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DISSERTATION THESIS

Genetic profile of genes involved in cell cycle control and the risk of sporadic colorectal cancer in the Czech Republic

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Content

List of Abbreviations

AIC	Akaike´s Information Criteria
APC	Adenomatosis Polyposis Coli
APE1	Apurinic endonuclease 1
ATM	Ataxiatelangiectasia Mutated
BER	Base Excision Repair
BMI	Body mass index
CCND1	Cyclin D1
CDKs	Cyclin-Dependent Kinases
CI	Confidence Interval
CIMP+	CpG Island Methylator Phenotype
CIN	Chromosomal instability
CRC	Colorectal Cancer
DDB	DNA Damage Binding protein
DNA	Deoxyribonucleic acid
DSB	Double Strand Break
EPIC	European Prospective Investigation
FAP	Familial Adenomatous Polyposis
FOBT	Fecal Occult Blood Test
GWAs	Genome wide association studies
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
hOGG1	Human oxoguanine glycosylase 1
HR	Homologous Recombination
HRT	Hormone Replacement Therapy
LD	Linkage Disequilibrium
MAF	Minor Allelic Frequency
MGMT	O^6-methylguanine methyltransferase
MMR	Mismatch Repair
MSI	Microsatellite Instability
NBN	Nibrin
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O6-MeG	O^6-methylguanine
OR	Odds Ratio
pRb	Retinoblastoma Protein
RNA	Ribonucleic acid
SCCHN	Squamous Cell Carcinoma of Head and Neck
SNPs	Single-Nucleotide Polymorphisms
SSB	Single Strand Break
UTR	Untranslated Region

Abstract

The Czech Republic has one of the highest incidence rates of colorectal cancer (CRC) worldwide. The vast majority of the CRC cases arises sporadically, with susceptibility determined by genetic factors in interaction with an environment. Cell cycle and DNA repair genes play a fundamental role in CRC development and presents many common variants.

In the present study, we genotyped common variants in cell cycle and DNA repair genes to assess the influence of genetic variation on the CRC risk, in 614 hospital-based CRC cases and 614 matched controls. Despite a tendency towards a differential distribution of the variant allele frequencies for some cell cycle polymorphisms, none was significantly associated with CRC risk. Similarly, none of the studied DNA repair polymorphisms was independently associated with CRC risk. The analysis of binary genotype combinations showed an increased CRC risk in individuals simultaneously homozygous for the variant alleles of apurinic endonuclease 1 (*APE1)* Asn148Glu and human oxoguanine glycosylase 1 (*hOGG1*) Ser326Cys (OR: 6.37; 95% CI: 1.40–29.02; P=0.02).

We observed a differential distribution between cases and controls of major haplotypes arising from the four analysed variants in the *TP53* gene (global P=0.0001). The two most common haplotypes, A_1GCG and A_2CCG, were present in 81% of the cases compared to 71% of the controls. In comparison to the most common haplotype (A_1GCG), the haplotype A_2CCG was associated with an increased risk (OR= 1.40; 95C=1.07–1.82), while the four other haplotypes A_1CCG (OR=0.60; 95%CI=0.45–0.79), A_2GCG (OR=0.53; 95%CI=0.35–0.81), A_1GTG (OR=0.31; 95%CI=0.15–0.64), and A_1GCA (OR=0.19; 95%CI=0.07–0.51) were associated with a decreased risk. The effect of haplotypes in the *TP53* gene was similar for colon (global P<0.0001) and rectal cancers (P=0.006). No association with the disease was observed for the haplotypes of the other analysed cell cycle gene variants. The results from this study suggest that prevalent haplotypes within the *TP53* gene may modulate CRC risk.

In addition to the high incidence of CRC, the Czech Republic, along with other central-eastern European countries, has a high incidence of Nijmegen breakage syndrome (NBS).

NBS is an autosomal recessive chromosomal instability disorder characterized by microcephaly, growth retardation, immunodeficiency and marked susceptibility to cancer. To assess whether *NBN* 657del5 is associated with an increased risk of sporadic CRC, we have screened this deletion in the same population of CRC cases and healthy controls for common variants and in an additional control group (818 healthy blood donors from the Czech Republic). There were no significant differences between the frequencies of heterozygous carriers among the three groups. The present results did not provide any evidence that the exceeding risk of CRC in this population is attributable to the high frequency of heterozygous carriage of the *NBN* 657del5.

1. Introduction

1.1. Colorectal cancer and its incidence

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer death worldwide. The incidence rates vary approximately 20-fold around the world, being higher in the industrialized regions of North America, Central Europe, New Zealand and Australia and lower in Asia, Africa and South America (IARC Cancer incidence 2007, **Figure 1**). In Western countries, the cumulative lifetime risk of CRC and death from CRC is approximately 5-6% and 2.5%, respectively (Sondergaard et al. 1991; Parkin et al. 2005). There were approximately 412 900 new cases of CRC, with 207 400 deaths in Europe only in 2006 (Ferlay et al. 2007)

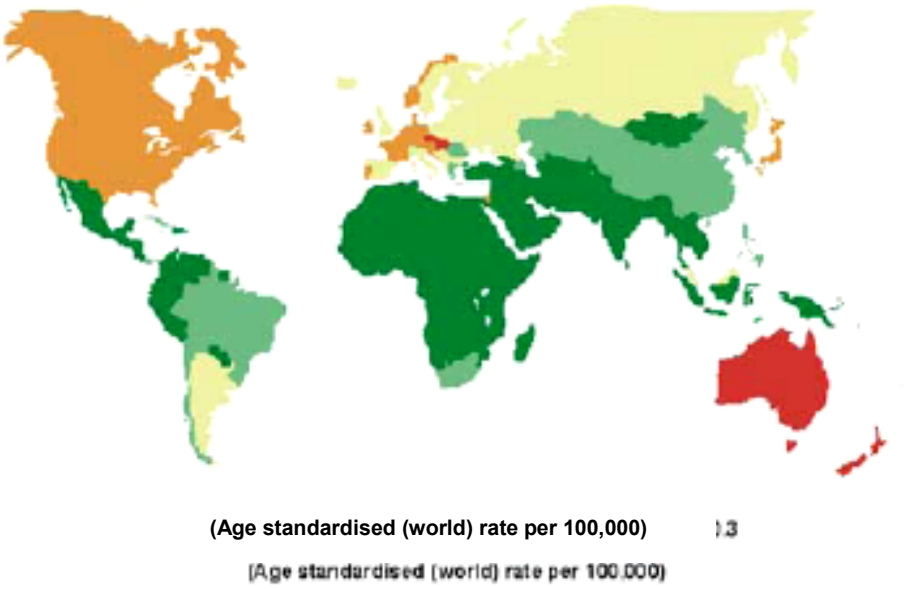


Figure 1. Worldwide incidence of colorectal cancer in males (*Modified from Parkin et al, 2005*)

1.2. Colorectal cancer in the Czech Republic: a negative record

CRC represents a serious health problem in the Central Europe and in particular in the Czech Republic, where the incidence for colon cancer ranks the third highest worldwide and the incidence of rectal cancer is the highest (Boyle and Langman, 2000; Janout and Kollarova, 2001; Parkin et al. 2005; Konecny et al. 2008). Compared to other European countries, the Czech Republic has a long recording of cancer incidence, thus the obtained and collected data represents a reliable source of information (**Figure 2**).

Colorectal malignant neoplasms were the third most frequent kind of cancer recorded in this country according to the last updated calculation by the end of 2005 (Konecny et al. 2008**)**. In 2007, 21 727 men and 16 111 women were affected with CRC (**Figure 2-4**), with reported almost 8 000 new cases (IARC Cancer incidence 2007). The gravity of the disease in this country is confirmed by the fact that it ranks second both in incidence and in mortality (15.6% of all deaths from cancer in the Czech Republic were associated with CRC). The mortality rate due to CRC among men is the highest worldwide. On the other hand, mortality among women is second worldwide, after the Netherlands (IARC Cancer Incidence 2007).

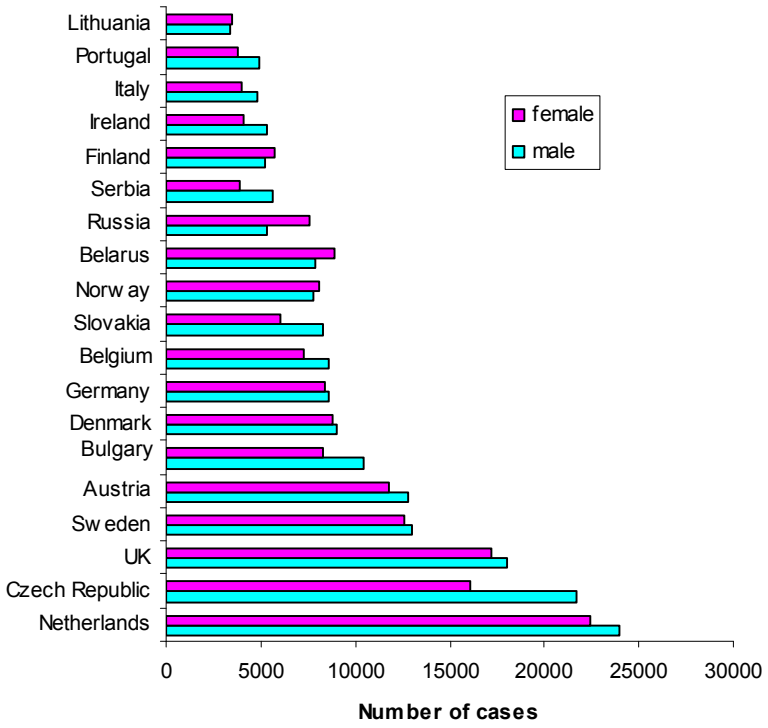


Figure 2. Age standardized incidence rates, CRC in EU countries, by sex, 2007 estimates (*Modified from IARC, GLOBOCAN 2007. Cancer Incidence, Mortality and Prevalence Worldwide (2007 estimates) 2007*)

1.3 Colorectal carcinogenesis

The development of CRC is characterized by a sequence of events during which normal colonic epithelium gradually transforms into carcinoma tissue, in most cases via the development of colorectal adenomas (Tanaka 2009). Adenomas, also known as polyps, are crypt epithelium that protrudes into the gut lumen. Adenomas are considered to be caused by a decreased apoptosis or by an increased cell proliferation in the gut epithelium (Makinen 2007).

The epithelium of adenomas can form glands or finger-like projections or combinations of both, which may later undergo a malignant transformation to CRC. This sequence of events is driven by an accumulation of molecular (epi)genetic alterations causing progressive disorders in cell growth, differentiation and apoptosis.

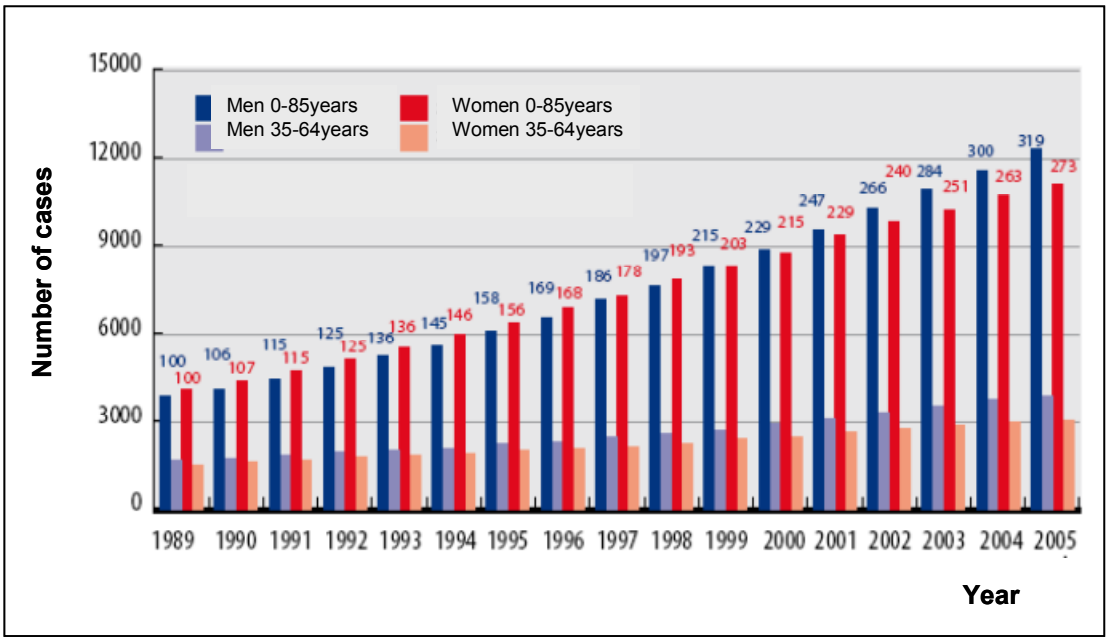


Figure 3. Prevalence of colon cancer cases in the Czech Republic (Konecny et al. 2008)

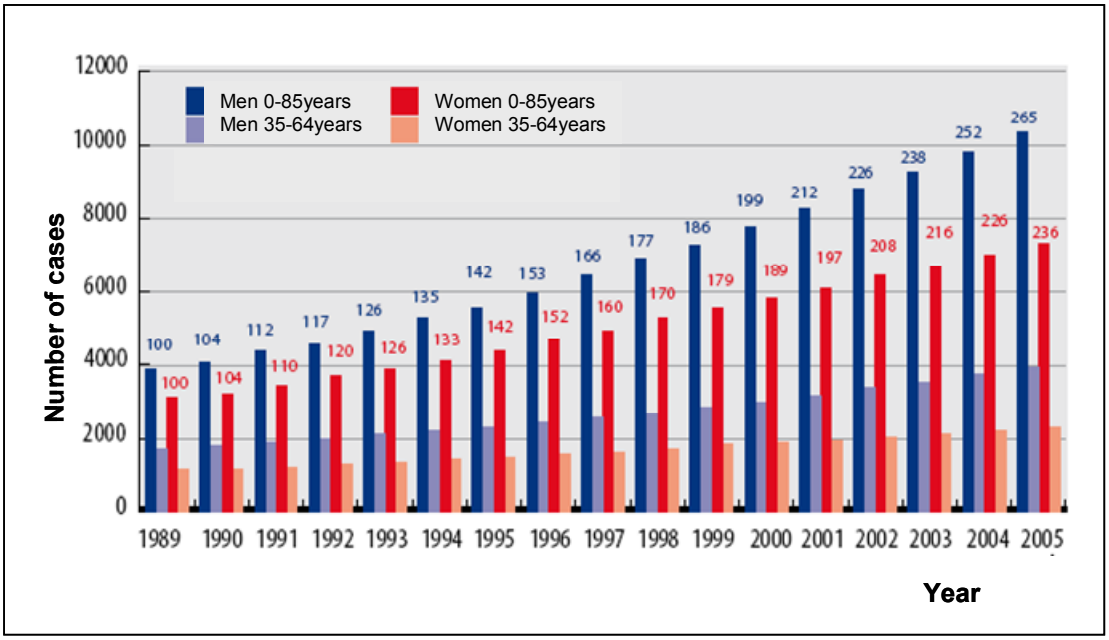


Figure 4. Prevalence of rectosigmoideal cancer cases in the Czech Republic (Konecny et al. 2008)

The multistep model of the adenoma-carcinoma transition was first proposed by Fearon and Vogelstein in 1990 (**Figure 5**) and is characterised by several events of chromosomal

changes (both numerical and structural) and hence referred to as the chromosomal instability pathway (CIN).

The earliest genetic events described in the adenoma-carcinoma transition are mutations and/or allelic losses of the Adenomatous Polyposis Coli (*APC*) gene and *K-ras* oncogene mutations. *APC* mutations or allelic losses at chromosome 5q are observed in up to 30%-70% of sporadic adenomas and in 34%-72% of sporadic cancers (Leslie et al. 2002; Takayama et al. 2006). Activating *K-ras* mutations are found in about 40%-65% of colorectal villous carcinomas and in 15%-68% of sporadic colorectal adenomas (Takayama et al. 2006).

Furthermore, allelic losses at chromosome 17p and 18q have been described in a large proportion of colorectal adenomas and carcinomas (Fearon et al. 1987; Fearon and Vogelstein, 1990; Lips et al. 2008; Pittman et al. 2009). Chromosome 18q, harboring the *SMAD2, SMAD4* and most recently identified *SMAD7* genes, is lost in 10-30% of early adenomas, 60% of late adenomas and 70% of carcinomas (Leslie et al. 2002; Pittman et al. 2009). Allelic loss of chromosome 17p, harboring the *TP53* gene, and mutations in this gene, have been reported in 50-75% of all colorectal carcinomas, but very rarely in benign lesions suggesting that functional inactivation of the *TP53* gene is a late genetic event associated with the transition from adenoma to carcinoma (Baker et al. 1989; Leslie et al. 2002).

