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Summary of the PhD Thesis

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Genetic variability in sporadic colorectal cancer: Searching for novel risk, prognostic and predictive biomarkers

Genetická variabilita u sporadické formy kolorektálního karcinomu:
Hledání nových diagnostických, prognostických a prediktivních biomarkerů

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ABSTRACT

Colorectal cancer (CRC) is a major public health problem worldwide. Despite improvements in the diagnostic process and advancement in the treatment methods, the prognosis remains poor. To improve survival rates, it is important to identify people with the predisposition for CRC and to detect the potentially curable early stage of the disease. Furthermore, identifying those who would have an adverse clinical outcome associated with a particular chemotherapy would help to avoid redundant chemotherapy burden in patients and contribute to enhanced therapeutic efficacy, while minimizing treatment-related toxicity.

The aim of the Thesis was to search for novel promising diagnostic, prognostic and predictive DNA-based biomarkers of sporadic form of CRC. As each patient is genetically unique, these biomarkers would aid clinicians in better diagnosis and/or in the selection of an optimal type of therapy for an individual CRC patient based on their molecular profile. In order to explore this issue, we investigated several candidate genes in healthy individuals as well as in newly diagnosed cancer patients.

The major outcomes of this PhD study, which were fully reported in seven publications included in the present Thesis, are 1) The observation of several candidate single nucleotide polymorphisms in microRNA target regions (miRSNPs) of double strand break repair genes, genes important for CRC etiology and mucin genes to be related either to CRC risk or to clinical outcome, 2) Evidence that miRSNPs in target genes modulate the efficiency of corresponding protein expression, 3) The revelation of genetic variants in NOD-like receptor (NLR) genes contribution to CRC onset and progression of the disease, 4) The identification of the association of several potential functional genetic variants in DNA repair genes with CRC.

Taken together, these studies suggested several novel potential biomarkers for clinical use. However, further studies in independent populations are needed to confirm their clinical significance and to decipher the biologic mechanisms underlying the associations.

Keywords: Colorectal cancer, biomarker, SNP, miRSNP, prognosis, chemotherapy response, DNA repair, CRC pathogenesis, mucin genes, NLR genes

ABSTRAKT

Rakovina tlustého střeva a konečníku (kolorektální karcinom, KRK) představuje celosvětově závažný zdravotní problém. I přes pokroky v diagnostice a v léčebných metodách zůstává prognóza onemocnění špatná. Pro zlepšení celkové míry přežívání je důležité umět rozpoznat jedince s vyšším rizikem vzniku KRK a odhalit onemocnění v rané potenciálně léčitelné fázi. Současně identifikace pacientů, kteří budou reagovat negativně na konkrétní léčbu, by přispěla k snížení nadbytečné chemoterapie a k minimalizaci toxicity související s léčbou.

Cílem této práce bylo hledání nových diagnostických, prognostických a prediktivních DNA-biomarkerů pro sporadickou formu KRK. Každý člověk je geneticky jedinečný a nalezení těchto biomarkerů by lékařům usnadnilo diagnózu a výběr optimální terapie pro každého pacienta s KRK na základě jejich molekulárního profilu. Pro dosažení tohoto cíle jsme zkoumali několik kandidátních genů u zdravých jedinců i u nově diagnostikovaných pacientů se sporadickou formou KRK.

Výsledky této PhD práce byly shrnuty v sedmi impaktovaných publikacích. Hlavními závěry jsou: 1) Genetické varianty v cílových oblastech pro vazbu microRNA (miRSNPs) v genech opravy dvouřetězcových zlomů, genech důležitých pro etiologii KRK a mucinových genech souvisí buď s rizikem KRK nebo s odpovědí na léčbu. 2) miRSNPs v cílových genech ovlivňují účinnost exprese odpovídajícího proteinu. 3) Genetické varianty NOD-like receptorů (NLR) přispívají ke vzniku a progresi onemocnění KRK. 4) Funkční genetické varianty v DNA opravných genech jsou asociovány s KRK.

Závěrem, tato disertační práce navrhuje několik nových biomarkerů pro klinické využití. Pro potvrzení klinického významu těchto biomarkerů jsou však nezbytné další studie na nezávislých populacích a porozumění s nimi spojených biologických mechanizmů.

Klíčová slova: rakovina tlustého střeva a konečníku, biomarker, genetické varianty, miRSNP, prongnóza, odpověď na léčbu, DNA reparace, patogeneze KRK, mucinové geny, NLR geny

1. INTRODUCTION

1.1 Colorectal cancer

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related death worldwide with an estimated 1.8 million new cases diagnosed and approximately 881,000 deaths every year [1]. The average lifetime risk for CRC is in the range of 3–5% [2].

The majority of CRC cases arise sporadically (up to 80%) with no specific cause for disease development. However, there are several independent risk factors involved such as age, male sex, diabetes mellitus, previous colonic polyps, intestinal inflammation, dietary and environmental factors and also common low-penetrant genetic variants [3].

CRC has a long preclinical stage of the disease (5–10 years) therefore identification of premalignant adenomas (polyps) or detecting potentially curable early stage cancers by screening can reduce CRC mortality [4-8]. Furthermore, the prognosis for patients with CRC is heavily dependent on the stage at diagnosis: 5-year survival is over 90% for patients with early stage cancer, compared with only 5-10% for patients diagnosed with an advanced stage of disease [9, 10].

