Inherited DNA repair capacity and individual responses to carcinogens

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Abstract

Humans are exposed to genotoxic carcinogens, such as polyaromatic hydrocarbons (PAHs), in their everyday lives. The human body has efficient means to deal with the adsorbed harmful compounds. However, wide inter-individual difference has been shown to exist in the capacity to resist the ill effects caused by environmental insults. These variations are therefore suggested as important modifiers of individual responses to chemical carcinogens. Many xenobiotic metabolizing enzymes and DNA repair proteins exhibit genetically determined polymorphism in their activities and expression. The aim of this thesis was to study the influence of polymorphisms in DNA repair genes *XRCC1*, *XRCC2*, *XRCC3*, *XPC*, *XPD*, and *XPG* on DNA damage (single-strand breaks [SSBs]), cytogenetic damage (mutagen sensitivity, chromosomal abberrations [CAs], and sister chromatid exchanges), and the risk of cancer in the upper aerodigestive tract (UAT). The *XRCC1* codon 280 variant allele was associated with elevated number of chromosome breaks induced by bleomycin *in vitro* (mutagen sensitivity), and with a higher frequency of chromosome breaks in peripheral blood lymphocytes of individuals with low exposure levels. Carriers of *XRCC1* codon 399 variant allele displayed a higher frequency of chromatid breaks and SSBs, and a diminished irradiation-specific repair activity. The *XPD* codon 23 variant allele was associated with a decreased capacity to repair gamma-irradiation-induced DNA strand breaks in smokers, and with elevated levels of chromatid-type aberrations. The *XPC* exon 15 wild type allele was associated with a lower level of SSBs, abasic sites and 8-oxoguanosines. Carriers of *XPG* exon 23 variant allele exhibited higher levels of SSBs. The *XRCC2* variant allele and the *XRCC3* wild type allele were associated with the elevated risk of UAT cancers in smokers. These findings substantiate the earlier studies where phenotypic effects were discovered for DNA repair gene polymorphisms. In conclusion, the present results show that polymorphisms of DNA repair genes are able to influence individual responses to genotoxic and carcinogenic exposures. This should facilitate a better characterization of risks and eventually enable the protection of people most vulnerable to adverse effects. Methodological improvements may also be indicated, if the DNA repair gene genotypes are taken into account when using cytogenetic methods for human biomonitoring and cancer risk assessment.

List of original publications

This dissertation is based on the following original publications referred to in the text by the Roman numerals I–IV as indicated below.

I. **Tuimala, J.**, Szekely, G., Gundy, S., Hirvonen, A., and Norppa, H. (2002) Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: role in mutagen sensitivity, Carcinogenesis, 23, 1003–1008.

II. **Tuimala, J.**, Szekely, G., Wikman, H., Järventaus, H., Hirvonen, A., Gundy, S., and Norppa, H. (2004) Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: effects on levels of sister chromatid exchanges and chromosomal aberrations, Mutation Res., in press.

III. Benhamou, S., **Tuimala, J.**, Bouchardy, C., Dayer, P., Sarasin, A., and Hirvonen A. (2004) DNA repair gene *XRCC2* and *XRCC3* polymorphisms and susceptibility to cancers of the upper aerodigestive tract, Int. J. Cancer, in press.

IV. Vodicka, P., **Tuimala, J.**, Stetitina, R., Kumar, R., Manini, P., Naccarati, A., Maestri, L., Vodicková, L., Kuricová, M., Järventaus, H., Majvaldova, Z., Hirvonen, A., Imbriani, M., Mutti, A., Migliore, L., Norppa, H., and Hemminki, K. (2004) Cytogenetic markers, DNA single-strand breaks, urinary metabolites, and DNA repair rates in styrene-exposed lamination workers, Environ. Health Perspect., 112, 867–871.

In addition, some unpublished data on the modulating effect of smoking, alcohol consumption and DNA repair genotypes on the risk of upper aerodigestive tract cancers is presented (Chapter 3.3). Data on effects of DNA repair gene polymorphisms on the level of SSBs and irradiation-specific DNA repair rates are also currently unpublished (Chapter 3.4).

Abbreviations

AP	apurinic / apyrimidinic site
BER	base-excision repair
BLHX	bleomycin hydrolase
CA	chromosomal aberration
CI	confidence interval
DNA	deoxyribonucleic acid
DSB	DNA double-strand break
ERCC	excision repair cross complementing group
GST	glutathione S-transferase
HR	homologous recombination
MN	micronuclei
NAT	N-acetyltransferase
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
OR	odds ratio
PAH	polyaromatic hydrocarbon
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RI	relative risk
ROS	reactive oxygen species
SCE	sister chromatid exchange
SO	styrene-7,8-oxide
SSA	single-strand annealing
SSB	DNA single-strand break
TCR	transcription coupled repair
UAT	upper aerodigestive tract
XME	xenobiotic metabolizing enzyme
XP	xeroderma pigmentosum
XRCC	X-ray cross complementing group

I Review of the literature

1. Introduction

All humans are exposed to various natural and artificial sources of potentially harmful compounds and radiation. People are also frequently exposed to chemicals in their working environment (factory workers, cleaning technicians, *etc.*), and everyday life (smoking, drinking alcohol), and hobbies (gardening, construction work, *etc.*). For people living in cities and other municipalities, a large portion of the exposure comes from atmospheric particles, radon, and UV-light. Exposures to genotoxic compounds can induce mutations. Even a low exposure of cancer initiating chemicals, such as polyaromatic hydrocarbons (PAHs), if prolonged, may lead to accumulation of mutations in the somatic and germ cells and, eventually, to development of tumors and cancer (Amdur *et al.*, 1991).

Genotoxic carcinogens affect the genome. Genetic factors are thought to play a central role in determining individual susceptibility to genotoxins; similar exposure will result in different genotoxic response in different individuals. The factors responsible for difference between individuals include genetic polymorphisms of enzymes functioning in the metabolism of xenobiotics (substances foreign to the organism) and DNA repair.

After initial adsorption into the body, most xenobiotics go through a two-phase metabolism. Because well-adsorbed xenobiotics are usually not very soluble in water, they have to be converted into a water-soluble form to make them more excretable. This is the so-called metabolic activation step (Phase I), where hydroxyl groups are added to the xenobiotics (catalyzed by cytochrome P450 oxidases and haemoglobin). The resulting compounds are often more reactive than their parent compounds. The next step is called the inactivation or detoxification phase (Phase II), where the reactive compounds are conjugated with innate molecules, for example, glutathione conjugation is catalyzed by glutathione *S*-transferases (GSTs) and acetylation is mediated by *N*-acetyltransferases (NATs). In the process, the reactivity of the compound is diminished and it is flagged to excretion. However, during the process, some of the products can react with DNA in the cells, and produce potentially lethal DNA lesions, strand breaks, and adducts.

Cellular DNA damage can be repaired. In fact, cells have very powerful repair mechanisms readily at their disposal; DNA strand breaks can be sealed by homologous or non-homologous methods, so that transcription and DNA replication can continue seamlessly. DNA adducts can be cleaved from the DNA either in short (base excision repair [BER]) or long patches (nucleotide excision repair [NER]). If these measures fail, the cell can go to apoptosis to preserve the parent organism. (Amdur

et al., 1991) Because both the xenobiotic metabolism and DNA repair may affect the level of genotoxic damage in cells, both should be considered when genotoxic effects are assessed in humans.

To date, several biological markers of exposure, effects, and susceptibility have been developed. For biological monitoring of exposure, peripheral blood or other tissues, such as adipose tissue, can directly be sampled, or the excretion of metabolic products of adsorbed xenobiotics can be quantified from urine or faeces. Many genotoxic chemicals bind to DNA and form DNA-adducts, whose amount can be quantified. Cytogenetic biomarkers, *e.g.*, chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MNs), can be detected from peripheral blood leukocytes. Their frequency is a suitable marker for genotoxic exposure and effects. Such biological markers are considered superior to chemical markers, since they reflect the biologically relevant dose (Amdur *et al.*, 1991). In addition, the relation of certain enzymatic polymorphisms and cancer can be studied in an epidemiological framework.

A schematic presentation of the metabolic and DNA repair pathways leading to various outcomes (outlined above) is presented in Figure I.

In this thesis, data about individual DNA repair capacity are presented on three different reductionistic levels (cell, individual and population):

1. The effect of polymorphic DNA repair genes on the frequency of spontaneous chromosomal aberrations and bleomycin sensitivity.
2. The relationships between DNA repair gene polymorphisms and markers of styrene exposure.
3. DNA repair gene polymorphisms as risk factors for upper aerodigestive tract cancers.

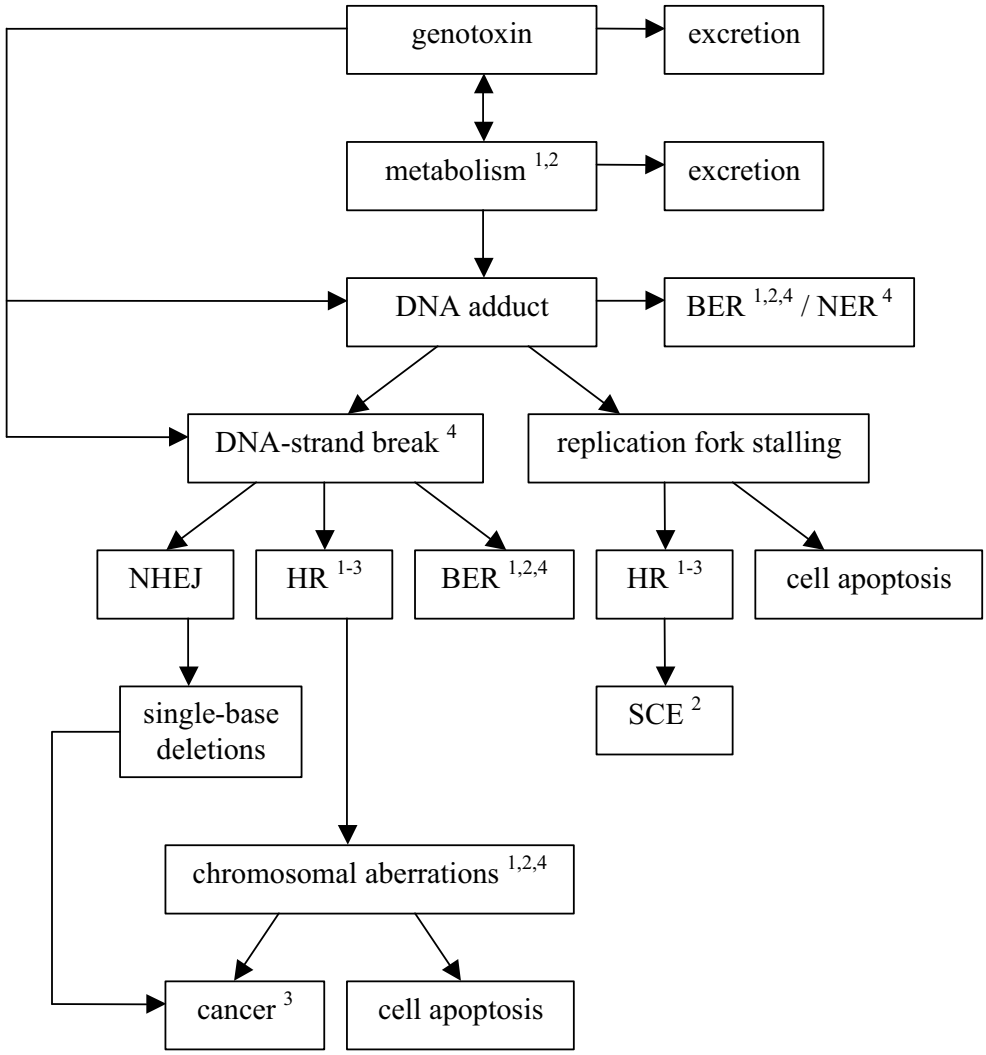

Figure I. Current view of genotoxin metabolism, repair of induced DNA lesions, and relationships between the measurable end-points or possible outcomes. BER, base excision repair; NER, nucleotide excision repair; HR, homologous recombination repair; NHEJ, non-homologous end-joining; and SCE, sister chromatid exchange. Superscripts refer to the articles included in this study.

2. Cytogenetic damage

2.1 Chromosome aberrations

CAs are present in normal circulating lymphocytes of healthy individuals, but in rather low frequencies. Several models of radiation-induced CA formation have been deduced, as reviewed by Savage (1998). Put briefly, the breakage-and-reunion theory ("Classic theory") assumes that radiation induces breaks in "chromonema", the chromosome backbone, and if two breaks exist close in space and time, they can interact to produce visible structural exchange aberrations (Lea *et al*., 1942; Lea 1946). The exchange theory ("Revell theory"), which challenged the classic theory a few decades back, suggests that the primary damage induced by radiation is not a break in the chromosome backbone, but an unstable lesion, the nature of which is undefined (Revell, 1955). If these unstable lesions interact in a suitable manner, they may enter into a state of exchange initiation, which in turn can lead to the formation of aberrations. One of the differences between the models is that the exchange theory predicts that the reversal of exchange is possible, something neither permitted nor possible with the Classic theory. In summary, the Classic theory states "no break–no exchange" whereas the Revell theory contradicts it by claiming "no exchange–no break". The molecular theory (Chadwick *et al*., 1978) combines the earlier statistical and cytological observations with modern knowledge of chromosome structure. This theory argues that the primary unstable lesion in the DNA is a double-strand break (DSB). This forms CAs by a recombinational process, which enzymatically induces a second DSB at the recombination point leading to reciprocal translocations. However, emergency situations may force the cell to use partial homology, which can lead to the formation of incomplete (non-reciprocal) translocations. Furthermore, the use of ultrasoft carbon X-rays has suggested that only one DSB is needed for the formation of simple aberrations (Savage, 1998). Ultrasoft carbon X-rays deposit their energy in the range of the diameter of one DNA double helix, and are very unlikely to induce DSBs in more than one DNA helix. However, the exact mechanisms of aberration formation are still largely unknown. For example, it is not known whether the break is a true discontinuity in the DNA, whether some material is missing or whether it is just an uncondensed region of DNA possibly undergoing DNA repair.

The mechanisms for radiation- and chemical-induced CAs may be different, although they may look similar under the microscope. Most chemicals do not directly induce DSBs, which must, therefore, be caused by the actions of the cell itself. Therefore, the most probable mechanism for DSB formation is disturbance in the DNA repair system.

CAs, as viewed through a microscope on a metaphase preparation, are end products of complex cellular systems. Metaphase chromosomes can be produced by colchicin (or its analogue Colcemid) arrest during cell division of cycling cells; CAs are normally scored from non-banded ("solid"), stained metaphase preparations of lymphocytes stimulated to divide by a mitogen. Radiation-induced damage can be observed as chromosome- or chromatid-type aberrations, depending on whether the cell resided in G0/G1 or G2 phase of the cell cycle, respectively, at the time of irradiation.

According to the most cited theory, breakage-and-reunion, misrepair of illegitimate rejoining of break-ends produces structural aberrations between two different chromosomes (interchanges) or within one chromosome (intrachanges) (Savage, 1975). In the case of an asymmetrical interchange, the centromeres of both chromosomes are located on the same piece, and an accompanying acentric fragment is present. This dicentric chromosome is easily recognizable after conventional staining. Reciprocal translocation involves mutual exchange of chromatin pieces, and only large rearrangements can be detected in solid stained preparations. If two breaks are located within one chromosome, one on each side of centromere, an easily observable ring chromosome can be produced. Pericentric inversion formed from the same lesion cannot usually be distinguished by solid staining. In cases where breaks remain dissociated, terminal or interstitial deletions are formed. The terminal deletions can be detected as acentric fragments, whereas interstitial deletions appear as either small double minutes or acentric rings.

The radiation- or chemical-induced CAs can be classified as chromatid-type or chromosome-type aberrations (Savage, 1975). Deletions are seen as chromatid- and chromosome-type breaks (Figure II), the former affecting one chromatid and the latter both sister chromatids at the same site. Dicentrics, ring chromosomes and double minutes are classified as chromosome-type aberrations, whereas chromatid-type aberrations include symmetrical and asymmetrical exchanges within a chromosome (intrachanges) and between two or more chromosomes (interchanges).

Several polymorphisms of xenobiotic metabolizing enzyme (XME) genes have been reported to affect spontaneous CA frequency in human peripheral blood lymphocyte cultures. Smokers lacking the *GSTM1* gene (*GSTM1* null individuals) appeared to have higher CA frequencies than those with the gene; the influence of *GSTM1* genotype was restricted to chromatid-type aberrations (Scarpato *et al*., 1996; Scarpato *et al*., 1997). In addition, four individuals deficient for both *GSTM1* and *GSTT1* genes showed significantly higher CA counts than the *GSTM1* positive and *GSTT1* null individuals (Scarpato *et al*., 1997). These findings may be explained by the reduced detoxification capacity of individuals lacking both of the genes.

