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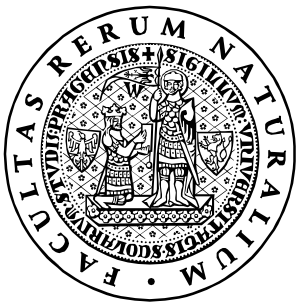

MSc. Jana Slyšková

The application of functional tests to measure DNA repair capacity in molecular epidemiological studies

Uplatnenie funkčných testov na meranie DNA reparačnej kapacity v molekulárne epidemiologických štúdiách

Dissertation Thesis

Supervisor: MD. Pavel Vodička, PhD.
Institute of Experimental Medicine, AS CR, v.v.i.

Prague, 2012

Personal statement

I have prepared this dissertation thesis by myself, referring to all cited and used literature sources. I claim that this work has not been used for any other purposes.

Prague,

Jana Slyšková

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Contents

List of Abbreviations

AP	apurinic and apyrimidinic site / abasic site
BER	base excision repair
BER-DRC	base excision repair capacity
BPDE	benzo[a]pyrene 7,8-diol 9,10-epoxide
CRC	colorectal cancer
Cq	quantification cycle
DDR	DNA damage response
DRC	DNA repair capacity
DSBR	double strand break repair
Endo V	T4 endonuclease V
Fpg	formamidopyrimidine DNA glycosylase
GGR	global genome repair
IARC	International Agency for Research on Cancer
MAF	minor allele frequency
MMR	mismatch repair
NER	nucleotide excision repair
NER-DRC	nucleotide excision repair capacity
OGG1	8-oxoguanine DNA glycosylase 1
Ox-DRC	oxidative repair capacity
PBMC	peripheral blood mononuclear cell
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PolyPhen	polymorphism phenotyping
RT-qPCR	real time-quantitative PCR
SBs	strand breaks
SB-DRC	strand break repair capacity
SIFT	sorting intolerant from tolerant
SNP	single nucleotide polymorphism
TCR	transcription-coupled repair
UV	ultraviolet light
XP	xeroderma pigmentosum
8-oxoG	7,8-dihydro-8-oxoguanine

Abstract

DNA repair is a vital process of a living organism. Inherited or acquired defects in DNA repair systems and cellular surveillance mechanisms are expected to be important, if not crucial factors in the development of human cancers. DNA repair is a multigene and multifactorial process which is most comprehensively characterized by the phenotypic evaluation of DNA repair capacity (DRC). DRC represents a complex marker with high informative value, as it comprises all genetic, epigenetic and non-genetic factors, by which it is modulated. Accordingly, DRC reflects the actual capability of the cell, tissue or organism to protect its DNA integrity.

The present PhD study was focused on investigating DRC, which specifically involves base and nucleotide excision repair pathways, in human populations with different characteristics. The main aim was to answer substantial questions on the possible use of DRC as biomarkers in epidemiological studies. The study was in fact designed to understand the extent of physiological variability of DRC in a population, its modulation by genetic and non-genetic factors, tentative adaptability to high genotoxic stress and, finally, its involvement in cancer aetiology. In order to explore these issues, DRC, in respect to genetic and environmental variability, was investigated in healthy subjects as well as in individuals with higher requirement for DNA stability maintenance, i.e. workers occupationally exposed to carcinogens, and in newly diagnosed cancer patients. Additionally, from a methodological aspect, the study was also aimed to ameliorate the comet-based repair assays for their wider applicability in human epidemiological studies.

The major outcomes of the PhD study, which are fully reported in the five publications included in the present Thesis, are: 1) The demonstration of feasibility to study phenotypically DRC in large-scale epidemiological studies on different types and quality of biological material, 2) Evidence that the marker of DRC provides fundamental information that cannot be obtained by single gene or single transcript analysis, 3) The observation of substantial biological variability in the DNA repair processes among healthy individuals which is modulated by the genetic variability in DNA repair genes and by the inter-sexual differences and lifestyle factors, 4) Lack of alteration of DRC by means of potential adaptive response to chronic exposure to high doses of carcinogens, 5) Proof of suboptimal activity of DNA repair and high level of DNA damage in cancer

patients, showing the significance of DRC in the individual susceptibility to cancer, 6) An upgrade of comet repair assay by its optimization for DRC measurement in human solid tissues with 6-times higher yield number of samples per analysis, which was finally followed by 7) The achievement of a first study on DRC in cancer-target tissues and tumors.

Abstrakt

DNA opravy sú životne dôležitým procesom živých organizmov. Zárodočné alebo somatické defekty DNA opráv sú pravdepodobným, ak nie nevyhnutným javom podmieňujúcim rozvoj nádorových ochorení. DNA opravy, ako multigénne a multifaktoriálne procesy, je možné fenotypovo charakterizovať pomocou komplexného parametru DNA reparačnej kapacity (DRC). DRC reprezentuje marker s vysokou informačnou hodnotou, pretože v sebe zahŕňa všetky genetické, epigenetické a negenetické faktory, ktoré úroveň DNA opráv modulujú. DRC tak reflektuje aktuálnu kapacitu bunky, tkaniva alebo celého organizmu chrániť integritu svojej DNA.

Táto doktorská práca bola zameraná na výskum DRC bázovej a nukleotidovej excíznej dráhy v rôznych typoch ľudských populácií s odlišnými charakteristikami. Cieľom práce bolo reflektovať niektoré podstatné otázky týkajúce sa využitia DRC v ľudských epidemiologických štúdiách a tým zistiť úroveň variability DNA opráv u zdravej populácie, odhaliť genetické a negenetické faktory, ktoré túto variabilitu podmieňujú, zistiť či dochádza k adaptačnej zmene DRC v reakcii na vysokú genotoxickú záťaž a, napokon, odhaliť vzťah medzi individuálnou hladinou DRC a rizikom vzniku nádorových ochorení. Za účelom naplnenia stanovených cieľov, DRC, s ohľadom na individuálnu genetickú a environmentálnu variabilitu jedincov, bola vyšetrovaná u zdravých ľudí, u pracovníkov chronicky exponovaných karcinogénom a u pacientov s novodiagnostikovaným sporadickým karcinómom. Z metodického hľadiska mala práca ďalší cieľ, ktorým bolo modifikovať a optimalizovať metódu kométového testu na meranie DRC v pevných tkanivách a pre jej širšie využitie vo veľkých epidemiologických štúdiách.

Hlavnými prínosmi doktorskej práce, ktorá je postavená na piatich publikáciách, sú novozískané poznatky o biologickej podstate DRC a rozšírenie metodických postupov jej stanovovania. Najdôležitejšími výstupmi sú: 1) Demonštrácia možnosti fenotypovo analyzovať DNA opravy aj vo veľkých epidemiologických štúdiách a na rôznom type a kvalite biologického materiálu, 2) Dôkaz o tom, že analýza DRC poskytuje komplexnú informáciu o aktivite DNA opráv, ktorá nie je v rovnakej komplexnosti detekovateľná analýzou jedného génu či transkriptu, 3) Zistenie významnej biologickej variability v

DRC u zdravých ľudí, ktorá je ovplyvnená genetickou variabilitou v DNA reparačných génoch, intersexuálnymi rozdielmi a faktormi životného štýlu, 4) Pozorovanie nevýznamného rozdielu v DRC u ľudí chronicky exponovaných karcinogénom v porovnaní s kontrolnou populáciou, 5) Odhalenie nízkej opravnej aktivity a vysokého DNA poškodenia u pacientov s rakovinou, poukazujúceho na význam DRC v individuálnej vnímavosti na nádorové ochorenia a napokon 6) Aktualizácia kométového testu na meranie DRC v pevných tkanivách s 6-násobne vyššou výťažnosťou počtu vzoriek na analýzu nasledovaná 7) Vypracovaním prvej štúdie pojednávajúcej o DRC priamo v nádorovom tkanive.

List of Manuscripts

This Thesis consists of an overview of the studies I have been involved in during my PhD and which were published between 2007 and 2012. I have included all publications in which I am the first author (**Manuscript I**, **III, IV** and **V**), and one study on which I have participated as a co-author (**Manuscript II**). **Manuscripts I-V** comprises studies evaluating DNA repair capacity (in particular, focused on base and nucleotide excision repair pathways) as a part of molecular epidemiological studies on different human populations.

Manuscript I	DNA damage and nucleotide excision repair capacity in healthy individuals. Slyskova J et al. (2011) *Environmental Molecular Mutagenesis* 27: 225-32.
Manuscript II	Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. Vodicka P et al. (2007) *Carcinogenesis* 28: 657-64.
Manuscript III	Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers. Slyskova J et al. (2007) *Mutation Research* 634: 101-11.
Manuscript IV	Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls. Slyskova J et al. (2012) *Mutagenesis* 27: 225-32.
Manuscript V	Functional, genetic and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas. Slyskova J et al. *accepted for publication in Clinical Cancer Research on 5.9.2012*

1. Introduction

1.1. DNA damage and its repair

The human organism is constantly exposed to a wide range of agents that are able to bind to DNA and disrupt its structure and/or function. The genome of each cell is attacked by reactive cellular metabolites and by a spectrum of mutagens from the environment that impair its integrity. Generated DNA lesions can block genome replication and transcription and, if they are not eliminated from DNA, they lead to mutations or aberrations that threaten viability of cell. DNA, in contrast to other biomolecules, cannot be replaced, it can only be repaired. Therefore, cells have evolved a variety of DNA repair mechanisms with a broad substrate specificity to maintain the stability of the genome (**Figure 1**).

For convenience, DNA damage can be arbitrarily divided according the source of genotoxic agents into two major classes, endogenous and environmental (or exogenous). The former category includes mainly hydrolytic and oxidative reactions that arise as a consequence of life surrounded by water and reactive oxygen species. The latter class includes a variety of DNA lesions induced by physical and chemical agents generated outside the cells. All components of a DNA molecule are vulnerable to harmful alterations: nitrogenous bases (e.g. to DNA adducts, crosslinks), sugar groups and phosphodiester linkages (e.g. to DNA breaks). However, the bases are the most reactive components and there is considerable knowledge about their modifications.

Endogenous DNA damage

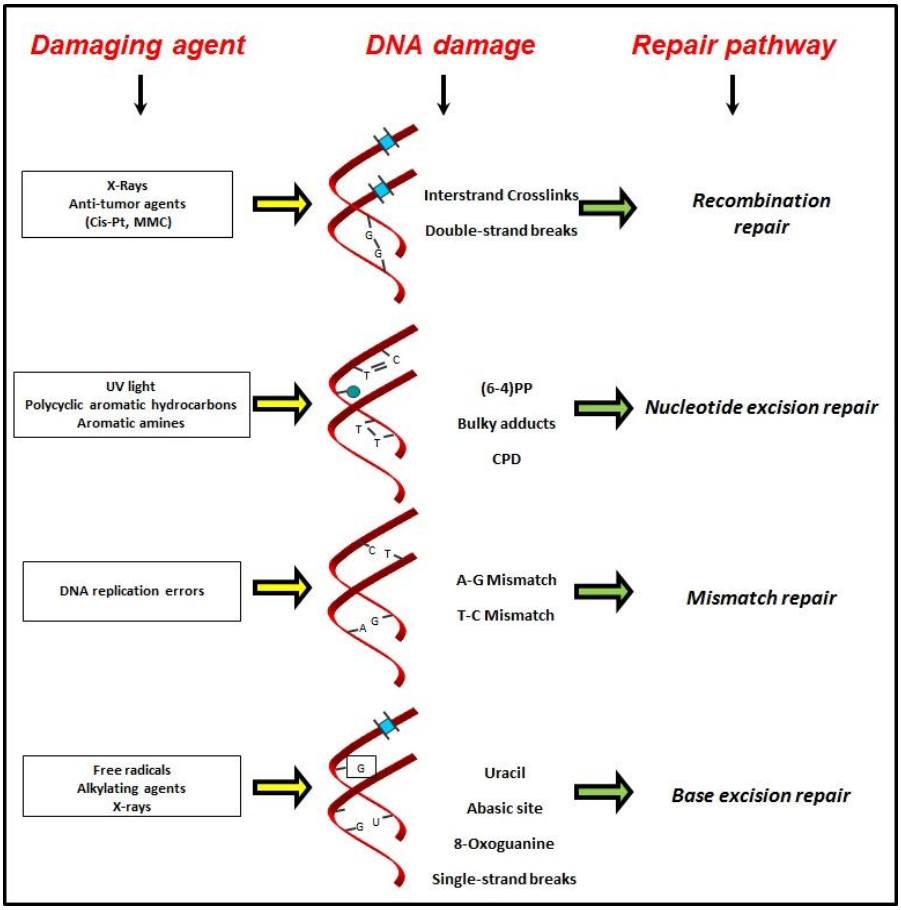
Hydrolyses (depurinations, depyrimidinations, deaminations), oxidations and methylations are daily occurring chemical modifications of stable DNA structure. Some chemical bonds in DNA tend to disintegrate spontaneously under physiological conditions or are constantly attacked by the presence of oxygen free radicals, frequent by-products of mitochondrial metabolism. As an illustration, about 18 000 purine and 600 pyrimidine residues are lost in each cell everyday by hydrolysis. Spontaneous transformation of cytosines to uracils by deamination occurs 100 to 500 times per day in each cell. The most frequent base oxidation, which gives rise to 8-oxoguanine production, occurs at 1000 to 2000 guanines per cell each day [1]. Furthermore, DNA

polymerases work in an error-prone manner and, even though they have proofreading activity, there is still some physiological level of DNA damage left after each DNA replication which has been calculated for 1 error per 10^9 bases per one cell cycle [2].

Environmental DNA damage

A further threat to genomic integrity is posed by genotoxins present in the environment. In human populations, except for cases of chronic occupational exposures, the contribution of these exogenous sources to the total DNA damage is typically small in comparison to endogenous sources of DNA damage, although it is not negligible [3]. In this respect, there is evidence that environmental risk factors can trigger or accelerate the onset of most cancers, most likely via damage to DNA.

The natural physical agents from external sources, UV and ionizing radiations, are known factors that assault DNA stability; UV by induction of strand crosslinks, whereas ionizing radiation by generation of DNA strand breaks. Many reactive chemicals, predominantly generated by man-made industrial activities, can alter DNA structure as well. Compounds that can covalently bind to DNA include alkylating agents (methylating and ethylating agents; e.g. methylnitrosourea, dimethyl nitrozamine, methyl chloride, streptozotocin), crosslinking agents (intra and interstrand crosslinking agents; e.g. nitrogen mustard, cisplatin, psoralen), metabolically activated electrophilic reactants (e.g. aromatic amines, nitrosamines, polycyclic aromatic hydrocarbons, aflatoxins). Additionally, several chemicals are able to induce breaks in DNA, like bleomycin, topoisomerase inhibitors, and some bacterial toxins.



Modified from J.H. Hoeijmakers [4]

Figure 1. Overview of various DNA damaging agents, types of DNA lesions induced by them and DNA repair mechanisms involved to restore original DNA structure.

DNA damage response

All the above examples illustrate that the ability of cell to protect its genomic integrity against large variety of DNA disruptions is a vital process of living organisms known as DNA damage response (DDR). DDR can be divided into several distinct, but functionally interwoven, pathways which are defined by the type of DNA lesions that they process. Most DDR pathways encompass a similar set of tightly coordinated processes: the detection of DNA damage, the accumulation of DNA repair factors at the damage site and finally the physical repair of the lesion. To use a simplistic stratification, DNA repair can occur by one of two fundamental mechanisms that involve either the reversal of DNA damage or the excision of the damaged site. The former mechanism has evolved a single enzyme that can repair lesions without affecting surrounding bases and incising the DNA sugar-phosphate backbone with, however, a very narrow substrate range (repair of alkylating damage by alkyltransferases). The latter one removes DNA lesions usually

with the whole base or even with several surrounding nucleotides and resynthesizes the gap copying the information from the intact complementary strand [5]. For a comprehensive overview, a list of all described DDR pathways is given in **Table 1**. Nevertheless, the present Thesis will particularly focus on base and nucleotide excision repairs, as these were pathways of interest within the experimental part of the doctoral work and which are described in more details in the following chapters.

Table 1. Classification of major cell response mechanisms dealing with the whole spectra of DNA damage. Recent reviews on the topic are reported in squared brackets.

Biological responses to DNA damage
Reversal of base damage [6]
Excision of damaged, mispaired, or incorrect bases
Base excision repair (BER) [7]
Nucleotide excision repair (NER) [8]
Mismatch repair (MMR) [9]
Double-strand break repair (DSBR) [10]
Homologous recombination
Nonhomologous end joining
Tolerance of base damage
Translesion DNA synthesis
Postreplicative gap filling
Replication fork progression
Cell cycle checkpoint activation [11]
Apoptosis [12]

Modified from E.C Friedberg et al. [3]

The core DDR machinery does not work alone but is coordinated with a set of complementary mechanisms that are also crucial for maintaining the integrity of the genome. For example, chromatin-remodeling proteins allow the DNA repair apparatus to gain access to the damage site [13]. DDR components also interact with cell cycle checkpoints and chromosome segregation machinery. The cascade of ATM/ATR - p53/Chk1/Chk2 - cyclin dependent kinases inhibition is important in DNA damage-triggered cell cycle arrest. These interactions allow DNA repair to occur before mitosis takes place and ensure that the intact genetic material is passed on to daughter cells [14]. Alternatively, if the damage cannot be removed, chronic DDR signaling activates cell death by apoptosis or cellular senescence [12].

1.2. Excision repair pathways

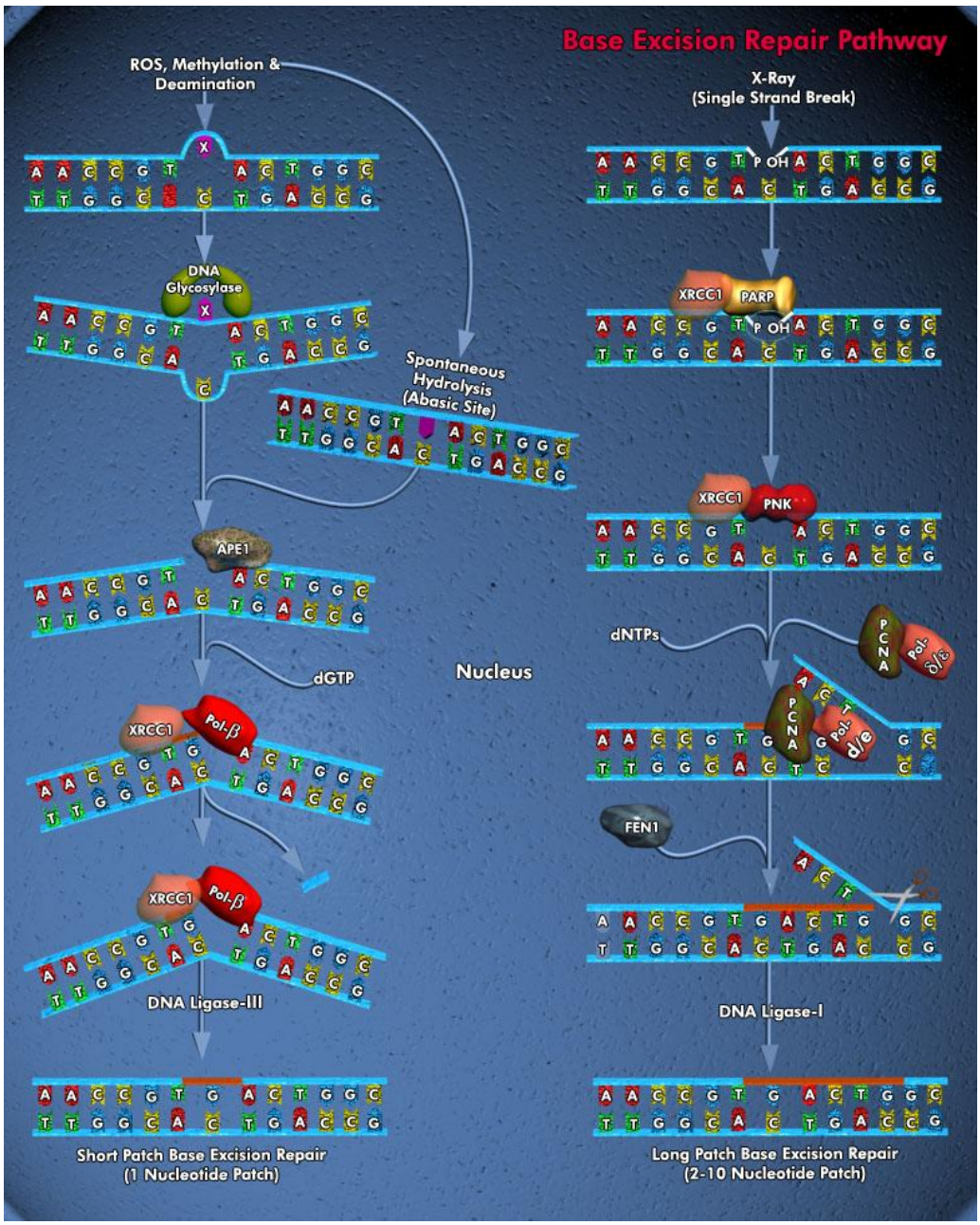
Base excision repair (BER) and nucleotide excision repair (NER) belong to the subgroup of DDR mechanisms that are activated mainly by structural modifications of DNA bases. Both excision pathways act according to a common pattern: recognition of the DNA lesion, excision of the damage and resynthesis of the removed sequence. While BER recognizes mostly subtle changes, such as oxidative damages, alkylations, abasic sites or single-strand breaks, NER is activated by helix-distorting damage, such as bulky adducts or strand crosslinks. Generally, most NER lesions arise from exogenous sources (except for some oxidative lesions), whereas BER is mostly, but not exclusively, concerned with damage of endogenous origin.

Base excision repair

Over 100 different oxidative base modifications in DNA have been detected in human cells. The best-known and most widely studied oxidative DNA base lesion is 7,8-dihydro-8-oxoguanine (also known as 8-oxoguanine; 8-oxoG). The presence of an 8-oxoG is often used as a cellular biomarker for indicating the extent of oxidative stress. In the present PhD work, the removal of this particular lesion has been used as a marker of BER activity (**Manuscript II, III and V**). 8-oxoG is one of the most deleterious products of oxidative DNA damage for the following reasons: (i) 8-oxoG is a very stable lesion; (ii) 8-oxoG●C base pair induces template/DNA polymerase distortions during replication, resulting in a stable 8-oxoG●A mismatch; (iii) unrepaired mismatch gives rise to G→T transversions [15].

In BER, four different types of enzymes are required: DNA glycosylases, DNA endonucleases, DNA polymerases and DNA ligases. The first ones in the row are responsible for recognizing their substrate and catalyzing the cleavage of an N-glycosidic bond, releasing the damaged base and creating an abasic site (AP). Some glycosylases are bifunctional and have also an AP-endonuclease activity. If this is not the case, the specialized AP-endonuclease APE1 has to take over. To date, 11 different mammalian glycosylases have been described, each with a specialization for different substrates. Out of three of them, MTH1, MUTYH and OGG1, are specialized to recognize 8-oxoG itself, or paired with adenine. However, a predominant role in removing oxidative DNA damage is played by 8-oxoguanine DNA glycosylase (OGG1), whose activity is usually

measured and considered as a representative biomarker for BER. After the initial recognition/incision step, the strand break is filled with new nucleotide(s) by DNA polymerases and the free ends are reconnected by DNA ligases. The scheme of the whole BER process and differences between its short (removal of one nucleotide only) and long (removal of 2-10 nucleotides) patch are presented in **Figure 2**.



Adapted from https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=53

Figure2. Scheme of short and long patch of BER.

Only recently has a clear link between BER deficiency and a human disease been found, referred to as MUTYH-associated polyposis. Germ-line mutations in BER gene *MUTYH*,

reducing its functional capacity, cause polyposis that regularly develops into colorectal carcinoma. Furthermore, inactivation of BER core proteins was shown to induce embryonic lethality which highlights the vital importance of this process as a whole [16].

Nucleotide excision repair

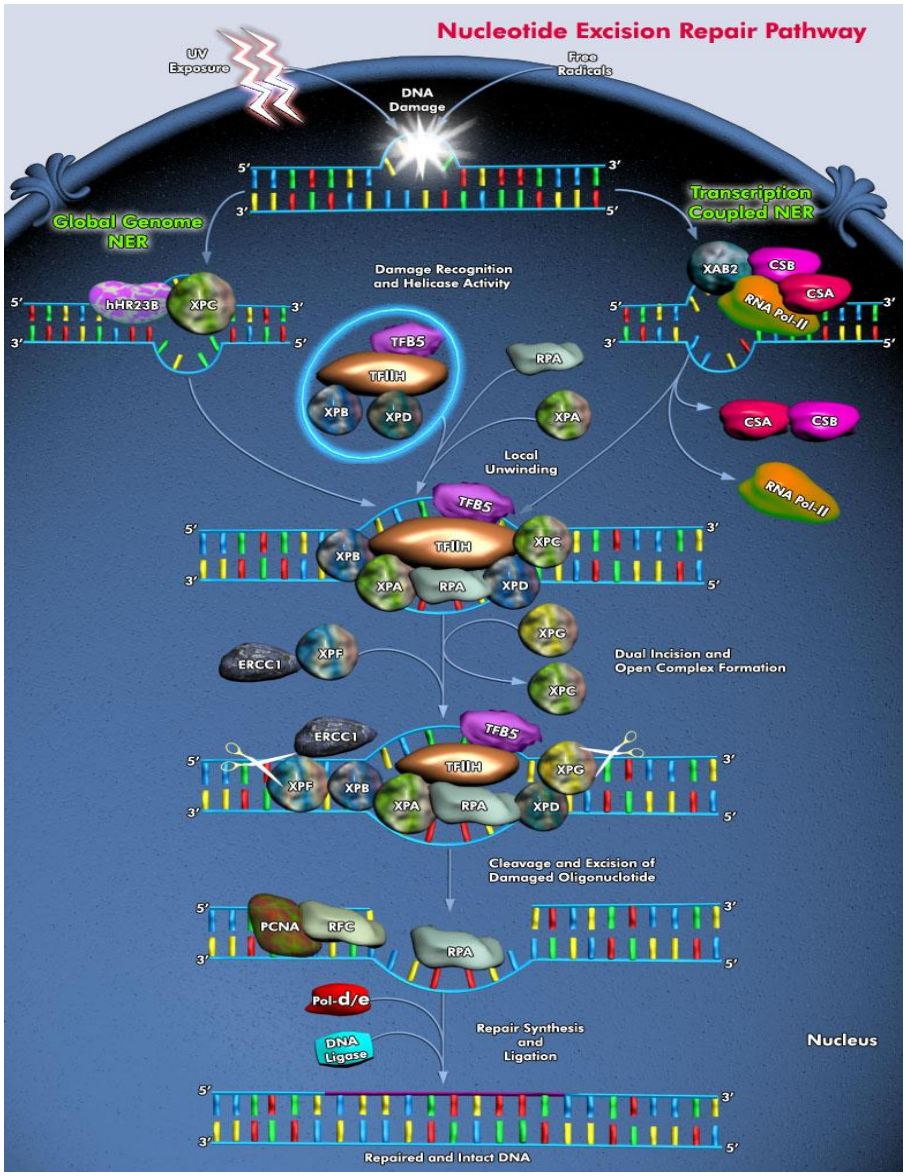
Of all repair systems, NER appears to be the most versatile in terms of lesion recognition. It can repair UV-induced lesions, a wide array of chemically induced bulky adducts or DNA crosslinks and even some types of oxidative damage. Experimentally, the most studied is the repair of UV-induced cyclobutane pyrimidine dimers and 6-4 photoproducts (**Manuscript V**) and benzo[a]pyrene diolepoxide (BPDE)-induced adducts, represented mainly by N^2-deoxyguanosine adducts (**Manuscript I, IV**).

In NER, there is no lesion specialization like in BER, but rather a universal mechanism of recognition of a wide spectrum of the damage takes place. To ensure this versatility, NER enzymes work in large complexes. At minimum, 30 proteins are involved in NER pathway in total. Xeroderma pigmentosum protein complementation group genes *XPA-XPG* perform the initial recognition/incision part: each of those genes act in complexes with some other NER factors, which are *in extenso* illustrated in **Figure 3**. The incision step of NER is known to be a rate-limiting [17,18] and as such it is used for study as a representative of overall NER capacity. The minimal requirement for the incision is at least 16 proteins but some of them have been recognized to be key players in the whole process. The most reasonable evidence was collected for XPA which was shown to be activated by the ATR/p53 sequence and thus activates the NER process [19]. ERCC1 protein was suggested to coordinate the whole recognition/excision phase [20]. After the removal of a damaged site, NER is finished by filling the excised gap of 18-24 nucleotides by DNA polymerases and DNA ligases.

Two NER subpathways exist with partly distinct substrate specificity. Global genome NER (GGR) surveys the entire genome for distorting injury, and transcription-coupled NER (TCR) focuses on damage that blocks elongating RNA polymerases which involves only about 1% of the genome that is transcriptionally active at the time. Thus, terminally differentiated cells display lower NER activity, which is performed by TCR, while GGR is downregulated in this case [21].

The importance of NER for genomic stability is demonstrated by several cancer-prone or neurodegenerative disorders caused by defects in enzymes of this pathway. Xeroderma pigmentosum with very high incidence of skin cancer results from a deficient NER at

global genomic level due to a mutation in *XPA-XPG* genes. Some others, not associated with increased risk to cancer, but often with premature ageing, such as Cockayne syndrome, trichothiodystrophy, UV-sensitive syndrome or cerebro-occulo-facio-skeletal syndrome are caused by defects predominantly in TCR [22].



