Charles University

First Faculty of Medicine

Summary of the Dissertation Thesis

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

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2023

Doctoral programs in Biomedicine

Charles University in Prague and Academy of Sciences of Czech Republic

Study Program: Molecular and Cell Biology, Genetics and Virology

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The printed version of the thesis will be available at the Department for Science and Research and International Relations of the Dean's office of the First Faculty of Medicine at least five days before the date of defense.

ABSTRACT

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

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

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

ABSTRAKT

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

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

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

INTRODUCTION

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

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

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

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

Distinguishing between the intrinsic and the acquired resistance is less clinically significant than understanding the cellular mechanisms of resistance. There are several mechanisms that underlay the patient's resistance towards chemotherapeutic treatment (Fig. 1).

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

Alteration of the drug target during targeted therapy is caused by secondary mutations in the target protein genes or their altered expression, resulting in drug resistance (Dong et al. 2019). For instance, EGFR inhibitors used in the treatment of non-small cell lung cancer (NSCLC) show initially good response rates, however, almost 50% of responsive patients

develop a T790M mutation in *EGFR* within one year, resulting in resistance towards 1st and 2nd generation of EGFR inhibitors (Wang, Schmid-Bindert, and Zhou 2012; Gridelli et al. 2011; Tang et al. 2013; Bell et al. 2005; Ma, Wei, and Song 2011). Another example is the development of resistance towards estrogen receptor (ER) inhibitors (Tamoxifen), used in the treatment of ER-positive breast cancers. Mutations in ER gene (*ESR1*) are significantly enriched in endocrine therapy resistant metastatic BC while being rare or non-existent in treatment naïve, primary tumors. The development of novel targeted inhibitors is therefore inevitable (Alluri, Speers, and Chinnaiyan 2014).

Senescence escape is another mechanism of chemoresistance. Chemotherapy-induced senescence (CIS) was initially seen as favorable outcome of chemotherapy, as it leads to arrest of the cell proliferation (Guillon et al. 2019). However, studies have shown that CIS may provide an oncogenic niche, enabling certain populations of tumor cells to perform senescence escape, gain stem-cell properties, restart their proliferation, and become more aggressive and less responsive towards chemotherapeutic treatment (Milanovic et al. 2018). This possesses a great challenge in cancer therapy, as conventional chemotherapeutics are not efficient towards non-proliferating senescent cells. Lately, there is an increasing interest in agents capable to induce apoptosis in senescent cells, known as senolytic drugs that may be combined with conventional or targeted therapies (Kirkland and Tchkonia 2020; Carpenter, Saleh, and Gewirtz 2021).

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

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

Tumor microenvironment (TME) may contribute to intrinsic chemoresistance. More acidic pH in tumor compared to normal tissues (pH 6.5-7.1 vs pH 7.3-7.5) impairs the distribution of weak base chemotherapeutics, such as anthracyclines, anthraquinones, and

vinca alkaloids, leading to physiological drug resistance (referred as "ion trapping") (Taylor et al. 2015; Webb et al. 2011; Wojtkowiak et al. 2011). On the other hand, acidic pH may be a potential target for anticancer therapy as proton pump inhibitors have been developed to shrink and sensitize tumors to chemotherapeutic drugs. Besides the acidic pH, fluctuating hypoxia is another characteristic of TME, causing oxidative stress. This may induce DNA damage, genetic instability and arise of new mutations, contributing to the genetic divergence of tumor cells (Bindra and Glazer 2005).

