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INFLUENCE OF GENETIC POLYMORPHISMS ON DNA REPAIR, *P53* MUTATIONS AND CANCER RISK

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Do not go where the path may lead, go instead where there is no path and leave a trail.

Ralph Waldo Emerson (1803-1882)

To my family

Abstract

Individuals have different susceptibility towards environmental exposures that may cause or contribute to disease. This inter-individual variation is partly due to genetic polymorphisms in genes involved in DNA repair and metabolism, which have potential to modulate the function of the encoded proteins. This thesis is a contribution to the knowledge about the importance of polymorphisms, and how they might interfere with health. More specifically, the aims have been to investigate (1) if and how the mutational spectra of the tumour suppressor gene *p53* in lung and bladder tumours is affected by genetic polymorphisms, (2) if polymorphisms can influence lung cancer risk in a study population with a high frequency of women and non-smokers, and (3) to develop a method to study the functionality of DNA repair polymorphisms in vitro.

Papers I-II are based on a lung cancer case-control study population, with approximately 50 % non-smokers and 70 % women. Papers III-IV are based on a bladder cancer cohort that includes all bladder cancer cases in the Stockholm county between 1995-1996. In paper V, lymphocytes from healthy non-smoking volunteers were collected to study the functionality of DNA repair polymorphisms, using the Comet assay. PCR-RFLP and Taq-Man allelic discrimination were used for genotyping, SSCP and sequencing for identification of *p53* mutations in tumours.

The impact of genetic polymorphisms on the *p53* gene was investigated in **study I, III and IV**. In study I we observed an association between the *XPD* 751Gln allele and an increased frequency of *p53* transversion mutations in lung tumours, especially among smokers, suggesting that *XPD* 751Gln carriers have a reduced DNA repair proficiency. In study III we found that *p53* transversion mutations were more common in bladder tumours of *GSTM1* null individuals, and also the *GSTP1* 105Val allele was associated with a higher frequency of *p53* transversion mutations than the wild type allele. *GSTP1* 105Val was also associated with a higher frequency of *p53* transitions at CpG sites. These results indicated that an impaired glutathione conjugation might affect the *p53* mutational spectrum. In study IV, the *cyclin D1* variant allele homozygotes and the *XRCC3* 241Met homozygotes were associated with an increased *p53* mutation frequency, while the *NQO1* 187Ser and 139Trp alleles and the *XPC* 939Gln allele were associated with an increased frequency of *p53* transversion mutations. Thus, in general, these polymorphisms seem to have an impact on the type of mutations in the *p53* gene in lung- and bladder- tumours, while their impact on the frequency of mutation is small. This increased risk for specific types of mutations probably reflects a higher susceptibility to environmental exposures following the impaired metabolism (glutathione conjugation), or less efficient DNA repair, among carriers of the susceptible alleles.

The influence of polymorphisms on lung cancer risk was investigated in **study II**, where the *XRCC1* 399Gln and the *XRCC3* 241Met alleles were found to be associated with decreased risk for lung cancer among non-smoking- and smoking- women respectively. The *NBS1* 185Gln allele was associated with an increased risk among non-smoking and low-dose smoking women. The effects of the two HR-proteins, XRCC3 and NBS1 enhanced each other. Our results suggest that these polymorphisms influence the cancer risk through their effect on the repair capacity, which may alter the risk of having mutations fixed in the genome.

In **study V**, we used a modified comet assay to study the influence of DNA repair polymorphisms on repair of MMS-induced DNA damage in peripheral blood lymphocytes in vitro. The investigated *APEX1* Asp148Glu, *XRCC3* Thr241Met and *NBS1* Glu185Gln polymorphisms were shown to have effect on the early stages of DNA repair, and were associated with the rate of transitions of cells from a more damaged to a less damaged state. This type of functional study of DNA repair enzyme polymorphisms will contribute to a better understanding of the significance of gene association studies in cancer research.

List of Publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals. Accepted and published papers are reprinted with permission from the copyright holders.

I. Hou S, **Ryk C**, Kannio A, Angelini S, Fält S, Nyberg F, Husgafvel-Pursiainen K. Influence of common *XPD* and *XRCC1* variant alleles on *p53* mutations in lung tumours. *Environmental and Molecular Mutagenesis 41:37-42 (2003).*

II. **Ryk C**, Kumar R, Thirumaran K.R, Hou S. Polymorphisms in the DNA repair genes *XRCC1*, *APEX1*, *XRCC3* and *NBS1*, and the risk for Lung Cancer in never- and ever-smokers. *Accepted for publication in Lung Cancer (2006).*

III. **Ryk C**, Berggren P, Kumar R, Hemminki K, Larsson P, Steineck G, Lambert B, Hou S. Influence of *GSTM1*, *GSTT1*, *GSTP1* and *NAT2* genotypes on the *p53* mutational spectrum in bladder tumours. *International Journal of Cancer 113, 761-768 (2005)*

IV. **Ryk C**, Kumar R, Sanyal S, Berggren P, Hemminki K, Larsson P, Steineck G, Hou S. Influence of Polymorphism in DNA Repair and Defence genes on *p53* mutations in Bladder Tumours. *Cancer Letters, Epub ahead of print, Dec 9, (2005).*

V. **Ryk C**, Routledge MN, Allan JM, Wild CP, Kumar R, Lambert B, Hou S. Influence of polymorphisms in DNA repair genes on repair kinetics, as measured by the Comet assay. *Submitted.*

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List of abbreviations

ALDH2	Aldehyde dehydrogenase 2
AP	Abasic site
APEX1	Apurinic/apyrimidinic endonuclease-1
BER	Base excision repair
BRCA1	Breast cancer 1
BRCT	BRCA1 carboxyl-terminal protein interaction domain
CA	Chromosome aberrations
CCNH	Kinase subunit cyclin H
ERCC1	DNA excision repair gene
FEN1	Flap-endonuclease 1
GSH	Glutathione
GST	Glutathione-S-transferase
HR	Homologous recombination repair
MLH1	MutL protein homolog 1
MMR	Mismatch repair
MMS	Methylmethane sulphonate
MSI	Microsatellite instability
MTHFR	Methylene-tetrahydrofolate reductase
NAT2	N-acetyltransferase-2
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NQO1	NAD(P)H dehydrogenase quinone 1
P53	Protein 53
PAH	Polycyclic aromatic hydrocarbon
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PHA	Phytohemagglutinin
Pol	DNA polymerase
PS	Pseudo score
py	Packyears
RPA	Replication protein A
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism analysis
TFIIH	Transcription factor II H
VS	Visual score
XPC	Xeroderma pigmentosum complementation group C
XPD	Xeroderma pigmentosum complementation group D
XPG	Xeroderma pigmentosum complementation group G
XRCC1	X-ray cross-complementing group 1
XRCC3	X-ray cross-complementing group 3

1. Introduction

There is an ongoing debate about genes and environment, their interactions, and the extent of their relative impact on life and health. However, most common diseases involve not only discrete genetic and environmental causes, but also interactions between the two [1-9]. Although any two unrelated people share about 99.9% of their DNA sequences, the remaining 0.1% is important because it contains the genetic variants that influence how people differ in their risk of disease or their response to drugs and environmental exposures. Discovering the DNA sequence variants that contribute to common disease risk offers one of the best opportunities for understanding the complex causes of disease in humans.

In a few cases single genes may have a high impact on the life and health of humans. These are major genes that have been strongly linked to specific diseases and affect families where a mutated variant of such a disease gene is inherited. For example, inherited mutations of the *BRCA1* gene are responsible for approximately 40-45% of hereditary breast cancers [10]. However, major, heritable disease genes are uncommon in the general population, and in fact only 2-3% of the total number of breast cancer cases is due to BRCA1 mutations, since this gene is rarely mutated in sporadic cancers [10].

Much more common are variants of genes, termed polymorphisms, which influence various metabolic processes or our susceptibility to different types of environmental exposures [11]. The genetic polymorphisms that may have impact on our susceptibility to environmental exposures are found among others, in genes that affect the metabolism of substances and toxins that enters the body and among genes that are important for the repair of genetic damage, which arises in the genome after exposure to genotoxic agents. These genetic polymorphisms will not modify an individual's risk of developing disease by themselves, but rather modify the effect of the exposure and the damage. One example of a genetic polymorphism that leads to a phenotype that is easily observed is the reproducible flushing response after alcohol ingestion in individuals with the low-activity polymorphisms in the aldehyde dehydrogenase gene (ALDH2) [4,12]. An individual carrying this genetic variant will experience flushing after exposure to an aldehyde dehydrogenase substrate. Thus, the importance of low-penetrance polymorphisms depends on exposure. If there is no

exposure that could be associated to the polymorphism in question, the possible difference in susceptibility of the alleles is less important, and the disease risk of the individual will not be affected.

In this thesis, I have studied how genetic polymorphisms in genes involved in DNA repair and metabolism may be associated with the risk of getting lung cancer, and with the frequency and type of mutations found in the *p53* gene of lung-and bladder-tumours. Moreover, I have aimed at developing a method to study the effect of DNA repair gene polymorphisms on the recovery of cells from experimentally induced DNA damage.

2. Polymorphisms

2.1 What is a polymorphism?

A polymorphism is a germline variation in the base sequence of the genetic code. As a rule of thumb, a heritable variation is termed a polymorphism if it is present at an allele frequency higher than 1% in the general population, otherwise, at lower frequencies, it is termed a germline mutation [13]. Also, mutations are associated with severe diseases, while polymorphisms generally are not. Thus, polymorphisms can remain and spread within a population, either because they have no major effect or because they were associated with a beneficial effect that may be counteracted by modern lifestyle. Mutations, at least those that exert a deleterious influence before reproduction, tend to be selected against by evolution, even if they do not disappear completely since they may arise spontaneously also in new generations. Also some deleterious mutations persist in populations because they are associated with a selective advantage under certain environmental conditions (e.g. sickle cell anaemia mutation in areas with malaria) [14]. Polymorphisms and germline mutations are present in the genetic code of every cell of an individual. There are also non-heritable alterations in the genetic code, acquired during the lifetime of an individual. These are termed somatic mutations and are present within the affected tissue only. Thus, somatic mutations can proliferate by clonal expansion of the mutated cell, but cannot be transmitted to the offspring of the affected individual.

Genetic polymorphisms are common throughout the genome. The most common type of polymorphisms, single nucleotide polymorphisms (SNPs), can occur as frequently as 1 out of every 300 base pairs and there are probably more than 10 million SNPs in the human population [11]. Polymorphisms can occur in both introns and exons of the genes and may sometimes, especially those within exons, have an impact on the structure and function of the protein coded for by the gene, especially in those cases when the polymorphisms leads to an amino acid exchange in evolutionarily conserved domains.

The exact frequencies of many polymorphisms vary with the ethnicity of the population. For example, the low-activity polymorphism in the ALDH2 gene mentioned before, is more common in Asia than in other parts of the world [12], and because of the discomfort of the flushing, many Asian people refrain from ingesting

alcoholic beverages. Another example is the GSTM1 null-allele, which occurs in about 50% of an ordinary European population (Table 1), but is present in only 22% of an African (Nigerian) population [15]. For each polymorphism, one of the alleles is considered to be the main allele, and this is usually named the wild-type allele. The wild-type is supposed to be the most common ("normal") genetic variant, but since the frequencies varies, this need not to be true for all populations.

Alleles of SNPs that are close together tend to be inherited together. A set of such alleles in a region of a chromosome is called a haplotype. Most chromosome regions have only a few common haplotypes [16], which together account for most of the genetic variation from person to person in a population.

2.2 Why are polymorphisms important?

Life on Earth, and evolution, are based on small genetic variations that occasionally arise and give the carrier an increased ability to survive and reproduce in the specific environment in which the organism exists. These genetic variations are transferred to the offspring and will spread during the following generations and increase the possibility for survival for the whole population in it's present environment. Among these genetic variations are the polymorphisms. Presumably, many polymorphisms spread in populations because they have no, or very little, effect. However, some may also spread because they confer an advantage in certain situations.

A polymorphism that leads to an amino acid exchange, and is found within an active site of an enzyme, at a DNA binding site, a substrate binding site or in other areas of importance for the protein function, may influence the activity of the encoded protein. This is especially true if the new amino acid has a different three dimensional form or electrical charge than the original amino acid, as this will change the structure or affinity of the protein, and make it non-functional, or more or less efficient than the original one [17-26]. If the protein is involved in processes such as DNA repair, cell cycle control or metabolism of toxic substances, a change in function may be associated with a different susceptibility to disease in a carrier of the variant allele. For example, a loss of function polymorphism, or a decreased efficiency in a metabolically active enzyme may after an exposure lead to accumulation of toxic metabolites, that the body will not be able to excrete before toxic levels are reached.

Thus, the susceptible individuals, carrying the polymorphic variant, will develop symptoms of intoxication at lower doses of exposures than individuals not carrying this allele [4,27,28]. Likewise, a less efficient DNA repair may increase the risk of having a permanent damage, for example a mutated DNA base, CA or micronuclei [29], if the cell has not had enough time to repair before going through mitosis.

Since the mid-20th century, it has been known that people react differently to certain drugs, even if they have the same diagnosis [27]. This was later associated with genetic polymorphisms in the genes that metabolised the active substances of the medicine. Today, there are a number of studies that indicate associations between polymorphic variants of certain genes and the medical outcome of a treatment, for example chemotherapy treatment of cancer patients [30-32]. Polymorphisms may also be of help to identify the genetic traits of different diseases. If, for example, a polymorphism that changes the function of a protein can be associated with a certain disease, there is a high probability that this polymorphic gene is of importance for that disease. Another important aspect of studying polymorphisms is that they may help us to identify at risk groups of individuals that are more susceptible to certain environmental exposures than others. This type of knowledge may in the future allow us to give individualized preventive advice before disease diagnosis, or offering personalized treatment after the disease has been diagnosed.

In most cases the effect of a single polymorphism is weak. Individuals with a variant allele leading to an increased susceptibility to certain exposures will, if they are exposed, be at an increased risk for diseases associated with this polymorphism, but the risk increase is usually small. Also, if the individual is not exposed to a substance associated with the susceptibility, the polymorphism will probably not yield any effect at all [4]. The impact of polymorphisms on health is, therefore, complex. Not only the environment, but also other genes that may interact have to be taken into consideration, and it should not, therefore, be surprising that many studies of polymorphisms show conflicting results [33]. However, even though the polymorphic effect on individuals is modest, it may be rather large at a populational level. There are a number of studies that indicate a possible association between polymorphic variants of certain genes and, for example, cancer risk [7,34-41]. Thus, a functional polymorphism that increases the susceptibility for example for lung cancer after tobacco exposure may have an impact on the total frequency of new lung cancer cases

in a population if the polymorphism is common [42] even if the effect on a single individual is weak.

The frequency of different polymorphic variants varies with the ethnicity of a population, and the same exposure may therefore not give the same result in different parts of the world (for Caucasian frequencies, see Table 1 and 2), as well as for different ethnical groups within the same country. Knowledge of the frequency of polymorphisms in a population could therefore be of importance for understanding the impact of certain exposures on a population, and for planning of health care.