Nonsmoking bus drivers concurrently lacking the *GSTM1* gene and having the *NAT2* slow acetylator genotype had an increased frequency of cells with CAs. Postal

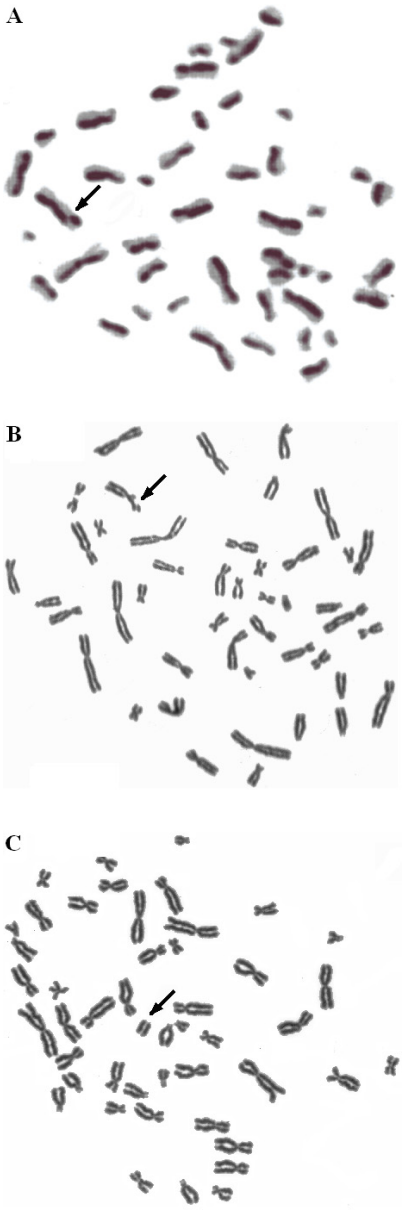

Figure II. Microphotographs of sister chromatid exchanges (A), a chromatid break (B) and a chromosome break (C) in metaphase chromosomes. The arrows indicate the diagnostic lesions in the chromosomes. Images B and C, courtesy of Hilkka Järventaus.

workers (low exposure group, nonsmokers) with slow acetylator *NAT2* genotype also showed elevated CA counts (Knudsen *et al*., 1999). Smokers with *NAT2* rapid acetylator genotype displayed more CAs (translocations) in their cultured lymphocytes than the slow acetylator genotype carriers (Pluth *et al*., 2000). This difference was observed in six individuals over 60 years of age of whom four were *GSTM1* null. The findings on *NAT2* may, at first sight, seem contradictory, but it is possible that the difference in the alleged risk genotype reflects the difference in genotoxic exposure between smokers and nonsmokers.

Based on two reviews addressing the effect of different XME polymorphisms on cytogenetic damage, *GSTM1* seems to affect CA frequency especially in smokers and individuals exposed to polyaromatic hydrocarbons or polluted air, while *NAT2* and possibly *GSTT1* could affect the baseline level of CAs (Norppa, 1997; Norppa, 2000). This reflects the importance of activation/inactivation metabolism in genotoxicity as well as the functionality of *XME* polymorphisms.

2.2 Sister chromatid exchanges

SCEs occur normally in replicating cells (Tice *et al*., 1976). They originate from apparently homologous intrachromosomal recombination involving both chromatids (Figure II). SCEs are induced, *e.g*., by UV-irradiation (Vogel *et al*., 1978), alkylating agents and other adduct-forming chemicals, and virus infections (Latt, 1981). Also DSBs produced by restriction enzymes electroporated into cells are potent SCE inducers (Natarajan *et al*., 1985). Enzymes leaving a cohesive end into DNA are less potent inducers of SCEs than enzymes leaving a blunt DNA end (Darroudi *et al*., 1989), probably because cellular repair systems may be more efficient in fixing cohesive DSBs.

The exact mechanism of SCE formation is still unclear, but some success has recently been achieved in unraveling the problem. In a murine Ku70-deletant fibroblast cell line, SCE frequency was significantly increased compared with the wild type cell line (Li *et al*., 1998). Ku70 is a DNA end-binding protein involved in DSB repair. Abolishment of homologous recombination in vertebrate cells leads to a decrease in SCE frequency (Sonoda *et al*., 1999), suggesting that SCEs are formed by homologous recombination. However, the SCE formation was not entirely blocked by the Rad-gene deletion constructed in the cell line. These data indicate that SCEs are probably initiated by unrepaired DSBs formed during the G1 phase of the cell cycle. It is also plausible that unrepaired DNA adducts induce DSBs during DNA replication when the replication machinery gets stuck in the adduct in the S phase of the cell cycle. DSBs are then repaired during the S phase by homologous recombination repair, which leads to the formation of SCEs. It has been proposed that

homologous recombination may reactivate stalled replication forks (Cox *et al*., 2000). Thus, SCEs may actually be an indirect measure of successful DNA repair.

2.3 Bleomycin sensitivity assay

Cultured lymphocytes of normal individuals and cancer patients differ in mutagen sensitivity: a higher percentage of cancer patients than normal individuals have been shown to display a high frequency of chromatid breaks (Figure II) in cultured lymphocytes when treated with the cancer drug bleomycin (Hsu *et al.*, 1985). However, some recent observations indicate that this is not necessarily the case in all conditions (Szekely *et al.*, 2003). Bleomycin sensitivity does not correlate with the spontaneous frequency of CAs, indicating that different mechanisms are involved in the formation of bleomycin-induced and spontaneous CAs (Hsu *et al*., 1985).

Bleomycin sensitivity is normally expressed as a frequency of chromatid breaks per cell (b/c). Donors are classified as non-sensitive, sensitive or hypersensitive on the basis of individual b/c. The b/c ratio is elevated in colon, head and neck, and lung cancer, but not in breast cancer. Old smokers are less sensitive than young smokers, but in general, smoking habits do not affect the test outcome (Hsu *et al*., 1989).

2.3.1 Genetic basis of bleomycin sensitivity assay

It has been hypothesized that bleomycin sensitivity is an inherited trait and could thus be used to assess individual cancer risk (Hsu *et al*., 1985). This hypothesis has been supported by a twin study reporting a heritability of 75% for the bleomycin sensitivity (Cloos *et al*., 1999). Because b/c distribution is continuous, it is conceivable that bleomycin sensitivity is a multigenic trait possibly characterized by a single major gene effect.

Bleomycin needs to be metabolically activated to be able to exert its genotoxic effects. Bleomycin is probably activated in a cytochrome P450 reductase-dependent reaction to a reactive Fe(II)·bleomycin complex (Scheulen *et al*., 1981; Kilkushie *et al*., 1984; Povirk *et al*., 1991). Its inactivation is mainly catalyzed by bleomycin hydrolase, a cysteine proteinase enzyme. It has been postulated that in some cancers resistance to bleomycin treatment results from overactive bleomycin hydrolase (Umezawa *et al*., 1974; Sebti *et al*., 1991). Furthermore, in the bacterium *Streptomyces verticillus*, the bleomycin-producing species, bleomycin is inactivated by NAT (Matsuo *et al*., 1997; Sugiyama *et al*., 1994). In Chinese hamster ovary cells, the lack of functional GST alpha may result in hypersensitivity to bleomycin (Giaccia *et al*., 1991). This lead Kocabas *et* al. (2000) to hypothesize that *GSTM1* defect in humans

might predispose to hypersensitivity to bleomycin. However, their findings were negative.

2.3.2 Bleomycin-induced DNA damage

The activated Fe(II)·bleomycin complex reacts with oxygen to form an extremely short-lived product that is believed to abstract hydrogen from C-4' of deoxyribose. Hydroxylation of this initial lesion results in sugar ring opening and base release leaving a chemically modified apurinic/apyrimidinic (AP) site. In addition to these single lesions, bleomycin specifically induces DSBs. DSBs are probably formed with single-hit kinetics (Povirk *et al*., 1991). The bleomycin-induced DSB possesses a complex structure similar to that produced by oxidative processes and ionizing radiation. Bleomycin DSBs are composed of blunt ends or ends containing a single 5'-base overhang. Regardless of the 5'-end structure, all bleomycin-induced DSBs possess 3'-ends blocked by phosphoglycolate (Pastwa *et al.*, 2001).

The repair of bleomycin-induced base damage is complicated. The AP site is probably first cleaved by human AP endonuclease Ape1 (Xu *et al*., 1998), which generates 3'-OH and 5'-terminal deoxyribose-5-phosphate (5'-dRp) (Chen *et al.*, 1991; Wilson *et al*. 1995). The resulting 5'-dRp can be removed by intrinsic activity of DNA polymerase beta (Matsumoto *et al*., 1995), followed by DNA repair synthesis to fill the gap, and ligation to complete the repair process. Thus, bleomycin-induced base-damage is repaired by base-excision repair.

DSBs are rejoined probably by various mechanisms. In brief, bleomycin forms OH-radicals that damage DNA, inducing single-strand breaks (SSBs) similarly to the X-ray induced DNA damage. Bleomycin may thus induce primarily base damage or SSBs, and only rarely DSBs; the probability for two SSBs localizing closely together (to form a DSB) is very small. The DSBs formed by bleomycin are probably generated by enzymatic reactions from primary lesions, *i.e*., SSBs.

Bleomycin-induced strand breaks are probably repaired at least by non-homologous end joining (NHEJ). When the expression of Ku80 (Belenkov *et al.*, 2002) or Ku70 (Omori *et al.*, 2002) was blocked in cultured human malignant glioma and lung carcinoma cells, respectively, the cells were sensitized to the effects of bleomycin. In asyncronized cell cultures, most cells are in the cell cycle phase G1, where NHEJ is active. Therefore, the results do not exclude the possibility that homologous recombination is also actively repairing bleomycin induced damage in the G2 phase.

Two Chinese hamster cell lines, xrs5 and xrs6, were both found to be more sensitive than wild-type cells to the induction of CAs by bleomycin (Darroudi *et al*., 1989). These cell lines are defective in NHEJ of DSB repair (Jeggo, 1998), missing essential proteins Ku70 and Ku80, respectively. Cells treated with bleomycin have

numerous single-base deletions at the sites of DSBs. This further indicates that the bleomycin-induced DSBs are repaired via the NHEJ route (Yu *et al.*, 2002). Thus, DSB repair, especially NHEJ, seems to participate in the repair of bleomycin-induced DNA lesions.

2.3.3 Bleomycin-induced chromosomal damage

The effect of bleomycin on chromosomes has been extensively reviewed (Vig *et al.*, 1978; Povirk *et al.*, 1991). Briefly, bleomycin was found to produce both chromosome- and chromatid-type aberrations (deletions, dicentrics, rings, exchanges, breaks and gaps) in all systems studied. Based on the reviewed results, it was concluded that bleomycin acted on chromosomes in an S-independent fashion since bleomycin produced aberrations regardless of the moment it was administered during the interphase. Furthermore, the types of aberrations produced by treatment in various phases of the cell cycle were those expected to result from the production of both double- and single-strand breaks by bleomycin. In the bleomycin sensitivity assay, bleomycin induces practically only chromatid breaks and gaps (Hsu *et al.*, 1989), probably because most of the cells are in the G1 phase of the cell cycle when treated with bleomycin for 6 hours at 66 hours of culture; the time period may be too short for effective induction of other types of CAs.

3. Cytogenetic damage and cancer risk

3.1 Chromosomal aberrations and cancer risk

An inter-European prospective study has assessed the possible cancer risk predictivity of cytogenetic endpoints, namely SCEs, micronuclei (MNs) and CAs. After trichotomization (high, medium, low), a high frequency of CAs predicts increased cancer risk. In different countries, the risk of cancer (standardized incidence or mortality ratio) varied from 1.8 to 2.4 for individuals with a high frequency of CAs compared with low-frequency individuals. MN and SCE frequencies were not predictive of cancer risk (Hagmar *et al*., 1994). MN and SCE frequencies were more difficult to trichotomize than CAs, because their variation between different samplings from the same individual was big compared with that observed in the CA frequencies. Furthermore, CAs in lymphocytes predicted human cancer risk independently of exposure to carcinogens (Bonassi *et al*., 2000). Also, the time elapsed between sampling for the CA-test and the cancer diagnosis did not affect the cancer predictivity. It is not resolved which CA type is the best predictor of cancer risk, but a Taiwanese case-control study (22+22 subjects) suggested that chromosome-type aberrations are a better predictor than chromatid-type aberrations (Liou *et al*., 1999). Especially chromosome breaks had good predictive power. The recent report from the inter-European prospective study including data on 3554 individuals also indicates that both chromatid- and chromosome-type aberrations are predictive of future cancer risk, although chromosome-type aberrations have a better predictive power (Hagmar, 2004).

3.2 Bleomycin sensitivity and head and neck cancer

Primary cancers of pharynx, larynx and oral cavity are classified as head and neck cancers. In the original study of Hsu *et al.* (1989), 72.75% of head and neck cancer patients were sensitive to bleomycin, whereas only 22.69% of controls were classified as sensitive. In further studies, 53.8% of cancer patients and 25.7% of healthy individuals were considered sensitive.

Environmental factors modified the cancer risk in the case-control study of Cloos *et al.* (1996). Smokers (more than 20 cigarettes a day for 25 years, *i.e*., 25 pack-years) and alcoholics (more than 75 ml of absolute ethanol daily) with a high b/c value were at the highest risk of head and neck cancer. Smokers using alcohol and displaying a high b/c frequency were at almost 60-fold risk of this malignancy compared with non-smokers not using alcohol and having a low b/c value. However,

alcohol consumption and smoking are well-established risk factors for head and neck cancers. Without environmental exposure the cancer risk for bleomycin sensitive individual varied from 3.6 to 5.8 (Spitz *et al*., 1989). In a group of head and neck cancer patients, never-smokers were significantly more likely (61.1%) to be mutagen sensitive than current smokers (35.6%) (Spitz *et al*., 1997).

Bleomycin sensitivity can also be used to predict tumor recurrence. In patients with high b/c, cancer was more likely (11.5%) to recur than in individuals with a lower b/c value (5.3%) (Spitz *et al*., 1998).

4. DNA repair

Because the viability of the cell is dependent on the physical integrity of its DNA, many versatile DNA repair pathways capable of repairing nearly all kind of damages have evolved. Many of them are best characterized in bacteria, like *Escherichia coli*, but have begun to be better understood also in mammalian cells. Some of the possible pathways do not even exist in bacterial cells. The same is also true *vice versa*, the existence of several pathways in mammalian cells is a highly disputed topic (Lewin, 1999).

If the DNA lesion is not repaired and the cell does not undergo apoptosis, the damage can lead to mutations in crucial genes and ultimately to cancer initiation and uncontrolled growth. In the following chapters, the DNA repair pathways relevant to the thesis are discussed in more detail.

4.1 Base excision repair

Base excision repair (BER) is the main pathway for the repair of simple DNA lesions that do not distort the DNA structure, unlike most lesions caused by reactive oxygen species. Thus, BER enzymes recognize relatively few but frequent lesions (Parikh *et al*., 1999). There are at least three pathways for BER coupled repair (Klungland *et al*., 1999; Lindahl, 2000).

The first step in all BER pathways is the cleavage of the base-sugar bond (catalyzed by DNA glycosylase), followed by DNA incision by a separate repair enzyme (AP endonuclease) at the resulting abasic site (Lindahl, 1976). In mammalian cells, DNA polymerase beta, X-ray cross complementing group 1 (XRCC1), and DNA ligase III are responsible for the dominating one-nucleotide replacement pathway. In the main pathway, DNA polymerase beta accounts both for the gap-filling synthetic step and the removal of a 5'-terminal abasic sugar-phosphate residue; the latter excision function appears to be a rate-limiting step in the repair reaction (Srivastava *et al*., 1998).

Long-patch BER uses an alternating pathway. After the removal of the damaged nucleotide, flap endonuclease (FEN1) and proliferative cell nuclear antigen (PCNA) proceed with the polymerase beta (gaps of 2–6 bps) or polymerase gamma (gaps of 2–13 bps) to synthesize new DNA. The nicks in DNA are sealed by XRCC1 and DNA ligase III complex (gaps of 2–6 bps) or DNA ligase I (gaps of 2–13 bps) (Wilson, 1998).

The repair of oxidative damage in pyrimidines (pyrimidine glycols) probably uses another pathway where a spesific DNA glycosylase called hNth1 removes the cyto-

toxic lesion. XPD protein is probably needed in this reaction (Klungland *et al*., 1999) as a loading factor for hNth1 in human cells (Lindahl, 2000). Also another kind of oxidative damage, 8-hydroxyguanine, has its own glycosylase, 8-oxoguanine glycosylase (OGG1), which removes damaged bases from DNA (Monden *et al*., 1999).