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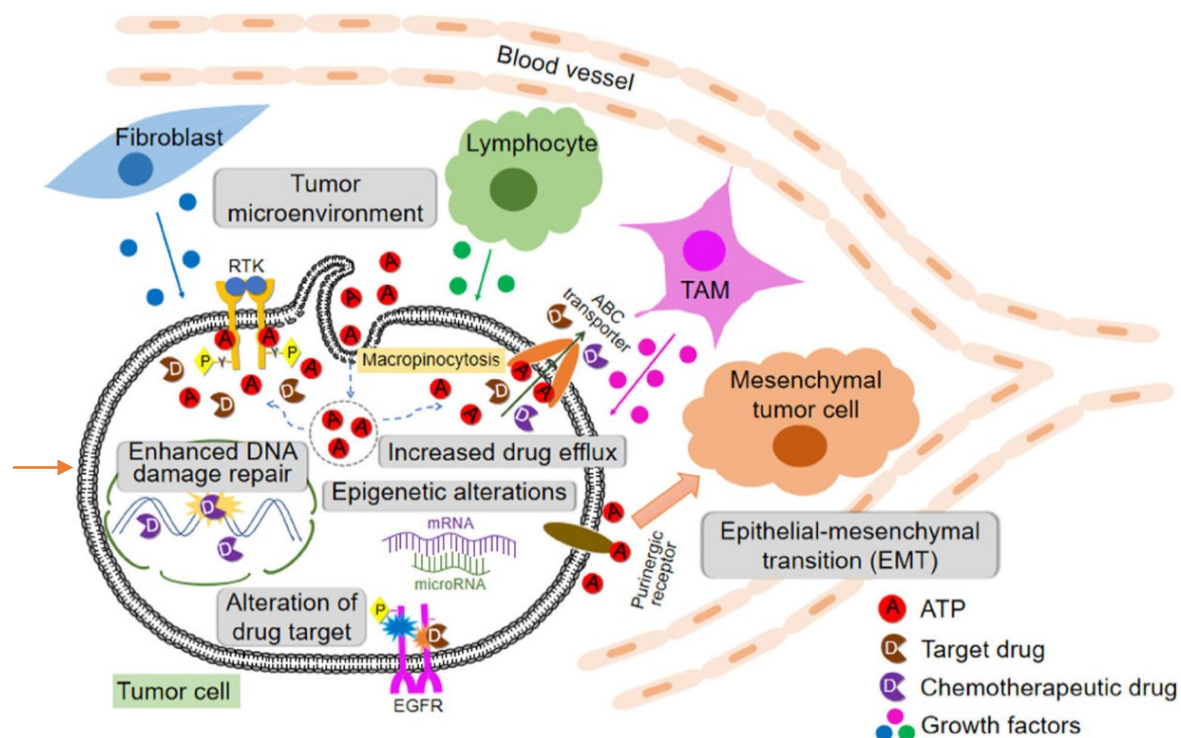

Fig. 1.: Cellular mechanisms of chemoresistance. From (Wang, Zhang, and Chen 2019). Dysregulations of DNA repair are one of the mechanisms of chemoresistance.

Dysregulated DNA repair and damage response (DDR) is another key mechanism involved in resistance and sensitivity towards chemotherapeutic drugs and it is the main topic of this PhD Thesis. Most anticancer therapies (including chemotherapeutics and ionizing radiation) induce cell death by causing direct or indirect DNA damage. Therefore,

dysregulation of DNA repair machinery may contribute to resistance or increased sensitivity to chemotherapeutic agents. It is widely acknowledged that enhanced DNA repair is associated with resistance to chemotherapeutics and, on the other hand, defects in DNA repair usually predispose cancer cells to higher sensitivity to DNA-damaging agents (Brandsma and Gent 2012).

Consequently, targeting DDR is one of the key therapeutical strategies to overcome resistance and increase sensitivity to cancer treatment. DNA repair and damage response may be modulated with a variety of molecular mechanisms including 1.) targeting DNA repair via epigenetic regulation; 2.) inhibition of core DNA repair proteins with inhibitors; 3.) developing new combination therapies with natural compounds that will enhance the effect of conventional chemotherapeutics.

Epigenetic regulation of DNA repair

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

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

MiRNAs are short (average length 18-23 nucleotides) non-coding RNAs that modulate the expression of protein-coding genes at post-transcriptional level via degradation or inhibition of translation of specific target mRNA. More than 2600 miRNAs have been predicted to be encoded by the human genome, with the ability to modulate the expression of about 60% of human genes (Chou et al. 2018). Deregulated miRNA expression has been observed in numerous cancers in association with cancer susceptibility, progression, metastatic formation and in therapy sensitivity/resistance (Melo and Esteller 2011). MiRNAs as regulatory elements can modulate the cancer cell sensitivity towards DNA-damaging agents by regulating the expression in DNA repair and damage response genes (Jurkovicova et al. 2022). Therefore, miRNAs represent promising therapeutic tools for improving the therapy response in chemoresistant cancers. Among numerous miRNAs associated with therapy response, we studied the role of miR-140 in oxaliplatin resistance **Manuscript 3** and also the role of miRSNPs (SNP polymorphisms in miRNA-binding sites in 3'UTRs of protein-coding genes) in therapy response in BC patients in **Manuscript 1**. Elucidating the role of