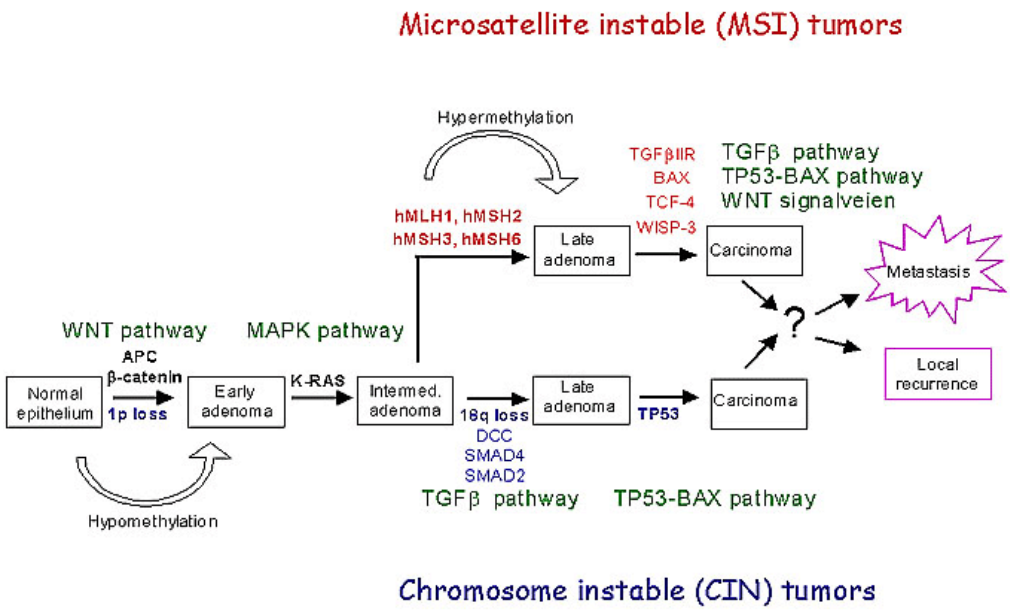


Figure 5. Sequential genetic and epigenetic changes leading to the evolution of CRC (*Modified from Fearon and Vogelstein, 1990* and *Fodde et al. 2002*)

In addition to simple growth advantage, the "successful" pre-cancerous clone must develop a cellular environment permissive of future mutations (Worthley et al. 2007). This process is called genomic instability and it ensures that subsequent strategic mutations occur at increasingly greater likelihood. Genomic instability is therefore critical in carcinogenesis. It accelerates the neoplastic evolutionary process, by increasing the mutation rate induced by the background mutagenic challenge. There are two main recognized pathways of genomic instability in CRC. The most common is the CIN pathway, in which the required genetic events occur through the accumulation of numerical or structural chromosomal abnormalities (aneuploidy). The other main type of genomic instability is microsatellite instability (MSI), which is a consequence of impaired recognition and repair of mismatched bases in the daughter strand of DNA during DNA replication. The mutual exclusivity of the pathways associated with CIN or MSI suggests that genomic instability is necessary and that either pathway is sufficient to lead to colorectal carcinogenesis (Walther et al. 2008; **Figure 5**).

More recently, epigenetic factors have been implicated in the development of certain subsets of cancers and polyps. Epigenetic events refer to modifications in gene expression, without a change in the DNA sequence. Such modifications are controlled by heritable but potentially reversible changes in DNA methylation and/or chromatin structure. Careful characterization of the epigenetic events, particularly gene promoter sequence methylation, has led to the definition of the CpG Island Methylator Phenotype (CIMP+) and a proposed novel pathway, the serrated neoplasia pathway. Moreover, in the progression and metastasis steps of CRC, gene alterations may be also involved, mainly including epigenetic inactivation of DNA mismatch repair (MMR) genes (*hMSH2, hMLH1, hPMS1, hPMS2*, and *hMSH6*) (Takayama et al. 2006; Worthley et al. 2007).

Genetic analyses have shown that mutations or deregulation of the genes involved in spindle assembly and dynamics, cell cycle regulation, and checkpoint control can result in human CIN (Jallepalli et al. 2001). In this respect the genes of interest are *BUB1, BUBR1, MAD2, Aurora A, Securin, Survinin, Cyclin E, ZW10, TP53, ATM, CHK2, BRCA1* and *BRCA2* (Hollstein et al. 1991; Li et al. 1997; Zhou et al. 1998; Bell et al. 1999; Tutt et al. 1999; Boultwood 2001; Jallepalli et al. 2001; Weaver et al. 2002; Schmidt and Medema 2006; Lentini et al. 2007).

1.4 Non-genetic predisposition to CRC

Epidemiologic studies conducted in the last 40 years have helped to highlight many of the major risk factors that are associated with colorectal neoplasia, including positive family history of cancer, obesity, diabetes, meat intake, smoking habit, and alcohol consumption. These studies have also identified several protective factors such as vegetable intake, calcium and folate status, together with other factors such as hormone replacement therapy (HRT), nonsteroidal antiinflammatory drugs (NSAIDs) and physical activity (de la Chapelle 2004).

Diet has undoubtedly a main influence on CRC development with many studies identifying specific nutrients and components of various foods that may play a role in the development or prevention of CRC (Heavey et al. 2004; Arasaradnam et al. 2008). The World Cancer Research Fund (1997) reported that a diet rich in vegetables decrease the risk of CRC and as well as one rich in fiber and starch, whereas high meat and alcohol consummption probably increases the risk. According to these guidelines, the panel estimated that the incidence of CRC might be decreased by 66-75%. As recently shown by Pufulete (2008) the dairy intake may also moderately decrease risk of CRC. A more striking protective effect was found for Calcium, where its supplementation at a level of 1 000–2 000 mg/day may reduce adenoma recurrence in individuals with a previous adenoma, but has no effect on CRC incidence (Pufulete 2008).

One of the latest prospective studies, the European Prospective Investigation into Cancer (EPIC), was designed to investigate the relationships between diet, nutritional status, lifestyle, environmental factors and the incidence of CRC. The results of this study suggest that there is a positive relationship between red meat intake, particularly processed meat, and CRC risk compared with any other food or nutrient (Boker et al. 2001). EPIC has also reported preliminary evidence of a moderate protective effect of vegetables and an even stronger protective effect of fiber against CRC onset (Boker et al. 2001; Riboli et al. 2002).

Alcohol consumption has been shown to contribute to MSI, unlike any other dietary factors usually associated with CRC risk (Slattery et al. 2001). On the other side, alcohol consumption in relation to CRC risk remains controversial, with some studies reporting a positive association (Barbou et al. 2002; Sharpe et al. 2002; Shimizu et al. 2003), whereas others do not (Breuer-Katschinski et al. 2000; Ye et al. 2003).

Cigarette smokers, in particular those with a long history of smoking, appear to be at increased risk for colorectal adenoma and CRC (Botteri et al. 2008; Liang et al. 2009). It

has been estimated that approximately 21% of MSI in colon tumors may be attributable to cigarette smoking (Slattery et al. 2000).

Some defined risk factors for CRC development are summarized in **Table 1**.

Table 1. Risk factors and causes of sporadic CRC

Older age
Male sex
Cholecystectomy
Uterocolic anastomosis
Hormonal factors: nulliparity, late age at first pregnancy, early menopause
Personal history of sporadic tumours
History of colorectal polyps
History of colorectal cancer (risk is 1.5–3% for developing a second cancer in first the 5 years)
History of small bowel, endometrial, breast, or ovarian cancer
Environmental and lifestyle factors
Diet rich in meat and fat, and poor in fibre, folate, and calcium
Sedentary lifestyle
Obesity
Diabetes mellitus
Smoking
Previous irradiation (eg, X rays, UV, etc.)
Occupational hazards (eg, asbestos exposure)
High alcohol intake
Colorectal cancer in inflammatory bowel disease
Ulcerative colitis
Crohn´s colitis

1.5 Genetic predisposition to CRC

CRC is traditionally divided into sporadic and familial (hereditary) forms. A majority of CRC is sporadic or shows a pattern of familial aggregation not fitting into Mendelian model of inheritance (de la Chapelle 2004). Typical clinical signs of families prone to cancers are multiple affected close relatives, an early age of onset and multiple primary tumors (Olsson 2003). Familial risk is increased if two or more family members are affected. When having an affected first-degree relative the increased risk of CRC is about two–fold as compared to the general population (Johns et al. 2001; Hemminki et al. 2008). According to a twin study combining data on 44 788 pairs of twins listed in the Swedish, Danish, and Finnish twin registries, 35% of variation in CRC was assigned to heritable factors (Lichtenstein et al. 2000). The genetic basis of familial CRC has been actively investigated in the last decades. The two main autosomal dominantly inherited

CRC forms known are: Familial Adenomatous Polyposis (FAP) and Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) syndromes. *APC* and mismatch repair (MMR) genes were found to initiate FAP and HNPCC, respectively (Cheach 2008). The Amsterdam criteria (I and II) identify families likely to have FAP and HNPCC.

However, despite a relatively large estimated genetic contribution, mutations in single high penetrance genes have been identified in approximately only 5% of all CRC, leaving the majority of the genetic burden unexplained. In the case of sporadic CRC forms, the remaining genetic predisposition might be attributable to low penetrance genes acting in concomitance with environment and lifestyle factors.

1.5.1 Importance of low-penetrance alleles in CRC risk

While high penetrance mutations have been identified in the hereditary forms of the disease, the greater part of inherited predisposition to sporadic CRC probably involves interactions between low penetrance susceptibility alleles and environmental factors (de la Chapelle 2004; Ahmed et al. 2006; Houlston et al. 2008). The identification of common variants (i.e. occurrence in the same population of multiple discrete allelic states of which at least two have high frequency, conventionally of 1% or more), also called genetic polymorphisms, has stimulated hypotheses to explain the high degree of observed individual variability in cancer susceptibility (Vineis 2004). When the penetrance of an allele (i.e. the frequency of expression of an allele, when it is present in the genotype) is low (i.e. less than 25%) the locus is usually difficult or impossible to identify by linkage analysis, because too many unaffected individuals who carry the disease allele will confound the calculations. Indeed, when the penetrance is very low (for example, conferring a two fold-risk), there are almost no families in which the mutated allele co-segregates with the phenotype and evidence for low penetrance alleles is therefore usually investigated by genetic association studies (de la Chapelle 2004).

Some high penetrance CRC predisposing genes most certainly remain to be yet detected. However, it is of general belief that low penetrance variants are responsible for a large proportion of the genetic predisposition to CRC. Numerous single nucleotide polymorphisms (SNPs) and other common variants have been investigated for association with an increased risk for CRC, according to the common variant-common disease model for polygenic predisposition. An association has been found in approximately one third of all published studies, however the replication rate has been very low for most significant

findings (Houlston and Tomlinson 2001). Genome-wide association (GWAs) studies have identified multiple loci at which common variants modestly influence the risk of developing CRC (Houlston et al. 2008). While individual alleles exert only small a effect, much larger risk is seen in carriers of multiple risk alleles (Houlston et al. 2008).

1.5.2 Identification of low penetrance genes

Association studies are performed to compare the frequency of a genetic variant in affected individuals and individuals without the disease, and they have been initially considered as a promising method for identification of low penetrance alleles (Cardon et al. 2001). The case-control study is the most commonly used population-based study design for searching allelic associations. To date, most association studies were based on the candidate gene approach and have only evaluated a restricted number of polymorphisms, primarily in genes implicated in the metabolism of dietary carcinogens and protection of DNA. Reports from these studies have largely been disappointing with numerous positive associations initially reported being unconfirmed by subsequent analyses. The chance of success for candidate gene association studies can be greatly improved by careful selection of both candidate genes and candidate polymorphisms (Pico et al. 2009). The dbSNPs database (http://www.ncbi.nlm.nih.gov/projects/SNP) now contains nearly 9 million SNPs, including most of the around 11 million SNPs with minor allele frequency of 1% or greater that are estimated to exist in the human genome (Crawford and Nickerson 2005). It has been known that many SNPs have alleles that show strong linkage disequilibrium (LD) with other nearby SNP alleles (see **Appendix**). One tagged SNP can thereby serve for many others in an association screen. Once the patterns of LD are known for a given region, a few tagSNPs can be chosen, individually or in multimarker combinations (haplotypes), to capture most of the common variation within that region (Crawford and Nickerson 2005). Characterization of patterns of LD across the human genome is an area of highly active research and it has been shown that, for case-control studies, haplotype-based methods can be more powerful than single-locus analyses when the SNPs are in LD with causative diallelic locus (Crawford and Nickerson 2005; Lee 2006).

However, the candidate-gene association study will, at best, identify only a fraction of genetic risk factors even for diseases in which the pathophysiology is relatively well understood (Hirschhorn et al. 2005). To fully understand the allelic variation that underlies complex diseases, a complete genome sequencing for many individuals with

and without disease is required (Wang et al. 2005). The discovery, through GWA scans, of a number of polymorphisms and loci that are associated with the disease susceptibility has provided an indication about the role of low-penetrance variants in the disease etiology (Kemp et al, 2006; Zanke et al, 2007; Houlston et al. 2008; Jaeger et al. 2008; Tomlinson et al, 2007; 2008; Tenesa et al. 2008).

Several studies have shown that the genes with pivotal roles in cell cycle regulation and DNA repair may modulate the risk and onset of sporadic CRC (Koushik et al. 2006; Naccarati et al, 2007; Houlston et al. 2008).

1.6 Cell cycle and DNA repair

The cell cycle comprises a series of tightly controlled events that drive the replication of DNA and cell division (**Figure 6**). It is divided into several phases: preparation for (G1 phase), DNA synthesis (S phase), a second gap phase (G2), and mitosis (M). Quiescence (G0) is a biochemically distinct state from which cells can re-enter the cell cycle and proceed to DNA replication and mitosis. Resting (nondividing) cells are in the G0 stage of the cell cycle and need to be recruited into the G1 stage and beyond in order to undergo replication. The transitions between these phases are regulated by changes in the activity of specific cyclin-dependent kinases (CDKs), with Cdk1/Cdk2 and Cdk2/Cdk4/Cdk6 controlling the transitions from G2 to mitosis and G1 to S phase, respectively. CDK proteins generally remain at constant levels throughout the cell cycle, while binding partners (such as cyclins) and post-translational modifiers (including kinases and phosphatases) undergo periodic fluctuations to regulate DNA synthesis and cell division. The sequential accumulation of different cyclins allows the formation of specific cyclin-CDK complexes that target substrates involved in transitions between the cell cycle phases (Caldon et al, 2006).

Deregulation of the cell cycle and cell proliferation mechanisms have an important role in carcinogenesis. A number of cell cycle genes, such as cyclins, CDKs, and CDKs regulators, are found frequently mutated in many types of cancer, for example, in breast (Vallian et al. 2009), non-small cell lung (Gautschi et al. 2007), and pancreatic cancers (Chen et al. 2008). In addition, germ-line mutations in several cell cycle control genes such as *Rb1* (Knudson 2002; Pietruszewska et al. 2008), *BRCA1* and *BRCA2* (Venkitaraman 2002; Antoniou et al. 2008), *TP53* (Robles 2001), *NF2* (Reed and Gutmann 2001) and *CHECK2* (Chrisanthar et al. 2008; Kleibl et al. 2008) have been found to cause a strong genetic predisposition to cancer. Several genes (*ATM, BRCA1*

and *BRCA2, NBS1*) mediate close link between the DNA repair and cell cycle. Although thw control of the G2/M transition is implicated in cancer resulting in chromosomal aberrations, the G1/S transition encompasses many of the important cell cycle events that might be specifically altered in CRC, including the actions of the oncogenes/tumor suppressors cyclin E, cyclin D1, and p27 (Caldon et al, 2006).

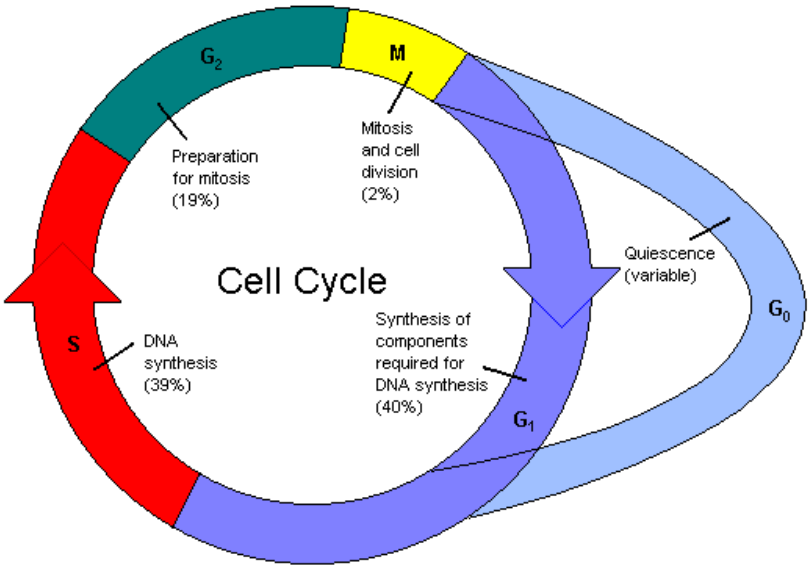


Figure 6. Scheme of cell cycle
(http://www2.mrc-lmb.cam.ac.uk/personal/sl/html/Graphics/CellCycle.gif)

In particular, abnormal expression of the regulatory proteins that control G1/S phase transition, a critical rate-limiting step in cell cycle progression, is frequently observed. G1/S transition requires phosphorylation of the retinoblastoma protein (pRb), which results in the release of the E2F family of transcription factors that in turn activate essential genes for entry into S phase. Phosphorylation of pRb is initiated by cyclin D1/(CDK)4-6 complexes and completed by cyclin E/CDK2 in late G1. Alterations in cyclins and/or CDKs expression result in an increased cell proliferation and are thought to contribute to malignancy (Kumar et al. 2002). CDK inhibitors, including $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p16^{Ink4a}$, normally cause G1 arrest by binding to cyclin-CDK complexes. Down-regulation or inactivation of the CDK inhibitors, are often observed in diverse human tumors, further rendering the cell susceptible to uncontrolled extracellular proliferation signals. p53 is a negative regulator of cell cycle control. It inhibits the cell cycle progression by activating p21 expression (**Figure 7**), and also controls the exit cells

from the cell cycle into programmed cell death (Bali et al, 2004). The gene encoding for p53 is frequently mutated in a wide range of human cancers.