Adapted from https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=328

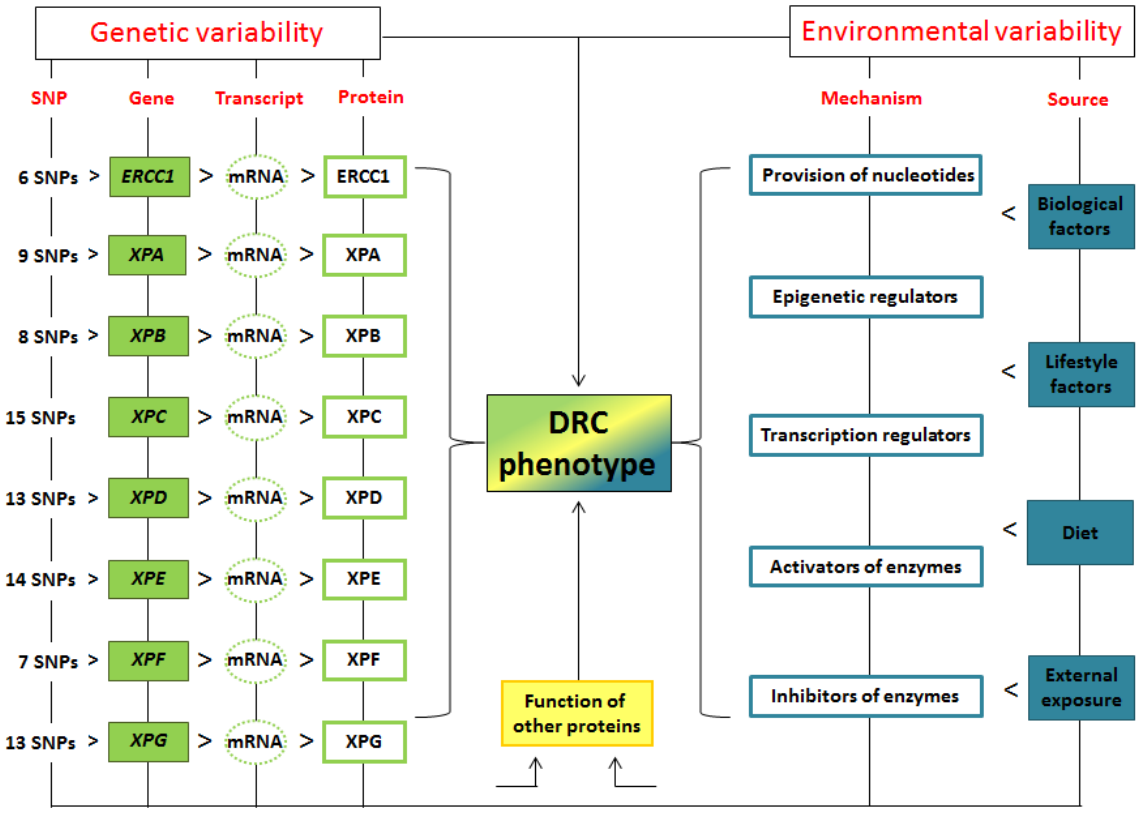
Figure 3. Scheme of global genome and transcription-coupled NER.

1.3. Phenotyping DNA repair

As obvious from current knowledge on DNA repair, this process involves many genes which have to work in a synchronized and coordinated way. The simultaneous participation of other processes like DNA damage signaling, cell cycle controls and other (presumably even unknown) genes makes the DNA repair a multigene and even a multipathway process. DNA repair machinery involves at least 150 genes, many of them being polymorphic in the human population [23]. However, the functional consequences of the majority of DNA polymorphisms in DNA repair genes have not been fully characterized thus far [24,25]. Associations that have been found between common gene variants and altered functionality of repair proteins are often ambiguous or results of different research groups are not in conformity. Thus, in the current state of the art, DNA analysis does not provide sufficient information in the prediction the overall DNA repair activity. Gene expression analysis has shown to be another misleading source of information, because changes in mRNA levels do not necessarily reflect changes in enzyme activity and, conversely, changes in enzyme activity are not inevitably accompanied by changes in the mRNA copies number [26-29]. Moreover, it is not exclusively genetic predisposition that modulates DNA repair activity of individuals. Family-based studies on homozygote twins showed that DNA repair is a phenotype with a heritability estimated in the range of 48-75% [30]. The rest is influenced by environmental and lifestyle factors via several possible mechanisms, such as activation/inhibition of repair enzymes, the pool of DNA precursors, regulation of expression of repair and other genes etc. [31]. Summarizing all of the above, the multifactorial process of DNA repair might be better characterized by functional analysis of DNA repair capacity (DRC), the true phenotypic endpoint that comprises the variability of both hereditary and environmental components, and as such gives information of actual DNA repair activity of the cell/tissue/organism (**Figure 4**).

DNA repair can be phenotypically characterized by a modification of the microgel electrophoresis technique, the comet assay, which is a rapid, sensitive and visual tool for DRC assessment. It can quantify the DNA strand breaks (SBs) that are generated as intermediates during the DNA repair of excision type, in particular BER and NER. Both repair pathways, fundamental for the removal of a broad spectrum of DNA lesions, process DNA damage in a similar way, by cleaving the damaged site and leaving DNA

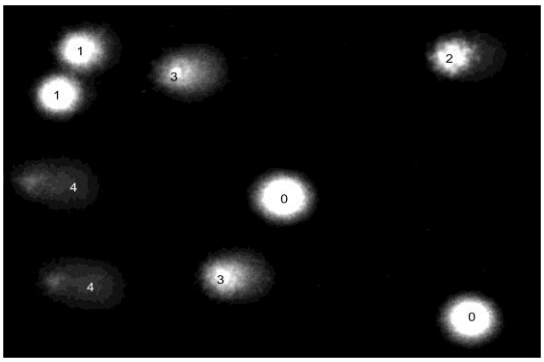
breaks behind. These breaks reflect incision phase of repair process that, as mentioned above, has been recognized as a rate-limiting step.



Modified from Ch. Li et al. [32]

Figure 4. A simplified scheme of multifactorial nature of DNA repair capacity phenotype.

Comet assay, known also as a single cell gel electrophoresis, was originally established to measure steady-state level of damage in DNA at the single-cell level. Östling and Johanson [33] described for the first time the behavior of DNA containing SBs under an electric field. The principle is that, in these conditions, free strand ends are released from the DNA loops and allow DNA fragments to migrate towards an anode, thus forming comet-like formations (**Figure 5**). Briefly, cells embedded in agarose gels on microscope slides are lysed in the presence of high salt concentration and detergents to eliminate cell debris. After lysis, most histones are removed, and nucleosomes are disrupted, but the DNA remains supercoiled



Adapted from A.R. Collins et al. [40]

Figure 5. Examples of comets

in nucleoids. The presence of a SBs will relax the supercoiling in the loop in which it occurs, enabling that loop to extend under electric field. For this purpose, agarose-embedded nucleoids are subjected to high pH, allowing DNA unwinding, and subsequent alkaline electrophoresis. Following electrophoresis, the slides are neutralized, stained with a DNA binding dye and the comets visualized by fluorescence microscopy. The extent of comet tail formation, expressed as tail DNA %, is proportional to the amount of DNA damage [34]. **Figure 6** describes, in a simplified way, the main steps of the comet assay. The standard alkaline version is actually able to detect SBs and alkali-labile sites, i.e. apurinic/apyrimidinic (AP) sites. The specificity for DNA damage might be increased using lesion-specific enzymes in the assay. Among others, formamidopyrimidine DNA glycosylase (Fpg) is used to recognize 8-oxoG and formamidopyrimidines. Endonuclease III recognizes oxidized pyrimidines and T4 endonuclease V (Endo V) converts cyclobutane pyrimidine dimers into SBs and is often used to determine UV-induced damage [35]. SBs and AP-sites occur also as intermediates during BER and NER processes and this makes both excision repair pathways as candidates for DRC evaluation by methods based on comet assay principles.

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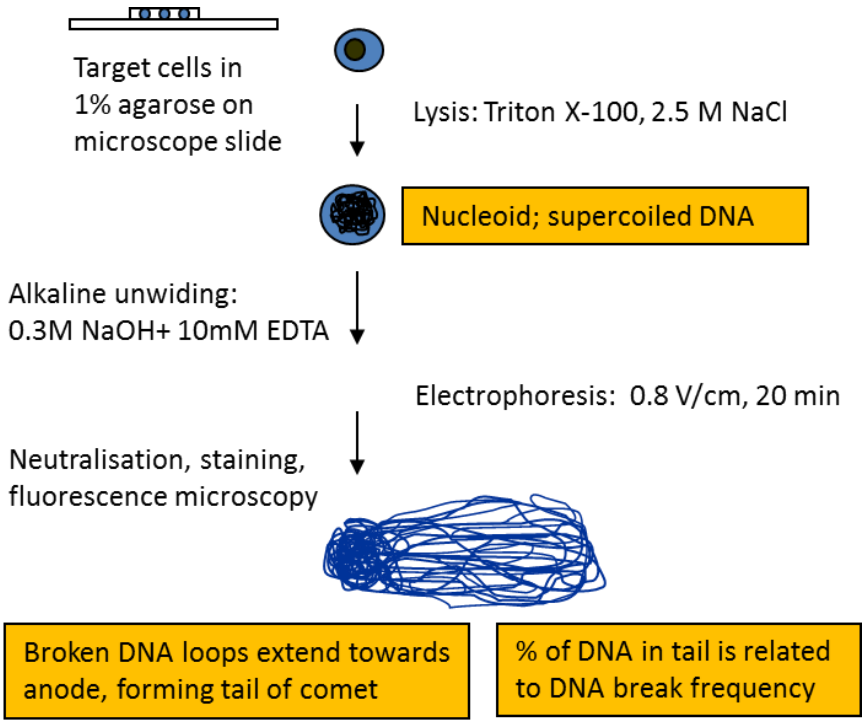


Modified from A.R. Collins et al. [36]

Figure 6. The principle of the comet assay.

In order to assess BER- and NER-specific DRC in various types of tissues, several different modified versions have been developed (**Table 2**). Moreover, depending on the viability of the cells, two slightly different approaches have been employed:

Challenge assay requires viable cells, usually obtained from fresh blood, and is considered to be the most straightforward approach to measure kinetic of DNA repair. Examined cells are cultured in the medium and mitogen stimulated. They undergo a treatment with specific DNA damage-inducing agents that introduce specific DNA lesions. Cells are allowed to repair this damage and afterwards they are collected and processed by comet assay to measure the amount of SBs. This repair assay might also be called "cellular repair", because it measures repair activity in intact cell system.

In vitro repair assay is a solution for cells with lower viability or for already frozen solid tissues. From collected cells or tissues a protein lysate is extracted. Whole protein extract is further incubated with a damage-containing DNA substrate to allow recognition and incision of damage. The DNA substrate is subsequently processed by the comet assay to quantify the amount of generated SBs. This assay is called *in vitro*, because the reaction takes place on the microscope slide in an environment imitating the temperature and humidity of living systems.

Table 2. Overview of types of biological material suitable for DRC measurement by comet-based repair assays with relevant methodological reports. Table also shows BER- and NER-specific DNA damage-inducing agents with their recommended concentrations to be used in the assays.

Pathway	Cell type [methodological report]	Lesion-inducing agents (concentrations)
BER	Peripheral blood cells [37] Animal solid tissues [38] Human solid tissues [Manuscript V]	γ-irradiation (2 - 5 Gy) Photosensitizer Ro 19-8022 (0.1 - 2μM) H_2O_2 (100-300μM)
NER	Peripheral blood cells [39] Animal solid tissues [40] Human solid tissues [Manuscript V]	UV (1 - 5 Jm^{-2}) BPDE (0.5 - 2μM)

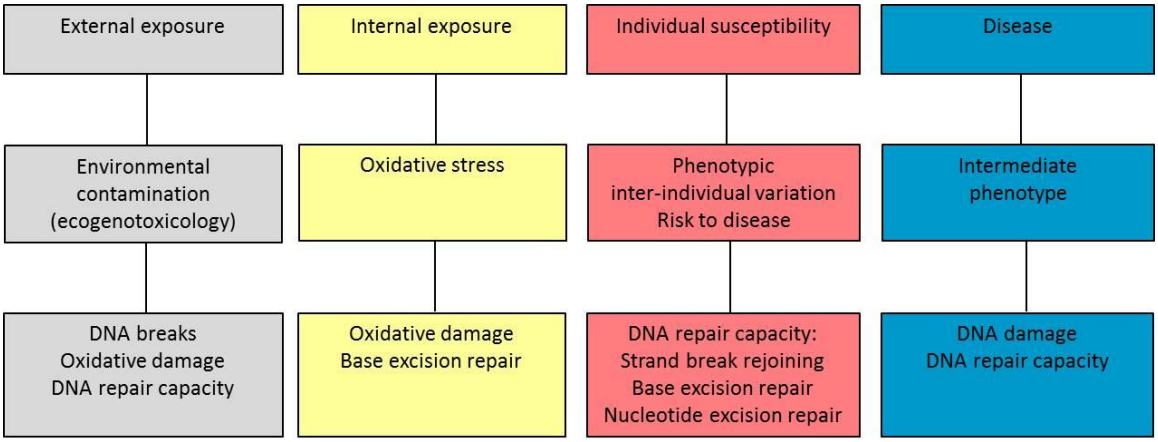
Concerning the source of cells for repair analyses, human blood has so far been prevalently used in vast majority of studies examining the DRC parameter. Understandably, blood is very convenient, non-invasive and often the exclusive source of cells for biomonitoring purposes. Peripheral blood mononuclear cells (PBMC) are easy to isolate, obtainable in relatively large numbers, they are viable even after 24 hour storage

at 4°C, and they are almost all in the same phase (G0) of cell cycle. Although PBMC are, like all tissues, highly specialized but due to their circulation throughout the whole body, their cellular, nuclear and metabolic state reflects overall body exposure [41,42]. Therefore, the use of PBMC is widely accepted in human biomonitoring studies on occupationally exposed populations.

1.4. DNA repair capacity in human molecular epidemiological studies

The molecular epidemiological approach, measuring molecular or cellular indicators of disease risk or exposure to causative factors, is valuable tool in addition to conventional epidemiology. Its main advantages are that it requires far smaller numbers of subjects than conventional epidemiology and the biomarkers, if carefully chosen, can give useful information about molecular mechanisms involved in the disease aetiology [42].

The comet assay, determining the level of DNA damage or DRC, has a large potential to be used in a wide range of applications, which are suggested in the **Figure 7**. So far, comet assay is most often applied in the nutritional trials to determine the positive/protective effect of various nutritional factors expected to reduce DNA damage and enhance DNA repair. Further, the comet assay has also been used to evaluate genotoxic effects of various chemicals, usually potential carcinogens that are used in the industrial production. In this context, DNA damage measured by comet assay and an evaluation of DNA repair-mediated unscheduled DNA synthesis represents a complementary genetic toxicology assays to the standard panel of assays for assessment the risk from exposure to xenobiotic agents, which nowadays comprises the Ames test, *HPRT* mutations test, chromosomal aberration and micronuclei assays [43]. Finally, comet assay techniques has been utilized for measuring the individual susceptibility to various diseases, most often in association to cancer risk, or recently even with response to anticancer treatment.



Modified from M. Dusinska et al. [42]

Figure 7. The possible use of comet assay to measure DNA damage and DNA repair at different stages of biomonitoring. Figure shows the range of applications, its informative potentials and experimental endpoints.

This section will provide a brief overview of the studies that underlie the scope of the Thesis and provide a theoretical basis for its experimental outcomes. To date, there are few reports investigating BER- or NER-specific DRC in general healthy population in order to estimate physiological inter-individual variability of DRC and its relation to genetic, epigenetic and biological or lifestyle factors (background for **Manuscripts I, II**). DRC has also been evaluated in case-control studies as a biomarker of genotoxic exposure in individuals occupationally exposed to carcinogens (**Manuscript III**) and as a cancer-related biomarker in patients with malignant disease (**Manuscript IV, V**). Despite numerous available literature on these topics, the majority studies is based only on a very small number of subjects (e.g. ≤10 individuals per group) and those will not be included into the overview.

General healthy population

Genetic and phenotypic variability in human populations is remarkable and this most likely applies also to the capacity to maintain DNA stability. However, it is not known yet how large is the inter-individual variability of pathway-specific DRC and in what range might be this variability still considered as normal/physiological. Until now, inter-individual variability for BER- and NER-DRC in the general population was evaluated in few studies by different methodological approaches. Four-fold differences were observed for BER-DRC, while NER-DRC variability was larger, up to 10-fold differences among studied individuals [44]. However, the study was performed on about 30 subjects only. In

another study, variability of OGG1-specific repair was estimated as 2.8-fold among 120 healthy individuals [29]. Inter-individual difference of NER-DRC, on the other hand, was found to be 11-fold on 57 individuals [45].

There are many studies focused on the relation between DRC and age, following the hypothesis that ageing is driven by accumulation of errors in macromolecules, including DNA, which might be also accompanied with the reduction of the DNA repair activity. Nevertheless, age-dependent decreasing of the DRC remains still on the level of hypothesis because there is inconsistency between the observations. Some groups reported negative correlation of DRC with age; some positive and some groups did not observed any relationship, as reviewed in [36].

Another branch of the research is represented by trials investigating whether DRC is modulated by the components of diet following diet supplementation with phytochemicals, antioxidant-rich food or other dietary supplements. In this respect, supplementation by mixture of antioxidant-rich plant-based food [46,47], kiwifruit only [48], or coenzyme Q10 [49] showed the effect on enhancement of DRC and/or reduction of the level of DNA damage. In some other cases, supplementation with selenium plus vitamins A, C and E [50], fruit juice [51], or broccoli [52] did not provide any significant influence on the level of DRC.

Many genes involved in BER and NER and their common allelic variants were studied in relation to cancer risk, and, less frequently, in relation to overall DRC. The effort to find strong associations of particular genotype with an overall DRC, or with a risk of cancer, is fostered by (i) the simplicity of the genotyping method; (ii) its applicability to large scale studies and (iii) plausibility to conduct the analysis on DNA isolated from any kind of tissue (which is usually and most conveniently blood) because of the germline nature of the genetic changes. Using the PolyPhen (Polymorphism Phenotyping) algorithm, by which it is possible to identify functional domains and structures within proteins to evaluate the potential functional significance of DNA repair gene polymorphisms, it was estimated that between 35-45% of all repair gene polymorphisms may have an impact on the function of protein [53]. Obviously, such an *in silico* approach cannot be taken for granted and needs to be verified in molecular epidemiology and subsequently in mechanistic proteomic studies. Combination of *in silico*, *in vitro* and epidemiologically-based (including ours) studies highlight the possible functional relevance of some particular polymorphisms, namely *OGG1* Ser326Cys, *XRCC1* Arg399Gln in BER pathway and *XPD* Lys751Gln, *XPA* A23G, *XPC* deletion of exon 12 and *XPC*-PAT in

genes of NER pathway, as reviewed in [3,54,55]. Nevertheless, it is becoming more and more apparent that the complexity of the biological processes and the etiology of the diseases cannot be targeted by single SNP analyses. On the other hand, although functional DNA repair tests are fundamentally more powerful, they suffer from two main disadvantages: they are more laborious and complicated than SNP analysis, and cannot be usually done in target tissues.

Similarly as for SNPs, the same effort to seek for potential predictors of DRC was made to find out whether mRNA level may reflect the activity of translated protein. Although quantitative PCR is not as simple method as SNP analysis, it has longer tradition and is more unified than functional assays, and, moreover, detailed guidelines for its performance are already available [56]. However, the level of mRNA does often not reflect the activity of translated protein, [26-29], which again underlines the relevance of protein functional studies.

Our understandings of the range and regulation of human DNA repair and, specifically, of the interplay between genes and the environment, are still at an early stage [57]. The studies presented in **Manuscript I** and **II** were aimed to bring some new evidence to this research topic or to support previously reported observations.

Occupationally exposed population

For environmentally induced diseases, molecular biomarkers play a key role in understanding the relationship between exposure to toxic chemicals and the development of chronic diseases and in identifying individuals at increased risk. They can be used to monitor levels of exposure to some disease-causing agents, or they may inform about inter-individual variation in response to these factors. Finally, they can be used in relation to genotype, which is increasingly seen as a crucial factor in influencing individual responses to exposure and susceptibility to disease of various kinds [42]. In this respect, biomarkers of DNA damage and, less often, also DRC have been used as biomarkers of genotoxic exposure to industrially produced chemicals in several epidemiological studies. The approach of measuring levels of DNA damage and DNA repair in human biomonitoring was applied in relation to smoking [58,59], exposure to pesticides [60], asbestos [61,62], mineral fibers [63], heavy metals [64,65], traffic pollution [66,67], in a rubber tire factory [68] or in workers from nuclear power plant [69]. No consistent overall picture arises from these studies, some agents seem to enhance DRC, other seem

to reduce its level, and sometimes no effect of exposure is observed. In some cases, even if the same agent is subject of investigation by different groups, results are not replicated.

Occupational exposure to styrene in lamination factories represents one particular topic extensively studied by the group of Dr. Pavel Vodicka for a considerable time [70-74]. In one of the latest studies conducted by our group on workers exposed to styrene for a period of 4 years on average, it was postulated that DRC, which was enhanced in workers as compared to controls, might be regulated in the sense of adaptive reaction of the organism against chronic genotoxic attack [75]. In **Manuscript III**, built on previous studies published by group of Pavel Vodicka, the effect of long-term (14 years on average) exposure to styrene on the level of DRC has been assessed, to further test this hypothesis.

Cancer patients

A fundamental feature of cancer is genomic and chromosomal instability. Furthermore, most agents recognized to be potential carcinogens operate via generating DNA damage and causing mutations. It is also known that inherited DDR defects commonly predispose to cancer, enhancing mutation rates, tumor cell survival and proliferation rate. The capacity of DNA repair is therefore one of the important determinants of susceptibility to cancer.

Polymorphisms in genes encoding DNA repair proteins are increasingly investigated in human epidemiological studies. There was some evidence reported on the association of several SNPs with occurrence of cancer. However, searching for candidate SNPs that would be clearly related to a higher or lower risk to malignant disease is producing inconsistent results. It is striking that even now, in the age of genome-wide association studies, very few of original candidate polymorphisms can be regarded as being convincingly associated with cancer risk [76]. Lack of unambiguous candidates is interpreted by rather additive effect of multiple low-penetrance variants and by multi-factorial pattern of the disease, following the paradigm common disease-common variant [77]. Moreover, studies of this design require a large sample size, given by the minor allelic frequency in the population, which can be serious limiting factor. This stimulated an interest for investigating the phenotypic measurement of DRC in association with cancer. A number of epidemiologic studies have been conducted to compare levels of DRC between cancer patients and healthy controls to assess the role of repair in the development of various types of human cancer. Berwick and Vineis [78] have reviewed

all published reports linking altered BER-DRC to an increased risk of cancer. Reviews focused on NER-DRC and cancer risk are currently missing, therefore a survey of case-control studies on this topic have been included into the **Manuscript IV, Table I**. Reduced NER-DRC has been observed in patients with bladder, breast, skin, head and neck, lung and prostate cancer. A sufficient body of evidence is now available to support the theory that individuals with suboptimal DRC (relative to control individuals) might be at higher risk to malignant disease.

Surprisingly, there is very limited information about BER- and NER-DRC in relation to sporadic colorectal cancer (CRC) in particular. Moreover, all available studies were examining the DRC in blood cells and almost nothing is known about the BER- and NER-specific DRC in cancer target tissues or in tumor tissue. In this respect, the **Manuscript IV and V** are novel and of particular importance.

2. Aims

The aims of this doctoral Thesis reflect the still growing interest in the nature and biological regulation of DNA repair in the human population both under physiological and pathological conditions. For this purpose, specifically BER- and NER-DRC were explored in healthy individuals. Furthermore, DRC was studied in two other different groups of subjects, both with possible alterations of DNA repair, i.e. workers occupationally exposed to constant high genotoxic stress and patients with colorectal carcinoma. These different categories of individuals were selected to answer the following questions:

- What is the normal variability of DRC in healthy humans?
- What are the genetic and non-genetic factors that might modulate DRC?
- Does long-term exposure to genotoxins trigger an adaptive induction of DRC?
- Is a high DRC associated with lower risk of cancer?

This Thesis also aimed to optimize methodologies for DRC evaluation in following aspects:

- To implement comet-based repair assays for DRC measurement in human solid tissues
- To optimize higher-throughput version of the repair assay to meet criteria required by large epidemiologic studies

3. Material and Methods

3.1. Study populations

Manuscript I: This study was conducted on 100 healthy individuals from the Czech Republic (52 women and 48 men; age range 21-86 years, mean age 41.6±17.5), who provided basic information about age, body weight and height, occupational and medical history and lifestyle habits. Subjects were sampled for peripheral blood.

Manuscript II: This study included 244 healthy individuals from the Czech and Slovak Republic (61 women and 183 men; age range 19-59 years, mean age 41.3±11.3), who were interviewed for detailed questionnaire. Subjects were sampled for peripheral blood.

Manuscript III: This biomonitoring study was performed on 24 workers exposed to styrene (16 women, 8 men, mean age 39.1±6.1) and 15 unexposed clerks (9 women, 6 men, mean age 41.3±8.3), all from the same factory located in the Slovak Republic. Subjects were sampled for peripheral blood.

Manuscript IV: In this case-control study 70 newly diagnosed patients with sporadic CRC (24 women and 46 men; mean age 65.4±10.1) were compared to 70 age-matched healthy controls (36 women and 34 men; mean age 62.1±12.7), all from the Czech Republic. All subjects provided necessary information requested in the questionnaire. Subjects were sampled for peripheral blood.

Manuscript V: A hospital-based study involved 70 incident patients (17 women and 53 men, mean age 66.2±10.6) diagnosed for sporadic CRC from the Czech Republic, who underwent surgical resection. Patients were sampled for colon/rectal tissue (both tumor and normal) and peripheral blood.

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

3.2. DNA damage assay

Comet assay is a popular and commonly used method to determine basal DNA damage at a single cell level. It quantifies the damage that is spontaneously transformed into SBs. Thus, it can detect true SBs and alkali-labile sites (AP sites). Classical comet assay protocol was published by Collins [79]. Briefly, isolated cells are embedded in the

agarose on the microscope slide; they undergo lysis to expose DNA. Further alkali unwinding cause denaturation of DNA and the electrophoresis create comet-like formations due to the present of SBs. The extent of DNA in the tail of the comet is directly proportional to the extent of damage. Results are expressed in tail DNA % (**Figure 6**).

3.3. DNA repair assays

Modifications of the described standard comet assay have been developed to measure SBs not only as a steady-state level of DNA damage, but as an intermediates of excision repair pathways, i.e. incision repair activity. There are two comet-based techniques, *ex vivo* (challenge assay) and *in vitro* (*in vitro* repair assay) that use different approach to quantify the DRC. Both have been used in the studies which constitutes the present Thesis.

Challenge assay

This assay was applied for measuring DRC in viable PBMC isolated from fresh blood. PBMC, mitogen-stimulated in culture medium, were treated with a DNA-damaging agent. After the treatment, the agent was washed out and cells were further cultured to allow them to incise the damage from DNA. Level of incurred breaks, reflecting the actual DRC, was measured. This approach was used for NER-specific DRC evaluation in the **Manuscripts I** and **IV**. In both studies, the identical protocol was applied. PBMC were treated with 1μM BPDE for 30 min to induce BPDE-adducts. The assay measures the accumulation of repair intermediates (incisions) within 4 hours of incubation. Final BPDE-induced DRC reflects the difference between the level of SBs measured immediately after the treatment with BPDE at time 0, and the maximum level of SB detected within 4 hr of culturing. For each experimental point, the background SBs level of untreated control cells was subtracted.

***In vitro* repair assay**

This assay was applied for measuring DRC in frozen PBMC, or solid tissues, i.e. colorectal mucosa. Cells, in which DRC was measured, were lysed and cellular protein extract was prepared. Extract was incubated with a substrate DNA which contained

artificially induced DNA damage, specifically recognizable by the DNA repair pathway of interest. Protein extract removes DNA damage from the substrate DNA and creates DNA breaks that are measured by the assay. This approach was used to measure BER-DRC in **Manuscripts II**, **III** and **V** and for NER-DRC in **Manuscript V**. However, the used protocols differed in various parameters like in e.g. DNA-damaging agents, their concentration or time of incubation.

BER-specific repair:

- PBMC of investigated subjects were embedded in the agarose and irradiated with 5 Gy of γ-rays to induce SBs. Afterwards, during the 40 min incubation at 37°C, SBs were repaired, according to the individual DRC. This assay reflects strand break repair (the long patch of BER, **Manuscript II**).
- In **Manuscripts II**, **III** and **V** another approach to measure BER-DRC was applied. Substrate cells were treated with photosensitizer (Ro 19-8022) and visible light to induce 8-oxoG. Different photosensitizer concentrations and time of illumination were used (0.1μM for 2 min, 0.1μM for 3 min or 2μM for 5 min, see for details in the enclosed manuscripts). Protein extract from cells of investigated subjects were incubated with substrate DNA for 45, 10 or 20 min, respectively, to recognize and incise 8-oxoG. This assay reflects the recognition and incision phase of BER.

NER-specific repair:

- Substrate DNA was irradiated with 5 Jm^{-2} of UV light to induce cyclobutane pyrimidine dimers and 6-4 photoproducts. Incubation of substrate DNA with protein extracts took 30 min (**Manuscript V**).