The main BER pathway repairs SSBs formed by enzymatic cleavage, gamma-rays, or chemicals like bleomycin, H_2O_2, nitrogen oxide (NO), and etoposide. XRCC1 has a central role in the main pathway. It has no enzymatic activity by itself, but rather functions as a loading factor for polymerase beta and ligase III (Thompson *et al*., 2000). BER is active in the G1 phase of cell cycle, but also in the G2 phase (Otterlei *et al*., 1999).

4.2 Nucleotide excision repair

In contrast to BER, one protein complex in NER is probably responsible for repairing a large number of different DNA lesions. NER is thought to be mainly involved in the repair of bulky lesions, such as benzo[*a*]pyrene (B[*a*]P) adducts, which distort the conformation of the DNA helix. NER is so far the only known mechanism in the human body that removes the major UV-induced photo lesions (Wood, 1999). Also small lesions caused by reactive oxygen species (ROS) can possibly be repaired by NER (Reardon *et al*., 1997).

NER is not essential for cellular or animal viability, and individuals with the inherited syndrome, xeroderma pigmentosum (XP), have a total deficiency in this pathway. Such patients usually have roughly a 1,000-fold increase in the incidence of skin cancers and their mean life span is only about 50 years. XP patients have impairing mutations in one (or more) of the *XP* genes. Another disease, Cockayne syndrome, is also characterized by cutaneous photosensitivity. Cockayne syndrome is a rare disorder in which people are sensitive to sunlight, have short stature, and appearance of premature aging. The genetic defect seems to be in the transcription coupled NER (Lawrence *et al*., 1999; Boer *et al*., 2000). Trichothiodystrophy (TTD) is the third syndrome classified under the same family of diseases. It is characterized by photosensitivity, ichthyosis, brittle hair, intellectual impairment, decreased fertility, and short stature. The genetic defect of TTD is localized in the *XPD* (*ERCC2*), but the involved gene region differs from that mutated in XP (Boer *et al*., 2000).

In humans, more than 25 different proteins involved in NER have been described to date. The separate steps in NER can be divided into DNA damage recognition, DNA unwinding, 3'- and 5'-incision, patch repair synthesis, and ligation. The main proteins include XPA and RPA (replication protein A, damage recognition), TFIIH (transcription factor II H) complex including XPB and XPD (DNA unwinding helicase and ATPase), XPC (DNA binding), XPF (5' nuclease), XPG (3' nuclease), RFC

(replication factor C), PCNA and polymerase epsilon (repair synthesis), and ligase I (nick sealing) (Lehmann, 1995; Lehmann, 1998; Sancar, 1996; Wood, 1996).

4.3 Transcription coupled repair

Transcription coupled repair (TCR) repairs DNA lesions preferentially located in the transcribed strand. TFIIH, which is central to NER, is also essential for the TCR pathway. The role of TFIIH is to guide the repair to the transcribed DNA strand. Furthermore, TFIIH probably also loads RNA polymerase II onto the damaged DNA, which is an initiative step in the TCR repair pathway (Mullenders, 1998).

TCR is usually classified as a subpathway of NER, but this view is changing. It is now thought that Cockayne syndrome patients, who have a defect in TCR, are unable to repair oxidative DNA damage irrespective of its nature. Thus, it was hypothesized that TCR might be the main pathway for recognition of oxidative DNA damage, directing the further repair of either BER or NER pathways (Hanawalt, 2000). This explanation is also consistent with the clinical symptoms (photosensitivity) displayed by Cockayne syndrome patients.

4.4 Double-strand break repair

Double strand break (DSB) repair corrects DNA breaks affecting both strands of the double helix. Three different DSB repair pathways have been identified in mammalian cells. Their relative importance for cell survival is probably significant, because DSBs are difficult lesions for cells to repair, and small differences in this ability may make the difference between cell survival and apoptosis. DSBs are very rarely, if at all, generated by endogenous oxidative damage and are therefore unlikely to represent a spontaneous lesion of any significance. DSBs are, however, sometimes introduced by endonucleases into specific sites and are repaired by specific DSB repair mechanisms. Mutants defective in defined repair mechanisms may therefore be defective in these normal metabolic processes. These repair mechanisms and their potential to induce CAs has recently been reviewed (Pfeiffer *et al*., 2000).

4.4.1 Non-homologous end-joining (NHEJ)

Non-homologous end-joining (NHEJ) is a repair pathway using a short stretch of homology as a guide to ligate the cleaved DNA ends together. A blunt ligation is also possible, but less efficient. About ten proteins are known to participate in the NHEJ repair. The process can be divided into three steps. First, the DSB is recog-

nized by Ku70 and Ku80 proteins, and the signal of DNA lesion is mediated by DNA-protein kinase (DNA-PK) to begin the repair processes. In the next step, MRE11 and NBS1 are recruited to the DNA lesion and in the last phase, DNA ligase IV stimulated by XRCC4 seals the DNA ends together (Critchlow *et al*., 1998; Jeggo 1998; Singleton *et al*., 1999).

In mammalian cells, NHEJ is probably the main repair pathway for DSBs, at least in the G1 phase of the cell cycle. Because cells rather use sister chromatids than homologous chromosomes for recombinational repair, it is conceivable that the activity of homologous recombination repair is low in the G1 phase. It has been shown that non-homologous recombination occurs between 100 to 10,000 times more frequently than homologous mechanisms in mammalian cells (Singleton *et al*., 1999). NHEJ is not a totally accurate repair pathway. It can lead to small deletions or insertions of a few base pairs. These mutations are probably much less critical for cell survival than a persisting DSB.

Two genetic disorders are characterized by the lack of effective NHEJ. Nijmegen breakage syndrome patients display an elevated level of CAs and other clinical symptoms. Mutations in the *NBS1* (Nijmegen breakage syndrome 1) gene are responsible for the syndrome (Ito *et al*., 1999). An ataxia telangiectasia-like disorder is caused by a missense mutation in another crucial NHEJ gene, *MRE11* (meiotic recombination 11) (Michelson *et al*., 2000).

4.4.2 Single-strand annealing (SSA)

Single-strand annealing (SSA) is involved in the DSB-induced recombination between long direct repeats, *e.g*., short (SINE) or long (LINE) interspersed nuclear elements. SSA in mammalian cells was first proposed to account for non-conservative recombination products detected after the transfection of DNA into mouse cells (Lin *et al*., 1984). The details of SSA are poorly characterized in mammalians, but are better known in yeast, *Saccharomyces cerevisiae* (Singleton *et al*., 1999, Jeggo 1998). SSA in yeast is independent of RAD50 (radiation sensitivity 50), which is essentially needed for efficient homologous recombination. SSA recombination is initiated by a DSB, but the break does not need to be within the region of homology. The DNA ends are degraded by a strand-specific endonuclease to reveal single-stranded direct repeat sequences. The homologous regions realign and the 3' single-stranded overhanging tails are removed. In *S. cerevisiae* this is performed by RAD1/RAD10 complex. Recombination is completed by repair synthesis and ligation (Singleton *et al*., 1999). Mammalian homologs of RAD1 and RAD10, XPF (ERCC4) and ERCC1, have been identified and are likely to operate in a similar way (van Duin *et al*., 1986; Brookman *et al*., 1996; Sijbers *et al*., 1996).

An important feature of the SSA pathway is that sequences are always lost during the process, in contrast to the conservative nature of homologous recombination. Therefore, the SSA mechanism is very error-prone.

4.4.3 Homologous recombination repair (HR)

Homologous recombination repair (HR) has two totally different roles in cells. It can be used to increase genetic variation in meiosis and also to maintain genomic stability in mitotic cells by repairing chromosome breaks using the sister chromatid as a template. The existence of HR in mammalian cells has been disputed until lately. Recent studies with chicken B lymphocyte line DT40 have shown that HR is an essential DNA repair pathway (Sonoda *et al*., 1998). RAD51-deficient cells accumulate chromosomal damage, especially breaks, before stalling at the G2/M phase of the cell cycle and going into apoptosis. In yeast, the HR process is dependent on the RAD52 epistasis group of genes (Singleton *et al*., 1999). Recently, mammalian homologs (*XRCC2* and *XRCC3*) of *RAD50*, *RAD51*, *RAD52*, and *RAD54* have been identified and cloned, underlining the evolutionary importance of homologous recombination (Shinohara *et al*., 1993; Muris *et al*., 1994; Petrini *et al*., 1995; Dolganov *et al*., 1996; Kanaar *et al*., 1996; Baumann *et al*., 1998).

DSB is processed to reveal single-stranded regions of DNA. RAD51p together with the single-stranded DNA-binding protein (SSB) coats the single-stranded DNA to form a nucleoprotein filament. This filament searches for a homologous sequence in the genome and invades the donor duplex to form a joint molecule. The DNA gap is repaired by fill-in synthesis using the donor strand as template, followed by branch migration, resolution of Holliday junction, and the release of DNA molecules (Singleton *et al*., 1999). In general, HR repair works the same way as normal HR (Lewin, 1999), but the proteins involved are somewhat different.

Expression of human RAD51p and RAD52p appears to be the lowest during the G1 phase of the cell cycle and upregulated during late S/G2 (Chen *et al*., 1997). Targeted knockout of *rad51* in mouse results in early embryonic lethality, indicating an essential role in mouse development in addition to a role in DSB repair (Lim *et al*., 1996). HR has also other important functions in cells than just the repair of replicated DNA. It has been hypothesized that HR may be mainly involved in the reactivation of stalled replication forks (Cox *et al*., 2000).

4.5 DNA repair genes *XRCC1*, *XRCC2*, *XRCC3*, *XPC*, *XPD* and *XPG*

4.5.1 *XRCC1*

XRCC1 (X-ray cross complementing group I) was the first cloned mammalian gene involved in correcting DNA damage from X-rays or ionizing radiation (Thompson *et al*., 1990). The *XRCC1* gene is located on chromosome 19q13.2. The gene is spread over a region of more than 32 kbs, and it has 17 exons. Human XRCC1 protein is composed of 633 amino acids.

Three polymorphisms (*Arg*^{194}*Trp*, *Arg*^{280}*His*, *Arg*^{399}*Gln*) resulting in amino acid substitutions were originally identified in the coding region of the *XRCC1* gene (Shen *et al*., 1998). The substitutions are located in amino acids conserved during evolution. The polymorphism of codon 399 is located in the BRCT1 interaction domain of the XRCC1 protein, but others are situated in the spacer regions (Figure III).

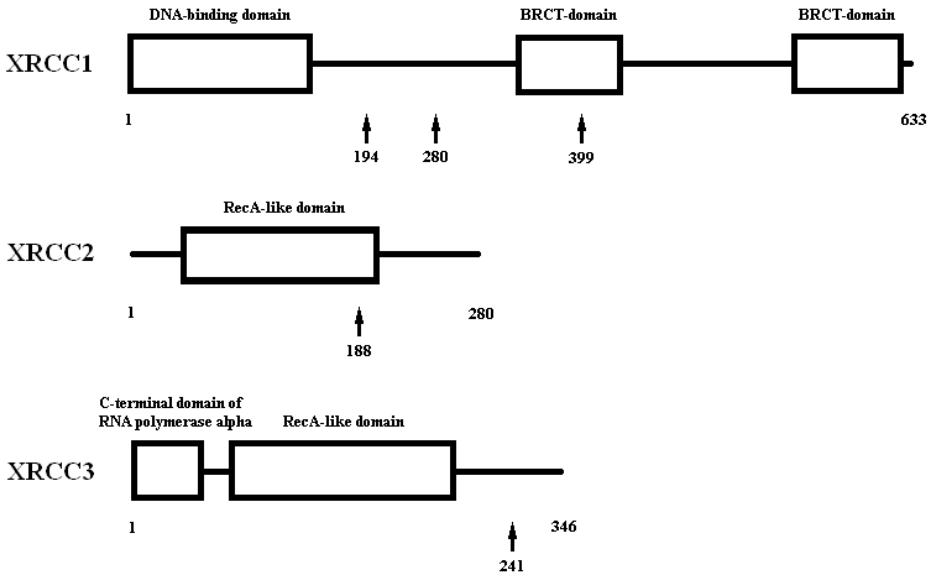

Figure III. The domain structure of the DNA repair proteins XRCC1, XRCC2, and XRCC3. The positions of the best characterized amino acid substitution polymorphism have been marked with arrows.

Several novel non-synonymous amino acid substitution polymorphisms have recently been shown to exist on the coding region of *XRCC1* gene. *Val*^{72}*Ala* polymorphism was initially identified in sequence databases (Tuimala *et al*., 2001), and quickly verified by laboratory studies (Mohrenweiser *et al.*, 2002, and Ruttan *et al.*, 2002). The ^{72}*Ala* variant allele frequency in Caucasian population has been reported to be 2–11%, making it the fourth most common amino acid polymorphism in XRCC1. It is located in the *N*-terminal part of XRCC1, and has potential phenotypic effects. With the exception of the tentative *Lys*^{51}*STOP* polymorphism (2%), the frequency of all other substitutions has been reported to be less than 1% (Mohrenweiser *et al.*, 2002, and Ruttan *et al.*, 2002).

XRCC1 is implicated in DNA repair based on its DNA repair capacity in the Chinese hamster ovary cell line EM9 (Thompson *et al*., 1990). EM9 is hypersensitive to ethyl methanesulfate (EMS) and X-rays, exhibits elevated levels of SCEs, and has a longer half-life of SSBs induced by EMS (Thompson *et al*., 1982), and a reduced efficiency of HR (Hoy *et al*., 1984). *XRCC1* expression has to date been described in murine, rat, and baboon tissues but not in human tissues (Walter *et al*., 1994; Yoo *et al*., 1992; Zhou *et al*., 1995). Testis was found to express *XRCC1* at significantly higher levels than other tissues in mouse and rat. In baboon, testis and ovary expressed the *XRCC1* gene in large amounts, lymph nodes in intermediate, and liver and kidney in small amounts.

Human XRCC1 exerts no enzymatic activity by itself (Thompson *et al*., 2000), but directs the assembly of short patch base-excision repair machinery on DNA (Rice, 1999). Three interaction domains have been identified in the human *XRCC1* gene (Figure III). They bind DNA polymerase beta (Kubota *et al*., 1996), poly (ADP-ribose) polymerase (PARP) (Masson *et al*., 1998), and DNA ligase III (Nash *et al*., 1997). Furthermore, the *N*-terminal end of XRCC1 binds specifically SSBs in DNA (both gapped and nicked) (Marintchev *et al*., 1999).

XRCC1 has a central role in short patch base-excision repair. After damage recognition and initial incision of DNA by monofunctional glycosylases, XRCC1 binds to nicked or gapped DNA (Rice, 1999; Thompson *et al*., 2000). XRCC1 can probably bind to nicks or gaps induced by various chemicals, *e.g.*, bleomycin, directly without the incision step. XRCC1 loads DNA polymerase beta on the nick or the gap, the polymerase synthesizes new DNA, and DNA ligase III recruited by XRCC1 seals the resulting nick (Thompson *et al*., 2000).

A recent study suggested that XRCC1 regulates the activity of PARP (Masson *et al*., 1998). The binding of PARP and XRCC1 to damaged DNA may therefore be mutually competing processes. If XRCC1 binds to single-stranded DNA, it inhibits PARP activity; PARP needs to bind to nicked or gapped DNA in order to be activated. This hypothesis is supported by findings of Masson *et al.* (1998).

The role of PARP in base excision repair is not well characterized. PARP is a zinc-finger DNA-binding protein that detects and signals DNA strand breaks generated directly or indirectly by genotoxic agents. In response to these lesions, the immediate poly(ADP-ribosylation) of nuclear proteins converts DNA interruptions into intracellular signals that activate DNA repair or programmed cellular death. Studies on PARP knockout mice demonstrate the importance of PARP in recovery from DNA damage that triggers BER (Dantzer *et al*., 1999). Thus, the role of PARP in BER may be to regulate the repair activity.

To date, several reports on the phenotypic significance of the *XRCC1* polymorphisms have been published. Healthy carriers of the *XRCC1* ^{399}*Gln* variant allele displayed an elevation in the levels of both the aflatoxin B_1-DNA adducts in blood lymphocytes and the glycophorin A variant erythrocytes (Lunn *et al*., 1999), spontaneous frequency of SCEs (Duell *et al*., 2000), higher smoking-related SCEs (Lei *et al*., 2002), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) -induced SCE frequencies in cultured lymphocytes (Abdel-Rahman *et al*., 2000). Heterozygous carriers of ^{194}*Trp* variant allele also showed slightly elevated NNK-induced SCE frequencies, but the difference did not reach statistical significance. However, the variant allele carriers had lower SCE levels at all three NNK concentrations, which implies that also codon 194 polymorphism may have a modulating effect on the XRCC1 activity (Abdel-Rahman *et al*., 2000).

