crosslinks of two nonadjacent guanines. Arising crosslinks have inhibitory eects on transcription and replication. Platinum derivatives may also bind to nucleophilic centers of proteins, forming various crosslinks that aect further their function [2,124].

Epigenetic events underlying MMR deficiency have also been investigated. As for sporadic cancers (OvC included), compromised MMR function due to promoter hypermethylation is known in MLH1 and MSH2. Resistance to platinum in EOC has been associated with hypermethylation of the MSH2 upstream region [125]. Thus, lower expression of MSH2 may indicate the poor prognosis in EOC patients [125]. In secondary EOC, MLH1 hypermethylation was found to be a cause of acquired platinum resistance as well, and it occurred more frequently in tumors treated with four or more courses of platinum-based chemotherapy [126]. The exact role of MLH1 and MSH2 in the platinum resistance is not yet clear. Watanabe et al. suggest that methylation of MLH1 during the platinum chemotherapy may be a temporary change protecting cancer cells from cytotoxic agent-induced apoptosis because after a platinum-free interval of 6- to 12- months, they become sensitive to platinum agents again [126]. Moreover, Zhao et al. reported that a sucient MMR system, defined in their study by high mRNA levels of MSH6, MLH1, and PMS2, may indicate better OS in OvC treated with platinum-based chemotherapy [127].

### 3.4. Base Excision Repair

BER is an essential part of DNA repair machinery, which is responsible for repairing small base lesions (alkylations, oxidations, deaminations, depurinations or single-strand breaks (SSBs)) resulting from endogenous (products of metabolism) as well as exogenous (radiation, chemicals, drugs) sources of damage. BER consists of several components; DNA glycosylases, apurinic endonucleases (such as apurinic/apyrimidinic endonuclease 1 (APE1)), DNA polymerases (such as DNA polymerase beta (POLB)), Flap endonuclease 1 (FEN1) and DNA ligase (DNA ligase 1 or 3 (LIG1 or 3)). Other important

players participating in BER are PARP1 or X-ray repair cross-complementing 1 (XRCC1) (as is illustrated in Figure 5).

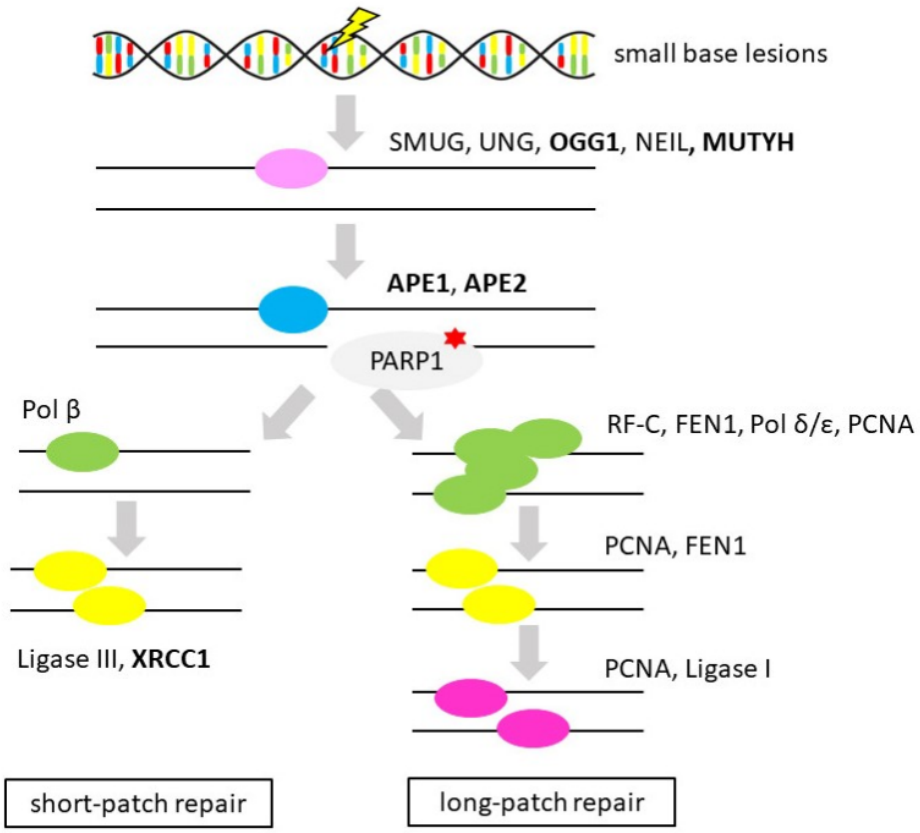



**Figure 5.** Base excision repair. Simplified scheme of base excision repair of small base lesions (gene alternations in OvC in bold, therapeutic interventions considered in OvC therapy marked by red star (PARP inhibitors)) [128]. Protein names: single-strand selective monofunctional uracil DNA glycosylase (SMUG), uracil DNA glycosylase (UNG), 8-oxoguanine DNA glycosylase 1 (OGG1), endonuclease VIII-like (NEIL), MutY DNA glycosylase (MUTYH), apurinic/apyrimidinic endonuclease 1 and 2 (APE1 and 2), poly(ADP-ribose) polymerase 1 (PARP1), DNA polymerase beta, delta and epsilon (POLB, -D, -E), DNA ligase 1 and 3 (LIG1 and 3), X-ray repair cross-complementing 1 (XRCC1), replication factor C (RFC), Flap endonuclease 1 (FEN1), proliferating cell nuclear antigen (PCNA) [44].

DNA glycosylases (e.g., single-strand selective monofunctional uracil DNA glycosylase (SMUG), uracil DNA glycosylase (UNG), 8-oxoguanine DNA glycosylase 1 (OGG1) or endonuclease VIII-like (NEIL) DNA glycosylases) initiate the BER pathway. Depending on the type of lesion, one of the 11 glycosylases is used to excise the aected base. Impairment of these glycosylases is often linked with various cancers, such as colorectal, oesophageal, gastric, ovarian or lung cancer [129,130]. In the term of OvC, SNPs in the OGG1 gene were described to increase the risk of OvC (for more details see Table 1). OGG1 is responsible for the excision of 8-oxoguanine, which is the result of damage caused by reactive oxygen species. Polymorphism Ser326Cys (rs1052133) was identified as a risk factor in 720 OvC patients compared to 720 healthy controls from Poland [90] and in a Chinese population using 420 patients and 840 controls, where they also linked it with type II EOC [91]. It is also known that Ser326Cys is linked with decreased repair capacity to oxidative damage [131]. Another polymorphism in OGG1 (rs2304277) increased the risk of OvC in BRCA1 mutations carriers [92]. In the following work they described rs2304277 role in OGG1 downregulation and a possible contribution to telomere shortening [132]. Other studies support that OGG1 downregulation leads to telomere shortening [133]. MutY DNA glycosylase (MUTYH) excises adenine, which is inappropriately paired. It is known

especially for its role in an increased risk of colorectal cancer [134], but its biallelic mutation is also a risk factor for OvC [135]. The role of DNA glycosylases in the therapy outcome of OvC remains unknown.