For colon cancer, the cornerstone of treatment is surgical resection and for early stage cancers (I and II), surgery alone may cure the disease. Unfortunately, more than 50% of cases are diagnosed at the higher stage of CRC (III and IV) and the only improvement of the prognosis can be achieved by appropriate 5-fluorouracil (5-FU) based adjuvant therapy [10-12]. Treatment of rectal cancer is more complex. Patients with stages II and III first undergo radiation therapy (usually simultaneously with 5-FU based chemotherapy) to improve local control of the disease and subsequent surgery is applied (Figure 4) [13]. For stage IV in both, colon and rectum cancer patients, targeted agents are used for treatment alongside 5-FU-based chemotherapy (e.g. cetuximab, panitumumab, bevacizumab, and regorafenib).

1.2 Biomarkers

A molecular marker (biomarker) is defined as a biological molecule which can be objectively measured and evaluated in blood, and other body fluids, or tissues. They can be used as an indicator of particular physiological or pathological processes, or pharmacological response to a specified therapeutic intervention [14]. According to their application, we can categorize biomarkers into three main types as diagnostic, prognostic and predictive (Figure 1) [15-17].

Since screening is expected to have further impact on CRC management, development of sensitive and specific biomarkers associated with the risk of CRC are being investigated for decades. Little invasive and inexpensive DNA-based tests of blood would be an ideal future possibility.

Furthermore, although advancements in CRC treatment have been made, relapse is still a major factor for the unsatisfactory outcome of the disease. Moreover, many CRC patients undergo the systemic chemotherapy without any benefit and even suffer from severe side effects [10, 18]. Novel prognostic and predictive biomarkers would aid oncologists in selection of optimal type, combination and dose of drugs for an individual patient to improve the outcome based on their molecular profile.

The aim of the current research of biomarkers is to identify and develop highly accurate, non-invasive, rapid and cost-effective biomarkers which could be easily translated into clinical practice. Many genetic and epigenetic biomarkers have been studied, but still none have been validated for clinical use.

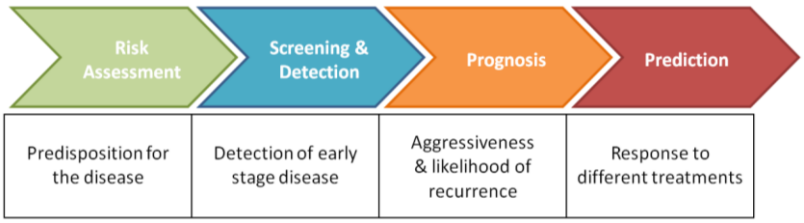

Figure 1: Major types of biomarkers in cancer detection [*modified from https://www.provistadx.com/blog/6-types-of-biomarkers-in-cancer-detection*].

SNPs are the most frequently studied type of DNA-based biomarkers. They refer to a substitution of a single nucleotide that occurs at a specific position in the genome, where each variation is presented in > 1% within a population. Genetic variants may be distinguished according to their position in the genome (non-coding or coding SNPs). Understanding the effects of SNPs is a difficult process and alteration in the final protein caused by a base change may be indicated as benign, pathogenic, or of unknown significance.

1.3 Candidate genes for CRC biomarkers

Studies included in this Thesis are of a candidate gene approach and the investigated genes were selected according to published studies on CRC, providing a tremendous amount of information on genes, pathways, and chromosomal regions that appear to be linked to disease. Genes involved in CRC mutagenesis were naturally prime candidates.

1.3.1 DNA repair genes

The human genome is constantly attacked by a plethora of mutagens that impact its stability. A continuous surveillance by DNA repair systems is therefore essential for the maintenance of genome integrity. Unrepaired damages may affect cell metabolism, trigger cell-cycle arrest or contribute to oncogenesis. When damage is too significant, a cell may opt for initiating the apoptosis.

The importance of proper DNA repair may be also illustrated by the fact that mutations in a number of DNA repair genes are known to be associated with several hereditary syndromes which are characterised by increased incidence of multiple cancers, immunodeficiency and multiple metabolic alterations [19].

At least 150 genes have been identified to be associated with DNA repair machinery in humans, many of them being polymorphic in the human population [20, 21]. As carcinogenesis generally depends on the acquisition of mutations in the cellular DNA, inter-individual differences in DNA repair systems caused by common SNPs in corresponding genes may be expected to play a role in modulating the individual risk of developing cancer or sensitivity of tumor cells to survive DNA damage induced by chemotherapeutic agents.

1.3.2 Genes involved in CRC pathogenesis

In 1990, Fearon and Vogelstein described the development of sporadic CRC as a multistep process of accumulation of activating mutations in oncogenes and deactivating mutations in tumor-suppressor genes [22, 23]. As the intestinal epithelium has a high turnover rate, the constant proliferation in the normal mucosa has to be maintained by the equilibrium between growth promoting oncogenes and growth limiting tumor suppressor genes [24]. Thus each of genetic events in these genes confers a selective growth advantage to an affected colon/rectal epithelial cell and may ultimately result in uninhibited cell growth, proliferation, and clonal tumor development [25]. This adenoma-carcinoma sequence model, which can proceed for more than 10 years, observes a slow development from aberrant crypt proliferation to adenomatous polyps, then to carcinomas *in situ* and finally to malignant tumors [9].

Given that the majority (55–70%) of CRC tumors arises via this pathway, a large number of previous studies focused on the above mentioned genes [26]. Furthermore, according to this model of CRC carcinogenesis, subtle effects caused by SNPs in genes involved in the CRC pathogenesis may contribute to the disease onset and patient's prognosis.