2.3 Polymorphisms in metabolic pathways

2.3.1 Metabolism

In general, the biotransformation of a substance through metabolism is a transformation of a lipophilic parental substance into a hydrophilic metabolite, which is easily excreted through the renal system. The enzymes involved in metabolism possess broad and overlapping specificity to be able to deal with newly exposed chemicals. The metabolism can be divided in two phases, I and II. Phase I involves reduction, hydrolysis, or oxidation of the parental (non-metabolized) substrate. This phase usually leads to an activation of the substance that make them more water soluble, but also more reactive [43,44]. The phase II proteins conjugates the activated substances (metabolites) from phase I. The attachment of an ionised group, like Glutathione (GSH), methyl or acetyl, usually makes the metabolite less reactive and more water soluble, which facilitates excretion [45,46]. The activity of the phase II system is important, since the activated metabolites from phase I may react with macromolecules, like proteins, RNA and DNA in the cell, if not conjugated and detoxified [47,48] (Figure 1). Given the importance of the phase II conjugation enzymes in detoxification and drug metabolism, and also how the balance between phase I and phase II enzymes can significantly alter the pharmacokinetics for a given drug or toxicant, this area deserves increased attention [48].

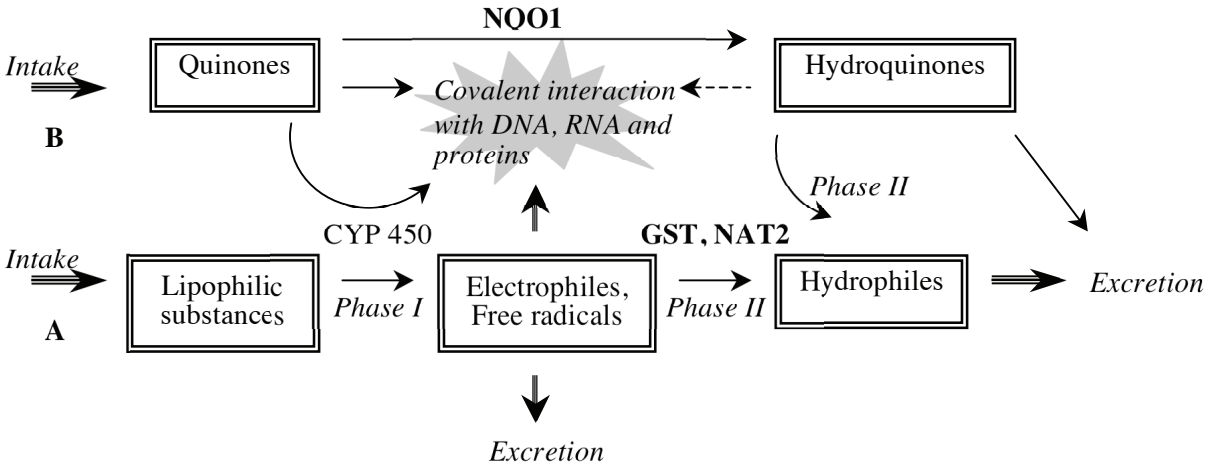


Figure 1. Metabolism of xenobiotics. A: Generalized picture of phase I and II. B: Metabolism of quinones, catalysed by NQO1, and the competing metabolic reaction of quinones, yielding free radicals, catalysed by e.g. CYP 450 reductase. Hydroquinones may be excreted, conjugated or through auto oxidation become reactive again [49].

Many chemicals can accelerate their own biotransformation and excretion by inducing the enzymes that metabolize them, a process called enzyme induction. The reverse is also true, where some chemicals cause enzyme inhibition. Other factors that alter enzyme levels include sex, age and genetic predisposition [22,24,27,45,46,50-52].

The effect of a functional polymorphism of a metabolic gene depends partly on which metabolic phase the gene is involved in, and also on the effectiveness of the other phase [48]. For example, a rapid phase I combined with a slow phase II metabolism of a certain substance may accumulate reactive metabolites in the body, yielding a toxic response, while a slow phase I may allow enough time for the phase II enzymes, and for the excretion, to take place, before toxic levels of the metabolite is reached. For example, when investigating phase I and phase II polymorphisms of genes involved in the metabolism of polycyclic aromatic hydrocarbons (PAHs), some individuals have been shown to have defective enzymes for activation, but normal enzymes for deactivation of PAHs. In contrast, the opposite has also been true for other individuals [46]. However, a slow phase I enzyme may also lead to toxic damage if the parental substance is more toxic than the metabolites. If the parental substance is mutagenic this may lead to an increased cancer risk. In most cases the

activated metabolites of phase I are the most reactive and dangerous substances and therefore the ability of the phase II enzymes to detoxify these metabolites efficiently is important to prevent DNA mutations and damage to cellular proteins [43,46-48].

2.3.2 Investigated polymorphisms in metabolic genes

In this thesis five metabolic genes, *GSTM1*, *GSTT1*, *GSTP1*, *NAT2* and *NQO1* were studied in the bladder cancer cohort (study III and IV). This included the *GSTP1* Ile105Val polymorphism, and the Arg139Trp and Pro187Ser polymorphisms of the *NQO1* gene. The *NQO1* gene is active in phase I of the metabolism, while the other metabolic polymorphisms investigated here are involved in phase II. The possible association between these polymorphisms and the type and frequency of mutations found in the *p53* oncogene of the bladder tumour was analysed. The frequencies of these polymorphisms in Caucasian populations are shown in table 1.

The super family of glutathione S-transferases (GSTs) is composed of multiple isozymes. Based on biochemical, immunologic, and structural properties, the soluble human GSTs are categorized into 7 main classes: alpha, mu, omega, pi, sigma, theta and Zeta [45]. The GSTs are phase II metabolic enzymes that conjugate reactive metabolites to glutathione (GSH) and detoxify a variety of potential carcinogens, exogenous compounds and endogenous metabolites. Thus, genetic polymorphisms in these enzymes may have high relevance in cancer susceptibility [35]. Data from cancer studies have also linked aberrant expression of GST isozymes with the development of resistance to a variety of chemicals, including cancer drugs [45].

For **GSTM1** (class mu) a homozygous deletion of parts of the gene, present in approximately 50% of the Caucasians results in a lack of the GSTM1 protein [15,21,53]. This so called *GSTM1*-null genotype has been associated with increased risk of lung, colon, colorectal and bladder cancer, and has also been associated with response to chemotherapy [38,40,53,54]. For example, *GSTM1*-null patients with acute myeloid leukaemia (AML) have been shown to have a better response to adriamycin and cyclophosphamide treatment, although the mechanism is not clear [55].

Also in the ***GSTT1*** gene (class theta) there is a common null genotype resulting in an inactive protein [21]. A homozygous deletion of the *GSTT1* gene results in diminished ability to detoxify a wide range of environmental carcinogens, including ethylene oxide and monohalomethanes. On the other hand, functional

GSTT1 conjugation can also form mutagenic metabolites, for example with dichloromethane. The null genotype of *GSTT1* has been reported to increase the risk of head, neck, oral cavity, colorectal, gastric and bladder cancer [34,40,41,56], though not all studies did observe any associations [42]. Individuals with homozygous deletions of both *GSTM1* and *GSTT1* are thought to be at increased risk for malignancies as a consequence of a decreased capacity to detoxify possible carcinogens.

In the ***GSTP1*** gene (class pi), two closely linked amino acid substitutions, Ile105Val and Ala113Val, have been identified [17]. The *GSTP1* Ile105Val polymorphism modifies the kinetic properties of the encoded enzyme [50,51]. The Valine allele has been associated with a substantial reduction in the catalytic activity [26], and some studies have shown a higher susceptibility for cancer in individuals carrying the variant Valine allele [17,57]. However, it is also well established that GSTP1 is overexpressed in a wide variety of solid tumours, and contradictionary results, with a protectional effect of the Valine allele have also been reported [40,58,59].

The ***NAT2*** polymorphism was discovered over 50 years ago when individual variability in isoniazid neurotoxicity was attributed to genetic variability in N-acetylation [27]. The importance increased when it was discovered that many aromatic amine and hydrazine drugs are subject to the acetylation polymorphism, thus affecting therapeutic efficacy and toxicity [28]. The N-acetyltransferase-2 (NAT2), is involved in the acetylation of aryl amines to aryl amides, including some well-known carcinogens [60]. Several studies have demonstrated an association between the NAT2-slow acetylator genotype and increased risk for different types of cancers, for example bladder cancer [3,37,61] and prostate cancer [62]. The NAT2-slow acetylator genotype has also been associated with increased 4-ABP DNA adducts in bladder cells [5], although not all studies have confirmed these results [63].

NQO1 is an inducible enzyme that protects cells from oxidative damage by catalyzing the reduction of toxic and carcinogenic quinoid compound, like benzene metabolites and related chemicals, to hydroquinones, bypassing the production of DNA-damaging semi-quinones and reactive oxygen species [49,64-66]. The 187Ser allele of the *NQO1* exon 6 has been associated with lower enzyme activity compared to the more common allele (Pro187) [22,24,52]. Is has been reported that the

Pro187Ser polymorphism may affect the individual susceptibility to lung and colorectal cancer [36], as well as bladder cancer [1,36] and other urological malignancies (renal cell carcinoma and urothelial carcinoma) [67]. The Arg139Trp polymorphism is less well studied. However, it has recently been reported to be associated with an increased risk of infant acute lymphoblastic leukaemia (ALL) in a Japanese population [68]. The 139Trp allele has also been associated with lower enzyme activity [69].

Gene	Polymorphism	Functional effect	Variant allele in Caucasians	References
GSTM1	Null	Absence of function	0.50 [a]	[15,70,71]
GSTT1	Null	Absence of function	0.20 [a]	[70,71]
GSTP1	Ile105Val	Change in affinity/activity	0.35 [b]	[17,26,50,58,70,71]
NAT2	Slow	Lower enzyme activity	0.45 [c]	[70,71]
NQO1	Arg139Trp	Lower enzyme activity	0.05 [b]	[69]
NQO1	Pro187Ser	Lower enzyme activity	0.15 [b]	[22,24,52,69,72]

Table 1. *Variant alleles of the investigated metabolic polymorphisms in Caucasian populations. Approximate frequency of:* [a] *deletion homozygotes;* [b] *the variant allele;* [c] *individuals with two slow acetylator alleles, yielding a slow phenotype.*

2.4 Polymorphisms with impact on DNA damage and repair

2.4.1 DNA repair pathways

Approximately 10^4 spontaneous oxidative damages are known to occur in every human cell every day [73]. If a cell is not able to repair, this may lead to cell death or mutations and malignant transformation (cancer). To protect the genome a number of highly efficient DNA-repair systems have evolved. These systems can be divided into different pathways depending on the type of DNA lesion they repair. A polymorphism leading to a decreased efficiency of a repair enzyme will increase the susceptibility for the type of damages handled by the repair pathway in which that single enzyme is involved. Cells with defective DNA-repair mechanisms generally show increased sensitivity to genotoxic agents, and increased mutation rate. Individuals with inherited disorders of DNA repair display increased sensitivity toward genotoxic exposure, increased levels of chromosomal aberration and mutation in somatic cells, and predisposition to cancer [74-77].

The polymorphic DNA repair genes investigated in this thesis are involved in four DNA repair pathways, the base excision repair (BER), the nucleotide excision repair (NER), the homologous recombination repair (HR) and the mis-match repair (MMR) pathway.

Damage of single DNA bases, for example as a result of oxidative processes (e.g. ionizing radiation), is repaired by the **BER pathway**. The BER process starts with the enzymatic removal of the damaged base by DNA glycosylases, which creates an abasic site (AP). AP can also be generated spontaneously or following exposure to radiation or chemicals. Some glycosylases (the bifunctional glycosylases) have an associated apurinic/apyrimidinic lyase activity and further catalyze the cleavage of the sugar-phosphate chain and excision of the abasic residue, leaving a single nucleotide gap. Other glycosylases (the monofunctional DNA glycosylases) have no associated lyase activity. When such enzymes initiate repair, the phosphodiester bond at the 5' side of the intact apurinic/apyrimidinic site is incised by apurinic/apyrimidinic endonuclease (APEX1) [73]. After this cleavage of the phosphodiester bond, the BER mechanism can proceed through two different sub-pathways: the short-patch BER and the long-patch BER. The first sub-pathway is characterised by the insertion of a single base at the lesion site. The long-patch BER involves the re-synthesis of a longer oligonucleotide spanning two to seven nucleotides in length. The gap is filled in and sealed by DNA polymerases and DNA ligases [73,78,79] (Figure 2). The BER process can also be initiated by binding of the XRCC1-PARP (Poly(ADP-ribose)polymerase) complex to single strand breaks [80]. No human disorders have been related directly to inherited BER deficiencies, and knockout mice that lack core BER proteins die as embryos, attesting to the important role of the BER process [73].

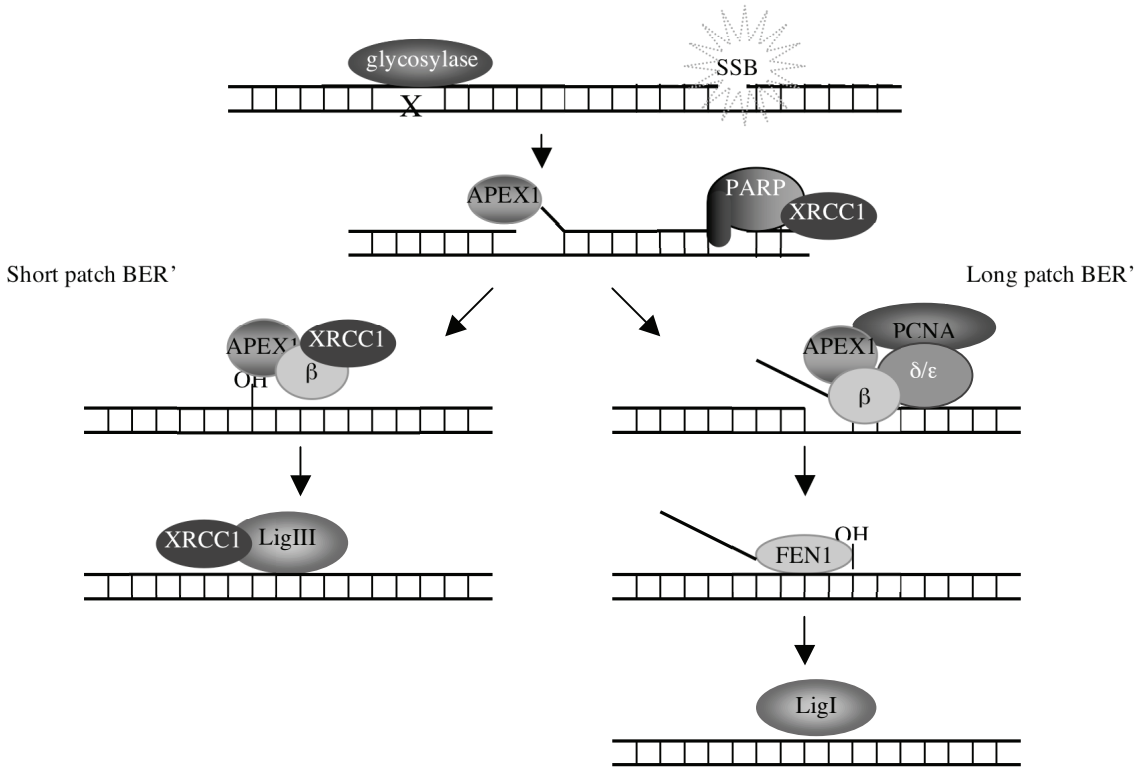


Figure 2. After cleavage of the phosphodiester bond, the BER mechanism can proceed through either the short or long patch sub-pathways. They differ through the repair gap size and enzymes involved. The short patch is characterised by the insertion of a single base by DNA polymerase β (Polβ). In the long patch Pol β or the replicative Pol δ/ε re-synthesise an oligonucleotide, two to seven nucleotides in length. The long patch also requires PCNA, which is loading clamp for the replicative Polymerases, and a flap-endonuclease, FEN1 that recognise and cleaves at the base of the flap structure. The nick is sealed by the DNA ligase III/x-ray repair cross-complementing group 1 (XRCC1) complex.