Moreover, *in vitro* repair assay can be performed in classic 2-gel per microscopic slide format system (**Manuscript II, III**), or by an advanced 12-gel per microscopic slide format system (**Manuscript V**). A 12-gel has been introduced at Prof. Andrew Collins's laboratory (Laboratory of DNA damage and DNA repair, University of Oslo) and was employed and optimized on human tissue samples during my 2 month stay fellowship in his laboratory.

3.4. Genotyping analysis

SNPs in genes encoding DNA repair proteins or xenobiotic-metabolizing enzymes were selected according the effect on the amino acid sequence in the protein (missense, deleterious) and according the minor allele frequency (MAF > 3%). SNPs were determined by a common PCR-RFLP based method or with TaqMan allelic discrimination assay. Following polymorphic sites were investigated in genes involved in BER: *hOGG1* Ser326Cys, *APE1* Asn148Glu, *XRCC1* Arg194Trp, *XRCC1* Arg280His, *XRCC1* Arg399Gln; NER: *XPA* G23A, *XPC-PAT*, *XPC* Lys939Gln, *XPC* Ala499Val, *XPD* Lys751Gln, *XPG* Asn1104His; Homologous repair: *NBS1* Glu185Gln, *XRCC3* Thr241Met; metabolism of xenobiotics: *EPHX1* activity, *GSTM1* deletion, *GSTT1* deletion, *GSTP1* Ile105Val (**Manuscripts I-IV**).

3.5. Gene expression analysis

The expression of target DNA repair genes was examined by RT-qPCR. Total RNA was transcribed into cDNA, which copy number was expressed by relative quantification. Ninety six well plates or 96x96 array platform was applied. Results were normalized to the mean of Cq values or according to reference genes, which depended on the normalization procedure recommended by Genorm and Normfinder algorithms. Data were expressed as relative to maximum quantities (lowest expression was considered as 1) and they were log2 transformed (**Manuscript IV, V**).

4. Results and Discussion

The subject of this Thesis was to investigate the biological variability of DRC in healthy individuals, as well as DRC behavior during the chronic exposure to xenobiotics and its role in human carcinogenesis. The working hypotheses and the experimental work were driven by several major starting points: (i) DNA repair is a vital process of organism that might play a significant role in the individual susceptibility to DNA-damage driven diseases; (ii) DNA repair can be phenotypically characterized by the modification of the microgel electrophoresis technique, comet assay, which is a rapid, sensitive and visual tool for DRC assessment; (iii) DRC is a promising complex marker of the actual capability of the cell, tissue or organism to protect its DNA integrity. DRC comprises all individual factors that are influencing the actual level of cellular DNA repair activity, such as genetic variability, variability in gene expression or in stability of proteins, effect of inhibitors/stimulators, individual lifestyle and influence of other environmental factors. In this section, the major findings from each publication representing the PhD study are discussed.

Manuscript I:

The study *"DNA damage and nucleotide excision repair capacity in healthy individuals"* was performed to assess the range of basal DNA damage and NER-specific DRC in healthy individuals, on the background of individual genetic and non-genetic factors presumably modulating DNA stability.

In our study group of 100 subjects, the observed inter-individual variability was remarkably large, and some individuals were characterized by only a negligible repair activity. The variability within the 5 and 95 percentile (i.e. by excluding outliers) represented a 16-fold difference in BPDE-induced NER repair. Gaivao et al [44] observed a 10-fold inter-individual variability in 30 subjects while Tyson et al. [45] found 11-fold variability in 57 subjects, both for UV-induced NER repair. It remains to be explored whether larger variability is affected by the larger size of examined population, or whether there is different range in the repair of UV versus BPDE-induced damage. In the same context, our study group exhibited an average DNA damage level of 0.1 SBs/10^6 nucleotides (~300 breaks/cell). In a meta-analysis based on 125 studies, the average SBs across several healthy populations was reported to be 0.09 SBs/10^6

nucleotides (~270 breaks/cell) [80]. The consistency of the available data based on different populations may approve this level of SBs as a reference value. Additionally, both, gender and alcohol consumption contributed as independent factors modulating the level of DNA damage. Women showed approximately 50% lower damage than men. This tendency was reported also by [81,82], and can be partially explained by action of estrogens which upregulate the expression of antioxidant enzymes [83-85]. The positive correlation of DNA damage with the amount of consumed alcohol was also observed by other groups [86,87]. This might be the effect of highly reactive, oxygen-containing molecules, generated during alcohol metabolism, e.g. acetaldehyde [88,89].

When DNA damage and DRC were stratified for DNA repair polymorphisms, some interesting associations were discerned: the homozygous variant genotype in *XPC* Ala499Val predisposed to the highest DNA damage level, as observed also by other authors [90,91], while the presence of variant allele in *XPA* G23A was associated with reduced NER-DRC [51,92,93]. This SNP has been further reported to modulate the risk of cancer [94,95]. The conformity of available data suggests that *XPA* G23A might be relevant for modulation of NER-DRC outcome.

In this study, we have reported the range of variability of DNA damage and NER-DRC in the general population and specified the genetic, biological and lifestyle characteristics that modulate its level. A similar study design was applied in **Manuscript II** to explore DRC specific for BER pathway.

Manuscript II:

The study *"Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects"* was aimed at evaluating the BER-specific DRC in 244 healthy individuals, in respect to genetic variability in several DNA repair genes.

In this study, two types of genotoxic agents were used to induce specific DNA damage repair, followed by the comet assay; γ-irradiation to follow strand break repair (SB-DRC) and photosensitizer Ro 19-8022 to induce 8-oxoG repair (ox-DRC), i.e. the long and the short patch of BER, respectively (see Figure 2, page 12). The observed inter-individual variability was 9-fold and 21-fold for SB-DRC and for ox-DRC, respectively. The lowest SB-DRC was detected in carriers of *XRCC1* 399Gln homozygous genotype and the lowest ox-DRC in the homozygotes for *OGG1* 326Cys. *XRCC1* Arg399Gln has been recognized via *in silico* approach, performed by SIFT (Sorting Intolerant from Tolerant) algorithm to have a high probability of being functionally significant [96,97]. Indeed, in

the area of molecular epidemiology, there is ample evidence that this particular polymorphism might influence the quality of functional outcome of the *XRCC1* gene [98]. *XRCC1* 399Gln was described to be a risk allele for tobacco- and age-related DNA damage [99] and allele increasing risk to cancer [100]. This data seems biologically plausible, as XRCC1 acts as a coordinator of SB repair proteins in the BER pathway [101]. The phenotypic influence of the *OGG1* Ser326Cys to ox-DRC is also biologically plausible. The ox-DRC reflects predominantly the activity of OGG1 enzyme, which is a glycosylase specialized for recognizing 8-oxoG, studied in the assay. Several studies which analyzed the biochemical properties of the OGG1 enzyme suggest that the *OGG1* 326Cys genotype may represent a phenotype with delayed or deficient repair of 8-oxoG [102-106]. A meta-analysis of 27 studies evaluating the role of this polymorphism in association with lung cancer risk indicated the variant Cys allele as a risk allele to develop this disease [107]. However, this relationship might be cancer-specific, as it was not seen in the meta-analyses of CRC [108] or breast cancer studies [109].

Studies reported in **Manuscript I** and **II,** conducted on disease-free populations, were designed to provide a background data for further applications of DRC as biomarker of susceptibility to deal with constant genotoxic stress induced by exposure to genotoxic agents, or susceptibility to cancer, as published in **Manuscript III and IV**.

Manuscript III:

The study *"Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers"* explored BER-DRC and biomarkers of genotoxicity (SBs, chromosomal aberrations, *HPRT*-mutations and DNA adducts) in relation to the genetic variability background of 24 workers occupationally exposed to styrene and 15 unexposed clerks from the same factory. Styrene and butadiene were previously and extensively studied in the laboratory of my supervisor Dr Vodicka, as model compounds to study the genotoxic effects of occupational exposure to monomers used in the plastics industry [55,70,75,110].

Workers in this study were exposed on average for 14±5.6 years to 98.1±98.9 mg/m^3 of styrene which is near to the permissible exposure limit (100 mg/m^3) in both the Czech and Slovak Republics [111]. This limit is in agreement with limits in other countries, e.g. in USA [112]. Styrene was classified by IARC as a possible human carcinogen and was observed to induce various DNA and chromosomal damage in humans. However, less is known about the involvement of DNA repair in its genotoxicity. Styrene is metabolized

into styrene-oxid that covalently binds to DNA and forms DNA adducts, which are recognised by BER pathway.

All examined biomarkers of genotoxic effect were significantly higher in exposed workers than unexposed clerks. Workers exhibited only a non-significant increase in BER capacity in comparison with controls. In a previous investigation on a different styrene-exposed study population, BER-DRC in workers was significantly increased and the level of SBs was associated with higher repair rates [75]. In that context, it was postulated that the lack of accumulation of genotoxic damage over time in exposed individuals could be due to the induction of adaptive DNA repair processes. However, despite a similar trend, this hypothesis was not strongly confirmed by the present study. This discrepancy might be due to the different characteristics and size of the two study populations, or there might be different mechanisms involved in the adaptation to the constant long-term genotoxic stress (~10 years longer than in previous study). On the other hand, our results suggest that individual BER-DRC might be significantly modulated by smoking and by gender.

BER-DRC was also modulated by SNPs in three DNA repair genes. A significantly lower BER-DRC in *XRCC1* 399Gln and *OGG1* 326Cys allele carriers was found. Although the population size of this study was small, the same relationships were also observed in our additional study (**Manuscript II)** on larger population of 244 subjects, and these associations were comprehensively discussed there. Interestingly, homozygous variant genotype 939Gln of *XPC* was associated to higher BER-DRC. The reason for interaction of a NER-involved gene with BER pathway remains unclear at present. However, it has recently been reported that SNPs in genes involved in NER, in particular *XPA*, might modulate the activity of the OGG1 enzyme [113]. Involvement of NER enzymes in the repair of 8-oxoG has also been demonstrated *in vitro* [114].

In this study a large pallet of examined biomarkers was employed to evaluate the effects of occupational exposure to styrene. The small size of the subjects recruited in this study population was due to the special category of workers in the lamination factory exposed to very high concentrations of styrene. This particular situation is very important for studying the subtle effects of genotoxicants like styrene, however, it is fortunately less and less retrievable at present, where strong regulations are imposed on occupational exposure.

Manuscript IV:

The case-control study entitled "*Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls"* reports a comparison of the level of DNA damage and NER-DRC between 70 patients with sporadic CRC and 70 age-matched healthy controls. Patients were recruited at the time of the first cancer diagnosis and were not treated before the sampling. DNA repair was investigated by functional assay, gene expression and genotype analysis in PBMC of studied subjects. The investigation of genetic variability in DNA repair genes in association to CRC risk was previously investigated in the laboratory of my supervisor Dr. Vodicka [54,115]. Thus, the present study was a natural continuation of these studies.

Significantly higher DNA damage and lower NER-DRC were observed in patients as compared to controls. Both parameters represented independent risk factors for CRC development. Deficient NER-DRC was previously reported for several other types of cancers, including bladder, breast, skin, head and neck, lung and prostate (see Table 1 in **Manuscript IV**). Our results contribute to the list of evidence on the importance of NER in carcinogenesis, showing the same relevance also for sporadic CRC. It is important to highlight the fact that all studies examined DRC in blood cells, assuming that this tissue may be informative about the general condition of the repair system in the organism and might suggest the level of individual susceptibility to DNA damage-driven diseases such as cancer [116]. Therefore, we might interpret low DRC in cancer patients as a biomarker of higher susceptibility to cancer development. The level of DNA damage in patients as compared to controls was 0.37 versus 0.18 SBs/10^6 nucleotides, respectively (~1113 versus 540 breaks/cell). A meta-analysis pooling the data from 119 publications showed that cancer-free population of the same age as ours bears 510 breaks/cell [80], which is in full agreement with our data. Thus, CRC patients had >2-fold higher level of SBs in DNA, which additionally demonstrates a general alteration of the DNA repair status in PBMC.

Surprisingly, expression profile of 9 core NER genes did not follow the same pattern as overall DRC. Although 2/3 of studied genes (6) were differently expressed in patients, their expression was not solely reduced (*XPB* and *XPF*), as it would have been expected from low DRC, but some were upregulated in patients (*XPA*, *XPG*, *ERCC1* and *RAD23B*). Moreover, expression level of neither gene correlated with the DRC. This phenomenon has been observed by many other studies as well [26-29,71], and although it is surprising, it is partially explainable. Most probably, in some cases it might be the

absence rather than the relative content of a functional protein that is important for cellular activity. Moreover, genes act in interactive networks and alterations of each of them might have different impact on the overall cell function [117]. Finally, communication between several pathways, e.g. in our case with cell cycle regulation or DNA damage signaling, may also play a critical role [118]. Thus, the present study further showed the usefulness of DRC analysis, which measures the real outcome of a complex multigene process like DNA repair. In this context, the question whether DRC in surrogate tissue fully reflects DRC in target tissue still remained to be addressed. This aspect has been subsequently afforded in **Manuscript V**, where a modified repair assay was employed to study DRC in tumor tissue.

Manuscript V

The study "*Functional, genetic and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas"* describes the level of both BER- and NER-specific DRC in tumor tissues of 70 patients with sporadic CRC in comparison to adjacent healthy tissues. In a subgroup of 28 patients, DRC in colon tissue was compared with DRC in blood cells. Additionally, expression profiling of 8 BER and 17 NER genes in both tissues was performed.

This study was also partially dedicated to the optimization of the comet-based repair assay for higher-throughput version and for measurement of DRC in human solid tissues. There is no other study that analyzed both repair pathways in human tumor tissues. The large amount of analyzed samples led us to the need of adopting the 12-gel format system as an upgrade of classic 2-gel format to increase throughput of the assay. These methodological implementations were performed in the collaboration with Prof. Andrew Collins during my fellowship period at the University of Oslo in Norway.

A moderate increase of NER-DRC but not BER-DRC was observed in tumor tissue in comparison to adjacent healthy tissue. To the best of our knowledge, there is only one study that previously investigated NER-DRC in tumor and healthy tissues of 23 CRC patients. In agreement with our findings, the authors have also reported a strong correlation of DRC between both colon tissues, however higher NER activity was found in tumors [119]. Several other studies have inferred higher BER or NER activity in tumors via an indirect approach of measuring the steady state level of DNA damage, assuming that a low damage level reflects a high repair rate [120-122]. No study has reported deficiency of excision repair in tumors so far. Consistency of observations might

lead to the conclusion that excision repair is not a factor contributing to the malignant transformation, but rather contributes to the growth advantage of existing tumor mass by decreasing the vulnerability to DNA damage accumulation which ultimately results in cell death.

Interestingly, even though PBMC had 2.5-fold lower repair out of all studied tissues, they positively correlated with the DRC of healthy mucosa for both excision repairs. This shows that DRC measured in blood may reflect the repair potential of the colonic mucosa. The same phenomenon was seen by [119], and suggested by [123]. PBMC might be therefore considered to be an appropriate surrogate for cancer-target tissue.

Four BER genes (*NEIL1*, *APEX1*, *OGG1* and *PARP1*) and four NER genes (*CSB, CCNH*, *XPA* and *XPD*) were deregulated in tumors, showing 1.08-1.28-fold differences against healthy tissues. Individual gene expression levels did not correlate with overall DRC. As shown by us, quantitative differences in gene copy numbers were not reflected by corresponding changes in enzymatic activity of coded proteins. In another words, measuring the transcript copy numbers is not a substitutive method for evaluation of the activity of the protein or pathway, which highlights the relevance and informative value of functional studies.

5. Conclusions

This work, based on five manuscripts, was performed in response to several unsolved concerns about the biology of DNA repair in humans, which were not satisfactorily explored yet. In this section, each question postulated in the Aims of the Thesis will be provided by an answer, summarizing thus the main outcomes of own experimental work in light of existing literature/knowledge.

- What is the normal variability of DRC in healthy humans?

We have observed a substantially large inter-individual variability in DNA integrity, as well as in the DRC rates, among healthy individuals. The range of DNA damage, representing SBs and AP-sites in DNA, varied of 25-fold. The average level was 1 SB/10^7 nucleotides and, as supported by the data from the literature, this level might be considered as a reference value for healthy individuals. Concerning the BER machinery, the variability of recognizing and incising the damage from DNA was observed to be much larger (21-fold) than the capacity to resynthesized and ligate the originated SBs (9-fold). Incision capacity was indeed recognized as a rate-limiting step of repair process. The variability to remove bulky adducts from DNA by NER pathway was 16-fold.

- What are the genetic and non-genetic factors that might modulate DRC?

We have observed that the BER-DRC was modulated by the presence of allele variants in the *OGG1* Ser326Cys and *XRCC1* Arg399Gln polymorphic BER gene sites. NER-DRC, on the other hand, was influenced by NER gene *XPA* G23A. For all polymorphisms, the variant allele was found to be less efficient. Our results, in combination with other *in silico*, *in vitro* and epidemiologically-based studies, highlight the possible relevance of non-synonymous polymorphisms in *XPA*, *XRCC1* and *OGG1* genes to the function of the coded protein.

Our results also show that DNA stability might be further modulated by inter-sexual differences and by lifestyle factors.

- Does long-term exposure to genotoxins trigger an adaptive induction of DRC?

We have observed clear genotoxic effect of long-term exposure to high doses of styrene. Genotoxic markers, reflecting DNA and chromosomal damage were significantly increased in exposed workers in comparison to unexposed controls. However, a clear influence of exposure on DRC was not observed.

- Is a high DRC associated with lower risk of cancer?

It is known that ~15% of sporadic CRC is deficient in MMR pathway. Our expectations that tumor cells might be also deficient in BER or NER, which would contribute to the malignant transformation of the epithelium, were not fulfilled. Nonetheless, we have shown that sporadic CRC patients are generally less active in NER (as measured in blood), which predispose them to higher genotoxic stress and as such might increase their susceptibility to cancer. This phenomenon was observed for several other types of malignancies, as apparent from the literature.

Despite the undeniable relevance of functional approaches to study DNA repair, DRC is still not routinely included as a biomarker in human biomonitoring studies. This is partially due to the fact that it is rather laborious method, especially when large amount of samples are analyzed. Therefore, the last two aims of this Thesis were of methodological character and were motivated by the need to upgrade the methodology for its wider applicability.

- To implement comet-based repair assays for DRC measurement in human solid tissues

For the first time, we employed and optimized repair assays for both excision repair pathways, BER and NER, on solid tissues of human origin. Our effort enables future addressing of tissue specificity of DNA repair.

- To optimize higher-throughput version of the repair assay to meet criteria required by large epidemiologic studies

We have adopted and optimized the modification of classical 2-gel slide format into 12-gel slide format, which increases 6-times the yield of analyzed samples per microscopic slide. This method can be strongly recommended for routine use.

6. Perspectives

It is becoming more apparent that the complexity of the DNA repair processes and its participation to the individual susceptibility to malignant diseases cannot be easily targeted by single SNP analysis, on a genomic level, or by gene expression analysis, on transcriptome level. Individual susceptibility is assumed to be formed by multiple additive effects of many medium- and low-penetrance gene variants, combined with the influence of the environmental components, which are very variable at the individual level. A common quest for developing the phenotyping approaches that would be able to directly quantify the activity of proteins or activity of whole pathways is inevitably growing. Generally, *in vitro* repair assay is becoming a methodological tool with a clearly high potential for a wide applicability. The method is now, thanks also to the contribution of the present PhD work, universally optimized for any kind of biological specimen: blood, cell suspensions, and solid tissues of any kind and source. There are no special requirements for quality of material; the method might be performed on fresh as well as deeply frozen samples. It is relatively high-throughput (as with the 12-gel format system optimized by us) and not excessively laborious. The epidemiological data obtained by the new modification of the method are reported in **Manuscript V**. Moreover, the detailed methodological protocol, in collaboration with the leader persons in the field, Prof. Andrew Collins and Dr. Sabine Langie, will be presented and discussed in a further manuscript, at present in preparation.

The inter-individual variability of DNA repair seems to be substantially large. Nevertheless, at present we still cannot derive any absolute reference values characterizing the physiological range of DRC in healthy individuals, to be used for further comparisons with other populations with different characteristics (affected by disease, occupationally or environmentally exposed to toxicants or simply of different ethnicity or age/sex composition). The reason is that the studies so far available have used slightly different protocols to measure DRC, with different DNA damage-inducing agent, concentrations, time intervals etc. On the other hand, the average level of SBs in DNA, which was 1 SB/10^7 nucleotides, can be accepted as a reference basal level in cancer-free healthy population, as observed in our studies and by many other groups. SBs are measured by a classic standard protocol for comet assay, which is the same across

laboratories. In the last period, however, there has been an effort dedicated towards an optimization and inter-laboratory validation of the methods to measure DNA repair as well. Prof. Andrew Collins established the ComNet group (Comet Assay Network group) for developing commonly accepted protocols for DRC measurement. Our laboratory participates on this project, which is described in [124].

It is apparent now that the cancer patients may generally suffer from a suboptimal DRC, as shown for BER and NER in many studies. We have demonstrated that the same tendency is valid for sporadic CRC. It might be postulated though that individuals with a DRC far below the population mean are at an increased risk to develop cancer, apparently due to a higher mutational frequency. The disadvantage of these molecular epidemiological studies is that they all investigated DRC in peripheral blood, not showing the same relevance for cancer-target tissues. We have, therefore, conducted a pilot experiment on matched pairs of blood cells and colon mucosa to compare and correlate their levels of DRC, showing that these two tissues positively correlated for BER and NER capacity. This means that if the individual's level of repair in PBMC is below the mean of the studied population (or is lower than the mean of control group), it can be expected that the level of repair in the colon mucosa of this individual will be also lower than would be the population mean. Some other investigators observed the same tendency, however, on a limited study group as was ours. More data on larger populations are needed to solve this important issue, since blood often remains the only tissue available from investigated individuals in human molecular epidemiological studies. The tissue specificity of DNA repair will be of the priority interest in our future studies.

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7. Manuscripts I-V *in extenso*

Manuscript I

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DNA damage and nucleotide excision repair capacity in healthy individuals

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Research Article

DNA Damage and Nucleotide Excision Repair Capacity in Healthy Individuals

Jana Slyskova,[1,2] Alessio Naccarati,[1] Veronika Polakova,[1] Barbara Pardini,[1] Ludmila Vodickova,[1,3] Rudolf Stetina,[4] Jana Schmuczerova,[1] Zdenek Smerhovsky,[3,5] Ludmila Lipska,[6] and Pavel Vodicka[1]*

[1]*Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Science of the Czech Republic, Prague*
[2]*Department of Genetics and Microbiology, Faculty of Science, Charles University, Prague, Czech Republic*
[3]*Department of Toxicogenomics, National Institute of Public Health, Prague, Czech Republic*
[4]*Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic*
[5]*Institute of Epidemiology, 2nd Medical Faculty, Charles University, Prague, Czech Republic*
[6]*Faculty Thomayer Hospital, Prague, Czech Republic*

Interindividual differences in DNA repair capacity (DRC) represent an important source of variability in genome integrity and thus influence health risk. In the last decade, DRC measurement has attracted attention as a potential biomarker in cancer prediction. Aim of the present exploratory study was to characterize the variability in DNA damage and DRC on 100 healthy individuals and to identify biological, lifestyle, or genetic factors modulating these parameters. The ultimate goal was to obtain reference data from cancer-free population, which may constitute background for further investigations on cancer patients. The endogenous DNA damage was measured as a level of DNA single-strand breaks and DRC, specific for nucleotide excision repair (NER), was evaluated using modified comet assay, following the challenge of peripheral blood mononuclear cells with benzo[a]pyrene diolepoxide. Additionally, genetic polymorphisms in NER genes (*XPA*, *XPC*, *XPD*, and *XPG*) were assessed. We have observed a substantial interindividual variability for both examined parameters. DNA damage was significantly affected by gender and alcohol consumption ($P = 0.003$ and $P = 0.012$, respectively), whereas DRC was associated with family history of cancer ($P = 0.012$). The stratification according to common variants in NER genes showed that DNA damage was significantly modulated by the presence of the variant T allele of *XPC* Ala499Val polymorphism ($P = 0.01$), while DRC was modulated by the presence of the A allele of *XPA* G23A polymorphism ($P = 0.048$). Our results indicate the range of endogenous DNA single-strand breaks and capacity of NER in healthy volunteers as well as the role of potentially relevant confounders. Environ. Mol. Mutagen. 52:511–517, 2011.

Key words: BPDE-induced DNA repair capacity; comet assay; interindividual variability; biological and lifestyle characteristics; genetic polymorphism

INTRODUCTION

DNA repair is a crucial mechanism in maintaining genomic stability. Defects in the DNA repair machinery increase cell vulnerability to DNA-damaging agents, accumulation of mutations in the genome, and finally lead to the development of various disorders. The importance of

Abbreviations: BPDE, benzo[a]pyrene diolepoxide, DRC, DNA repair capacity, GM, geometric mean, GSD, geometric standard deviation, MAF, minor allele frequency, NER, nucleotide excision repair, PBMC, peripheral blood mononuclear cells, RT, room temperature, SNPs, single-nucleotide polymorphisms, SSBs, single-strand breaks, TD, tail DNA %.

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*Correspondence to: Pavel Vodicka, Department of Molecular Biology of Cancer, Inst Expt Med, ASCR,, Videnska 1083, 14220 Prague, Czech Republic. E-mail: pvodicka@biomed.cas.cz

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DNA repair is clearly illustrated by monogenic DNA repair deficiency syndromes (such as Xeroderma pigmentosum or Cockayne syndrome etc.); however, mutations responsible for these syndromes are very rare. It is currently estimated that ~150 genes are directly involved in the DNA repair mechanisms [Friedberg, 2003; Hakem, 2008], many of them being polymorphic in the human population. This knowledge has stirred up enthusiastic research to determine whether different genotypes are associated with pathological phenotype, including cancer [Naccarati et al., 2007]. Functional consequences of the majority of DNA repair polymorphisms, mainly single-nucleotide polymorphisms (SNPs), have not been fully characterized yet [Goode et al., 2002; Xi et al., 2004].

Besides heritable polymorphisms in DNA repair genes, DNA repair capacity (DRC) represents an additional source of interindividual variability and therefore it has become attractive as a biomarker in human population studies [Benhamou and Sarasin, 2000; Vodicka et al., 2007]. DRC represents a complex marker comprising the sum of several factors such as gene variants, gene expression, stability of gene products, effect of inhibitors/stimulators, lifestyle and environmental factors [Paz-Elizur et al., 2007].

Nucleotide excision repair (NER) is one of the major repair pathways in humans, responsible for the removal of helix-distorting base lesions produced by ultraviolet light (UV) and a variety of chemical agents [Gillet and Scharer, 2006]. At present, scant data are available on background DRC levels in healthy individuals [Gaivao et al., 2009; Wei et al., 2003; Zhu et al., 2008]. More than 10-fold interindividual differences in NER capacity have been reported, even though these data were collected from relatively small study groups (33 and 57 subjects) [Gaivao et al., 2009; Tyson et al., 2009]. Considering the important role of the DNA repair system, such variability may have a substantial influence on individual susceptibility to sporadic forms of cancer, characterized by gene-environment interactions. Thus, the assessment of DRC levels in healthy populations provides the essential background data for a comparison with potentially altered DRC in cancer patients. Cellular DRC can be measured by using several methods, as reviewed by [Benhamou and Sarasin, 2000; Spitz and Bondy, 2010]. One of the most straightforward approaches for evaluating DNA repair in human cells is the use of a challenge assay, i.e., the determination of the kinetics of DNA breaks or enzyme-sensitive sites disappearance during incubation of cells after treatment with specific damage-inducing agent [Dusinska and Collins, 2008].

We have employed a comet-based challenge assay to measure nucleotide excision DRC in peripheral blood mononuclear cells (PBMC), following chemical stress induced by benzo[a]pyrene diolepoxide (BPDE). The aim of this exploratory study was to analyze BPDE-induced DRC as well as endogenous DNA damage in a group of healthy individuals to assess the background variation in the population. Moreover, routinely interviewed biological and lifestyle characteristics and common variants in important NER genes (*XPA, XPC, XPD,* and *XPG*), considered to have a possible impact on DRC phenotype, have been concurrently investigated.

MATERIALS AND METHODS

Study Subjects and Data Collection

The study was conducted on biological material from healthy Caucasian volunteers who live and have been living in the area of Prague (Czech Republic). The condition for inclusion to the study was a healthy status and the absence of any manifest disease. Additionally, these individuals have not been exposed to any potentially genotoxic or carcinogenic agents from other than environmental sources. No other selection criteria have been applied.

Using detailed questionnaires, all volunteers provided information about gender, age, body weight and height (BMI index), occupational and medical history and lifestyle habits, such as smoking, alcohol consumption (intake of alcohol was calculated as a sum of drinks consumed per day, considering that 25 g of alcohol correspond to 4 mL of spirit, or 2 dL of wine, or 0.5 L of beer). The study population consisted of 52 women and 48 men with a mean age ± SD 41.6 ± 17.5 years, and with a range between 21 and 86 years. Twenty-eight individuals were smokers. Characteristics of the study population are shown in Table I. Each person included in the study signed an informed consent. The Ethics Committee of the Institute for Clinical and Experimental Medicine and Faculty Thomayer Hospital (Prague, Czech Republic) provided ethical approval, based on the Helsinki declaration.