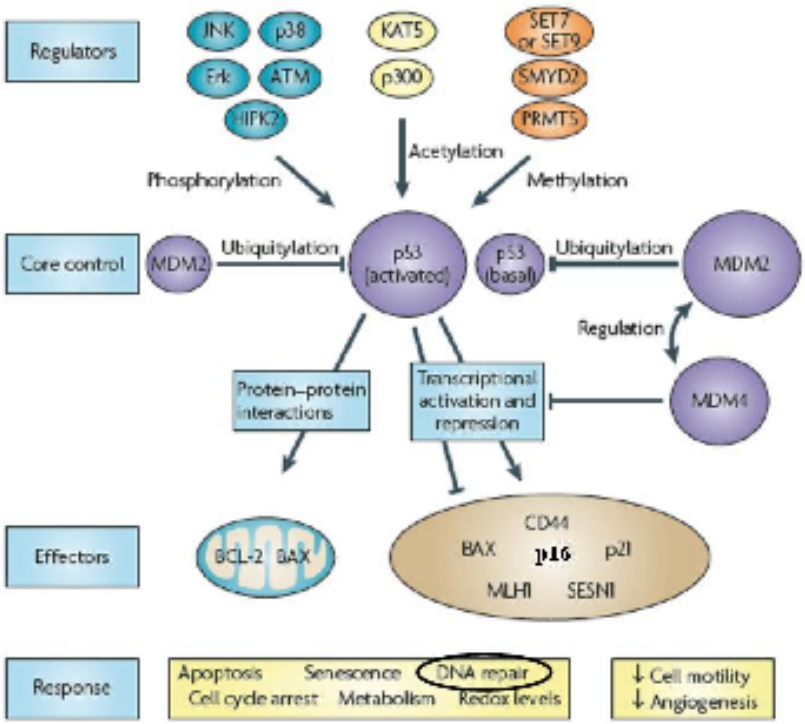


Figure 7. The p53 pathway (adapted from Whibley et al. 2009)

Unrepaired damage may lead to unregulated cell growth and cancer or otherwise can ultimately result in apoptosis. When the DNA damage is recognized by cell machinery, several responses may occur to prevent replication in the presence of genetic errors: cell cycle checkpoints can be activated to arrest the cell cycle, transcription can be up-regulated to compensate for the damage, or the cell can undergo apoptosis. Alternatively, the damage can be repaired at the DNA level enabling the cell to replicate (Branzei and Foiani 2008).

DNA repair is commonly divided into five major pathways: direct damage reversal operated by O(6)-methylguanine-DNA methyltransferase gene (MGMT), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double strand break repair (DSB repair), each dealing with specific types of lesions (Gillet and Schaerer, 2006). Coordination of the DNA repair pathway is controlled through different CDKs (Branzei and Foiani 2008). However, the choice of which repair system to use

depends both on the type of lesion and on the cell cycle phase. The function of direct damage repair pathway throughout the cell cycle is not well understood. Chemical alterations of nucleotide bases are often removed by BER in G1 phase. BER is also involved in removing misincorporated uracils during S phase (Sancar et al. 2004). NER plays an important role during G1 phase to remove bulky lessions, such as those caused by ultraviolet irradiation and polycyclic aromatic hydrocarbons. If left unrepaired during G1 phase, bulky DNA lesions can block DNA polymerases (Branzei and Foiani 2008). Base-base mismatches and small insertion and/or deletion loops that are generated by faulty replication are corrected by the MMR pathway, which functions mainly during S phase (Jiricny 2006). Double-strand breaks (DSBs) that occur during G1 phase are mainly repaired through non-homologous end-joining (NHEJ), whereas DSBs that are formed during S and G2 phase are predominantly repaired by homologous recombination (HR) mechanisms (Branzei and Foiani 2008).

1.7 Cell cycle genes

The regulatory pathways controlling cell cycle phases include several oncogenes and tumor suppressor genes that display a range of abnormalities. It was estimated that some 800 cell cycle-regulating genes might exist (Spellman et al. 1998). The genes were divided into the following five groups: M/G_1 (113 genes), G_1 (300 genes), S (71 genes), G_2 (121 genes), and M (195 genes). Hereby, we will provide a brief description of some of them.

1.7.1 *TP53*

TP53 (MIM# 191170), located at chromosome region 17p13.1, represents one of the most studied tumor suppressor genes. The *TP53* gene contains eleven exons which encode for a 2.8 kb mRNA, translated into a 53kDa protein (Matlashewski et al. 1984, Harlow et al. 1985). Its product, the p53 protein, is referred to as 'the guardian of the genome', and acts as a key regulator of cellular growth control (Pietsch et al, 2006). p53 is a phosphoprotein, encoded by 393 amino acids forming five highly conserved regions and four functional domains (Harris and Hollstein, 1993). In response to a variety of stress signals (including genotoxic stress, and oncogene activation), the p53 protein is post-translationally stabilized, leading to its activation as a sequence-specific transcription factor. This stabilization can then result in different programs, depending on the cell of origin or cellular context, and include cell cycle arrest, senescence, or

apoptosis (Pietsch 2006). As a tumor suppressor, p53 is essential for preventing inappropriate cell proliferation and maintaining genome integrity in relation to genotoxic stress. Following various intracellular and extracellular insults, such as DNA damage (induced by ionizing radiation, UV radiation, xenobiotics, application of cytotoxic drugs or chemotherapeutic agents, and various viruses), heat shock, hypoxia, and oncogene overexpression, wild-type p53 is activated and emerges as a pivotal regulatory protein which triggers diverse biological responses, both at the level of a single cell as well as in the whole organism. p53 activation involves an increase in overall p53 protein level as well as qualitative changes in the protein through extensive post-translational modification, thus resulting in activation of p53-targeted genes. For example, in response to DNA DSBs, ataxiatelangiectasia mutated (ATM) protein kinase is activated and this in turn activates Chk2 kinase. Both ATM and Chk2 then phosphorylate p53 at distinct sites, leading to p53-dependent cell cycle arrest or apoptosis (Bai and Zhu, 2006). The ability of p53 to prevent cell growth is pivotal to its tumor suppressor functions. p53 can induce cell cycle arrest in the G1, G2 and S phases of the cell cycle. The induction of cell cycle arrest at G1 and G2 by p53 provides additional time for the cell to repair genomic damage before entering the critical stages of DNA synthesis and mitosis. The arrested cells can be released back into the proliferating pool through p53's biochemical functions that facilitate DNA repair (Bai and Zhu, 2006).

TP53 is well-known as the most frequently mutated gene in human cancer, i.e. in more than 50% of tumors, including CRC (Ilyas and Tomlinson, 1996; Losi et al. 2005). The mutations are usually single base substitutions that disrupt function, and some of them confer new oncogenic (gain-of-function) properties. Some of these mutations have already been correlated to specific clinical phenotypes, such as specific mutation site of p53 gene may be important in assessing recurrence risk in bladder cancer (George et al. 2007). It is therefore conceivable that the existence of natural variants of *TP53* could be linked with the development of specific diseases, owing to differences in the activity of variant proteins in this pathway, and could then represent an interesting predictive marker for CRC susceptibility.

TP53 common variants

The *TP53* gene represents one of the most studied tumor suppressor genes in biology and over 200 SNPs in *TP53* have been identified. In contrast to tumor-associated mutations, most of these *TP53* SNPs are unlikely to have biological effects (http://www-

p53.iarc.fr/). A great number of these natural variants is localized in non-coding regions (introns) of the gene (**Figure 8**). The most widely studied polymorphisms in *TP53* are a 16bp duplication in intron 3 (PIN3) and the *TP53* Arg72Pro.

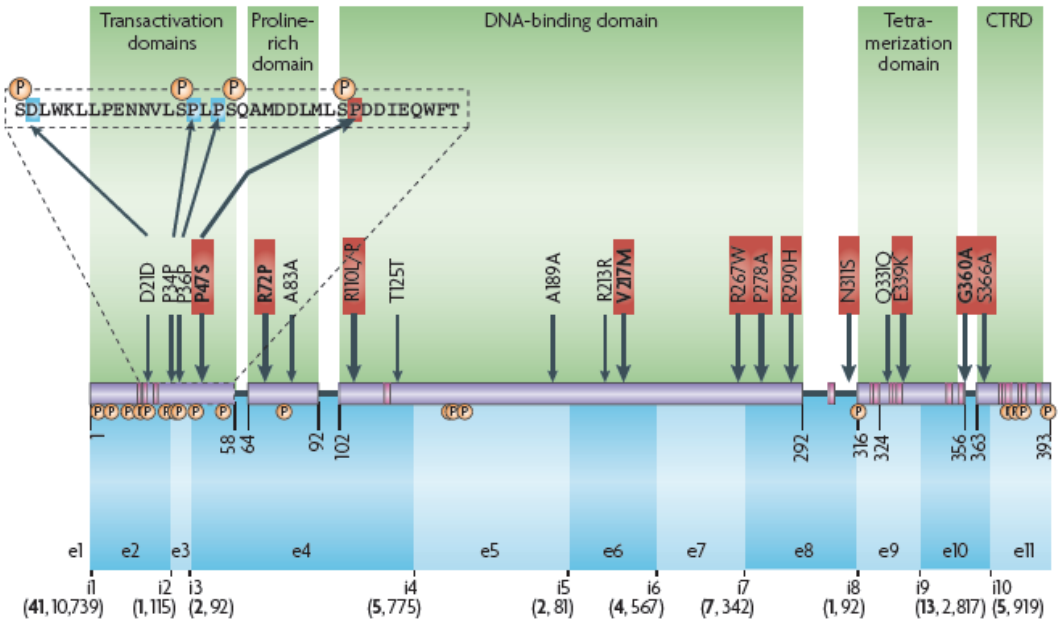


Figure 8. *TP53* single nucleotide polymorphisms: locations in the p53 protein and DNA sequences (Whibley et al. 2009)

Some of these germ-line variations have also been associated with an increased risk of cancer development. The functional impact of individual *TP53* polymorphisms may be a consequence of a particular variant or may appear as a result of the linkage to other, functionally significant, polymorphisms of *TP53*.

Below we will briefly describe the two most studied genetic polymorphisms of *TP53*.

TP53 Arg72Pro polymorphism

This common SNP occurs in a non-conservative G to C transversion in codon 72 of exon 4, with an amino acidic change from arginine to proline that results in a structural change of the protein giving rise to variants of distinct electrophoretic mobility (Harris et al, 1986; Matlashewski et al, 1987). This polymorphism occurs in a proline-rich region of p53, which is known to be important for the growth suppression and apoptotic functions of the protein (Walker and Levine, 1996; Soussi and Lozano 2005). Beckman and co-workers (1994) demonstrated that the frequency of the 72Pro allele differs with geographic latitude, increasing in a linear manner in populations near the equator. These observations led the authors to suggest that the codon 72 variants differed in biological

activity, and further that these differences in activity might be subject to selection in areas of high ultraviolet light exposure.

The Arg72 and 72Pro isoforms of p53 differ from the biochemical and biological point of view. Arg72 variant of p53, when in *cis* form with certain tumor-derived mutations, might have enhanced tumor suppressive function owing to increased ability to inactivate p73. Subsequent studies suggest that the ability of Arg72 to target and inhibit p73 may be cell-type dependent (Vikhanskaya et al. 2005). Specifically, these authors demonstrated that some of the p53 tumor derived mutants that are unable to bind and inhibit p73, are still able to confer resistance to drug treatment. This result may suggest that Arg72-containing mutants may possess other mechanisms to disrupt chemotherapy-induced apoptosis (Pietsch 2006).

In summary, the combined data from several investigators have confirmed the altered apoptotic potential of the codon 72 polymorphic variants, with the Arg72 variant demonstrating enhanced apoptotic ability, and the 72Pro variant showing enhanced growth arrest capacity (Bonafe et al. 2002; Dumont et al. 2003; Pim and Banks 2004). Based on these findings, a number of studies have tried to establish a correlation between the *TP53* Arg72Pro polymorphism and the risk to develop certain types of cancer. In general, these studies have not yielded consistent results. This may be due to the fact that the simultaneous presence of *TP53* Arg72 allele in mutated form of *TP53* may be an enhanced tumor development predictor (increasing inactivation of p73). On the other hand, when this allele is found alongside with not mutated form of *TP53*, it might increase apoptotic ability.

The other cancer-related phenotype that has been studied in relation to the *TP53* Arg72Pro polymorphism is prognosis or response to treatment. An earlier median age of onset of squamous cell carcinoma of the head and neck, HNPCC and oral cancer has been reported in patients homozygous for the *TP53* 72Pro allele (Shen et al. 2002; Jones et al. 2004). Patients with breast, lung or head and neck cancer and homozygous for the *TP53* Arg72 allele were reported to have higher response rates and survival after receiving chemotherapy and radiotherapy (Sullivan et al. 2004; Nelson et al. 2005; Tommiska et al. 2005; Xu et al. 2005). Many studies on CRC patients have shown evidence of the prognostic value of *TP53* mutations/loss of function and its association with worse survival (Etienne et al. 2002; Russo et al. 2005; Iacopetta et al. 2006). No robust evidence or consensus about predictive role of p53 in relation to treatment is yet available, and therefore p53 is not routinely used in clinical practice.

Intron 3 (PIN3) polymorphism

Among all the other polymorphisms identified in the *TP53* gene, only the polymorphism in intron 3 (PIN3, A1 allele is the common one and allele A2 is referred to a 16 bp duplication) has been frequently studied. However, only a single study has demonstrated an altered activity of this natural variant. Harboring the assumption that the *TP53* PIN3 A2 variant allele might influence alternative splicing of p53, Gemignani et al. (2004) reported a reduced amount of steady-state RNA for this allele in immortalized lymphoblastoid cell lines, relative to wild type. These results were re-capitulated with mRNA extracted directly from patient lymphocytes. Other investigators have reported that the A2 variant allele is associated with decreased apoptotic and DNA repair capacity in lymphoblastoid cell lines (Wu et al. 2002). Consistent with these altered functional activities, several studies have correlated the intron 3 duplication with an increased risk of various cancers, including CRC (Gemignani et al. 2004), lung (Wu et al. 2002), breast (Weston and Godbold 1997; Wang-Gohrke et al. 1999; Powell et al. 2002), and ovary (Runnebaum et al. 1995; Wang- Gohrke et al. 1999). However, other groups have failed to confirm these results (Khaliq et al. 2000; Mitra et al. 2005).

1.7.2 *p21 (CDKN1A)*

p21 (also known as *Waf1* or CIP1) gene, (MIM# 116899) located on chromosome region 6p21.2, encodes a potent cyclin-dependent kinase inhibitor and plays an important protective role, being involved in the apoptotic pathway, regulating cellular arrest in the presence of DNA damage. p53 up-regulates *p21* expression in response to DNA damage leading to cell cycle arrest at the G1 checkpoint (Xiong et al. 1993). An unstable or altered p21 protein could modify the cellular response to genomic injury and abolish the effect of *p21*. *p21* expression results in inhibition of the cyclin-dependent kinases (CDKs), that are essential for cell division. Consequently, cell cycle is arrested at the G1 phase, until genome repair is established (Huang et al. 2004).

As genes with growth suppressive function are frequently prone to mutation in human cancers, several studies have investigated the possibility that the *p21* gene may be mutated in malignancies. With the exception of a few rare cases, including Burkitt´s lymphoma (Bhatia et al. 1995), primary prostate cancer (Gao et al. 1995), primary cervical cancer (Harima et al. 2001) and breast cancer (McKenzie et al. 1997), mutations in *p21* are generally rare (Shiohara et al. 1994; Mousses et al. 1995; Pietsch et al. 2006).