The NER pathway corrects many different types of damages that cause distortions of the DNA double helix, including UV-induced pyrimidine dimers and bulky adducts. The process consists of recognition of the damaged DNA, excision of an oligonucleotide of twenty-four to thirty-two residues containing the damaged DNA, filling in of the resulting gap by DNA polymerase, and finally ligation of the nick (Figure 3) [81-84]. The NER process consists of two sub-pathways, the global genome repair (GG-NER) and the transcription-coupled repair (TCR-NER). The GG-NER surveys the entire genome for lesions which distort the DNA, while TCR-NER focuses on DNA lesions that block the activity of RNA polymerases and overall

transcriptional activity, and rapidly repairs damage on the transcribed strand of active genes [81,85,86].

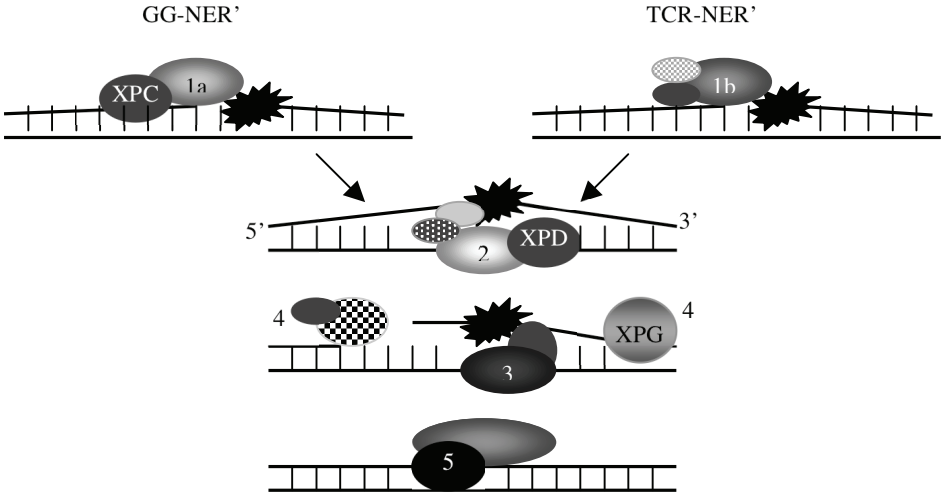


Figure 3. Nucleotide excision repair of a bulky adduct. (1a) DNA damaged-induced helical distortion is recognised by the XPC-hHR23B complex. (1b) Stalled RNA polymerase II acts as a marker for lesion recognition by CSA/CSB. (2) TFIIH, a nine-subunit complex of helicases, XPB, XPD and CCNH, carry out DNA unwinding. (3) RPA stabilises the unwound DNA, and XPA assemble DNA repair factors. (4) The damaged DNA is removed by XPG and the XPF-ERCC1 complex, which make 3' and 5' incisions. (5) The gap is filled by DNA polymerase δ/ε, and sealed by DNA ligase.

The cellular response to DNA double strand breaks (DSB) is to slow down or inhibit progression through the cell cycle, and to induce and activate DNA repair systems. There are two complementary pathways for DNA DSB repair**, the HR repair**, and the **non-homologous end joining (NHEJ)**, which both are highly conserved throughout eukaryotic evolution [87,88]. The HR results in error-free repair, which uses the intact undamaged DNA strand as a template to replace the damaged strand. The NHEJ is error-prone and the proteins involved simply trim the ends of a DNA break and link them together, which may result in loss of a few nucleotides. It appears that one or the other pathway is preferred depending on tissue type, extent of DNA damage, cell cycle phase and relative need for repair fidelity [89]. Also, competition between the NHEJ Ku protein and HR active RAD52 protein is suggested to determine which of the two DSB repair pathways is employed [90]. Defects in DSB repair proteins result in increased sensitivity to ionizing radiation, and there are also diseases that are associated with impaired DSB repair, e.g. the

Nijmegen breakage syndrome [91,92]. The initiation of the HR is thought to be the processing of the 3'end of the DNA DSB by the MRE11/RAD50/NBS1 complex, followed by assemble of the RAD51 complex. Thereafter follows alignment of the sister chromatides and DNA strand exchange. A DNA polymerase copies the DNA sequence of the undamaged template molecule, the ends are ligated, and finally the DNA crossovers (Holliday junctions) are resolved [93] (Figure 4). The HR pathway is thought to predominate in repair of DNA double strand breaks in germline tissues, during the DNA synthesizing phase (S-phase), and in the following G2-phase of the cell cycle [94].

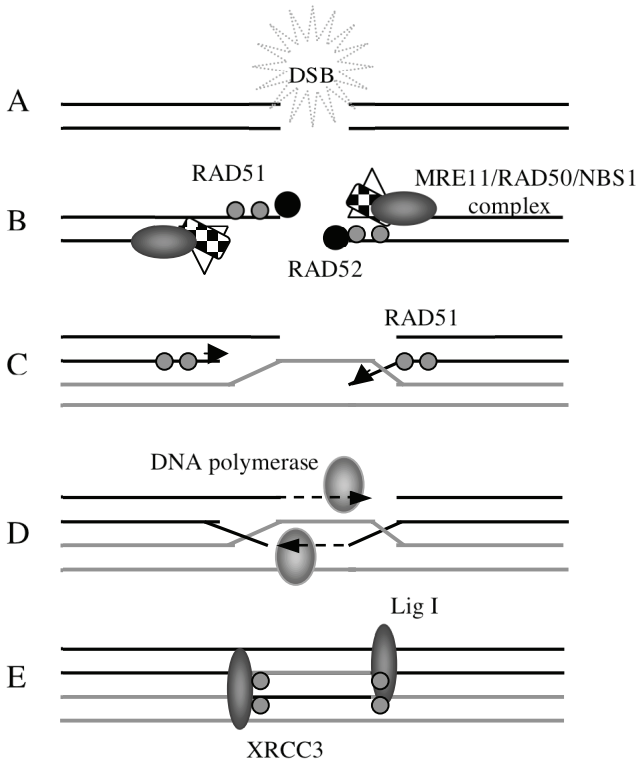


Figure 4. Repair of a DNA DSB through the HR pathway. A: DSB occur; B: 5' to 3' resection by MRE11/RAD50/NBS1 complex. Binding of RAD52 stimulates filament assembly, and the DNA ends are then bound by RAD51 and associated proteins (e.g. RPA, XRCC3); C: The RAD51 and associated proteins interacts with an undamaged DNA molecule (sister chromatide or homologue) and catalyses strand exchange events; D: The 3' end of the damaged DNA molecule is extended by DNA polymerase that copies information from the undamaged partner; E: The ends are ligated by DNA ligase I and Holliday junctions are resolved (e.g. XRCC3).

The MMR system is critical to correct mis-paired bases and small insertions and deletions that are occasionally left behind after the DNA polymerase proofreading. Microsatellites are particularly prone to slippage and inefficient proofreading by DNA polymerase, thus an inactivated MMR system is associated with an elevated mutation rate and microsatellite instability phenotype (MSI), indicating the importance of this repair system for protection against mutation [95].The MMR system can be divided into four phases; (i) recognition of mismatches, (ii) recruitment of repair enzymes, (iii) excision of the incorrect sequence, and (iv) re-synthesis by DNA polymerase using the parental strand as a template (Figure 5). There are different models suggested for how the MMR can discriminate the parental DNA-strand from the damaged DNA-stand. These models include recognition through DNA bending at the mismatch, methylation pattern or recognition through Okizaki-fragment [95,96]. MMR is conserved through evolution from bacteria to man, although the eukaryotic system has more components involved.

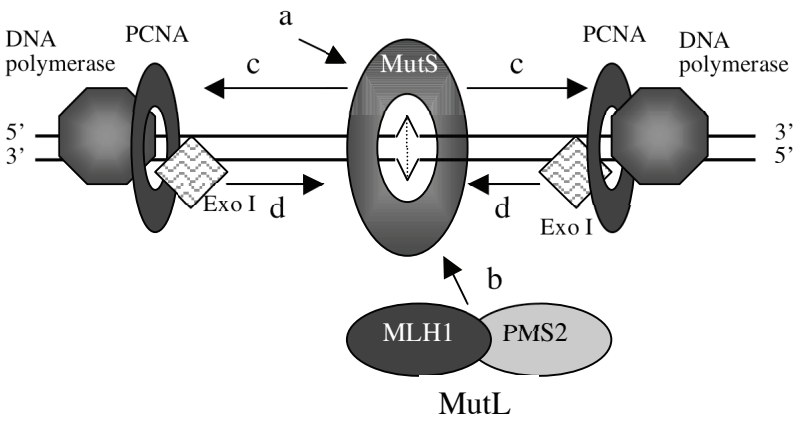


Figure 5. Repair of a single nucleotide mismatch. (a) MutS heterodimer recognises mismatch. (b) MutL heterodimer (MLH1/PMS2) is recruited. (c) The MutS sliding DNA clamps can migrate in either direction. MutL works as a matchmaker, displacing the PCNA/DNA polymerase complex when encountered (i.e. when the DNA clamp moves in 5'>3' direction). (d) MutL recruits exonuclease 1 (Exo I), which excise up to a kilobase of DNA back to the site of the mismatch. The error is corrected by re-synthesis. (Adapted from Jascur & Boland, Int J Cancer, 2006, [95]).

2.4.2 Investigated polymorphisms in DNA repair genes

The repair of damaged DNA is essential to prevent mutations to become fixed in the genome of a cell, and thus, prevent mutagenic transformation. Individuals with repair capacity below the population mean may accumulate genetic alterations, and may be at increased risk of developing different kinds of cancer. Polymorphism can result in subtle structural alteration of the repair enzymes and modulation of cancer susceptibility [86]. Functional polymorphisms in DNA repair genes are thought to be responsible for inter-individual differences in repair capacities that exist within populations. In this thesis ten polymorphisms in eight DNA repair genes, *XRCC1*, *APEX1*, *XRCC3*, *NBS1*, *hMLH1*, *XPD*, *XPC* and *XPG* were studied. Possible associations with lung cancer risk and with the type and frequency of mutations found in the *p53* oncogene were analysed. The frequencies of these polymorphisms in Caucasian populations are shown in Table II.

X-ray cross complementing group 1 protein (XRCC1) is important for genetic stability and for embryonic viability [25] and is involved in the repair of DNA single-strands breaks and base damage from a variety of endogenous and exogenous oxidants. *XRCC1* acts as a scaffold protein or coordinator in single strand break repair in BER through its interaction with at least three other enzymes, poly-ADP-ribose polymerase (PARP), DNA ligase III and DNA polymerase β [19,97] (Figure 2). Three coding polymorphisms that could alter the *XRCC1* function have been identified, at codon 194 (Arg to Trp) within a hydrophobic core in exon 6, at codon 280 (Arg to His) in exon 9, and at codon 399 (Arg to Gln) in exon 10 [97]. Although all of the three polymorphisms lead to amino acid substitutions, there is no direct data on their functional consequences. Nevertheless, the relatively common Arg399Gln variation, which occurs within the PARP binding domain is believed to affect complex assembly and repair efficiency. This polymorphism is also located within the central BRCA1 (breast cancer susceptibility gene 1 product) carboxyl-terminal protein interaction domain that is required for efficient single-strand break repair [98].

The AP endonuclease, APEX1, is the rate-limiting enzyme in the BER pathway [99], where it assembles pol ß onto AP sites and allows pol ß and DNA ligase III to enter DNA repair sites [100] (Figure 2). It is abundant in human cells and accounts for nearly all of the abasic site cleavage activity observed in cellular extracts. *APEX1* expression is elevated in a variety of cancers and a high *APEX1* expression

has been associated with poor outcome to chemoradioterapy. There are a number of polymorphisms identified in the *APEX1* gene. Three of them show reduced endonuclease activity [18], but occur at a rather low frequency. The *APEX1* Asp148Glu polymorphism has not been shown to result in reduced endonuclease activity, but a reduced ability to communicate with other BER proteins giving rise to reduced BER efficiency has been suggested [18]. The variant 148Glu allele has also been reported to be associated with a higher sensitivity to ionizing radiation [19]. An association between the Glu allele and an increased risk of lung cancer has been reported in a Japanese study [2], but no association was reported in a study with Caucasian subjects [101].

The *XPC* (Xeroderma Pigmentosum complementation group C) gene is involved in damage recognition in the global genome repair NER pathway (Figure 3). The XPC protein has been shown to rapidly localize with sites of UV induced DNA damage [102], and the p53 dependent up-regulation of NER [103] includes control of expression of the *XPC* gene, an inducible response that is essential for efficient DNA repair [104]. There are several polymorphisms in this gene, including the Lys939Gln polymorphisms studied in paper IV of this thesis, and associations between polymorphic variants and different types of cancers have been reported [7,39,41,105].

The XPD (Xeroderma Pigmentosum complementation group D) protein is involved in DNA unwinding during NER (Figure 3). The *XPD* gene has several single nucleotide polymorphisms, including those in exon 10 (G→A, Asp312Asn) and exon 23 (A→C, Lys751Gln) [97], studied in this thesis (study I and IV). The amino acid replacement in exon 10 occurs in a region that has been highly conserved during evolution [97]. The exon 23 polymorphism is close to the putative nuclear location signal at codon 683 that is believed to be responsible for XP-D symptoms [106]. These variant alleles exist at frequencies of around 30% in the US Caucasians and are closely linked to each other [107].

The *XPG* (Xeroderma Pigmentosum complementation group G) gene, responsible for XP-G symptoms [108] is essential for the incision of the damaged strand during NER (Figure 3) [109]. The XPG protein has well established catalytic and structural roles in NER and it acts as a cofactor for a DNA glycosylase that removes oxidised pyrimidines from DNA. It also interacts with other NER proteins, for example the TFIIH subunit, during the NER process [110]. Polymorphisms in the

XPG gene, including the Asp1104His polymorphism investigated in this thesis (study IV), has earlier been associated with cancer susceptibility [41,111,112].