APE1 and apurinic/apyrimidinic endonuclease 2 (APE2) cleave the apurinic/apyrimidinic sites left by the glycosylases or by spontaneous depurination [136,137]. APE1 is the major apurinic endonuclease in humans with more than 95% total cellular activity leaving the rest for APE2 [138]. There was identified SNP (rs1130409), which was significantly associated with risk for OvC [93]. The higher level of APE1 was reported in serous and mucinous tumors. Moreover, APE1-positive cases had a lower chance of ideal debulking surgery with consequent worse OS, implicating a more aggressive phenotype [139]. Cellular localization of APE1 had its impact on disease prognosis as well. Cytoplasmatic localization was higher in EOCs stages III and IV in comparison with lower stages in FIGO classification and it was, also, linked with lower survival rate [140]. Moreover, the abnormal cytoplasmatic level of APE1 with an abnormal level of nucleophosmin (NPM1) is associated with poor prognosis and higher chemoresistance of HGSOC [141].

XRCC1 is a scaolding protein, which interacts with PARP1 and LIG3 in BER pathway. It has no enzymatic activity but acts as a scaold allowing other repair proteins to carry out their enzymatic work [138]. Several studies studied OvC risk and polymorphisms in the XRCC1 gene. Polymorphism Arg399Gln (rs25487) is linked with higher susceptibility to OvC development [94]. Association between the same polymorphism [95] along with Arg194Trp (rs1799782) [95,96] and worse clinical outcome and prognosis was also found. The expression of XRCC1 was significantly linked with a higher stage of the illness, serous histological type of tumor, sub-optimal debulking surgery, and platinum resistance. All of these lead to a higher risk of death and worse prognosis [142].

PARP1 has become one of the major topics in BER in the last decade. Its role in BER is the detection of single-strand breaks and PARP1 acts as a signal for the repair machinery, which consists of scaolding protein XRCC1, LIG3, and POLB. In 2014, PARPi were approved by FDA and European Medicine Agency (EMA) for use in OvC. PARPi eect is mediated by its synthetic lethality concept in HR deficient cells. Inhibition of PARP1 promotes SSBs, which, if unrepaired, consequently lead to DSBs. HR deficiency causes reliance on error-prone NHEJ pathway, therefore with PARPi together lead to genetic damage followed by cell death [143]. As for OvC, present approved application of PARPi is for patients with germline BRCA1/2 mutations, for patients with germline or somatic mutation BRCA1/2 with relapsed illness or patients with relapsed illness sensitive to platin-derivate chemotherapy regardless to BRCA status. Other indications are under clinical trials and have not been approved yet. There is a growing number of studies pointing out the potential benefit of PARPi treatment in other DDR genes deficiency outside BRCA mutations (e.g., ATM, ATR, BARD1, BRIP1, CHK1, CHK2, PALB2, RAD51 or FANC) or combination treatment with other chemotherapeutics and targeted therapy. For more information, the reader is referred to other excellent up to date reviews focused on PARP and its inhibitors [144–148]. However, in vitro and in vivo evidence suggest that mutations in PARP1 abolishing the DNA binding cause the resistance towards PARPi [149].

### 3.5. Nucleotide Excision Repair

NER recognizes bulky, helix distorting DNA damage, the main of which include UV photoproducts, polycyclic aromatic hydrocarbons, aromatic amines, platinated products, and several others (repair mechanism is illustrated in Figure 6). The deficiency of several proteins in the NER pathway is tied to three rare autosomal recessive syndromes: Cockayne syndrome, Xeroderma pigmentosum and the photosensitive form of the trichothiodystrophy [150]. Cockayne syndrome is a neurodegenerative disease caused by the mutation in either Cockayne syndrome A (CSA/ERCC8) or Cockayne syndrome B (CSB/ERCC6) genes, which leads to impaired transcription-coupled NER [151]. Xeroderma pigmentosum, characterized by extreme photosensitivity to UV radiation, results from the mutations in any of genes xeroderma pigmentosum complementation group A, B, C, D, E, F, G (XPA, -B/ERCC3, -C, -D/ERCC2, -E, -F/ERCC4, -G), xeroderma pigmentosum variant (XPV), or excision repair cross-complementation group 1 (ERCC1) [152]. Trichothiodystrophy belongs to ectodermal

disorders. About half of the patients are photosensitive because they bear the mutation in XPB/ERCC3, XPD/ERCC2, or general transcription factor IIH subunit 5 (GTF2H5) [153].