Three studies assessed the frequency of B[a]P or smoking-related DNA adducts in the carriers of *XRCC1* ^{399}*Gln* allele (Matullo *et al*., 2001a, 2001b; Pastorelli *et al*., 2002). All the studies reported negative results, although there was a tendency for higher DNA adduct levels in the carriers of ^{399}*Gln* allele (Pastorelli *et al*., 2001; Matullo *et al*., 2001b). The negative results may partly be due to small sample sizes (small number of carriers of variant allele) in these studies. The ^{399}*Gln* allele may also be associated with ionizing radiation sensitivity (Hu *et al*., 2001).

In conclusion, *XRCC1* codon 399 polymorphism might be associated with elevated levels of SCEs, which are probably formed by homologous recombination from DNA double-strand breaks, but not with the frequencies of bulky DNA adducts, which are probably more efficiently repaired by NER or HR.

The phenotypic effects on DNA repair rates or association with cancer risk of the novel polymorphisms, like *Val*^{72}*Ala*, has not been studied.

4.5.2 *XRCC2*

XRCC2 (X-ray cross complementing group II) is a Rad51 paralogue, and plays a pivotal role in the homologous recombination repair machinery (Cartwright *et al*., 1998; Dosanjh *et al*., 1998). *XRCC2* consists of three exons, which are distributed

over a 30 kb region in the chromosome 7q36.1. The length of the transcript is 307 amino acids.

To date, one amino acid substitution polymorphism has been described on the coding region of *XRCC2* gene (Mohrenweiser *et al.*, 2002), resulting in $Arg^{188}His$ amino acid substitution (Figure III). The potential phenotypic effects of this polymorphism are currently unknown.

The Chinese hamster ionizing radiation-sensitive cell line (irs1) lacking *XRCC2* was shown to display extreme sensitivity to DNA cross-linking agents and genomic instability (Cartwright *et al.*, 1998). The XRCC2 therefore seems to promote chromosomal stability in cells, but is not essential for viability of cultured cells (Liu *et al.*, 1998; Cui *et al.*, 1999). On the other hand, *XRCC2*-defective mice do not develop normally (Deans *et al.*, 2000).

XRCC2 promotes the repair of DSBs by HR (Johnson *et al.*, 1999). It is also of paramount importance in the repair of DNA cross-links. NER repair is capable of removing cross-links from DNA, but HR, not non-homologous end-joining, is needed to repair the NER-induced DSBs (De Silva *et al.*, 2000). XRCC2 might also be involved in the control of genomic rearrangement processes leading to chromatid breaks (Mozdarani *et al.*, 2001). There is, however, some controversy on the methodology of the study, specifically on the problems in scoring radiation-induced chromatid breaks (Thacker *et al.*, 2002).

4.5.3 *XRCC3*

XRCC3 (X-ray cross complementing group III) gene was originally identified by its ability to complement the DNA repair defect in a Chinese hamster cell line (Jones *et al*., 1987; Fuller *et al*., 1988, Tebbs *et al*., 1995). It is located on chromosome 14q32.3, where its seven exons lie in the region taking 13.5 kbs. *XRCC3* is a small protein of 346 amino acids.

A substitution of C to A has been characterized in the coding region of human *XRCC3* (Shen *et al*., 1998). It results in $Thr^{241}Met$ amino acid change (Figure III). The *XRCC3 Met/Met* genotype has been associated with higher DNA adduct levels in lymphocytes of healthy subjects (Matullo *et al.*, 2001). However, this effect is likely not caused by a defect in homology-directed DSB repair; individuals with ^{241}Met or ^{241}Thr allele repaired the DSBs to the same extent (Araujo *et al.*, 2002).

The XRCC3-deficient Chinese hamster cell line irs1SF is moderately sensitive to X-ray, gamma and UV-radiation, and EMS (Jones *et al*., 1987; Fuller *et al*., 1988). The cell line also shows extreme sensitivity to DNA cross-linking agents, such as mitomycin C (Jones *et al*., 1987; Tebbs *et al*., 1995) and has increased rates of spontaneous and X-ray induced chromatid and chromosome breaks (Fuller *et al*., 1988; Tebbs

et al., 1988). Sequence analysis of the *XRCC3* gene has revealed a homology to the recombinational repair gene *RAD51* (Liu *et al*., 1998).

XRCC3 is required for chromosome stability in Chinese hamster cells (Cui *et al*., 1999). Cell lines without XRCC3 activity displayed highly elevated levels of deletions, breaks, and especially translocations. The number of deletions and translocation was also cumulative through extended passage of the cell lines. *XRCC3* is required for the efficient repair of DNA and chromosome breaks by homologous recombination (Brenneman *et al*., 2000). These findings support the original hypothesis that *XRCC3* is involved in homologous recombination repair of DSBs. Furthermore, *XRCC3*, and therefore also HR, is required for efficient and correct chromosome segregation (Griffin *et al*., 2000). It is probable that *XRCC3* is an assessory protein required for efficient HR repair.

4.5.4 *XPC*

XPC (xeroderma pigmentosum group C complementing protein), a protein consisting of 937 amino acids, guides the NER machinery to the damaged site by binding to the DNA. The *XPC* gene is located in chromosome 3p25. Its 19 exons are distributed over a region of 33.4 kbs.

In addition to alternative splicing forms of mRNA (Khan *et al*., 2002), a $A^{2920}C$ polymorphism in exon 15, resulting in $Lys^{939}Gln$ amino acid substitution, has been described in the *XPC* gene (Khan *et al*., 2000). The $Lys^{939}Gln$ substitution does not appear to affect the DNA repair capacity measured by a post–UV host cell reactivation assay (Khan *et al*., 2000). Post-UV host cell reactivation assay measures how effectively cells remove UV-damaged nucleotides from DNA.

4.5.5 *XPD*

XPD (xeroderma pigmentosum group D complementing protein), an enzyme consisting of 759 amino acids, acts as a DNA unwinding helicase and ATPase during nucleotide excision repair. The *XPD* gene consists of 22 exons, and takes about 18.8 kbs in chromosome 19q13.3.

Several polymorphisms are located in the *XPD* gene (Broughton *et al*., 1996; Shen *et al*., 1998). The A to C base change in exon 23, resulting in $Lys^{751}Gln$ amino acid substitution, is especially interesting; this substitution appears to have clear phenotypic consequences. The $Lys^{751}Gln$ polymorphism is in strong linkage disequilibrium with the $Asp^{312}Asn$ polymorphism located in the seven-motif helicase domain of the RecQ family of DNA helicases (Shen *et al*., 1998). Thus, for evaluation of the effects of these substitutions, investigation of the codon 751 polymorphism is anticipated to be sufficient.

After X-ray treatment, individuals, or rather their cells, with the *Lys/Lys* genotype exhibited about four times as many chromatid aberrations (breaks and gaps) as other individuals. The effect was equally profound in both breaks and gaps (Lunn *et al.*, 2000). The *CAT* (a bacterial drug resistance gene) -gene-based host reactivation assay of B[*a*]P-treated plasmids indicates that lung cancer patients having the *Gln/Gln*-genotype might have lower DNA repair capacity (Spitz *et al.*, 2001). A similar study conducted on healthy subjects showed that the ^{751}*Gln* allele delays the rate of DNA adduct removal (Qiao *et al.*, 2002). Studies based on healthy subjects exposed to traffic pollution or city air suggest that the carriers of at least one ^{751}*Gln* allele have higher levels of bulky DNA adducts, although smoking might regulate the effect (Matullo *et al.*, 2001; Palli *et al.*, 2001).

4.5.6 *XPG*

XPG (xeroderma pigmentosum group G complementing protein) acts as a 3'-endonuclease in the nucleotide excision repair. *XPG* gene is located on chromosome 13q22, where its two exons occupy 3.4 kbs. The transcript is 67 amino acids long.

In addition to six splice variants, three amino acid substitution polymorphisms have been identified in the coding region of the *XPG* gene. Only one of the substitutions, *i.e.*, the Asp^{1104}His substitution resulting from *C*^{3507}*G* base change in exon 15, has been experimentally verified, and occurs in polymorphic frequencies (>1%) in the Caucasian population (Emmert *et al.*, 2001).

Because XPG acts as a 3'-endonuclease in the nucleotide excision repair, it might have profound effects on the capacity to repair bulky DNA adducts, but the ^{3507}*C* and ^{3507}*G* alleles do not seem to differ in their capacity to remove crosslinking dimers (TT=C or TT=T) from the DNA (Kumar, *et al.*, 2003).

4.6 DNA repair genes and cancer risk

Since most of the DNA repair genes and their polymorphisms have been identified only lately, their relevance to cancer risk has been quite scarcely explored to date. *XRCC1*, polymorphisms of which were among the earliest reported, is an exception in this regard. The results of numerous molecular epidemiological studies conducted on XRCC1 genotypes and susceptibility to cancer have been summarized in Table I.

A recent epidemiological meta-analysis review revealed that only a few of the so far published studies are large and population-based. The *XRCC1* codon 194 variant allele appeared to be consistently associated with decreased cancer risk, whereas the results for the *XRCC1* codon 399 genotypes were inconclusive (Goode *et al.*,

2002). When a combined risk for *^{399}Gln* allele was calculated on the basis of the published studies (Table I, unpublished data), a study-corrected (combined risk divided by the number of studies) odds ratio (OR) was estimated to be about 1.4 (statistically not significant). If the number of subjects included in the studies was also considered (combined risk divided by the number of subjects), the risk was practically abolished (OR=1.04, statistically not significant).

Recent studies have reported a positive association between *XRCC3 ^{241}Met* allele and melanoma (Winsey *et al*., 2000), bladder cancer (Matullo *et al.*, 2001; Stern *et al.*, 2002), squamous cell carcinoma of head and neck (Shen *et al.*, 2002), and breast cancer (Smith *et al.*, 2003). In contrast, the findings on lung cancer have been negative (David-Beabes *et al.*, 2001; Hu *et al.*, 2002; Misra *et al.*, 2003).

Two studies linked the *XRCC2 ^{188}His* allele to increased risk of breast cancer with ORs of 2.6 (95% CI=1.0–6.7) and 1.3 (95% CI=1.0–3.8), respectively (Kuschel *et al.*, 2002; Rafii *et al.*, 2002).

The epidemiological approaches may contain some uncertainties in sorting out the risk alleles that possibly affect also DNA repair activity. For instance, if the DNA of the cell is badly damaged and cannot be repaired, another solution for the cell is to go into apoptosis. Therefore, if the defect in the DNA repair gene preferentially results in apoptosis of damaged cells, the risk allele may be falsely deduced from the epidemiological data.

The aforementioned epidemiological studies suggest that polymorphisms in *XRCC1*, *XRCC2*, and *XRCC3* genes may contribute to individual cancer susceptibility. Even more interesting would be to know the impact of combined effects of variant alleles in, *e.g*., both the BER and NER enzyme genes. These studies would, however, require very large study sizes due to the rarity of some of the variant alleles in both repair pathways.

To date there are no published data available on the potential association between *XPC* exon 15 polymorphism and individual cancer proneness. In contrast, the *XPD* exon 23 *Lys*-genotype has been linked to elevated risk of basal cell carcinoma (Dybdahl *et al.*, 1999), head and neck cancer (Sturgis *et al.*, 2000), and lung cancer (Hou *et al.*, 2002). The *Lys* allele may also be a risk factor for basal cell carcinoma (statistically non-significant findings, [Dybdahl *et al.*, 1999]). The *Gln* allele seems to predispose individuals to cancers of head and neck and lung.

The *XPG* exon 15 polymorphism has been linked to susceptibility to breast cancer (Kumar, *et al.*, 2003). Carriers of the *XPG ^{1104}Asp* allele exhibited a slightly elevated risk of breast cancer (OR=1.33, 95% CI=1.0–1.8). The combined risk for homo- and heterozygous carriers of the *Asp* allele was even a bit higher (OR=1.50, 95% CI=1.04–2.16).

Table I. Summary of the results from studies assessing the role of XRCC1 genotypes in the risk of different cancers

	Codon 194		Codon 280		Codon 399	
	Arg	Trp	Arg	His	Arg	Gln
Lung cancer[1]	-	-	-	+	-	-
Lung cancer[14]	-	-	-	-	-	-
Lung cancer[18]	+	-	o	o	-	-
Lung cancer[19]	o	o	o	o	-	+
Lung cancer[22]	-	-	o	o	-	-
Non-melanoma skin cancer[2]	o	o	o	o	-	+
Head and neck cancer[3]	+	-	o	o	-	+
Head and neck cancer[4, 20]	-	-	o	o	+	-
Adenocarcinoma of lungs[5]	o	o	o	o	-	+
Bladder cancer[6]	-	-	o	o	-	+
Colorectal cancer[7]	-	+	o	o	-	+
Prostate cancer[8]	-	-	o	o	-	-
Breast cancer[9]	-	-	o	o	-	+
Breat cancer[10]	-	-	o	o	-	+
Esophageal cancer[11]	-	-	-	-	+	-
Esophageal cancer[12]	+	-	o	o	-	-
Gastric cancer[13]	+	-	o	o	-	-
Gastric cancer[15]	-	-	-	-	-	-
Pancreatic adenocarcinoma[16]	o	o	o	o	-	+
Myoloblastic leukemia[17]	-	-	o	o	+	-
Lymphoblastic leukemia[21]	-	-	o	o	o	o

+ denotes a risk allele for the cancer, and – denotes a (concomittant) non-risk allele. o denotes the allele not included in the study.

[1] Ratnasinghe *et al.*, 2000, 2001;[2] Nelson *et al.*, 2000, 2002; [3] Sturgis *et al.*, 1999; [4] Watson *et al.*, 2000; [5] Divine *et al.*, 2000, 2001; [6] Stern *et al.*, 2000, 2001, 2002; [7] Abdel-Rahman *et al.*, 2000; [8] Hu *et al.*, 2000; [9] Duell *et al.*, 2000, 2001; [10] Kim *et al.*, 2002; [11] Lee *et al*., 2001; [12] Xing *et al*., 2002; [13] Shen *et al*., 2000; [14] Butkiewick *et al*., 2001; [15] Lee *et al*., 2002; [16] Duell *et al*., 2002; [17] Seedhouse *et al*., 2002; [18] David-Beabes; [19] Park *et al*., 2002; [20] Olshan *et al*., 2002; [21] Krajinovic *et al*., 2002; [22] Chen *et al*., 2002.

5. Xenobiotic metabolism

Enzymes involved in the metabolism of foreign compounds are classically divided into phase I and phase II enzymes. Phase I XMEs including, *e.g.*, cytochrome P450 mono-oxygenases (CYPs), introduce or release a functional group of metabolized compound by an oxidation, hydroxylation, or reduction reaction so that the original compound becomes more water-soluble. Phase II XME enzymes further metabolize these compounds by conjugating a sulfate-, glutathione-, amino acid-, acetyl-, or glucuronide group to the metabolized compound. Phase II enzymes are usually classified as detoxifying enzymes, because the end products are generally less reactive and can be more easily excreted (Amdur *et al.*, 1991).

5.1 Glutathione S-transferases

Glutathione *S*-transferases (GSTs) form a superfamily of enzymes catalyzing conjugation of the tripeptide glutathione with compounds containing electrophilic carbon atoms (Strange *et al.*, 2000). GSTs have been further divided into several families; to date, six cytosolic (alpha, mu, phi, theta, sigma and zeta), and two membrane-bound (microsomal and leukotriene C4) GST families have been identified (Strange *et al.*, 2000). Because glutathione conjugation can prevent potentially harmful xenobiotics from binding to cellular constituents, it has a central role in the antioxidant defence. Sometimes the cellular glutathione balance can be altered by toxic compounds, which can lead to drastic deleterious results like lipid peroxidation and cellular death (Amdur *et al.*, 1991).

To date, genetic polymorphisms have been found in six of the cytosolic GSTs, *i.e.*, in *GSTA2*, *GSTM1, GSTM3, GSTP1, GSTT1* and *GSTZ1* (Tetlow *et al.*, 2001; Strange *et al.*, 2000). The most studied polymorphic *GST* genes are *GSTM1* and *GSTT1*; about 50% of Caucasians completely lack *GSTM1* activity due to a homozygous deletion of the gene (Board, 1981), and 10–20% are carriers of a homozygous deletion of *GSTT1* gene (Pemble *et al.*, 1994). Both GSTs are supposedly expressed in human peripheral blood lymphocytes, but only *GSTT1* is expressed in red blood cells (Schroder *et al.*, 1996). However, GSTM1 and GSTP1 predominate in hematopoietic lymphoid cells, whereas GSTT1 expression is relatively the greatest in erythroid lines but absent in 60% of presumably (by genotype) positive erythroid cell lines (Wang *et al.*, 2000). How these findings correlate with expression in mature blood cells is still unclear.