The X-ray cross-complementing group 3 protein (XRCC3) participate in the HR pathway [113,114], where it is a member of the Rad51-related proteins [115]. During HR, XRCC3 is required for accumulation of RAD51 protein at sites of DNA damage [116], and it is also involved in the resolution of Holliday Junctions during the later stages of HR [117] (Figure 4). The polymorphism *XRCC3* Thr241Met (study II, IV, V) is a non-conservative change, which does not reside in the functional ATP-binding domains of the enzyme [97] and the role of the *XRCC3* gene in cancer development is still uncertain. The variant 241Met allele has been associated with reduced repair of bulky DNA adducts [20], and predisposition to breast cancer [118], melanoma, bladder cancer and head and neck cancer [8,119-121]. Thus this polymorphism may have biological implications for the functionality of the enzyme and the interaction with other proteins involved in the repair pathway.

The Nijmegen breakage syndrome 1 protein (NBS1) is a member of the MRE11/RAD50 complex, which forms in response to a DNA damage, and actively participates in HR [122] (Figure 4). This complex is also involved in checkpoint signalling and DNA replication. *NBS1* contains two domains in the N-terminal region of the gene, that are found in many DNA damage-responsive cell cycle checkpoint proteins [123], a fork-head-associated (FHA) domain, which is thought to be important for the interactions between phosphorylated proteins, and a BRCT domain. The function of NBS1 is not completely clear, and the only biochemical activity assigned to NBS1 so far is stimulation of the unwinding and hairpin cleavage activities of the MRE11/RAD50 complex [124]. However, individuals with a homozygous germ-line mutation in the *NBS1* gene develop Nijmegen breakage syndrome, which gives, among other characteristics, a predisposition to several types of cancer [91,92]. Also, a null mutation in the *NBS1* gene has been shown to be lethal [125]. The variant allele of the Glu185Gln polymorphism (study IV and V) has previously been associated with a higher frequency of G:C-T:A transversion mutations in the *p53* gene [126].

The MutL protein homolog 1 (MLH1) is involved in DNA mismatch repair where it is one of the components in the MutL heterodimer (Figure 5). MutL dimers appear to function as molecular matchmakers that displace the DNA polymerase/

Proliferating cell nuclear antigen (PCNA) complex during mismatch repair [95], where PCNA function as a the loading clamp for replicative DNA polymerases. The polymorphism investigated in this work (-93 A/G) is found in the transcription factor binding site of the *MLH1* gene, 93 base pairs downstream of the promotor and may affect the gene expression. It has also been associated with an increased risk for colorectal cancer among smokers [127]. Epigenetic silencing of MLH1 expression has earlier been demonstrated to lead to MSI in sporadic colorectal cancer [128,129].

Gene	Polymorphism	Functional effect	Variant allele in Caucasians	References
APEX1	Asp148Glu	Reduced BER efficiency	0.20	[18,78,79]
Cyclin D1	G870A, Exon 4	Increased half-life of the protein. Modulate cell cycle entry	0.42	[23,130]
MLH1	-93 A/G	Change in gene expression	0.22	[127]
MTHFR	Ala222Val	Reduced enzyme activity, increased thermolability	0.40	[6,131-133]
MTHFR	Glu429Ala	Reduced enzyme activity	0.25	[134,135]
NBS1	Glu185Gln	Decreased repair capacity	0.35	[126,136]
XPC	Lys939Gln	Reduced single-strand break repair	0.40	[7,137,138]
XPD	Arg312Asn	Reduced NER efficiency	0.40	[139-141]
XPD	Lys751Gln	Reduced NER efficiency	0.32	[139-141]
XPG	Asp1104His	Associated with single-strand break levels	0.21	[138,142]
XRCC1	Arg194Trp	Increased BER efficiency	0.10	[20,78,79,143,144]
XRCC1	Arg399Gln	Affect BER complex assambly	0.30	[78,79,98,143]
XRCC3	Thr241Met	Reduced repair of bulky DNA adducts	0.40	[20,145,146]

Table 2. *Variant alleles of the investigated polymorphisms concerning DNA repair, the cell cycle, and folate metabolism, in Caucasian populations. Approximate frequency of the variant allele.*

2.5 Other investigated polymorphisms

2.5.1 Cell cycle polymorphism

Cells multiply through the cell cycle process. The cell cycle is divided into four main phases; the G1 (G, gap), S (DNA synthesis, replication of the genetic code), G2 and M (mitosis, segregation of cell components into daughter cells) [147]. There is also the G0 phase for cells that are not actively dividing. Most cells in living

organisms are found in the quiescent G0 state and only begin to proliferate if instructed by extracellular signals like growth factors or hormones. Transitions between the cell cycle phases are governed by check points, and progression through the cell cycle beyond a check point is tightly regulated by a number of regulatory proteins [148]. The regulation of the cell cycle is usually disturbed in malignant cells, which may result in loss of control of the cell proliferation. Cells with unrepaired DNA damage may then proliferate and multiply into tumourous cell clones.

The *Cyclin D1* gene in the retinoblastoma (RB) pathway is involved in a complex network that controls cell proliferation and DNA synthesis at the G1-S checkpoint of the cell-cycle [149]. Over expression of the *Cyclin D1* gene may lead to an increase in cancer risk since the cells may be entering the S-phase while still carrying damaged DNA [150,151]. The polymorphism at the splice site in exon 4 (G870A) of the *Cyclin D1* gene influences the ratio of the two alternatively spliced *Cyclin D1* transcripts, which have been shown to modulate entry into the cell cycle differently [23]. *Cyclin D1* variant allele has been associated with an increased risk of bladder cancer, suggested to be caused by the difference in ratio of the two alternatively spliced forms due to polymorphism [152]. Other studies, however, did not show any association between *Cyclin D1* and cancer risk [153].

2.5.2 Folate metabolism polymorphism

Folate is a water soluble B-vitamin that modulate the DNA methylation [154], and is important for DNA synthesis, stability and repair [155]. Deficiency has been associated with anaemia, cardiovascular diseases, neural tube defects, adverse pregnancy outcome and cancer [156,157]. There are several polymorphisms among the genes involved in the folate metabolic pathway, though those in **the Methylenetetrahydrofolate reductase (*MTHFR*)** gene have had most attention [6,133].

The MTHFR is a critical enzyme in the folate metabolism, where it catalyses the irreversible conversion of the 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The Ala222Val polymorphism of this gene is rather common, with around 15% homozygous and 50% heterozygous for the variant Val allele among Caucasians [6,133]. The Ala222Val polymorphism reduces the activity and increases the thermolability of the MTHFR protein [131,132], and it has been associated with similar symptoms as folate deficiency [133].

The Glu429Ala polymorphism of the *MTHFR* gene is less well studied and the functional significance of this polymorphism is not yet well characterized [135,158,159]. It has been associated with a decreased enzyme activity, but unlike the Ala222Val polymorphism the encoded enzyme is not thermolabile [134,135]. There are approximately 7-12% homozygous and 33% heterozygous for the variant 429Ala allele among north Americans, which includes a majority of Caucasians [134,135].

3. Genetic polymorphisms and p53 gene mutations

3.1 p53 structure and function

The *p53* gene was discovered in 1979 [160,161]. It was originally thought to be an oncogene, but this view was later revised and the gene is now established as a tumour suppressor gene. Mutated versions of the *p53* gene have been observed in the majority of human cancers [162-164]. The gene is located on chromosome 17p13.1 and contains eleven exons, interrupted by ten introns. Exon 2-11 code for the 53kDa nuclear p53 protein, consisting of 393 amino acids [165] (Figure 6). The p53 protein is tetrameric and binds to four repeats of a consensus DNA sequence, 5'-PuPuPuCA/T-3', arranged as inverted repeats [166], which is very common in the human genome [167]. The *p53* gene structure is well conserved in all vertebrates [163], and the protein contains five evolutionarily conserved domains, of which four are located in the DNA-binding core. Early studies noted that most *p53* mutations occurred in these regions, mainly in exon 5-8 (also studied in paper I, III and IV in this thesis) [168,169].

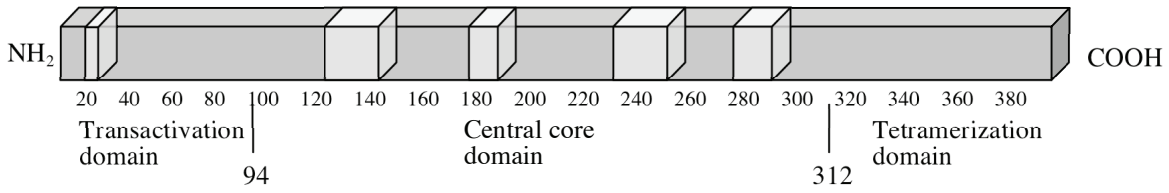


Figure 6. Schematic figure of the p53 protein. The light squares represent the evolutionarily conserved domains, four of which are located in the DNA-binding core domain. The numbers represent the codons of the protein. (Adapted from Hollstein et al 1998 [170]).

The p53 protein has many different biological functions. It is involved in DNA replication, transcription, cell cycle regulation, differentiation and development, DNA repair, maintenance of genomic stability, senescence and apoptosis [163]. Stabilisation and activation of the p53 protein can be triggered in response to several stimuli, like DNA damage or aberrant growth signals. An activated *p53* pathway will lead to cell cycle arrest followed by DNA repair, or apoptosis if the damage of the cell is irreparable [171]. Thus, mutations in the *p53* gene may disrupt many important cellular processes, which in the end may lead to malignant transformation of the cell. To evaluate the cancer risk associated with the *p53* gene it is important to learn more about how the mutations arise. Associations with environmental factors and with polymorphisms in susceptibility genes then become interesting.

3.2 Associations between genetic polymorphisms and p53 mutations

Somatic mutations in the human *p53* tumour suppressor gene are found in almost all types of cancer [162-164], although the mutation frequency varies a lot between different cancer forms. Approximately three quarters of the described *p53* mutations occur as single missense mutations. More than 90% of these are found within the sequences coding for the highly conserved DNA-binding hydrophobic core domain [163] and may prevent or inhibit *p53*-mediated cell responses to cellular stress and DNA damage [166,172,173].

In some human cancers it has been possible to link the mutational pattern at the *p53* locus with environmental exposures like tobacco smoke, aflatoxin B1 and UV radiation [59,174,175]. Among the different *p53* mutation subtypes, transversions has been most strongly associated with environmental exposures [174-176]. There are, for example, a higher frequency of *p53* mutations in tumours among smokers than among non-smokers, suggesting that *p53* inactivation is a key event in smoking induced carcinogenesis [177]. In smokers, there is also an increased frequency of *p53* G:C-T:A transversion mutations, which are known to be induced by the major tobacco smoke carcinogen metabolite benzo[a]pyrene diol-epoxide (BPDE) in vitro and in animal models [168,174,178] (Figure 7). Also G:C-C:G transversions can result from tobacco smoke, through formation of oxygen radicals [178]. These mutations are thus, at least in part, consequences of DNA damage that are induced by exogenous agents.

Hypothetically, the mutational spectra of *p53* in environmentally associated cancers may therefore be influenced by the properties of xenobiotic metabolising enzymes, as well as by the efficiency of the DNA repair system. This has been further investigated in papers I, III and IV of this thesis.

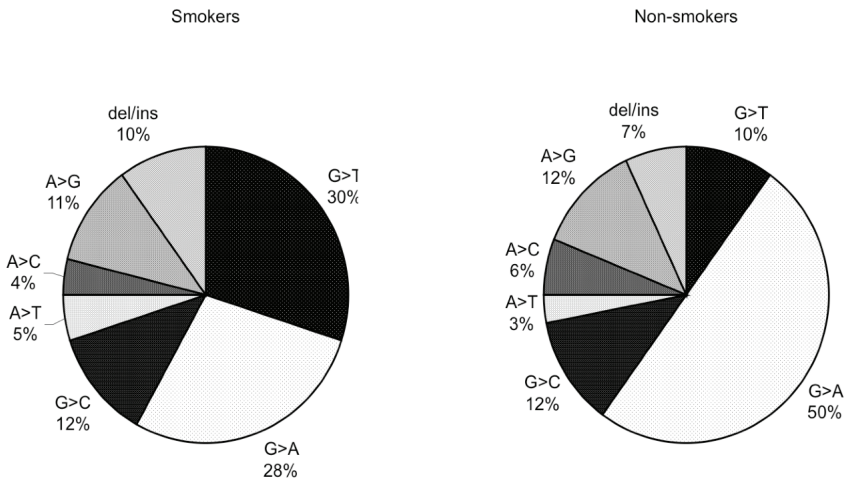


Figure 7. The types of mutations in the p53 gene of human lung cancers (smokers, n=1598; non-smokers, n=99). The mutational pattern differs between smokers and non-smokers. (Adapted from Hainaut and Pfeifer, Carcinogenesis, 2001 [174]).

4. Cancer

Human tumourigenesis involves several steps, and may be initiated by a mutation that increases the genetic instability of a cell (promotion step). The instability increases the risk of acquiring other mutations and genetic alterations, which can give the cell a growth advantage over the surrounding cells, and lead to tumour progression. During the development of a cancer cell, the cell has to overcome the normal need for growth stimuli to proliferate, develop resistance to growth regulators and apoptosis, and be able to induce blood vessel formation [179]. For malignant transformation, the cell has to be able to invade other tissues and to metastasise.

The rise of mutations in a cell depends on both endogenic and exogenic factors. Polymorphisms as risk factors in cancer development are probably more associated with exogenic factors, since they may influence the metabolism of the mutagenic substances, as well as the efficiency of the repair of the damages from such substances. The two cancer forms investigated in the studies of this thesis, lung- and bladder- cancer, are both strongly influenced by environmental exposures.

4.1. Lung cancer

Over the past century, lung cancer has become the leading cause of cancer death worldwide [180]. Lung cancer incidence largely mirrors smoking prevalence, with a latency period of several decades. Based on current and projected smoking patterns, it is anticipated that lung cancer will remain the leading cause of cancer death in the world for decades to come [181]. Important risk factors for the development of lung cancer, other than smoking, include environmental exposure to tobacco smoke, radon, occupational carcinogens, and pre-existing non-malignant lung disease [181]. Studies in molecular biology and epidemiology have elucidated the role that genetic factors play in modifying an individual's risk for lung cancer. The role of genetic susceptibility in lung cancer is indicated by studies showing increased risk of lung cancer in relatives of patients with the disease [180,182]. Only a fraction of smokers, and a low number of non-smokers, develop lung cancer, which implies influence of host factors in individual susceptibility.

4.2. Bladder cancer

Urinary tract tumours, typically originating from in situ precursor lesions in the bladder, is the fifth most common type of cancer [183,184]. Bladder cancer develops via two distinct but somewhat overlapping pathways, where tumours of high grade/stage follow a *p53* dependent pathway and tumours with low grade/stage follow a *p53* independent pathway [185-187]. Approximately 15% to 20% of the bladder tumours are following the first pathway, and are high grade, solid, non-papilloma tumours that aggressively invade the bladder wall and have a high propensity for distant metastasis. These tumours often show loss of important tumour suppressor genes, like the retinoblastoma (*RB1*) and *p53* genes. The remaining 80% represent superficial papillary lesions, typically of low grade, that rarely invade the bladder wall or metastasize. However, about 10% to 15% of the low grade papillary lesions may eventually progress into high grade invasive carcinomas [188].