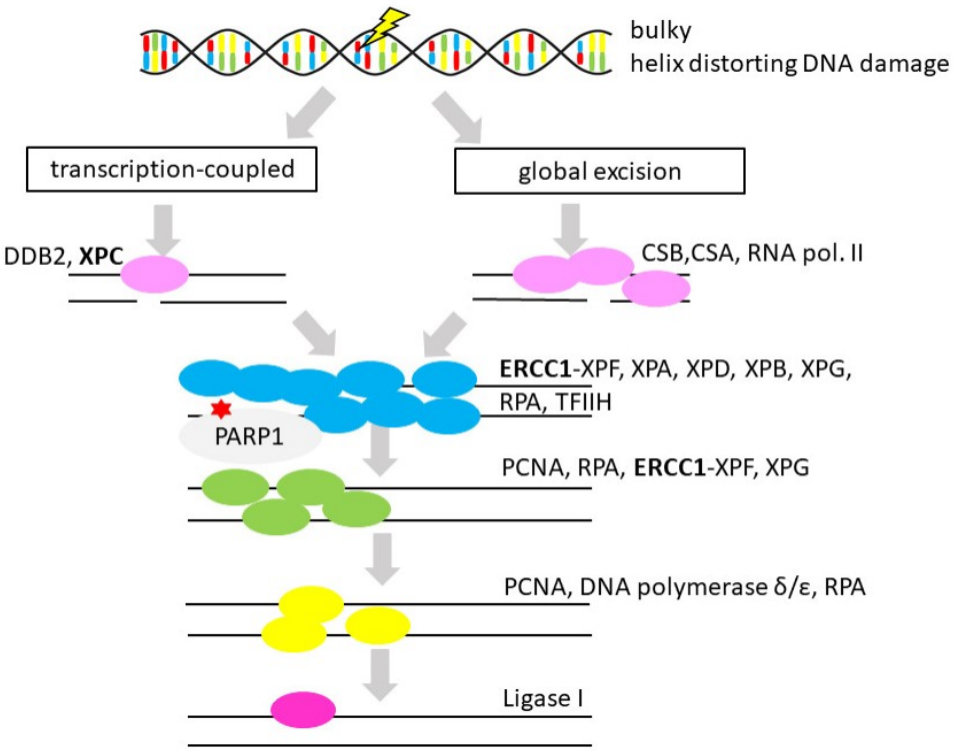



**Figure 6.** Nucleotide excision repair. Simplified scheme of nucleotide excision repair of bulky lesions and helix distorting DNA damage DNA (gene alternations in OvC in **bold,** therapeutic interventions considered in OvC therapy marked by a red star (PARP inhibitors)) [150]. Protein names: damage specific DNA binding protein 2 (DDB2), xeroderma pigmentosum complementation group A, B, C, D, F, G (XPA, -B/ERCC3, -C, -D/ERCC2, -F/ERCC4, -G), Cockayne syndrome A and B (CSA and B), RNA polymerase II (RNA pol. II), excision repair cross-complementation group 1 (ERCC1), replication protein A (RPA), transcription factor II Human (TFIIH), poly(ADP-ribose) polymerase 1 (PARP1), proliferating cell nuclear antigen (PCNA), DNA polymerase delta and epsilon (POLD and E), DNA ligase 1 (LIG1) [44].

Zhao et al. analysed 17 SNPs in NER genes XPA, XPC, XPD/ERCC2, XPF/ERCC4, XPG, and ERCC1 in 89 OvC cases and 356 controls, and their results suggested that ERCC1, XPC, and XPD/ERCC2 may be linked to OvC susceptibility [154]. Although this study for the first time explored the association of core genes in NER pathway with OvC, it should be pointed out that the sample size was insucient to link OvC susceptibility to particular genetic variations and further, authors were not able to measure the mRNA expression of ERCC1, XPC, and XPD/ERCC2 to validate their findings. However, the study by Sun et al. associated higher XPC mRNA expression with poor OvC prognosis [155]. ERCC1 is a non-catalytic subunit of $5^0$ endonuclease which in complex with XPF/ERCC4 (a catalytic subunit) incises the damaged DNA strand on the $5^0$ side of the lesion. XPC initiates NER reaction by detecting the DNA damage. XPD/ERCC2 is a helicase that, as a part of TFIIH core complex, unwinds (together with other TFIIH helicase XPB/ERCC3) DNA around the site of the lesion to enable its subsequent incision.

Cisplatin regimen is a standard chemotherapeutic procedure for OvC patients [156]. The most prominent damage the cisplatin introduces in DNA are 1,2- and 1,3-intrastrand crosslinks, which can be removed by NER. It is hardly surprising, therefore, that upregulation of NER mediates resistance to cisplatin-based therapy [2,157]. Ishibashi et al. reported that in OvC cell lines, tyrosine kinase with immunoglobulin-like and EGF like domains 1 (TIE1) promotes XPC-dependent NER and this leads to decreased susceptibility to cisplatin-induced cell death [158]. Last but not least, a study in 559

EOC patients showed an association of ERCC1 polymorphisms rs11615 and rs3212986 with cisplatin resistance [159]. Beyond the PARP1 well-established role in BER, this protein is also known to regulate the NER system by its association with XPA (for more details, see Section 4) [160].

### 3.6. Direct Repair

Unlike other DNA repair mechanisms, a direct reversal of a lesion represents a relatively simple way to remove some DNA and RNA modifications, e.g., at guanine $O^6$ position, without incision of phosphodiester backbone, DNA synthesis, and ligation. The base damage is eliminated in single enzyme reactions, allowing error-free repair (illustrated in Figure 7). The most common modifications involve DNA alkylation damage or RNA methylation arising by epigenetic mechanisms [44,161].

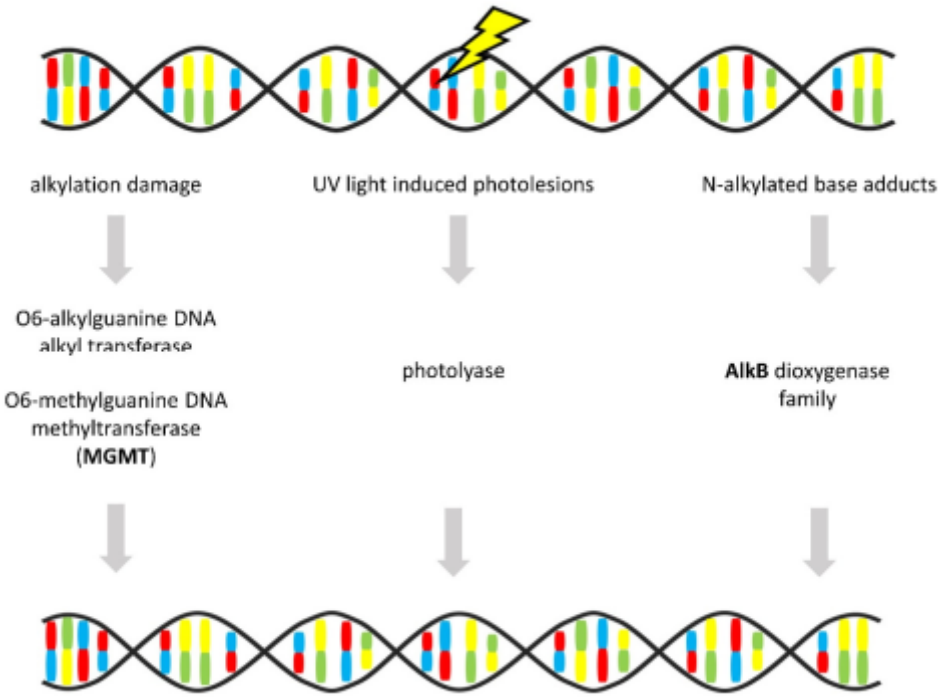



**Figure 7.** Direct repair. Simplified scheme of direct lesion reversal removing alkylation and UV-induced damage, and N-alkylated base adducts (gene alternations in OvC in **bold**) [162]. Protein names: $O^6$-methylguanine DNA methyltransferase (MGMT), alpha-ketoglutarate-dependent dioxygenase AlkB (AlkB) [44].