Four polymorphisms in the *p21* gene have been identified and investigated with respect to their effects on cancer susceptibility. Two of these polymorphisms are non-synonymous SNPs located in the *p21* coding region, at codons 31 and 149. The other two polymorphisms are located in the 3´untranslated region (UTR), 20bp from the translation stop codon and in intron 2, 16bp from the 5´splice site (Pietsch et al. 2006).

p21 Ser31Arg polymorphism

This common SNP occurs in a C to A transversion in codon 31 of exon 2 and results in an amino acid change from serine to arginine. Interestingly, like for the polymorphisms in the coding region of *TP53*, the frequency of the Arg allele varies dramatically between major ethnic groups (Birgander et al. 1996; Roh et al. 2004). In Caucasians, the frequency of the Arg allele ranges from 4 to 19% (Koopmann et al. 1995; Birgander et al, 1996; Sjalander et al. 1996; Facher et al. 1997; Lukas et al. 1997; Keshava et al. 2002) while in African and Asian populations the frequency ranges from 22 to 55% (Koopmann et al. 1995; Birgander et al. 1996; Hachiya et al. 1999).

Su et al. (2003b) reported that the Ser31Arg polymorphism may not affect the functional activity of the protein, but may rather alter mRNA expression of *p21*. Authors discussed that the Ser and Arg polymorphic variants have very similar kinase inhibitory activity and growth suppression abilities, therefore they suggested that the Ser31Arg SNP may not affect the structure or the function of the protein (Su et al. 2003b).

The link between this *p21* polymorphism and modulation of cancer risk has been only seldom analysed (Hachiya et al. 1999; Sjalander et al. 1996; Chen et al. 2002). The results so far support the idea that Ser31Arg polymorphism alone is not sufficient to assert cancer risk (Xi et al. 2004).

Since *p21* is a downstream target of *TP53*, several studies have investigated if there is a correlation between the effect of the polymorphisms in these genes. It seems that for a few tumor types, a combination of *TP53* and *p21* polymorphisms correlates with an increased risk of cancer development. For example, the combination of the *p21* Ser31 and the *TP53* 72Pro was found to be associated with an increased susceptibility for development of endometrial cancer in a Korean population (Roh et al. 2004).

p21 C70T polymorphism

This conventional SNP, located in the 3´UTR, results in a transition of C to T. It may have an effect on the protein function and may thus play a role in the development of cancer (Li et al. 2005), but no functional studies have been done yet. Authors observed a nearly 1.5-fold increased risk in squamous cell carcinomas of the head and neck (SCCHN) associated with the combined *p21* TC/TT genotype. The contribution of this polymorphism to genetic susceptibility to SCCHN may be due to an alteration of the mRNA stability, thereby affecting intracellular levels of p21 protein (Li et al. 2005).

1.7.3 *p16* (*CDKN2A*)

The $p16^{INK4}$ gene (*CDKN2A,* MIM# 600160), located at the chromosome region 9p21, encodes for the p16 protein, a member of the INK4 family of CDK inhibitors. p16 competitively inhibits the formation of active cyclin D/CDK4 complexes, thus maintaining the growth inhibitor pRB in an active hypo-phosphorylated state and the cell cycle arrest in G1 (Zhang et al. 2000). The importance of disruption of *p16* expression in cancer has been well documented (Ruas and Peters 1998; Ma et al. 2005). In colorectal tumours, *p16* expression has been shown to be silenced through promoter hypermethylation (Nakayama et al. 2007). However, silencing of expression by hypermethylation of the promoter region can also activate the *p16* gene. For instance, an overexpression of *p16* in the cytoplasm, as well as loss expression of *p16* in the nucleus might be important in the development of normal epithelia to adenoma and adenoma to colorectal carcinoma, respectively (Zhao et al. 2006).

Recent research has shown that the methylation status of *p16* plays an important role in the regulation of angiogenesis (Wettergren et al. 2008). Methylation of *p16* associated with silenced transcription has been found in head and neck, lung, brain, colon, esophagus and bladder cancers (Rocco and Sidransky 2001; Yoo et al. 2008).

Polymorphisms of p16

Whilst somatic disruption of *p16* in tumour cells is well documented (Laud et al. 2006), germline mutations in CRC patients are rare (Zhao et al. 2006). These include a nucleotide 442 G>A change of codon 148 in exon 2, encoding an Ala to Thr change in the C-terminus of the translated protein. Further studied variants are a C>G

polymorphism at nucleotide 500, and a C>T variant at nucleotide 540 in the 3'UTR of the gene (Hussussain et al. 1994; Harland et al. 1997).

p16 polymorphisms have been associated with tumour progression in patients with melanoma: the prevalence of the *p16* G500 allele increased linearly with increasing family risk of melanoma (Aitken et al. 1999; Berggren de Verdier et al. 2006). Although C540G polymorphism is not leading to an amino acid sequence change, it has been associated with tumor susceptibility in a small number of studies. Thus, it has been related to a low expression of *TP53*, more aggressive course of malignant melanoma (Sauroja et al. 2000) and a shortened tumor-specific survival in bladder carcinomas (Sakano et al. 2003), suggesting that this polymorphism may have some functional relevance in other tumor entities, too. Geddert et al. (2005) observed that polymorphisms in *p16* are unlikely to be associated with risk of adenocarcinomas of the upper gastrointestinal tract. In sporadic melanoma patients, the presence of *p16* G500 allele has been correlated to shorter progression time from primary to metastatic disease (Sauroja et al. 2000). McCloud et al. (2004) observed that *p16* polymorphisms were associated with tumour progression in patients with sporadic CRC. In particular, patients with the Ala148Ala and CC500 genotypes were more commonly associated with decreased tumour differentiation and advanced Dukes' stage. No associations between patient carrying C/T540 genotype and clinical prognostic parameters were found.

1.7.4 *Cyclin D1 (CCND1)*

Cyclin D1 gene (*CCND1*; MIM# 168461) located at chromosome 11q13, is involved in the normal regulation of the cell cycle, playing an important role in the transition from the G1 to the S phase. Expression of *CCND1* is induced as a delayed early response to many mitogenic signals, and is universally associated with the transition from the quiescence into the proliferative cycle of the cells (reviewed by Knudsen et al. 2006). Among the cyclins that regulate G1 progression, it is hypothesized that stimulation of *CCND1* expression represents the point at which mitogenic signal of transduction cascades are integrated to mediate engagement of the cell cycle machinery (Knudsen 2006).

Amplification or overexpression of the *CCND1* gene is frequently observed in a variety of cancers, where it may induce proliferation (Kong et al. 2001, Ahmed 2006). The *CCND1* locus is known to be amplified in specific tumor types and it is thought that this

event determines a net increase in the proto-oncogenic functions of the *CCND1* protein (Knudsen et al. 2006).

CCND1 Pro870Pro polymorphism

Over 100 single nucleotide polymorphisms have been identified spanning the *CCND1* locus and catalogued in public SNP databases (dbSNP: www.ncbi.nlm.nih.gov/SNP/ HapMap: www.hapmap.org; or GeneSNPs: www.genome.utah.edu/genesnps/). However, the *CCND1* G870A (Pro241Pro) polymorphism, is the most thoroughly investigated so far. This common SNP in exon 4 is associated with the presence of 2 distinct mRNA transcripts for CCND1 protein (Sawa et al. 1998). This polymorphism could then act both as a modifier of phenotypic expression in the inherited CRC and as a low-risk susceptibility factor in the sporadic CRC.

The frequency for this polymorphism in the Caucasian population is approximately 44% for the A allele and 56% for the G allele (Simpson et al, 2001; Sanyal et al, 2004), but a large variation has been reported between ethnic groups (Pakakasama et al. 2004).

Owing to the significance of *CCND1* in human cancer, a large number of epidemiological studies have investigated the influence of this particular polymorphism on cancer susceptibility and disease outcome. These studies generally compared the allelic frequency of G870A polymorphism in disease affected or unaffected individuals, and assessed correlations of genotypes with clinical parameters (e.g. stage at diagnosis or overall survival). The majority of studies related the A-allele to an increased cancer risk and a poor disease outcome, with the largest associations observed for the AA genotype. In these studies, relative risks were significant but typically modest, with many of them reporting less than a two-fold effect. However, results have been inconsistent and some studies have also related the G-allele to an increased cancer risk, while others have ascribed no significant value to any allele of the G870A polymorphism, as reviewed by Pabalan et al. (2008). Combined, these results indicate that individual alleles may harbor differential effects in distinct tumor types. However, even within a specific tumor type (e.g. CRC) there have been different conclusions regarding the role of this polymorphism. These discrepancies could reflect either the characteristic of patient under study or the possible involvement of external factors (e.g. smoking or obesity) that have been suggested to interact with the polymorphism in specific studies (Buch et al, 2005; Shu et al, 2005).

Important issues that remain to be solved are whether the G870A polymorphism is strongly associated with CRC per se, or due to the linkage to other biologically relevant polymorphisms. Such a possibility may in part explain some of the discrepancies associated with the results of G870A in cancer risk in different study populations and may emphasize the importance of conducting LD or haplotype-based investigations of common genetic variation across the entire locus.

One group has analysed both the G870A and the G1722C polymorphism of *CCND1*. This study indicated that the two polymorphisms are in LD and individuals harboring the A870-allele most likely also carry the C1722 allele. In spite of this observation, each polymorphism had a distinct influence on head and neck cancer, suggesting that other variants in addition to the G870A variant in *CCND1* may be important (Holley et al, 2001).

Although there is significant a evidence that G870A polymorphism may alter cancer risk, more exhaustive studies are required to fully understand the involvement of this polymorphism in cancer (reviewed by Knudsen et al, 2006).

1.8 Susceptibility to CRC: the role of common variants in cell cycle and DNA repair genes

A small proportion of all CRC cases is due to inherited mutations in genes. Familial forms of CRC are likely to be due to a combination of environmental factors, rare gene mutations with high penetrance and more common low penetrance gene variants acting together to alter disease susceptibility.

The identification of genetic polymorphisms has stimulated hypotheses to explain the high degree of observed individual variability in cancer susceptibility. The successful sequencing of the human genome has provided the identification of a large number of low-penetrance alleles and loci.

Most association studies have focused on polymorphisms in genes involved in biologically defined mechanisms such as cell cycle and DNA repair pathways.

The deregulation of the cell cycle leads to uncontrolled cellular proliferation (Malumbres and Barbacid, 2001; 2007; 2009). Inappropriate expression (and/or mutations) in cell cycle genes occurs frequently in human disease, such as cancer (Savas et al. 2005). As previously observed, the putative functional effects of several polymorphisms in cell cycle genes may influence susceptibility to CRC, during transition from normal epithelium to CRC (**Figure 9**).

Genetic polymorphisms involved in different DNA repair pathways may modulate the individual repair capacity in response to DNA damage, and then may have also an impact on the individual genetic susceptibility to sporadic CRC (Naccarati et al. 2007).

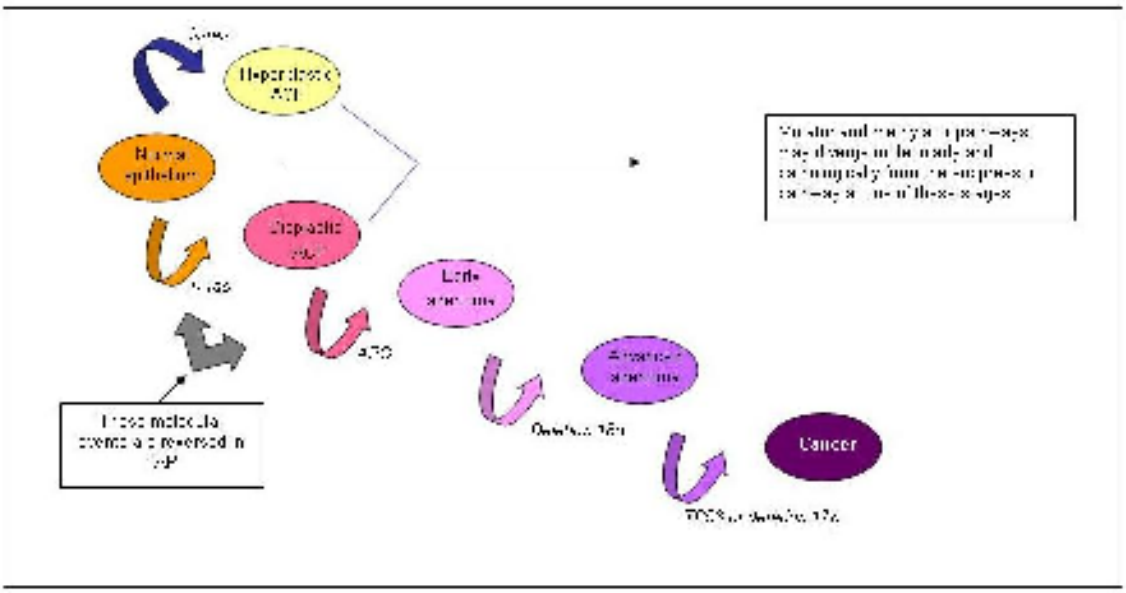


Figure 9. Sequential pathological stages and molecular events of sporadic CRC with indication of involved genes; ACF – aberrant crypt focus (adapted from Worthley 2007)

Results from previous studies, based on association between the most investigated *TP53* polymorphisms (PIN3 and Arg72Pro) and the risk of CRC, have been mainly inconsistent.

Sjalander et al. (1995) found a significant reduction of CRC risk in the *TP53* PIN3 A_2 carriers, and a non-significantly increased risk in carriers of variant 72Pro allele of the Arg72Pro polymorphism. On the contrary, A_2 allele of the PIN3 polymorphism was found to be associated with an increased risk in a Spanish population and the 72Pro allele was associated with a marginally increased CRC risk (Gemignani et al, 2004). Other recent studies on *TP53* Arg72Pro, in relatively small populations (not exceeding 120 individuals per group), have reported an association of the 72Pro allele with increased CRC risk (Goodman et al. 2006; Perfumo et al. 2006; Jia et al. 2007; Zhu et al. 2007; Cao et al. 2009). This pattern of risk should be interpreted with some caution as much larger study populations are required to confirm this observation. Whereas, results obtained by Gemignani et al. (2004), Jia et al. (2007) and Zhu et al.(2007) could be taken as reliable due to large and well-characterized population. Jia et al. (2007) observed that the carriers of 72Pro allele had a higher CRC risk than the 72Arg allele carriers.

In several studies the Arg allele has been found associated with an increased CRC risk (Schnieder-Stock et al. 2004; Lima et al. 2006; Perez et al. 2006; Dakouras et al. 2008; Wang et al. 2008). However, all these studies were performed on study population with a small number of patients (from 53 to 121 patients with sporadic CRC).

Three larger and well-designed studies (Koushik et al. 2006; Tan et al. 2007 and Polakova et al. 2009) have reported discordant results. Koushik et al. (2006) and Polakova et al. (2009) did not find any association between *TP53* Arg72Pro polymorphism and CRC risk, but Koushik et al. (2006) observed a moderate association of 72Pro allele with the risk of adenoma. Conversely, Tan et al (2007) reported a decreased CRC risk for the carriers of the 72Pro allele in 467 cases and 563 controls. The same authors suggested that genetic factors such as *TP53* polymorphisms may play a role in predicting response to chemotherapy and that there is a need to determine the mechanism by which *TP53* haplotypes may modify the CRC risk (Tan et al. 2007).

Polymorphisms in other cell cycle control genes have been studied less extensively. Some studies have previously investigated the epigenetic changes in various genes, including *CDKN2A,* in CRC patients (Miranda et al. 2006; Kawakami et al. 2006). The role of polymorphisms in these genes for CRC susceptibility has not been found conclusive (McCloud et al. 2004; Polakova et al. 2009). As mentioned previously, the *CCND1* G870A polymorphism could act both as a modifier of phenotypic expression in inherited CRC and as a low-risk susceptibility factor in sporadic CRC. Although still no clear and unambiguous conclusions can be drawn so far, several studies have observed an association between 870A variant and an increased risk of adenomas (Lewis et al. 2003) and CRC (Kong et al. 2001; Porter et al. 2002; Le Marchand et al. 2003; Jiang et al. 2006), despite in different ethnic groups. On the contrary, three studies did not find any association (Grieu et al. 2003; Schernhammer et al. 2006; Probs-Hensch et al. 2006), and one observed an inverse association with the 870G allele (Hong et al. 2005b). The recent results suggested that SNPs around p14 promoter region may be responsible for the interindividual susceptibility to p14 promoter methylation among individuals with CRC (Kang et al. 2008). Germline variant in MDM2 SNP309 gene did not play a role in the development of very early onset of CRC (Khan et al. 2008).

Over the last 10 years, a growing number of studies have investigated the role of DNA repair in the CRC onset (Naccarati et al. 2007). The identification of germline mutations in BER gene *MUTYH* in individuals with a predisposition to multiple colorectal adenomas and carcinomas has highlighted the relevance of DNA repair in CRC

development. Moreover, mutations in MMR genes are known to segregate in families with HNPCC.

Hereby we summarize the main results from association studies between genetic polymorphisms in the main DNA repair pathways and risk of CRC/adenomas. The outcomes from the association studies on polymorphisms of NER genes do not show any strong and straight association with CRC risk. The most frequently studied polymorphism, *XPD* Lys751Gln, provided significant associations only with adenoma risk (Bigler et al. 2005; Skjelbred et al. 2006).