In the Middle East, the most important risk factor of bladder tumours is infestation of the bladder by *Schistosoma hematobium*. This type of bladder cancer arise from squamous dysplasia as a result of chronic irritation of the bladder [189]. Almost all bladder cancers in Western countries are urothelial cell carcinomas (UCC). Cigarette smoking, industrially related aromatic amines and exposure to the drugs phenacetine, chlornaphazine and cyclophosphamide, are associated with UCC occurrence [190-192]. Smoking accounts for up to 50% of the cases in western countries, and occupational and environmental exposure to aromatic amines yields another 25% [188]. The combination of cigarette smoking and exposure to petrochemical carcinogens combined with individual genetic predisposition has been shown to have synergistic effects [193,194].

5. Aim of this thesis

This thesis is a contribution to the knowledge about the importance of polymorphisms in risk assessments of human cancers. The aim has been to investigate the influence of polymorphisms in genes involved in xenobiotic metabolism, DNA repair and cell cycle regulation on (1) the frequency and type of somatic *p53* mutations in lung and bladder tumours, (2) lung cancer risk in a well defined population, and (3) to develop a method by which the functional effect of DNA repair gene polymorphisms can be studied.

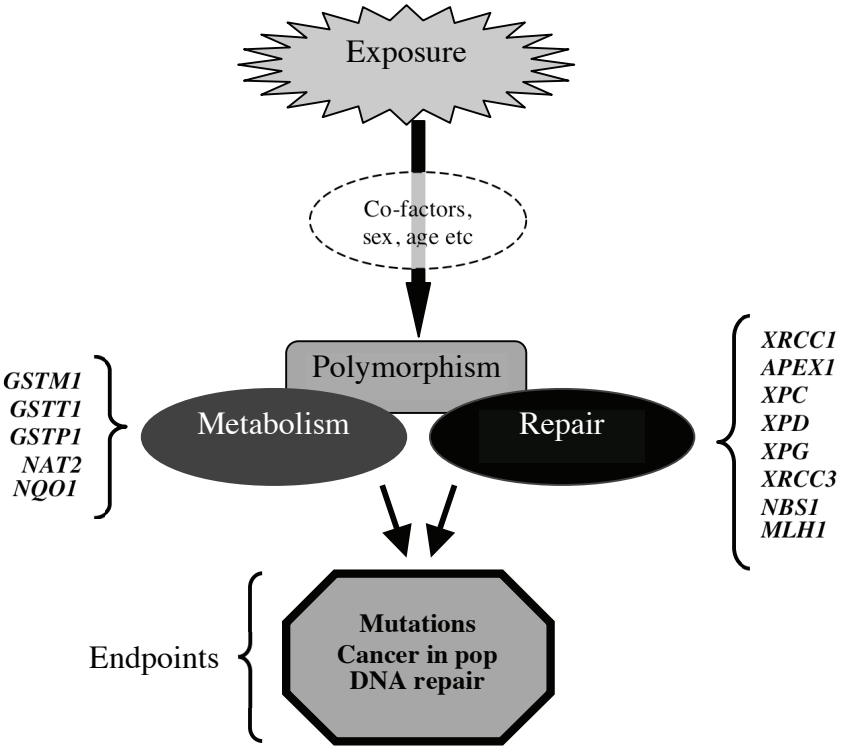


Figure 8. Study Design. *As endpoints we have studied the frequency and type of mutations in genes related to cancer (the p53 gene)(study I, III and IV); risk variations in populations in case control studies (study II); and cell survival and repair after DNA damage, with the Comet assay method (study V).*

6. Comments on methodology

There are different ways to analyse genetic polymorphisms, and new variants develop all the time as the techniques are improving. Today, with access to the right equipment a high number of samples can be analysed in a relatively short time. The large amounts of data that in this way will be made accessible are going to improve our possibilities to analyse the complex web of gene-environment interactions.

6.1. Genotyping methods

The base for genotyping is to amplify a piece of DNA (a gene or part of a gene) of interest. When there is enough genetic material the sample can be analysed with regard to what genotype it represents. There are different methods of analysis, two of which have been used in this work.

6.1.1. PCR-RFLP

This method is rather inexpensive and easy to use. The genes of interest are amplified with a thermocycler until the number is large enough to be visualised on an agarose or acrylamide gel, stained with ethidium bromide (Figure 9). Restriction enzymes that cleave the DNA specifically for different alleles are used, and the alleles of each sample can be observed as a specific band pattern on the gel. Negative controls were included in all PCR-runs to prevent misjudging following contamination of the samples. The genotyping results were confirmed by repeated analysis of approximately 10% of all samples, randomly chosen. For two of the investigated genotypes (*GSTT1* and *XRCC1*) my results were also later confirmed in a repeated analysis, by a second independent researcher.

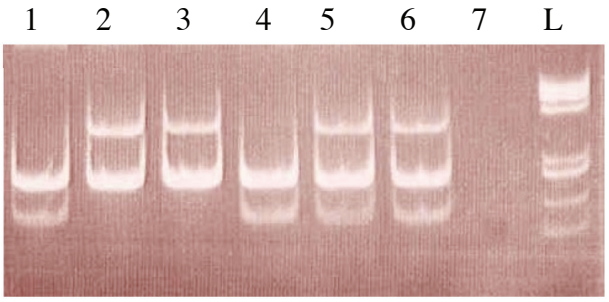


Figure 9. Gel-picture of cleavage products from a duplex PCR for analysis of the GSTT1 and GSTP1 polymorphisms. Line 1 shows a sample heterozygous for the GSTP1polymorphism (Ile/Val), and with a GSTT1 null genotype (no GSTT1 line visible). Line 2 shows a sample homozygous for the Ile allele of GSTP1, and with the normal GSTT1 gene. Line 7 represents the negative control and L is the DNA size-ladder.

6.1.2. Taq-Man allelic discrimination method

This method is more expensive than PCR-RFLP, and more advanced equipment is needed. However, there are many advantages compared to the previously described PCR-RFLP. It is a closed tube system that does not require post PCR handling, and this minimises the risk of contamination. The system has high throughput, and the same amount of samples that would take months to run by the PCR-RFLP may with this method be handled in about a week. In short the method is as follows: A fluorogenic probe, consisting of an oligonucleotide labelled with both a fluorescent reporter dye and a fluorescent quencher is used. The probe is cleaved by the 5' nuclease activity of Taq DNA polymerase, but only if the probe target is being amplified. Cleavage of the fluorogenic probe during the PCR assay liberates the reporter dye from the effect of the quencher (Figure 10).

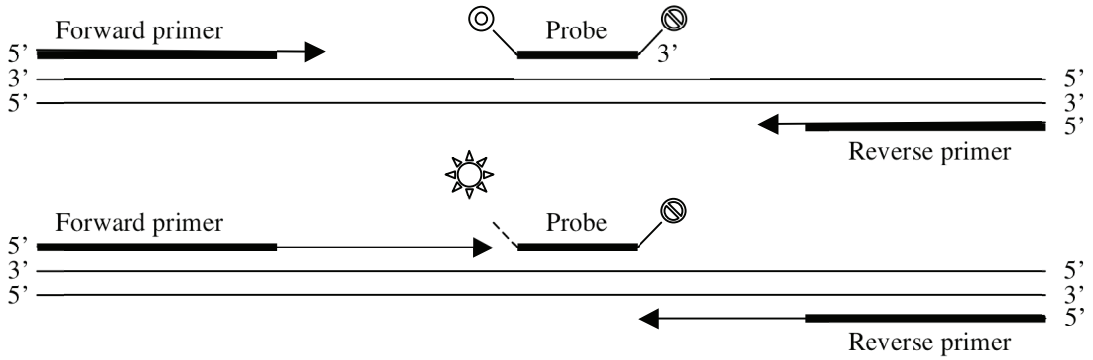


Figure 10. A: The fluorescence is initially silenced by a quencher. B: During the extension phase of the PCR cycle, the probe gets cleaved by a 5'>3' nuclease activity, which separates the reporter dye from the quencher and results in an increase in fluorescence.

Probes labelled with different fluorescent reporters, specific for each allele, are included in the PCR assay. The assay is performed under competitive conditions with both probes present in the same reaction tube, and mismatched probes are prevented from binding due to the more stable binding of exact-matched probes. A substantial increase in dye-specific fluorescence indicates homozygosity for the dye-specific allele and an increase in both signals indicates heterozygosity (Figure 11). In this thesis, the results were confirmed by random sequencing of 8% of the samples.

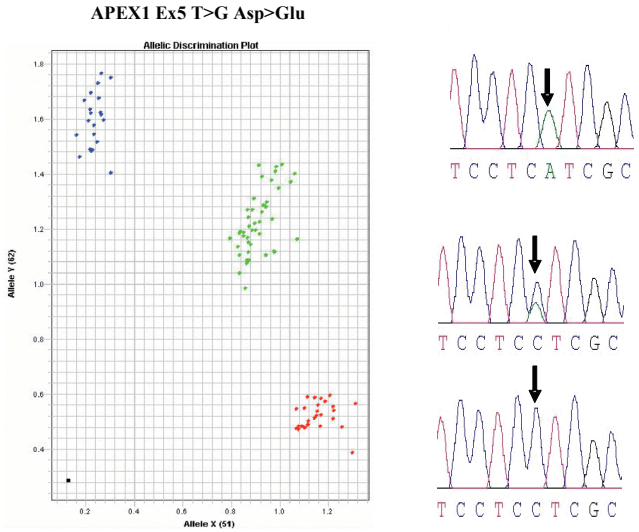


Figure 11. Examples of allelic discrimination analyses. Samples marked with red or blue are homozygotes for wild type or variant alleles. Samples marked with green are heterozygotes

6.2. Mutation screening by SSCP

One commonly used technique for detection of point mutations, deletions and insertions is the single cell conformation polymorphism (SSCP) analysis [195-197]. The method uses the fact that each single stranded DNA molecule has a characteristic secondary structure in a non-denaturating gel, depending on the nucleic acid sequence. If the nucleic acid sequence is changed the conformation of the folded structure will also change, which can be observed as a mobility shift in the gel. The impact on the mobility shift is dependent on the change itself, as well as the structure of the neighbouring bases [198,199]. Different gel conditions can detect different types of changes in the nucleic acid sequence and the method therefore needs a lot of in-tuning before use. For example, temperature, presence of glycerol, acrylamide concentration, cross linking acrylamide/bis-acrylamide and size of the analysed fragment are important gel parameters [200]. Smaller changes, like SNP are detected by some gel conditions and not by others. Thus, samples should be analysed with several different gel conditions, and all mobility shifts should by sequenced to confirm and identify suspected polymorphisms. Is has been estimated that the SSCP method is capable of detecting over 90% of the base changes in any given DNA sequence [201].

6.3. Single cell gel electrophoresis (SCGE) - The Comet assay

The SCGE method, or the comet assay, was developed during the late 1980's [202], and during the past decade it has become one of the standard methods for assessing DNA damage. The assay attracts adherents by its simplicity, sensitivity, economy and flexibility with regard to the type of target cells. It is performed on single cells, and not much material is needed. There are a number of slightly modified comet assays for detection of different types of lesions. For example, the neutral comet assay for detection of DNA double strand breaks [203], the alkaline comet assay, which is used to detect single/double strand breaks [202,204,205] and comet assay with enzymes for more specific analyses of different types of lesions [206-208].

The comet assay is ideally suited for human investigations because it requires no prelabeling with radioactivity or other harmful procedures and can be applied to easily obtainable cells. Usually, white blood cells are used, since blood is easy to sample and blood cells are exposed to almost all sorts of environmental toxins that enters the body. However, there are some disadvantages as well. For example, white blood cells are normally not the target tissue for cancer and it is not clear that the damage detected in these cells reflects the damage in the actual target tissue.

There are many different ways to analyse the results after the electrophoresis step has been performed. There are numerous software packages that compute fluorescence parameters for comets, usually as tail length, relative tail intensity (%tail DNA) or tail moment (tail length x tail intensity). It is also possible to compute DNA damage from comets without image analysis programs by visual scoring (VS), which is a rapid as well as an easy method to perform.

In this thesis the common alkali version of the comet assay was used to monitor the speed with which the cells removed DNA lesions, as a measure of DNA repair capacity. The assay was performed on lymphocytes that had been cultured for 48 hours in an PHA containing medium and then exposed to 0.055 mM methylmethane sulphonate (MMS). This mutagen was chosen because it does not require metabolism to react with DNA to form adducts. The slides were rinsed three times for a total of six minutes, followed by incubation at 37 °C, for 0, 15, 40 or 120 minutes, lysed, and unwinded in cold alkaline buffer. The amount of DNA strand breaks at the different time points after MMS exposure was analysed (Figure 12).

DNA damage was computed by VS. It has been shown that VS and computer image analysis (percent tail DNA) are in a very close agreement and highly correlated [209].

Each cell with a visible head was scored, and the cells were classified as normal cells, mild, moderate or severe comets. The VS was calculated [209,210] by assigning each cell a value according to category; 0 for normal cells, 1.33 for mild comets, 2.67 for moderate comets, and 4 for severe comets [211]. With a total of 100 cells, the total score for a slide was between 0 and 400. By division by four, the VS is converted to a pseudo-percentage score (PS=VS/4), comparable to % tail DNA [209-211].

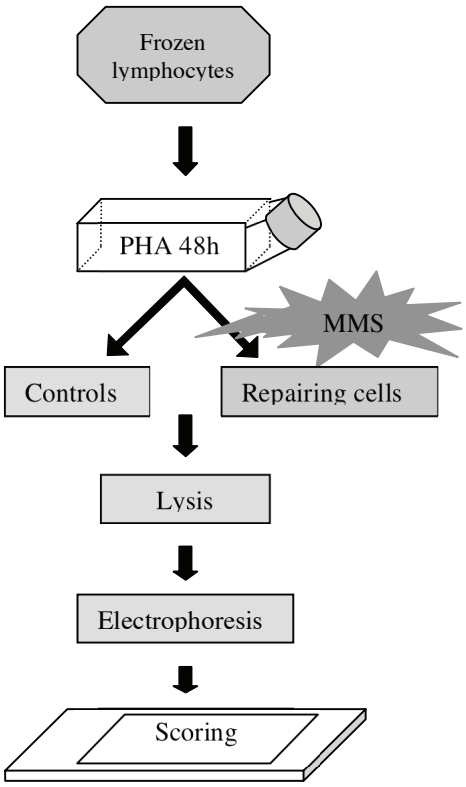


Figure 12. Experimental design.

7. Study populations

The papers in this work are based on three different study populations. Two of these are Swedish populations, one lung cancer case control population (study I and II), and one bladder cancer cohort (study III and IV) in the Stockholm city county. The third study population, recruited to obtain material for the polymorphism functionality study (study V) is based on healthy volunteers recruited from non-smoking students and personnel at Leeds University in Leeds, England. All three study populations consist of a vast majority of white Caucasians.