cancer-associated miRNAs in carcinogenesis is also vital for developing new promising strategies of anti-cancer treatment by either restoring their function by applying miRNA mimics or inhibiting them with inhibitors or antagomiRs (Fu et al. 2021). Additionally, their good accessibility and high stability in body fluids is making them suitable as non-invasive predictive and prognostic biomarkers in cancer therapy (He et al. 2020).

Novel DNA repair and DNA damage response inhibitors

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

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

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

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

New combination therapies with natural compounds

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

One of the most studied natural compounds in cancer therapy is curcumin, isolated from *Curcuma longum*. It has been proven to reverse the chemoresistance of various agents *in vitro, in vitro,* and even in clinical trials, e.g., cis-platin resistance in OvC cells (Muhanmode et al. 2021), 5-FU resistance in CRC cells (Li et al. 2021), doxorubicin, and etoposide resistance in gastric cancer cells (Yu et al. 2011), paclitaxel resistance in hepatocellular carcinoma (Tian et al. 2019) or oxaliplatin resistance in CRC both *in vitro* and *in vivo*

(Ozawa-Umeta et al. 2020). Another extensively studied natural compound is resveratrol, which naturally occurs in about 70 plant species. It has been observed to suppress platin resistance in several cancers or resistance to docetaxel in BC (Muhanmode et al. 2021; Ferraresi et al. 2021; Vinod et al. 2015). In our study (**Manuscript 2**), we investigated the effect of *Ganoderma lucidum* extract (GLC) on 5-FU efficacy. GLC is a popular over-the-counter supplement with numerous anti-proliferative effects on cancer cells (Sohretoglu and Huang 2018; Rejhova et al. 2018). Our study confirmed its enhancing effect on 5-FU in CRC *in vitro* and *in vivo*.

HYPOTHESES AND AIMS

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

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

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

MATERIAL AND METHODS

POPULATION STUDY - MANUSCRIPT 1

Study population

Study included 673 incident BC patients and 675 controls (343 healthy blood donor volunteers and 332 individuals who underwent colonoscopy examination with negative results and did not have any malignancy at the time of the sampling).

Clinical information

The following data on BC patients were retrieved from medical records: date of cancer diagnosis, age, menopausal status, family history of cancer (number of relatives affected by BC, ovarian cancer, or other malignant diseases), tumor size, International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification, histological type and grade of the tumor, expression of ER, PR and HER2; expression of the Ki-67 protein; chemotherapy and hormonal regimen.

SNP selection and genotyping

SMUG1 rs2233921 G>T, *SMUG1* rs971 G>A and *NEIL2* rs6997097 T>C polymorphisms were analyzed by using the KASP^{TM} chemistry (LGC Genomics, UK), a competitive allele-specific PCR-based SNP genotyping system, described in (Pardini et al. 2008).

Bioinformatic and statistical analyses

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

IN VITRO STUDIES - MANUSCRIPTS 2,3,4, UNPUBLISHED STUDY

Cell Cultures

Cell lines were cultured in appropriate media, supplemented with 10% fetal bovine serum (Merck, Germany), 1 mM L-glutamine (Biosera, France), 1 mM sodium pyruvate (Biosera, France) and 1mM penicillin/streptomycin (Biosera, France). Cells were cultured in a humidified incubator at 37°C, 5% CO_2.

Manuscript 2

The study was performed using human colorectal cancer cell lines HCT116, HT29, HCT116$^{P53-/-}$ (obtained from ATCC, USA) and non-cancer human colon mucosal epithelial cell line NCM460 cells (originally obtained from INCELL Corporation, USA by Prof. Sliva).

Manuscript 3

The study was performed using human CRC cell lines HCT116, HT29 and DLD1 (Merck, Germany).

Manuscript 4

The study was performed using the human ovarian carcinoma cell line OVCAR3 (Merck, Germany).