Regarding BER pathway, the majority of studies analyzed variants in *XRCC1* gene. Two recent large studies, comprising 980 cases and 1200 controls, reported a decreased risk for adenomas in association with variant allele of *XRCC1* Arg399Gln (Stern et al. 2005; Skjelbred et al. 2006), whereas in considerably smaller populations CRC risk was moderately increased in association with this variant allele (Abdel-Rahman et al. 2000; Hong et al. 2005a). Discrepancies between premalignant adenoma and CRC are difficult to explain, but adenoma risk was studied on a significantly larger and better characterized populations. Although *hOGG1* Ser326Cys is one of the most frequently analysed BER polymorphisms, the outcomes remain inconclusive (Kim et al. 2003; Hansen et al 2005). In addition, there are few reports on *APE1* polymorphism and CRC risk, where no significant association was found (Moreno et al. 2006).

Regarding the DSB pathway, results based on association between *XRCC3* polymorphisms and the risk of CRC have mainly been inconsistent (Mort et al. 2003; Krupa and Blasiak 2004; Jin et al. 2005; Stern et al. 2005; Goodman et al. 2006). No association of *NBS1* (recently known as *NBN*) polymorphism with colon cancer risk was reported by Goodman et al. (2006).

Tranah et al. (2006) observed that *MGMT* 143V allele was associated with decreased risk of CRC in cohort of women, however no association was found in other studies on *MGMT* polymorphisms (Moreno et al. 2006; Goodman et al. 2006; Bigler et al. 2005.

There are still limited data on MMR polymorphisms and CRC risk for drawing any conclusion at present. Berndt et al. (2007) found significant associations with *hMSH3* SNPs and Yu et al. (2006) found a possible modifying effect of smoking for hyperplastic polyp risk in *hMLH1* -93A carriers. Otherwise, the studies did not reveal clear positive associations. Interestingly, polymorphisms in *EXO1* seem to modulate inversely CRC risk, but only one study is available (Yamamoto et al. 2005). These results should be confirmed on larger, ethnically homogeneous populations.

In spite of the fact that DNA repair and cell cycle regulating systems act in concordance to preserve genome integrity, few studies so far have addressed polymorphisms in candidate genes covering above important pathways.

2. Hypothesis and aims and of the study

The hypothesis of this PhD Thesis is based on the following assumptions: A) The deregulation of cell cycle and alterations in DNA repair play an important role in carcinogenesis. Inappropriate expression of the genes involved in these pathways occurs frequently in various cancers, including CRC. Thus, common variants in the genes with pivotal roles in cell cycle regulation and DNA repair may modulate the risk and onset of sporadic CRC, especially as there is a close interplay between these two important pathways. B) CRC represents a serious health problem in the Czech Republic, as its incidence ranks the fourth highest worldwide and the incidence of rectal cancer is the highest.

The present PhD work has aimed to investigate the following topics:

1. The risk of sporadic CRC in association with common variants in relevant cell cycle control genes *TP53, p21*, *p16* (**Polakova et al. 2009 Publication 1**), and *CCND1*.

2. The risk of CRC in association with common variants in important DNA repair genes *XPD*, *XPG*, *XPC*, *XRCC1*, *hOGG1*, *XRCC3*, *APE1* and *NBN1* (**Pardini et al. 2008 Publication 2**).

3. The role of common haplotypes within the analysed cell cycle genes reconstructed from selected variants on the risk of sporadic CRC (**Publication 1, Polakova et al., manuscript in preparation**).

4. The modulating role of particular binary genotype combinations of variants in genes involved in cell cycle regulation and in DNA repair.

5. The frequency of *NBN* 657del5 mutation, high in the Slavic population, in association with CRC susceptibility (**Pardini et al. 2009, Publication 3**).

3. **Material and Methods**

3.1 Study population

The study population initially comprised 532 patients with CRC and 532 hospital-based healthy controls frequency matched for sex and age (**Publication 2**) and rose to 614 patients with CRC and 614 controls frequency matched for sex and age (**Publication 1**) over the time of the PhD. thesis. Eligibility criteria for participation in the study included cases and controls that were aged 29 years or more, were of Czech origin, and consented to provide biological samples for genetic analysis. Cases with histologically confirmed CRC diagnosis were recruited (between September 2004 and February 2006) among patients, visiting nine oncological departments all over the Czech Republic (two in Prague, the others in the towns of Benesov, Brno, Liberec, Ples, Pribram, Usti nad Labem, and Zlin), as representative of the entire country. During the study period, a total of 968 cases with CRC provided blood samples from the above mentioned hospitals. Sixteen individuals were initially excluded because they met the Amsterdam criteria I and II (Vasen et al, 1991, 1999) for hereditary CRC.

Controls were selected from individuals admitted to five large gastroenterological departments (Prague, Brno, Jihlava, Liberec, and Pribram) all over the Czech Republic at the time when cases were being recruited (Control Group I). The control subjects were undergoing colonoscopy for various gastrointestinal complaints. The reasons for colonoscopical investigation were i) macroscopic bleeding; ii) positive Fecal Occult Blood Test (FOBT); iii) abdominal pain of unknown origin. Due to the high incidence of CRC in the Czech Republic, colonoscopy is largely recommended and practiced. The control group was composed of subjects with negative colonoscopic results for malignancy or idiopathic bowel diseases (Landi et al. 2008). To reduce selection bias, only those subjects with no previous diagnosis were included into the study. This criterion was used to avoid inclusion of patients with chronic diseases who might be repeatedly admitted to hospital and modify their habits because of their disease. This procedure paralleled the criterion for cases. Among 739 recruited controls, a total of 663 (89.7%) were used for matching for sex and age with CRC patients. As a result, 614 case-control pairs were formed. Thus, 338 cases and 49 controls not fitting as the pairs or with

incomplete lifestyle and potential risk factor information were excluded from initial groups.

The distribution of the considered covariates did not differ between the patients and the controls, with the exception of a small difference in smoking status and education.

Table 2. Characteristics of CRC patients and control subjects

	Cases (*n*=614)	**Controls I (*n*=614)**	**Controls II (n=818)**
Gender			
Males	343	343	473
Females	271	271	345
Age at diagnosis (years)			
Mean±SD	58.63±10.45	57.83±12.42	45.39±23.33
Median	58	58	46
Range	26-84	29-85	30-63
Diagnosis			
Colon cancer	217	-	-
Sigmoidal	156	-	-
Colon + Sigmoidal	373	-	-
Rectal cancer	241	-	-
CRC family history (%)			
Positive CRC history in the family	18.7	16.6	-
Negative CRC history in the family	81.3	83.4	-
Smoking status (%)			
Non-smokers	51.1	53.4	58.68
Ex-smokers >5 years	22.1	20.9	14.43
Ex-smokers <5 years	10.6	4.4	2.69
Current smokers	16.2	21.3	24.20

No of Cigarettes (%)			
< 20 cig/day	58	59.4	-
> 20 cig/day	42	40.6	-
Alcohol (%)			
No	46.5	42.6	-
Yes	53.5	57.4	-
Place of residence (%)			
City	56.7	54.6	-
City + Country	14.7	19.5	-
Country	28.6	25.9	-
Education (%)			
Basic School	34.8	28.5	-
High School	51.5	53.2	-
University	13.7	18.3	-
Body Mass Index			
Mean±SD	26.74±4.39	27.00±4.49	29.80±3.83
< 18.5 (%)	1.5	0.4	0.4
18.5 – 24.9 (%)	36.6	35	40.4
25.0 – 29.9 (%)	43.1	43.1	45.3
30.0 – 39.9 (%)	17.8	19.9	13.5
> 40 (%)	1	1.6	0.4

Recently, only for the **Publication 3,** we used further control population (Control Group II) which has been collected among healthy individuals from a blood donor center in Prague. All individuals were subjected to standard examinations to verify the health status for blood donation (detailed blood count, urinary examination, blood pressure, and general examination). For this study, besides the willingness to participate to it and the healthy status, the collection of samples was limited only due to practical reasons (circa 20 persons per day). The sample collection was performed at the same time as that of the other two study groups previously described. The mean age at the time of sampling was

45 years (median 46; range 30–63). Main characterisctics of the Control Group II are showed in **Table 2**.

The participating subjects were properly informed, signed a written consent and the approval for genetic analysis in accord with the Helsinki declaration. The Ethical Committee of the Institute of Experimental Medicine, Prague, Czech Republic, approved the design of the study.

3.2 Interviews

Cases and controls were personally interviewed by trained personnel using a structured questionnaire to determine demographic characteristics and potential risk factors for CRC. Study subjects provided information on their lifestyle habits, body mass index (BMI), diabetes, and family/personal history of cancer. Lifelong or long-term (at least six consecutive months) drug use questions were also included in the questionnaire.

3.3 Selection of polymorphisms

The polymorphisms within the *TP53* gene were selected using a tag-SNP approach. Phased SNP dataset was obtained from the SNP500 cancer project (http://snp500cancer.nci.nih.gov), where 19 SNPs with minor allele frequency (MAF)>5% have been genotyped in over 30 Caucasians. Phased haplotypes were analysed with Haploview software (http://www.broad.mit.edu/mpg/haploview; (Barrett et al. 2005)). Using the "four gamete rule", the *TP53* locus showed two blocks of LD: one spanning more than half of the first intron and the other encompassing all the remaining parts of the gene. We focused on the latter LD block as it contains all the exons where usually somatic mutations are occurring in cancers. Thus, we analysed the SNPs from rs8079544 (located at the end of the intron 1) to rs35659787 (located at the beginning of the 3' UTR), for a total of 12 SNPs. Setting the minor allele frequency (MAF) at 0.03 with a r^2 threshold at 0.7, three tag SNPs were obtained that included Arg72Pro, IVS7+72 C>T, and Ex11-363 G>A. In the study, we also included the 16bp ins/del polymorphism within the intron 3 (allele A_2 carries the 16bp insertion within the intron 3). For *p21, p16* and *CCND1* genes, we selected SNPs that were common in Caucasians. Within *p21* we chose Ser31Arg polymorphism, a C-to-A transversion within the exon 2 causing an amino acid substitution at codon 31, and Ex3+70 C>T. For *p16* we chose the common nucleotide variant G-to-A in the exon 2, coding for an amino acid change Ala148Thr, and

two other SNPs in 3`UTR region: 3`UTR 500C>G, and 3`UTR 540C>T. For *CCND1* we chose the common nucleotide variant G-to-A in exon 4, Pro242Pro.

The selection of polymorphism of genes involved in the main DNA repair pathways was done on the basis of our recent investigations in healthy population on functional effects on DNA repair capacities (Vodicka et al. 2007), and DNA and chromosomal damage (Musak et al. 2008).

3.4 Genotyping

DNA was isolated from coded blood samples and stored at −80ºC. Genetic polymorphisms of DNA repair and cell cycle were genotyped using either PCR-RFLP or TaqMan allelic discrimination assay.

The polymorphisms in the *TP53* (PIN3 rs17878362, Arg72Pro rs1042522, IVS7 +72C>T rs12947788, Ex11 -363G>A rs17884306), *p16* (3´UTR 500C>G rs11515, 3´UTR 540C>T rs3088440, Ala148thr rs3731249) and *CCND1* (Pro242Pro rs9344) genes were genotyped by PCR-RFLP method (**Publication 1**). The amplified fragments were digested with appropriate restriction endonucleases (**Table 3**). The digested PCR products were resolved and analysed on 3% agarose gels containing ethidium bromide and visualized under UV light.

Table 3. PCR primers and restriction enzymes

Genetic polymorphism	Primer sequence	Restriction enzyme
***TP53* PIN3** (Ins11951_11966)	F CCC CTC TGA GTC AGG AAA CA R GGG ACA GCA TCA AAT CAT CC	—
***TP53* Arg72Pro** (Ex4+119G>C)	F TTG CCG TCC CAA GCA ATG GAT GA R TCT GGG AAG GGA CAG AAG ATG AC	*BstU*I
***TP53* IVS7 +72C>T**	F GTT GGC TCT GAC TGT ACC ACC R GCC GGA AAT GTG ATG AGA	*Eco47I*
***TP53* Ex11-363G>A**	F CTC TTG TAT ATG ATG ATC TG R TCA AAC TCC TGG GCT CAG GC	*NlaIII*
***p16* Ala148Thr** (Ex2-16G>A)	F TGG ACG TGC GCG ATG CCT GG R TCC TCA CCT GAG GGA CCT TC	*SacII*
***p16* rs11515:C>G** (3`UTR 500C>G)	F TTT TCT TTC TGC CCT CTG CA R GAC CTT CGG TGA CTG ATG AT	*MspI*
***p16* rs3088440:C>T** (3`UTR 540C>T)	F GCC TGT TTT CTT TCT GCC CTC TG R CGA AAG CGG GGT GGG TTG T	*HaeIII*
***CCND1* Pro242Pro** (Ex4 -1 G>A)	F GTG AAG TTC ATT TCC AAT CCG C R GGG ACA TCA CCC TCA CTT AC	*MspI*

For the gene polymorphisms of DNA repair: for *XPD* Lys751Gln (rs28365048), *XPG* Asn1104His (rs17655), *XPC* Lys939Gln (rs2228001), *XRCC1* Arg194Trp (rs1799782) and Arg399Gln (rs25487), *hOGG1* Ser326Cys (rs1052133), *XRCC3* Thr241Met (rs861539) polymorphisms PCR was carried out using primers and conditions described by Pardini et al. (2008). The presence of *NBN* 657del5 was tested by allele-specific PCR as described by Pardini et al. (2009).

Genetic polymorphisms in the *p21* gene, the Ser31Arg (rs1801270) and Ex3+70 C>T (rs1059234) were analysed with TaqMan allelic discrimination assay (Applied Biosystems, Foster City, USA, Assay-on-demand, SNP Genotyping products: C_14977_20 and C_7514111_10). The TaqMan genotyping reaction was amplified on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA) (95°C for 10 min, 92°C for 15 sec, and 60°C for 1 min for 40 cycles).

The *APE1* Asn148Glu (rs1130409) and *NBS1* Glu185Gln (rs1805794) polymorphisms were also analysed using the TaqMan allelic discrimination assay (Applied Biosystems, assay-on-demand, SNP genotyping products: C_26470398_10 for *NBS1* and C_8921503_10 for *APE1*).

The genotype screening was performed simultaneously for cases and controls. The results were regularly confirmed by random re-genotyping of more than 10% of the samples for each polymorphism, which yielded concordant results. The genotypes with unclear results were excluded from the data.

3.5 Statistical analyses

Genotype distribution for each polymorphism was tested in controls for Hardy-Weinberg equilibrium and differences in expected and observed frequencies were tested for statistical significance by Pearson chi-square test. Differences in baseline socio-demographic characteristics between cases and controls were analysed using chi-square test and Student's t-test. Multivariate logistic regressions were used to examine the association between variant alleles, genotypes and risk of CRC as well as after stratification for colon and rectal cancers. Odds ratio (OR), 95% confidence intervals (CI) and P-values calculated for risk associated genotypes and variant alleles were adjusted for age and gender. The haplotype frequencies in cases and controls, and the haplotypes carried by each individual (diplotype) were estimated with the SAS/Genetics software module. The analysis was carried out to examine the phase of *TP53*, *p21* and *p16*

polymorphisms using the expectation–maximization algorithm to generate maximum likelihood estimates of haplotype frequencies. Relationships between genotype/haplotypes and the disease risk were summarized as global P-values. Linkage disequilibrium was calculated with Haploview software (www.broad.mit.edu/mpg/haploview/documentation.php). For the haplotypes in the *TP53* gene, selection of genetic models that included one to four polymorphisms was based on Akaike's information criteria (AIC; Akaike 1973). Further, the possible interactions between loci in the same or different genes were explored by multi-dimensionality reduction method (Ritchie et al. 2001).

4. Results

4.1 Allele frequencies

Results on allele frequency analyses for cell cycle genes are reported in **Table 4**. The frequency of variant allele for the *TP53* polymorphism, IVS +72C>T, was lower in CRC cases than in controls, however, the difference observed was not significant. On the other hand, the frequencies of variant alleles for two polymorphisms in the *p16* gene, Ala148Thr and 3´UTR 500C>G, were higher in cases than in controls.

The analyses stratified for a specific cancer site (**Table 5**) showed a non-significantly decreased frequency of the variant allele for the IVS +72C>T polymorphism in *TP53* in colon cases compared to controls, while a significantly increased frequency of the variant allele for the *p16* 3´UTR 500C>G (OR=1.43, 95%CI 1.07-1.91, P=0.02) was observed in rectal cases (**Table 5**).

Moreover, a significantly decreased risk in rectal cases was observed for the variant allele of *CCND1* Ex4 -1G>A (OR=0.82. 95%CI 0.68-1.00, P=0.05) (**Table 5**).

Results on allele frequencies analyses for DNA repair genes are reported in **Table 6**. No significant associations were observed for any of the studied polymorphism, even when stratified for the cancer site (**Table 7**).