Principle of the Assay

Endogenous DNA damage was determined as a basal level of single-strand breaks (SSBs) in DNA of untreated cells analyzed by alkaline comet assay based on routinely used protocol [Collins, 2004]. The reported parameter represents the mean value of five independent experimental points. BPDE-induced DRC was analyzed as a level of intermediate SSBs in DNA of BPDE-treated cells, originated during the repair of BPDE-DNA adducts by NER pathway. The increase in DNA breaks reflects the ability of NER machinery to recognize and incise corresponding adducts from DNA. We have employed a challenge assay, in which isolated PBMC, mitogen-stimulated in culture medium, were treated with BPDE. After challenge, PBMC were cultured in the medium and harvested after 1, 2, and 4 hr of culturing for being processed by comet assay analysis. Concentrations of genotoxic agent as well as repair time intervals were chosen according to our pilot experiments (data not shown) and [Zheng et al., 2005]. BPDE-induced DRC is reflecting the difference between the level of SSBs measured immediately after the treatment with BPDE at time 0, and the maximum level of SSBs detected within 4 hr of culturing. For each experimental point, the background SSBs level of untreated PBMC was subtracted.

PBMC Culture

Totally, 8 mL of peripheral venous blood from each subject were drawn into heparinised tubes, mixed 1:1 with RPMI 1640 medium (HEPES modification, containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 1.5% phytohemaglutinin and 0.2% penicillin/streptomycin, Sigma-Aldrich), layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 320*g* for 40 min at room temperature (RT). Isolated PBMC were counted and checked for cell viability by trypan

TABLE I. Characteristics of the Study Population

Characteristic	Number of individuals
Gender	$n = 100$
Males	48
Females	52
Age	$n = 100$ (years)
Mean ± SD	41.6 ± 17.5
Median (25–75 percentile)	34 (27–56)
Range	21–86
Smoking status	$n = 100$ (%)
Non-smokers + Ex-smokers	72
Current smokers	28
Number of cigarettes/day[a]	$n = 26$ (%)
≤5	38.5
6–10	38.5
>10	23
Alcohol consumption	$n = 100$ (%)
No	36
Yes	64
Alcohol in grams/day[a]	$n = 98$ (%)
<25	67.2
25–49.9	20.3
50–74.9	10.9
75–100	1.6
Body Mass Index[a]	$n = 91$ (%)
Mean ± SD	24.5 ± 4.2
<18.5	4.4
18.5–24.9	60.4
25.0–29.9	28.6
30.0–39.9	6.6
>40	0
Family history of cancer[a]	$n = 99$ (%)
Positive	48.5
Negative	51.5
Place of residence[a]	$n = 95$ (%)
City	71.6
City + Country	12.6
Country	15.8
Education	$n = 100$ (%)
Basic school	7
High school	35
University	58

[a]Information was not provided by all participants.

blue exclusion. When viability was higher than 95%, cells were aliquoted into cultivation tubes with medium ($\sim 10^5$ cells per 5 mL medium). Tubes were incubated at 37°C. After a mitogen-stimulation period of 20 hr, PBMC were processed for further endogenous DNA damage analysis or challenge assay.

BPDE Challenge and Comet Assay

PBMC cultured in medium were treated by adding (+)-anti-Benzo[a]-pyrene-7,8-dihydrodiol-9,10-epoxide (from A. Seidel, BIU, Germany) in a 1 μM concentration for 30 min at 37°C. After treatment, the medium containing BPDE was removed by a centrifugation at 320*g*, 10 min, RT and fresh medium was added to the pellet. PBMC were further cultured at 37°C for up to 4 hr. After that, cells were separated from the medium by centrifugation (320*g*, 10 min, RT), rewashed with PBS, resuspended in low melting point agarose and layered on microscope slides, followed by lysis for ≥1 hr at 4°C (lysis solution: 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10). In the next step, all slides were treated with alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. Ongoing electrophoresis was carried out at 25 V, 300 mA for 20 min. All slides were then washed twice with neutralizing buffer (0.4M Tris, pH 7.5). For the scoring, slides were stained with ethidium bromide (0.01 ng/μL, 20 μL per slide) and evaluated with fluorescence microscope (N-400 series, Optika microscopes, Italy) using an image analysis software (Lucia 4.82, Laboratory Imaging, Czech Republic). Data are reported as tail DNA% (TD), which was determined for 50 randomly selected cells from two parallel slides per experimental point.

Genotyping

All subjects were genotyped for six polymorphisms in four NER genes: *XPA, XPC, XPD,* and *XPG*. For genotyping *XPD* Lys751Gln (rs28365048), *XPG* Asn1104His (rs17655) and *XPC* Lys939Gln (rs2228001), PCR-RFLP procedure was carried out using primers and conditions described previously [Vodicka et al., 2007]. For *XPC* Ala499Val (rs2228000), primers and conditions are described in [Hu et al., 2005] and for *XPC*-PAT +/− (poly-AT insertion/deletion of 83 bases in intron 9; GenBank accession number AF076952) in [Shore et al., 2008]. *XPA* G23A (rs1800975) has been analyzed with TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA; Assay-on-demand, SNP genotyping products: C_482935_1). The results were regularly confirmed by random re-genotyping of more than 10% of the samples for each polymorphism and yielded concordant results. The genotypes with ambiguous and/or no results were excluded from the data set. The set of investigated SNPs was not feasible or successful to analyze for all individuals, the final number of observations for each SNP is shown in related table.

Statistical Analysis

DRC net data (background level of SSBs was subtracted at each experimental point) are expressed as geometric mean (GM) ± geometric standard deviation (GSD). The asymmetric distribution of the investigated DNA damage and repair parameters in the study population was normalized by logarithmic transformation. Genotype frequencies for each polymorphism were tested for compliance with Hardy-Weinberg equilibrium. The relationships between variables of interest at the bivariate level were studied by means of T-test, ANOVA and Pearson correlation. The multivariate linear regression was employed to study the simultaneous effect of genotypes, age, gender, smoking habit, alcohol consumption, BMI and family history of cancer on the logarithmically transformed endogenous DNA damage and DRC. All statistical tests were performed at 5% level of statistical significance. The SPSS analytical package version 16.0 (Chicago, IL, USA) and SAS JMP 8 (NC, USA) were employed for all statistical analyses.

RESULTS

Interindividual Variability in the Study Population

We have observed a large interindividual variability of both analyzed parameters within the study group. For endogenous DNA damage, the level of SSBs ranged from 0.3 to 26.5 TD, with GM ± GSD being 7.4 ± 6.5 TD. Individual levels of SSBs, reflecting BPDE-induced DRC, ranged from 0 to 76.0 TD, the GM ± GSD values were 19.2 ± 14.0 TD (Fig. 1).

Relations to Biological and Lifestyle Characteristics

The stratification of endogenous DNA damage for main biological and lifestyle characteristics (Table II) showed a

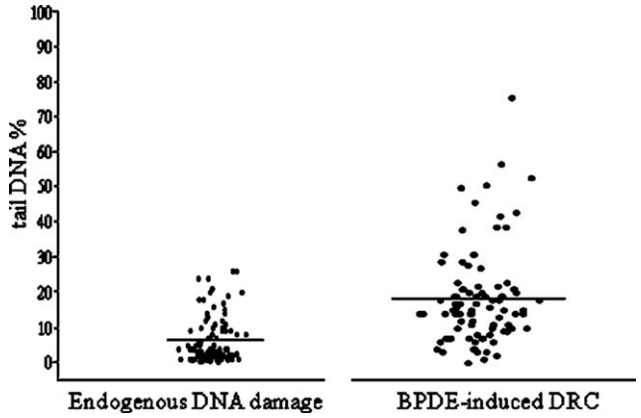

Fig. 1. Interindividual variability of endogenous DNA damage and BPDE-induced DNA repair capacity of 100 individuals. The lines represent geometric mean in the study population.

significant difference between men and women ($P = 0.003$): the GM for DNA damage in women (4.6 ± 5.0 TD) was lower than that in men (8.4 ± 7.2 TD). A significant difference was detected between alcohol consumers and teetotalers (GM 7.8 ± 7.0 TD vs. 4.0 ± 4.6 TD; $P = 0.012$). Moreover, there was a positive correlation between alcohol intake (in grams per day) and DNA damage ($R = 0.225$, $P = 0.023$). Distribution of gender among groups characterized for alcohol consumption was as follows: among alcohol consumers ($N = 64$), 37.5% were women and 62.5% men, whereas among teetotalers ($N = 36$), 77.8% were women and 22.2% men. Gender and alcohol contributed as independent predictors of the outcome, as proved by multivariate regression model. There was no significant difference of DNA damage between smokers and nonsmokers (GM 6.9 ± 6.0 TD vs. 6.3 ± 6.7 TD, respectively) and no association with age ($R = -0.078$, $P = 0.448$).

BPDE-induced DRC was neither affected by age ($R = -0.051$, $P = 0.137$) nor was associated with any of the investigated factors, except for family history of cancer (FHC). Individuals with a positive FHC exhibited higher BPDE-induced DRC than individuals without any cancer in the family (positive FHC: GM 21.8 ± 15.3, negative FHC: 15.1 ± 12.5; $P = 0.012$).

Relations to the Genotype Background

We examined whether endogenous DNA damage and DRC were modulated by any of six polymorphisms in four NER genes: *XPA* G23A, *XPC* Lys939Gln, *XPC* Ala499Val, *XPC*-PAT +/−, *XPD* Lys751Gln and *XPG* Asn1104His (Table III). Considering the size of our study population, SNPs were chosen according to the minor allele frequency (MAF > 0.25), and according to the expected effect on DRC phenotype based on [Friedberg, 2006]. Distribution of the genotypes was in agreement with the Hardy-Weinberg equilibrium. We have observed

TABLE II. Endogenous DNA Damage and BPDE-Induced DNA Repair Capacity Stratified for Biological and Lifestyle Characteristics

	Endogenous DNA damage[a]				BPDE-induced DRC[a]			
Characteristic	*N*	GM	GSD	P^b	*N*	GM	GSD	P^b
Gender								
Women	52	4.6	5.0		52	18.0	14.0	
Men	48	8.4	7.2	0.003	48	18.3	14.5	0.857
Age								
≤34 (below median)	51	7.9	7.7		51	19.8	12.6	
>34 (above median)	49	6.9	7.3	0.422	49	18.5	17.3	0.654
Smoking status								
Smokers	28	6.9	6.0		28	21.3	15.3	
Nonsmokers	72	6.3	6.7	0.445	72	17.0	13.4	0.147
Alcohol consumption								
Consumers	64	7.8	7.0		64	19.2	13.6	
Teetotalers	36	4.0	4.6	0.012	36	16.4	15.0	0.258
Family history of cancer								
Positive	48	7.4	7.0		48	21.8	15.3	
Negative	47	5.7	6.2	0.256	47	15.1	12.5	0.012

[a]Data are expressed as geometric mean (GM) and geometric standard deviation (GSD) of tail DNA% values.
[b]ANOVA for differences in mean values in studied variables.

a significant association between endogenous DNA damage and *XPC* Ala499Val (C→T) polymorphic site. Individuals with variant TT genotype exhibited the highest DNA damage ($P = 0.042$), but even the presence of one copy of the T allele was significantly associated with an increased DNA damage ($P = 0.011$). This finding was also supported by a regression model, where *XPC* Ala499Val significantly affected the DNA damage along with gender ($P = 0.03$ and $P = 0.0007$, respectively). BPDE-induced DRC was associated with *XPA* G23A genotype; the presence of the variant A allele was associated with reduced DRC ($P = 0.048$) (Table III).

DISCUSSION

DRC can be considered as a useful marker for studying the maintenance of DNA integrity in biomonitoring studies, as well as a transient susceptibility marker in carcinogenesis. Several approaches have been conducted to explore DNA repair process in relation to certain diseases, exposure to environmental or occupational pollution or in healthy populations. However, there are still scant data addressing substantial questions, such as how much healthy individuals vary in their repair capacity, what range of variability can still be considered as a normal distribution, and whether the variation is influenced by genetic polymorphisms [Collins and Gaivao, 2007].

In this study, we report an evaluation of DNA damage and NER capacity in 100 healthy individuals, characterized for major biological and lifestyle factors and genetic background. Our experimental protocol, based on a modi-

TABLE III. Endogenous DNA Damage and BPDE-Induced DNA Repair Capacity Stratified for Analyzed NER Gene Polymorphisms

		Frequency		Endogenous DNA damage[a]			BPDE-induced DRC[a]		
Polymorphism	Genotype	*N*	%	GM	GSD	*P*[b]	GM	GSD	*P*[b]
XPD Lys751Gln (*N* = 94)	*AA*	23	24.5	6.5	7	0.546	15.0	10.1	0.373
	AC	48	51	5.5	5.3		18.5	14.8	
	CC	23	24.5	7.9	7.3		22.7	16.1	
	AC+CC	71	75.5	6.3	6.1	0.820	19.8	15.2	0.347
XPG Asn1104His (*N* = 98)	*GG*	54	55	6.9	6.2	0.378	20.5	15.9	0.492
	GC	39	40	5.8	6.6		15.7	11.7	
	CC	5	5	8.8	8.7		14.7	7.8	
	GC+CC	44	45	6.2	6.5	0.476	15.6	11.3	0.239
XPC Lys939Gln (*N* = 98)	*AA*	38	38.8	6.6	6.3	0.538	19.1	14.7	0.893
	AC	46	47	6.9	6.8		18.8	15.3	
	CC	14	14.2	5.3	6.0		14.5	7.6	
	AC+CC	60	61.2	6.5	6.6	0.767	17.8	13.9	0.806
XPC Ala499Val (*N* = 91)	*CC*	57	62.6	5.3	5.2	0.042	16.6	12.4	0.369
	CT	32	35.2	8.6	7.6		22.7	17.4	
	TT	2	2.2	10.0	11.3		16.7	3.6	
	CT+TT	34	37.4	8.7	7.6	0.011	22.3	16.9	0.156
XPC-PAT +/− (*N* = 88)	+/+	48	54.6	7.4	7.1	0.396	19.9	14.9	0.306
	+/−	32	36.4	6.4	5.9		17.7	13.6	
	−/−	8	9	4.0	3.9		11.0	6.3	
	+/− and −/−	40	45.4	7.1	5.6	0.405	16.4	12.7	0.395
XPA G23A (*N* = 98)	*GG*	47	48	6.9	6.8	0.720	19.9	14.0	0.084
	GA	40	41	6.3	6.2		16.6	15.3	
	AA	11	11	6.1	6.6		17.6	10.9	
	GA+AA	51	52	6.2	6.2	0.420	16.8	14.3	0.048

[a]Data are expressed as geometric mean (GM) and geometric standard deviation (GSD) of tail DNA% values.
[b]ANOVA for differences in mean values among studied genotypes.

fied version of the comet assay, introduces some positive aspects. DRC was analyzed from fresh blood, thus the impact of cryopreservation does not interfere with the results, and was examined within an intact cell system. Furthermore, a rather low intraindividual variability in DRC was detected, when assayed for eight individuals sampled twice within six months period ($R = 0.762$, $P = 0.028$).

We have observed a substantial interindividual variability in both examined parameters, in DNA damage as well as DRC. The levels of SSBs that represents NER-mediated breaks originated from BPDE-adducts removal varied from 0 to 76 TD. To our knowledge, there is no study using similar experimental conditions reporting DNA repair variability on sufficiently large population to be confronted with our findings. However, even if some variability may arise from interexperimental discrepancies, observed differences in BPDE-induced DRC may reflect the normal variation in healthy population. In such a case, DNA adducts recognition and removal may be expected to play a serious role in maintaining the homeostasis of the organism.

The mean level of SSBs reported as endogenous DNA damage was 7.4 ± 6.5 TD. By expressing TD value in SSBs/10^6 nucleotides [Collins et al., 1996; Collins et al., 2008], we may assume that healthy individuals in our study group bear 0.1 SSBs/10^6 nucleotides in their DNA (~300 breaks/cell). These breaks, measured by alkaline comet assay, are of different origin and might represent single-strand breaks, alkali-labile DNA adducts, oxidized bases, abasic sites, repair intermediates or breaks associated with replication [Dusinska and Collins, 2008]. Moller [2006] pooled results from 125 studies to assess reference level of SSBs in healthy populations, measured by comet assay in human blood cells, and estimated the median being 8.6 TD. As DNA damage was found to be different according to the geographical latitude, the comparison with data obtained from the Czech population alone is more relevant to our study and is in accordance with those reported (6.5 TD; 0.09 SSBs/10^6 nucleotides; 270 breaks/cell) [Moller, 2006].

In our study, the level of endogenous DNA damage was significantly affected by gender. A lower DNA damage in women, reported also by [Bajpayee et al., 2002; Hofer et al., 2006], can partially be explained by action of estrogens which bind to estrogen receptors and increase the expression of various genes, including those encoding antioxidant enzymes. As a result, mitochondria in females produce fewer reactive oxygen species [Baltgalvis et al., 2010; Strehlow et al., 2003; Vina et al., 2005].

Endogenous DNA damage also positively correlated with alcohol consumption and this is in agreement with previous studies [Weng et al., in press; Zhu et al., 2000]. Interestingly, the levels of DNA damage increased with the increasing quantity of consumed alcohol, as estimated in grams per day. This might be the effect of the highly

reactive, oxygen-containing molecules, generated during alcohol metabolism, e.g., acetaldehyde. Acetaldehyde is a possible human carcinogen, acting through multiple mechanisms, such as induction of DNA damage and interference with DNA replication [IARC, 1999; Seitz and Becker, 2007]. The role of polymorphisms in alcohol metabolizing genes (*ALDH* and *ADH* families) would also be of interest in this context. However, in this study with a limited number of volunteers consuming alcohol, screening of variations in above genes would have inevitably resulted in reduction of statistical power of the outcome. Therefore, study remained focused on genetic variation in NER genes in particular.

The levels of endogenous DNA damage were not affected by smoking habit, as reported also by [Hecht, 1999]. On the contrary, several recent studies reported an effect of smoking on the levels of DNA damage [Hoffmann et al., 2005; Kopjar et al., 2006]. The lack of association in our population could be caused by a low number of strong smokers, whereas sporadic and moderate smokers prevailed.

DRC was not significantly affected by any of the investigated biological and lifestyle factors. However, individuals with cancer family anamnesis exhibited significantly higher DRC. Only scarce and contradictory data are available investigating this parameter along with DNA repair, at present. In contrast with our findings, Li and colleagues [Li et al., 2009] reported lower DRC in individuals with positive FHC, suggesting that this subgroup may in particular be susceptible to cancer. Interestingly, in our study population neither DNA damage, nor DRC were affected by age. Although the influence of age on investigated parameters is often inconclusive, several studies have suggested inverse relation between age and DRC [Moller, 2006; Weng et al., 2009]. Even if the age range in our study population was large, 50% of individuals were 34 years old or younger, and such distribution might have obscured an effect of age on the DRC.

Significant associations have emerged between DNA damage, BPDE-induced DRC and variants in *XPC* and *XPA* genes, both encoding enzymes involved in the preincision complex of NER [Nouspikel, 2009]. In our study, carriers of variant T allele in *XPC* Ala499Val polymorphism exhibited higher DNA damage, but no significant association of this SNP with DRC was observed. However, a more efficient DRC in association with the T allele of this particular polymorphism was found by other groups [Shen et al., 2006; Zhu et al., 2008]. The variant A allele of the *XPA* G23A SNP was related with a reduced DRC and the same observation was reported by other investigators [Langie et al., 2010; Lin et al., 2007; Wu et al., 2003]. Moreover, homozygous AA genotype for this SNP was associated with an increased risk for lung cancer [Butkiewicz et al., 2004; Kiyohara and Yoshimasu, 2007]. All these observations may define the *XPA* 23A allele as a low activity allele. The associations between NER polymorphisms and DRC are still difficult to interpret at present, since few DRC studies have been carried out on sufficiently large populations, enabling detection of subtle (if any) effects of individual SNPs. Besides, different approaches of addressing DRC have been employed so far, hampering a straight comparison of results among studies. The involvement and relevance of genetic variation in NER phenotype remain to be further explored.

CONCLUSIONS

We have observed a relevant variability in response of PBMC to BPDE-induced DNA damage, most likely reflecting interindividual differences in DRC among individuals, which deserves to be further investigated. The level of endogenous DNA damage found in our study population is in agreement with results reported by other laboratories and might be considered as a reference background. Possible modulating effects of biological factors, lifestyle habits and SNPs in NER genes have been addressed. Interestingly, the conformity of available data investigating *XPA* G23A polymorphism along with DRC may determine this particular SNP as a predictor for NER capacity. We postulate that BPDE-induced NER capacity may serve as a useful complex biomarker for providing background data on healthy individuals. This may represent a starting point for assessing DRC in association with cancer risk and, finally, for an estimation of the efficiency of the anticancer therapy and prognosis. In this context, the question whether DRC in surrogate tissue reflects DRC in target tissue should also be addressed.

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Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects

Pavel Vodicka[1,*], Rudolf Stetina[2], Veronika Polakova[1], Elena Tulupova[1], Alessio Naccarati[1], Ludmila Vodickova[1,3], Rajiv Kumar[4,5], Monika Hanova[1], Barbara Pardini[1,6], Jana Slyskova[1], Ludovit Musak[7], Giuseppe De Palma[8], Pavel Soucek[3] and Kari Hemminki[4,5]

[1]Institute of Experimental Medicine, Videnska 1083, 14200, Academy of Sciences of the Czech Republic, Prague, Czech Republic, [2]Purkynje Military Medical Academy, Hradec Kralove, Czech Republic, [3]Center of Occupational Medicine, National Institute of Public Health, Prague, Czech Republic, [4]Division of Molecular Genetic Epidemiology, German Cancer Research Center, Heidelberg, Germany, [5]Department of Biosciences at Novum, Karolinska Institute, Huddinge, Sweden, [6]Department of Biology, University of Pisa, Italy, [7]Department of Medical Biology, Jessenius Medical Faculty, Comenius Univesity Martin, Slovak Republic and [8]Laboratory of Industrial Toxicology, Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, Italy

*To whom correspondence should be addressed. Tel: +420 2 41062694; Fax: +420 2 41062782;
Email: pvodicka@biomed.cas.cz

We investigated association between polymorphisms in DNA repair genes and the capacity to repair DNA damage induced by γ-irradiation and by base oxidation in a healthy population. Irradiation-specific DNA repair rates were significantly decreased in individuals with *XRCC1* Arg399Gln homozygous variant genotype (0.45 ± 0.47 SSB/10^9 Da) than in those with wild-type genotype (1.10 ± 0.70 SSB/10^9 Da, $P = 0.0006$, Mann–Witney *U*-test). The capacity to repair oxidative DNA damage was significantly decreased among individuals with *hOGG1* Ser326Cys homozygous variant genotype (0.37 ± 0.28 SSB/10^9 Da) compared to those with wild-type genotype (0.83 ± 0.79 SSB/10^9 Da, $P = 0.008$, Mann–Witney *U*-test). Investigation of genotype combinations showed that the increasing number of variant alleles for both *XRCC1* Arg399Gln and *APE1* Asn148Glu polymorphisms resulted in a significant decrease of irradiation-specific repair rates ($P = 0.008$, Kruskal–Wallis test). Irradiation-specific DNA repair rates also decreased with increasing number of variant alleles in *XRCC1* Arg399Gln in combination with variant alleles for two other *XRCC1* polymorphisms, Arg194Trp and Arg280His ($P = 0.002$ and $P = 0.005$, respectively; Kruskal–Wallis test). In a binary combination variant alleles of *hOGG1* Ser326Cys and *APE1* Asn148Glu polymorphisms were associated with a significant decrease in the capacity to repair DNA oxidative damage ($P = 0.018$, Kruskal–Wallis test). In summary, *XRCC1* Arg399Gln and *hOGG1* Ser326Cys polymorphisms seem to exert the predominant modulating effect on irradiation-specific DNA repair capacity and the capacity to repair DNA oxidative damage, respectively.

Abbreviations: BER, base excision DNA repair; SSB, single-strand breaks; SNP, single nucleotide polymorphism; PBL, peripheral blood lymphocytes.

Introduction

In the recent years, several studies have investigated polymorphisms in DNA repair genes and their possible links to the risk of various cancers. Sequence variants in DNA repair genes are assumed to modulate DNA repair capacity and, therefore, are associated with the altered cancer risk. As an example, the *hOGG1* Cys/Cys genotype has been associated with an increased lung cancer risk (1). In tobacco-related cancers a protective effect of *XRCC1* Arg194Trp variant allele was shown, while variant allele in *XRCC1* Arg399Gln polymorphism was associated with an increased risk among light smokers only (2). An increased risk of colorectal cancer was recently reported for *XRCC1* Arg399Gln variant allele (3). Statistically significant associations have been found between *XPD* polymorphisms and skin, breast and lung cancers [reviewed by ref. (4)]. Increasing number of studies relating genetic polymorphisms in DNA repair genes and various kinds of cancer in the past 5 years do not provide unambiguous consistent associations, mainly due to low statistical power for detecting a moderate effect, false-positive results, heterogeneity across study populations (5), failure to consider effect modifiers such as environmental exposures (6) and, most importantly, due to the virtually unknown relationship between the genotype and the functional outcome (phenotype) (7).

An analysis of SNPs in 88 DNA repair genes and their functional evaluation, based on the conservation of amino acids among the protein family members, shows that ~30% of variants of DNA repair proteins are likely to affect substantially the protein function. It applies particularly for polymorphisms in *XRCC1* Arg280His and Arg399Gln, and *XRCC3* Thr241Met (8). Susceptibility towards ionizing radiation, as measured by prolonged cell cycle G_2 delay, was determined in relation to *XRCC1* Arg194Trp, Arg399Gln and *APE1* Asn148Glu genotypes. Ionizing radiation sensitivity was significantly affected by amino acid substitution variants in both *XRCC1* and *APE1* genes (9). Using the cytogenetic challenge assay, *XRCC1* 399Gln and *XRCC3* 241Met alleles were associated with significant increase in chromosomal deletions as compared with the corresponding homozygous wild-types. Authors concluded that *XRCC1* 399Gln and *XRCC3* 241Met are significantly defective in base excision repair (BER), while *XPD* 312Asn and *XPD* 751Gln are significantly defective in nucleotide excision repair (NER) (10). Individuals with the wild-type Arg/Arg genotype in *XRCC1* Arg194Trp polymorphism exhibited significantly higher values of chromosomal breaks, as assessed by the mutagen sensitivity assay, than those with variant Trp allele, suggesting a protective effect of this allele. On the other hand, variant Gln allele in *XRCC1* Arg399Gln was significantly associated with an increase in chromosomal breaks per cell. These data are biologically plausible, since codon 399 is located within the *BRCA1* C-terminus

functional domain and codon 194 is in the linker region of the *XRCC1* N-terminal functional domain (11). Three studies using different approaches have found a functional impact of *hOGG1* Ser326Cys polymorphism (12–14), but other studies [reviewed by ref. (1)] did not find any conclusive result for *hOGG1* genetic polymorphisms. *hOGG1* Ser326Cys polymorphism has also been described to affect the glycosylase function due to the localization and phosphorylation status (15). The results of such tests allow a more meaningful choice of genes for association studies, though they are still not sufficient for an accurate prediction for the DNA repair capacity.

In the present report we attempt to investigate associations between DNA repair genetic polymorphisms (*XPD* Lys751Gln, *XPG* Asn1104His, *XPC* Lys939Gln, *XRCC1* Arg194Trp, Arg280His and Arg399Gln, *APE1* Asn148Glu, *hOGG1* Ser326Cys *XRCC3* Thr241Met and *NBS1* Glu185Gln) and individual DNA repair activity in a general healthy population from the Central Europe, assessing *in vitro* the capacity to repair both irradiation-specific induced- and oxidative-induced DNA damage. In the former case, the comet assay (single cell alkaline gel electrophoresis) has been modified to measure the ability of lymphocytes to repair γ-irradiation induced single-strand breaks (SSBs) after 40 min of incubation (16), and in the latter, to measure the ability of a subcellular extract of lymphocytes to carry out the initial incision step of repair on a DNA substrate carrying specific lesions-namely, oxidized bases introduced by visible light in the presence of photosensitizer (17).

Materials and methods

Study population

The study was conducted on 244 healthy individuals (183 men and 61 women, mean age 41.3 ± 11.3 years, 90 individuals were smokers and 154 non-smokers) employed in local administration, medical centers and various branches of plastic industry. The investigated population was recruited in the regions of western Slovakia and eastern Bohemia, which exhibit close similarities in socio-economical conditions. Confounding factors, like X-rays, medical drug treatments, dietary (vitamins intake, particular diets) and lifestyle habits (smoking, alcohol and coffee consumptions) and possible exposure-related effects were recorded in detailed questionnaires and considered in the statistical analyses. Present cohort is representative, ethnically homogenous population and therefore suitable for the determination of relationships between DNA repair genetic polymorphisms and DNA repair rates. Lower number of observations for DNA repair rates, in comparison to that given in Table I, were due to methodological limitations (i.e. successful processing of the fresh material). The study design was approved by the local Ethical Committee and the participants provided their informed consent to be included in the study. The sampling of blood was carried out according to the Helsinki Declaration.

DNA repair polymorphisms

Single nucleotide polymorphisms (SNPs) in genes encoding DNA repair enzymes were determined by a PCR–RFLP based method. PCR products were generated using 50 ng of genomic DNA in 25 μl volume reactions containing 20 mM Tris–HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.3 mM each dNTP, 0.3 μM each primer (Table I) and 0.2 U *Taq* DNA polymerase. The temperature conditions for PCR were established as denaturation at 94°C for 30 s, annealing (given in Table I) for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 5 min. The amplified fragments were digested with appropriate restriction endonucleases (Table I) and analyzed. The digested PCR products were resolved on 3% agarose gels containing ethidium bromide and visualized under UV light. The genotype results were regularly confirmed by re-genotyping (10% of samples) and by TaqMan allelic discrimination assay (Assay-on-Demand', Applied Biosystems, Foster City, USA), using Real-Time Gene Amp PCR system on AB 7500 equipment (Applied Biosystems, Foster City, USA). The concordance rate was 100%.