1.3.3 Mucin genes

Mucins are high molecular weight, heavily glycosylated extracellular proteins produced by epithelial cells [27, 28]. Under physiological conditions, mucins play an important role in the maintenance of homeostasis by covering epithelial surfaces, including human colorectal epithelium, by gel mucus layer [29]. Their function lies in limiting the activation of inflammatory responses at the interface with the environment, therefore deregulation of mucin production is an important link between inflammation and cancer [30].

Aberrant expression of mucins was found in diverse human adenocarcinomas (in gastric, esophageal, breast, endometrial and lung cancer), while increased levels of mucin production have been associated with higher risk of cancer as well as with worse patient prognosis [31-34]. Concerning CRC, an overexpression of *MUC1* and *MUC5AC* and a downregulation of *MUC2* have been described to be involved in the development and progression of the disease [16] and the upregulation of *MUC20* have been observed as a predictor of poor prognosis [35, 36].

As miRNAs have emerged as important regulators responsible for an altered mucin expression [37], miRSNPs located in mucin genes may play a role in cancer susceptibility, efficacy of chemotherapy, and survival.

1.3.4 NOD-like receptor genes

Highly conserved nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are cytosolic pattern recognition receptors that play a crucial role in mucosal immune defense [38].

NLRs are activated by recognizing a wide range of pathogens or damage-associated molecular patterns and trigger sequential activation of intracellular signalling pathways that initiate the innate response and the subsequent adaptive immune response [39]. Therefore a dysregulated expression of NLR genes, due to functional or genetic defects, may lead to an excessive or uncontrolled signalling of underlying regulatory pathways. Consequently, this may result in the development of local and chronic inflammation, inflammatory bowel disease (such as ulcerative colitis and Crohn's disease) and/or CRC [38, 40]. As the inflammation affects all stages of tumorigenesis, SNPs located in NLRs genes may also play a role in cancer susceptibility.

2. AIMS

The main goal of the thesis was to identify potential novel biomarkers for sporadic CRC. For this purpose, we studied the association of genetic variants with CRC susceptibility and patient's clinical outcome.

We stated the following aims:

- To test whether SNPs in miRNA target regions of selected genes affect cancer susceptibility, survival and response to therapy in CRC patients.
- To examine whether miRSNPs modulate the efficiency of corresponding proteins translation.
- To investigate whether coding SNPs in NLR genes contribute to human CRC development or progression.
- To search whether SNPs causing amino acid substitution in DNA repair genes influence the risk of CRC and modulate the clinical outcome after CRC diagnosis.

3. MATERIAL & METHODS

3.1. Study populations

Manuscript I: Study was conducted on 1126 histologically confirmed CRC patients and 1469 healthy controls from the Czech Republic. Subjects were sampled for peripheral blood.

Manuscript II: 1111 CRC patients and 1469 healthy controls, that provided peripheral blood samples, were tested in the study.

Manuscript III: Study was carried out on the same group of patients and healthy controls as in **Manuscript II**.

Manuscript IV: A discovery cohort from the Czech Republic (1237 CRC cases and 787 healthy controls) and replication cohorts from Germany (1798 CRC cases and 1810 healthy controls) and Scotland (2210 CRC cases and 9350 healthy controls) were included. Blood samples were collected from all study participants.

Manuscript V: 1424 newly diagnosed patients with sporadic CRC were compared to 1114 age-matched healthy individuals. All study participants provided blood samples.

Manuscript VI: 589 CRC patients from the Czech Republic were tested in the study. All subjects were sampled for peripheral blood. For expression analyses, patients provided biopsy material and healthy controls providing buffy coats or whole blood.

Manuscript VII: A discovery cohort from the Czech Republic (1832 CRC cases and 1172 healthy controls) and replication cohort from Austria (950 CRC cases and 820 healthy controls) were tested in the study. Blood samples were collected from healthy controls. Patients were sampled for healthy colon/rectal tissue or peripheral blood.

All individuals included in the above studies provided informed consent and the particular studies have obtained appropriate approval from Ethic committees.

3.2. Selection of candidate SNPs

To select the candidate SNPs, several *in silico* prediction software programs were implemented. The SNPs were filtered for their minor allele frequency (MAF > 1-10% depending on the Manuscript) in Caucasian populations to reach an appropriate representation of all genotypes in our set of cases and controls (Source: 1000Genomes, dbSNP, HapMap). SNPs with the required MAF were tested for the possibility to be in linkage disequilibrium (LD) (Source: HaploView and HapMap).

Detailed workflow for the SNPs selection is described in the Manuscripts.

3.3. Genotyping analysis

Genomic DNA was isolated from peripheral blood lymphocytes using standard procedures. When blood was not available, nonmalignant colon/rectal tissue was used to obtain DNA by using the DNeasy Blood and Tissue Kit (Qiagen).

SNPs were determined by TaqMan SNP Genotyping Assays (Thermo Fisher Scientific), KASP Genotyping Assays (LGC genomics), the Illumina HumanCytoSNP or Illumina HumanOmniExpress Platform [41], the Infinium Human Exome BeadChip (Illumina), OmniExpressExome BeadChip (Illumina) and Axiom Genome-Wide CEU 1 Array (Affymetrix). In manuscript IV, imputation was performed for autosomal SNPs to the CEU population in HapMap and in manuscript VII, genotype assignment was performed as described in [42].

The genotypes with unclear results were excluded from the studies.