4.2 Genotype frequencies

Cell cycle

The distribution of genotypes within the selected genes in the controls was in agreement with the Hardy-Weinberg equilibrium (**Table 4**). No significant differences were found between cases and controls in the genotype frequencies for any of the analysed polymorphisms (**Table 4**). Similarly, the analyses stratified for a specific cancer site (**Table 5**) showed no significant association of the studied polymorphisms with risk of colon cancer. However, we observed that the individuals with the variant A allele for *TP53* Ex11 -363G>A polymorphism were at a decreased risk of rectal cancer (OR=0.58, 95%CI 0.34-0.99, P=0.05), while individuals with the variant G allele genotypes for *p16* 3´UTR 500C>G were at an increased risk of this kind of cancer (OR=1.40, 95%CI 1.00-1.95, P=0.05) (**Table 5**).

DNA Repair

None of the studied DNA repair gene polymorphisms was independently associated with CRC risk in either dominant or recessive model of inheritance (**Table 6).**

The analyses of specific cancer sites (**Table 7**) showed that the carriers homozygous for variant allele of the *APE1* Asn148Glu polymorphism were associated with an increased risk of colon cancer (OR 1.50; 95% CI=1.01–2.22; P=0.05). When similar analyses were performed on patients with rectal cancer, no independent association with any polymorphism was found (**Table 7**).

NBN 657del5

We have assayed for a 5-basepair-deletion in gene encoding nibrin (*NBN* 657del5), since this alteration may result in non-fully functional protein product. Additionally, *NBN* 657del5 carriage may predispose to an elevated risk of various cancers. In the same population of CRC cases and in two groups of controls (607 from Control Group I and 818 from Control Group II), three of the CRC patients were heterozygote carriers of the *NBN* 657del5, while in the control groups five carriers had been reported. The present results do not show any evidence for an increased risk of CRC in individuals carrying this specific *NBN* mutation (**Publication 3**).

Table 4. Distribution of cell cycle genotypes and results of unconditional logistic regression analysis[a], considering all cases together

Genotypes	Controls[b] (n=614)	Cases[b] (n=614)	OR	95% CI	P-value	X^2,P-value HWE[c]
***TP53* PIN3**						
A_1A_1	447	429	1.00	Referent	0.27	2.08, 0.35
A_1A_2	158	168	1.12	0.86 – 1.45		
A_2A_2	8	15	1.91	0.80 – 4.55		
A_1A_2+ A_2 A_2	166	183	1.16	0.90 – 1.48	0.25	
A_1 allele	1052	1026	1.00	Referent		
A_2 allele	174	198	1.17	0.94-1.46	0.16	
***TP53* Arg72Pro**						
GG	326	327	1.00	Referent	0.49	0.41, 0.82
GC	237	225	0.95	0.75 – 1.21		
CC	49	60	1.22	0.81 – 1.85		
GC+CC	286	285	0.99	0.79 – 1.24	0.94	
G allele	889	879	1.00	Referent		
C allele	335	345	1.04	0.87-1.24	0.61	
***TP53* IVS7 +72C>T**						
CC	548	565	1.00	Referent	0.25	0.09, 0.95
CT	59	43	0.72	0.48 – 1.09		
TT	2	1	0.48	0.04 – 5.36		
CT+ TT	61	44	0.71	0.47 – 1.07	0.10	
C allele	1155	1173	1.00	Referent		
T allele	63	45	0.77	0.48-1.06	0.09	
***TP53* Ex11-363G>A**						
GG	533	553	1.00	Referent	0.08	2.77, 0.25
GA	77	52	0.65	0.45 – 0.95		
AA	0	4	-	-		
GA+AA	77	56	0.70	0.49 – 1.01	0.06	
G allele	1143	1158	1.00	Referent		
A allele	77	60	0.77	0.54-1.09	0.14	

***CDKN1A* Ser31Arg**						
CC	530	542	1.00	Referent	0.22	1.28, 0.53
CA	80	66	0.81	0.57 – 1.15		
AA	1	4	4.18	0.46 – 37.65		
CA+AA	81	70	0.85	0.60 – 1.20	0.36	
C allele	1140	1150	1.00	Referent		
A allele	82	74	0.90	0.65-1.25	0.38	
***CDKN1A* Ex3 +70C>T**						
CC	520	537	1.00	Referent	0.11	1.97, 0.370
CT	89	69	0.75	0.53 – 1.05		
TT	1	4	4.13	0.46 – 37.21		
CT+TT	90	73	0.79	0.56 – 1.10	0.16	
C allele	1129	1143	1.00	Referent		
T allele	91	77	0.84	0.61-1.15	0.27	
***CDKN2A* Ala148Thr**						
GG	579	565	1.00	Referent	0.35	0.36, 0.83
GA	29	40	1.45	0.88 – 2.39		
AA	0	2	-	-	-	
GA+AA	29	42	1.52	0.93 – 2.50	0.10	
G allele	1187	1170	1.00	Referent		
A allele	29	44	1.57	0.97-2.56	0.06	
***CDKN2A* 3´UTR 500C>G**						
CC	457	431	1.00	Referent	0.16	0.46, 0.80
CG	138	159	1.24	0.95 – 1.61		
GG	13	18	1.59	0.76 – 3.35		
CG+GG	151	177	1.27	0.98 – 1.64	0.07	
C allele	1052	1021	1.00	Referent		
G allele	164	195	1.25	1.00-1.57	**0.05**	
***CDKN2A* 3´UTR 540C>T**						
CC	526	528	1.00	Referent	0.61	2.89, 0.23
CT	76	80	1.05	0.75 – 1.47		
TT	6	3	0.51	0.13 – 2.06		
CT+TT	82	83	1.01	0.73 – 1.41	0.95	
C allele	1128	1136	1.00	Referent		

T allele	88	86	0.97	0.72-1.33	0.87	
***CCND1* Pro242Pro**						
GG	159	157	1.00	Referent	0.56	2.72, 026
GA	325	301	0.94	0.72 – 1.23		
AA	127	154	1.23	0.89 – 1.69		
GA+AA	452	455	1.02	0.79 – 1.32	0.88	
G allele	643	615	1.00	Referent		
A alele	579	609	1.01	0.94 – 1.29	0.26	

OR, odds ratio; CI, 95% confidence interval. Significant P-values are in bold

[a] Adjusted for age and sex.

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

[c] X^2 and P-values for the deviation of observed and the numbers expected from the Hardy-Weinberg equilibrium (HWE) in the controls

[d] Allele A_2 carries the 16bp insertion within the intron 3

Table 5. Distribution of cell cycle genotypes and results of unconditional logistic regression analysis[a] for CRC patients according to stratification for tumor location

Genotypes	Controls (n=614)[b]		Colon cases (n=373)[b]	OR	95% CI	P-value		Rectal cases (n=241)[b]	OR	95% CI	P-value
***TP53* PIN3**											
A_1A_1	447		260	1.00	Referent			169	1.00	Referent	
A_1A_2+ A_2 A_2	166		112	1.17	0.88 – 1.56	0.29		71	1.13	0.81 – 1.58	0.46
A_1 allele	1052		623	1.00	Referent			403	1.00	Referent	
A_2 allele	174		121	1.18	0,92-1.52	0.20		77	1.11	0.87-1.40	0.41
***TP53* Arg72Pro**											
GG	326		189	1.00	Referent			138	1.00	Referent	
GC+CC	286		183	1.12	0.86 – 1.45	0.41		102	0.82	0.61 – 1.11	0.21
G allele	889		523	1.00	Referent			356	1.00	Referent	
C allele	335		221	1.12	0.91-1.37	0.28		124	0.91	0.72-1.66	0.47
***TP53* IVS7 +72C>T**											
CC	548		347	1.00	Referent			218	1.00	Referent	
CT+ TT	61		25	0.65	0.40 – 1.07	0.09		19	0.80	0.47 – 1.37	0.42
C allele	1155		718	1.00	Referent			455	1.00	Referent	
T allele	63		26	0.67	0.42-1.07	0.09		19	0.78	0.46-1.32	0.36
***TP53* Ex11-363G>A**											
GG	533		333	1.00	Referent			220	1.00	Referent	
GA+AA	77		37	0.78	0.52 – 1.19	0.25		19	0.58	0.34 – 0.99	**0.05**
G allele	1143		702	1.00	Referent			456	1.00	Referent	
A allele	77		38	0.82	0.55-1.22	0.36		22	0.70	0.43-1.15	0.15
***CDKN1A* Ser31Arg**											
CC	530		330	1.00	Referent			212	1.00	Referent	
CA+AA	81		42	0.82	0.55 – 1.23	0.34		28	0.88	0.56 – 1.40	0.59
C allele	1140		699	1.00	Referent			451	1.00	Referent	
A allele	82		45	0.89	0.61-1.30	0.54		29	0.91	0.60-1.42	0.69
***CDKN1A* Ex3 +70C>T**											
CC	520		326	1.00	Referent			211	1.00	Referent	
CT+TT	90		44	0.77	0.52 – 1.14	0.19		29	0.80	0.51 – 1.26	0.33

C allele	1129		693	1.00	Referent			450	1.00	Referent	
T allele	91		47	0.83	0.58-1.20	0.32		30	0.84	0.55-1.29	0.43
***CDKN2A* Ala148Thr**											
GG	579		344	1.00	Referent			221	1.00	Referent	
GA+AA	29		26	1.52	0.87 – 2.65	0.14		16	1.52	0.80 – 2.86	0.20
G allele	1187		713	1.00	Referent			457	1.00	Referent	
A allele	29		27	1.56	0.91-2.70	0.11		17	1.59	0.86-2.94	0.13
***CDKN2A* 3´UTR 500C>G**											
CC	457		266	1.00	Referent			165	1.00	Referent	
CG+GG	151		104	1.19	0.89 – 1.60	0.24		73	1.40	1.00 – 1.95	**0.05**
C allele	1052		629	1.00	Referent			392	1.00	Referent	
G allele	164		111	1.15	0.88-1.49	0.31		84	1.43	1.07-1.91	**0.02**
***CDKN2A* 3´UTR 540C>T**											
CC	526		322	1.00	Referent			206	1.00	Referent	
CT+TT	82		50	1.00	0.69– 1.47	0.98		33	1.03	0.67– 1.60	0.88
C allele	1128		691	1.00	Referent			445	1.00	Referent	
T allele	88		53	0.99	0.70-1.42	0.96		33	0.96	0.63-1.45	0.84
CCND1 Pro242Pro											
GG	159		95	1.00	Referent			62	1.00	Referent	
GA+AA	452		277	1.03	0.77 – 1.38	0.88		178	1.01	0.72 – 1.43	1.00
G allele	643		374	1.00	Referent			214	1.00	Referent	
C allele	579		370	0.93	0.78 – 1.11	0.31		239	0.83	0.68 – 1.00	**0.05**

OR, odds ratio; CI, 95% confidence interval. Significant P-values are in bold

[a] Adjusted for age and sex.

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

[c] Allele A_2 carries the 16bp insertion within the intron 3

Table 6. Distribution of DNA repair polymorphisms and risk of CRC

Genotypes	**Controls (n=532)**	**Cases (n=532)**	**OR**	**95% CI**	**P value**	X^2,**P-value HWE**[b]
***XRCC1* Arg194Trp**						
CC	466	454	1.00	Referent		3.80, 0.14
CT	59	72	1.24	0.86-1.80	0.25	
TT	5	6	1.17	0.35-3.87	0.80	
CT+TT	64	78	1.24	0.87-1.77	0.24	
C allele	991	980	1.00	Referent		
T allele	69	84	1.23	0.88-1.71	0.25	
***XRCC1* Arg399Gln**						
AA	219	229	1.00	Referent		0.31, 0.86
AG	240	233	0.93	0.72-1.21	0.60	
GG	73	68	0.88	0.60-1.29	0.52	
AG+GG	313	301	0.92	0.72-1.18	0.51	
A allele	678	691	1.00	Referent		
G allele	386	369	0.94	0.79-1.12	0.51	
***hOGG1* Ser326Cys**						
CC	331	336	1.00	Referent		0.60, 0.74
CG	181	168	0.91	0.70-1.18	0.47	
GG	20	28	1.43	0.79-2.59	0.24	
CG+ GG	201	196	0.96	0.75-1.23	0.74	
C allele	843	840	1.00	Referent		
G allele	221	224	1.02	0.83-1.25	0.92	
***APE1* Asn148Glu**						
TT	157	140	1.00	Referent		0.15, 0.93
TG	267	261	1.10	0.83-1.47	0.50	
GG	106	130	1.39	0.98-1.96	0.06	
TG+GG	373	391	1.18	0.91-1.55	0.22	
T allele	581	541	1.00	Referent		
G allele	479	521	1.17	0.98-1.39	0.08	
***XPD* Lys751Gln**						
AA	174	189	1.00	Referent		0.13, 0.94
AC	264	258	0.89	0.68-1.17	0.41	
CC	94	85	0.82	0.57-1.18	0.28	
AC+CC	358	343	0.87	0.68-1.13	0.30	

A allele	612	636	1.00	Referent		
C allele	452	428	0.91	0.77-1.08	0.31	
***XPG* Asn1104His**						
GG	356	334	1.00	Referent		1.57, 0.46
GC	153	177	1.25	0.96-1.63	0.10	
CC	23	21	0.99	0.54-1.83	0.98	
GC+CC	176	198	1.22	0.94-1.57	0.13	
G allele	865	845	1.00	Referent		
C allele	199	219	1.12	0.91-1.40	0.30	
***XPC* Lys939Gln**						
CC	189	171	1.00	Referent		1.92, 0.38
CA	243	268	1.23	0.94-1.61	0.14	
AA	100	93	1.02	0.72-1.45	0.90	
CA+AA	343	361	1.17	0.90-1.50	0.23	
C allele	612	610	1.00	Referent		
A allele	444	454	1.04	0.88-1.24	0.68	
***XRCC3* Thr241Met**						
CC	219	203	1.00	Referent		0.43, 0.81
CT	250	264	1.14	0.88-1.48	0.32	
TT	63	65	1.11	0.75-1.65	0.61	
CT+TT	313	329	1.13	0.89-1.45	0.32	
C allele	668	670	1.00	Referent		
T allele	376	394	1.04	0.88-1.25	0.66	
***NBS1* Glu185Gln**						
GG	239	246	1.00	Referent		3.15, 0.21
GC	220	234	1.03	0.80-1.33	0.83	
CC	71	52	0.71	0.48-1.06	0.10	
GC+CC	291	286	0.95	0.75-1.21	0.68	
G allele	712	726	1.00	Referent		
C allele	362	228	0.92	0.76-1.10	0.36	

[a] Unconditional logistic regression analysis adjusted for age and sex.

[b] X^2 and P-values for the deviation of observed and the numbers expected from the Hardy-Weinberg equilibrium (HWE) in the controls

Table 7. Distribution of DNA repair polymorphisms and risk of CRC after the stratification for tumor location

Genotypes	Controls (n=532)		Colon cases (n=335)	OR	95% CI	P value		Rectal cases (n=197)	OR	95% CI	P value
***XRCC1* Arg194Trp**											
CC	466		288	1.00	Referent			166	1.00	Referent	
CC+TT	64		47	1.19	0.79-1.78	0.41		31	1.30	0.82-2.07	0.27
C allele	991		621	1.00	Referent			359	1.00	Referent	
T allele	69		49	1.13	0.78-1.66	0.58		35	1.40	0.92-2.14	0.15
***XRCC1* Arg399Gln**											
AA	219		152	1.00	Referent			77	1.00	Referent	
AG+GG	313		183	0.83	0.63-1.09	0.19		120	1.14	0.81-1.59	0.46
A allele	678		450	1.00	Referent			243	1.00	Referent	
G allele	386		22	0.86	0.70-1.05	0.16		151	1.09	0.86-1.39	0.51
***hOGG1* Ser326Cys**											
CC	331		225	1.00	Referent			111	1.00	Referent	
CG+ GG	201		110	0.80	0.60-1.07	0.14		86	1.28	0.92-1.79	0.15
C allele	843		540	1.00	Referent			300	1.00	Referent	
G allele	221		130	0.92	0.72-1.17	0.53		94	1.20	0.91-1.57	0.23
***APE1* Asn148Glu**											
TT	157		82	1.00	Referent			58	1.00	Referent	
TG+GG	373		153	1.30	0.95-1.77	0.10		139	1.05	0.73-1.50	0.81
T allele	581		335	1.00	Referent			207	1.00	Referent	
G allele	479		335	1.21	1.00-1.47	0.06		187	1.10	0.87-1.38	0.48
***XPD* Lys751Gln**											
AA	174		118	1.00	Referent			71	1.00	Referent	
AC+CC	358		217	0.89	0.67-1.89	0.43		126	0.85	0.60-1.20	0.36
A allele	612		398	1.00	Referent			238	1.00	Referent	
C allele	452		272	0.93	0.76-1.13	0.47		156	0.89	0.70-1.12	0.35
***XPG* Asn1104His**										Referent	
GG	356		213	1.00	Referent			121	1.00		
GC+CC	176		122	1.19	0.89-1.59	0.24		76	1.26	0.89-1.77	0.19
G allele	865		539	1.00	Referent			306	1.00	Referent	
C allele	199		131	1.06	0.83-1.35	0.71		88	1.25	0.94-1.66	0.14

***XPC* Lys939Gln**											
CC	189		105	1.00	Referent			66	1.00	Referent	
CA+AA	343		230	1.21	0.90-1.61	0.21		131	1.11	0.78-1.57	0.56
C allele	621		386	1.00	Referent			224	1.00	Referent	
A allele	444		284	1.03	0.85-1.25	0.81		170	1.06	0.84-1.34	0.66
***XRCC3* Thr241Met**											
CC	219		133	1.00	Referent			70	1.00	Referent	
CT+TT	313		202	1.07	0.81-1.41	0.64		127	1.26	0.90-1.78	0.18
C allele	668		428	1.00	Referent			242	1.00	Referent	
T allele	376		242	1.00	0.82-1.23	1.00		152	1.12	0.88-1.42	0.40
***NBS1* Glu185Gln**											
GG	239		154	1.00	Referent			92	1.00	Referent	
GC+CC	291		181	0.97	0.73-1.27	0.81		105	0.93	0.67-1.29	0.67
G allele	712		459	1.00	Referent			267	1.00	Referent	
C allele	362		211	0.90	0.74-1.11	0.37		127	0.94	0.73-1.20	0.64

[a] Unconditional logistic regression analysis adjusted for age and sex.