As an alternative to complex NER mechanism works photoreactivation [163]. This direct repair mechanism is mediated by photolyases and removes ultraviolet light-induced modifications of DNA, namely cyclobutane pyrimidine dimers and pyrimidine-pyrimidone photoproducts. Placental mammals possess however no class of photolyases and are reliant only on NER [164].

In humans, enzymes directly employed in DNA repair of alkylation damage are $O^6$-methylguanine DNA methyltransferase (MGMT), which erases alkylations at the $O^6$ position of guanine and thus prevents DNA cross-links, and alpha-ketoglutarate-dependent dioxygenase AlkB (AlkB) homologs, which oxidatively dealkylate e.g., $N^1$-methyladenine, $N^6$-methyladenine or $N^3$-methylcytosine [161,165].

Downregulation of MGMT expression due to hypermethylation of its CpG islands located in the promoter region of MGMT and its probable relation to OvC carcinogenesis was firstly described by Roh et al. [166]. MGMT promoter hypermethylation was detected in 12 of 86 (14.0%) EOCs and strongly negatively correlated with MGMT expression. These data suggest that in sporadic OvC, MGMT is repressed mainly due to methylation of its promoter. Meta-analysis of 10 studies comprising 910 ovarian tissue samples by Qiao et al. concluded that the inactivation of MGMT might

be associated with carcinogenesis in specific histological types of EOC [26]. Aberrant MGMT promoter methylation appears also in other human cancers such as cervical cancer [167], lung cancer [168], and glioblastoma [169].

As a DNA repair protein, MGMT seems to be also implicated in OvC chemoresistance. It was found to transcriptionally activate deubiquitinating enzyme 3 (DUB3) which stabilizes myeloid cell leukemia 1 (MCL1), an anti-apoptotic protein belonging to B-cell lymphoma 2 (BCL2) protein family [170]. This upregulation of MCL1 prevents apoptosis and is essential for tumors to evade anti-cancer drugs and become resistant. To suppress the growth of MGMT-DUB3-MCL1-overexpressing cells may be useful a combined therapy with histone deacetylase inhibitors and $O^6$-(4-bromothenyl)guanine (PaTrin-2).

Altered expression of some members of the AlkB human homolog family, the latter mentioned group of dealkylating enzymes, has been related to OvC at the level of post-transcriptional modification to mRNA. Methyl modifications to mRNA allow post-transcriptional control of gene expression by altering the mRNA interactions with other cell components [171]. Demethylation of the $N^1$ atom of adenine by AlkB homolog 3 (AlkBH3) was found to increase the half-life of colony-stimulating factor 1 (CSF1) mRNA without affecting the translation efficiency [172]. The expression of cytokine CSF1 predicts poor prognosis in ovarian and breast tumors [173]. Also, another AlkB homolog 5 (AlkBH5) was found to enhance the stability of BCL-2 mRNA through demethylation of $N^6$ atom of adenine in EOC [174].

## 4. Interplay of DNA Repair Pathways

In our recent review, we have presented DNA repair as a complex biological process that ensures cellular integrity and genomic stability [3]. It has been known for long that DNA repair consists of several distinct pathways restoring dierent types of DNA damage [175]. In recent years, there is growing evidence of interactions among proteins involved in distinct DNA repair pathways. Regarding OvC, these interactions are illustrated in Figure 8.

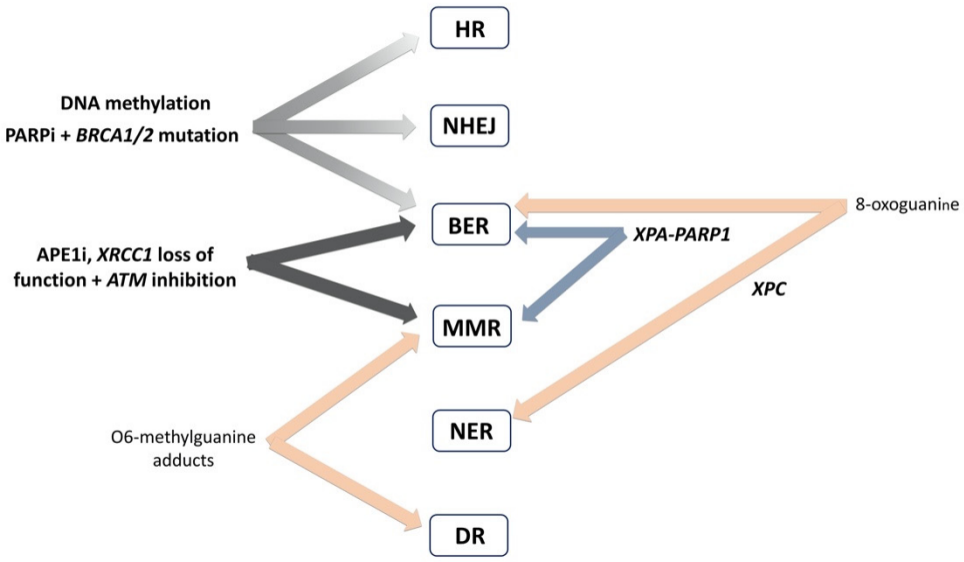



**Figure 8.** Interplay of DNA repair pathways. Simplified scheme of interactions between proteins from distinct DNA repair pathways (genes interacting in OvC in **bold**). Gene names: breast cancer 1 and 2 (BRCA1 and 2), X-ray repair cross-complementing 1 (XRCC1), Ataxia telangiectasia mutated (ATM), xeroderma pigmentosum complementation group A and C (XPA and C), poly(ADP-ribose) polymerase 1 (PARP1). Protein inhibitors: apurinic/apyrimidinic endonuclease 1 inhibitors (APE1i), poly(ADP-ribose) polymerase inhibitors (PARPi).