The Caucasian frequencies, according to literature, of the polymorphisms investigated in this thesis, are shown in Table 1 and 2.

7.1. The lung cancer study population

The lung cancer study population in this thesis originates from a study of lung cancer risk in relation to genetic polymorphisms of metabolic phase II enzymes [212]. The lung cancer patients were recruited during 1992-1995 at the three major county hospitals responsible for diagnosis and treatment of lung cancer in Stockholm County. Controls from the Stockholm County population register were frequency matched to cases by hospital catchment area, gender, age group and smoking category.

Patients were asked to participate in the study after being diagnosed with lung cancer. The majority of the cases included in the population had adenocarcinoma (51%) or squamous cell carcinoma (22%). For each never-smoking case, the next diagnosed ever-smoking case of the same gender and age group was recruited, in the same hospital, yielding a study population with an unusually high frequency of non-smokers, approximately 50%, and a median age of 69 and 68 years among cases and controls respectively. Compared to other studies [9,213] the smoking doses among the included smokers were also rather low.

More than 70% of the index cases (non-smoking patients) in our study population were women, reflecting the fact that women usually are overrepresented among non-smoking lung cancer cases [214]. The high number of women and non-smokers in this population is unusual, since most other studies focus mainly on male smokers. There is only one other study with a similar high number of non-smokers included [215], but in that study gender was not specified. Our unequal gender

distribution gave us a unique opportunity to analyse effects on women. However, the statistical power to do analyses on men was reduced.

7.2. The bladder cancer study population

The bladder cancer study population in this thesis originates from a collaboration between seven hospitals in Stockholm county 1995-1996. This collaboration led to collections of tumour tissue and blood samples from almost all new cases of bladder cancer in the region. Tumour tissue from a total of 563 patients of Caucasian origin were collected [216], and demographic and exposure data were obtained from a structured questionnaire which was answered by about 50% of the participants. None of the patients had been given treatment before analysis. Blood samples were available from 327 patients, of whom 35% were women. The median age of the patients studied was 71 years. No selection of patients towards high grade tumours was made for the initial recruitment [216] or inclusion of patients in the studies of this thesis. Only 36.1% of the samples had tumour grade 3 or higher.

7.3. The comet assay study population

In order to perform the DNA repair polymorphism functionality study (study V) peripheral blood lymphocytes from 52 volunteers were drawn and used as tissue samples. The volunteers were recruited from students and personnel at the University of Leeds, Leeds, United Kingdom. Since both smoking and disease may influence the DNA repair capacity of an individual, only healthy non-smokers (n=52) were included. All subjects provided 10 ml of whole blood. The only information kept about the volunteers was their sex, and a confirmation of their non-smoking status. An informed consent was obtained from all subjects.

8. Ethical issues

Functional genetic polymorphisms or haplotypes differ in frequency among populations, and association studies, similar to those performed in the present work, will allow comparisons between different groups. This could raise risks of group discrimination. For example, researchers may find that a genetic allele associated with an increased risk of disease is more common in one population than another. This information can be misinterpreted to mean that all members of that group has an increased risk for the disease, even though this is only true for individuals, inside the group or out, who carries the risk allele. Also, information from genetic association studies may be misinterpreted and used by for examples insurance companies or employers. This would be an unfortunate outcome, since the polymorphic effect is difficult to predict at the individual level.

One positive ethical outcome of genetic studies could be that they might help to demonstrate that common ideas about “race” emerge largely from social and cultural interactions and are only loosely connected to biological ancestry.

9. Summary of results and discussions

9.1. Paper I. Influence of common XPD *and* XRCC1 *variant alleles on p53 mutations in lung tumours.*

Less efficient repair of DNA damage is hypothesised to affect the number of mutations that are fixed in the genome of a cell. The type of mutations could also be affected since different pathways repair different types of damage. In this study, genetic polymorphisms in *XPD* and *XRCC1* involved in NER and BER respectively, were investigated in 97 lung cancer patients (56 never-smokers and 41 ever-smokers) from the lung cancer study population. The possible association between the polymorphisms and the number and type of mutations in the *p53* gene in lung cancer tumours was investigated. In particular transversion type mutations that are considered to be more associated with external exposures, like tobacco smoke, were of interest.

We found that none of the investigated variant alleles (*XPD* Asp312Asn, *XPD* Lys751Gln or *XRCC1* Arg399Gln) altered the overall frequency of *p53* mutations. However, among patients with at least one *XPD* variant allele, *p53* transversion mutations were marginally increased compared to wild-type homozygotes (P=0.085). We also observed that six of seven smokers who carried at least one *XPD* 751Gln allele had *p53* transversion in their tumours. This is consistent with an earlier study that reported a decreased repair capacity associated with the *XPD* 751Gln allele [141]. An increased risk for different cancers has also been associated with this allele [217-219].

The *XRCC1* variant allele did not show any effect on the type or frequency of *p53* mutation, which is consistent with a previous report showing that the two different alleles of the *XRCC1* Arg399Gln polymorphism support DNA single strand break repair equally well [98].

Our results indicate that individuals carrying at least one *XPD* 751Gln allele had an increased frequency of smoking-related *p53* mutations in lung tumours. Thus, it seems possible that smokers with this allele are more susceptible and have an increased risk of getting *p53* mutations, than smokers with the wild type allele, or non-smokers in general. Presumably this is due to the reduced DNA repair

proficiency of the *XPD* 751Gln allele [141]. A mutated *p53*-gene is strongly associated with human cancer tumours [162-164], and thus, our results indicate a possible increased lung cancer risk in smoking individuals with a decreased NER repair capacity.

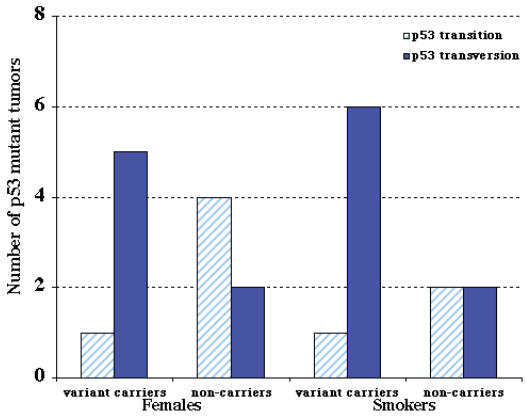


Figure 13. *Number of p53 transitions (striped bars) and transversions (filled bars) among female or smoking patients carrying at least one variant allele in XPD exon 23 (751Gln), compared to those carrying the wild type allele (XPD 751Lys) only.*

9.2. Paper II. Polymorphisms in the DNA repair genes XRCC1*,* APEX1*,* XRCC3 *and* NBS1*, and the risk for Lung Cancer in never- and ever- smokers*

Depending on the polymorphism, the type of cancer studied, and the study population, contradictory results concerning possible effects of low-penetrance polymorphisms on cancer risk have been reported. The aim of this study was to investigate possible effects of BER- and HR-gene polymorphisms, on lung cancer risk in a well-defined study population, characterized by a high percentage of non-smokers (50%) or low-dose smokers, as compared to other populations studied. Since women are overrepresented among non-smoking lung cancer cases [214], our study population also had an unusual high percent of women (70%).

The results from this study showed that variant alleles in different DNA repair genes can modulate lung cancer risk. The *XRCC1* 399Gln allele was associated with a significantly decreased risk for lung cancer among non-smoking women (OR 0.4, 95% confidence interval (CI) 0.2-0.9) (Figure 14). A similar protective effect of the *XRCC1* 399Gln allele was reported in a multicenter study including 116 informative

non-smoking lung cancer cases [215], although in that case gender was not specified. In other studies, with a predominance of high-dose smoking men, the *XRCC1* 399Gln allele was rather associated with an increased cancer risk [2,9,101,213], which may suggest a possible gender-specific variation in the repair efficiency associated with this allele. No significant effect was seen with the *APEX1* polymorphism, which is in agreement with other studies [101,136].

Women smokers carrying the *XRCC3* 241Met allele showed a significantly decreased risk for lung cancer (OR 0.3, CI 0.2-0.7) (Figure 14). Compared to other studies where this allele rather was associated with an increased risk [146,220,221], the smoking doses in our study population were much lower, which may explain the difference in results [221].

The *NBS1* 185Gln allele was significantly associated with an increased risk for lung cancer among non-smoking women (OR 2.2, CI 1.0-4.8) and low-dose smoking women (OR 4.8, CI 1.5-15.7) (Figure 14). This is consistent with a previously reported higher frequency of G:C-T:A transversion mutations in the *p53* gene and a putative decrease in DNA repair capacity associated with this allele [126].

The protective effect of the variant *XRCC3* 241Met allele was strengthened when combined with the low-risk Glu185 allele of the *NBS1* gene, which is active in the same DNA repair pathway (HR). Smokers (OR 0.38, CI 0.16-0.90) and women (OR 0.42, CI 0.21-0.85) with at least 3 low-risk alleles in these two HR genes showed a significantly decreased risk for lung cancer.

Our results show that the investigated polymorphisms of the *XRCC1*, *XRCC3* and *NBS1* genes may influence lung cancer susceptibility among women, giving an altered risk for lung cancer in carriers of the variant allele, and that combination of risk alleles in the two HR genes can enhance the effects. This is probably due to impact of the polymorphisms on the repair capacity, which may alter the risk of having mutations fixed in the genome.

The number of men in the lung cancer study population was too low to yield any statistically significant results about the associations between the investigated polymorphisms and lung cancer risk among men. However, there were some indications that the result in men would be different from that in women. Even if it was not possible to draw any conclusions about gender-specific effects due to the

unequal number of men and women in our study population, it would be very interesting to investigate this more closely in future studies.

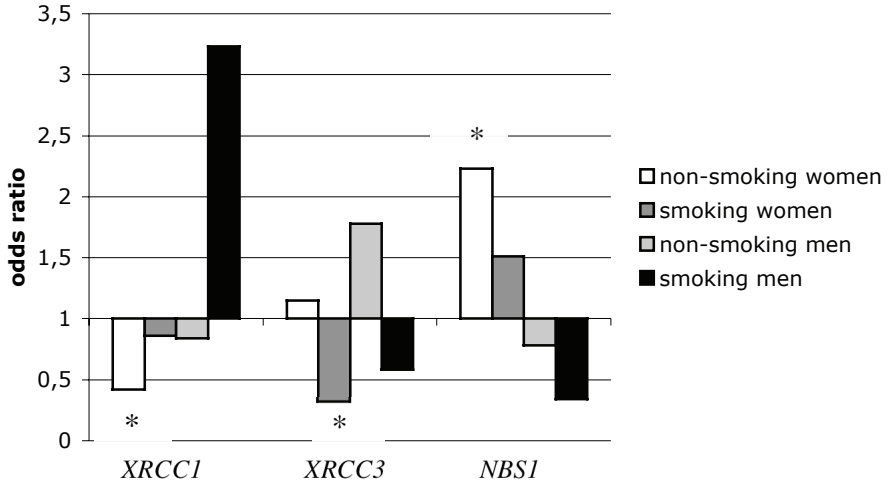


Figure 14. *Odds ratios for the XRCC1, XRCC3 and NBS1 after stratification into smoking/non-smoking men and women. The common allele of each polymorphism is used as the reference value (OR=1.0).*

9.3. Paper III. Influence of GSTM1*,* GSTT1*,* GSTP1 *and* NAT2 *genotypes on the* p53 *mutational spectrum in bladder tumours*

Impaired or less efficient metabolism of reactive substances is hypothesised to affect the number and type of mutations that a cell acquires. In this study we investigated four metabolic genes that are involved in a number of detoxification processes and in which functional and relatively common polymorphisms have been identified in human populations. The aim was to investigate if groups of individuals with different genotypes differed also in the frequency and type of mutations that existed in the *p53* gene in their tumours.

A two-pathway model for UCC pathogenesis has been proposed by earlier studies [185-187], and according to this model, tumours of high grade/stage follow a *p53* dependent pathway, while tumours with low grade/stage follow a *p53* independent pathway. In this study we found that *p53* mutations were more common in advanced tumours (P≤0.002), and that these tumours were more frequent in patients

with at least one *GSTP1* 105Val allele (P=0.057). However, no significant difference in the frequency of *p53* mutations among patients with different genotypes was observed overall.

A high proportion of G:C-C:G transversions in the *p53* gene of bladder tumours has been reported in many studies [178,222-225]. This was also the case in our study. A possible connection of G:C-C:G transversions to oxidative DNA damage has been suggested [222], and since GSTs, besides providing protection against xenobiotics, also are involved in protection against oxidative stress [226], it could perhaps be expected that, with one exception, all tumours with this type of transversion occurred in *GSTM1* negative patients. *p53* transversion mutations overall were also significantly more common in *GSTM1* negative compared to *GSTM1* positive individuals (OR 5.26, CI 1.07-25.82).

Among smokers, carriers of the *GSTP1* 105Val allele had a higher proportion of *p53* transversions than non-carriers (P=0.022). This could be explained by a difference in substrate affinity by the two alleles of the *GSTP1* Ile105Val polymorphism, leading to a less efficient detoxification by the Val allele in our study population. Earlier studies have shown that the two alleles of differ in enzymatic activity [17,50,58], and it may be possible that the two alleles have different efficiency for detoxification of different carcinogens.

These findings, taken together, suggest that impaired glutathione conjugation may affect the mutation spectrum in critical target genes like *p53* in bladder cancer. An interesting question would be if some types of mutations in the *p53*-gene may lead to more aggressive cancer tumours. Polymorphisms that influence the mutation type could then be important for the cancer progression and diagnosis of cancer patients.

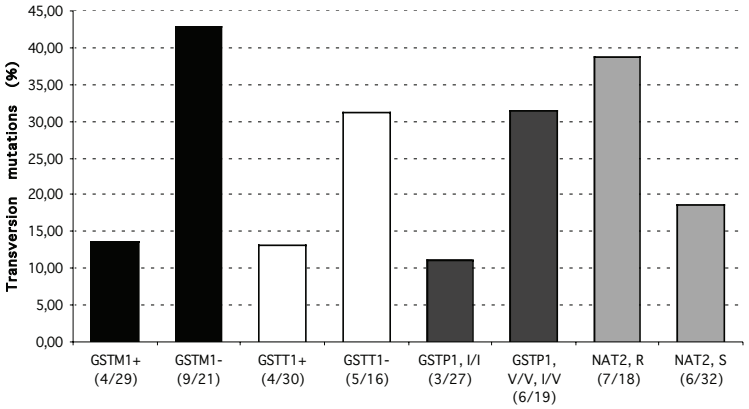


Figure 15. *Percent p53 transversions found in tumour samples with different metabolic genotypes. (Number of transversion mutations divided with the total number of p53 mutations within each genotype subgroup). Calculated according to number of mutations rather than number of individuals. Samples with the GSTM1 null genotype had a significantly higher frequency of transversions than samples carrying the functional GSTM1 gene.*

9.4. Paper IV. Influence of polymorphism in DNA repair and defence genes on p53 *mutations in bladder tumours*

Only a few studies have so far been conducted to study the effect of polymorphisms in DNA repair genes on the frequency and type of *p53* mutations in tumours. In addition to the increased frequency of *p53* mutations in lung tumours of patients carrying the *XPD* 751Gln allele, reported in study I in this thesis, the *XRCC1* Arg399Gln [227], the *NBS1* Glu185Gln [126] and the *XPD* Asp312Asn [228] polymorphisms have previously been associated with an increased *p53* mutation frequency.