γ-irradiation DNA repair test

Peripheral blood lymphocytes (PBL), isolated using Ficol gradient, were used to test individual DNA repair capacity as described previously (18,19). Briefly, cells embedded in agarose on slides were irradiated with 5 Gy of γ-rays (0.42 Gy/min) and either lysed immediately or incubated at 37°C for 40 min before the lysis. The DNA breaks induced by γ-rays are repaired during the 40 min of incubation period, according to the individual repair capacity. The results (i.e. the amount of repaired SSBs) are calculated as a difference between the initial levels of SSBs, measured immediately after irradiation, and the levels of SSBs detected after 40 min of incubation. The repaired DNA damage is subsequently expressed as SSB/10^9 Da. Consequently, higher values of repaired SSBs reflect higher DNA repair activity. The detailed description of the tentative origin γ-ray-induced DNA

Table I. Details on investigated SNPs in DNA repair genes

Genetic polymorphism	Exon	Primer sequence	Annealing temp. (°C)	Restriction enzyme	Genotype distribution	Frequency of variant allele
Base-excision repair						
XRCC1 Arg194Trp	6	F GCC CCG TCC CAG GTA R AGC CCC AAG ACC CTT TCA CT	63	*Msp*I	CC TC TT 184 30 2	qT = 0.078
XRCC1 Arg280His	9	F TTG ACC CCC AGT GGT GCT R CCC TGA AGG ATC TTC CCC AGC	57	*Rsa*I	GG GA AA 202 13 1	qA = 0.035
XRCC1 Arg399Gln	10	F GCC CCT CAG ATC ACA CCT AAC R CAT TGC CCA GCA CAG GAT AA	65	*Msp*I	GG GA AA 104 112 18	qA = 0.316
hOGG1 Ser326Cys	7	F AGT GGA TTC TCA TTG CCT TCG R GGT GCT TGG GGA ATT TCT TT	59	*Fnu*4HI	CC CG GG 154 75 13	qG = 0.209
APE1 Asn148Glu	5	F CTG TTT CAT TTC TAT AGG CTA R AGG AAC TTG CGA AAG GCT TC	59	*Bfa*I	TT TG GG 88 112 35	qG = 0.387
Nucleotide-excision repair						
XPD Lys751Gln	23	F CCC CTC TCC CTT TCC TCT GTT R GCT GCC TTC TCC TGC GAT TA	60	*Pst*I	AA AC CC 65 138 36	qC = 0.439
XPG Asn1104His	15	F TGG ATT TTT GGG GGA GAC CT R CGG GAG CTT CCT TCA CTG AGT	56	*Hsp*92II	GG GC CC 114 102 11	qC = 0.273
XPC Lys939Gln	15	F GAT GCA GGA GGT GGA CTC TCT R GTA GTG GGG CAG CAG CAA CT	61	*Pvu*II	AA AC CC 83 110 41	qC = 0.410
Double-strand break repair						
XRCC3 Thr241Met	7	F GCT CGC CTG GTG GTC ATC R CTT CCG CAT CCT GGC TAA AAA	59	*Hsp*92II	CC CT TT 71 121 36	qT = 0.423
NBS1 Glu185Gln	5	F GGA TGT AAA CAG CCT CTT G R CAC AGC AAC TAT TAC ATC CT	59	*Hinf*I	GG GC CC 89 122 25	qC = 0.364

damage as well as the calibration and optimization of the repair test have already been described in details elsewhere (17).

Oxidative DNA repair test

The repair capacity of PBL extracts towards repairing 8-oxoguanine was determined as previously described (20). Briefly, isolated lymphocytes from each individual were collected and divided into aliquots and stored in liquid nitrogen at −80°C, until experiment. Before an assay, a frozen aliquot was thawed and washed with 1% Triton X-100 in a lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8) and the lysate was centrifuged to remove nuclei and cell debris. The supernatant was mixed with a reaction buffer (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml BSA pH 7.8) and kept on ice until use.

A substrate of HeLa cells (2×10^5 per dish) was prepared and pretreated with 2 ml 0.1 μM phosphosensitizer Ro 19-8022 (Hoffmann-La Roche, Basel, Switzerland) and PBSG, and irradiated with a fluorescent lamp (2 min on ice from a 1000 W tungsten halogen lamp, to induce 8-oxoguanines). HeLa cells were successively washed, removed from dishes by gentle trypsinization and embedded in agarose on slides and placed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris made to pH 10 with NaOH, and 1% Triton X-100) for 1 h at 4°C. After lysis slides are incubated either with individual PBL extracts or with buffer alone at 37°C for 45 min, followed by electrophoresis and neutralization according to comet assay standard protocol (20).

The results (i.e. the amount of repaired oxidative DNA damage, reflecting the removal of 8-oxoguanines) are calculated as a difference between the levels of SSBs, measured in slides with PBL extract and the levels of SSBs measured in slides with buffer only. The level of SSBs, is expressed as SSB/10^9 Da.

Statistical analyses

Statistical calculations were performed using Statgraphics, version 7 (Manugistics Inc., Cambridge, MA). Hardy–Weinberg equilibrium was tested using the chi-square 'goodness-of-fit' test. The data for both DNA repair assays, given in Tables II–VI, are expressed as mean ± SD. For testing significant differences between groups, specifically Table II, the non-parametrical Mann–Whitney *U*-test was applied. Associations between the combined genotypes and DNA repair rates were tested by Kruskal–Wallis test (as shown in Tables III–VI). Simple linear regression analysis was used to estimate the correlation between confounder and DNA repair rates, whereas multifactorial regression analysis was applied to discern the major influencing factors on the DNA repair rates (i.e. analyzing main confounding factors and DNA repair polymorphisms simultaneously).

For statistical analyses non-smokers as well as males were assigned as '0', while smokers and females as '1', age was calculated as continuous variable. Similarly, for statistical analyses the wild-type genotype was assigned as '0', heterozygous variant allele bearers as '1' and homozygous variant allele bearers as '2'.

Evaluation of DNA repair rates in relation to gene–gene interactions, when three and more polymorphisms were considered, was based on the construction of arbitrary score for variant allele. Wild-type allele was assigned as '0', heterozygous variant allele as '1' and homozygous variant allele as '2'. The above approach does not discriminate all possible genotype combinations (i.e. 27 theoretically possible outcomes in ternary and 81 in quaternary combinations), but takes into account a number of variant alleles in particular combination and the higher score reflects the higher number of variant alleles in either genes.

Results

Genotype distribution

The genotype distributions for individual DNA repair genes are shown in Table I. Allelic frequencies in *XPD* Lys751Gln, *XPG* Asn1104His, *XPC* Lys939Gln, *XRCC1* Arg399Gln, *XRCC3* Thr241Met, *NBS1* Glu185Gln and *APE1* Asn148Glu are in agreement with those earlier described for the central European population (21,22), while allelic frequencies in *hOGG1* Ser326Cys, *XRCC1* Arg194Trp and Arg280His for the same population are shown for the first time (Table I). The genotype distribution for all investigated polymorphisms, except for *XPD* Lys751Gln ($\chi^2 = 7.0$, $P = 0.01$), was in the Hardy–Weinberg equilibrium.

Table II. Irradiation-specific DNA repair rates and oxidative DNA damage repair rates stratified for individual DNA repair polymorphisms

Genotypes	Irradiation-specific DNA repair rates (SSNs/10^9 Da)	Oxidative DNA damage repair rates (SSBs/10^9 Da)
XRCC1 Arg399Gln		
GG	1.10 ± 0.70[a] ($n = 92$)	0.75 ± 0.69 ($n = 88$)
GA	0.76 ± 0.69[a] ($n = 103$)	0.76 ± 0.87 ($n = 95$)
AA	0.45 ± 0.47 ($n = 17$)	0.75 ± 0.41 ($n = 14$)
XRCC1 Arg280His		
GG	0.86 ± 0.74 ($n = 183$)	0.72 ± 0.69 ($n = 169$)
GA	0.90 ± 0.65 ($n = 12$)	0.44 ± 0.49 ($n = 12$)
AA	1.34 ($n = 1$)	0.5 ($n = 1$)
XRCC1 Arg194Trp		
CC	0.88 ± 0.73 ($n = 167$)	0.68 ± 0.63 ($n = 155$)
CT	0.87 ± 0.74 ($n = 27$)	0.77 ± 0.95 ($n = 26$)
TT	0.89 ± 0.72 ($n = 2$)	0 ($n = 1$)
APE1 Asn148Glu		
TT	0.95 ± 0.82 ($n = 75$)	0.79 ± 0.85 ($n = 72$)
TG	0.83 ± 0.65 ($n = 100$)	0.73 ± 0.68 ($n = 96$)
GG	0.86 ± 0.61 ($n = 32$)	0.68 ± 0.69 ($n = 26$)
hOGG1 Ser326Cys		
CC	0.88 ± 0.68 ($n = 143$)	0.83 ± 0.79[a] ($n = 130$)
CG	0.90 ± 0.78 ($n = 63$)	0.61 ± 0.67[a] ($n = 64$)
GG	0.66 ± 0.76 ($n = 12$)	0.37 ± 0.28 ($n = 13$)
XPD Lys751Gln		
AA	0.87 ± 0.63 ($n = 57$)	0.61 ± 0.66 ($n = 55$)
AC	0.86 ± 0.72 ($n = 123$)	0.86 ± 0.84 ($n = 119$)
CC	0.91 ± 0.81 ($n = 35$)	0.52 ± 0.47 ($n = 29$)
XPG Asn1104His		
GG	0.83 ± 0.70 ($n = 98$)	0.79 ± 0.80 ($n = 90$)
GC	0.94 ± 0.73 ($n = 95$)	0.72 ± 0.72 ($n = 93$)
CC	0.83 ± 0.45 ($n = 11$)	0.76 ± 1.01 ($n = 10$)
XPC Lys939Gln		
AA	0.82 ± 0.70 ($n = 77$)	0.86 ± 0.87 ($n = 67$)
AC	0.86 ± 0.65 ($n = 96$)	0.68 ± 0.69 ($n = 94$)
CC	1.00 ± 0.86 ($n = 36$)	0.73 ± 0.72 ($n = 38$)
XRCC3 Thr241Met		
CC	0.89 ± 0.66 ($n = 65$)	0.79 ± 0.69 ($n = 57$)
CT	0.85 ± 0.70 ($n = 105$)	0.76 ± 0.79 ($n = 101$)
TT	0.77 ± 0.74 ($n = 34$)	0.73 ± 0.84 ($n = 35$)
NBS1 Glu185Gln		
GG	0.89 ± 0.60 ($n = 79$)	0.82 ± 0.75 ($n = 73$)
GC	0.81 ± 0.72 ($n = 111$)	0.66 ± 0.69 ($n = 102$)
CC	1.12 ± 0.99 ($n = 23$)	0.68 ± 0.72 ($n = 25$)

The results are presented as mean ± SD.
[a]$P < 0.05$. The comparison between individual genotypes was performed by Mann–Whitney *U*-test.

DNA repair rates and confounders

Both irradiation-specific DNA repair rates and the capacity to repair of oxidative DNA damage were not affected by age and there was no significant difference in both DNA repair rates between men and women. Irradiation-specific DNA repair rates were significantly higher among smokers (1.05 ± 0.81 SSB/10^9 Da) as compared to non-smokers (0.77 ± 0.62 SSB/10^9 Da, $P = 0.014$, Mann–Whitney *U*-test), while the capacity to repair of oxidative DNA damage was not affected by smoking habit. By investigating simultaneous influence of genotypes in DNA repair and recorded confounders (age, sex, exposure status and smoking), irradiation-specific DNA repair rates were mainly affected by polymorphism in *XRCC1* Arg399Gln gene ($t = -4.54$, $P < 0.001$), and also by smoking ($t = 2.92$, $P = 0.004$, $R^2 = 0.132$; multiple regression analysis). Figure 1 shows the lowest irradiation-specific DNA repair rates being associated with homozygous variant *AA XRCC1* Arg399Gln genotype both in smokers and non-smokers, although only in non-smokers the difference in

Table III. Effect of selected binary combinations of SNPs of BER genes on irradiation-specific DNA repair rates (expressed as SSBs/10^9 Da)

	APE1 Asn148Glu genotype			
	TT	TG	GG	χ^2, *P*
(A) *XRCC1* Arg399Gln genotype				
GG	1.14 ± 0.77 (46)	1.00 ± 0.67 (30)	1.10 ± 0.59 (14)	0.77, 0.682
GA	0.81 ± 0.86 (28)	0.78 ± 0.64 (58)	0.68 ± .059 (17)	0.37, 0.831
AA	0.57 ± 0.43 (6)	0.42 ± 0.47 (12)	0.28 ± 0.28 (2)	0.96, 0.619
χ^2, *P*	6.98, 0.03	8.35, 0.015	3.76, 0.154	Overall $\chi^2 = 20.87$, $P = 0.0008$
	XRCC1 Arg280His genotype			
	GG	GA	AA	χ^2, *P*
(B) *XRCC1* Arg399Gln genotype				
GG	1.11 ± 0.73 (74)	0.98 ± 0.80 (7)	1.34 (1)	0.43, 0.807
GA	0.75 ± 0.72 (91)	0.84 ± 0.47 (4)	— (0)	0.20, 0.652
AA	0.44 ± 0.48 (16)	0.60 ± 0.06 (2)	— (0)	0.34, 0.563
χ^2, *P*	18.32, 0.0001	0.94, 0.627	—	Overall $\chi^2 = 14.91$, $P = 0.002$
	XRCC1 Arg194Trp genotype			
	CC	CT	TT	χ^2, *P*
(C) *XRCC1* Arg399Gln genotype				
GG	1.12 ± 0.73 (71)	1.00 ± 0.83 (10)	1.40 (1)	0.68, 0.711
GA	0.74 ± 0.73 (91)	0.81 ± 0.64 (18)	— (0)	0.20, 0.651
AA	0.49 ± 0.47 (16)	0.00 (1)	0.38 (1)	1.22, 0.543
χ^2, *P*	16.90, 0.0001	2.27, 0.321	1.00, 0.317	Overall $\chi^2 = 15.00$, $P = 0.005$

The results are presented as mean ± SD; in parentheses are reported the number of individuals with the particular genotype combination. Comparisons were performed by Kruskal–Wallis test.

comparison to wild-type *GG* genotype was statistically significant.

DNA repair rates and genotype analyses

Irradiation-specific DNA repair rates were significantly decreased in individuals with the homozygous variant (*AA*) in *XRCC1* Arg399Gln than those with the wild-type (*GG*) and heterozygous (*GA*) genotypes, (Mann–Whitney *U*-test: $P = 0.0006$ and $P = 0.002$, respectively; Table II).

We did not observe any significant influence on irradiation-specific DNA repair rates in the *XRCC1* Arg194Trp and Arg280His and *APE1* Asn148Glu polymorphisms. Similarly, no association between the genetic polymorphism in *hOGG1* Ser326Cys and irradiation-specific DNA repair rates was observed (Table II).

Our results did not show any effect on irradiation-specific DNA repair rates by genetic polymorphisms in genes involved either in NER (*XPD* Lys751Gln, *XPG* Asn1104His and *XPC* Lys939Gln) or DNA recombination repair (*XRCC3* Thr241Met and *NBS1* Glu185Gln) (Table II).

Combinations of different polymorphisms in BER genes were investigated in relation to irradiation-specific DNA repair rates. By testing all genotype combinations of *XRCC1* Arg399Gln and *APE1* Asn148Glu the irradiation-specific repair rates significantly decreased with increasing number of variant (*A*) allele in *XRCC1* Arg399Gln, whereas *APE1* Asn148Glu genotype contributed moderately (Kruskal–Wallis test: $\chi^2 = 20.87$, $P = 0.008$, Table III).

A significant decrease in irradiation-specific DNA repair rates was also constantly observed in association with variant allele (*A*) in *XRCC1* Arg399Gln, whereas no contribution of the two other investigated polymorphisms in *XRCC1* gene (Arg280His and Arg194Trp) was observed [Kruskal–Wallis test: $\chi^2 = 14.91$, $P = 0.002$ and $\chi^2 = 15.00$, $P = 0.005$, respectively; (Table III, B and C)].

When *XRCC1* Arg399Gln polymorphism was not taken into consideration, binary genotype combinations in BER genes did not significantly affect the level of irradiation-specific DNA repair rates (data not shown).

Similar results were observed for combinations of 3 and 4 polymorphisms in BER genes assessed using a score system that reflects the number of variant alleles in particular combination (the data are shown in Table V), revealing again that the predominant effect on the irradiation-specific DNA repair rates is associated with variant (*A*) allele in *XRCC1* Arg399Gln, and this significant tendency persists in spite of increasing number of genes analyzed in combination.

The capacities to repair oxidative DNA damage were significantly decreased in individuals with the homozygous variant (*GG*) genotype in *hOGG1* Ser326Cys as compared to those with wild-type (*CC*) and heterozygous (*CG*) genotypes (Mann–Whitney *U*-test: $P = 0.008$ and $P = 0.041$, respectively; Table II).

A significant decrease in the capacity to repair DNA oxidative damage was also associated with combination of variant alleles in *hOGG1* Ser326Cys and *APE1* Asn148Glu, (Kruskal–Wallis test: $\chi^2 = 8.84$, $P = 0.018$, Table IV). As evident from Table IV, the predominant effect is due to the variant *G* allele in *hOGG1* Ser326Cys.

Table IV. Effect of a selected binary combination of SNPs of BER genes on the capacity to repair oxidative DNA damage (expressed as SSBs/10^9 Da)

	APE1 Asn148Glu genotype			
	TT	TG	GG	χ^2, *P*
(A) *hOGG1* Ser326Cys genotype				
CC	0.84 ± 0.83 (48)	0.81 ± 0.75 (59)	0.63 ± 0.46 (19)	0.62, 0.734
CG	0.60 ± 0.78 (22)	0.61 ± 0.54 (30)	0.51 ± 0.39 (8)	0.85, 0.654
GG	0.54 ± 0.20 (5)	0.48 ± 0.36 (6)	0.23 ± 0.23 (2)	1.25, 0.535
χ^2, P	4.43, 0.097	4.10, 0.129	1.80, 0.408	Overall $\chi^2 = 8.84$, $P = 0.018$
	XRCC1 Arg194Trp genotype			
	CC	CT	TT	χ^2, *P*
(B) *hOGG1* Ser326Cys genotype				
CC	0.71 ± 0.59 (95)	0.96 ± 1.00 (20)	0.00(1)	2.96, 0.227
CG	0.66 ± 0.31 (50)	0.35 ± 0.48 (6)	— (0)	1.21, 0.271
GG	0.38 ± 0.31 (10)	0.28 ± 0.21 (2)	— (0)	0.06, 0.830
χ^2, *P*	4.02, 0.094	3.37, 0.186	—	Overall $\chi^2 = 8.85$, $P = 0.045$
	XRCC1 Arg280His genotype			
	GG	GA	AA	χ^2, *P*
(C) *hOGG1* Ser326Cys genotype				
CC	0.78 ± 0.69 (108)	0.26 ± 0.23 (7)	0.50 (1)	6.09, 0.048
CG	0.63 ± 0.71 (51)	0.68 ± 0.69 (5)	— (0)	0.01, 0.920
GG	0.36 ± 0.29 (12)	— (0)	— (0)	—
χ^2, *P*	7.71, 0.021	0.83, 0.370	—	Overall $\chi^2 = 8.13$, $P = 0.017$

The results are presented as mean ± SD; in parentheses are reported the number of individuals with the particular genotype combination. Comparisons were performed by Kruskal–Wallis test.

Binary combinations of polymorphisms in *hOGG1* Ser326Cys and *XRCC1* Arg194Trp, and Arg280His showed that the predominance of the variant *G* allele in *hOGG1* Ser326Cys is associated with the lower capacity to repair DNA oxidative damage (Tables IV and IV).

By testing the effect of all analyzed polymorphisms in BER genes, the capacity to repair DNA oxidative damage decreased with increasing number of variant alleles in *hOGG1* Ser326Cys in combination with the increasing number of variant alleles in the other investigated polymorphisms (Kruskal–Wallis test: $\chi^2 = 11.07$, $P = 0.050$, Table VI).

Discussion

Age-related decrease has been observed for hOGG1 activity in PBL from healthy individuals (13) as well as for irradiation-specific repair rates (16), whereas both irradiation-specific DNA repair rates and the capacity for the repair of oxidative DNA damage were not affected by age in our study. Interestingly, irradiation-specific DNA but not oxidative DNA damage-related DNA repair rates were significantly higher in smokers than non-smokers. In previous studies xenobiotic exposure-related increase in BER capacities has been recorded in individuals occupationally exposed to styrene (16) and xenobiotics in the tire plant, suggesting possible induction of DNA repair (23). Approximately 2-fold higher irradiation-specific DNA repair rates were found in smokers than in non-smokers (23). These findings may have be consequential, since exposure to potentially carcinogenic industrial chemicals as well as to the complex mixture of carcinogens in cigarette smoke seems to result in an increased BER capacity in healthy, cancer-free population. Whether this increase is due to an induction, or to a process of adaptation, remains to be clarified.

Irradiation-specific DNA repair rates were significantly higher among individuals with the wild-type genotype in *XRCC1* Arg399Gln as compared to those with homozygous variant genotype. Because most of the DNA damage induced by γ-irradiation is repaired in a short time (<1 h), the measured DNA repair activity is attributable mainly to the BER pathway (16,18), in agreement with the role of the *XRCC1* gene. An observation of the decreased DNA repair capacity in individuals bearing variant *A* allele in *XRCC1* exon 10 (codon 399) is additionally supported by the cytogenetic challenge assay (10), protein conservation analysis (8) and by increased irradiation sensitivity (9). These data seem to be biologically plausible, as XRCC1 protein acts as a coordinator of single strand break repair proteins in the base excision repair pathway with polymorphic codon 399 located within the *BRCA1* C-terminus functional domain (11). By testing the effect of other genetic polymorphisms in individual genes involved in BER, i.e. *XRCC1* Arg194Trp and Arg280His and *APE1* Asn148Glu, we did not observe any significant influence on irradiation-specific DNA repair rates. Although the highest DNA repair rate was seen in just one individual with homozygous variant genotype in *XRCC1* Arg280His, no conclusion may be drawn on the base of our present study. On the contrary, irradiation hypersensitivity was observed in 135 women with homozygous variant Glu/

Table V. Irradiation-specific DNA repair rates in relation to combinations of SNPs in BER genes[a]

Score	*n*	Irradiation-specific DNA repair rates (SSBs/10^9Da)
(A) *XRCC1* Arg399Gln, Arg280His and Arg194Trp		
0	66	1.14 ± 0.71
1	90	0.77 ± 0.75
2	36	0.72 ± 0.60
3	3	0.40 ± 0.35
4	1	0.38
$\chi^2 = 15.29$, $P = 0.004$		
(B) *XRCC1* Arg399Gln, Arg280His and *APE1* Asn148Glu		
0	37	1.20 ± 0.81
1	51	0.83 ± 0.79
2	75	0.87 ± 0.65
3	33	0.55 ± 0.52
4	2	0.18 ± 0.18
5	1	0.38
$\chi^2 = 16.81$, $P = 0.005$		
(C) *XRCC1* Arg399Gln, Arg194Trp and *APE1* Asn148Glu		
0	33	1.22 ± 0.82
1	55	0.92 ± 0.82
2	71	0.82 ± 0.60
3	28	0.55 ± 0.60
4	7	0.55 ± 0.45
5	1	0.38
$\chi^2 = 14.94$, $P = 0.011$		
(D) *XRCC1* Arg399Gln, Arg280His, Arg194Trp and *APE1* Asn148Glu		
0	29	1.23 ± 0.83
1	52	0.88 ± 0.84
2	71	0.87 ± 0.62
3	34	0.60 ± 0.57
4	7	0.54 ± 0.50
5	2	0.47 ± 0.13
$\chi^2 = 12.77$, $P = 0.026$		

The results are presented as mean ± SD. Comparisons were performed by Kruskal-Wallis test.
[a]Combinations were constructed on the base of an arbitrary score for variant alleles (See Materials and Methods for details). The higher score means the higher number of variant alleles in combined SNPs.

Glu genotype in *APE1* (9). It becomes more apparent in the light of the occurrence of the variant allele in the general population, which slightly exceeds 3%. The functional significance of *XRCC1* Arg280His polymorphism is not yet known (2). The data from the literature indicate that individuals with the wild-type Arg/Arg genotype in *XRCC1* Arg280His exhibit significantly higher chromosomal breaks per cell than those with variant His allele. We did not observe any association of polymorphism in codon 194 in *XRCC1* Arg194Trp, probably due to low occurrence of the variant allele in our studied population (29 individuals with at least one variant allele). The lack of observed effect of *APE1* polymorphism on BER is in agreement with the outcome of computational functional test, which suggested that this SNP is unlikely to exhibit an effect on the protein function (8).

Table VI. The capacity to repair oxidative DNA damage in relation to combinations of SNPs in BER genes[a]

Score	*n*	Oxidative DNA repair rates (SSBs/10^9 Da)
hOGG1 Ser326Cys, *XRCC1* Arg194Trp, Arg280His, Arg399Gln and *APE1* Asn148Glu		
0	14	1.05 ± 0.70
1	45	0.65 ± 0.59
2	58	0.72 ± 0.69
3	41	0.67 ± 0.69
4	17	0.49 ± 0.45
5	4	0.25 ± 0.22
$\chi^2 = 11.07$, $P = 0.050$		

The results are presented as mean ± SD. Comparisons were performed by Kruskal–Wallis test.
[a]Combinations were constructed on the base of an arbitrary score for variant alleles (See Materials and Methods for details). The higher score means the higher number of variant alleles in combined SNPs.

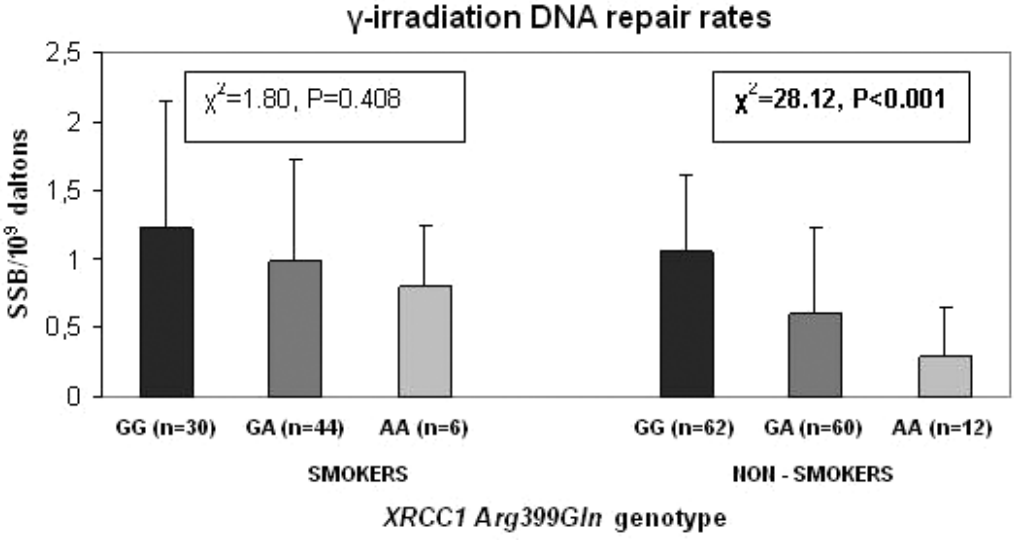

Fig. 1. The γ-irradiation DNA repair rates (expressed as SSB/10^9 Da) in individuals stratified for smoking habit (smokers $n = 80$, non-smokers $n = 134$) and for *XRCC1* Arg399Gln polymorphism. The results are presented as mean ± SD, statistical comparison was performed by Kruskal–Wallis test.

A significant decrease in irradiation-specific DNA repair rates was apparently associated with the binary combination of variant alleles in *XRCC1* Arg399Gln and *APE1* Asn148Glu polymorphisms. Although polymorphism in *APE1* Asn148Glu has no significant effect on irradiation-specific DNA repair, it seems to augment the effect exerted by *XRCC1* Arg399Gln polymorphism. An effect of various BER gene polymorphisms in combination on irradiation-specific DNA repair rates was also tested using an attributed score, reflecting a number of variant alleles in the particular combination, since all existing allele combinations could not be tested due to the low frequency of variant allele, particularly in *XRCC1* Arg194Trp and Arg280His. Apparently, the highest irradiation-specific DNA repair rates were associated with the lowest score, i.e. with the predominance of wild-type alleles in particular combinations. The results suggest that the main effect is due to the *XRCC1* Arg399Gln variant allele.

The capacity to repair oxidative DNA damage was 2-fold higher among individuals with the wild-type genotype (*CC*) in *hOGG1* Ser326Cys as compared to those with homozygous variant genotype. Although the larger functional studies also suggest reduced repair function with variant alleles in *hOGG1* (13,24), the evidence is generally inconclusive. On the other hand, variant *G* allele in *hOGG1* Ser326Cys was suggested to affect the glycosylase function due to the localization and phosphorylation status (15). Our data on the *hOGG1* Ser326Cys polymorphism and the capacity to repair oxidative DNA damage may provide more quantitative data on the decrease of oxidative damage repair in association with the variant allele in the above gene.