3.4. Gene expression analysis

In Manuscript I, a Dual-Luciferase reporter assay was used to investigate whether the *MRE11A* rs2155209 alleles were associated with a differential gene expression. The assays were carried out using the Dual-Luciferase Reporter Assay Kit (Promega) and three replicates

of all experimental points were performed in each experiment. Luminescence intensity was evaluated by a luminometer (Optima FluoStar) and luciferase activities were averaged from four measurements.

In Manuscipt IV, gene expression analysis was carried out using single-gene TaqMan1Gene Expression Assays (Applied Biosystems). mRNA was isolated from samples of two donors or cell lines (THP-1, HCT116, DLD-1 or CaCo2) by RNeasy Mini Kit (Qiagen). Commercially available RNA samples for human ovary, duodenum, ileum, rectum and colon adenocarcinoma were also used (Agilent). RNA from ileum or colon biopsies was isolated using TRIzol Reagent (Life Technologies) according to standard protocols. The samples were analyzed in triplicate using the 7500fast Real-Time System (Applied Biosystems).

3.5. Statistical analysis

Genotype frequencies in healthy controls were tested for Hardy-Weinberg equilibrium (HWE; Pearson's goodness-of-fit $\chi 2$ test). Odds ratios (ORs) and 95% confidence intervals (CIs) for associations between genotypes and risk of CRC were estimated by logistic regression.

The outcome variables measured were OS and EFS. The relative risk of death was estimated as hazard ratio (HR) using Cox regression. The survival curves for OS and EFS were derived by the Kaplan–Meier method.

In Manuscript I, the multifactor analysis of variance with interactions (MANOVA) was performed in the *in vitro* assays to compare the ratios of the measurements of luminescence between genotypes. In Manuscript V, additive influence of the risk alleles on CRC risk and patient's survival was estimated. In Manuscript VII, a multivariate analysis, referred to as a classification and regression tree (CART), was used to assess the prognostic value of interactions between the standard clinico-pathological variables and the genetic variants in relation to their impact on five-year survival in CRC patients.

Multiple testing corrections were performed using the Bonferroni test or the Benjamini-Hochberg false discovery rate.

Detailed information about individual patients, methods of sample processing and methods of individual analyzes are reported in the enclosed publications.

4. RESULTS & DISCUSSION

In this section, the major findings from each publication representing the PhD study are discussed.

Manuscript I:

The study "*Double-strand break repair and colorectal cancer: gene variants within 3′ UTRs and microRNAs binding as modulators of cancer risk and clinical outcome*" explored the association of 21 polymorphisms in 3′UTRs in relevant DNA repair genes of DSB repair pathway with CRC susceptibility and prognosis.

The major finding of the study comprising 1126 cases and 1469 controls identifies the carriers of the variant CC genotype in *MRE11A* rs2155209 as strongly associated with a decreased risk of CRC ($p = 0.0004$). In the survival analyses among CRC patients, and specifically among those with colon cancer, carriers of the variant CC genotype in *MRE11A* rs2155209 showed a worse survival ($p = 0.03$). *MRE11A* expression was further investigated by a Dual-Luciferase Reporter Assay. Between the two constructs carrying the different alleles of the SNP a statistically significant difference was observed ($p = 0.007$).

Since miRNAs have been recognized as pivotal players in diverse biologic processes (including DNA repair and DNA damage response [43, 44]), the presence of SNPs within the 3′UTRs of target DNA repair genes might cause an altered binding of specific miRNAs, and thus modulate gene expression and ultimately affect cancer susceptibility [45-47], therapy outcomes [48] and survival [49].

In this study, we have reported a significant role of SNP rs2155209 in miRNA target site of DSB repair gene *MRE11A* in CRC risk and clinical outcome, and our results support the idea of miRNAs and miRSNPs contribution to CRC [50]. Since the interest on miRNAs has lately increased for the possibility to use them as diagnostic, prognostic and predictive clinical biomarkers [51], a similar study design was applied in **Manuscript II and III** to explore miRSNPs in genes frequently mutated in CRC pathogenesis and in mucin genes.

Manuscript II:

The study "*MicroRNA-binding site polymorphisms in genes involved in colorectal cancer etiopathogenesis and their impact on disease prognosis*" was performed to test 8 genetic variants in the 3′UTRs of 5 highly penetrant genes known to be frequently mutated in CRC pathogenesis in association with CRC risk and clinical outcome.

Selected miRSNPs were tested in the same cohort of patients and controls as described in **Manuscript I**. In a case-control study, the polymorphism rs8679 in *PARP1* gene (C allele) was associated with a risk of CRC (p = 0.05). The CC genotype in *PARP1* rs8679 was also associated with patient's survival when patients undergoing 5-FU-based chemotherapy were at increased risk of recurrence/progression (p = 0.03).

As already mentioned in the previous **Manuscript I**, the ability of miRNAs to locate and bind a target mRNA has been found to be critical for regulating transcripts level and protein expression necessary for a proper DNA repair and DNA damage response [52]. Thus inherited genetic variants in miRNA target sites are suggested to affect miRNA function and may have an important role in human disease susceptibility and progression [48, 53, 54].

Understanding the modulating effect of miRNAs on PARP1 protein levels in CRC tumors is particularly important because of the current interest in the use of *PARP1* inhibitors as a single agent or as a chemo- or radiosensitizer in cancer treatment [55]. In the present study, we provide evidence that variations in potential miRNA-binding target sites in the 3′ UTR of PARP1 gene may modulate CRC risk and prognosis after therapy.

Manuscript III:

The study "*Polymorphisms in microRNA binding sites of mucin genes as predictors of clinical outcome in colorectal cancer patients*" describes the association of 13 miRSNPs of 9 mucin genes with CRC risk and clinical outcome.