Unpublished Study

The study was performed using human CRC cell line DLD1 (Merck, Germany).

Cell treatments

Manuscript 2

Cells were treated with *Ganoderma lucidum* extract (GLC, Pharmanex, USA), 5-FU (Sigma Aldrich, USA) and their combination.

Manuscript 3

Cells were treated with oxaliplatin (Merck, Germany).

Manuscript 4

Cells were pretreated for 1 hour with a MRE11 inhibitor Mirin (Sigma-Aldrich, Germany). After the pretreatment, cells were treated with carboplatin (Sigma-Aldrich, Germany).

Unpublished Study

Cells were treated with an increasing concentration of 5-FU (Sigma-Aldrich, USA) following protocol from Coley (Coley 2004).

Viability, proliferation and growth assays

Manuscript 2,3,4, Unpublished Study

Colony formation assay (CFA) was used to determine the clonogenicity potential and viability of cells in association with studied agents.

Manuscript 2,3,4, Unpublished Study

WST-1 cell proliferation assay (Roche, Switzerland) was used to measure cell proliferation after exposure to studied agents.

Manuscript 4, Unpublished Study

Cell growth was analyzed by seeding cells on 12 well plates and after corresponding treatment, viable cells were counted after 24, 48 and 72hrs using Trypan blue.

Migration assay

Manuscript 2

Cell migration was measured using Corning Transwell Permeable Supports 8.0 μm (Sigma Aldrich, USA) according to the manufacturer's manual.

Cell cycle analysis

Manuscript 2,3,4, Unpublished Study

Samples were analyzed using a flow cytometer Apogee A-50 micro (Apogee, UK). Obtained data were analyzed with FlowLogic^{TM} software (Inivai Technologies, Australia).

Tumor samples

Manuscript 3

Tumor and non-malignant adjacent mucosa paired samples were obtained from 50 CRC patients (26 males and 24 females) who underwent surgical tumor resection.

Reverse transcription and quantitative PCR (qPCR)

Manuscript 3,4, Unpublished Study

Total RNA from cells or tumor samples was isolated using Qiagen miRNeasy Mini Kit (Qiagen, Germany). Reverse transcription to cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Expression of studied

protein-coding genes was measured using qPCR SybrMaster (Jena Bioscience, Germany). Expression of miRNA coding gene was measured using TaqMan MicroRNA Assay. All proceedings were done according to manufacturer's protocols. QPCR analyses were run on 7500 Real Time PCR System (Thermo Fisher Scientific, USA). Data were subsequently analyzed by the $2^{-\Delta\Delta Ct}$ method.

Protein isolation, SDS-PAGE and Western blot analysis

Manuscript 2,3,4

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

Western blot analysis was performed by separating proteins in 10% SDS-PAGE gels at 15 mA for 60min and transferring proteins to 0.45 μm Amersham ™ Protran ® Western blotting membranes (Cytiva Life sciences, UK) in methanol transfer buffer using Mini Trans-Blot Cell (Bio-Rad Laboratories, USA). After blocking, membranes were incubated with the corresponding primary antibodies from Cell Signaling Technology (USA) or Abcam (UK) overnight at 4°C and incubated with a secondary antibody conjugated with horseradish peroxidase (Abcam, UK). Membranes were then incubated with Immobilon western Chemiluminescent HRP Substrate (EMD Millipore Corporation, USA) and visualized by Azure c600 (Azure Biosystems, USA).

Transient transfection

Manuscript 3

To study the effect of miRNA overexpression, MISSION miRNA mimics technique was performed according to the manufacturer's manual.

MRE11 silencing

Manuscript 3

ShRNAs technique was employed to develop HCT116 cells with silenced MRE11. Briefly, HEK293FT cells (Thermo Fisher Scientific, USA) were co-transfected with pLKO1 mission MRE11 shRNA plasmids and helper plasmids psPax2 and pMD2.g (Addgene, USA) using Lipofectamine 3000 (Thermo Fisher Scientific, USA). Culture media containing recombinant lentiviruses were added to HCT116 cells. Colonies containing integrated lentiviruses were selected by cultivating cells with 2 μg/ml of Puromycin (Sigma Aldrich, USA) for 4–5 days. Successful MRE11 silencing was confirmed using PCR and Western blot.