A significant decrease in the capacity to repair DNA oxidative damage was also associated with variant alleles in *hOGG1* Ser326Cys and *APE1* Asn148Glu polymorphisms, when this binary gene–gene interaction was investigated. Our data suggest that *APE1* Asn148Glu polymorphism contributes to highlight an effect of variant *G* allele in *hOGG1* Ser326Cys, although *APE1* Asn148Glu polymorphism itself did not influence the oxidative DNA damage repair capacity.

Binary combinations of polymorphisms in *hOGG1* Ser326Cys and *XRCC1* Arg194Trp, and Arg280His showed that the predominance of wild-type *C* allele in *hOGG1* Ser326Cys is associated with the higher capacity to repair DNA oxidative damage. The proper investigation of gene–gene interactions should be based on substantially larger population and the present data should be cautiously interpreted. Additionally, some other polymorphisms, such as those involved in nucleotide excision repair, may modulate levels of DNA damage as well as activity of OGG1 repair enzyme [higher activity was reported to be associated with the wild-type A allele in *XPA* gene, (25)].

By investigating simultaneous influence of genotypes in genes coding for BER enzymes and recorded confounders (age, sex, exposure status and smoking), irradiation-specific DNA repair rates were significantly affected by polymorphism in *XRCC1* Arg399Gln and by smoking. These data suggest the importance of gene–environment interactions and the research in this direction should be continued. Similarly, the capacity to repair DNA oxidative damage was significantly modulated by tentative exposure status and by *hOGG1* Ser326Cys polymorphism. A participation of environmental and occupational exposure factors on both irradiation-specific DNA repair rates as well as on the capacity to repair oxidative DNA damage has been reported earlier, suggesting that the particular DNA repair pathways may be induced by the exposure to xenobiotics (16,23).

An understanding of the relationships between DNA repair polymorphisms and corresponding functional reflections may contribute to the interpretation of results obtained from case–control association studies on various types of cancer. In order to clarify the roles of DNA repair polymorphisms and DNA repair capacities, as important susceptibility factors affecting the onset of cancer, both markers need to be analyzed first in general healthy population (background levels) and subsequently compared with those found in newly diagnosed, untreated cancer patients.

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Manuscript III

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Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers

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Relationship between the capacity to repair 8-oxoguanine, biomarkers of genotoxicity and individual susceptibility in styrene-exposed workers

J. Slyskova [a,b], M. Dusinska [a,c], M. Kuricova [d], P. Soucek [e], L. Vodickova [b,e], S. Susova [e], A. Naccarati [b], E. Tulupova [b], P. Vodicka [b,*]

[a] *Department of Experimental and Applied Genetics, Research Base of Slovak Medical University, Bratislava, Slovak Republic*
[b] *Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague 4, Czech Republic*
[c] *Norwegian Institute for Air Research (NILU), Kjeller, Norway*
[d] *Department of Immunology and Immunotoxicology, Research Base of Slovak Medical University, Bratislava, Slovak Republic*
[e] *Group for Biotransformations, Center of Occupational Medicine, National Institute of Public Health, Prague, Czech Republic*

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Abstract

Genotoxic effects related to exposure to styrene have been a matter of investigation for many years by employing markers of exposure, effect and susceptibility. The role of individual DNA-repair capacity in response to exposure to styrene may explain the controversial results so far obtained, but it is still scarcely explored. In the present study, we measured capacity to repair oxidative DNA damage in cell extracts obtained from 24 lamination workers occupationally exposed to styrene and 15 unexposed controls. The capacity to repair oxidative DNA damage was determined by use of a modified comet assay, as follows: HeLa cells, pre-treated with photosensitizer and irradiated with a halogen lamp in order to induce 7,8-dihydroxy-8-oxoguanine, were incubated with cell extracts from mononuclear leukocytes of each subject. The level of strand breaks reflects the removal of 7,8-dihydroxy-8-oxoguanine from substrate DNA by the enzymatic extract.

In styrene-exposed subjects a moderate, non-significant increase in oxidative DNA repair was observed. Stratification for sex and smoking habit showed that unexposed males ($P = 0.010$) and unexposed smokers ($P = 0.037$) exhibited higher DNA-repair rates. The repair capacity did not correlate with parameters of styrene exposure and biomarkers of genotoxic effects (DNA strand breaks, N1-styrene-adenine DNA adducts, chromosomal aberrations and mutant frequencies at the *HPRT* locus). Significantly higher levels of DNA-repair capacity were observed in carriers of *GSTM1-plus*, compared to those with a deletion in *GSTM1*. The DNA-repair capacity was significantly lower in individuals with variant Gln/Gln genotype in *XRCC1* Arg399Gln than in those with heterozygous Arg/Gln and wild-type Arg/Arg genotypes. Significantly lower repair capacity was also found in individuals with the wild-type Lys/Lys genotype in *XPC* Lys939Gln as compared with those homozygous for the Gln/Gln variant genotype.

Keywords: Occupational exposure; Styrene; DNA-repair capacity; Oxidative damage; Comet assay

* Corresponding author. Tel.: +420 241 062 694; fax: +420 241 062 782.
E-mail address: pvodicka@biomed.cas.cz (P. Vodicka).

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1. Introduction

Styrene is an important industrial chemical that is widely present in the environment due to emissions from industrial processes and vehicles, cigarette smoke and combustion of styrene polymers. Occupational exposure in hand-lamination work may entail a daily intake of several grams of styrene via inhalation, while absorption through the skin occurs at a lower rate. The IARC has classified styrene as a possible human carcinogen (Group 2B) and its main intermediary metabolite, styrene-7,8-oxide, as a probable human carcinogen (Group 2A) [1].

Styrene-induced genotoxicity has extensively been studied *in vitro*, in experimental animals as well as in humans for over two decades, often with controversial or inconclusive results [2–4]. Pero et al. [5] reported for the first time that styrene exposure increased unscheduled DNA synthesis (UDS) in leukocytes obtained from styrene-exposed workers and treated *in vitro* with 2-acetylaminofluorene, but no increase in UDS was recorded after UV irradiation. Since then, only few other studies addressed the relationship between styrene exposure and DNA-repair capacity [6], subsequently with attention to a possible modulating role of polymorphisms in the relevant genes [7,8]. *In vitro* DNA-repair capacity, based on measurement of residual strand breaks after a styrene-oxide challenge, was influenced by the intensity of recent styrene exposure and, inversely, by the duration of exposure. In the same group, polymorphisms in *GSTT1* and *XRCC1* Arg194Trp seemed to modulate the DNA-repair capacity [9]. Recently, increased irradiation-induced DNA-repair capacity was recorded in styrene-exposed workers in comparison with unexposed controls. A significant positive correlation was observed between DNA-repair rates and styrene-exposure parameters, whereas lower levels of strand breaks were associated with the higher DNA-repair rate. It was postulated that the lack of accumulation of genotoxic damage over time in exposed individuals could be due to the induction of adaptive DNA-repair processes [10].

The role of oxidative stress related to styrene exposure may be an important factor, as indicated by increased levels of 8-hydroxyguanine DNA adducts among exposed workers [11]. A recent hypothesis has postulated that oxidative stress may arise as a result of imbalance between oxidants and antioxidants, and contribute to the genotoxic effects of styrene exposure [12]. In concordance with this hypothesis an increased capacity to incise 8-oxoguanine was recorded among styrene-exposed workers in an exposure-related manner [10]. In the present study, we analysed the capacity to repair oxidative DNA damage, which is part of the base-excision repair (BER) pathway, as well as individual susceptibility in relation to various parameters of genotoxicity in styrene-exposed individuals. A comparison of the present data with those from a previous communication [10] may provide interesting clues on the role of exposure pattern, since subjects in the earlier study who showed an adaptive induction of BER were exposed to styrene for a short period (4 years on average), whereas in the present study we investigated a population exposed to styrene for a considerably longer period of time (14 years on average). In this case, an equilibrium between DNA-damage formation and removal may well be established, as we postulated earlier [13]. Although this study has been conducted on a relatively small cohort, its main value is the large and comprehensive spectrum of various biomarkers that were studied in relation to the DNA-repair capacity.

2. Materials and methods

2.1. Subjects

All samples were obtained from workers employed in a plastics lamination plant in western Slovakia. The styrene-exposed group consisted of 41 workers, who were employed in the lamination plant for several years (14.6 ± 5.6): *hand laminators* directly exposed to high styrene concentration and medium-exposed *sprayers*. This group counted 27 females and 14 males, among whom were 17 smokers and 24 non-smokers. The control group consisted of 18 clerks from the same factory (10 females and 8 males, 7 smokers and 11 non-smokers). Occupational exposure to styrene was determined by measuring styrene concentration-in-air at the workplace and styrene concentration in blood. These were determined at the time of the original study [14]. On the above population the following parameters were previously analysed: strand breaks in DNA, N1-(2-hydroxy-2-phenylethyl)-adenine adducts (1-Ade-adducts), chromosomal aberrations (CAs) and *HPRT* mutant frequency (*HPRT* MF). The biomarkers measured as well as the number of individuals investigated for each particular biomarker are shown in Table 1a. Furthermore, the genetic polymorphisms included in this study are presented in Table 1b, with the data on DNA-repair polymorphism in *APE1, NBS1* and *hOGG1* genes being shown for the first time. Blood samples were collected during the original sampling and DNA-repair capacities and polymorphisms were assayed subsequently on stored material.

The study was conducted on healthy individuals, interviewed about possible confounding factors such as smoking habit, alcohol consumption and medication. Each person included in the study signed an informed consent form. The Ethical committee of National Institute of Public Health,

Table 1a
Summary of the parameters of styrene exposure and genotoxic effects in the study population

	Age (years)	N	Styrene at workplace (mg/m^3)	*N*	Styrene in blood (mg/l)	*N*	SBs (tail DNA%)[a]	*N*	CAs (%)[a]	*N*	1-Ade-adducts (No./10^9 dNp)[b]	*N*	*HPRT* MF (No./10^6 cells)[c]	*N*
All subjects	38.8 ± 7.5	59	68.2 ± 94.6	59	0.53 ± 0.95	54	23.8 ± 10.2	42	2.7 ± 1.3	58	1.5 ± 4.3	25	17.1 ± 19.3	36
Exposure														
Exposed	38.2 ± 6.8	41	98.1 ± 98.9 [d]	41	0.70 ± 1.06[d]	40	30.4 ± 8.2 [e]	24	3.2 ± 1.0 [f]	40	2.0 ± 5.0 [d]	18	20.8 ± 26.3	18
Controls	40.1 ± 9.1	18	b.d.[*]	18	0.04 ± 0.09	14	15.0 ± 4.3	18	1.4 ± 0.8	18	0	7	13.3 ± 6.7	18
Sex														
Women	40.1 ± 6.3	37	92.4 ± 111.2 [e]	37	0.80 ± 1.11 [d]	34	25.1 ± 10.9	27	2.9 ± 1.3	36	0.7 ± 1.0	19	19.4 ± 22.1	26
Men	36.6 ± 9.0	22	27.4 ± 27.8	22	0.06 ± 0.06	20	21.4 ± 8.7	15	2.2 ± 1.0	22	3.7 ± 8.7 [d]	6	10.9 ± 5.4	10
Smoking habit														
Smokers	37.9 ± 8.0	24	59.7 ± 87.8	24	0.35 ± 0.76	20	24.6 ± 10.7	17	2.7 ± 1.2	24	2.4 ± 6.7	10	23.2 ± 28.0 [g]	12
Non-smokers	40.0 ± 6.8	35	74.1 ± 99.9	35	0.63 ± 1.05	34	23.3 ± 10.0	25	2.6 ± 1.3	34	0.8 ± 1.1	15	14.0 ± 12.8	24

Data are expressed as mean ± S.D.

[a] The data on SBs and CAs were reported in [14].

[b] The data on 1-adenine DNA adducts[2] in [22].

[c] The data on *HPRT* MF[3] in [7].

[d] $P < 0.0001$.

[e] $P < 0.001$.

[f] $P < 0.05$.

[g] $P = 0.044$.

[*] b.d.—Below the detection limit.

Slovak Republic, provided ethical approval, based on the Declaration of Helsinki.

2.2. Preparation of extracts from mononuclear leukocytes

Cell extracts were prepared from human mononuclear leukocytes isolated immediately from freshly collected whole blood samples and stored frozen at −80 °C, using the method described by Wood et al [15]. Briefly, blood was mixed 1:1 with PBS and added to Lymphoprep. After centrifugation (200 × *g*, 30 min, RT), mononuclear cells were washed with PBS and spun down again (180 × *g*, 20 min, RT). The supernatant was removed and 15 mL of solution A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM ditiotreitol, 10% glycerol, pH = 7.8, 3 × diluted with H_2O) was added to the pellet and centrifuged again (180 × *g*, 15 min). For every 10^7 cells, 50 μL of solution A was added and cells were stored at −80 °C. Before use, thawed samples were mixed with 12 μL of 1% Triton X-100 in solution A and centrifuged at 160 × *g* (5 min, 4 °C). The supernatant was removed and the remaining pellet was mixed with solution B (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 1% BSA, pH = 8.0). For each individual, the same number of cells was used to prepare the cell extract.

Table 1b
Genotype distribution and allele frequencies in the study population for XME and DNA-repair polymorphisms

Polymorphism	Genotype	*N*	Frequency of variant allele
XME polymorphisms			
EPHX1 deduced activity[a]	Low	10	
	Medium	27	
	High	2	
GSTM1 deletion	Plus	20	$q = 0.460$
	Null	17	$(q = 0.483)^*$
GSTT1 deletion	Plus	30	$q = 0.210$
	Null	8	$(q = 0.190)^*$
GSTP1 Ile105Val	Ile/Ile	20	$q = 0.284$
	Ile/Val	13	$(q = 0.293)^*$
	Val/Val	4	
DNA-repair polymorphisms			
XPD Lys751Gln	Lys/Lys	11	$q = 0.410$
	Lys/Gln	24	$(q = 0.405)^*$
	Gln/Gln	4	
XPG Asn1104His	Asn/Asn	24	$q = 0.205$
	Asn/His	14	$(q = 0.207)^*$
	His/His	1	
XPC Lys939Gln	Lys/Lys	12	$q = 0.462$
	Lys/Gln	18	$(q = 0.431)^*$
	Gln/Gln	9	
XRCC1 Arg399Gln	Arg/Arg	14	$q = 0.372$
	Arg/Gln	21	$(q = 0.327)^*$
	Gln/Gln	4	
XRCC3 Thr241Met	Thr/Thr	22	$q = 0.243$
	Thr/Met	15	$(q = 0.293)^*$
	Met/Met	2	
APE1 Asn148Glu	Asn/Asn	12	$q = 0.450$ $(q = 0.440)^*$
	Asn/Glu	19	
	Glu/Glu	8	
NBS1 Glu185Gln	Glu/Glu	18	$q = 0.307$
	Glu/Gln	18	$(q = 0.302)^*$
	Gln/Gln	3	
hOGG1 Ser326Cys	Ser/Ser	24	$q = 0.167$ $(q = 0.190)^*$
	Ser/Cys	12	
	Cys/Cys	0	

* Signifies reported frequencies of variant alleles in the whole cohort.

[a] *EPHX1* activity genotype was deduced from the combination of *EPHX1*, exons 3 and 4 genotypes.

Technical and methodological requirements enabled processing of 24 workers (16 females and 8 males, 11 smokers and 13 non-smokers) and 15 clerks (9 females and 6 males, 5 smokers and 10 non-smokers) as control individuals.

2.3. Induction of 7,8-dihydroxy-8-oxoguanine in HeLa cells

Nuclei of HeLa cells pre-treated with photosensitizer Ro 19-8022 and irradiated with a halogen lamp to induce 7,8-dihydroxy-8-oxoguanine (8-oxoG), were incubated with enzymatic extracts prepared from mononuclear cells of each subject as described above, and analysed by use of the comet assay. The level of strand breaks reflects the removal of 8-oxoG from substrate DNA by the enzymatic extract.

HeLa cells (human transformed endothelial cells derived from a human carcinoma) were used as a source of DNA. Cells were grown in Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (10,000 U/ml) and streptomycin (10 mg/ml). The cultures were incubated at 37 °C in a water-saturated atmosphere containing 5% CO_2. To prepare substrate DNA with specific oxidative damage, for measurement of the activity of the repair enzyme 8-oxoguanine DNA glycosylase (OGG1), HeLa cells were treated with the photosensitizer Ro 19-8022 (Hoffmann La Roche, Basel, Switzerland), and illuminated with visible light from a 1000-W tungsten halogen lamp, to oxidise guanine to 8-oxoG. Cells growing on the culture dish were washed with cold PBS and then 5 ml of cold PBS containing Ro 19-8022 (0.1 μM) was added. The dish was placed on ice and irradiated for 3 min with the halogen lamp, the distance being 33 cm. The photosensitizer solution was removed and the monolayer of cells was washed with PBS and trypsinized to detach the cells. After counting, the samples were centrifuged at 400 × *g* for 5 min at 4 °C, and prepared for the repair assay.

2.4. Measurement of repair rates of oxidative DNA damage by a modified comet assay

HeLa cells were mixed with the appropriate volume of 1% LMP agarose in PBS and embedded on agarose-coated microscope slides. Cover slips were then placed on top and slides were kept in the cold to set for 5 min. After the cover slips were removed, the slides were immersed in ice-cold cell lysis solution (4 °C) for at least one hour. Lysis solution contained 2.5 M NaCl, 100 mM EDTA and 10 mM Tris (pH 10.0), with 1% Triton X-100 added immediately before use.

After cell lysis, the slides were washed two times (15 min each) with buffer (40 M HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8). Slides were then kept in the cold and 45 μl of extract (or buffer) was added to each, and immediately covered with a cover slip. Slides were incubated at 37 °C for 10 min in a humidified atmosphere, then treated with 300 mM NaOH, 1 mM EDTA (pH 13) for 40 min. Electrophoresis was carried out at 25 V, 300 mA for 20 min. All slides were then washed two times with neutralising buffer (0.4 M Tris, pH 7.5) for 5 min each.

Samples were stained with DAPI (20 μl of a 1-μg/ml DAPI solution in distilled water). Comets were analysed by visual scoring under a fluorescence microscope (Olympus) using 5 classes of comets according the extent of DNA damage. One hundred comets were selected at random from each gel, so the total score from one gel ranged from 0 to 400 arbitrary units [16]. The increase of DNA breaks, detected as the difference between the score obtained from cells incubated with extract and the score from those incubated with buffer only (with a time interval of 10 min), was taken as a rate of repair-incision for statistical analysis.

2.5. Analysis of other biomarkers and genetic polymorphisms

The levels of strand breaks in DNA were detected in lymphocytes by use of single-cell gel electrophoresis (comet assay) as described earlier [17]. The methodology used for measuring chromosomal aberrations (CAs) is described in [14], for 1-Ade-adducts in [18] and for *HPRT* mutant frequency (MF) in [7]. SNPs in genes encoding biotransformation enzymes (*CYP1A1* 3′-flanking region, *Msp*I site *1A/*2A, *CYP2E1* 5′-flanking region, *Rsa*I site *1A/*5B and intron 6, *Dra*I site, *1A/*6, *EPHX1* Tyr113His and His139Arg, *GSTP1* Ile105Val) and DNA-repair enzymes (*XPD* Lys751Gln, *XPG* Asn110His, *XPC* Lys939Gln, *XRCC1* Arg399Gln, *XRCC3* Thr241Met, *APE1* Asn148Glu, *NBS1* Glu185Gln and *hOGG1* Ser326Cys) were determined by PCR/RFLP using a method described earlier [19,20]. The allelic frequencies of all three-genotype SNPs were in concordance with Hardy-Weinberg equilibrium and are shown in Table 1b. Deletions in *GSTM1* and *GSTT1* were assessed by allele-specific multiplex method, as described in [21].

2.6. Statistics

Statistical analyses were performed with SPSS software, version 10, LEAD Technologies, Inc., USA. Non-parametric tests were employed for statistical evaluation of the data. To test significant differences between groups, the Mann–Whitney *U*-test was used. Spearman coefficient was used to estimate the correlation between parameters; when more than two parameters were evaluated the Kruskal–Wallis test was applied. Inter-relationship between various variables and DNA-repair capacity was analysed by regression, stepwise selection, analysis. *P*-values below 0.05 were considered to correspond with statistical significance. Data are expressed as mean ± S.D. and as median (bottom, upper quartile) for DNA-repair capacities.

3. Results

3.1. Characterisation of the study group

In our study, parameters of both external (concentration of styrene at the workplace) and internal exposure

(styrene concentration in blood) were analysed to characterise occupational exposure to styrene. The analysis of workplace air by GC, performed at the same time of blood sampling, did not reveal any contamination by other chemicals than styrene. Styrene concentration at the workplace was 98.1 ± 98.9 mg/m^3. Styrene concentration in blood was 0.70 ± 1.06 mg/l among the exposed individuals and 0.04 ± 0.09 mg/l among the unexposed controls ($P < 0.0001$).

3.2. Markers of genotoxic effects

Several parameters of genotoxic effects, such as DNA strand breaks, CAs, 1-Ade-adducts, *HPRT* MF were determined (data summarised in Table 1a). These data have previously been published separately [7,14,22]. Briefly, strand breaks, CAs and 1-Ade-adducts were significantly higher in the exposed than in the controls. All these parameters correlated with styrene exposure [14,22]. The mutant frequencies in *HPRT* were moderately higher in individuals exposed to styrene as compared with controls, but this difference was not significant. No correlation was found between *HPRT* MF and parameters of styrene exposure [7].

3.3. Markers of individual susceptibility

The data on *XME* polymorphisms have previously been published [7]. Allelic frequencies of the genetic polymorphisms in genes encoding XME and DNA-repair enzymes did not differ from published data on the Central-European population [19,20] and complied with Hardy–Weinberg equilibrium. Genotype distribution and allelic frequencies in *APE1*, *NBS1* and *hOGG1* genes (shown for the first time in this cohort) as well as in genes showing an association with DNA-repair capacity are presented in Table 1b.

3.4. DNA-repair capacity

We measured the ability of an enzymatic extract obtained from mononuclear leukocytes of exposed and control subjects to remove oxidized purines, especially 8-oxoG, from damaged DNA. Individual repair capacities ranged from 11 to 277 arbitrary units (Fig. 1), with median 119, and bottom and upper quartile, respectively, at 82 and 185. The DNA-repair capacity was moderately higher in the exposed group than in controls, but this difference was not significant. Borderline significant differences were found between males and females ($P = 0.055$) and between the exposed females and control females ($P = 0.051$). Significantly higher repair capacity was found in control males when compared with control females ($P = 0.010$) and in control smokers in comparison with control non-smokers ($P = 0.037$; Table 2).

3.5. DNA-repair rates in relation to other biomarkers and genetic polymorphisms

In the present study, we investigated possible relationships between the capacity to repair oxidative DNA damage, main confounders, parameters of exposure and parameters of genotoxic effects (SBs, CAs, 1-Ade-adducts and *HPRT* MF). In general, there was no significant correlation between the DNA-repair capacity and any biomarkers studied both in the whole group as well as after the stratification for styrene exposure. The only positive correlation was found between DNA-repair capacity and SBs in females ($R = 0.417$, $P = 0.038$, $N = 25$).

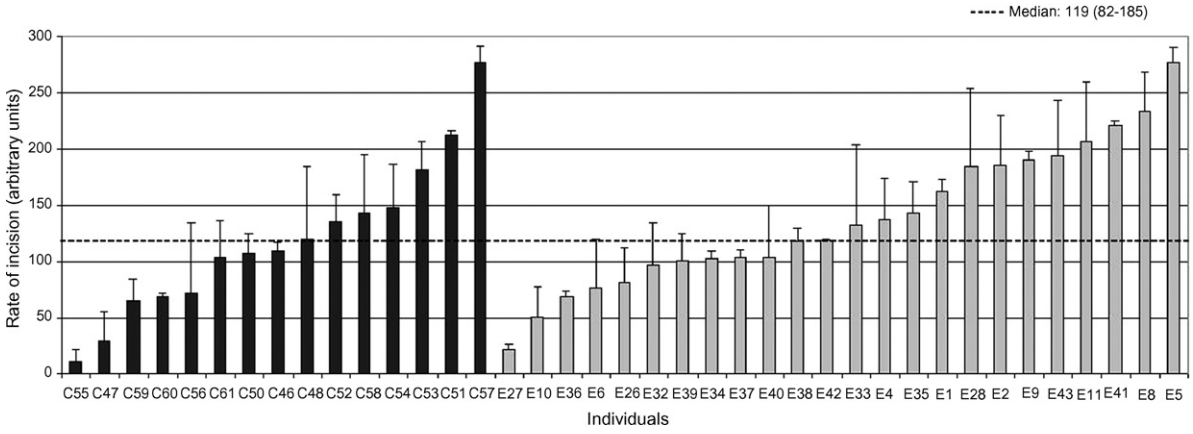

Fig. 1. Inter-individual variability in repair of oxidative DNA damage in exposed workers (grey bars) and unexposed controls (black bars). Data are expressed as arbitrary units and represent mean ± S.D. of 100 scored comets from each of two parallel slides.

Table 2
DNA-repair capacity of lymphocyte extract in the whole group and subgroups

	All	*N*	Exposed	*N*	Controls	*N*
All	119(82; 185)	39	126 (99; 188)	24	109 (69; 149)	15
Females	108 (71; 143)[a]	25	126 (99; 188)[b]	16	72 (47; 115)[b,c]	9
Males	165 (104; 194)[a]	14	152 (91; 192)	8	165 (135; 212)[c]	6
Smokers	146 (91; 190)	16	144 (77; 194)	11	149 (122; 197)[d]	5
Non-smokers	108 (72; 143)	23	119 (103; 188)	13	88 (65; 121)[d]	10

Table shows medians (bottom, upper quartile) of data evaluated by visual scoring (in arbitrary units, see Material and Methods for details).
[a] $P = 0.055$.
[b] $P = 0.051$.
[c] $P = 0.010$.
[d] $P = 0.037$.

Possible associations between the capacity to repair oxidative DNA damage and polymorphisms in genes encoding bio-transformation enzymes and DNA-repair enzymes were analysed. Polymorphism in *GSTM1* was significantly associated with DNA-repair capacity: higher DNA-repair rates were observed in carriers of *GSTM1-plus* genotype, expressing functional enzyme, compared with those with a deletion in *GSTM1*

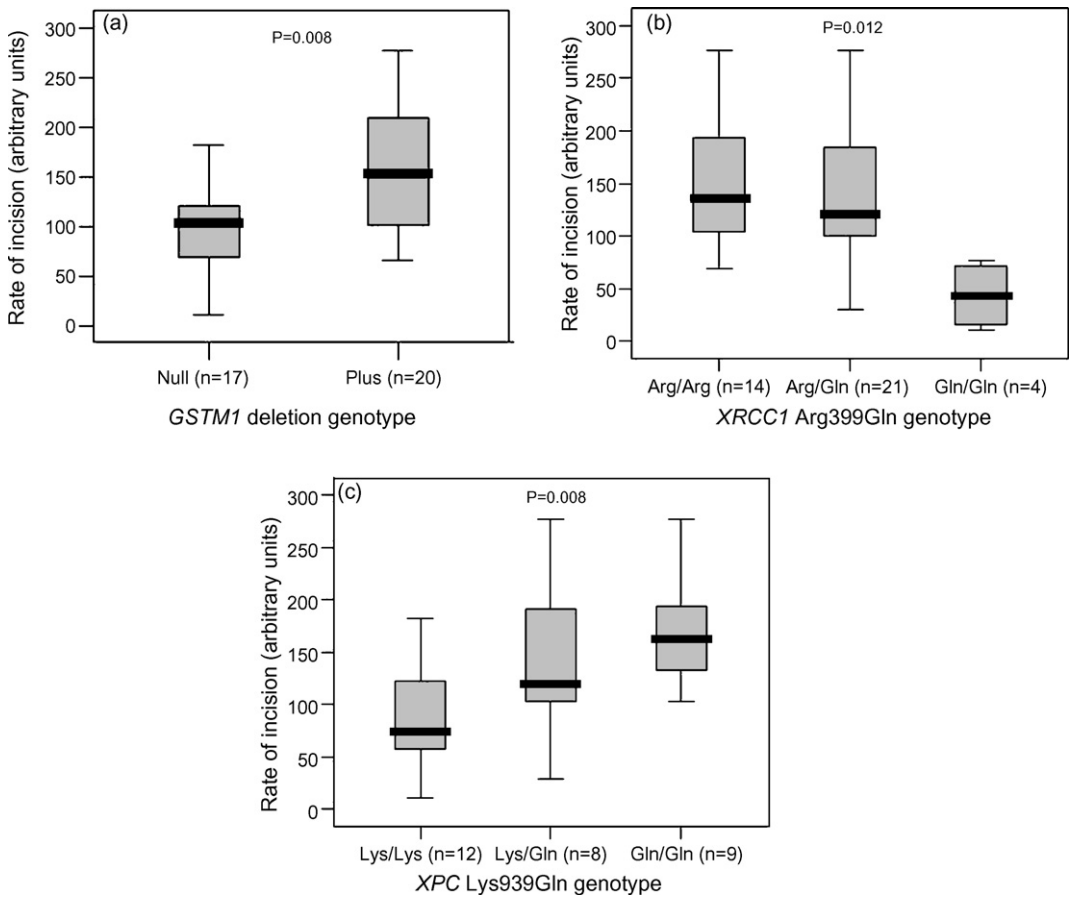

Fig. 2. (a) DNA-repair capacity stratified in the study population for genotypes in *GSTM1* deletion. Boxplots show the median, interquartile range and extreme cases of individual variables within a category. (b) DNA-repair capacity stratified in the study population for genotypes in *XRCC1* Arg399Gln. Boxplots show the median, interquartile range and extreme cases of individual variables within a category. (c) DNA-repair capacity stratified in the study population for genotypes in *XPC* Lys939Gln. Boxplots show the median, interquartile range and extreme cases of individual variables within a category.