Selected miRSNPs were assessed in the same cohort of patients and controls as in **Manuscript I and II**. Overall, no strongly significant associations were observed in the case-control study. Among the strongest associations with patient's survival, the carriers of the CC genotype in *MUC21* rs886403 were associated with a worse survival and a higher recurrence risk in CRC patients (OS: p = 0.01 and EFS: p = 0.0002). In contrast, rectal cancer patients carrying the variant CC genotype in *MUC17* rs4729655 displayed a better survival (p = 0.0002). Finally, CRC and colon cancer patients carrying the variant GG genotype of in *MUC20* rs6782006 showed a worse OS (p = 0.02).

Mucins are glycoproteins predominantly expressed at the epithelial part of tissues and provide a protection for colon surface. Under physiological conditions, mucins maintain a homeostasis by covering human colon surface by gel mucous layer [29]. During the malignant development, miRNAs have emerged as important regulators responsible for an altered mucin expression [37]. Therefore, miRSNPs located in mucin genes may play a role in cancer susceptibility, efficacy of chemotherapy and survival.

In the present study, plausible candidate miRSNPs potentially affecting miRNA binding to mucin genes were identified as related to either CRC susceptibility or patient´s survival. Our results support the idea that a 'miRNA network' may contribute to CRC pathogenesis. Expanding our knowledge on mucins may help us to better understand the etiopathogenesis of CRC and thereby contribute to the development of new treatment strategies.

Manuscript IV:

The study "*Coding variants in NOD-like receptors: An association study on risk and survival of colorectal cancer*" was aimed at evaluating 41 non-synonymous SNPs in 21 NLR genes for their association with CRC risk and clinical outcome.

In this study, a discovery cohort from the Czech Republic (1237 cases and 787 controls) and two large GWAS data sets for a replication analysis were included (Germany: 1798 cases and 1810 controls and Scotland: 2210 cases and 9350 controls). The major findings in a discovery set describe five SNPs to be significantly associated with CRC risk and eight with patient´s survival however the associations were not confirmed in a replication analysis. mRNA levels were measured in primary tissue samples and cell lines and divergent expression patterns of *NLRP2*, *5*, *6* and *13* were found in hematopoietic and non-hematopoietic cells.

NLRs are important innate pattern recognition receptors and regulators of inflammation and play an important part in the homeostasis of the immune system [56, 57]. A different expression of NLR genes may lead to a disruption of the underlying regulatory pathways and result in the development of local and chronic inflammation, inflammatory bowel disease and/or CRC [58, 59].

In spite of the *in silico* predictions about the functionality of studied SNPs the promising results from the Czech cohort could not be confirmed in the two GWAS data sets. However, our expression analysis showed the NLRs associated with CRC risk or survival in the discovery set as expressed in primary human colon or rectum cells, CRC tissue and cell lines, providing preliminary evidence for a potential involvement of NLRs in CRC development and progression. Furthermore, the expression of development-related *NLRP5* was undetectable in nonmalignant colon tissue but was upregulated in colon cancer tissue and cell lines, suggesting a potential novel role beyond developmental control for this NLR in humans [60]. Induced expression of *NLRC5* in HCT116 cells may have a functional outcome by modulating major histocompatibility complex (MHC) class I expression [61] which correlates with survival due to its effect on CD8 cytotoxic T-cell and natural killer cell immuno-surveillance [62].

To further uncover the poorly understood role of NLRs in CRC development and survival, the effect of regulatory variants of *NLRC5* on CRC susceptibility and clinical outcome were explored in **Manuscript V and VI**.

Manuscript V:

The study "*Investigation of single and synergic effects of NLRC5 and PD-L1 variants on the risk of colorectal cancer*" reports the influence of 16 potential regulatory variants in the *NLRC5* and Programmed death-ligand 1 (*PD-L1)* genes selected by several *in silico* tools on CRC susceptibility.

This case-control study comprising 1424 cases and 1114 healthy controls from the Czech Republic reports a moderate association between rectal cancer risk and two *NLRC5* SNPs. Eighteen pair-wise interactions within and between the *NLRC5* ad *PD-L1* genes were obtained. Six more interactions appeared when the previously genotyped *IFNGR1* and *IFNGR2* variants were added to the analysis [63]. The main interactions included three *NLRC5* SNPs (rs289747, rs289748, rs56315364) located in the upstream and promoter region with the same *PD-L1* promoter SNP rs2890657.

NLRC5 gene, a member of a NLR family, plays a prominent role in antitumor immunity while *PD-L1* acts as a physiological feedback mechanism necessary for terminating the immune responses and for maintaining self-tolerance [64]. Changes in these genes expression may lead to a disrupted anti-tumor immune response, which in turn may influence CRC susceptibility [65-67]. SNPs located in regulatory regions of *NLRC5* and *PD-L1* may thus be actively involved in the regulation of gene expression and have an impact on CRC development [68].

Our data suggest that the interaction between the inherited genetic variants within genes involved in immune surveillance contributes to signaling defects, which in turn may lead to alteration in the anti-tumor immune response and further play an important role in the onset of CRC. Expanding our knowledge on regulatory variants in the *NLRC5* and *PD-L1* genes could eventually improve CRC risk management but also *PD-L1*-based immunotherapy in CRC. The association of *NLRC5* with regard to a therapy response as well as survival of the CRC patients is further explored in **Manuscript VI.**

Manuscript VI:

The study "*Influence of regulatory NLRC5 variants on colorectal cancer survival and 5-fluorouracil-based chemotherapy*" was preformed to evaluate the effect of 11 potential

regulatory polymorphisms in *NLRC5* selected by several *in silico* tools on overall and event-free survival of patients with sporadic form of CRC.