As postulated by Nagel et al., no single pathway eciently repairs all types of DNA lesions and some lesions serve as substrates for more than one pathway [176]. Another evidence of the interplay of dierent DNA repair pathways was found for $O^6$-methylguanine adducts, which may be removed by

both direct reversal repair or converted by MMR in DSBs [124]. Furthermore, Melis et al. indicated that XPC is involved in the initiation of several DNA damage-induced cellular responses and functions in the removal of DNA oxidation damage, redox homeostasis, and cell cycle control [177]. In our study on chromosomal aberrations in healthy individuals, we documented several DNA repair gene–gene combinations evinced either in enhanced or decreased frequencies of chromosomal aberrations [178]. In the frame of OvC, the attention is paid mainly to PARP1. Interestingly, polymerase PARP1 that detects SSBs within BER, has been found to regulate the NER system by its association with XPA [160]. The XPA-PARP1 non-covalent interaction reduces the XPA binding anity to DNA, whereas XPA directly stimulates PARP1 enzymatic activity. On the other hand, PARP1 inhibition suppressed the recruitment of XPA to sites of laser-induced damage [179]. Likewise, PARPi decrease PARP1-XPA associations and reduce chromatin binding of XPA, suggesting the close relationship of both BER and NER pathways [160]. There is emerging evidence of extensive interactions among proteins involved in distinct DNA repair pathways and it needs to be reflected when evaluating the cancer etiology, prognostic and predictive factors based on DNA repair and DDR [180,181]. Although the concerted action of various DNA repair pathways in tumorigenesis is postulated, there is however scarce experimental evidence on this interplay.

The interaction of various DNA repair pathways found its application in cancer therapy [182]. Targeted therapy based on inhibiting DNA repair/DDR pathways enables tailoring the treatment of patients with tumors lacking functions in above pathways (e.g., inhibition of a complementary DDR pathway selectively kills cancer cells with a defect in a particular DNA repair pathway, i.e., concept of synthetic lethality; [183]). The concept of synthetic lethality has been utilized mostly in BRCA1/2 mutated OvC patients, treated with PARPi [184]. The complexity of DNA repair/DDR, as nicely illustrated by Brown et al. 2017 [185], oers the use of other inhibitors as well. For instance, inhibitors of CHK1 and CHK2 appeared as promising therapeutics for OvC, both as monotherapy or in combination with PARPi [186,187]. The other example is based on the interaction of ATM inhibition in combination with APE1 inhibitors (APE1i) or XRCC1 loss of function [188,189]. Furthermore, nicotinamide adenine dinucleotide may aect DNA methyltransferase 1 through the regulation of BRCA1 in OvC [190]. A contemporary study provides evidence that oxidative DNA damage can cause dynamic changes in DNA methylation in the BRCA1 gene due to the crosstalk between BER and de novo DNA methylation [191]. Interactions between DNA repair and DNA methylation may impact cellular regulatory mechanisms and epigenetic regulations in general and their understanding may contribute to the understanding of the carcinogenic process. The interaction of various DNA repair pathways and also DNA methylation present very promising applications in cancer therapy and OvC treatment in particular.

## 5. Therapeutic Perspectives–Targeting of DNA Repair System in Ovarian Cancer

As stated earlier, first-line treatment of OvC is based on surgery, followed by combination therapy of platinum derivatives and taxanes (usually carboplatin with paclitaxel) [17,18]. However, despite the initial remission of the disease, 70–85% of patients will experience relapse with a median survival of the recurrent OvC being 12–24 months [192]. Currently, new therapeutic approaches are directly aimed at molecular targets and pathways, e.g., anti-angiogenic agents such as bevacizumab or pazopanib, inhibitors of growth factor signaling, folate receptor inhibitors, inhibitors of AKT signaling, immunotherapeutic approaches and PARPi [19,20]. Targeting DNA repair has become a contemporary treatment option in OvC and it is aimed at DNA damage sensing, coordination of DNA repair, initiation of signaling pathways to promote cell cycle checkpoint activation, and triggering apoptosis [185].

PARPi have recently emerged as a promising class of new anti-cancer therapeutic agents. The employment of PARPi is a modern example of a synthetic lethality concept, based on alterations in DNA repair pathways. For instance, inhibition of PARP1 enzyme, a part of BER, results in persistent SSBs, the subsequent collapse of the replication fork, and the ultimate formation of DSBs. If this inhibition is applied in OvC tumors with defective HR, tumor cells utilize error-prone NHEJ, leading

to the accumulation of DNA damage and cell death [144]. Since 2014, three PARPi have been approved by FDA and EMA for use in OvC–olaparib, rucaparib, and niraparib [144]. Another PARPi—veliparib and talazoparib—are showing promising clinical results and facing FDA and EMA approvals in the treatment of OvC shortly [193–195].

Among the other DNA repair system targets, cell cycle checkpoints as an essential part of DDR machinery are the most promising targets. They provide cell cycle arrest during which cells activate appropriate DNA repair mechanisms and efficiently repair damaged DNA. Since defects in DNA repair pathways are a prominent feature of OvC tumors, targeting DDR is nowadays one of the most extensively studied therapeutic approaches. However, the current lack of impressive clinical responses to DDR inhibitors, in general, would presumably make DDR inhibitors a part of cancer combination therapy (with either pharmacological treatment and/or radiotherapy), with only limited use as single agents [185,196].

Into common DNA damage caused by irradiation comprises base damage, crosslinks, SSBs and mostly DSBs. Therefore, targeting DDR may lead to potentiation of radiotherapy. There are several studies showing the potential of combination therapy based on irradiation and various DDR inhibitors (DNA-dependent protein kinase (DNA-PK), ATM/ATR, LIG4, PARP1, CHK1) but mainly in other types of cancer [197]. Radiotherapy is one of the least used therapeutic methods in OvC treatment at present. Although majority of the OvC is radiosensitive, the topographical position of ovaries in peritoneal cavity with other organs, which are rather radiosensitive, limits the applications of radiotherapy. High rates of both acute and chronic toxicity, especially gastrointestinal, lead to abandoning the treatment. With the discoveries of more potent chemotherapy drugs, radiotherapy is left for inoperable chemoresistant cases or for metastases [198].

Over 96% of HGSOC tumors are harboring gain-of-function or loss-of-function mutations in TP53 (encoding p53 protein) leading to the disfunction of the G1/S phase checkpoint [199]. HGSOC tumors cells than heavily rely on G2/M checkpoint making it a possible target of anti-cancer therapy [186]. Inhibition of essential proteins involved in G2/M checkpoint may be exploited in anti-cancer therapy. Disabling of cell cycle arrest followed by mitosis may result in a mitotic catastrophe due to the lack of DNA repair and excessive DNA damage. Several DDR inhibitors have been studied in connection to OvC therapy, encompassing CHK1, ATR, ATM or Wee1-like protein kinase 1 (WEE1) inhibitors.