($P = 0.008$; Fig. 2a). This relationship was significant also when only the exposed group was considered (12 individuals with *GSTM1-plus* and 10 with *GSTM1-null* genotype, $P = 0.004$), males only (7 with *GSTM1-plus*, 6 with *GSTM1-null* genotype, $P = 0.032$), and smokers only (9 and 6, respectively, $P = 0.018$). In the whole group, a significant difference in the individual capacity to repair oxidative DNA damage was also found in relation to *XRCC1* Arg399Gln genotypes. The DNA-repair capacity was significantly lower in individuals with variant Gln/Gln genotype than in those with heterozygous Arg/Gln and wild type Arg/Arg genotypes ($P = 0.012$; Fig. 2b). In females only, the same association was significant (10 individuals with Arg/Arg, 11 with Arg/Gln and 4 with Gln/Gln genotypes, $P = 0.008$). A different trend was observed for *XPC* Lys939Gln polymorphism, where higher DNA-repair capacity was found in individuals with the homozygous Gln/Gln variant genotype as compared with those with the wild-type Lys/Lys genotype. This association was found in the pooled group ($P = 0.008$; Fig. 2c), in the exposed group (5 individuals with Lys/Lys, 11 with Lys/Gln and 8 with Gln/Gln genotypes, respectively, $P = 0.025$), in females (8, 10 and 7, respectively, $P = 0.008$) and in non-smokers (5, 11 and 7, respectively, $P = 0.005$). Individuals with the wild-type Ser/Ser genotype for the *hOGG1* Ser326Cys polymorphism showed a higher DNA-repair capacity ($N = 24$, median = 134 and quartiles = 103–185) than those with heterozygous Ser/Cys genotype ($N = 12$, median = 102 and quartiles = 38–137). This difference was of borderline significance ($P = 0.054$). This association is hampered by the low frequency of the variant Cys allele, as evident from data provided in Table 1b.

Association between DNA-repair capacity and the above-mentioned polymorphisms was also analysed by using a general linear model, with polymorphisms in *GSTM1* and *XRCC1*, sex, smoking and exposure as variables. Simultaneous analysis revealed that the *GSTM1* genotype, along with sex, are the most significant factors modulating DNA-repair capacity ($P = 0.002$, $P = 0.042$, respectively). As apparent from Table 3, the *XRCC1* genotype significantly affects DNA-repair capacity as well ($P = 0.050$).

4. Discussion

Several studies described genotoxic effects of styrene in occupationally exposed workers, but still limited information is available on the effects of styrene exposure on DNA-repair capacity [2,4].

We studied the individual capacity to repair oxidative DNA damage (incision step in removal of oxidized bases, mainly 8-oxoG) in workers occupationally exposed to styrene and in unexposed clerks from the same factory. The results were analysed in relation to data on various biomarkers of styrene genotoxicity determined in the same cohort. The exposed individuals showed a moderate, although non-significant increase in the capacity to incise oxidized purines compared with unexposed controls. This increase is subtle compared with the results from a previous study, where significantly higher DNA-repair capacity in styrene-exposed individuals in comparison with the control group was observed [10]. Simultaneously, lower strand-break levels were associated with a higher DNA-repair rate. It was postulated that the lack of accumulation of genotoxic damage over time in exposed individuals could be due to the induction of adaptive DNA-repair processes [10]. Although on average the repair rates of oxidative DNA damage are fairly comparable between both cohorts studied, the absence of significant differences between exposed and controls in the present study is most likely related to pronounced inter-individual variability in DNA-repair rates, differences in the levels and/or the duration of exposure, and the smaller size of the population investigated. In contrast to the previous study, the present data on individual DNA-repair capacity were obtained in a population exposed to styrene for a relatively long period (14 years on average). Differences in the exposure patterns between the two styrene-exposed cohorts are clearly shown from evaluating the cumulative exposure factor (styrene concentration in air multiplied by

Table 3
Influence of *GSTM1* deletion and *XRCC1* Arg399Gln genotypes, sex, smoking habit and exposure status on oxidative DNA-repair capacity

Model	Beta ln	*t*	Sig.	Co-linearity statistics tolerance
GSTM1 deletion	0.479	3.276	0.002	
XRCC1 Arg399Gln	−0.288	−2.024	0.050	0.971
Sex	−0.295	−2.109	0.042	0.999
Smoking habit	0.156	1.068	0.293	0.994
Exposure	0.149	1.017	0.316	0.999

$R^2 = 0.230$.

duration of exposure [10]), which is on average 1340 in the present and 238 in the previous study. Different exposure patterns between the two populations may additionally implicate a different balance between DNA-damage formation and removal, as we postulated earlier [14].

Interestingly, unexposed smokers incised oxidative DNA damage significantly more effectively than unexposed non-smokers, and the same trend, although non-significant, was found considering the whole study group or the exposed workers only. This may be due to the generation of oxidative stress by smoking and subsequent stimulation of cellular DNA-repair activity. As a conclusion of these findings, exposure to genotoxic xenobiotics, including the mixture of chemicals in cigarette smoke, seems to result in an increased DNA-repair capacity – i.e. base-excision repair – as we have shown previously [23]. Our results reveal moderately higher repair capacity in males as compared with females. In agreement with recent data, similar modulation of 8-oxoG incision rates by sex were discovered in occupationally exposed populations (rock wool, asbestos). The authors concluded that various factors may also contribute to these sex-related differences, such as life style, nutrition and smoking habits, hormonal status, etc. [24,25].

No clear relationship between DNA-repair capacity and age, length of exposure, measures of both internal and external styrene exposure, DNA strand breaks, CAs or 1-Ade-adducts was observed. The lack of correlation between the DNA-repair capacity and other parameters of genotoxicity is probably due to the fact that the particular DNA-repair pathway studied here is not responsible for removal of either primary DNA damage (strand breaks, 1-Ade-adducts) or chromosomal damage. Primary DNA damage may be repaired by other BER glycosylases, although there is scarce information on the repair of specific DNA adducts in general. Markers of biological effects such as CAs and *HPRT* mutant frequency arise as consequence of multiple events. In these instances other DNA-repair pathways, such as double-strand break repair, are assumed to be more relevant.

In the present study, we also report associations between DNA-repair capacity and polymorphisms in genes encoding the xenobiotic-metabolising enzyme GSTM1 and the DNA-repair enzymes XRCC1 and XPC. Interestingly, individuals with *GSTM1*-plus genotype had significantly higher capacity to repair oxidative DNA damage, particularly 8-oxoG, than those with *GSTM1*-null genotype. Our hypothesis that functional *GSTM1* contributes to a reduction of oxidative damage should be further tested, since *GSTM1* is encoding an important detoxification and antioxidant enzyme. It is difficult to discern whether *GSTM1* polymorphism modulates the rate of oxidative DNA-damage induction rather than its repair. However, by analysing the relationship between the levels of endonucleaseIII-sensitive sites and *GSTM1* polymorphisms (data not shown), the levels of this non-specific marker for oxidative DNA damage appeared not to be affected by either *GSTM1* genotype. There is a scarcity of data on the relationship between the levels of 8-oxoG and *GSTM1* genotype. In general, higher levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the trabecular meshwork region among glaucoma patients [26], as well as in leukocytes from 105 healthy volunteers [27] were significantly associated with *GSTM1*-null genotype. Higher levels of urinary 8-OHdG in relation with *GSTM1*-null genotype were described among 81 Korean pregnant women [28]. We also observed a significant association between DNA-repair capacity and polymorphism in *XRCC1* Arg399Gln. Subjects with Arg/Arg or Arg/Gln genotypes showed higher repair capacity than carriers of the variant Gln/Gln genotype. This trend was apparent in the whole group, as well as after stratification for exposure and sex. In mammalian cells, the XRCC1 protein is essential for the maintenance of genomic stability and is involved in BER and single-strand break repair. XRCC1 is crucial in the last phase of the BER, but also participates in the first step by interacting with human glycosylases hOGG1 and NEIL1 [29]. Thus, the XRCC1 product contributes to the overall BER process by interplaying with other enzymes engaged in this repair pathway. As some authors assume, levels of available XRCC1 protein in the cell may influence the overall efficiency of BER [30,31]. DNA-repair capacity also seemed to be associated with *XPC* Lys939Gln polymorphism: significantly higher rates were found in individuals with variant Gln/Gln genotype than in those with the wild-type Lys/Lys genotype. This effect was observed both in the whole group and in the exposed group, females and non-smokers considered separately. The reason for interaction of *XPC* with the BER pathway remains unclear at present, since this gene codes for a DNA damage-recognising enzyme active in DNA nucleotide-excision repair (NER) [32]. It has recently been reported that polymorphisms in genes involved in NER may modulate levels of DNA damage as well as activity of the OGG1 repair enzyme. Higher OGG1 activity was associated with the wild-type genotype in *XPA* in 389 exposed and control subjects [33]. In the present study, increased repair capacity was also associated with *hOGG1* Ser/Ser wild-type genotype, compared with the heterozygous Ser/Cys genotype. This associ-

ation should not be over-interpreted, since it was of borderline significance and the size of the study population was small (e.g. there were no carriers of the homozygous variant genotype). However, the present findings are in agreement with our recent data on the relationship between DNA-repair polymorphisms and outcomes from functional DNA-repair studies in 210 healthy human subjects. We observed significantly higher (approximately two-fold) DNA-repair capacity in individuals carrying the wild-type *hOGG1* genotype, compared with those carrying the homozygous variant genotype. A significant difference in the capacity to repair oxidative damage was also found by comparing individuals with heterozygous Ser/Cys and homozygous variant Cys/Cys genotypes [7]. Considering the size of the cohort, which becomes substantially reduced after stratification, it is difficult to clearly discern any possible interaction between the analysed genotypes and styrene exposure (and also the confounders). From the general linear model analysis (Table 3) the DNA-repair capacity appears to be significantly modulated by polymorphisms in *GSTM1* and *XRCC1* and by sex, while exposure status and smoking did not have any effect in this particular study.

In studies associating various genotypes with biomarkers, the statistical power is dependent on the size of the cohort. In case of a limited population (a necessary compromise in assaying for specific markers, which cannot be measured in large groups) data on genotypes are informative as additional factors characterizing the population. On the other hand, relevant polymorphisms related to the corresponding functional tests (genotype-phenotype relationships) are still of importance, since such data are scarce at present [34].

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Manuscript IV

Slyskova J, Naccarati A, Pardini B, Polakova V, Vodickova L, Smerhovsky Z, Levy M, Lipska L, Liska V, Vodicka P

Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls

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Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls

Jana Slyskova[1,2,3], Alessio Naccarati[1], Barbara Pardini[1], Veronika Polakova[1,3], Ludmila Vodickova[1,3,4], Zdenek Smerhovsky[4,5], Miroslav Levy[6], Ludmila Lipska[6], Vaclav Liska[7] and Pavel Vodicka[1,3,*]

[1]Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Science of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic, [2]Department of Genetics and Microbiology, Faculty of Science, Charles University, Albertov 6, 120 00 Prague 2, Czech Republic, [3]Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Albertov 4, 120 00 Prague 2, Czech Republic, [4]Departments of Toxicogenomics and Occupational Health, National Institute of Public Health, Srobarova 48, 100 00 Prague 10, Czech Republic, [5]Institute of Epidemiology, 2nd Medical Faculty, Charles University, V uvalu 58, 160 00 Prague 6, Czech Republic, [6]Department of Surgery, 1st Medical Faculty of Charles University and Thomayer University Hospital, Videnska 800, 142 00 Prague 4, Czech Republic and [7]Department of Surgery, Teaching Hospital and Medical School Pilsen, Charles University, Alej Svobody 80, 304 00 Pilsen, Czech Republic.

[*]To whom correspondence should be addressed. Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Science of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic. Tel: +420 241 062 694; Fax: +420 241 062 782; Email: pvodicka@biomed.cas.cz

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Alteration of DNA integrity is a potential cause of cancer and it is assumed that reduced DNA repair capacity and accumulation of DNA damage may represent intermediate markers in carcinogenesis. In this case-control study, DNA damage and nucleotide excision repair capacity (NER-DRC) were assessed in association with sporadic colorectal cancer (CRC). Both parameters were quantified by comet assay in blood cells of 70 untreated incident patients and 70 age-matched healthy controls. mRNA expression and polymorphisms in relevant NER genes were concurrently analyzed. The aim of this study was to characterize incident CRC patients for NER-DRC and to clarify possible relations between investigated variables. Comet assay and mRNA expression analysis showed that CRC patients differ in repair capacity as compared to controls. Patients had a lower NER-DRC and simultaneously they exhibited higher endogenous DNA damage (for both $P < 0.001$). Accumulation of DNA damage and decreasing NER-DRC behaved as independent modulating parameters strongly associated with CRC. Expression levels of 6 out of 9 studied genes differed between groups ($P \leq 0.001$), but none of them was related to DRC or to any of the studied NER polymorphisms. However, in patients only, *XPC* Ala499Val modulated expression levels of *XPC*, *XPB* and *XPD* gene, whereas *XPC* Lys939Gln was associated with *XPA* expression level in controls (for all $P < 0.05$). This study provides evidence on altered DRC and DNA damage levels in sporadic CRC and proposes the relevance of the NER pathway in this malignancy. Further, alterations in a complex multigene process like DNA repair may be better characterized by functional quantification of repair capacity than by quantification of individual genes transcripts or gene variants alone.

Introduction

Colorectal neoplasia is the third most common cancer worldwide and the fourth leading cause of cancer death (1). Recently, decreasing colorectal cancer (CRC) mortality rates have been recorded in developed countries, most likely due to larger efforts in cancer screening and/or improved treatment (2). Since an early diagnosis of CRC is associated with substantially better prognosis and curability, early biomarkers and intermediary end points are called for to dissect the complex process of colorectal carcinogenesis (3).

While in the etiopathogenesis of inherited CRC forms the influence of particular genetic features prevails, sporadic forms (around 80% of all cases) show a multifactorial pattern and are determined by an interplay of multiple genetic and environmental/lifestyle factors (4). Well-established risk factors for sporadic CRC are obesity, diet low in vegetables/fruits and rich in meat, physical inactivity and smoking (5,6). Concurrently, the specific nature of colon epithelium, which is a dynamically changing system, makes it prone to genotoxic attacks of external source and/or spontaneous genetic changes. The above reasons underline the importance of efficient DNA repair machinery to maintain the cellular genomic integrity. Indeed, well-supported associations exist between DNA repair insufficiency and inherited forms of CRC: hereditary nonpolyposis CRC is caused by deficient mismatch repair and MUTYH-associated polyposis is associated with deficient mutation in the base excision repair (BER) gene *MutY* (7).

In sporadic CRC, there is no single germ-line mutation causing a strong deficiency in DNA repair activity. However, alterations of the individual DNA repair capacity (DRC) may significantly modulate the susceptibility to this cancer, especially in the context of gene–environment interactions. Family-based studies have suggested that DRC is a phenotype with a strong genetic basis, estimating heritability in the range of 48–75% (8). DNA repair may be further modulated by environmental/lifestyle factors via several possible mechanisms, such as activation/inhibition of repair enzymes, different provision of building blocks (nucleotides) for the repair machinery or regulation of repair gene expression (9). Thus, DRC, comprising the effect of both hereditary components and variable environmental factors, characterizes the actual phenotype of the cells. Due to the above aspects, estimation of DRC is naturally becoming a representative biomarker and an integral part of modern molecular epidemiological studies on cancer.

Nucleotide excision DNA repair (NER), one of the major players in maintaining genomic integrity, may modulate predisposition to various cancers as well as response to subsequent treatment (10). So far, reduced NER-DRC was

Table I. Reduced NER capacity detected in blood cells of patients with various types of sporadic cancer—an overview of the literature

Cancer	Cases	Controls	Damage-inducing agent	Assay	References
Bladder	106 incident cancer, previously untreated	137 matched by age, sex and ethnicity	BPDE	Comet assay	Schabath *et al.* (11)
Breast	33 breast carcinoma patients	47 matched by age	UVC	HCR	Ramos *et al.* (12)
	69 newly diagnosed, previously untreated	79 matched by age and ethnicity	BPDE	HCR	Shi *et al.* (13)
Colorectal	40 sporadic CRC 28 hereditary predisposition to CRC	39 not specified	N-AcO-2-FAA	UDS	Pero *et al.* (14)
Cutaneous melanoma	312 newly diagnosed, previously untreated	324 matched by age, sex and ethnicity	UV	HCR	Wei *et al.* (15)
Head and neck	55 newly diagnosed, previously untreated	61 matched by age, sex, ethnicity and smoking status	BPDE	HCR	Cheng *et al.* (16)
	123 newly diagnosed, previously untreated	136 matched by age and sex	BPDE	Comet assay	Xiong *et al.* (17)
	744 newly diagnosed, previously untreated	753 matched by age, sex and ethnicity	BPDE	HCR	Wang *et al.* (18)
Non-melanoma skin	88 primary cancer	135 matched by sex and age	UV	HCR	Wei *et al.* (19)
	255 newly diagnosed, previously untreated	333 unmatched	UV	HCR	Wang *et al.* (20)
Non-small cell lung	316 newly diagnosed	316 matched by age, sex, and smoking status	BPDE	HCR	Wei *et al.* (21)
	467 newly diagnosed, previously untreated	488 matched by age, sex, ethnicity and smoking status	BPDE	HCR	Shen *et al.* (22)
	108 with second primary cancer	99 matched by age and sex	BPDE	Comet assay	Orlow *et al.* (23)
	271 lung carcinoma patients	271 matched by age and smoking	BPDE	HCR	Deng *et al.* (24)
Prostate	75 newly diagnosed, previously untreated and 65 prevalent cancer	96 matched by age and race	UV	HCR	Hu *et al.* (25)

UV, ultraviolet light; HCR, host-cell reactivation assay; N-AcO-2-FAA, N-acetoxy-N-2-fluorenylacetamide; UDS, unscheduled DNA synthesis.

associated with several types of sporadic malignancies (summarized in Table I). However, there is a lack of data on NER-DRC in association with sporadic CRC. Additionally, the characterization of individual DNA repair profiles may be of further importance for the implementation of targeted therapies. For instance, platinum-based compounds are first-line cytostatic drugs employed in CRC treatment and variability in NER pathway has been linked to the different sensitivity towards above chemotherapeutics (26,27).

Based on the above considerations, we have focused this case-control study on evaluating NER-DRC and endogenous DNA damage in peripheral blood mononuclear cells (PBMC) from 70 newly diagnosed CRC patients and 70 healthy age-matched controls from the Czech Republic. In this country, CRC constitutes a serious health problem as it has among the highest rates of incidence and mortality worldwide (28). Simultaneously, we have also profiled mRNA expression levels of nine genes involved in the incision phase of NER, whose rate is measured by our functional assay. Further, we have genotyped common single nucleotide polymorphisms (SNPs) in four NER genes. Additionally, the above variables were also analyzed in the context of individual biological and lifestyle factors and principal clinical characteristics.

The strength of our approach was that we included in the study only patients with newly diagnosed, histologically confirmed sporadic CRC. The homogeneity of the study group was further potentiated by the fact that all patients were sampled prior to any surgical intervention or therapy, thus eliminating the effects of these factors on the individual level of investigated parameters and minimizing the bias on the results.

Materials and methods

Study population

The study population comprised 70 patients with sporadic CRC and 70 healthy controls frequency matched for age. All participants were of Caucasian origin.

Incident cases were recruited at the time of the diagnosis among patients visiting two surgical departments (Thomayer Teaching Hospital in Prague and Teaching Hospital and Medical School in Pilsen, Czech Republic) between 2008 and 2010. Only new histologically confirmed CRC cases who did not receive any surgery or other specific treatment prior to sampling were included into the study. Collaborating clinicians provided clinical and pathological characteristics for each CRC patient: presence of inflammatory processes, number of polyps at colonoscopy, tumor localization, stage of the malignancy [tumor, nodes, metastases staging system (TNM) according to Union of International Cancer Control] and microsatellite instability (MSI) status.

Controls were selected from healthy individuals of similar age distribution who provided blood samples voluntarily. Only subjects with no previous diagnosis and without manifestation of any disease were included into the study. Controls have not been exposed to any potentially harmful chemicals except for those from environmental sources. No other selection criteria have been applied.

Participating subjects were properly informed about the aim of the research; they signed a written consent and the approval for genetic analysis, in accord with the Helsinki declaration. The Ethics Committees of the Thomayer Teaching Hospital in Prague and Teaching Hospital and Medical School in Pilsen (Czech Republic) approved the design of the study. Trained personnel

Table II. Characteristics of the study population

Characteristics	Category	Controls[a]	CRC patients	*P*-value[b]
Sex		*n* = 70	*n* = 70	
	Female	36	24	
	Male	34	46	***P* = 0.042**
Age		*n* = 70	*n* = 70	
	Mean ± standard deviation	62.1 ± 12.7	65.4 ± 10.1	
	Median (quartiles)	60 (52–74)	66 (58–74)	
	Range	39–86	39–84	*P* = 0.079
Diagnosis			*n* = 70	
	Colon		38 (54.3%)	
	Rectum		32 (45.7%)	
TNM			*n* = 69	
	I		7 (10.2%)	
	II		25 (36.2%)	
	III		16 (23.2%)	
	IV		21 (30.4%)	
Smoking status		*n* = 69	*n* = 66	
	Non-smokers	54 (78.3%)	50 (75.8%)	
	Smokers	15 (21.7%)	16 (24.2%)	*P* = 0.732
Number of cigarettes/day		*n* = 14	*n* = 14	
	≤10	9 (64.3%)	6 (42.9%)	
	>10	5 (35.7%)	8 (57.1%)	*P* = 0.592
Alcohol consumption		*n* = 69	*n* = 65	
	No	26 (37.7%)	18 (27.7%)	
	Yes	43 (62.3%)	47 (72.3%)	*P* = 0.222
Alcohol intake (grams/day)		*n* = 43	*n* = 43	
	<25	24 (55.8%)	28 (65.1%)	
	25–49.9	12 (27.9%)	80 (18.6%)	
	50–74.9	40 (9.3%)	50 (11.6%)	
	75–100	3 (7%)	2 (4.7%)	*P* = 0.342
Body mass index		*n* = 61	*n* = 54	
	Mean ± standard deviation	26.1 ± 4.2	27.5 ± 4.5	
	<18.5	1 (1.7%)	2 (3.7%)	
	18.5–24.9	29 (47.5%)	14 (25.9%)	
	25.0–29.9	24 (39.3%)	23 (42.6%)	
	30.0–40.0	7 (11.5%)	15 (27.8%)	***P* = 0.039**
Family history of cancer		*n* = 68	*n* = 66	
	Negative	41 (60.3%)	35 (53%)	
	Positive	27 (39.7%)	31 (47%)	*P* = 0.400
CRC in family		*n* = 61	*n* = 63	
	No	53 (86.9%)	56 (88.9%)	
	Yes	8 (13.1%)	7 (11.1%)	*P* = 0.735
Diabetes		*n* = 66	*n* = 65	
	No	59 (89.4%)	53 (81.5%)	
	Yes	7 (10.6%)	12 (18.5%)	*P* = 0.205

[a]Data were not available for all study participants for some parameters.
[b]One-way ANOVA for categorical variables, Kruskal–Wallis test for continuous variables. Significant *P*-values shown in bold.

interviewed patients and controls, using a structured questionnaire. Study subjects provided information on their lifestyle habits, body mass index (BMI), diabetes and family/personal history of cancer (Table II). Lifelong or long-term (at least six consecutive months) drug use or exposures to genotoxins were also investigated by the questionnaire.

Isolation of PBMC

Eight milliliters of peripheral venous blood were drawn from each subject into heparinized tubes, mixed 1:1 with RPMI 1640 medium (HEPES modification, containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 1.5% phytohemagglutinin and 0.2% penicillin/streptomycin, Sigma-Aldrich), layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 320g for 40 min at room temperature (RT). Isolated PBMC were counted and their viability was checked by trypan blue exclusion. When viability was higher than 95%, cells were aliquoted into cultivation tubes with medium ($\sim 10^5$ cells per 5-ml medium). Tubes were incubated at 37°C. After a mitogen-stimulation period of 20 h, PBMC were further processed for the challenge assay.

Challenge assay for evaluating NER capacity

NER-DRC was analyzed as a level of intermediate single-strand breaks (SSBs) in DNA of (+)-anti-Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)-treated cells, originated during the incision of BPDE-DNA adducts by NER pathway. The increase in DNA breaks reflects the ability of NER machinery to recognize and remove corresponding adducts from DNA.

The methodology is described in detail in Slyskova *et al.* (29). Briefly, BPDE was added into the medium with mitogen-stimulated PBMC at a concentration of 1 μM for 30 min at 37°C. After challenge, old medium was replaced by a new one to remove BPDE excess. PBMC were harvested immediately after the treatment (time 0) or further cultured and harvested at 1, 2 and 4 h. Separated from medium by centrifugation, cells from each experimental point were rewashed with PBS and further processed using a standard comet assay protocol.

In parallel, culturing, harvesting and processing of PBMC of the same individual and in the same conditions but without any BPDE treatment were performed. Untreated PBMC represented the basal control DNA damage.

Comet assay

SSBs in DNA were analyzed by the alkaline comet assay based on a routinely used protocol (30). Experimental conditions for lysis, alkali treatment, electrophoresis, neutralization and scoring are presented in (29). Data are reported as tail DNA%, determined in 50 randomly selected cells from two parallel slides per experimental point.

DRC and endogenous DNA damage calculation

For each experimental point, the net DNA damage value was calculated by subtracting the basal control tail DNA% of untreated cells from the tail DNA% of treated cells. Final and reported DRC value was obtained as a difference between the net level of tail DNA% measured immediately after the treatment

with BPDE at time 0, and the net value at the maximal increase of tail DNA% detected up to 4 h of culturing.

The parameter reported as 'endogenous DNA damage' represents the mean value of all independent measurements of tail DNA% of the untreated control PBMCs.

SSBs in DNA per 10^9 dalton can be derived from tail DNA% by multiplying by a conversion factor of 0.042 based on a calibration curve (over the range of damage detected in the current study, the calibration curve is linear) as reported in (31).

Expression analysis

RNA isolation and quality control. Total RNA from 2 ml of fresh peripheral blood sampled into EDTA vacutainers was isolated using TRIzol according to the procedure supplied by manufacturer (Invitrogen, Paisley, UK) and was kept at −80°C. RNA integrity (RIN) was measured using capillary electrophoresis performed on Agilent Bioanalyzer 2100, with RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RIN of all samples were in the range between 8.0 and 10.0. RNA quantity and purity was measured using ASP-3700 Micro-volume UV/Vis Spectrophotometer (Avans-Biotechnology, Taiwan). $OD_{260/280}$ ratios for all samples were between 1.8 and 2.0. Inhibition testing was performed for all samples by adding internal control template DNA (spike DNA), using Internal DNA extraction control kit (Primer Design, Southampton, UK) and following manufacturer's instructions.

cDNA synthesis and RT-qPCR. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA by using a RevertAid™ First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers in a final volume of 40 µl following manufacturer's instructions. cDNA was stored at −20°C. qPCR was performed on 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using chemicals produced by Primer Design Ltd. Precision™ 2× qPCR Mastermix and custom designed real-time PCR assays with PerfectProbe™ were used. All target genes (*ERCC1*, *RAD23B*, *RPA1*, *XPA*, *XPB*, *XPC*, *XPD*, *XPF*, *XPG*) assays were individually designed and were fully validated, with guaranteed priming specificity (BLAST screening) and > 90% of efficiency. Primer sequences are shown in Supplementary Table I, available at *Mutagenesis* Online. The PCR reactions were performed in a volume of 20 µl, containing 25 ng of cDNA for each sample. Cycling program was set at initial hold at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 32 sec and 72°C for 15 sec. Each run contained positive (interplate calibrator, 25 ng of human cDNA) and negative (no template) control. Results were analyzed using integrated 7500 System SDS Software version 1.3.1 (Applied Biosystems).

Reference genes were selected from a geNorm™ housekeeping gene selection kit of 12 genes with PerfectProbe™ and analyzed by both Genorm and Normfinder algorithms (GenEx Professional, MultiD Analyses AB, Göteborg, Sweden). Two combinations of selected reference genes (*TOP1*, *EIF4A2* and *B2M*, *CYC1*) were tested for stability in all study samples, but none of them proved to be a reliable normalization factor, and the same was observed for total RNA amount. Therefore, Cq values of target genes were normalized to mean expression of all genes, as it was shown to be the best normalization factor, applying both Genorm and Normfinder algorithms. *M*-value for mean expression of all genes was 0.1, when ignoring groups, or 0.02, when stratified for groups (patients versus controls). Data are expressed as relative to maximum quantities (lowest expression was considered as 1). Expression analyses were performed following MIQE guidelines (11).

Genotyping analysis

Considering the size of our study population, SNPs were chosen according to the minor allele frequency (MAF > 0.25) and according to the expected effect on DRC phenotype based on (12). All subjects were genotyped for five polymorphisms in four NER genes: *XPA*, *XPC*, *XPD* and *XPG*. For *XPD* Lys751Gln (rs28365048), *XPG* Asn1104His (rs17655) and *XPC* Lys939Gln (rs2228001) genotyping a PCR-RFLP procedure was carried out using primers and conditions previously described (13). For *XPC* Ala499Val (rs2228000), primers and conditions of reaction have been described in (15). *XPA* G23A (rs1800975) has been analyzed with TaqMan allelic discrimination assay (Applied Biosystems; Assay-on-demand, SNP genotyping products: C_482935_1). The results were regularly confirmed by random regenotyping of >10% of the samples for each polymorphism and showed concordant results. The genotypes with ambiguous and/or no results were excluded from the data set. Distribution of genotypes in the study group is shown in Supplementary Table II, available at *Mutagenesis* Online.