The case-only study was carried out on 589 CRC cases from the Czech Republic (232 patients received 5-FU-based therapy). Minor alleles of two SNPs (rs27194 and rs289747) were significantly associated with a decreased survival in all patients and metastasis-free patients at the time of diagnosis. Among CRC patients receiving a 5-FU-based adjuvant therapy, rs12445252 was associated according to the dosage of the minor allele T with OS and EFS.

As previously mentioned *NLRC5* is a transactivator of MHC class I molecules [69] and plays a pivotal role in immune-surveillance with a potential influence on cancer patient's survival. It is an interferon gamma (IFNγ)–inducible nuclear protein and due to the link with the IFNγ system, *NLRC5* might also play a role in the 5-FU-based therapy.

Our results indicate that polymorphisms in immune surveillance genes, such as *NLRC5*, may be used as prognostic markers for clinical outcome in CRC, as well as for survival of CRC patients in response to 5-FU-treatment. Our study also adds a new layer on the complex function of *NLRC5* in the innate immune system.

Manuscript VII:

The study "*Functional polymorphisms in DNA repair genes are associated with sporadic colorectal cancer susceptibility and clinical outcome*" evaluated the relevance of 16 functional genetic variants in 12 DNA repair genes on the risk of CRC development and modulation of the clinical outcome after cancer diagnosis.

In the discovery set of 1832 patients and 1172 controls from the Czech Republic, the carriers of the variant *AA* genotype in *REV3L* rs3204953 were observed as associated with an increased risk of CRC ($p = 0.006$). The valine to isoleucine substitution has been recognized via *in silico* approach, performed by F-SNP database, to have a high probability of being functionally significant [70] and in the area of a molecular epidemiology, there is evidence that polymorphisms in *REV3L* are associated with different malignancies. Unfortunately, despite the promising results in the Czech population, an association of *REV3L* SNP with CRC risk could not be confirmed in the Austrian replication set comprising 950 patients and 820 controls. However, since *REV3L* was observed as significant in the Austrian survival analyses and according to all of the available data, we suggest that the *REV3L* gene may impact CRC susceptibility, survival, and therapy outcomes and warrants further investigation.

The CART analysis, investigating the interactive effects of genotypes and clinico-pathological parameters in association with five-year OS and EFS, showed a prognostic utility of several investigated DNA repair gene polymorphisms. Only a few of these were shown as significant more than once in the final structure of the tree, suggesting their potentially greater relevance on patient's survival. *POLQ* gene polymorphisms appeared four times as the optimal split factor in the Czech CART analyses (rs1381057, rs3218649 twice, and rs3218651) and four times in the Austrian CART analyses (rs1381057 twice and rs3218651 twice). Apart from the deleterious nature of the protein function of all studied *POLQ* SNPs predicted by F-SNP database, ELASPIC estimated the substitution of glutamine to arginine of *POLQ* SNP rs1381057 to decrease the final protein stability. The accurate level of the functional protein in cells has been reported as important in a complementary body of literature.

Based on the information from published studies we consider the significance of adequate *POLQ* functioning and regulation for tumor suppression.

In the five-year EFS CART analysis *NEIL3* gene SNP rs7689099 emerged twice as the optimal split factor in the Czech cohort. Likewise in previously mentioned *REV3L* and *POLQ*, significantly elevated expression levels of *NEIL3* were reported in tumors of 20 cancer sites, including CRC [71, 72]. The association of *NEIL3* SNP with the survival of CRC patients was not replicated in the Austrian sample set. However, considering the available data, we suggest that the variation of the *NEIL3* gene also has relevance for CRC susceptibility, survival, and therapy outcome.

In this study, we evaluated the association of genetic variants in DNA repair genes selected by likely functional relevance with CRC. Our data suggest that even subtle alterations in the specific proteins that function in DNA repair pathways may lead to inaccurate DNA repair and thus play a role in CRC pathogenesis.

5. CONCLUSIONS

The main results which were obtained during the work on this thesis are summarized in the following paragraphs:

- We have observed several candidate SNPs in miRNA target regions related either to CRC risk or to clinical outcome. In DSB repair genes, we identified a miRSNP *MRE11A* rs2155209 as strongly associated with a decreased risk of CRC and worse survival (Manucript I). In genes important for CRC etiology, an association between *PARP1*

rs8679 and either a decreased risk of CRC or an increased risk of recurrence or progression in patients that received 5-FU-based chemotherapy was observed (Manuscript II). In mucin genes, *MUC21* rs886403 was associated with a worse survival and a higher recurrence risk in CRC patients, *MUC20* rs6782006 showed a worse OS in CRC patients and *MUC17* rs4729655 displayed a better OS in rectal cancer patients (Manuscript III).

- We have found that genetic variations in the 3′ UTR of target genes modulate the efficiency of corresponding protein expressions. We investigated a role of miRSNP *MRE11A* rs2155209 in modulating *MRE11A* expression by a Dual-Luciferase Reporter Assay and a statistically significant difference was observed between two constructs carrying the different alleles of the SNP. One allele was related to a reduced activity of the reporter gene by 14% (Manuscript I).
- We have demonstrated that genetic variants in NLR genes contribute to CRC onset and progression of the disease. In Manuscript IV, 5 SNPs were described to be associated with CRC risk, and eight with CRC survival in the Czech population. Also an additive effect on CRC risk and survival was detected, resulting in a 2-fold increased risk and a 3-fold worse survival for carriers of 6 and 8 risk alleles, respectively. However, the results could not be confirmed in the German and Scottish GWAS data sets and future studies are needed to validate the results.