The correlation between genetic polymorphisms and respective enzyme activity is naturally best characterized for *GSTM1* and *GSTT1*; lack of the gene (deletion)

results in total absence of the respective enzyme activities. For other GSTs the genotype-phenotype relationships are much less accurate.

5.2 *N*-acetyltransferases

N-acetyltransferases (NATs) are cytosolic enzymes catalyzing the acetylation of amines. Especially important substrates for NATs are aromatic amines present, *e.g.*, in dyes, pesticides, food, and tobacco smoke. Depending on the metabolic balance of the target tissue, NATs may either activate (*O*-acetylation) or inactivate (*N*-acetylation) arylamine carcinogens. In humans, two genes, *NAT1* and *NAT2* are responsible for *N*-acetylation of xenobiotics. Both NAT1 and NAT2 catalyze *N*- and *O*-acetylation and the enzymes have partly overlapping substrate specificity (Kadlubar *et al*., 1992). In humans, NAT2 is the predominant form in liver, whereas NAT1 dominates in nearly all other tissues. NAT1 activity in whole blood is about 9–10 times higher than NAT2 activity (Risch *et al*., 1996). Both *NAT1* and *NAT2* are polymorphic in humans. Several polymorphisms have been idenfied in the *NAT1* gene (for nomenclature see Vatsis *et al*., 1995), but since only polymorphisms of the *NAT2* gene were considered in this thesis, they will not be discussed in more detail here.

The *NAT2* genotype can be used to interprete the individual enzymatic acetylator status (slow/rapid) (Cascorbi *et al*., 1995 and 1999). Carriers of at least one copy of the *NAT2* wild type allele (*NAT2*4*) are considered as rapid acetylators. About 50% of Caucasians have two *NAT2* variant alleles (slow acetylator genotype) and therefore exhibit decreased NAT2 enzyme activity. To date, several defective *NAT2* variant alleles have been described (Vatsis *et al*., 1995; Grant *et al*., 1997; Hein, 2000). Five of the variant alleles (**5A*, **5B*, **5B*, **6A* and **7A*) account for over 95% of the slow *NAT2* acetylator genotypes in Caucasians (Cascorbi *et al*., 1995; Cascorbi *et al*., 1999).

5.3 Bleomycin hydrolase

Bleomycin hydrolase (BLHX) is a proteinase belonging to the papain superfamily (Sebti *et al*., 1989). It was originally isolated from rabbits and mice based on its enzymatic activity to render bleomycin to its inactive metabolite (Umezawa *et al*., 1974). These findings led the authors to suggest that drug inactivation could play a major role in tumor resistance to bleomycin treatment (Sebti *et al*., 1991).

Because *BLHX* is a housekeeping gene (GC-rich promoter sequence, no TATA-box), it is widely expressed in all tissues (Brömme *et al*., 1996; Ferrando *et al*. 1996). It has been shown to be especially heavily expressed in head and neck cancers

(Ferrando *et al*., 1996). However, results on BLHX expression in human blood leucocytes are inconsistent. While Ferrando *et al.* (1996) reported a strong expression of BLHX in healthy donor's leukocytes, Brömme *et al.* (1996) found no expression at all. BLHX is expressed in human leukemias (Brömme *et al*., 1996), which may reflect the presense of the enzyme only in stimulated leukocytes.

So far one polymorphisms in the coding region of *BLHX* gene has been identified (Brömme *et al*., 1996). The $A^{1450}G$ substitution results in Val to Ile amino acid change in the carboxy-terminal end of the protein. Val in this position is highly conserved during evolution; it is the normal amino acid in humans, rat, chicken, yeast, and even a few bacterial species (Koldamova *et al*., 1998). Furthermore, the last eight amino acids are essential for the BLHX and aminopeptidase activity. The Val to Ile substitution takes place only four amino acids upstream from the essential amino acids (Koldamova *et al*., 1998). The C-terminus is also required for protection of bleomycin-induced chromosomal damage (Lefterov *et al*., 1998). The phenotypic changes induced by substitution in the *BLHX* gene have not been studied, but it is conceivable that the substitution may affect the enzyme activity by interrupting the essential C-terminal amino acid sequence. Furthermore, the variant allele was associated with the risk of sporadic Alzheimer's disease (Montoya *et al*., 1998). Because BLHX also seems to have a regulatory role in the metabolic cascade possibly leading to Alzheimer's disease (Lefterov *et al*., 2000), the polymorphism may indeed affect the enzyme activity.

6. Exposures studied

Two model compounds of occupational exposures are introduced here, styrene and tobacco-smoke. Styrene is a good model compond for genotoxicology studies, because it is widely used, exposures remain high in reinforced plastics industry, and its toxicology, metabolism and other properties are very well characterized. Tobacco-smoke is a common (occupational) exposure, and an often used model mixture for genotoxicology studies.

6.1 Styrene

Styrene (ethenylbenzene, C_8H_8, CAS no. 100-42-5) is one of the most important organic chemicals, and is widely used in the production of plastics, synthetic rubber, and polyester resins. Styrene is mainly produced to prepare solid polystyrene foam, which is used as an insulation material in buildings and water pipes. Styrene is also an environmental contaminant. It can enter the environment during production, use or disposal of styrene-based products. It is present in small quantities in food items, tobacco smoke, engine exhausts, air, water, and soil (IARC, 1994).

6.1.1 Styrene carcinogenicity and genotoxicity

Although the evidence of styrene's carcinogenicity in humans is either inadequate or contradictory, and the evidence for animal carcinogenicity limited, IARC has classified styrene as a possible human carcinogen (group 2B). The principal styrene metabolite, styrene-7,8-oxide (SO), is classified as a probable human carcinogen (group 2A). Styrene gives positive results in many genotoxicology assays (Norppa *et al.*, 1988; Barale, 1991; Tates *et al.*, 1994; Vodicka *et al.*, 1995; Somorovská *et al.*, 1999).

Most studies concerning reinforced plastic workers exposed to styrene have reported an increased frequency of CAs in peripheral blood lymphocytes (WHO 2000). Cohort studies have not been able to demonstrate an association between styrene exposure and cancer, with the possible exception of leukemias and lymphomas (McMichael *et al.*, 1976; Hodgson *et al.*, 1985; Bond *et al.*, 1992).

6.1.2 Styrene exposure

Humans are exposed to styrene primarily via inhalation of contaminated air during the manufacturing processes of reinforced plastics (Pfäffli, 1982). Occupational ex-

posure in hand lamination work may intail a daily intake of several grams of styrene via inhalation. Styrene is also easily adsorbed through the skin. Styrene is soluble in water (or blood) and accumulates in adipose tissues (Wolff *et al.*, 1977).

6.1.3 Styrene metabolism

Styrene is genotoxic after metabolic activation and its most important reactive metabolite seems to be SO, an alkylating agent found to be genotoxic in several test systems. For example, styrene has given only positive results in genotoxicity assays with human whole-blood lymphocyte cultures. The effects are probably due to the metabolic activation of styrene to SO in the presence of erytrocytes (Belvedere *et al.*, 1981; Norppa *et al.*, 1983; Jantunen *et al.*, 1986).

Styrene metabolism has been extensively reviewed (IARC, 1994; Vodička, *et al.*, 2002). Briefly, in humans styrene is oxidized by cytochrome P450-enzymes or oxyhaemoglobin to SO, which is a strong reactive intermediate; SO reacts readily with nucleic acids to form various DNA adducts, mainly *O*^{6}-deoxyguanosine and *N*7-deoxyguanosine. SO is metabolically inactivated by microsomal epoxide hydrolase (EPHX1) and excreted into urine as mandelic and phenylglyoxylic acids. These acids are the primary metabolites quantitated in the biomonitoring of styrene exposed humans.

To a minor extent, SO is conjugated with glutathione, resulting in the subsequent formation of phenyl hydroxyethyl mercapturic acids (PHEMAS). An alternative minor route is oxidation on the aromatic ring, which may lead to the formation of 3,4-arene oxide, thought to contribute to styrene genotoxicity (Pfäffli *et al.*, 1981; Watabe *et al.*, 1982). The above described metabolic pathways may be followed by measuring the urinary concentrations of mercapturic acids and 4-vinyl phenol.

6.1.4 DNA repair and styrene

Styrene induces DNA strand breaks in human white blood cells (Brenner *et al.*, 1991; Walles *et al.*, 1993; Somorovská *et al.*, 1999; Vodička *et al.*, 1999). The repair of DNA strand breaks in cultured cells exposed to SO is rapid: the lesions are effectively removed from the DNA within a few hours after the treatment (Laffon *et al.*, 2002). On the other hand, DNA adducts, expecially at O^6 position of guanosine, formed by SO, are very persistent, and not very effectively repaired; they were detectable in cultured human lymphocytes even eight days after the treatment (Bastlova *et al.*, 1995). SO seems to induce many more strand breaks than DNA adducts at consentrations viable for cultured cells (Bastlova *et al.*, 1995). Cytogenetic damage (SCEs and CAs) is another consequence of styrene exposure (Tates *et al.*, 1994; Somorovská

et al., 1999). Base excision and oxidative lesion repair seem to be elevated in styrene exposed workers (Vodicka *et al.*, 2002), suggesting that DNA excision repair mechanisms may be critical in protecting cells from the adverse effects of styrene metabolites.

These data indicate that styrene and SO primarily induce strand breaks that are rapidly repaired. It is possible that some strand breaks remain, and in such cases CAs may be induced in order to sustain the unity of the damaged DNA. It is also plausible that some persistent bulky adducts formed by SO prevent the transcription of the DNA, which might lead to DNA strand breaks and subsequent CAs, or to the reactivation of damaged replication forks that might correspond to the formation of SCEs. This hypothesis is supported by the known mechanism of action of SO; CAs induced by SO are formed during the S-phase of the cell cycle.

6.2. Cigarette smoke

Cigarette smoke is a highly complex mixture of more than 3,800 compounds. There are at least 20 compounds that have been convincingly found to induce tumours, including benzene, aromatic amines, and PAHs (Hecht, 1999). PAHs and aromatic amines are probably the main carcinogens in tobacco smoke. Moreover, the tar content of cigarettes correlates with the risk of developing upper digestive tract neoplasms (Gallus *et al.*, 2003). It is presently unclear, whether tar is the principal source of genotoxic compounds in cigarette smoke.

Some compounds identified from cigarette smoke can generate reactive oxygen species, which are capable of inducing DSBs in the DNA (Jaloszynski *et al.*, 2003). The oxygen radicals are likely to play a central role in the development of lung cancer (Marnett *et al.*, 2000), and might also contribute to the formation of upper-aerodigestive tract cancers (UAT). PAHs and aromatic amines in cigarette smoke might also form DNA adducts, which can be converted into DNA strand breaks. Therefore, it is plausible that the DNA repair of both strand breaks and DNA adducts is implicated in cigarette smoke exposure, and therefore also in UAT cancers.

UAT cancers, *i.e.*, oral, pharyngeal, and laryngeal cancers, claimed 30,504 victims in the European Union during 1998 (IARC, 1998). The UAT cancers are more prevalent in men than in women (IARC, 1998) and are strongly associated with alcohol and tobacco consumption (Johnson, 2001; Risch *et al.*, 2003; Gallus *et al.*, 2003). The standardized incidence for all the three cancers was 20.51 / 100 000 individuals. The standardized mortality was 13.74 / 100,000 individuals. Laryngeal cancer has a higher mortality than the cancers of oral or pharyngeal origin, probably because its diagnosis is often delayed. For some reason, France has much higher UAT incidence and mortality rates than other European countries.

The UAT cancers comprise mostly of squamous cell carcinomas, which might partly explain the strong association of these cancers with the consumption of stimulants; epithelium is mainly of squamous type in mouth, pharynx and larynx.

Because PAHs and aromatic amines undergo an extensive metabolism, and most of the relevant XMEs are polymorphic, the possible role of the xenobiotic metabolism in the UAT cancers has been studied earlier. It has been shown, using the same French population that has been used in the epidemiological study included in this thesis, that certain polymorphisms of *GSTM3*, *P1*, and *T1*, *EPHX1*, and *NAT2* are risk factors for UAT cancers (Jourenkova-Mironova *et al.*, 1999a, 1999b, 1999c, 2000)

Since May 2000, tobacco smoke has been classified as an occupational cancer hazard in Finland. All workers who are occupationally exposed to tobacco smoke for more than 40 days annually must now be reported to the ASA register (Finnish register of workers exposed to carcinogens). ASA register holds records of workers exposed to cancer promoting chemicals in Finland.

II Present study

1. Aims of the study

The major objective of this thesis was to determine if DNA repair gene polymorphisms could affect individual sensitivity to genotoxic effects and cancer.

More specifically, the overall aims were:

1. To evaluate the influence of different xenobiotic metabolizing and DNA repair enzyme polymorphisms on the outcome of bleomycin sensitivity assay.
2. To evaluate the effect of genetic polymorphisms of *XRCC1* and *XRCC3* DNA repair genes on the frequency of spontaneous CAs in short-term culture of human lymphocytes.
3. To analyze whether DNA repair genotypes modify the risk of UAT cancers in smokers.
4. To investigate whether DNA repair gene polymorphisms affect the levels of genotoxicity biomarkers in occupational exposure to styrene.

2. Subjects and methods

2.1 Study subjects

Four different Caucasian study populations were investigated in this work (Table II). Finnish and Hungarian populations were used to study the effect of DNA repair gene polymorphisms on CAs and SCEs. A Czech cohort was used to study the effect of DNA repair gene polymorphisms on the genotoxicity end-points in styrene exposure. A French population was used to study the possible role of DNA repair gene polymorphisms in susceptibility to cancers of the upper aerodigestive tract in smokers. These populations were chosen for this work because of their relevance to the studied hypothesis, and because we had ongoing collaboration with the laboratories providing the material.

2.1.1 Finnish study population

The Finnish subjects, who were originally used as controls in a study on isocyanate asthma (Piirilä *et al*., 2001), were mainly office workers and secretaries, but also included were a few chemists, and other people who possibly had been exposed to isocyanates at very small amounts. Although relatively many of the study subjects (34.4%) were smokers, their smoking dose appeared to be rather low (mean 8.1 pack-years). Most (84%) of the subjects were males, because they acted as a control group for factory workers consisting mainly of men. All the subjects were healthy at the time of blood donation.

2.1.2 Hungarian study population

The Hungarian study subjects were recruited at the Hungarian National Cancer Institute, Budapest. Originally, they had participated in a case-control study of head and neck cancer (Szekely *et al*., 2003). Smoking was about as frequent in the Hungarian controls (39%) as in the Finnish controls. Most of the Hungarian subjects (73.0%) were males.

2.1.3 Czech study population

The styrene-exposed group consisted of 86 workers employed at three different plants located in the same area. The control group consisted of employees of a Regional Hygienic Station and unexposed maintenance workers from one of the three plants. Smoking was more common in the exposed group (51.2%) than in the control group (19.1%).

2.1.4 French study population

The French study subjects were recruited from 10 French hospitals between 1988 and 1992. Of these subjects, 250 were cancer patients. Peripheral blood samples were available from 121 patients with cancers of the oral cavity or pharynx, and 129 patients with cancers of the larynx. Only incident cases with histologically confirmed squamous cell carcinoma were included. The control group consisted of individuals without previous or current malignant disease. The control group was age- sex-, and hospital-matched with the cancer patients. Only regular smokers, defined as people having smoked at least five cigarettes a day for at least five years, were included in the study. Most of the study subjects were men (95–96% of cases and controls). A more detailed description of the study population has been published previously (Jourenkova-Miranova, 1999).

2.2 Blood samples and DNA isolation

For cell culture purposes, 10ml of peripheral blood was collected into heparinized Vacutainer® tubes, and whole-blood lymphocyte cultures were established within 24 hours of blood sampling. For genotyping purposes, DNA was isolated by standard phenol-chloroform method (Blin *et al.*, 1976) from buffy coats of peripheral blood samples collected into EDTA Vacutainer® tubes and stored at –20^{O}C until use.

2.3. Styrene exposure

To assess personal exposure to styrene, ambient air was sampled by personal dosimeters (Paper IV) (Vodička *et al.*, 1995 and Somorovská *et al.*, 1999). In addition, the concentration of styrene in blood was determined (Vodička *et al.*, 2001).

2.4 Chromosomal aberrations and mutagen sensitivity assay

The mutagen (bleomycin) sensitivity assay (Paper I) was performed for the Hungarian study group essentially as described in previous work (Schantz *et al.*, 1989; Hsu *et al.*, 1989). In brief, standard lymphocyte cultures were established in RPMI 1640 medium. On the third day of the culture, the cells were treated with bleomycin (30 mg/ml) for five hours. During the last hour, the cells were treated with Colcemid

Table II. Populations studied, their age, gender and smoking behaviour distributions.