Reactive oxygen species (ROS) measurement

Manuscript 2

Levels of ROS were measured using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Thermo Fisher Scientific, USA) and measured using fluorescent reader Biotek (Vermont, VT, USA) at excitation/emission wavelengths Ex/Em: 485nm/538nm.

Comet assay

Manuscript 2

DNA damage associated with studied agents was measured using Comet assay, a single cell electrophoresis assay according to standard protocol. Comets were visualized using a fluorescence microscope Olympus BX63 (Olympus, Japan) and scored using semi-automated Lucia Comet Assay™ software (Laboratory Imaging, Czech Republic).

In vivo experiment

Manuscript 2

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

Bioinformatical analyses

Manuscript 4, Unpublished Study

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

Statistical analyses

Manuscript 2,3,4, Unpublished Study

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

RESULTS AND DISCUSSION

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

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

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

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

therapy), we observed that the TC genotype of *NEIL2* rs6997097 in patients receiving only hormonal-based therapy was associated with shorter OS both in the co-dominant and the dominant model (HR=4.15, 95% CI=1.7-10.2, p=0.002; HR= 3.52, 95% CI=1.4-8.6, p=0.006, respectively). The same group of patients showed also a shorter DFS in the co-dominant model, moderately exceeding the Bonferroni-adjusted threshold of significance (HR=2.56, 95% CI=1.5-5.7, p=0.02).

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

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

Our results showed a strong association between the *NEIL2* rs6997097 TC genotype and shorter survival in patients receiving hormonal therapy (inhibitors of aromatases and tamoxifen). However, the low frequency of the C allele (only 3 controls and 2 patients with the CC genotype in our studied population) precluded evaluation of the genotype dosage and computing HR for CC bearers. Unfortunately, no significant associations were found between analyzed miRSNPs and the survival of patients receiving 5-FU-based chemotherapy or other types of chemotherapy.

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

Both SMUG1 and NEIL2 have been studied for their role in cancer (Vodicka et al. 2020). *In vivo* experiments showed that SMUG1 effectively collaborate with UNG to eliminate incorporated uracil in the genome and is important for preventing the accumulation of spontaneous mutations in DNA (Alsoe et al. 2017). Moreover, low *SMUG1* expression is linked to aggressive clinicopathological phenotypic features of BC (like the absence of hormonal receptors, EGFR overexpression, the presence of basal-like phenotype and triple-negative phenotype) and poor prognosis. Low *SMUG1* expression was associated also with aberrant expression of several other DNA repair, cell-cycle control and apoptosis genes and overall genomic instability in SMUG1-low tumours. This endorses the important role of SMUG1 in breast carcinogenesis (Abdel-Fatah et al. 2013). Regarding NEIL2, the minor allele of *NEIL2* rs1466785 associates with increased BC risk in *BRCA2* mutation carriers (Osorio et al. 2014). Loss of *NEIL2* expression, simultaneously with alterations of nucleotide excision repair genes *CETN2* and *ERCC1,* was associated with resistance to endocrine treatment for ER+ breast tumours (Anurag et al. 2018).

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

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

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

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

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

GLC treatment alone showed a number of anticancer effects. To study its inhibitory effect, we measured the cell proliferation after GLC treatment for 24, 48 and 72 hours. The most significant decrease in proliferation was observed after 48 hours of treatment with 0.5 mg/ml concentration of GLC. The proliferation of HCT116 decreased by 27% ($p<0.05$) and HT29 by 39% ($p<0.05$). The proliferation of non-malignant NCM460 cells was not affected.

Results from CFA confirmed the inhibitory effect of GLC, as the number of colonies after 0.5 mg/ml GLC treatment significantly decreased by 46% and 45% in HCT116 and HT29 cells, respectively (p<0.05). CFA did not show any effect of GLC on non-malignant cells. GLC also showed inhibitory effect on invasive cancer behavior. Migratory properties of HCT116 decreased by 57% (p<0.05) and of HT29 by 14% (not significant). Cell cycle analysis indicates that GLC induced G1/S cycle arrest both in HCT116 (p<0.001) and HT29 cells (p<0.05). GLC treatment increased the amount of oxidative DNA damage in both cancer cell lines (p<0.05), however, we did not find any decrease in DNA strand breaks and oxidative DNA damage in non-malignant NCM460 cells. GLC also caused a significant decrease in ROS accumulation in non-malignant cells by about 20% after 6h (0.25 mg/ml, p<0.05; 0.5 mg/ml, p<0.01) and by about 17% after 24h (0. 25 mg/ml, p<0.05; 0.5 mg/ml, p<0.001).