4.3 Haplotype analyses of cell cycle genes

The analysis of linkage disequilibrium (LD) for the four loci in the *TP53* gene showed that the D' value for linkage between PIN3 and Arg72Pro loci was 0.61; 0.63 between PIN3 and IVS +72C>T. Similarly, the D' value for linkage between PIN3 and Ex11 -363G>A was 1.00; 0.48 between IVS7 +72C>T and Arg72Pro; 0.66 between Ex11 -363G>A and Arg72Pro; and 0.76 between IVS7 +72C>T and Ex11 -363G>A. The r^2 values ranged between 0.002 and 0.17.

Out of the 16 possible haplotypes, 10 were detected in the controls and 9 in the cases. The number of polymorphisms included for haplotype analysis was based on AIC value. Comparison of haplotypes with different combinations of 1 to 4 polymorphisms showed that the model with the lowest AIC (3294.31) was the one that included all the investigated polymorphisms in the *TP53* gene. AIC values for all other models were higher than the one selected for analysis. A multidimensional reduction method also identified the best model, which included four polymorphisms in the *TP53* gene. Different configurations resulted in testing balanced accuracies higher than 0.57 and cross validation consistencies higher than 10/10. The adequacy of the model was also suggested by permutation testing, which resulted in a critical value lower than 0.001.

The difference in distribution of the *TP53* haplotypes between cases and controls was statistically significant (global P-value for haplotype effect <0.0001; **Table 8**). The two haplotypes (A_1GCG and A_2CCG) were present in 81% cases and only in 72% controls. In comparison to the most common haplotype with only common alleles A_1GCG, the A_2CCG haplotype was associated with a statistically significant increased risk of CRC (OR=1.40, 95%CI 1.07-1.82, P<0.0001). On the other hand, four haplotypes (A_1CCG, A_2GCG, A_1GTG, and A_1GCA) were associated with statistically significant decreased risk (P<0.0001) (**Table 8**). Interestingly, the most common haplotype A_1GCG when compared to all other haplotypes was associated with an increased risk (OR 1.24, 95% CI 1.04-1.47).

The analysis of haplotype distribution for *TP53* after the stratification for tumour localization revealed consistently statistically significant differences between the controls and both colon and rectal cancer patients (global P-value for haplotype effect <0.0001 for colon, and global P-value for haplotype effect =0.003 for rectal cancer). In particular, the haplotype A_2CCG resulted at increased risk of colon cancer, while the haplotypes A_1CCG, A_2GCG, A_1GTG, and A_1GCA were associated with a decreased risk (OR=0.66;

0.49; 0.21 and 0.13, respectively) (**Table 8**). However, for rectal cancer the increased risk due to the haplotype A_2CCG was not statistically significant. The haplotypes A_1CCG and A_1GCA were associated with decreased risk in rectal cancer as well (OR=0.50 and 0.19, respectively).

Haplotype analysis of the two *p21* polymorphisms and the three *p16* polymorphisms did not show any difference between cases and controls (**Table 9 and 10**). Separate analysis for colon and rectal cancer also did not reveal any significant association (**Table 9 and 10**). The values of LD for the two polymorphic loci of *p21* (D'=0.96, R^2=0.85) and the three loci of *p16* (between 3´UTR 500C>G and Ala148Thr D'=0.98, r^2=0.17; between 3´UTR 540C>T and Ala148Thr D'=1, r^2=0.002 and between 3´UTR 540C>T and 3´UTR 500C>G: D'=1, r^2=0.01) suggested a strong linkage.

4.4 Gene-gene interactions

Gene-gene interactions were tested for association with CRC risk for selected SNPs in genes involved in the same DNA repair pathway. We found significantly increased risk of CRC in individuals carrying variant allele homozygous genotypes for both *APE1* Asn148Glu and *hOGG1* Ser326Cys polymorphisms (OR 6.37; 95% CI=1.40–29.02; P=0.02). The same genotype combination also showed an increased risk for colon cancer (OR 7.14; 95% CI=1.49–34.38; P=0.01).

We have stratified the population for the most common *TP53* haplotypes (A_1GCG and A_2CCG). In the bearers of above haplotypes we analysed the *TP53* and DNA repair gene-gene interaction (**Table 11**). No significant associations were observed with the three most relevant genes (*APE1, hOGG1* and *NBS1*). We have chosen these particular genes due to the observed association with the risk of CRC in **Publication 2**.

Table 8. Haplotype distribution of the four investigated *TP53* polymorphisms in CRC patients, also according to stratification for tumor location, and control subjects

Haplotypes[a]	Controls n[b]	All Cases n[b]	OR (95% CI)[c]	Colon cases n[b]	OR (95% CI)[c]	Rectal cases n[b]	OR (95% CI)[c]
A_1-G-C-G	763	819	Referent	493	Referent	326	Referent
A_2-C-C-G	106	159	**1.40 (1.07-1.82)**	100	**1.46 (1.09-1.96)**	59	1.30 (0.92-1.83)
A_1-C-C-G	141	90	**0.60 (0.45-0.79)**	60	**0.66 (0.48-0.91)**	30	**0.50 (0.33-0.75)**
A_1-C-C-A	51	54	0.99 (0.66-1.47)	36	1.09 (0.70-1.70)	18	0.82 (0.47-1.43)
A_2-G-C-G	63	36	**0.53 (0.35-0.81)**	20	**0.49 (0.29-0.82)**	16	0.59 (0.34-1.04)
A_1-C-T-G	32	34	0.99 (0.61-1.62)	22	1.06 (0.61-1.85)	12	0.88 (0.45-1.72)
A_1-G-T-G	29	10	**0.31 (0.15-0.64)**	4	**0.21 (0.07-0.59)**	6	0.48 (0.20-1.17)
A_1-G-C-A	24	5	**0.19 (0.07-0.51)**	2	**0.13 (0.03-0.55)**	3	**0.19 (0.05-0.83)**
A_2-C-T-G	2	1	-	0	-	1	-
A_2-G-C-A	1	0	-	0	-	0	-

OR, odds ratio; CI, 95% confidence interval. Significant P-values are in bold.

[a] Loci: *TP53* PIN3, Arg72Pro, IVS7+72C>T, Ex11-363G>A

[b] n is the number of alleles. Because each individual has two alleles, the total number of alleles will be twice the total number of individuals. Individuals with missing haplotyping data were not included in the analyses

[c] Global P-value for haplotype effect calculated from χ^2 test shows for all CRC P<0.0001, for colon cases P<0.0001 and for rectal cases P=0.006.

Table 9. Haplotype distribution of the four investigated *p21* polymorphisms in CRC patientsand control subjects

Haplotypes[a]	Controls n[b]	All Cases n[b]	OR (95% CI)[c]	Colon cases n[b]	OR (95% CI)[c]	Rectal cases n[b]	OR (95% CI)[c]
C-C	1133	1149	Referent	688	Referent	461	Referent
A-T	78	72	0.91 (0.65-1.27)	42	0.89 (0.60-1.31)	30	0.95 (0.61-1.46)
C-T	13	5	0.38 (0.13-1.07)	4	0.51 (0.16-1.56)	1	0.19 (0.03-1.45)
A-C	4	2	0.50 (0.09-2.70)	2	0.82 (0.15-4.51)	0	-

OR, odds ratio; CI, 95% confidence interval.

[a] Loci: *p21* Ser31Arg, Ex3+70C>G

[b] n is the number of alleles. Because each individual has two alleles, the total number of alleles will be twice the total number of individuals. Individuals with missing haplotyping data were not included in the analyses

[c] Global P-value for haplotype effect calculated from χ^2 test shows for all CRC $P<0.0001$, for colon cases $P<0.0001$ and for rectal cases $P=0.006$.

Table 10. Haplotype distribution of the four investigated *p16* polymorphisms in CRC patients and control subjects

Haplotypes[a]	Controls n[b]	All Cases n[b]	OR (95% CI)[c]	Colon cases n[b]	OR (95% CI)[c]	Rectal cases n[b]	OR (95% CI)[c]
G-C-C	976	946	Referent	572	Referent	374	Referent
G-G-C	135	152	1.16 (0.90-1.49)	85	1.07 (0.80-1.44)	67	1.29 (0.94-1.78)
G-C-T	88	86	1.00 (0.74-1.38)	53	0.45 (0.28-0.72)	33	0.98 (0.64-1.49)
A-G-C	29	43	1.53 (0.95-2.47)	25	1.47 (0.85-2.54)	18	1.62 (0.89-2.95)
A-C-C	0	1	-	1	-	0	-

OR, odds ratio; CI, 95% confidence interval.

[a] Loci: *p16* Ala148Thr, 3`UTR 500C>G, 3`UTR 540C>T

[b] n is the number of alleles. Because each individual has two alleles, the total number of alleles will be twice the total number of individuals. Individuals with missing haplotyping data were not included in the analyses

[c] Global P-value for haplotype effect calculated from χ^2 test shows for all CRC P<0.0001, for colon cases P<0.0001 and for rectal cases P=0.006.

Table 11. *TP53* haplotype (A_1GCG and A_2CCG) and DNA repair genes (*APE1, hOGG1* and *NBS1*) interactions

Genotypes	Controls	Cases	OR	95% CI	P
***APE1* Asn148Glu**					
TT	130	130	1.00	Referent	
TG	230	254	1.10	0.82-1.50	0.57
GG	91	120	1.32	0.92-1.90	0.16
***hOGG1* Ser326Cys**					
CC	312	318	1.00	Referent	
CG	160	160	0.98	0.75-1.28	1.00
GG	21	27	1.26	0.70-2.28	0.53
***NBS1* Glu185Gln**					
GG	202	230	1.00	Referent	
GC	190	229	1.06	0.81-1.39	0.73
CC	60	45	0.66	0.43-1.01	0.07

5. Discussion

CRC is one of the most common cancers worldwide. It is also one of the most curable cancers if detected early. Most cases of CRC are sporadic, and genetic and environmental factors are important. Understanding the genes and pathways that cause CRC would contribute to better detection, early diagnosis and thus reduce cancer morbidity and mortality.

The development of CRC typically is a multi-factorial process with a number of alterations in the cascade of genes regulating cellular proliferation control, apoptosis and DNA repair. The aim of the present study was to determine the role of polymorphisms in cell cycle and DNA repair genes in relation to CRC risk in a Czech population. This population has not yet been comprehensively investigated for genetic susceptibility to CRC despite it has one of the highest reported incidences of CRC worldwide (Konecny et al. 2008).

The investigated case-control population study has several strengths that include (a) cases and controls are matched for age and sex (these covariates often introduce substantial bias in association studies, (Wacholder, 2004)); (b) representative character of the study population (the diet is typically homogeneous in the country); and (c) inclusion of colonoscopically negative individuals as controls. Though the selection of controls may not necessarily represent the general population, it does ensure disease-free control individuals. The negative result of colonoscopy serves as best available proof of CRC absence (Singh et al, 2006).

This Thesis includes a report which represents the first association study where simultaneous genotype and haplotype analyses for the *TP53*, *p21,* and *p16* genes vis-à-vis CRC risk have been carried out (Polakova et al. 2009; **Publication 1**). In this study, we observed that none of the genotypes of the investigated polymorphisms was significantly associated with overall risk of CRC. Data stratification for cancer site showed that significant associations with specific genotypes were observed for rectal cancer only; the genotypes with variant A-allele for the *TP53* Ex11 -363G>A polymorphism were associated with a significantly decreased risk, and the genotypes with variant G-allele for the *CDKN2A* 3´UTR 500C>G polymorphism were associated with an increased risk.

However, the low variant allele frequencies and reduction in population size due to the stratification of the cases into colon and rectal cancers may not allow us to make strong conclusions. Further, after taking into consideration the correction for multiple-hypothesis testing, the associations being due to mere chance cannot be ruled out.

We did observe a tendency towards an allele effect on the risk of CRC for some of *TP53* polymorphisms. The frequency of variant allele of the IVS7 +72C>T polymorphism was under-represented in CRC cases than in controls. A similar tendency without reaching statistical significance was discernible in colon cancer. In rectal cancer cases, in contrast to colon, the variant allele for the Ex11 -363G>A polymorphism was less frequent than controls. These results show that individual SNPs are not associated with strong modulation of CRC risk. This observation is in line with previous studies where results based on association between the most investigated *TP53* polymorphisms and the risk of CRC have been mainly inconsistent. However, most of the studies published so far are based on the analysis of small numbers of cases and controls (**Polakova et al.. manuscript in preparation**).

The functional effects of several polymorphisms in the *TP53* gene may influence CRC susceptibility. The majority of studies have investigated the *TP53* Arg72Pro because of its functional relevance due to a weaker *in vitro* affinity of the protein with the common 72Arg allele for several transcription-activating factors (Murphy, 2006). The functional significance of the PIN3 polymorphism remains unclear. The intronic sequences in the *TP53* have been implicated in the regulation of gene expression and in DNA protein interactions (Gemignani et al, 2004; Pietsch et al, 2006). Insufficient information is available for *TP53* polymorphisms in intron 7 and exon 11 (Berggren et al, 2000, 2001).

None of the allelic associations was statistically significant; nevertheless, we speculate that there is an accentuation of the observed effects in resultant haplotypes. As a new finding, we have observed that haplotypes based on the four analysed *TP53* polymorphisms (loci in the order: PIN3, Arg72Pro, IVS7 +72C>T, Ex11 -363G>A) showed a significant differential distribution between cases and controls. The two most frequent haplotypes A_1GCG and A_2CCG were more common in cases than in controls. While the A_2CCG haplotype was associated with a statistically significantly increased risk of CRC, the four less frequent haplotypes affected a decreased risk. Interestingly, the most common haplotype that comprised the common alleles for all polymorphisms imparted an increased risk compared to all other haplotypes. Based on the frequency of

variant alleles, the effect of the haplotype A_2CCG on the increased CRC risk, appears to be logical (common alleles in *TP53* IVS7 +72C>T, and Ex11 -363G>A polymorphisms contribute to non-significantly increased risk compared to the variant alleles). However, the relatively low numbers of observations preclude a similar plausible explanation for the less frequent, protective haplotypes. The effect of studied haplotypes was observed individually for both colon and rectal cancer. We used a set of statistical techniques, like goodness of fit based on AIC and multiple dimensionality reduction for analysis of locus-locus interactions, to determine the soundness of different haplotype models. Two statistical techniques, one based on haplotype determination followed by logistic regression and the other on a non-parametric model, suggested the adequacy of the model that included all the four polymorphisms within the *TP53* genes.

While selecting tagging SNPs, we carried out an extended tag-SNP analysis on the most relevant part of the gene, i.e. from exon 2 to 3'UTR. This region is where almost all of the somatic and germline mutations are encountered. In the process the genetic variability in the first haplotype block was not captured. Following this approach we captured the most common Caucasian haplotypes, with $r^2>0.7$ and MAF>0.03 and the tagging of *TP53* was done with the higher resolution than the previously published studies. A few studies have investigated also the haplotypes based on the *TP53* polymorphisms with the risk of CRC, that mainly included only PIN3 and Arg72Pro (Perfumo et al, 2006, Tan et al, 2007) or also *Msp*I RFLPs in intron 6 (Sjalander et al. 1995). Even though, the earlier data have been inconsistent (**Polakova et al. manuscript in preparation**). For other kinds of cancer, we can report two interesting studies on lung cancer risk (Wu et al. 2002; Jung et al. 2008). The first published study reported an association between an increased cancer risk in a population from USA and a specific haplotype constructed on three *TP53* polymorphims. Interestingly, a most recent study investigated *TP53* haplotypes constructed on five polymorphisms in a Korean population of smaller size but no association was observed (Jung et al. 2008).

Thus the analysis of haplotypes, representing more effective approach than single polymorphism investigations and integrating a number of common genetic variations, showed strongly significant associations with sporadic CRC (Tan et al. 2007; **Publication 1**). The analysis of haplotypes represents a much more powerful approach than only analysing individual polymorphisms as it is possible to observe from the recent study published (Gast et al. 2007). This approach also ensures an increased statistical power. Assignment of alleles to chromosomes/haplotypes moreover provides important

information on recombination (physical exchange of DNA during meiosis), which is fundamental for locating disease-causing mutations by linkage methods.