CHEK1 is a serine/threonine protein kinase which phosphorylates several downstream eectors including various proteins involved in cell cycle arrest, p53, DNA repair proteins, and proteins involved in cell death and transcription inhibition [200]. CHEK1 is an essential part of the G2/M checkpoint signaling pathway and it is overexpressed in almost all HGSOC [201], suggesting a need of cancer cells for G2/M checkpoint and arrest to essential DNA repair. Therefore, CHEK1 inhibitors (CHEK1i) are one of the most promising new therapeutic agents as suggested in Table 2. The CHEK1i V158411, PF-477736 and AZD7762 revealed eciency in ovarian carcinoma cell lines [202,203]. In vitro and in vivo (on patient-derived xenograft mice models) studies revealed an extensive activity of the other potent CHEK1 (and CHEK2) inhibitor prexasertib in HGSOC, both as a monotherapy and in combination with PARPi olaparib, with anti-tumor activity even in olaparib-resistant models [204]. At present, prexasertib is being clinically tested as a therapeutic for OvC [187,201].

ATR is a central checkpoint kinase activated by DNA SSBs which may also result from the processing of DSBs and stalled replication fork. After activation, ATR phosphorylates a series of substrates promoting a wide array of cellular responses including activation of cell cycle checkpoints (via CHEK1 and WEE1), cell cycle arrest, DNA repair, and eventually apoptosis [205]. Several potent small molecules have been discovered to be used as ATR inhibitors (ATRi). In vitro study on ATRi (VE-821, VE-822, AZ20) shows that inhibition of ATR may resensitize PARPi-resistant cell lines to PARPi [206]. Recent in vitro study on PARPi-resistant OvC cell lines from Burgess et al. [207] confirms these results with ATR inhibitor VE-821, making treatment with ATRi a new promising approach to overcome PARPi-resistance in HR-deficient OvC. ATR inhibitor AZD6738 in combination with PARPi has revealed higher eciency than PARPi alone [208,209].

**Table 2.** Current promising therapeutic approaches targeting DNA repair system in OvC.

| DNA Repair Pathway | Gene Targets | In Vitro/In Vivo Eciency | Pre-Clinical/Clinical Studies |
|---|---|---|---|
| Base Excision Repair | PARPi | Talazoparib and veliparib are in advanced clinical trials at the moment. Clinically available PARPi olaparib, rucaparib and niraparib are currently approved for the therapy of OvC on the basis of their BRCA1/2 status (summarized in [210]) | Olaparib-approved by FDA and EMA for use in OvC therapy [144]<br>Rucaparib-approved by FDA and EMA for use in OvC therapy [144]<br>Niraparib-approved by FDA and EMA for use in OvC therapy [144]<br>Veliparib–advanced clinical trials in combination with carboplatin and paclitaxel. Veliparib induction therapy followed by veliparib maintenance therapy led to significantly longer PFS than carboplatin plus paclitaxel induction therapy alone [193,194]<br>Talazoparib–ongoing advanced clinical trials [194,195] |
| Cell cycle checkpoints | CHEK1i | The CHEK1i V158411, PF-477736 and AZD7762 inhibited the proliferation of OvC cells [202]<br>AZD7762 in combination with cisplatin suggested synergistic eects in ovarian clear cell carcinoma cell lines in vitro and suppressed growth of tumors in vivo [203]<br>Prexasertib–eective in monotherapy in PARPi-resistant HGSOC cell lines and mouse xenografts [204]<br>Combination of prexasertib mesylate monohydrate (LY2606368), a CHEK1 and CHEK2 inhibitor, and a PARPi, olaparib synergistically decreased cell viability in HGSOC cell lines (OVCAR3, OV90, PEO1 and PEO4) cell lines and induced greater DNA damage and apoptosis than the control and/or monotherapies [204,211] | Prexasertib–eective in clinical phase II study in recurrent HGSOC [201] |
| | ATRi | ATRi (VE-821, VE-822, AZ20) resensitized PARPi-resistant BRCA1-mutated human OvC cell line to PARPi [206]<br>AZD6738 ecient in in ATM-deficient cells and in vivo in PDX mouse models with complete ATM loss [208]<br>Combination PARPi with ATRi (AZD6738) and CHEK1i (MK8776) is more eective than PARPi alone in reducing tumor burden in BRCA1/2 mutated HGSOC cells and PDX models [209] | Ongoing clinical PhaseII CAPRI Study of ATRi AZD6738 (ceralasertib) in combination with PARPi olaparib in HGSOC patients [212] |
| | ATMi | ATMi KU55933 enhanced the response to ionizing radiation in A2780 and OVCAR3 OvC cells [213] | |
| | WEE1i | Adavosertib (AZD 1775 alias MK1775)–ecient in vitro in SKOV-3 and ID8 OvC cell lines, ecient in vivo in ID8 ovarian tumors in monotherapy independent on TP53 or BRCA1 status [214] | AZD1775–active in phase I clinical study of monotherapy in OvC patients carrying BRCA mutations [215]<br>AZD1775–combination therapy with AZD1775 enhanced carboplatin ecacy in TP53-mutated ovarian tumors in phase II clinical study [216] |

Several clinical trials on the use of ATRi alone or in combination therapy (with PARPi or conventional chemotherapeutics) of OvC are in early initiation phases (for more see e.g., [212], Table 2) with results expecting in next few years.

By the presence of DSBs, ATM is activated as an essential part of DDR machinery. ATM phosphorylates hundreds of substrates to activate G1/S checkpoint, to induce intra-S and G2/M cell cycle arrest, DNA repair, chromatin remodeling, transcription, and apoptosis [205]. Mutations in ATM are known to cause Ataxia telangiectasia syndrome, a multisystem disorder characterized by progressive neurological impairment, immunodeficiency, hypersensitivity to X-rays, and predisposition to several cancers. Somatic mutations in ATM are present in several cancers including hematologic malignancies (e.g., are present in about 45% of mantle cell lymphoma cases), hepatocellular cancer, CRC, skin cancer, BC and others, however, only rarely mutated in OvC [217].

ATM inhibition has been shown to be synthetic lethal in vitro in combination with APE1i or functional loss of XRCC1 [188,189]. ATM inhibitors (ATMi) are known potent radio-sensitizers, studied currently on in vitro and in vivo models mainly for its potential use in brain-tumors cancer therapy [218–220]. However, in vitro results show that ATMi sensitize dierent gynecological cancer cell lines (e.g., A2780 and OVCAR3 ovarian cancer cells, Table 2) to ionizing radiation as well [213]. Additionally, a recent study from Riches et al. shows that AZD0156 (ATMi) enhances the eects of olaparib in lung, gastric and breast cancer cell lines and on triple negative breast cancer xenograft models [221], making it a potential tool in PARPi combination therapy in gynecological carcinomas. AZD0156 is currently being evaluated in phase I studies [221]. However, there is a limited amount of studies performed on OvC and further research is needed.