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

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

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

However, the particular cellular mechanism underlying the anticancer effects of GLC must be elucidated. Results from Jiang *et al.* suggest that GLC may restore the p53 function in

p53-mutated cancer cells (Jiang et al. 2017). Other results indicate that spore oil from *Ganoderma Lucidum* induces apoptosis by activating caspase-3 and caspase-9. Li *et al.* reported that ethanol extract of *Ganoderma* triterpenes upregulates E-cadherin and suppresses HCT116 migration (Li et al. 2017).

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

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

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

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

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

Firstly, we identified miR-140 as the best candidate for our study using TargetScan (McGeary et al. 2019) and TCGA database analysis ('The Cancer Genome Atlas Program (TCGA)'). MiR-140 showed the strongest statistically significant association ($p<0.01$) with PFS out of 187 predicted miRNAs targeting MRE11, where miR-140 overexpression was associated with better survival. Our results on 50 CRC patient's samples (tumor vs. adjacent non-malignant mucosa) were in concordance with TCGA analysis. Higher expression of miR-140 in tumors was associated with better PFS ($p=0.017$). Comparing tumors vs. non-malignant mucosa, levels of miR-140 were significantly lower in tumor tissues ($p<0.01$). Lower levels of miR-140 were also associated with the metastatic phenotype ($p<0.05$).

To confirm the MRE11 as a target of miR-140, we used miRNA mimics to increase the levels of miR-140 v DLD1 cells. After transfection, mRNA and protein levels of MRE11 were decreased. To evaluate the effect of miR-140 overexpression on DSBs, we measured the expression of gH2AX protein. Western blot analysis showed increased levels of gH2AX, a marker of DSBs damage, in DLD1 cells. Overexpression of miR-140 also resulted in decreased proliferation, measured with WST-1 assay.

For detailed figures on miR-140 overexpression effect on CRC cells see Manuscript 3 (Horak et al. 2022).

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

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

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

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

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

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

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

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

To assess the effect of Mirin on carboplatin (Cbpt) sensitivity in OVCAR3, cells with and without 1 h pre-treatment with 100 μM Mirin were treated with a 6 μM concentration of carboplatin. OVCAR3 responded only moderately to carboplatin alone. However, Mirin pre-treatment caused significant a decrease in cell viability and clonogenic potential. We also observed increased expression of γH2AX and S phase arrest after Mirin+Cbpt treatment, compared to Cbpt alone.