The observed differential distribution of the *TP53* haplotypes in the CRC cases and controls may also reflect a linkage of the disease to hitherto unknown functional polymorphism within *TP53* or in some neighboring genes. Thus, an identification of critical polymorphisms in proximity of *TP53* on chromosome region 17p deserves an attention in relation to sporadic CRC risk in the future. The chromosome region 17p (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid=9606&chr=17) hosts a number of genes other than *TP53* that could carry putative functional variant(s) linked to the detected haplotypes. This argument is supported by predictions of such associations and the existence of large haplotype blocks within the human genome (Crawford and Nickerson, 2005). Moreover, the haplotypes in the gene represent the entire haplotype block covering the DNA-binding domain of the *TP53* gene, as polymorphisms selected in the study were based on a tagging approach. Further, the analysis of genotype combination also did not show any effect. The reversal of modulation effect of the *TP53* polymorphisms within haplotypes also probably points to additional polymorphism(s) that cause differential cancer risk either directly or through interaction with environmental effects.

Haplotypes of polymorphisms in the *p21* and *p16* genes did not show any association either with CRC or separately with colon or rectal cancer risk. On the other hand, we observed increased variant allele frequencies in CRC cases for the Ala148Thr and 3′UTR 500C>G polymorphisms in the *p16* gene. The *p21* and *p16* genes encode functionally important cell cycle regulators; however, not much information on the variants in these genes is available vis-à-vis CRC risk. Mc Cloud et al. (2004) reported an association of the 3′UTR 540C>T polymorphism in the *p16* gene with a decreased risk of sporadic CRC and altered tumor progression. Two other studies did not report any association for polymorphisms in these cell cycle genes with colon cancer risk (Goodman et al, 2006) or CRC risk in the Israeli population (Starinsky et al, 2005). Since *p21* encodes a downstream affector of p53, several studies have investigated a possible correlation between *TP53* and *p21* polymorphisms. In endometrial cancer in the Korean population and gastric cancer among Chinese, various combinations of *TP53* and *p21* polymorphisms were shown to result in an increased risk (Roh et al, 2004; Xi et al, 2004).

Elucidating the effect of polymorphisms of *TP53* and cancer risk remains a challenge. Traditional studies that investigated individual *TP53* polymorphisms in case-control studies have not provided definite answers yet and new approaches are therefore required. High-throughput methodologies and consortium studies investigating large numbers of individuals will provide required power in more unbiased approach. Recent publications have proven on large examined populations that polymorphisms with a low but significant effect on cancer risk can be identified (Whibley et al. 2009).

The studies constituting this Thesis are a part of a large international collaboration investigating associations between low-penetrance genes involved in main regulation pathways (DNA repair, cell cycle control, metabolism, etc) and sporadic CRC risk. In one of the articles related to the Thesis, we tested the hypothesis, whether SNPs in genes encoding different DNA repair enzymes covering the main DNA repair pathways may influence the risk of CRC (Pardini et al. 2008; **Publication 2**). None of the investigated polymorphisms was associated with the modulation of CRC risk. However, individuals homozygous for variant allele of the *APE1* Asn148Glu polymorphism in the base excision repair pathway (BER) were at moderately increased risk of the disease. The stratification of cases according to cancer site pointed to the effect of the 148Glu homozygous genotype confined to colon cancer. Interestingly, a newly observed increased risk in individuals, simultaneously homozygous for variant alleles of the *APE1* Asn148Glu and *hOGG1* Ser326Cys polymorphisms in both CRC and colon cancer indicates a kind of multiplicative gene-gene interaction. This interaction between genes involved in BER is probably suggestive of a role for inflammatory processes and/or oxidative stress in the colon cancer. The *APE1* and *hOGG1* genes are known to repair oxidative DNA damage as a part of BER pathway (Weiss et al. 2005).

The above mentioned results and the close link between DNA repair pathways and cell cycle control inspired us to address possible interaction between SNP in genes involved in DSB and BER and cell cycle control (e.g. *NBS1-TP53*) on the risk of CRC. However, no significant interactions were observed to modulate this risk considering the two most common haplotypes of *TP53* and SNPs in the three most relevant DNA repair genes (*APE1, hOGG1* and *NBS1*).

NBS, more recently called *NBN*, is the gene mutated in Nijmegen breakage syndrome (an autosomal recessive chromosomal instability disorder) characterized by a marked susceptibility to cancer (van de Burgt et al. 1996). Nibrin, the product of this gene, is part of the MRE11/RAD50 complex, which is involved in the repair of DNA DSBs and in cell cycle checkpoints (Tauchi et al. 2002; Kracker et al. 2005). *NBS1* has proved to be an essential modulator in cell cycle checkpoint control, which is an important part of the DNA damage response (Zhang et al. 2006).

The most common NBS-causing mutation is a 5-basepair deletion of *NBN*, 657del5, which results in a truncated and non-fully functional protein. Mutation 657del5 is particularly frequent in Central and Eastern Europe (population frequency 0.6%) (Varon et al. 2000), but rarely found in other regions (Dzikiewicz-Krawczyk et al. 2008). Several studies have been conducted to evaluate if heterozygous carriage of *NBN* 657del5 predisposes to an increased risk of cancer of any type. Although these studies need further confirmation (Dzikiewicz-Krawczyk et al. 2008), an increased risk of lymphoma/leukemia (Chrzanowska et al. 2006), breast cancer (Bogdanova et al. 2008), and gastrointestinal lymphoma (Steffen et al. 2006) has been reported.

Accordingly, we investigated whether a high frequency of *NBN* 657del5 mutation in the Slavic population can modulate CRC susceptibility in patients of Czech origin. The results did not show any evidence for an increased risk of CRC in individuals carrying this specific *NBN* mutation (Pardini et al. 2009; **Publication 3**). For this study, two different control populations have been chosen for the following reasons. The inclusion of colonoscopy negative individuals as controls (Control Group I) ensures cancer-free control individuals, because the negative result of colonoscopy serves as best available proof of CRC absence (Singh et al. 2006). Since the selection of these controls may not necessarily represent the healthy general population, we decided to include also healthy individuals recruited from blood donor centers (Control Group II).

In summary, this study has highlighted population based differences in CRC risk, investigated by haplotypes based on four polymorphisms within the *TP53* gene. Importantly, the two most frequent haplotypes were associated with an increased CRC risk. However, the mechanism through which the risk modulation is affected remains yet to be understood. It is also possible that the haplotypes within the *TP53* gene along with SNPs in other genes in the cell cycle pathway may impact the development not only of CRC but also other cancers. Interestingly, the *TP53* haplotypes studied by us modulate

significantly both colon and rectal cancer risk suggesting general mechanisms in the genesis of colon and rectal cancers. On the basis of these results, the role of *TP53* common variants and reconstructed haplotypes in therapy response could be also taken into consideration for further investigations.

The associations observed between the polymorphisms in DNA repair genes and CRC risk were marginal. However, in view of a strong hypothesis for the role of DNA repair in CRC, larger populations enabling stronger statistical analyses, functional aspects of studied genes and the interaction with environmental factors should be taken into the account. For sporadic CRC, both genetic and environmental factors must be present to produce the disease and, consequently, both should be identified and investigated in the future studies. In fact, gene–environment interactions may obscure the detection of a genetic effect if they are not controlled for properly.

6. Conclusions

1. All the aims proposed for the PhD work have been fullfiled.

2. None of the investigated polymorphisms of cell cycle and DNA repair genes were independently associated with risk modulation of CRC in a hospital-based case-control association study.

3. A differential distribution between cases and controls of major haplotypes arising from four variants in the *TP53* gene has been observed in the study population.

4. The A_2CCG haplotype was associated with a significantly increased CRC risk, while four rare haplotypes (A_1CCG, A_2GCG, A_1GTG, and A_1GCA) were associated with a significantly decreased CRC risk, in comparison to the most common haplotype (A_1GCG).

5. The same tendency was observed for both colon and rectal cancer, when analyzed independently.

6. Haplotype analysis of two *CDKN1A* polymorphisms and three *CDKN2A* polymorphisms did not show any difference between cases and controls; separate analysis for colon and rectal cancer also did not show any association.

7. The analysis of binary genotype combinations showed an increased CRC risk in individuals simultaneously homozygous for the variant alleles of *APE1* and *hOGG1* polymorphisms.

8. The present results do not provide any evidence that the exceeding risk of CRC in this population is attributable to the high frequency of heterozygous carriage of the *NBN* 657del5 mutation.

7. Appendix

7.1 Genomic architecture

Genomic architetcure generally refers to the variable patterns of SNP correlations across the human genome. The various key concepts are: (a) Haplotypes, (b) Linkage Disequilibrium (LD), (c) Haplotype Blocks.

(a) **Haplotype**: A haplotype is a sequence of SNP alleles stretching along a segment of DNA. It is usually inherited as a single unit from parents. If SNPs are close enough in the genome, alleles will tend to be inherited as haplotypes more often than for SNPs which are more distant. Haplotype analyses represent a powerful tool, especially for populations in which an individual founder mutation accounts for a substantial percentage of all cases, or for any population with a susceptibility allele in a region of very low combination (Houlston and Peto, 2003).

(b) **Linkage Disequilibrium (LD)**: A non-random association of alleles of SNPs that are close together is called linkage disequilibrium (LD) (Lewontin 1964). In order to measure LD, we calculated the D´ value as follows: $D' = D/D_{max}$, where

D= is dependent on marginal allele frequencies in contingency table and D_{max} = the absolute max D value

D´is constrained between -1 and +1. D̀ coefficient may be interpreted as follows:

D´ = 1 (perfect positive LD between SNP alleles)

D´ = 0 (linkage equilibrium or no association between SNP alleles)

D´ = -1 (perfect negative LD between SNP alleles)

D´ = 0.87 (strong positive LD between SNP alleles)

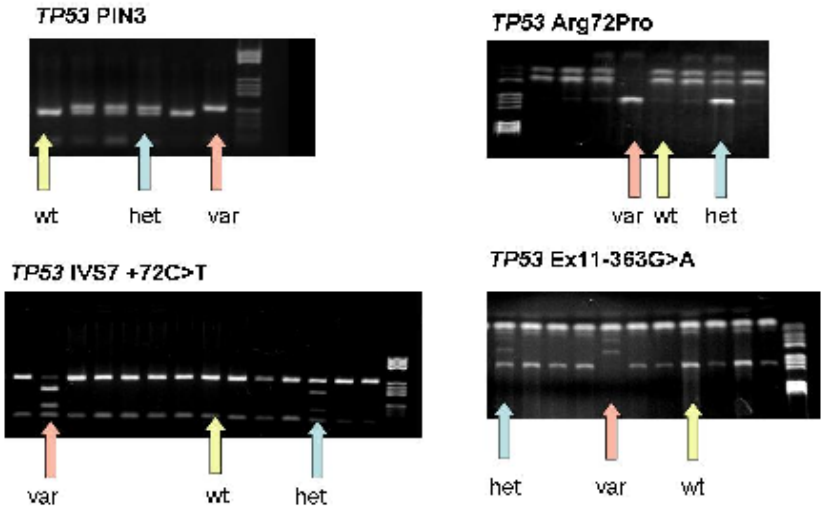
D´ = 0.12 (weak positive LD bewteen SNP alleles)

Factors, which affect LD, are: occurrence of new mutations, physical distance between SNPs (kb), differing recombination rates, and number of generations.

(c) **Haplotype blocks**: The human genome may be defined as regions of high LD, called haplotype blocks. These are separated by smaller regions of low LD, called recombinations hotspots. A haplotype block consists of a few common haplotypes that account for a large DNA segment. SNPs that uniquely represent haplotypes are called tagSNPs and they are used in genetic association studies of disease (Crawford and Nickerson 2005).

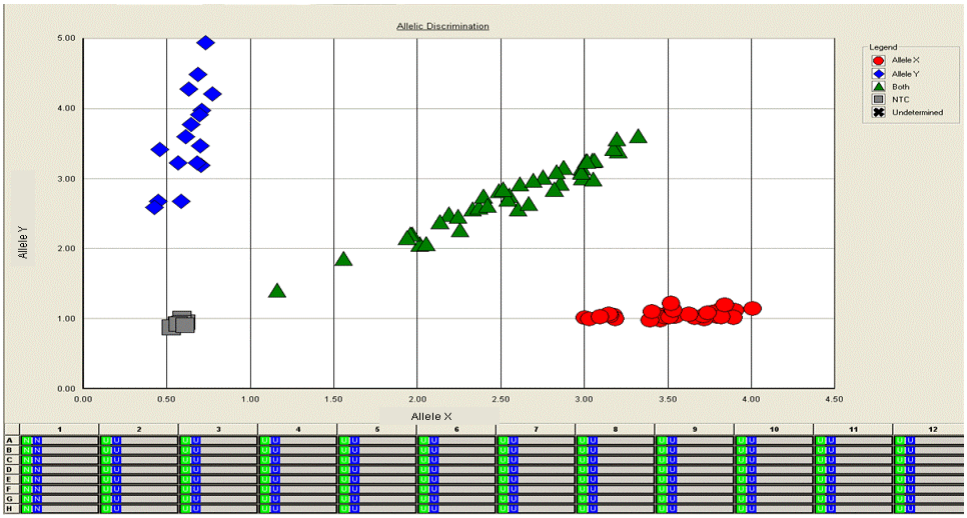
7.2 Examples of results of genotyping analyses performed in the study

Figure 10. Results of PCR-RFLP method for four analysed *TP53* polymorphisms. The amplified and digested PCR products are resolved and analysed on 3% agarose gels and visualized under UV light.



(wt – wild type, het- heterozygote, var- variant)

Figure 11. Results of *p21* Ser31Arg, analysed with TaqMan allelic discrimination assay. The TaqMan genotyping reaction was amplified on a 7500 Real-Time PCR system.



Allele X (red) wild type

Allele Y (blue) variant

Both (green) heterozygote

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8. Publications

Publications and manuscript in preparation, directly related to the Thesis:

1. **Polakova V**, Pardini B, Naccarati A, Landi S, Slyskova J, Novotny J, Vodickova L, Bermejo JL, Hanova M, Smerhovsky Z, Tulupova E, Kumar R, Hemminki K, Vodicka P. Genotype and haplotype analysis of cell cycle genes in sporadic colorectal cancer in the Czech Republic. Hum Mutat. 2009. 30(4):661-668. **IF=6.3**

2. Pardini B, Naccarati A, Novotny J, Smerhovsky Z, Vodickova L, **Polakova V**, Hanova M, Slyskova J, Tulupova E, Kumar R, Bortlik M, Barale R, Hemminki K, Vodicka P. DNA repair genetic polymorphisms and risk of colorectal cancer in the Czech Republic. Mutat Res. 2008. 638(1-2):146-153. **IF=4.1**

3. Pardini B, Naccarati A, **Polakova V**, Smerhovsky Z, Hlavata I, Soucek P, Novotny J, Vodickova L, Tomanova V, Landi S, Vodicka P. NBN 657del(5) heterozygous mutations and colorectal cancer risk in the Czech Republic. Mutat Res. 2009. 666(1-2):64-67. **IF=4.1**

4. **Polakova V**, Naccarati A, Pardini B, Vodickova L, Kumar R, Hemminki K, Vodicka P. Genetic variation in TP53 and sporadic colorectal cancer risk: a review. Manuscript before submission

Publications in loose relation with the Thesis:

a) Journals with IF

1. Vodicka P, Naccarati A, Vodickova L, **Polakova V**, Dusinska M, Musak L, Halasova E, Susova S, Soucek P, Hemminki K. Do GST polymorhisms modulate the frequency of chromosomal aberrations in healthy subjects? Environ Health Perspective. 2009. 117(9): A2-A3

2. Halasova E, Matakova T, Musak L, **Polakova V**, Vodicka P. Chromosomal damage and polymorphisms of DNA repair genes XRCC1 and XRCC3 in workers exposed to chromium. Neuro Endocrinol Lett. 2008. 29(5):658-662. **IF=1.4**

3. Tulupova E, Kumar R, Hanova M, Slyskova J, Pardini B, **Polakova V**, Naccarati A, Vodickova L, Novotny J, Halamkova J, Hemminki K, Vodicka P. Do polymorphisms and haplotypes of mismatch repair genes modulate risk of sporadic colorectal cancer?. Mutat Res. 2008. 648(1-2):40-45. **IF=4.1**

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5. Musak L, Vodicka P, Klimentova G, Soucek P, Hanova M, Mikulkova R, Buchancova J, Vodickova L, **Polakova V**, Pec M. Chromosomal damage and polymorphisms of DNA repair genes XRCC1 and XRCC3 in workers exposed to cytostatics. Neuro Endocrinol Lett. 2006. 2:57-60. **IF=1.4**

6. Vodicka P, Stetina R, **Polakova V**, Tulupova E, Naccarati A, Vodickova L, Kumar R, Hanova M, Pardini B, Slyskova J, Musak L, De Palma G, Soucek P, Hemminki K. Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. Carcinogenesis. 2007. 28(3):657-664. **IF=5.1**

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