In this study, eleven polymorphisms, *XPC* (Lys939Gln), *XPD* (Lys751Gln), *XPG* (Asp1104His), *XRCC1* (Arg3999Gln), *XRCC3* (Thr241Met), *NBS1* (Glu185Gln), *cyclin D1* (Pro241Pro), *MTHFR* (Ala222Val and Glu429Ala) and *NQO1* (Arg139Trp and Pro187Ser), involved in DNA repair, metabolism and cell cycle regulation were analysed. The aim was to investigate if groups of individuals with different genotypes differed in the frequency and type of mutations that existed in the *p53* gene in their tumours. The type of mutations could be affected since different pathways repair different types of damage.

An interesting observation in the present study is the overall increased risk of *p53* mutations in individuals with the variant allele of the *Cyclin D1* gene (OR 2.4, CI 0.8-6.7, for variant homozygotes). Although not statistically significant, the effect seems to be dependent on the number of variant alleles, with an OR of 1.8 and 2.4 among variant heterozygous and homozygous, respectively. It may be possible that the polymorphism in *Cyclin D1* exon 4 results in an overall higher expression of the gene, which then could increase the rate of cell proliferation and reduce the time for DNA repair [150,151], and thereby create an increased risk of *p53* mutations.

XRCC3 241Met homozygotes also had an overall increased risk for *p53* mutations among non-smokers (P=0.03), which is consistent with the notion that effects of genetic polymorphisms may be more prominent in groups with low-level exposure to carcinogens, since the activity of both alleles might be saturated in an environment heavily exposed to carcinogens [229].

Carriers of the *NQO1* 187Ser allele showed increased risk for *p53* transversions (OR 4.7, CI 0.9-26.1), and 2/2 *NQO1* 139Trp allele carriers but only 7/40 of the *NQO1* Arg139 homozygotes had *p53* transversions. Since transversions have been associated with exogenous exposures [174-176] including those causing oxidative DNA damage [230], our results are consistent with a less efficient reduction of carcinogenic quinoid compound among individuals carrying the 187Ser allele for *NQO1* polymorphism, leading to a higher risk of acquiring oxidative DNA damage and transversion mutations in the *p53* gene. In addition, 3/3 *p53* transversions, but only 5/12 *p53* transitions were found among smoking carriers of the *XPC* 939Gln allele.

Our findings suggest that altered repair and detoxification due to genetic polymorphism may influence the frequency and type of *p53* mutations in bladder cancer. This influence may result from individual modulation of mutagenic DNA adduct levels, and be of importance for the individual risk of developing urinary bladder cancer.

9.5. Paper V. Influence of polymorphisms in DNA repair genes on repair kinetics, as measured by the comet assay

The functional significance of specific polymorphisms at the cellular level is still unknown for several DNA repair genes. DNA strand breaks can be caused by a number of different factors, like direct breaks caused by ionising radiation or secondary breaks caused by the removal of bulky adducts or mis-paired bases during the repair process. A possible effect of a certain polymorphism may vary with the type of DNA damage.

The aim of this study was to explore the possibility of using DNA strand break repair kinetics in human lymphocytes, following methylmethane sulphonate (MMS) exposure, as an experimental method to investigate the functional importance of DNA repair gene polymorphisms. Seven polymorphisms, *XRCC1* Arg399Gln, *XRCC1* Arg194Trp *XPD* Lys751Gln, *hMLH1* –93 A/G, *APEX* Asp148Glu, *XRCC3* Thr241Met and *NBS1* Glu185Gln, were included. Lymphocytes were obtained from healthy non-smoking volunteers. DNA samples were genotyped and the cells were stimulated with PHA for 48 hours, treated with MMS, and then left to repair. DNA damage at four observation time points (6, 21, 46 and 126 minutes) after MMS treatment was evaluated with the Comet assay, both as % damaged cells and as a pseudo score (PS), comparable to % tail DNA (Figure 16).

An overall repair effect with increasing repair time, reflecting a steady increase in the number of normal comet-free cells derived from the repair of cells in other groups was observed during this study. Also, a steady increase in the number of severe comets was observed, which likely represents cells that, following damage, were destined to die (Figure 16). Our results indicate that the repair rates at early stages during the repair process are affected by the *APEX1*, *XRCC3* and *NBS1* polymorphisms.

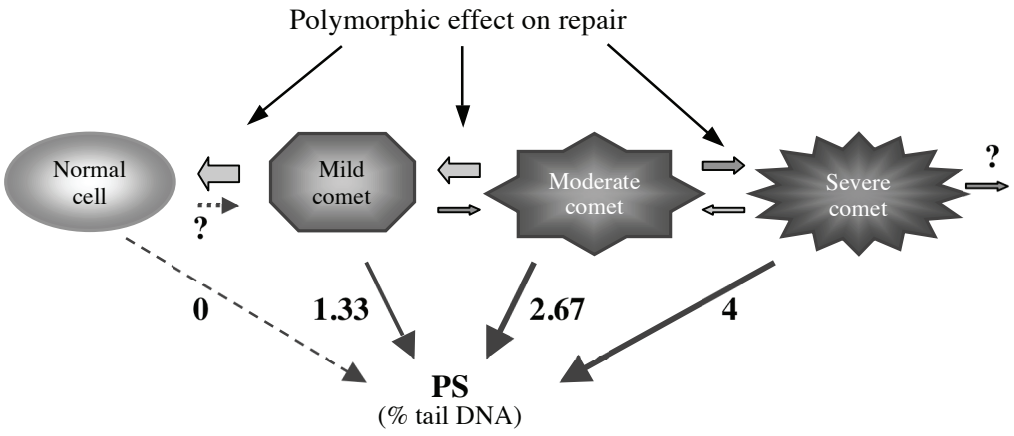


Figure 16. Model of repair. The frequency of normal and severely damaged cells is increasing during the observation time. The polymorphisms are hypothesized to influence the transition of cells between the different categories, i.e. the rate of repair. Each counted cell is assigned a value according to category. With a total of 100 cells, the total score for a slide is between 0 and 400 "arbitrary units", which may be converted to a pseudo score (PS). The PS is mainly influenced by the comets. The normal cells only influence by their number. Apoptotic and dead cells were not measured in the present study.

The main pathway for repair of MMS-induced DNA damage is the BER pathway. The rate limiting enzyme in BER is *APEX1* [99], and thus, a potential influence by *APEX1* on the repair could be predicted. As expected, the largest and most consistent effect in this study was observed for the *APEX1* polymorphism. An increased OR of a high PS-value at the first three time points was observed in cells from individuals with at least one *APEX1* Asp148 allele (OR 5.62, CI 2.04-15.48; OR 3.44, CI 1.35-8.78; OR 5.62, CI 2.04-15.48 respectively) and indicates that the rate of repair at early stages post-MMS treatment may be modified by the *APEX1* polymorphism.

Cells with at least one *XRCC3* 241Met allele were shown to have an increased level of DNA damage (high PS value) after MMS exposure at the first two time points (OR 3.57, CI 1.59-8.00, similar for both). This is consistent with an earlier study where the variant *XRCC3* 241Met allele was associated with a reduced repair of bulky DNA adducts [20].

The effect of the *NBS1* Glu185Gln polymorphism was inconsistent over time. The *NBS1* 185Gln allele appeared to increase the OR for a high PS value at the first

observation time point (OR 3.17, CI 1.39-7.19), followed by a significantly decreased OR at the next two time points (OR 0.44, CI 0.20-0.99; OR 0.32, CI 0.14-0.72 respectively). Taken together, these data make it difficult to interpret the effects of *NBS1* on repair of MMS-induced DNA damage.

In conclusion, we observed that the initial repair of MMS-induced DNA damage may be affected by the *APEX1* Asp148Glu, *XRCC3* Thr241Met and *NBS1* Glu185Gln polymorphisms. These polymorphisms were associated with the rate of transitions of cells from a more damaged to a less damaged state. The fact that the functional impact on repair is only observed at the first three observation time points probably reflects that we were only looking at one single exposure. Assuming that almost all cells will repair, the cells with a slower repair will eventually catch up with the more rapid ones during the course of the experiment. However, in vivo this would probably not be the case, since there is always a continuous exposure to all sorts of chemicals, and thus, individuals with a slow repair may not be able to ever catch up.

This type of functional study of DNA repair enzyme polymorphisms will contribute to a better understanding of the significance of gene association studies in cancer research, and help to clarify biological mechanisms behind that what is observed.

10. General Discussion and Conclusions

Much still remains to clarify regarding the functional consequences and impact on human health of known polymorphisms in metabolic and DNA repair genes. With this thesis I hope to contribute to the knowledge about the importance of polymorphisms, how they interact with the everyday environment, and how they might interfere with the health of populations and individuals.

There are several mechanisms by which polymorphisms may affect metabolic or DNA repair pathways and influence the cancer risk in a population. One possible outcome of a polymorphism that gives rise to a less efficient detoxification enzyme or repair protein is an increased level of DNA damage, which in turn may influence the frequency of mutation in specific disease genes. Also the type of mutation (transition, transversion etc.) may be influenced. The general aims of this thesis were to investigate if and how the mutational spectrum of the *p53* gene in lung- and bladder tumours was affected by polymorphisms, if polymorphisms could influence lung cancer risk, and to develop a method to study the functionality of polymorphisms.

The common aim of paper I, III and IV was to study the effect of different genotypes on number and type of somatic mutations in the *p53* gene in tumours. Some of the investigated polymorphisms were shown to be associated with the mutational pattern of the *p53* gene in both the lung cancer and the bladder cancer populations. In general, these polymorphisms seemed to have an impact on the type of mutations in the *p53* gene, while their impact on the frequency of mutations in this single gene is small. This is as expected, since the frequency is dependent on the number of mutations that become fixed in the specific gene studied, mutations in other critical genes, the apoptotic rate of the cell population, and probably also by many overlapping processes hiding the effects of each other. Thus, a larger study population would be needed, and a larger number of target genes, to identify differences in mutation frequency. Nevertheless, the increased risk for specific types of mutations in a specific gene probably reflects a higher susceptibility to specific environmental exposures among carriers of the susceptible polymorphic alleles, as a result of altered capacity in either detoxification or DNA repair. The influence on the type of mutation thus may result from individual modulation of mutagenic DNA adduct levels, either by metabolic or DNA repair processes, and may be of importance

for the individual risk of developing cancer. An interesting question is if some types of mutations in the *p53*-gene may lead to more aggressive cancer tumours. Polymorphisms that influence the mutation type could then be important for the cancer progression and diagnosis of cancer patients.

Three of the investigated polymorphisms, *XRCC1* Arg399Gln, *XRCC3* Thr241Met and *NBS1* Glu185Gln were also shown to be associated with the risk of developing lung cancer among women, and the effect of the protective *XRCC3* 241Met allele seemed to be enhanced if combined with the low-risk Glu185 allele of the *NBS1* gene involved in the same repair pathway (HR) (study II). It was only possible to draw conclusions about the impact of the polymorphisms on lung cancer risk in women, since the number of men included in the lung cancer study population was to few too give any statistical power to the analyses. However, there were indications of a possible gender-specific effect, which would be interesting to investigate in future studies.

The comet assay method for investigating the functionality of DNA repair polymorphisms proved to be a suitable method, and the first results show that three of the investigated polymorphisms, *APEX1* Asp148Glu, *XRCC3* Thr241Met and *NBS1* Glu185Gln may have an effect on the early stages of DNA repair after methylation damage by MMS (study V). The method could however be refined, for example by increasing the repair time to allow the cells to fully recover and reach approximately 100% normal cells, if that is possible. Closer observation time points would be interesting, especially in the beginning of the repair process, and also the concentration of MMS could be modified. Repeated exposure is another possibility, since this would make it possible to investigate long-term effects of the repair polymorphisms on the level of DNA damage in cells.

When interpreting the results of this work, the rather small sizes of the investigated study populations' needs to be taken into consideration. There may be large variations between different groups of individuals and therefore there are always stochastic factors that may impact and bias the results. These biases increase, and the power of the studies decrease, the smaller the number of individuals investigated. However, the lung cancer study population was very well defined, with the number of non-smokers, low-dose smokers and women being unusually large; the bladder cancer population included almost all new bladder cancer cases in the Stockholm county

during 1995-1996; and the healthy volunteer population used for study V was large enough for developing the methodology and getting preliminary results. Bigger resources and international collaboration are needed for larger populations, and this is also going to be the case in further studies.

10.1. Future perspective

As more and more information about the biological and chemical function of different genes is gathered, the possibilities to identify the role of different polymorphisms will also increase. In this context it will be important to consider the possible complexity of interactions between different polymorphisms, especially in different genes at different steps along the cancer pathway. Such complex interactions require bioinformatics approaches to understand the contribution of multiple polymorphisms to cancer risk.

High-throughput genotyping technologies such as whole-genome SNP scans hold the promise of finding the major genetic variants that contribute to the risk of common diseases over the next five to ten years. Once such variants have been discovered, researchers can learn much more about the origins of illnesses and about ways to prevent, diagnose, and treat those illnesses. However, since the number of SNPs in the human genome is very high, it seems likely that the research to a higher degree will focus on so called tag-SNPs, i.e. SNPs that may be used to identify different haplotypes. The tag-SNPs, which will contain most of the information about the genetic variation in a population is estimated to be about 300 000 to 600 000, which is far fewer than the 10 million common SNPs [11]. Researchers will then be able to compare whole haplotypes instead of single SNPs, and if for example a particular haplotype occurs more frequently in cases than in controls in a disease association study, it may be concluded that a gene influencing the disease may be located within or near that haplotype. This will greatly increase the probability of finding new associations, since the perspective will be much broader.

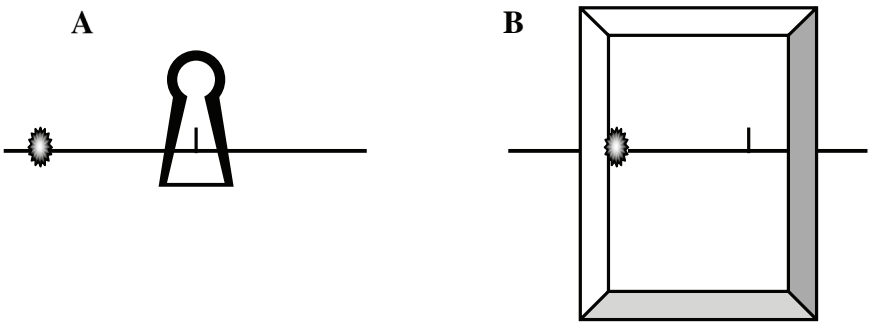


Figure 17. A: A view from a keyhole: common SNP-investigation. The SNP investigated (l) *is not associated with disease. The disease causing polymorphisms is not found. B: A view from a window: tag-SNP/haplotype investigation. Even though the investigated tag-SNP* (l) *is not the one causing disease, the disease causing polymorphisms is found within the same haplotype.*

11. Swedish summary/Populärvetenskaplig sammanfattning på svenska

Vad påverkar oss mest? Våra gener, eller den miljö vi lever i? I de allra flesta fall är det ett samspel och det är svårt att avgöra vilken enskild faktor som väger tyngst. Om livet när man föds sägs vara som ett oskrivet blad, kan generna sägas vara färgerna på våra pennor, medan miljön är bilden som vi målar med pennorna.