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

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

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

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

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

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

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

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

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

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

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

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

All 9 DDR and DNA repair genes, which expression was significantly changed and were associated with survival of CRC patients treated with 5-FU, were already studied in relationship with different cancers. Germline mutations in *RAD51L1* are rare but have been linked with breast and ovarian cancer risk (Buys et al. 2017; Song et al. 2015). RAD51L1 (also called RAD51B) is a paralog of RAD51 and a part of a multi-protein complex (BCDX2). Its inhibition leads to HR deficiency and higher sensitivity to DNA-damaging agents (Lee et al. 2014). RAD52 is another important protein in the repair of DSBs, responsible for promoting complementary ssDNA annealing (Nogueira et al. 2019). RAD52 has been proposed to be a new interesting target for synthetic-lethality-based therapies when resistance to PARP inhibitors occurs (Malacaria et al. 2020). XRCC2 is another paralog of RAD51, and its downregulation has already been linked to higher sensitivity to 5-FU in CRC (Zhang et al. 2017). TDG is a DNA-glycosylase involved in BER. Controversially, Miao *et al.* recently associated TDG overexpression with the better OS of CRC patients and suppression of invasive behavior. However, we observed its overexpression in both 5-FU resistant cell lines and, according to TCGA database, its overexpression is associated with worse disease-specific survival (DSS). TDP1 is a phosphodiesterase with a role not only in BER but also in NHEJ, which is another pathway for repairing of DSBs (Heo et al. 2015). DAPK1 protein, a member of the DAPK family, is involved in apoptosis, necrosis, and autophagy. It is considered a tumor-suppressor, which could indicate that its overexpression is not associated with chemoresistance, but rather with the cellular response to high doses of cytotoxic 5-FU (Bialik and Kimchi 2006). ATM kinase is an essential part of HR, responsible for sensing of DSBs and downstream activation of DDR pathways. ATM inhibitors are already studied for their utilization as chemo-/radio-sensing agents in cancer therapy, e.g., in combination therapy with Irinotecan and 5-FU in CRC (Davis et al. 2022). The loss of 53BP1 encoded in the *TP53BP1* gene is associated with resistance to 5-FU in CRC (Li et al. 2013). Interestingly, we observed the overexpression of *TP53BP1* in the 5FUR40 resistant cell line and its downregulation in the 5FUR160 cell line. *TOPBP1* encodes Topoisomerase IIβ binding protein 1 which interacts with Topoisomerase IIβ and is involved in processes of DDR, checkpoint activation, replication, and transcription (Wu et al. 2017; Lv et al. 2016). Its downregulation sensitizes cancer cells to a variety of genotoxic agents such as doxorubicin, cisplatin, or Mitomycin C. This protein interacts with NBS1 and is directly involved in HR (Morishima et al. 2007).

In this study, we showed that dysregulated expression of genes involved in DDR and DNA repair is crucial for the development of acquired resistance to 5-FU. It is noteworthy, that most of the relevant genes, that were significantly overexpressed in our resistant cell lines and simultaneously their expression was significantly associated with survival of 5-FU-treated CRC patients (TCGA database), were mostly related to HR repair pathway, either in sensing of DSBs, promoting the downstream reaction or as the core repair proteins. That underlies the fundamental importance of HR, not only in response to chemotherapeutics designed to cause

DSBs (such as platinum compounds) but also to other cytotoxic agents like 5-FU. 5-FU, when incorporated into DNA, is recognized with the BER pathway. These lesions, when unrepaired, give arise to DSBs, which are then repaired by HR.

We previously (Manuscript 4) studied Mirin, a HR inhibitor, and described its ability to sensitize OVC cells to carboplatin. Therefore, we focused on the effect of Mirin on 5-FU sensitivity in parental CRC DLD1 cells. Cells were treated with 5 µL 5-FU with an eventual 1hr of 100 µM Mirin pre-treatment. We observed a similar decrease in cell proliferation, compared to 5-FU treatment alone. These results point out the key role of HR in response to 5-FU. The addition of HR inhibitors, such as Mirin, is a promising tool for enhancing sensitivity to conventional chemotherapeutics. To evaluate the effect of Mirin on 5-FU response in CRC, further research is necessary, such as studying its effect on different parental and 5-FU resistant CRC cell lines or *in vivo* experiments on mice. HR inhibitors (such as RAD51, RAD52, MRE11, ATM, ATR inhibitors) and their possible utilization in a great variety of cancers and therapy regimes, are currently of great interest to researchers.

CONCLUSIONS

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

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

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

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

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

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

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

ZÁVĚRY

Cílem této disertační práce bylo objasnit roli DNA opravy a její modulace v odpovědi a v rezistenci na léčbu nejběžnějšími chemoterapeutiky.

Následující odstavce shrnují výsledky této práce:

1. Zabývali jsme se úlohou tří miRSNPs v DNA reparačních genech *SMUG1* a *NEIL2* v odpovědi na léčbu pacientů s BC. Zjistili jsme asociaci alely NEIL2 rs6997097 C s horším OS a DFS ve skupině pacientů, kteří dostávali pouze hormonální léčbu. U pacientů dostávajících konvenční chemoterapeutika nebyla nalezena žádná asociace těchto miRSNP, které naše skupina dříve popsala u pacientů s CRC. Tyto výsledky však potvrzují význam jemné regulace exprese DNA reparačních genů prostřednictvím miRNAs v odpovědi na protinádorovou léčbu.