  In manuscript V and VI, role of SNPs within *NLRC5* gene in CRC risk and clinical outcome was reported. The results suggest that genetic variants in immune surveillance genes, such as *NLRC5*, may serve as candidate prognostic and predictive markers of CRC.
- We have identified the association of several potential functional SNPs in DNA repair genes with CRC. *REV3L* rs3204953 was observed to be associated with an increased risk of CRC and several other SNPs were shown to be associated with OS and EFS in the CART analyses (Manuscript VII). Our data suggest that even subtle alterations of the final protein caused by amino acid substitution may lead to inaccurate DNA repair, and thus contribute to carcinogenesis.

This thesis suggested several potential candidate biomarkers for clinical use. However, further studies are needed to replicate our findings and assess the SNPs in independent populations, to functionally characterize the significant genetic variants and to find the biologic mechanisms underlying the associations.

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7. CURRICULUM VITAE

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BRIEF EDUCATIONAL AND PROFESSIONAL HISTORY

2012-now **PhD. student**
Department of Molecular Biology of Cancer, Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech Republic; http://www.iem.cas.cz/research/departments/molecular-biology-of-cancer.html

2013-2018 **Research Assistant**
Institute of Biology and Medical Genetics, 1st Medical Faculty, Prague, Czech Republic

2007 **Lab Assistant**
The Institute of Hematology and Blood Transfusion, Dept. of HLA Analysis, Prague, Czech Republic

2004-2007 **Diploma thesis**
Genetic polymorphism in Czech patients with narcolepsy and other sleep disorders, The Institute of Hematology and Blood Transfusion, Laboratory for DNA Diagnostics, Prague, Czech Republic

2002-2007 **University study**: Charles University, Faculty of Science in Prague, Department of Genetics and Microbiology, Czech Republic

RESEARCH FUNDING

Principal investigator 2015-2016 **Grant Agency of Charles University CR:** 112515
Project title: Non-synonymous functional variants in DNA repair genes in sporadic colorectal cancer: searching for predictive and prognostic markers

ACADEMIC RESEARCH TRAININGS

Internship **COST STSM** (Short Term Scientific Mission) - 10-14-2014 to 12-06-2014
Cost Action: "Cooperation Studies on Inherited Susceptibility to Colorectal Cancer" Topic: Functional assessment of variants in low penetrance DNA repair genes
Host: Dr. Stefano Landi, University of Pisa, Genetics - Department of Biology, Pisa, Italy

Courses **CNV analysis by NGS & Bioinformatic analysis workshop**
HPST, Deparment of genomics and diagnostics, Prague, 2017

Introduction to Genetic Models: from C.elegans to mouse,
Andalusian Center for Developmental Biology, Sevilla, Spain, July 2016

Digital PCR – Applications and Analysis,
TATAA Biocenter, Gothenburg, Sweden, March 2016

Elements of Science,
Science Communication, Paper & Grant Writing, Poster & Lecture Preparation, Institute of Molecular Genetics ASCR, Prague, March 2016

Statistical Analyses in Medical Research,
Acrea CR, Prague, November 2015

The Basics of Cellular and Molecular Immunology,
Institute of Physiology of the ASCR, Prague, 2013

Advances in the Molecular Biology and Genetics,
Institute of molecular genetics ASCR, Prague, November 2012 & 2013

CONFERENCES

Lecture

Genetic Toxicology and Cancer Prevention, Třeboň, Czech Republic, 2018
Functional polymorphisms in DNA repair genes are associated with sporadic colorectal cancer susceptibility and clinical outcome

Genetic Toxicology and Cancer Prevention, Telč, Czech Republic, 2016
Non-synonymous functional variants in DNA repair genes in sporadic colorectal cancer: searching for predictive and prognostic markers

20th World Congress on Advances in Oncology and 18th International Symposium on Molecular Medicine, Athens, Greece, 2015
Non-synonymous functional variants in DNA repair genes in sporadic colorectal cancer

Poster

Translating Colorectal Cancer Research, Porto, Portugal, 2017
Non-synonymous functional variants in DNA repair genes in sporadic colorectal cancer

3rd annual Circulating Biomarkers conference, Dundee, Great Britain, 2016
Circulating miRNAs: A Cancer Screening Biomarkers in Rectal Cancer (Best poster award)

AACR Annual Meeting, New Orleans, USA, 2016
Non-synonymous functional variants in DNA repair genes in sporadic colorectal cancer

AACR Annual Meeting, Philadelphia, USA, 2015
Excision DNA repair: a biomarker of colorectal cancer onset and its chemotherapy

LINGUISTIC KNOWLEDGE

English: Full Professional Proficiency, TOEFL 6/2014 (Total Score 104)

8. PUBLICATION ACTIVITY

This Thesis consists of an overview of research I have been involved in during the time of my PhD studies and which were published from 2016 to 2018. All manuscripts comprise molecular epidemiological studies on different human populations focused on genetic variants in candidate genes.