	Hungarian cohort	Finnish cohort	Czech cohort		French cohort	
	Controls n=84	Controls n=61	Controls n=42	Exposed n=86	Controls n=172	Patients n=250
Age						
Mean (SD)	41.7 (11.5)	40.1 (8.9)	45.2 (8.3)	36.5 (12.0)	54.9 (11.1)	54.7 (9.8)
Gender (%)						
Male	53 (63.1)	51 (83.6)	22 (52.4)	61 (70.9)	163 (94.8)	240 (96.0)
Female	29 (36.9)	10 (16.4)	20 (47.6)	25 (29.1)	9 (5.2)	10 (4.0)
Smoking (%)						
Never	32 (39.0)	21 (34.4)	34 (80.9)	42 (48.8)	116 (67.4)	184 (73.6)
Ever	50 (61)	40 (65.6)	8 (19.1)	44 (51.2)	56 (32.6)	66 (26.4)

(0.05 mg/ml) to accumulate mitosis prior to harvest for conventional air-dried metaphase preparations. Control cultures, from which the spontaneous CA-frequency was scored, received only the one-hour Colcemid treatment. The slides were coded and stained with Giemsa without banding. 50 metaphases per sample were scored for CAs (control cultures) or for bleomycin-induced chromosome breaks (chromatid breaks and exchanges). The frequency of bleomycin-induced breakage was expressed as breaks per cell (b/c) for purposes of comparison.

For the cytogenetic studies performed in Finland, peripheral blood cell cultures were set up (Papers I, II and IV) as had been previously described, with 48-hour and 72-hour culture times for CAs and SCEs, respectively (Valjus *et al*., 1993). One hundred cells per donor were microscopically scored for the CA analysis and 30 cells per donor for the SCE analysis.

2.5. Single-strand breaks and DNA repair capacities

Alkylation-specific DNA damage was detected by a modified single-cell gel electrophoresis (Comet) assay (Collins *et al.*, 1996). The comet assay is based on the alkaline lysis of labile DNA at sites of damage. The unwound, relaxed DNA is able to migrate out of the cell during electrophoresis and can be visualized with fluorescent DAPI-staining. Cells that have accumulated DNA damage appear as fluorescent comets with tails of DNA fragmentation or unwinding, whereas normal undamaged DNA does not migrate far from the origin. Abasic sites and oxopyrimidines were detected with the Comet assay after endonuclease III treatment (Collins *et al.*, 1996 and 2001).

Repair of irradiation-specific DNA strand breaks was performed according to Alapetite *et al.*, 1999. Agarose embedded cells were irradiated with γ-rays and either lysed immediately or incubated at +37°C for 40 minutes. The level of SSBs was determined at both time points by the Comet assay, and the decrease in SSBs occurring during the incubation was used as a measure of DNA repair capacity (Vodicka *et al.*, 2002).

The individual capacity of mononuclear leukocyte extracts to repair 8-oxoguanine, known to be removed from DNA by a specific glycosylase OGG1, was determined as had been previously explained (Collins *et al.*, 2001). In brief, HeLa cells were pretreated with the photosensitizer Ro 19-8022 (Hoffmann-La Roche) and irradiated with a fluorescent lamp to induce 8-oxoguanine. Aliquots of cell extracts prepared from mononuclear leukocytes were incubated on microscopic slides together with the pretreated HeLa cells, and mounted in agarose for the Comet assay. The level of SSBs in the HeLa cells reflected the removal of 8-oxoguanine from HeLa cell DNA by the leukocyte extract.

2.6 Genotype analyses

2.6.1 *BLHX*

The original genotyping method for *BLHX* was based on single-strand conformation polymorphism (SSCP) analysis (Montoya *et al*., 1998). This is a rather laborous technique if numerous samples are to be prosessed. Therefore, we designed a novel PCR and RFLP (PCR-RFLP) -based method for the genotype analysis (Paper I). The nucleotide substitution together with the mismatch incorporated in the forward primer creates a *Mun*I restriction digestion site. The primers used were BLHX-G (forward) CCT GGA TCT GTC CTT TGC AGC TAC G and BLHX11R (reverse) GCT GTG TTA GAG CAG GAA CCC AAT T. This primer pair (6 pmols of each primer) amplifies a DNA fragment of 130 bps under the standard PCR conditions with the following program: denaturing 94 ^{O}C 30 seconds, annealing 58 ^{O}C 30 seconds and elongation 72 ^{O}C 15 seconds for 35 cycles. After digestion with 5 U of enzyme and 3% agarose gel electrophoresis, the A/A genotype gives a fragment of 130 bps, the A/I genotype fragments of 130, 106 and 24 bps and the I/I genotype fragments of 106 and 24 bps.

2.6.2 *GST*

In *GSTM1* and *GSTT1* genotyping analysis (Paper I, II and IV), the gene-specific primer pairs were used together with a third pair for b-globin in a multiplex reaction. The absence of *GSTM1* and/or *GSTT1* specific PCR-product indicated the corresponding null genotype while the presence of β-globin specific fragment confirmed the proper functioning of the reaction (Chen *et al*., 1996; Hirvonen *et al*., 1996).

2.6.3 *NAT2*

The *NAT2* genotypes were determined by the method of Bell *et al*., 1993, which differentiates between the *NAT2 *4, *5, *6 and *7* alleles (Papers I, II, and IV). The absence of a specific restriction site indicated the presence of a specific variant allele. Individuals having inherited two defective alleles were considered as slow acetylators. Genotype-phenotype comparisons have indicated that by the method used, approximately 90% of all the subjects will be correctly classified according to the acetylation status (Cascorbi *et al*., 1995).

2.6.4 *XRCC1* and *XRCC3*

The *XRCC1* and *XRCC3* genotypes were detected by a PCR-RFLP (restriction fragment length polymorphism) -based technique (Papers I, II, and III). Codon 194 and 399 polymorphisms of *XRCC1* were sought as had been previously described (Lunn *et al*., 1999) with slightly modified primer sequences; three bases were added to the end of 26106F primer (codon 194-spesific forward primer) to unify the annealing temperatures in the multiplex PCR reaction. The absence of the *Msp*I restriction sites indicated the presence of variant alleles.

For the genotype analyses of *XRCC1* codon 280 and *XRCC3* codon 241, a multiplex PCR method was developed. The $A^{280}G$ ($Arg^{280}His$) nucleotide substitution abolishes a *Rsa*I cleavage site, and the $C^{241}T$ ($Thr^{241}Met$) substitution creates a *Nla*III restriction site. The following primers were used to amplify a 280 bp and a 335 bp fragment of *XRCC1* and *XRCC3*: XRCC1-280F TGG GGC CTG GAT TGC TGG GTC TG and XRCC1-280R CAG CAC CAC TAC CAC ACC CTG AAG G for codon 280 and XRCC3-241F GCT CGC CTG GTG GTC ATC GAC TCG and XRCC3-241R AAG AGC ACA GTC CAG GTC AGC TG for codon 241. PCR conditions consisted of 100–400 ng of genomic DNA, 1.67 mM $MgCl_2$, 200 mM each dNTPs, 1 unit of Taq polymerase (Promega), 10 pmols of each primer in 1 X PCR-buffer (Promega) in a 30ml volume. A 10 ml aliquot of PCR product was digested with *Rsa*I (New England Biolabs) and *Nla*II (New England Biolabs), as suggested by the manufacturer. The DNA fragments were resolved in 3% agarose. The resulting bands were as follows: codon 280, A/A genotype 280 bp, A/G genotype 280 bp and 140 bp and G/G genotype 140 bp; codon 241, C/C genotype 335 bp and 102 bp, C/T genotype 335 bp, 233 bp and 102 bp, and T/T genotype 233 bp and 102 bp.

A method described earlier (Matullo *et al.*, 2001) was used for *XRCC1* and *XRCC3* genotyping in Paper IV.

2.6.5 *XRCC2*

In the *XRCC2* genotyping analysis (a novel method, Paper III), a 200 bp PCR product was amplified from isolated DNA using gene-specific primers (5'-CAG TAG TAG CAC CCA CTT ACT TC-3' and 5'-GTT TGT GTC GTT GCA AAA AGA ACC-3'). The PCR was conducted as follows: 5 min of initial denaturation at 94°C, followed by 34 cycles of 30 sec at 94°C, 30 sec at 60°C, and 60 sec at 72°C, and a fival extension of 5 min at 72°C. Following the PCR, the variant *His* allele was differentiated from the wild type *Arg* allele by the presence of the *BstU*I (New England Biolabs) restriction site in the former, which resulted in 173 bp and 27 bp digestion products.

2.6.6 *XPC, XPD*, and *XPG*

The genetic polymorphisms in *XPC* (exon 15), *XPD* (exon 23), and *XPG* (exon 15) were determined as had been described earlier (Hemminki *et al*., 2001; Kumar *et al*., 2003; Khan *et al*., 2002).

2.7 Statistical analyses

Prior to statistical analyses, the variables were tested for normality by Kolmogorov-Smirnof test, normal probability plot and skewness, and kurtosis of the distribution. Outlying measurements were searched for, *e.g*., by leverage in a normal probability plot (Papers I, and II). Student's t-test and Mann-Whitney's U test (Papers I, II, and IV) were used to compare the means of the dependent variables. All statistical tests were two-sided. SPSS for Windows 9.0 and 10.1.7 (SPSS Inc., Chicago, USA) computer programs were used in the statistical analyses (Papers I and II).

The bleomycin-induced break frequency ratio was calculated by exponentiation (e^b) of the parameter estimate from a linear regression model (Paper I). The ratio compares the geometric mean break frequency in an exposed group with that in a referent (unexposed) group, and can be thought of as the adjusted proportional change in the geometric mean frequency of bleomycin-induced breaks between the two comparison groups. All final regression models of the mean break frequency were evaluated for model fit using regression diagnostics including graphical display of studentized residuals.

For Paper II, the adjusted SCE frequency was calculated by linear regression as described in Paper I. Because CA frequencies are neither linear nor continuous, but do not significantly deviate from the Poisson distribution, a log-linear Poisson regression (Bonassi *et al.*, 1994) was used for calculating the adjusted CA frequencies. Before regression analysis, the CA frequencies of the Finnish and Hungarian study populations were centered around the same positive integer. After centering, the populations were combined for succeeding statistical analysis. For Poisson regression, the relative risks (RIs) and their 95% confidence intervals were calculated using R 1.5.0 (http://www.r-project.org).

ORs and their 95% CIs were calculated by unconditional logistic regression (Paper III), with sex, age, daily consumption of tobacco, duration of smoking, and daily consumption of alcohol as confounding factors. The analyses were performed using STATA package 7.0 (StataCorp LP, College Station, USA). All statistical tests were two-sided.

Kruskall-Wallis test was employed to test for an association between biomarkers and the genotypes (Paper IV). The differences between group means were tested

for statistical significance by Mann-Whitney's U test. Certain confounders (age, sex, and smoking, *etc.*) were also controlled for by the Kruskall-Wallis models. For an evaluation of the modulating role of genetic polymorphisms on the levels of genotoxicity biomarkers, the statistical tests were performed on the whole cohort in order to increase the number of subjects, as suggested by Hemminki and Försti (2002). The statistical calculations were performed using Statgraphics 7.0 (Manugistics, In., Cambridge, USA).

The allele frequencies were tested for Hardy-Weinberg equilibrium by χ^2-test. Yates' correction for continuity was applied when appropriate.

2.8. Bioinformatics

Several sequence databases, computer programs, and search tools were utilized in this study. For example, PCR primers were hand-designed using complete gene sequences retrieved from EMBL database (http://srs.ebi.ac.uk). The binding sites of the designed PCR primers were verified by BLAST-searches against human sequences in NR-database (http://www.ncbi.nlm.nih.gov/blast).

3. Results

3.1 Bleomycin sensitivity studies (Paper I)

The effect of metabolic (*GSTM1, GSTT1, NAT2*, and *BLHX*) and DNA repair (*XRCC1* and *XRCC3*) gene polymorphisms on individual bleomycin sensitivity (mean b/c) was investigated among 80 healthy Caucasian controls. The allele frequencies of all the genes were in Hardy-Weinberg equilibrium, and did not significantly deviate from the frequencies reported earlier for Caucasian populations.

Multivariate linear regression model was fit to the data taking into account some variables relevant for the hypothesis (smoking, age, and sex) and the genotypes. Separate regression models were constructed for the metabolic and DNA repair genes. As only a few individuals were homozygous for the variant alleles of *XRCC1*, *XRCC3* or *BLHX*, they were combined with the heterozygous variant allele carriers for the statistical analyses in order to increase the power of the study. Based on their *NAT2* genotype, the individuals were classified as slow or rapid acetylators. The acetylator status was used as one variable in the regression models.

We compared regression models with and without one influential observation (b/c=1.91). After the removal of this influential observation, the b/c frequency distribution was normalized (skewness 0.22, kurtosis –0.32, Kolmogorov Smirnov 0.067, and p-value 0.2) which is an obligatory requirement for the statistical model used. However, the parameter estimates of both of the regression models were not found to differ materially, and therefore the results for all the 80 subjects are presented.

When bleomycin sensitivity studies are performed, individuals are normally classified as insensitive, sensitive, and hypersensitive, on the basis of the mean b/c frequency (Hsu *et al*., 1989). The selection of an appropriate cut-off point for sensitive and hypersensitive individuals on the basis of the mean b/c frequency is critical for the interpretation and comparitivity of the results. Usually, cut-off points of 0.8 (50^{th} percentile of b/c in controls) and 1.0 (75^{th} percentile of b/c in controls) are used for sensitive and hypersensitive individuals, respectively. We used the same cut-off points, because the real 50^{th} and 75^{th} percentile calculated from our study group were almost the same (0.79 and 0.99, respectively).

3.1.1 Metabolic gene polymorphisms and bleomycin sensitivity

Neither age, sex, smoking status, nor the *GSTM1*, *GSTT1* or *NAT2* genotypes were related to bleomycin sensitivity (see Table II in Paper I). Subjects with the *BLHX* normal allele displayed higher mean levels of bleomycin-induced breaks than variant allele carriers (0.89 versus 0.80, p=0.061, two-sided Mann-Whitney's test). When

the subjects were further stratified, the effect of the *BLHX* genotype appeared to concern only smokers (0.91 versus 0.78, p=0.036, two-sided Mann-Whitney's test). No effect was seen for non-smoking subjects.

3.1.2 DNA repair gene polymorphisms and bleomycin sensitivity

The statistical models were run twice, and all the *XRCC1* polymorphisms were taken into account independently or as genotype combinations. The results for the two models did not differ materially, and the results from the model with independent polymorphisms are presented.

Age, sex, smoking status, and the *XRCC1* and *XRCC3* genotypes were not related to the bleomycin sensitivity (Table IV). Elevated levels of bleomycin induced breaks were observed in subjects carrying the *XRCC1* codon 280 variant allele (1.01 versus 0.81, p=0.002, two-sided Mann-Whitney's test). When the effect of the *XRCC1* codon 280 polymorphism was adjusted for the age, sex, smoking, and the other genotypes, the finding was of borderline significance in the regression model (RR=1.18, 95% CI=0.98–1.41, see Table I in Paper I).

It is also possible that smoking interacts with the *XRCC1* codon 280 genotype. Smokers with the *XRCC1* ^{280}*Arg* allele had significantly lower levels of chromatid breaks in their cultured lymphocytes than the variant allele carriers who did not smoke (0.79 b/c versus 1.15 b/c, p=0.001, two-sided Mann-Whitney's test). However, the effect was only of borderline significance in the linear regression model (RR=0.68, 95% CI=0.52–1.01).

3.2 DNA repair gene polymorphisms and cytogenetic damage (Paper II)

The effect of genetic polymorphisms of *XRCC1* and *XRCC3* on individual spontaneous CA frequency was investigated among 84 and 61 healthy Hungarian and Finnish controls, respectively. The subjects were pooled for the multiple regression analyses.

Age, gender, and smoking were the most important non-genotype related variables affecting the CA frequency in both populations. Age and smoking influenced mainly the frequency of gaps and chromatid type aberrations. The frequency of SCEs was higher in women than in men.

We controlled for the genotypes of *GSTM1, GSTT1* and *NAT2*, because they might affect smoking-induced (*GSTM1)* or baseline frequency (*GSTT1, NAT2*) of SCEs or CAs. These genotypes were, however, mostly unrelated to the baseline frequency of SCEs (see Table I in Paper II), although the SCE frequency was slightly (9%) in-

creased in the *GSTT1* null individuals. The *GSTT1* and *NAT2* genotypes affected the frequency of chromosome breaks (see Tables IV and V in Paper II).