2. Zkoumali jsme možnost kombinace přírodních látek, jako je extrakt z *Ganoderma lucidum* (GLC), s konvenčními chemoterapeutiky, jako je 5-FU, za účelem zlepšení chemosenzitivity buněk a odpovědi na léčbu. Potvrdili jsme, že GLC má protinádorovou aktivitu, způsobuje oxidační poškození DNA a zvyšuje účinek 5-FU *in vitro* i *in vivo*. Zajímavé je, že GLC má spíše ochranný účinek na nenádorové buňky, což z něj činí slibný doplněk konvenční léčby pro snížení potřebné dávky cytotoxických chemoterapeutik a minimalizaci jejich vedlejších účinků.

3. Zkoumali jsme roli miRNA regulace MRE11, důležitého DNA reparačního proteinu zapojeného do homologních oprav, v citlivosti vůči oxaliplatině. Potvrdili jsme, že miR-140 cílí na MRE11, potlačuje jeho expresi a zvyšuje citlivost CRC buněk vůči oxaliplatině. Naše výsledky naznačují, že miR-140 působí jako nádorový supresor a hraje důležitou roli v HR a odpovědi CRC na léčbu.

4. Zkoumali jsme účinek inhibitoru MRE11, Mirinu, na chemorezistentní buňky OvC. Mirin významně zvýšil citlivost buněk ke karboplatině a dokázal obnovit citlivost ke karboplatině i u OvC buněk rezistentních ke karboplatině. Tyto výsledky potvrdily význam HR v odpovědi na platinové deriváty.

5. Zabývali jsme se úlohou dysregulované exprese DDR a DNA reparačních genů v procesu vzniku získané rezistence k 5-FU v *in vitro* modelu CRC. Tyto výsledky naznačují zásadní roli nadměrné exprese genů HR v chemorezistenci k 5-FU.

Naše zjištění poukazují na důležitou roli reparace DNA a odpovědi na poškození v odpovědi na léčbu, citlivosti a rezistenci nádorů. Věříme, že objasnění molekulárních mechanismů rezistence a citlivosti na chemoterapeutika by mohlo vést k účinnější a cílenější léčbě rakoviny.

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PUBLICATION ACTIVITY

MANUSCRIPTS RELATED TO THE THESIS

Original Research Articles: Manuscripts 1-4

Manuscript 1: Genetic variations in 3'UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response.
Cumova, A.; Vymetalkova, V.; Opattova, A.; Bouskova, V.; Pardini, B.; Kopeckova, K.; Kozevnikovova, R.; Lickova, K.; Ambrus, M.; Vodickova, L.; Naccarati A.; Soucek P.; Vodicka P. Genetic variations in 3'UTRs of *SMUG1* and *NEIL2* genes modulate breast cancer risk, survival and therapy response. *Mutagenesis* 2021, *36*, 269-279, doi:10.1093/mutage/geab017.
IF (2021) = 3.000

Manuscript 2: *Ganoderma Lucidum* induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo.*
Opattova, A.; Horak, J.; Vodenkova, S.; Kostovcikova, K.; **Cumova, A.**; Macinga, P.; Galanova, N.; Rejhova, A.; Vodickova, L.; Kozics, K., Turnovcova K.; Hucl T.; Sliva D.; Vodicka P. Ganoderma Lucidum induces oxidative DNA damage and enhances the effect of 5-Fluorouracil in colorectal cancer *in vitro* and *in vivo*. *Mutat Res Genet Toxicol Environ Mutagen* 2019, *845*, 403065, doi:10.1016/j.mrgentox.2019.06.001.
IF (2019) = 2.506

Manuscript 3: MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients.
Horak, J.; Dolnikova, A.; Cumaogullari, O.; **Cumova, A.**; Navvabi, N.; Vodickova, L.; Levy, M.; Schneiderova, M.; Liska, V.; Andera, L.; Vodicka P.; Opattova A. MiR-140 leads to MRE11 downregulation and ameliorates oxaliplatin treatment and therapy response in colorectal cancer patients. *Front Oncol* 2022, *12*, 959407, doi:10.3389/fonc.2022.959407.
IF (2022) = 5.738

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

Review Articles: Manuscripts 5 and 6

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

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

MANUSCRIPTS NOT DIRECTLY RELATED TO THE THESIS

Original Research Articles: Manuscript 7

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