Manuscript I	Double-strand break repair and colorectal cancer: gene variants within 3′ UTRs and microRNAs binding as modulators of cancer risk and clinical outcome. Naccarati A *et al.* 2016. *Oncotarget* 7(17):23156-69. **IF 5.008**
Manuscript II	MicroRNA-binding site polymorphisms in genes involved in colorectal cancer etiopathogenesis and their impact on disease prognosis. Schneiderova M *et al.* 2017. *Mutagenesis* 32(5):533-542. **IF 2.507**
Manuscript III	Polymorphisms in microRNA binding sites of mucin genes as predictors of clinical outcome in colorectal cancer patients. Vymetalkova V *et al.* 2017. *Carcinogenesis* 38(1):28-39. **IF 4.874**
Manuscript IV	Coding variants in NOD-like receptors: An association study on risk and survival of colorectal cancer. Huhn S *et al.* 2018. *PlosOne* 13(6). **IF 2.806**
Manuscript V	Investigation of single and synergic effects of NLRC5 and PD-L1 variants on the risk of colorectal cancer. Catalano C *et al.* 2018. *PLoS One* 13(2). **IF 2.806**
Manuscript VI	Influence of regulatory NLRC5 variants on colorectal cancer survival and 5-fluorouracil-based chemotherapy. Catalano C *et al.* 2018. *Eur J Gastroenterol Hepatol*. 30(8):838-842. **IF 2.152**
Manuscript VII	Functional polymorphisms in DNA repair genes are associated with sporadic colorectal cancer susceptibility and clinical outcome. Jiraskova K *et al.* 2018. *Int J Mol Sci* 20(1). **IF 3.687**

Papers not related to the PhD thesis, published in journals with IF:

1. Kunzmann AT, Proença MA, Vodicka P, Jiraskova K, Schneiderova M, Levy M, Liska V, Buchler T, Vodickova L, Vymetalkova V, Jordao H, McKenna G, Silva AE, Coleman HG and Hughes DJ. Fusobacterium nucleatum tumor DNA levels are associated with survival in colorectal cancer patients. *European Journal of Clinical Microbiology & Infectious Diseases,* 2019 Jul 31. doi: 10.1007/s10096-019-03649-1. **IF 2.591**

2. Kroupa M, Rachakonda SK, Liska V, Srinivas N, Urbanova M, Jiraskova K, Schneiderova M, Vycital O, Vymetalkova V, Vodickova L, Kumar R, and Vodicka P. Relationship of telomere length in colorectal cancer patients with cancer phenotype and patient prognosis. *British Journal of Cancer,* 2019 Aug;121(4):344-350. **IF 5.922**

3. Vodenkova S, Jiraskova K, Urbanova M, Kroupa M, Slyskova J, Schneiderova M, Levy M, Buchler T, Liska V, Vodickova L, Vymetalkova V, Collins A, Opattova A, Vodicka P. Base excision repair capacity as a determinant of prognosis and therapy response in colon cancer patients. *DNA Repair (Amst).* 2018 Dec;72:77-85. **IF 4.461**

4. Kral J, Korenkova V, Novosadova V, Langerova L, Schneiderova M, Liska V, Levy M, Veskrnova V, Spicak J, Jiraskova K, Opattova A, Vymetalkova V, Vodicka P, Jana Slyskova J. Expression profile of miR-17/92 cluster is predictive of treatment response in rectal cancer. *Carcinogenesis*. 2018 Dec 13;39(11):1359-1367. **IF 5.105**

5. Kroupa M, Polivkova Z, Rachakonda S, Schneiderova M, Vodenkova S, Buchler T, Jiraskova K, Urbanova M, Vodickova L, Hemminki K, Kumar R, Vodicka P. Bleomycin-induced chromosomal damage and shortening of telomeres in peripheral blood lymphocytes of incident cancer patients. *Genes Chromosomes Cancer*. 2018 Feb;57(2):61-69. **IF 3.696**

6. Carrai M, Campa D, Vodicka P, Flamini R, Martelli I, Slyskova J, Jiraskova K, Rejhova A, Vodenkova S, Canzian F, Bertelli A, Dalla Vedova A, Bavaresco L, Vodickova L, Barale R. Association between taste receptor (TAS) genes and the perception of wine characteristics. *Sci Rep.* 2017 Aug 23;7(1):9239. **IF 4.259**

7. Vodicka P, Musak L, Frank C, Kazimirova A, Vymetalkova V, Barancokova M, Smolkova B, Dzupinkova Z, Jiraskova K, Vodenkova S, Kroupa M, Osina O, Naccarati A, Palitti F, Försti A, Dusinska M, Vodickova L, Hemminki K. Interactions of DNA repair gene variants modulate chromosomal aberrations in healthy subjects. *Carcinogenesis*. 2015 Nov;36(11):1299-306. **IF 5.334**

8. Vymetalkova V, Soucek P, Kunicka T, Jiraskova K, Brynychova V, Pardini B, Novosadova V, Polivkova Z, Kubackova K, Kozevnikovova R, Ambrus M, Vodickova L, Naccarati A, Vodicka P. Genotype and Haplotype Analyses of TP53 Gene in Breast Cancer Patients: Association with Risk and Clinical Outcomes. *PLoS One.* 2015 Jul 30;10(7):e0134463. **IF 3.234**

9. Vodenkova S, Polivkova Z, Musak L, Smerhovsky Z, Zoubkova H, Sytarova S, Kavcova E, Halasova E, Vodickova L, Jiraskova K, Svoboda M,Ambrus M, Hemminki K, Vodicka P. Structural chromosomal aberrations as potential risk markers in incident cancer patients. *Mutagenesis*.2015 Jul;30(4):557-63. **IF 3.497**