The *XRCC1* and *XRCC3* genotypes did not affect the mean frequency of SCEs (Tables I and II in Paper II). Individuals with the *XRCC1* ^{194}*Trp* or ^{280}*His* alleles had about 20% less chromatid breaks than the respective homozygous wild type allele carriers (Table III in Paper II). A similar effect was also seen for chromosome breaks, but the variant allele carriers had 3–5 times less breaks than the carriers of the wild type allele (Table III in Paper II). After multiple Poisson regression adjustment for age, sex, smoking, and genotype, the effects of the *XRCC1* genotypes became significant for chromosome breaks only (see Table IV in Paper II). Subjects heterozygous for *XRCC3* codon 241 variant allele showed about 40% higher frequency of chromatid-type breaks than wild-type or variant homozygotes, and a statistically significant effect was obtained in the combined data (Tables III and IV in Paper II).

To further characterize the effect of smoking on CA frequencies, the subjects were divided into smokers and non-smokers. The *GSTM1* null allele was associated with a statistically significant decrease in the chromatid break frequency in non-smokers, but not in smokers, where it was linked to the increased number of chromatid breaks instead. Similarly, the *NAT2* slow acetylator genotype decreased the frequency of chromosome breaks in non-smokers.

The *XRCC1* ^{280}*His* allele decreased the number of chromosome breaks in both smokers and non-smokers, but the effect reached statistical significance only in smokers. *XRCC1* codon 399 variant allele was statistically significantly associated with a decreased number of chromatid gaps in non-smokers only.

3.3 DNA repair genes and risk of upper aerodigestive tract cancers (Paper III and unpublished data)

The association between risk of upper aerodigestive track cancers and the *XRCC2* and *XRCC3* polymorphisms was studied in a French population. There were 165 controls, 119 oral cavity or pharyngeal cancer patients, and 127 laryngeal cancer patients available for the study.

The *XRCC2 Arg*^{188}*His* and *XRCC3 Thr*^{241}*Met* genotype distributions (Table VII) were in Hardy-Weinberg equilibrium (p=0.88 and p=0.28, respectively). The frequency of the *XRCC2* ^{188}*His* allele (0.07) was comparable to the frequencies reported earlier (Kuschel *et al.*, 2002; Rafii *et al.*, 2002). The *XRCC3* ^{241}*Met* allele frequency (0.45) was similar to the frequency observed in an Italian population (0.42) (Matullo *et al.*, 2002), but higher than seen in a US (0.38) (David-Beabes *et al.*, 2001) or UK (0.36) (Kuschel *et al.*, 2002) population.

The *XRCC2 His* allele containing genotypes were associated with a significantly increased risk of pharyngeal cancer (OR=2.9, 95% CI=1.3–6.2) (Table 2 in article III), whereas the *XRCC2* polymorphism did not modify the risk for oral cavity or laryngeal cancers. Neither was any interaction found between alcohol consumption, smoking and the *XRCC2* genotype.

A reduced risk of supraglottic cancers was found in the carriers of *XRCC3* ^{241}*Met* allele (OR=0.1, 95% CI=0.2–0.8) (Table 2 in article III). Moreover, there was a significant interaction between smoking history and *XRCC3* genotype; individuals with the *XRCC3 Met* allele containing genotypes and a history of less than 30 years of smoking had a reduced risk of oral or pharynx cancers (OR=0.3, 95% CI=0.1-0.8). This effect was not seen in those with a history of more than 30 years of smoking (OR=1.0, 95% CI=0.5-2.1).

The relationships between cancers of the upper aerodigestive tract and the *XRCC2* ^{188} *His* allele containing genotypes were not modified by the *XRCC3* genotypes.

3.4 Styrene-exposed lamination workers (Paper IV and unpublished data)

Lamination workers had been exposed to rather high concentrations of styrene in air, determined by personal dosimeters (81.3 mg/m^3, 2*SD=56.3 mg/m^3, range=4-223 mg/m^3). Finnish occupational hygienic (HTP) value for an 8-hour exposure is 86 mg/m^3. Clearly, the HTP value was grossly exceeded in some workers, probably because of their individual tasks, while some workers were only lightly exposed to styrene during the measurements. Styrene concentration in blood, a marker of internal exposure, was on the average 0.56 mg/l (2*SD=0.43 mg/l) in the exposed group and 0.07 mg/l (2*SD=0.06 mg/l) in the controls (includes some maintenance workers from the styrene lamination plants). The styrene concentrations in the air and blood correlated significantly ($r=0.759$, $p<0.001$) with each other.

The mean frequency of CAs was the highest among the unexposed (external) controls (3.2, 2*SD=2.0) and the lowest in the exposed (plant) controls (1.7, 2*SD=0.9) (see Table II in Paper IV). Furthermore, there was no tendency for the more heavily exposed individuals to display elevated frequencies of CAs, nor did the frequency of CAs show significant correlation with exposure to styrene (styrene in air or blood). However, the frequency of CAs correlated positively, but not very strongly, with age ($R=0.215$, $P=0.018$). In non-smokers, the frequency of chromosome breaks was positively correlated with the leukocyte capacity to incise 8-oxoguanine in DNA ($R=0.388$, $P=0.005$).

The mean level of alkylation-induced single-strand breaks in the DNA was found to be significantly lower in the exposed group (0.29/10^9 daltons) than in the con-

trols ($0.57/10^9$ daltons, $p<0.001$). In addition, the frequency of SSBs correlated inversely with the concentration of styrene in the air or blood. In contrast, the level of abasic sites and oxopyrimidines did not differ between the exposed and unexposed subjects (0.12 and 0.16 single-strand breaks per 10^9 daltons, respectively), and did not significantly correlate with the styrene concentration in the air or blood.

Irradiation-specific DNA repair capacity, which is thought to reflect BER, was significantly higher in the exposed group than in the unexposed group (1.1 and 0.7 single-strand breaks per 10^9 daltons, respectively, p=0.002). The repair capacity correlated significantly with the styrene concentration in the air (r=0.337, p=0.006) and blood. The 8-oxoguanine-specific DNA repair capacity correlated positively with the styrene concentration in the air (r=0.375, p=0.001) and blood (r=0.311, p=0.019).

The following genotype results were removed from the published article, as requested by two anonymous referees. Therefore, the data is presented here. Smokers with the *XRCC1* ^{399}*Gln* variant allele displayed a higher frequency of CAs than carriers of the normal allele (r=-0.359, p=0.021; F=3.2, p=0.052). Non-smokers with the *XPD* codon 23 variant allele had higher frequencies of chromatid aberrations than those non-smokers who had the with wild-type allele (r=0.255, p=0.043; F=2.8, p=0.070).

The level of single-strand breaks was lower in individuals homozygous for the wild-type alleles of *XPC* exon 15 or *XRCC1* codon 399 (r=0.217, p=0.055 and r=0.251, p=0.034, respectively) (Figure IV). After stratification by smoking, the effect of *XPC* became significant only in smokers (r=0.342, p=0.016) and the effect of *XRCC1* only in nonsmokers (r=-0.483, p=0.029). The level of abasic sites and 8-oxopyrimidines was modulated by *XPG* (F=5.3, P=0.009) and *XPC* (F=4.0, P=0.025) genotypes.

The level of irradiation-specific DNA repair was significantly decreased in homozygous carriers of the *XRCC1* codon 399 variant allele and *XPG* exon 15 wild-type allele (Figure IV). The highest DNA repair capacity was found in individuals with the homozygous wild-type genotype and the lowest in individuals homozygous for the ^{399}*Gln* allele (R=-0.433, P=0.001). When the subjects were stratified by smoking, irradiation-specific DNA repair was even more clearly associated with the *XRCC1* codon 399 genotype in non-smokers (r=-0.525, p=0.001; F=5.9, p=0.007), and also showed an association with *XPD* (r=-0.400, p=0.012; F=3.5, p=0.040) and *XPG* (r=0.349, p=0.050; F=2.9, p=0.071) polymorphisms.

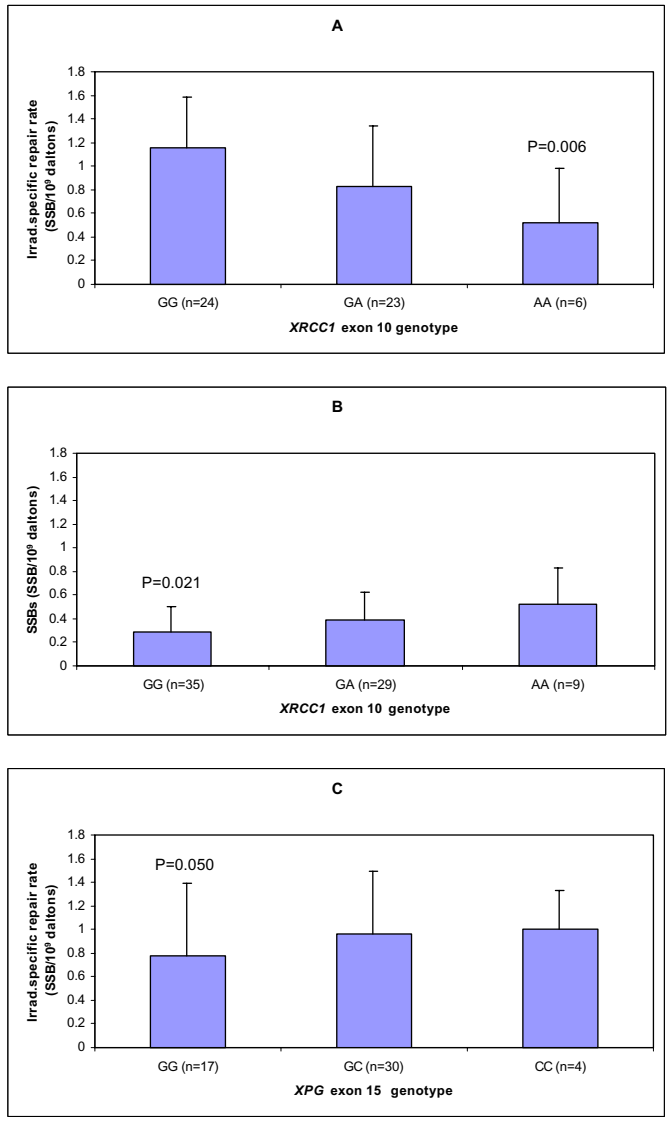

Figure IV. A: Mean values±SD of irradiation-specific DNA repair rates (expressed as SSB/10^9 daltons) stratified by *XRCC1* exon 10 genotype. B: Mean values±SD of SSBs (expressed as SSB/10^9 daltons) stratified by *XRCC1* exon 10 genotype. C: Mean values±SD of irradiation-specific DNA repair rates (expressed as SSB/10^9 daltons) stratified by *XPG* exon 15 genotype. Groups include both smokers and non-smokers as well as exposed and unexposed individuals.

4. Discussion

4.1 Bleomycin sensitivity studies

Bleomycin sensitivity has usually been used as a phenotypic test for increased cancer susceptibility. It is commonly thought that bleomycin sensitivity is a trait with a strong genetic component with an estimated heritability index of 75% (Cloos *et al*., 1999). On the basis of a test that measures bleomycin-induced breaks per cell (b/c), people are usually classified as nonsensitive, sensitive, or hypersensitive. However, if it were possible to identify the genetic factor(s) responsible for bleomycin sensitivity, easier tests could be constructed to assess the same outcome. One such possibility is to identify the gene polymorphisms that affect the test outcome.

In this thesis work, we studied the effect of some XME (*GSTM1, GSTT1, NAT2,* and *BLHX*) and DNA repair genes (*XRCC1* and *XRCC3*) on the test outcome. These genes are possibly involved in the bleomycin metabolism or the repair of bleomycin-induced DNA lesions. This issue has not been previously explored.

It has been hypothesized that the genetic polymorphism of bleomycin hydrolase may account, at least partially, for the observed interindividual variation in bleomycin sensitivity (Caporaso, 1999). Also thought to be important in bleomycin metabolism are *GSTs* and *NAT2*. Our results suggest that the *BLHX* normal genotype, especially combined with cigarette smoking, may contribute to a higher bleomycin-induced break frequency.

The interpretation of the results is not straighforward, since exact data on the *BLHX* genotype/phenotype correlation are not available. Although the variant allele seems to be more efficient in inactivating the bleomycin in cell cultures, the difference is quite small. Bleomycin is a DNA-intercalating chemical, and it can be removed from the DNA only if BLHX can effectively bind to the DNA (Koldamova *et al*., 1998 and Lefterov *et al*., 1998). Because DNA binding is required for effective inactivation of bleomycin by BLHX, the variant allele may increase enzyme activity by allowing a more efficient DNA binding than the normal allele.

We found a probable interaction between cigarette smoking and *BLHX* genotype. The difference in b/c between normal and variant allele carriers was higher in smokers than non-smokers. It may be possible that some constituents of cigarette smoke increase the expression of BLHX in smokers, although this has not been shown.

While the *BLHX* genotype had only a slight effect on bleomycin sensitivity, the *XRCC1* codon 280 polymorphism exhibited a more profound effect. Carriers of *XRCC1* ^{280}*Gln* variant allele displayed a higher b/c frequency than subjects homozygous for the normal allele. If the cut-off point of 0.8 and 1.0 b/c are considered for sensitive and hypersensitive individuals, the *XRCC1* codon 280 polymorphism seems to have a significant effect in predetermining whether the individual is sensitive.

DISCUSSION

Bleomycin-induced damage is not repairable with BER without the glycosylase and endonuclease activity. If the protein produced by the *XRCC1* ^{280}*Gln* allele binds to the DNA more efficiently than the protein produced by the normal allele, it could prevent the efficient binding of glycosylase and endonuclease to the damaged site, and slow down the repair process on bleomycin-induced lesions.

These results suggest that *XRCC1* codon 280 polymorphisms may demonstrate a major gene effect on the bleomycin sensitivity assay. On the basis of these results cigarette smoking and polymorphisms in other genes, such as *BLHX*, seem also to have a modulating role. The *XRCC1* codon 280 polymorphism has rarely been studied in molecular epidemiology settings where DNA repair gene genotypes have been determined. This is probably due to the low frequency of the variant allele. Our results warrant further studies of this polymorphism, because even heterozygocity for the variant allele seems to have profound effects on the level of bleomycin-induced chromosome damage.

Our results disagree with those from a larger study conducted in another ethnic (USA) group (Wang *et al.*, 2003), which reports a nonsignificant association of *XRCC1* codon 194 variant allele and codon 399 normal allele with a decreased number of chromatid breaks. In our study, the *XRCC1* ^{194}*Trp* variant allele was very rare, and no homozygous carriers of this allele were found; this might explain why we did not observe any effect for that particular polymorphism. Unfortunately, Wang *et al.* did not analyze *XRCC1* codon 280 polymorphism, and therefore no consensus can be drawn from these bleomycin sensitivity studies. Clearly, a larger study simultaneously addressing all the relevant polymorphisms is needed.

The drawback of the bleomycin sensitivity assay has been the lack of knowledge about its biological significance. The present results suggest that both metabolism of bleomycin and DNA repair capacity may alter the individual response to bleomycin treatment. DNA repair genotypes seemed to be the predominant factors affecting the test outcome.

Bleomycin sensitivity assay may now be used for cancer risk assessment with a greater certainty, because on the basis of these results it appears to measure the individual capacity of base excision repair. In the future, the mechanistic understanding of the mutagen sensitivity trait would allow a better characterization of environmental cancer risks. In addition, the identification of a set of genes (in addition to *XRCC1*) that provide the same information as the bleomycin sensitivity phenotype may be a significant methodological improvement in identifying mutagen sensitive individuals, because genotype analysis is less laborous to perform than the current bleomycin sensitivity assay based on cell culture and more or less subjective microscopic analysis.

4.2 Genotypes of metabolic and DNA repair genes and cytogenetic damage

Direct DSBs, which can induce both SCEs and CAs, are probably rare events in normal healthy cells. It has been estimated that about 50 DSBs are endogenously formed, mainly from single-strand lesions, during every S-phase of the cell cycle (Vilenchik *et al.*, 2003), and these DSBs seem to be very effectively repaired. However, CAs are present in certain amounts in peripheral blood leukocytes of healthy individuals, and a few SCEs seem to be found in almost all cells. One possible mechanism for their formation in healthy individuals in the absence of direct DSBs is enzymatic induction from SSBs (Vilenchik *et al.*, 2003). Alternatively, environmental exposure to genotoxins (DNA adducts) may induce DSBs during the S-phase of the cell cycle and chromosomal damage.

The people examined in the present study were mostly city dwellers, who were likely to have been exposed to urban air pollution. Thus, in addition to possible smoking, they probably had had some exposure to PAHs and other genotoxins in the air, which may contribute to the observed level of chromosomal damage. Furthermore, the human diet usually also contains a multitude of genotoxins, *e.g.*, heterocyclic amines formed from amino acids by heating. In fact, for all practical purposes, it is impossible to acquire a cohort, which has not been exposed to any genotoxins. Therefore, the current study does not actually assess how the polymorphisms affect the baseline frequency of chromosomal damage, but rather how they modulate the extent of damage in minimally exposed individuals.