WEE1 mediates the activation of CDK1 and CDK2 kinases. Its increased gene expression has been observed in several cancers including OvC. High WEE1 protein levels are associated with poor survival in OvC patients with post-chemotherapy eusions, suggesting WEE1 inhibition may be a novel therapeutic approach in OvC [222]. Several in vitro studies on the role of a specific WEE1 inhibitor (WEE1i) adavosertib (AZD1775, MK1775) in combination therapies of several cancer models have been conducted [223,224]. Preclinical models showed a possible benefit of using WEE1i also with PARPi [225]. A recent study from Zhang et al. documented anti-tumor eects of adavosertib as a single agent in OvC therapy both in vitro and in vivo [214]. Still, the potential benefits of using WEE1i in OvC therapy have not been well established. Those data of recent studies suggest a high potential of various players in DNA repair/DDR pathways in OvC therapy.

## 6. Conclusions and Future Perspectives

Long term outcomes for OvC remains unsatisfactory (with five-year survival rates ranging from 30% to 50%) irrespectively of the advent of new treatment strategies. Based on the recent research activities it is completely clear that DNA repair machinery is involved in the risk of OvC development, the profile of the disease, and also in the prediction of therapeutic outcome. The functional status of DNA repair along with DDR determines cancer onset and impacts prognosis and ecacy of chemotherapy (often acting via DNA damage generation). The high-throughput genetic profile of DNA repair system genes allows us to identify and select crucial genetic variants important for prognosis and therapeutic response of OvC. However, the information on the prediction of therapeutic ecacy remains still fragmental, since many elements in the complex puzzle are missing. We have recorded that scarce studies address DNA repair in relation to the disease prognosis.

Our current review disclosed the following gaps in our understanding of the role of DNA repair/DDR in the onset, development, and management of OvC:

(i) We are facing the lack of systematic knowledge of DNA repair at various levels (i.e., genetic, epigenetic, protein, and functional) and their dynamic in the course of the disease. No available complex functional studies are characterizing any of the DNA repair pathways, as they do exist for other malignancies [226–228].

(ii) Although genetic alterations in HR repair pathway and their role in OvC are characterized decently, very little is known about the main pathway restoring DSBs, NHEJ. What is its importance in OvC onset, prognosis, and prediction? In the context of the previous point, further studies are needed on mechanisms (involvement of DSB repair?) underlying chromosomal instability in OvC (such as amplifications, deletions, translocations).

(iii) There is limited knowledge on the interaction of MMR (substantial in OvC etiology) with other DNA repair pathways. In this context, generally, more eort should be dedicated to the links between MMR (and other DNA repair pathways?) with immune response and with the microenvironment. These aspects may impact the patient's prognosis, as they do in colon cancer.

(iv) In general, there is a poor understanding of interactions among individual DDR players.

(v) Contemporary studies illuminated interesting links between DNA damage, DNA repair, and DNA methylation/demethylation. This important aspect may exert future implications and consequences (epigenetic regulations).

(vi) Epigenetic regulation of DNA repair/DDR via non-coding RNAs should further be addressed in relation to the disease onset, prognosis, and therapy outcome.

(vii) There is a need to characterize OvC patients with a good and poor response with respect to the DNA repair system and its changes. Disclosure of critical determinants in DNA repair/DDR machinery could significantly contribute to the improvement of therapy success in OvC patients with multidrug-resistant tumors.

The imminent perspectives depend on addressing the above-listed points. The scientists/clinicians may reflect the axioms that alterations in DNA repair pathways (HR, MMR for instance) play a role in OvC, and targeting of DNA repair in a concept of synthetic lethality represents a beneficial therapeutic option. The most important genes of the DNA repair system in OvC (as illustrated in Figure 9 and described in Table 3) and their targeting in the frame of OvC will deserve further attention. The function of newly identified targets of DNA repair system in OvC therapy needs to be further defined. After that identification, targeted DNA repair gene manipulation may enable us to improve present clinically used regimens.

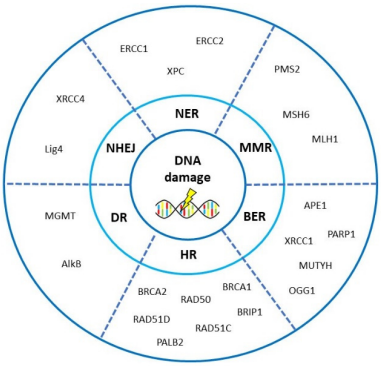



**Figure 9.** The most important genes involved in DNA repair pathways in ovarian cancer. Scheme of DNA damage and the most important genes playing role in ovarian carcinogenesis, prognosis and therapy response NER (nucleotide excision repair), BER (base excision repair), NHEJ (non-homologous end-joining repair), MMR (mismatch repair), HR (homologous recombination), DR (direct repair)). Protein names: PMS1 homolog 2 (PMS2), MutS homolog 6 (MSH6), MutL homolog 1 (MLH1), apurinic/apyrimidinic endonuclease 1 (APE1), poly(ADP-ribose) polymerase 1 (PARP1), X-ray repair cross-complementing 1 (XRCC1), MutY DNA glycosylase (MUTYH), 8-oxoguanine DNA glycosylase 1 (OGG1), breast cancer 1 and 2 (BRCA1 and 2), BRCA1-interacting protein C-terminal helicase (BRIP1), RAD50 homolog 1 (RAD50), RAD51 paralog C and paralog D (RAD51C and D), partner and localizer of BRCA2 (PALB2), alpha-ketoglutarate-dependent dioxygenase AlkB (AlkB), $O^6$-methylguanine DNA methyltransferase (MGMT), DNA ligase 4 (LIG4), X-ray repair cross-complementing 4 (XRCC4), excision repair cross-complementation group 1 (ERCC1), xeroderma pigmentosum complementation protein C and D (XPC and D/ERCC2).