Skillnader mellan olika individer beror förutom olika uppväxtmiljö och gener även på att det finns många olika varianter, s.k. polymorfier, av en och samma gen. T.ex. finns det inte bara en ögonfärgsgen, utan det finns flera polymorfa varianter. Kombinationen av dessa bestämmer vilken ögonfärg en människa får. Detsamma gäller för många andra gener i kroppen, t.ex. de gener som är viktiga för cellernas reparation av DNA skador som kan uppstå på arvsmassan efter exponeringar, och de gener som påverkar kroppens nedbrytning av olika ämnen och gifter. Kombinationen av polymorfa varianter av dessa gener avgör hur effektiv DNA-reparationen och giftnedbrytningen är, och polymorfierna kan därmed påverka sjukdomsrisken hos individer.

En polymorfi som t.ex. ger en något långsammare reparation av vissa typer av skador, eller en något sämre förmåga att ta hand om ett giftigt ämne, kan ge en ökad känslighet och en något ökad sjukdomsrisk hos individer som bär på den polymorfa varianten. Effekten av en enskild polymorfi är dock oftast svag. Riskökningen är vanligtvis marginell eller helt försumbar för den enskilda individen, och dessutom enbart associerad med exponeringar. D.v.s. om individen ej utsätts för det den är känslig för, t.ex. ett visst gift som den har svårt att bryta ner, så spelar arvsanlaget för den känsligheten mindre eller ingen roll, och risken för att utveckla sjukdom ökar inte.

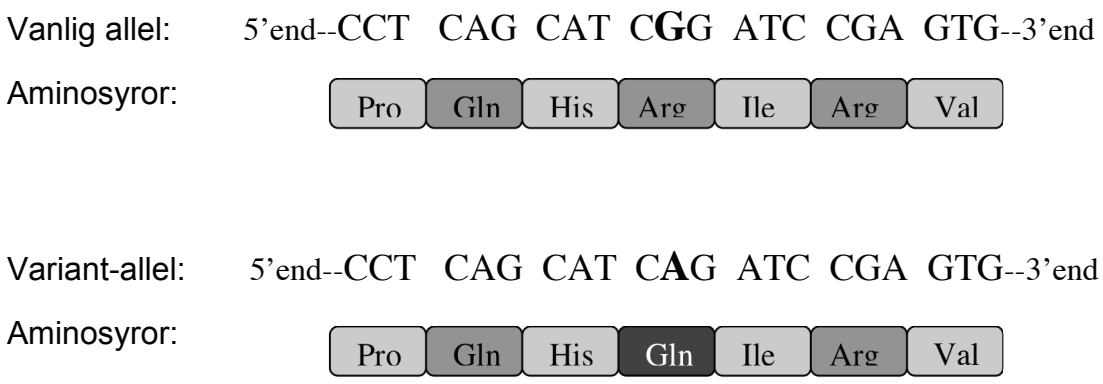
11.1 Vad är en polymorfi?

En polymorfi är en ärftlig variation i den genetiska koden, som förekommer hos mellan 1 och 50 % av individerna i en befolkning. Genetiska förändringar som förekommer i lägre frekvenser (<1%) kallas istället för mutationer. Till skillnad från polymorfier som normalt bara har mycket milda effekter på en individs hälsa och som

därför kan spridas i en befolkning, så är mutationer vanligtvis förknippade med någon sjukdom eller bristsymptom.

Polymorfier är vanliga i genomet (arvsmassan) och kan förekomma så ofta som i 1 av 300 baspar. Polymorfier som innebär att en enda DNA-bas är utbytt mot en annan kallas för ”Single nucleotide polymorphisms”, förkortat SNP. T.ex. kan en del individer ha basen Guanin (G) på en viss position där andra har basen Adenin (A). De olika polymorfa varianterna kallas för alleler. Polymorfierna hittas både i intron (icke-kodande del) och exon (kodande del) i gener och kan ibland, speciellt om de återfinns inom ett exon och leder till att en aminosyra blir utbytt mot en annan, påverka strukturen och funktionen hos det protein som genen kodar för (Figur 18). Denna påverkan beror på att proteinets förmåga att binda till DNA, till sitt substrat, eller till andra proteiner som det normalt interagerar med kan förändras om den nya aminosyran har en annan form eller laddning än den ursprungliga.

Om proteinet är aktivt i viktiga processer som t.ex. DNA-reparation, cellcykelkontroll eller nedbrytning av giftiga ämnen så kan en förändrad aktivitet leda till att bäraren av det förändrade enzymet blir känsligare, vilket i sin tur kan leda till en ökad sjukdomsrisk. T.ex. kan en långsammare nedbrytning leda till att giftiga ämnen ackumuleras (lagras) i kroppen och inte hinner utsöndras innan de når toxiska värden. De känsliga individerna kommer då att få symptom på förgiftning vid mycket lägre halter än icke-känsliga. En långsammare reparation kan leda till att skador som uppstått i en cell till följd av en exponering eller en endogen (kroppsegen) process, inte hinner repareras innan cellen delar sig. Det kan leda till att skadan permanentas, i form av t.ex. en muterad bas. Eftersom det finns olika reparationsvägar som tar hand om olika typer av skador så innebär en nedsatt reparationsförmåga i en reparationsväg att man blir extra känslig för just den typ av skador som hanteras av den vägen.



Figur 18. *SNP polymorfism i den genetiska koden som ger upphov till två olika alleler (varianter av DNA sekvensen). DNA-bas nummer två i ett kodon (tre baser som hör ihop) har ändrats, vilket leder till ett aminosyrautbyte. Proteinet (en kedja av aminosyror) förändras.*

11.2 På vilket sätt är polymorfier viktiga?

Sedan 50-talet har man sett att människor reagerar olika på medicinska behandlingar, trots att de har samma diagnos. Detta visade sig senare bero på att de bar på olika varianter av de gener som reglerade nedbrytningen av den medicin som användes. Polymorfier kan således ha betydelse för medicinska behandlingar av enskilda individer, eftersom de som snabbare bryter ner den aktiva substansen av en medicin kan behöva en högre dos för att kuren ska vara verksam, medan de som är långsamma att bryta ner samma ämne kanske måste få en sänkt dos för att ämnet inte ska ackumuleras och ge upphov till biverkningar. T.ex. kan polymorfier inverka på resultatet av medicinska behandlingar av cancerpatienter.

Vilka genetiska varianter som finns i en befolkning varierar beroende på befolkningens etnicitet. Varianter som är vanliga i en asiatisk befolkning kan vara betydligt ovanligare i en europeisk och det kan leda till att olika etniska grupper kan ha olika känslighet för exponering. Detta gäller inte bara mellan olika länder utan samma skillnader kan finnas mellan olika etniska grupper som lever i ett och samma land. Vilken betydelse polymorfierna får beror också på i vilken miljö som människorna befinner sig och vad de därför exponeras för. T.ex. brukar risken för olika cancerformer hos andragenerationens invandrare vara densamma som hos

landets befolkning i övrigt, även om riskbilden i föräldrarnas hemland var helt annorlunda. Sålunda kan polymorfier också vara viktiga då man ska göra hälsoriskbedömningar och planera för det framtida vårdbehovet hos olika befolkningsgrupper.

11.3 Polymorfier i den här avhandlingen

I den här avhandlingen har 19 polymorfier i femton olika gener analyserats. Bland annat har deras betydelse hos lung- och urinblåsecancerpatienter studerats. De analyserade generna är involverade i tre huvudprocesser; metabolism, reparation och cellcykelreglering. Alla tre processerna är viktiga för att hålla kroppens celler friska. Tre olika studiepopulationer, varav två svenska (en bestående av urinblåsecancerpatienter och en bestående av lungcancerpatienter+kontroller) och en brittisk (friska ickerökande universitetsstudenter och anställda forskare från Leeds i norra England) har undersökts. Alla tre grupperna räknas som vita kaukasier (européer) och förekomsten av dessa polymorfier bland kaukasier enligt den publicerade litteraturen kan ses i tabell 1 och 2.

Det finns olika sätt att analysera genetiska polymorfier (genotypning), och det kommer hela tiden nya varianter, då tekniken utvecklas. Med tillgång till rätt utrustning kan man idag analysera stora mängder prover på en relativt kort tid. Grunden för genotypning är att man mångfaldigar det DNA (den gen eller del av gen) som man är intresserad av, och sedan med olika metoder analyserar provets DNA-sekvens för att få fram vilken genotyp som provet representerar.

11.4 p53-genen

p53-genen är en s.k. ”tumörhämmande” gen, vilket innebär att den hindrar normala celler från att utvecklas till cancerceller. p53-proteinet som genen kodar för är involverat i många viktiga biologiska funktioner i cellen, b.la. cellcykelreglering (styr hur och när cellen delar sig och bildar s.k. dotterceller) och apoptos (celldöd). Då en DNA-skada uppstår aktiveras *p53* och cellcykeln avstannar så att reparationsprocesserna kan sätta igång. Skulle skadan vara för svår kan p53-proteinet starta en apoptotisk process, vilket leder till att cellen istället dör.

Om *p53*-genen muteras så att den inte längre fungerar normalt kan det leda till att skadade celler tillåts gå vidare i cellcykeln. Därmed kan fler skador uppstå utan att cellen stannar upp för att reparera eller dö. Skadorna i cellen fixeras och förs vidare

till dotterceller som därmed också saknar ett normalt p53-protein. Kloner av skadade celler kan så småningom leda till canceruppkomst. I nästan 50 % av mänskliga tumörceller bär cancercellerna på en muterad *p53*-gen. Både metabolismpolymorfier, som påverkar förekomsten av giftiga ämnen i en cell, vilka kan ge upphov till mutationer, och reparationspolymorfier som ger olika effektiv reparation av de mutationer som uppstår, skulle hypotetiskt sett kunna påverka risken för att *p53*-genen ska muteras.

11.5 Frågeställningar

Den här avhandlingen är ett bidrag till kunskapen om polymorfiers betydelse inom riskbedömningen. Den övergripande målsättningen har varit att undersöka hur polymorfier i gener involverade i metabolism och DNA reparation påverkar (1) förekomsten och typen av mutationer som kan uppstå i *p53*-genen hos lung- och urinblåsecancerpatienter, (2) lungcancerrisk, och (3) att utveckla en metod för att kunna studera funktionaliteten hos DNA-reparationsgener.

11.6 Resultat och diskussion

Studie I, III och IV: En del av de undersökta polymorfierna visade sig kunna associeras med mutationsmönstret i *p53*-genen hos både lung- och urinblåse-cancer populationerna. Generellt tycks polymorfierna kunna påverka typen av mutationer i *p53*-genen, men deras påverkan på frekvensen är marginell. Detta beror troligen på att den sämre förmågan att bryta ner vissa ämnen, eller den lägre effektiviteten på vissa reparationsprocesser som är förknippad med vissa av de undersökta polymorfierna kan ge upphov till speciella typer av skador som speglar den exponering som individen utsatts för. Dock är andra reparations- och metabolism-vägar inte påverkade och därmed förändras inte den övergripande frekvensen av mutationer.

En intressant frågeställning är om vissa typer av mutationer i *p53*-genen leder till en aggressivare tumörutveckling än andra. Polymorfier som har inverkan på mutationstypen skulle därmed kunna vara viktiga för sjukdomsutveckling och diagnos hos cancerpatienter. Detta är något som framtida studier får utvisa.

Studie II: Tre av de undersökta polymorfierna, *XRCC1* Arg399Gln, *XRCC3* Thr241Met och *NBS1* Glu185Gln tycks kunna påverka risken för att utveckla lungcancer hos kvinnor, såtillvida att individer som bar på den ena av allelerna hos respektive polymorfi hade en ökad eller minskad risk att utveckla lungcancer jämfört

med de individer som bar på den andra allelen. Effekterna av *XRCC3* och *NBS1*, två gener som deltar i samma reparationsprocess, tycks också kunna förstärka varandra, så att individer med lågriskalleler av båda generna har ett starkare skydd mot lungcancer än de som enbart bär på lågriskalleler av den ena genen. I den studiepopulation som deltog i studie II var andelen män inte tillräckligt stor för att det skulle vara möjligt att få fram några säkerställda resultat om polymorfiernas betydelse hos dem. Visa tendenser tyder dock på att resultaten hos männen skiljde sig från de hos kvinnorna, vilket skulle vara intressant att undersöka närmare i framtida studier.

Studie V: Den utvecklade metoden för att undersöka funktionaliteten hos DNA-reparationsgener fungerade bra och resultaten tyder på att tre av de genetiska polymorfierna i studien, *APEX1* Asp148Glu, *XRCC3* Thr241Met och *NBS1* Glu185Gln kan ha en viss effekt på reparationshastigheten i ett tidigt skede efter metyleringsskador på vita blodkroppar. Metyleringsskador repareras främst via ”base excision”-reparationsvägen, och i den är *APEX1*-genens funktion avgörande för reparationshastigheten. Den starkaste effekten sågs också just på denna gen, då celler som bar på den ena av *APEX1*-allelerna hade en mycket långsammare minskning av DNA-skador än celler med den andra allelen. Detta tyder på att *APEX1* polymorfien är funktionell och därmed troligen även kan ha inverkan på individers sjukdomsrisk.

Alla resultat av de fem studier som ingår i den här avhandlingen måste tolkas med viss försiktighet eftersom studiepopulationerna har varit relativt små. Då stora variationer kan förekomma mellan olika grupper av människor finns det alltid en slumpfaktor med som kan påverka de statistiska analyserna, och slumpfaktorn blir större då antalet individer som deltar i undersökningen är lågt. Denna brist finns i många studier av det här slaget och det är därför viktigt med verifikation av resultaten, samt att man kan koppla biologiska mekanismer till det som observerats.

11.7 Framtida reflektioner

Det finns mycket som man fortfarande inte vet om hur polymorfier kan påverka hälsan hos individer och befolkningsgrupper, men i och med den snabba teknikutvecklingen verkar det troligt att man inom den närmaste tioårsperioden kommer att ha hittat de flesta viktigare genetiska polymorfier som kan påverka vanliga sjukdomar. Därmed kommer vi förhoppningsvis också att kunna lära oss mycket om hur man kan förebygga och behandla sjukdomar på ett mer effektivt sätt

än idag. Det verkar också troligt att polymorfiforskningen alltmer kommer att fokuseras på analys av grupper av SNP som nedärvs tillsammans, vilket kommer att ge ett bredare perspektiv än analyser av enskilda SNP. Det kommer också att öka möjligheterna att hitta nya genetiska varianter som kan påverka individers känslighet och sjukdomsutveckling.

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