Our results indicate that neither the polymorphisms in the studied *XME* genes, with a possible exception of *GSTT1*, nor in the DNA repair genes significantly affect the frequency of SCEs in healthy Caucasians. Furthermore, the present study did not indicate any connection between smoking and the *XRCC1* codon 399 genotype in determining the frequency of SCEs, in contrast to previous findings (Duell *et al.*, 2000 and Lei *et al.*, 2002). One possible explanation for the divergence was the difference in sex distribution and smoking habits between our study population and earlier studies.

The *XRCC1*, *GSTT1*, and *NAT2* polymorphisms seem to affect the level of chromosome breaks in cultured lymphocytes. The association of the *GSTT1* null genotype and *NAT2* slow acetylator genotypes with the elevated levels of chromosome breaks is probably due to the diminished capacity of inactivation metabolism in the cells.

Among *XRCC3 Thr/Met* heterozygotes, the frequency of chromatid breaks was elevated in smokers but chromosome breaks were decreased in nonsmokers. These findings appear difficult to explain at present, although XRCC3 might be present in

trimeric complex of XRCC3, RAD51C and RAD51 (Liu *et al.*, 2002), and show overdominant behavior. Overdominance is a state in which the heterozygote has greater phenotype value and perhaps is more (or less) fit than the homozygous state for either of the alleles that it comprises. Overdominance is best documented for tetrameric proteins, like haemoglobin and alcohol dehydrogenase (Hall *et al.*, 1987), but there is no reason why it could not explain phenotypic effects observed in trimeric protein complexes, like XRCC3-RAD51C-RAD51, also.

Our findings indicate that the *XRCC1* codon 280 variant allele is associated with an increased capacity to repair DNA strand breaks. The *XRCC1* codon 280 is located near the sequences that mediate the protein-protein interactions with poly(ADB-ribose) polymerase (PARP) and DNA polymerase beta (Thompson, 2000). The ^{280}*Gln* variant allele may encode a twisted protein. This protein may have an increased or decreased affinity to other proteins (PARP) or an altered DNA repair capacity (better binding to DNA).

Our results also suggest that the link between chromosome breaks and predisposition to cancer (Hagmar *et al.*, 1998, Liou *et al.*, 1999 and Bonassi *et al.*, 2000) may be in the polymorphic XME and DNA repair genes; they seem to raise the level of cytogenetic damage, and have been associated, *e.g.*, with cancers of the head and neck (Sturgis *et al.*, 1999), lungs (Henning, *et al.*, 2000), and the larynx (Stucker *et al.*, 2002).

The current study extends our understanding about the inherited differences in the xenobiotic metabolism and DNA repair. Whether determination of DNA repair gene genotypes should be routinely incorporated into biomonitoring studies that are based on CA tests, needs to be further studied. It might also be worthwhile to include genotyping into studies of CAs and individual cancer risk.

4.3 DNA repair gene polymorphisms and the risk of upper aerodigestive tract cancers

Only two previous studies have investigated the role of *XRCC2* polymorphisms in cancer proneness (Kuschel *et al.*, 2002; Rafii *et al.*, 2002). In both studies the rare *XRCC2* ^{188}*His* allele was linked to an elevated risk of sporadic breast cancer. The results are consistent with our findings.

The *XRCC2* ^{188}*His* allele seems to have an effect on cell survival after treatment with cross-linking reagents, such as mitomycin C (Rafii *et al.*, 2002). Although this effect appears to be small in general, no major biological defects can be expected for low-penetrance genes. However, it may explain the slight but significant increase in cancer risk.

In the current study, the *XRCC2 Arg*^{188}*His* polymorphism affected the risk of pharyngeal cancers but not the risk of supraglottic cancers. Both cancers share the same, very high risk linked to alcohol and tobacco consumption (Pèquignot *et al.*, 1988). Therefore, it is difficult to understand why the elevated cancer risk should be limited only to supraglottic cancers.

There are at least two plausible explanations for the observation. In smokers, higher levels of aromatic DNA adducts have been characterized in the pharynx than in the larynx (Pabiszczak *et al.*, 2001). Higher levels of bulky DNA adducts might lead to higher levels of DNA double-strand breaks. This might explain why the effect of *XRCC2* is limited to pharyngeal cancers. It is also possible that the type of the DNA adducts differs between anatomical sites.

In contrast to an earlier report (Shen *et al.*, 2002), we found that the *XRCC3* ^{241}*Met* allele is linked to a decreased risk of cancer. The allele frequencies of *XRCC3* and several other genes that were previously investigated using the same population agreed well with the literature. However, we cannot totally exclude bias in the selection of the controls.

The present results provide some evidence to support the idea that polymorphisms in the DNA repair genes that are involved in recombinatorial repair may modulate susceptibility to the cancers of the upper aerodigestive tract.

4.4 Styrene-exposed lamination workers

In contrast to previous studies (Somorovská *et al.*, 1999; Artuso *et al.*, 1995), we did not observe any effect of styrene exposure on CA frequencies. The SSB levels were significantly lower among the exposed workers when compared with the controls, and their level correlated inversely with the parameters reflecting external and internal exposure. This is in striking contrast to several previous studies (Vodička *et al.*, 1995; Somorovská *et al.*, 1999; Brenner *et al.*, 1991; Walles *et al.*, 1993; Vodička *et al.*, 1999), where an exposure-related increase in the frequency of SSBs was found, and where the frequency of SSBs correlated significantly with the frequency of O^6-styrene-guanine DNA adducts (Vodička *et al.*, 1995) and the frequency of CAs (Somorovská *et al.*, 1999).

These discrepancies may be due to the differences between the populations studied. In the previous studies, styrene-exposed lamination workers had been regularly exposed for more than 14 years on average, while the present workers were exposed for four years on average, the majority of these had been employed in a plastics plant for 1–2 years. The duration of exposure and the cumulative exposure to styrene (and, presumably, to any chemical) may lead to a shift in the equilibrium between an induction and inhibition of biotransformation and repair enzymes.

Adaptation processes, as well as differences between long-term and short-term exposures to potentially carcinogenic chemicals, have to be properly addressed in future studies, so we can properly evaluate the genotoxic/carcinogenic risk of these xenobiotics.

In the present study, we employed two tests for the determination of DNA repair capacity in humans. Irradiation-specific DNA repair capacity, attributable to BER, was found to decrease moderately with age. It has previously been reported that the DNA repair capacity correlates negatively with the number of cells with gaps, and thus indicates a protective effect of DNA repair toward chromosome damage (Knudsen *et al.*, 1999). As DNA repair capacity seemed to increase with styrene exposure in our study, it is possible that the DNA repair machinery participating in the excision of the lesions from DNA had been effectively activated. DNA repair capacity (both irradiation- and oxidation-specific) correlated significantly with the concentration of styrene either in the air or blood, while the frequency of single-strand breaks correlated with these parameters inversely. Important questions about the true induction of DNA repair capacity, existence of a treshold in this induction, and the situation in the long-term exposure (exhaustion related to increasing index of cumulative exposure) remains to be studied.

In our study, the *XRCC1* codon 399 variant allele was linked to an increased number of SSBs and a decreased irradiation-specific DNA repair capacity, noticeable particularly in the non-exposed group. Thus, our results add evidence to the above observations that the *XRCC1* codon 399 polymorphism affects the efficiency of BER in human cells (Lunn *et al.*, 1999; Abdel-Rahmann *et al.*, 2002).

Interestingly, the frequency of SSBs, abasic sites, and 8-oxoguanosines were also associated with the *XPC* polymorphism, involved in NER. It has been reported that DNA repair capacity for the removal of UV photoproducts was the lowest in people bearing homozygous *XPC* variant allele genotypes (Qiao *et al.*, 2002). It is possible that the DNA damage in styrene-exposed individuals is, at least partially, repaired via NER. However, as the effect of the *XPC* polymorphism was not restricted to the styrene-exposed workers, the association could be attributed to styrene exposure or to other environmental/lifestyle factors.

In conclusion, the observed genotoxic effects in styrene-exposed workers probably depend on the extent of exposure, duration of exposure (Vodicka *et al.*, 2002), and individual susceptibility (complex combination of various XME and DNA repair gene polymorphisms), as well as adaptational processes.

4.5 Limitations and future perspectives

The recent completion of a draft sequence of the human genome has dramatically increased the number of known human polymorphisms. Availability of genomic

sequence also enables computational identification of previously unknown polymorphisms. At the time the laboratory work for this study was started (and completed), the draft sequence of the human genome was not yet available, and the polymorphisms in the DNA repair genes were mined from published articles. Today, a more comprehensive study of phenotypic consequences of the polymorphisms in human DNA repair genes would be accomplished, if polymorphisms situated in the promoter regions, introns and intron/exon borders were to be included. Such studies would also require large study populations.

Small sample size is the main limitation in current studies, and including more polymorphisms would make this even more pronounced. The diseases and exposures studied in this work are rare in Finland, and recruitment of larger study populations could be challenging. For example, cytogenetic analyses are laborous to perform, and it easily takes a whole day to score CAs for one individual only. Thus, recruitment of study populations large enough for very powerful studies could take years. Fortunately, CAs have already been scored for a number of people in cytogenetic laboratories in Finland and around Europe, but the availability of genetic material for genotyping could limit the usability of this data.

In the current study, PCR-RFLP-based methods were used for genotyping the individuals. This method is thought to be quite reliable, because it combines the specificity of PCR with the specificity of the enzymatic DNA restriction reaction. Unfortunately, the method is laborous, and not very fast to perform. Therefore, the genotyping methods developed in this study were carefully optimized: In order to speed up the analyses, the PCR programs were modified by removing the first denaturing step and the last elongation step. In addition, primers were optimized so that both the annealing and elongation steps could be performed at the same temperature. The amount of work was minimized by developing multiplex PCR reactions. These improvements made genotyping much faster. With the current protocols, genotypes of *XRCC1* and *XRCC3* can be determined from about 400 individuals in a day. With robotics this could be speeded up even more, although even faster and more easily automatized methods, such as PCR combined with denaturing high performance liquid chromatography (HPLC), could be used.

The statistical methods used in this study were selected on the basis that they are widely used standard methods. Multiple linear and Poisson regression are used, when the dependent variable is either normally (SCEs) or Poisson distributed (CAs). Logistic regression is usually used, when the effect of a genotype in combination with environment on the binomial disease outcome is analyzed. It would also be possible to use linkage disequilibrium (LD) in such cases, but construction of the disease model, especially when many environmental factors are analyzed, could be tricky. There are also non-parametric alternatives to most of the mentioned statistical methods.

This study furthers current knowledge about the possible cellular mechanisms involved in the formation of DNA and cytogenetic damage. The studied DNA repair genes seem to influence the level of cytogenetic damage, and they might also explain the observed link between chromosomal aberrations and increased cancer risk. Keeping in mind the small sample size of this study, it is possible that some potential effects of the DNA repair gene polymorphisms on the studied endpoints have been missed. Thus, current findings need to be verified in larger studies. This study also leaves some important questions unanswered. It might be interesting to see whether DNA repair polymorphisms could actually explain the observed link between the high frequency of chromosome aberrations and elevated cancer risk. Furthermore, the molecular mechanisms of chromosome aberration formation might be studied in more detail in controlled *in vitro* studies.

In conclusion, it is obvious that an individual's genotype for a number of detoxifying enzymes is likely to determine the individual's response to carcinogenic compounds. In the future, large homogeneous study populations are therefore needed to allow proper analysis of the combined impact of various relevant genes for a given exposure.

III General conclusions

The effects of polymorphisms in DNA repair genes *XRCC1*, *XRCC2*, *XRCC3*, *XPC*, *XPD*, and *XPG* on mutagen sensitivity, frequency of CAs, risk of head and neck cancers, and genotoxicity biomarkers in exposure to styrene were studied in four Caucasian populations. The work included genotyping for *XME* (*GSTM1*, *GSTT1*, and *NAT2*) and DNA repair gene polymorphisms as well as cytogenetic and statistical analyses. The results showed that:

1. Mutagen (bleomycin) sensitivity was partly determined by bleomycin hydrolase genotype in smokers, and *XRCC1* codon 280 polymorphism. The *XRCC1* codon 280 polymorphism also affected the sensitivity classification of the subjects.
2. The wildtype alleles of *XRCC1* codons 194 and 280 were linked with an elevated level of chromosome breaks in healthy, minimally exposed individuals. The *GSTT1* and *NAT2* genotypes were also found to affect the number of chromosome breaks in the healthy individuals. Heterozygosity for *XRCC3* polymorphism was associated with elevated level of chromatid breaks.
3. SCE frequencies were not statistically significantly modulated by any of the studied polymorphisms.
4. The *XRCC2* ^{188}*His* allele was associated with an increased risk of pharyngeal cancer. A reduced risk of supraglottic cancers was found for the carriers of the *XRCC3* variant allele.
5. The *XRCC1* codon 399 polymorphism was associated with an elevated level of SSBs in styrene exposed workers. The *XRCC1* codon 399 polymorphisms also affected the number of SSBs in cultured human lymphocytes after irradiation treatment.

Effective repair of DNA lesions is essential for cell viability. As demonstated here, the altered metabolism of xenobiotics and the reduced DNA repair capacity may express themself as an increased number of DNA strand breaks and CAs or as a higher cancer risk. It is also plausible that XME and DNA repair gene polymorphisms modify the relationship between the frequency of CAs and the risk of cancer.

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ERRATA

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The following page (page 2) was accidentally omitted from the book:

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The reference to the source of the following text on page 13 was accidentally omitted. The following chapters should read as:

CAs, as viewed through a microscope on a metaphase preparation, are end products of complex cellular systems. Metaphase chromosomes can be produced by colchicin (or its analogue Colcemid) arrest during cell division of cycling cells; CAs are normally scored from non-banded ("solid"), stained metaphase preparations of lymphocytes stimulated to divide by a mitogen. Radiation-induced damage can be observed as chromosome- or chromatid-type aberrations, depending on whether the cell resided in G0/G1 or G2 phase of the cell cycle, respectively, at the time of irradiation. (A slightly modified excerpt from Lindholm, 2000)

According to the most cited theory, breakage-and-reunion, misrepair of illegitimate rejoining of break-ends produces structural aberrations between two different chromosomes (interchanges) or within one chromosome (intrachanges) (Savage, 1975). In the case of an asymmetrical interchange, the centromeres of both chromosomes are located on the same piece, and an accompanying acentric fragment is present. This dicentric chromosome is easily recognizable after conventional staining. Reciprocal translocation involves mutual exchange of chromatin pieces, and only large rearrangements can be detected in solid stained preparations. If two breaks are located within one chromosome, one on each side of centromere, an easily observable ring chromosome can be produced. Pericentric inversion formed from the same lesion cannot usually be distinguished by solid staining. In cases where breaks remain dissociated, terminal or interstitial deletions are formed. The terminal deletions can be detected as acentric fragments, whereas interstitial deletions appear as either small double minutes or acentric rings. (A slightly modified excerpt from Lindholm, 2000)

The radiation- or chemical-induced CAs can be classified as chromatid-type or chromosome-type aberrations (Savage, 1975). Deletions are seen as chromatid- and chromosome-type breaks (Figure -II), the former affecting one chromatid and the latter both sister chromatids at the same site. Dicentrics, ring chromosomes and double minutes are classified as chromosome-type aberrations, whereas chromatid-type aberrations include symmetrical and asymmetrical exchanges within a chromosome (intrachanges) and between two or more chromosomes (interchanges). (A slightly modified excerpt from Lindholm, 2000)

Lindholm, C. (2000) Stable chromosome aberrations in the reconstruction of radiation doses, doctoral dissertation, STUK A176.

The following errors were found after printing:

Page 6, lines 12-15

The *XRCC1* codon 280 variant allele was associated with elevated number of chromosome breaks induced by bleomycin *in vitro* (mutagen sensitivity), and with a higher frequency of chromosome breaks in peripheral blood lymphocytes of individuals with low exposure levels.

Should be:

The *XRCC1* codon 280 variant allele was associated with elevated number of chromosome breaks induced by bleomycin *in vitro* (mutagen sensitivity), and with a lower frequency of chromosome breaks in peripheral blood lymphocytes of individuals with low exposure levels.

Page 52, paragraph 3, lines 5-6:

Similarly, the *NAT2* slow acetylator genotype decreased the frequency of chromosome breaks in non-smokers.

Should be:

Similarly, the *NAT2* slow acetylator genotype increased the frequency of chromosome breaks in non-smokers.

Page 59, paragraph 2, lines 4-7:

The ^{280}*Gln* variant allele may encode a twisted protein. This protein may have an increased or decreased affinity to other proteins (PARP) or an altered DNA repair capacity (better binding to DNA).

Should be:

The ^{280}*His* variant allele may encode a twisted protein. This protein may have an increased or decreased affinity to other proteins (PARP) or an altered DNA repair capacity (better binding to DNA).