**Table 3.** Overview of the most important DNA repair genes, their predisposition and prognostic impact and potential therapeutic use in targeted therapy for OvC. Symbols: " means higher, or better; # means lower, or worse. Protein names: breast cancer 1 and 2 (BRCA1 and 2), RAD51 paralog C and paralog D (RAD51C and D), RAD50 homolog 1 (RAD50), partner and localizer of BRCA2 (PALB2), BRCA1-interacting protein C-terminal helicase (BRIP1), PMS1 homolog 2 (PMS2), X-ray repair cross-complementing 4 (XRCC4), DNA ligase 4 (LIG4), MutS homolog 6 (MSH6), MutL homolog 1 (MLH1), PMS1 homolog 2 (PMS2), 8-oxoguanine DNA glycosylase 1 (OGG1), MutY DNA glycosylase (MUTYH), apurinic/apyrimidinic endonuclease 1 (APE1), X-ray repair cross-complementing 1 (XRCC1), poly(ADP-ribose) polymerase 1 (PARP1), xeroderma pigmentosum complementation protein C and D (XPC and D/ERCC2), excision repair cross-complementation group 1 (ERCC1), $O^6$-methylguanine DNA methyltransferase (MGMT), alpha-ketoglutarate-dependent dioxygenase AlkB (ALKB).

| DNA Repair Pathway | Gene | Predisposition Impact | Prognostic Impact | Therapeutic Potential (or Use) |
|---|---|---|---|---|
| Homologous recombination repair | BRCA1 | Mutations associated with " risk [45] and earlier onset [46] | " OS vs. non-carriers [50] | Better response to platinum-based chemotherapeutics [50,53], response to PARPi [55,229] |
| | BRCA2 | Mutations associated with " risk [45] and earlier onset [46] | " OS vs. non-carriers [50] | Better response to platinum-based chemotherapy [50,53], response to PARPi [55,229] |
| | RAD51C | Mutations associated with " risk [59,60] and earlier onset [60] | N/A | Response to PARPi (in vivo and in vitro evidence) [64,65] |
| | RAD51D | Mutations associated with " risk [9,61–63] and earlier onset [60] | N/A | Response to PARPi (in vivo and in vitro evidence) [65] |
| | RAD50 | Mutated in about 0.12% of tumors [66] | Copy number deletion associated with " OS and PFS [69] | In vitro knock-down associated with better response to PARPi [69] |
| | PALB2 | Mutations associated with " risk [73] | N/A | Response to PARPi (in vivo and in vitro evidence) [74,75] |
| | BRIP1 | Mutations associated with " risk [62,82–84] | N/A | Likely to predispose the response to PARPi and platinum [55]–needs further evaluation |
| Non-homologous end joining | XRCC4 | N/A | " expression associated with # OS [106] | N/A |
| | LIG4 | Possible involvement of SNPs needs further evaluation | N/A | N/A |
| Mismatch repair | MSH6 | N/A | N/A | Deficiency predisposes to platinum sensitivity in clear cell carcinoma [230] |
| | MLH1 | Mutations associated with " risk of Lynch syndrome-associated OvC [231] | # expression associated with " OS and PFS [232] | N/A |
| | PMS2 | Germline mutation associated with " risk of Lynch syndrome-associated OvC [233] | N/A | N/A |

**Table 3.** Cont.

| DNA Repair Pathway | Gene | Predisposition Impact | Prognostic Impact | Therapeutic Potential (or Use) |
|---|---|---|---|---|
| Base excision repair | OGG1 | SNPs associated with " risk [90,91,132] | N/A | N/A |
| | MUTYH | Biallelic mutation associated with " risk [135] | N/A | N/A |
| | APE1 | SNP associated with " risk [93] | " expression [139] and cytoplasmatic localization [140,141] have # prognosis and OS | N/A |
| | XRCC1 | SNP associated with " risk [94] | SNPs [95,96,234,235] and " expression [142] associated with # prognosis | N/A |
| | PARP1 | N/A | N/A | PARPi approved application for patients with germline BRCA1/2 mutations, with germline or somatic mutation BRCA1/2 with relapsed illness or with relapsed illness sensitive to platin-derivate chemotherapy regardless to BRCA status (FDA and EMA guidlines) |
| Nucleotide excision repair | XPC | N/A | SNPs associated with " PFS [236] | N/A |
| | XPD/ ERCC2 | SNP associated with " risk [237] | SNPs associated with prognosis [238] | SNP associated with severe neutropenia in patients treated by cisplatin-based chemotherapy [239] |
| | ERCC1 | N/A | SNPs associated with " OS [240] | SNP associated with " risk of nephrotoxicity in patients treated by cisplatin-based chemotherapy [239] |
| Direct repair | MGMT | N/A | N/A | Likely to drive chemoresistance [170] |
| | ALKB | N/A | N/A | ALKBH5 downregulation contributes to PARPi resistance in BRCA-deficient EOC [241] |

**Author Contributions:** Writing–original draft preparation, K.T., A.C., J.H., K.K.; writing-review and editing, R.V., L.V., P.V.; visualization, K.S.; supervision, R.V., LV, P.V. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the project of the Czech Science Foundation no.19-10543S, by the project of the Czech Health Research Council no. NV-18/03/00199 and by the projects of the Czech Ministry of Education, Youth and Sports: INTER-COST no. LTC19020 and the National Sustainability Program I (NPU I) Nr. LO1503. This project was also supported by the Research programs of Charles University, PROGRES Q 28 and UNCE/MED/006.

**Acknowledgments:** R.V. acknowledges the support of the European COST Action CA17104 through project INTER-COST LTC19020. P.V. acknowledges the support of the European COST Action CA17118 and COST Action CA15132.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

| | |
|---|---|
| APE1i | APE1 inhibitors |
| ATMi | ATM inhibitors |
| ATRi | ATR inhibitors |
| BER | base excision repair |
| CHEK1i | CHEK1 inhibitors |
| DDR | DNA damage response |
| DSB | double-strand break |
| EMA | European Medicine Agency |
| EOC | epithelial ovarian carcinoma |
| FDA | Food and Drug Administration U.S. agency |
| GWAS | genome wide association study |
| HGSOC | high-grade serous ovarian carcinoma |
| HR | homologous recombination repair |
| MMR | mismatch repair |
| MSI | microsatellite instability |
| NER | nucleotide excision repair |
| NHEJ | non-homologous end joining |
| OvC | ovarian cancer |
| OS | overall survival |
| PARPi | poly(ADP-ribose) polymerase inhibitors |
| PFS | progression-free survival |
| SNP | single-nucleotide polymorphism |
| SSB | single-strand break |
| WEE1i | WEE1 inhibitors |
| wt | wild-type |

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