Statistical analysis

Investigated parameters were normalized by logarithmic transformation due to their asymmetric distribution in the study population. The relationships between variables of interest at the bivariate level were studied by means of *T*-test, ANOVA and Pearson correlation. The strength of associations between CRC occurrence and categorical variables at binary level were tested by Chi-square test. The binary logistic regression was employed to study the simultaneous association of the DNA damage, NER-DRC and gene expression with CRC, adjusted for age, sex, smoking habit, alcohol consumption, BMI and family history of cancer. Genotype frequencies for each polymorphism were tested for compliance with Hardy–Weinberg equilibrium. All statistical tests were performed at 5% level of statistical significance; for expression data, correction for multiple testing analyses (significant *P*-value after correction being 0.005*)* was applied. The SPSS analytical package version 16.0 (Chicago, IL, USA) was employed for all statistical analyses.

Results

Study population

The study was carried out on 70 incident CRC patients and 70 healthy controls (mean age ± SD 65.4 ± 10.1 and 62.1 ± 12.7 years, respectively). In patients, malignancy in colon accounted for 54.3% of cases, whereas the rest were diagnosed for rectal cancer. The TNM staging was available for 69 patients: 7 were classified as stage I, 25 belonged to the stage II, 16 to stage III and stage IV was assigned for 21 individuals. In one patient, the pathologist failed to determine TNM. MSI status was available for 41 cases and 7 out of them (17.1%) were MSI unstable. The distribution of all clinical, biological or lifestyle characteristics is reported in Table II. No differences were observed between patients and controls, except for sex distribution (males prevailed among patients, $P = 0.042$) and for BMI (lower in controls, $P = 0.039$).

Endogenous DNA damage

Significantly higher endogenous DNA damage was observed in CRC patients, with median 25.9 (interquartile range 4.0–43.0) tail DNA% as compared to controls, median 9.3 (interquartile range 2.4–21.5) tail DNA%, ($P < 0.001$; Figure 1). After categorizing DNA damage into quartiles, we observed that incident CRC patients were over-represented in the fourth quartile category with the highest DNA damage (odds ratio [OR] 11.49, 95% confidence interval [CI] 3.36–39.34, $P < 0.001$), while the controls prevailed among those with the lowest level of DNA damage (first quartile). Investigated biological or lifestyle factors (age, sex, smoking status, alcohol consumption, BMI, family history of cancer and diabetes),

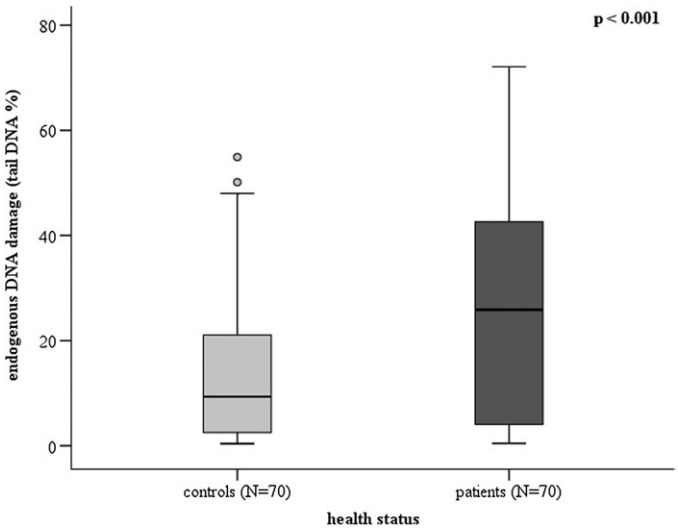

Fig. 1. Endogenous DNA damage level in CRC patients and healthy controls.

when included into the binary logistic regression model, did not significantly affect the value of regression coefficients.

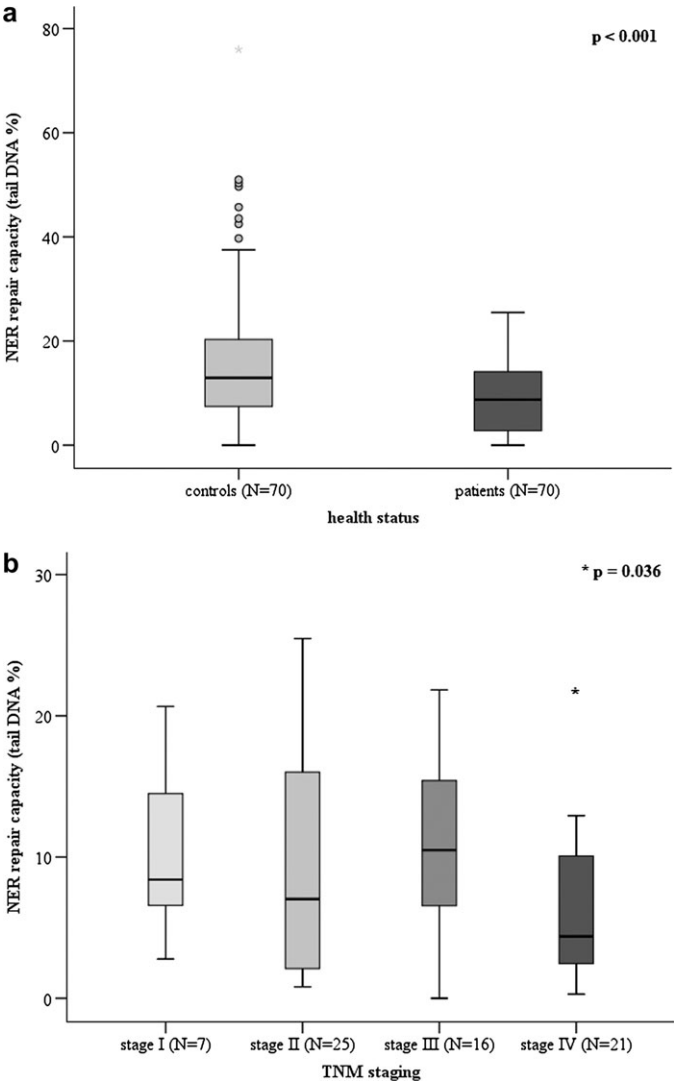

Fig. 2. (a) NER-DRC in CRC patients and healthy controls. (**b**) NER-DRC in CRC patients stratified according to TNM staging.

There was no association between DNA damage and TNM stage or localization of the tumor.

NER-DRC capacity

The CRC patients, with median 8.7 (interquartile range 2.7–14.3) tail DNA%, exhibited significantly lower NER-DRC than the controls with median 12.9 (interquartile range 7.4–20.6) tail DNA% ($P < 0.001$; Figure 2a). After categorizing NER-DRC into quartiles, the subjects with the lowest NER-DRC (the first quartile) comprised mainly CRC patients, whereas control subjects were the majority in the fourth quartile with the highest NER-DRC (OR 0.1, CI 0.03–0.32, $P < 0.001$). Similar to DNA damage, none of the investigated biological or lifestyle factors were associated with NER-DRC and their inclusion into the binary logistic model did not affect the value of regression coefficient. DNA damage and NER-DRC did not significantly correlate, either in the pooled study population or according to the diagnosis. Despite only a moderate decrease of NER-DRC in patients with TNM from I to III, a most pronounced reduction in NER-DRC was observed in patients with stage IV ($P = 0.036$, Figure 2b).

Expression and genotyping analyses

Expression profile was analyzed in a subgroup of 66 patients and 42 controls, for which RNA material was available and which pass the selection criteria for RNA purity and quality and control of PCR inhibition (as described in Materials and methods). Out of nine studied genes, mRNA levels of six of them significantly differed between patients and controls, also after applying correction for multiple testing analysis (significant *P*-value after correction being 0.005). Expression levels of *XPB* and *XPF* genes were higher in control group, while higher expression levels of *XPA*, *XPG*, *ERCC1* and *RAD23B* were detected in patients (for all $P \leq 0.001$, Figure 3). A strong relationship between expression levels of *RAD23B*, *XPG* and *ERCC1* genes was observed in the whole study group ($R = 0.98$, $P < 0.001$) and after stratification for patients and controls. Expression levels of any studied repair gene did not correlate with DNA damage or DRC and were not modulated by any of clinical, biological or lifestyle factors.

Distribution of the analyzed genotypes was in agreement with the Hardy–Weinberg equilibrium. None of the studied

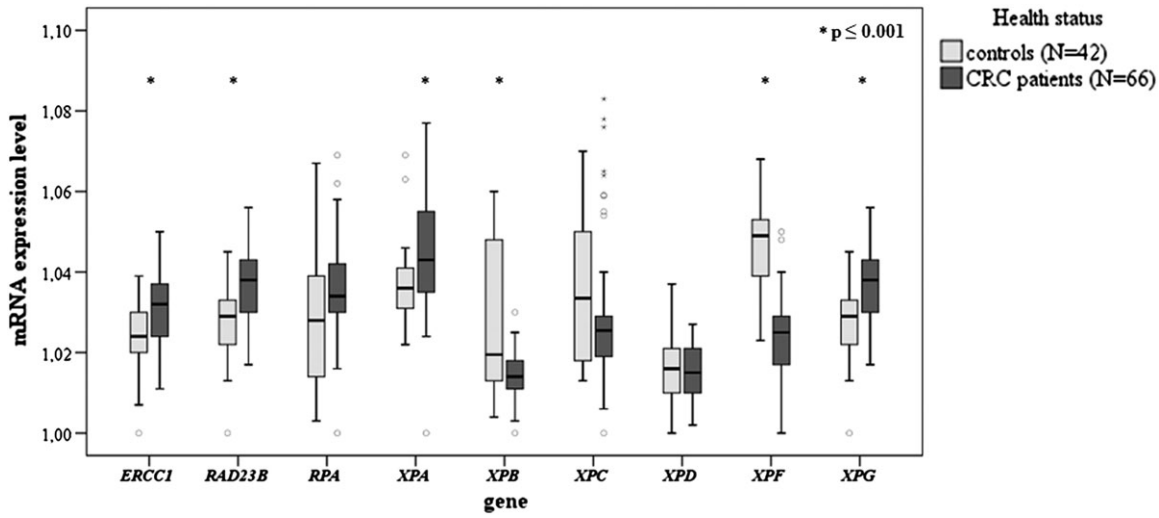

Fig. 3. mRNA expression levels of nine NER genes in CRC patients and healthy controls, expressed as quantities relative to the lowest detected expression assigned as value 1.

SNPs were significantly associated with DNA damage levels or DRC, either in patients or in controls. Expression levels were only moderately modulated by some SNPs. In cases only, variant allele of *XPC* Ala499Val was associated with lower *XPC* and higher *XPB* and *XPD* expression levels (for all $P < 0.05$). In controls, variant allele of *XPC* Lys939Gln was associated with higher *XPA* expression level ($P < 0.05$; data not shown).

Discussion

DRC reflects the actual capacity of the organism to maintain DNA integrity and constitutes an informative biomarker of intermediate cancer phenotype (19). Our study represents an investigation on basal DNA damage and DRC in relation to sporadic CRC. In particular, we have focused our interest on evaluation of individual DRC characterizing NER activity. To approach this, we have challenged PBMC of study subjects by BPDE and quantified the removal of BPDE adducts from DNA (which reflects rate-limiting incision step of NER pathway) by a modified version of comet assay. BPDE was chosen as a model compound for two main reasons. First, it is a metabolite of carcinogen benzo(a)pyrene (BaP), to which an organism is commonly exposed from various sources. Along with environmental or occupational pollution, BaP is generated also by pyrosynthesis in burning tobacco or in meat prepared at high temperatures. Second, BPDE binds to DNA, forming predominantly N^2-deoxyguanosine bulky adducts, which have been detected in colonic mucosa (20) and are specifically removed by NER (16). Generally, NER recognizes a wide spectrum of bulky DNA lesions induced by UV light and a variety of helix-distorting agents (17). A reduced NER capacity may consequently enhance the CRC risk due to a diminished protection of intestinal epithelium against genotoxic compounds, present in the lumen or transported by the blood.

In our study group, newly diagnosed CRC patients had significantly lower NER-DRC and higher levels of SSBs in DNA as compared to the age-matched healthy subjects. This was clearly documented after categorization of the above parameters into quartiles, where accumulation of SSBs and decreasing DRC were characteristic for CRC patients. Interestingly, the two parameters behaved as factors independently associated with sporadic CRC. Furthermore, when the cases were stratified according to the TNM staging, significantly lower NER-DRC was observed in patients with the stage IV in comparison to those with less severe/invasive stages of the disease. A gradual decrease of DRC with increasing TNM stage in a surrogate tissue possibly reflects important biological phenomenon in relation to progression of the disease. Deficient or reduced NER-DRC was already reported as a risk factor for several different cancers, including bladder (18), breast (21,22), skin (23–25), head and neck (14,32,33), lung (34–37) and prostate cancer (38), as summarized in Table I. Our results contribute to the list of evidences on the importance of NER-DRC in carcinogenesis, showing the same relevance also for sporadic CRC. To our knowledge, the only study investigating specifically NER capacity in sporadic CRC patients was performed almost 30 years ago by unscheduled DNA synthesis in smaller study group (39).

Simultaneously with reduced NER-DRC, CRC patients exhibited elevated endogenous DNA damage. The arbitrary unit of tail DNA% may be converted to SSBs/10^6 nucleotides (31,40). By expressing our data in this latter unit, we obtain 0.18 versus 0.37 SSBs/10^6 nucleotides in studied controls and patients, respectively which means ~540 versus 1113 breaks/cell. Pooling together data from 119 publications reporting endogenous DNA damage extent in cancer-free populations (41), a clear positive correlation was observed between level of SSBs and age. For individuals belonging to the 50- to 64-year-old group, an average SSB level of 510 breaks/cell was calculated, which is in full agreement with our data. This demonstrates that CRC patients bear >2-fold higher level of strand breaks in DNA than the reference value for healthy population of the same age. The enhanced levels of DNA damage represent an additional suggestion for a generally altered status of the DNA repair machinery among cancer patients. In agreement with our findings, several studies have shown that basal DNA damage is indeed increased in leukocytes of patients suffering from a variety of different forms of cancer, as reviewed by (42). On the other hand, we cannot rule out that the level of endogenous DNA damage may comprise various alkali-labile DNA lesions, converted during comet assay into SSBs. These lesions are a target for BER pathway, which was also reported to be deficient in cancer patients (43,44).

Additionally, mRNA quantity of nine genes involved in the recognition/incision step of NER was studied. Expression levels of individual genes were not significantly related to either NER-DRC or the extent of DNA damage. However, a coordinated expression of *RAD23B*, *XPG* and *ERCC1* genes was observed. This is an interesting finding considering that XPC-RAD23B complex acts in DNA damage recognition and subsequently recruits the XPG and ERCC1/XPF to the site of damage directly or via strong interactions with TFIIH nine subunits complex (45,46). Six NER genes were found to be differently transcribed between patients and controls. *XPB* and *XPF* had higher expression in controls, while *XPA*, *XPG*, *ERCC*1 and *RAD23B* were more expressed in patients. Such a finding is somehow surprising, as total DRC was shown to be lower in patients. Observed expression profiles and the lack of correlation between quantity of mRNA and DRC may be in concordance with previous observations showing that mRNA quantity does not necessarily reflect the activity of protein (47,48) or overall repair capacity (49,50). In some cases, it is the absence rather than the relative content of a functional protein that is important for cellular activity. Moreover, genes act in interactive networks and alterations of each of them might have different impact on the overall cell function (51). Communication between the NER system and DNA damage signaling may also play a critical role (52).

In the present study, we have also attempted to relate outcomes of functional tests and expression levels to relevant variation in some NER genes. The individual differences in DNA damage levels and DRC as well as expression levels have been hypothesized for many years to be associated to individual genetic background in DNA repair genes (13,29). In our hands, investigated SNPs were not significantly associated with either DNA damage or DRC after stratification for health status. However, there were some associations with NER gene expression, the most interesting being a modulation of expression levels of *XPC*, *XPB* and *XPD* by *XPC* Ala499Val variant allele in CRC patients. A potential functional effect of this SNP is supported by previous studies on susceptibility to cancer (53,54). A role of SNPs in NER pathway on sporadic CRC risk has been recently postulated (55,56), but the limited size of our currently studied population precludes evaluation of the association of individual SNPs with CRC risk. The actual

association of variation in DNA repair genes and CRC risk has not been clearly disclosed either in the context of recent genome-wide association studies (57).

In conclusion, our study provides evidence on alterations of cellular DRC among sporadic CRC patients and suggests the role of NER in its etiology. However, one of the main future challenges with intermediate biomarkers, like DNA damage and DRC, is to understand whether they belong to the causal pathway of a disease, whether they are simply a side effect of a disease or whether their measurement may be confounded by some other factors.

Genes involved in DNA damage recognition/incision phase of NER act in an interplay and in a synchronized way (45,58). Based on our findings, this process is more comprehensively characterized by functional quantification of repair capacity than by quantification of individual gene transcripts or gene variants. Overall, our observation points out the usefulness of DRC analyses, which measure the real outcome of a complex multigene process, as also recently concluded by Collins and Azqueta (59). Comet assay is a convenient methodology for DRC evaluation and high-throughput versions of it are currently under development. This will allow the simultaneous determination of DRC in multiple samples and thus making this assay suitable for large population screening minimizing inter-experimental variation (60).

Supplementary data

Supplementary Tables I and II are available at *Mutagenesis* Online.

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Erratum

Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls

Jana Slyskova, Alessio Naccarati, Barbara Pardini, Veronika Polakova, Ludmila Vodickova, Zdenek Smerhovsky, Miroslav Levy, Ludmila Lipska, Vaclav Liska and Pavel Vodicka

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In the published article listed above, the numbers of the references cited in the 'cDNA synthesis and RT-qPCR' and 'Genotyping analysis' subsections of the Materials and methods and in the Discussion section were incorrect. The corrected sections are reproduced below.

cDNA synthesis and RT-qPCR. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using a RevertAid™ First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers in a final volume of 40 μl following manufacturer's instructions. cDNA was stored at −20°C. qPCR was performed on 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using chemicals produced by Primer Design Ltd. Precision™ 2× qPCR Mastermix and custom designed real-time PCR assays with PerfectProbe™ were used. All target genes (*ERCC1*, *RAD23B*, *RPA1*, *XPA*, *XPB*, *XPC*, *XPD*, *XPF*, *XPG*) assays were individually designed and were fully validated, with guaranteed priming specificity (BLAST screening) and > 90% of efficiency. Primer sequences are shown in Supplementary Table I, available at *Mutagenesis* Online. The PCR reactions were performed in a volume of 20 μl, containing 25 ng of cDNA for each sample. Cycling program was set at initial hold at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 32 sec and 72°C for 15 sec. Each run contained positive (interplate calibrator, 25 ng of human cDNA) and negative (no template) control. Results were analyzed using integrated 7500 System SDS Software version 1.3.1 (Applied Biosystems).

Reference genes were selected from a geNorm™ housekeeping gene selection kit of 12 genes with PerfectProbe™ and analyzed by both Genorm and Normfinder algorithms (GenEx Professional, MultiD Analyses AB, Göteborg, Sweden). Two combinations of selected reference genes (*TOP1*, *EIF4A2* and *B2M*, *CYC1*) were tested for stability in all study samples, but none of them proved to be a reliable normalization factor, and the same was observed for total RNA amount. Therefore, Cq values of target genes were normalized to mean expression of all genes, as it was shown to be the best normalization factor, applying both Genorm and Normfinder algorithms. *M*-value for mean expression of all genes was 0.1, when ignoring groups, or 0.02, when stratified for groups (patients versus controls). Data are expressed as relative to maximum quantities (lowest expression was considered as 1). Expression analyses were performed following MIQE guidelines (32).

Genotyping analysis. Considering the size of our study population, SNPs were chosen according to the minor allele frequency (MAF > 0.25) and according to the expected effect on DRC phenotype based on (33). All subjects were genotyped for five polymorphisms in four NER genes: *XPA*, *XPC*, *XPD* and *XPG*. For *XPD* Lys751Gln (rs28365048), *XPG* Asn1104-His (rs17655) and *XPC* Lys939Gln (rs2228001) genotyping a PCR-RFLP procedure was carried out using primers and conditions previously described (34). For *XPC* Ala499Val (rs2228000), primers and conditions of reaction have been described in (35). *XPA* G23A (rs1800975) has been analyzed with TaqMan allelic discrimination assay (Applied Biosystems; Assay-on-demand, SNP genotyping products: C_482935_1). The results were regularly confirmed by random regenotyping of >10% of the samples for each polymorphism and showed concordant results. The genotypes with ambiguous and/or no results were excluded from the data set. Distribution of genotypes in the study group is shown in Supplementary Table II, available at *Mutagenesis* Online.

Discussion

DRC reflects the actual capacity of the organism to maintain DNA integrity and constitutes an informative biomarker of intermediate cancer phenotype (36). Our study represents an investigation on basal DNA damage and DRC in relation to sporadic CRC. In particular, we have focused our interest on evaluation of individual DRC characterizing NER activity. To approach this, we have challenged PBMC of study subjects by BPDE and quantified the removal of BPDE adducts from DNA (which reflects rate-limiting incision step of NER pathway) by a modified version of comet assay. BPDE was chosen as a model compound for two main reasons. First, it is a metabolite of carcinogen benzo(a)pyrene (BaP), to which an organism is commonly exposed from various sources. Along with environmental or occupational pollution, BaP is generated also by pyrosynthesis in burning tobacco or in meat prepared at high temperatures. Second, BPDE binds to DNA, forming predominantly N^2-deoxyguanosine bulky adducts, which have been detected in colonic mucosa (37) and are specifically removed by NER (38). Generally, NER recognizes a wide spectrum of bulky DNA lesions induced by UV light and a variety of helix-distorting agents (39). A reduced NER capacity may consequently enhance the CRC risk due to a diminished protection of intestinal epithelium against genotoxic compounds, present in the lumen or transported by the blood.

In our study group, newly diagnosed CRC patients had significantly lower NER-DRC and higher levels of SSBs in DNA as compared to the age-matched healthy subjects. This was clearly documented after categorization of the above parameters into quartiles, where accumulation of SSBs and decreasing DRC were characteristic for CRC patients. Interestingly, the two parameters behaved as factors independently associated with sporadic CRC. Furthermore, when the cases were stratified according to the TNM staging, significantly lower NER-DRC was observed in patients with the stage

IV in comparison to those with less severe/invasive stages of the disease. A gradual decrease of DRC with increasing TNM stage in a surrogate tissue possibly reflects important biological phenomenon in relation to progression of the disease. Deficient or reduced NER-DRC was already reported as a risk factor for several different cancers, including bladder (11), breast (12,13), skin (15,19,20), head and neck (16–18), lung (21–24) and prostate cancer (25), as summarized in Table I. Our results contribute to the list of evidences on the importance of NER-DRC in carcinogenesis, showing the same relevance also for sporadic CRC. To our knowledge, the only study investigating specifically NER capacity in sporadic CRC patients was performed almost 30 years ago by unscheduled DNA synthesis in smaller study group (14).

Simultaneously with reduced NER-DRC, CRC patients exhibited elevated endogenous DNA damage. The arbitrary unit of tail DNA% may be converted to SSBs/10^6 nucleotides (31,40). By expressing our data in this latter unit, we obtain 0.18 versus 0.37 SSBs/10^6 nucleotides in studied controls and patients, respectively which means ~540 versus 1113 breaks/cell. Pooling together data from 119 publications reporting endogenous DNA damage extent in cancer-free populations (41), a clear positive correlation was observed between level of SSBs and age. For individuals belonging to the 50- to 64-year-old group, an average SSB level of 510 breaks/cell was calculated, which is in full agreement with our data. This demonstrates that CRC patients bear >2-fold higher level of strand breaks in DNA than the reference value for healthy population of the same age. The enhanced levels of DNA damage represent an additional suggestion for a generally altered status of the DNA repair machinery among cancer patients. In agreement with our findings, several studies have shown that basal DNA damage is indeed increased in leukocytes of patients suffering from a variety of different forms of cancer, as reviewed by (42). On the other hand, we cannot rule out that the level of endogenous DNA damage may comprise various alkali-labile DNA lesions, converted during comet assay into SSBs. These lesions are a target for BER pathway, which was also reported to be deficient in cancer patients (43,44).

Additionally, mRNA quantity of nine genes involved in the recognition/incision step of NER was studied. Expression levels of individual genes were not significantly related to either NER-DRC or the extent of DNA damage. However, a coordinated expression of *RAD23B*, *XPG* and *ERCC1* genes was observed. This is an interesting finding considering that XPC-RAD23B complex acts in DNA damage recognition and subsequently recruits the XPG and ERCC1/XPF to the site of damage directly or via strong interactions with TFIIH nine subunits complex (45,46). Six NER genes were found to be differently transcribed between patients and controls. *XPB* and *XPF* had higher expression in controls, while *XPA*, *XPG*, *ERCC*1 and *RAD23B* were more expressed in patients. Such a finding is somehow surprising, as total DRC was shown to be lower in patients. Observed expression profiles and the lack of correlation between quantity of mRNA and DRC may be in concordance with previous observations showing that mRNA quantity does not necessarily reflect the activity of protein (47,48) or overall repair capacity (49,50). In some cases, it is the absence rather than the relative content of a functional protein that is important for cellular activity. Moreover, genes act in interactive networks and alterations of each of them might have different impact on the overall cell function (51). Communication between the NER system and DNA damage signaling may also play a critical role (52).

In the present study, we have also attempted to relate outcomes of functional tests and expression levels to relevant variation in some NER genes. The individual differences in DNA damage levels and DRC as well as expression levels have been hypothesized for many years to be associated to individual genetic background in DNA repair genes (29,34). In our hands, investigated SNPs were not significantly associated with either DNA damage or DRC after stratification for health status. However, there were some associations with NER gene expression, the most interesting being a modulation of expression levels of *XPC*, *XPB* and *XPD* by *XPC* Ala499Val variant allele in CRC patients. A potential functional effect of this SNP is supported by previous studies on susceptibility to cancer (53,54). A role of SNPs in NER pathway on sporadic CRC risk has been recently postulated (55,56), but the limited size of our currently studied population precludes evaluation of the association of individual SNPs with CRC risk. The actual association of variation in DNA repair genes and CRC risk has not been clearly disclosed either in the context of recent genome-wide association studies (57).

In conclusion, our study provides evidence on alterations of cellular DRC among sporadic CRC patients and suggests the role of NER in its etiology. However, one of the main future challenges with intermediate biomarkers, like DNA damage and DRC, is to understand whether they belong to the causal pathway of a disease, whether they are simply a side effect of a disease or whether their measurement may be confounded by some other factors.

Genes involved in DNA damage recognition/incision phase of NER act in an interplay and in a synchronized way (45,58). Based on our findings, this process is more comprehensively characterized by functional quantification of repair capacity than by quantification of individual gene transcripts or gene variants. Overall, our observation points out the usefulness of DRC analyses, which measure the real outcome of a complex multigene process, as also recently concluded by Collins and Azqueta (59). Comet assay is a convenient methodology for DRC evaluation and high-throughput versions of it are currently under development. This will allow the simultaneous determination of DRC in multiple samples and thus making this assay suitable for large population screening minimizing inter-experimental variation (60).

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Supplementary Table I. Description of primers and amplified sequences of target genes

Gene	Sequence accession number	Amplicon length (bp)	Distance from 3′UTR (bp)	Primer	Sequence of primer
ERCC1	NM_202001	124	776	Forward	CAATCCCGTACTGAAGTTCGT
				Reverse	TGGGTGCAGGTTGTGGTAG
RAD23B (*HR23B*)	NM_002874	144	2929	Forward	TTTACGGAATCAGCCTCAGTTT
				Reverse	TGAATAAAATGCTCCTGGTGTTG
RPA1	NM_002945	100	3294	Forward	GAAAGACTCACTTGTAGACATCATC
				Reverse	ATTCCTCTTGGCAACTTCTCTG
XPA	NM_000380	102	850	Forward	GGGGTGATATGAAACTCTACTTAAAG
				Reverse	CCTGTCGGACTTCCTTTGC
XPB (*ERCC3*)	NM_000122	122	2124	Forward	GAAAGTTGCCACCCTGATGTA
				Reverse	GATTTGCTTGTGAAAGTCTCTGT
XPC	NM_004628	102	1910	Forward	TCCGAGATGTCACACAGAGG
				Reverse	TCTGGTATGGTCTCAAGGTCTC
XPD (*ERCC2*)	NM_000400	130	1289	Forward	CCAAAGGCTTCACCATCATCA
				Reverse	ACAGACTGGAAACGCTCAAAATA
XPF (*ERCC4*)	NM_005236	89	3436	Forward	CTGGAACACTGAGAAGAAACATC
				Reverse	GCAGACAGGCAGAAGTATGG
XPG (*ERCC5*)	NM_000123	126	2452	Forward	AAGTGTGTGCTGGGGGATGA
				Reverse	CAGTAAACGGTATTCCTTTTCCAT

Supplementary Table II. Distribution of genotypes in the study group

Polymorphism	Genotype	Controls		CRC patients		X^2, P-value [a]
		Frequency [b]		Frequency [b]		
		N	%	N	%	
		68	100	70	100	
	GG	33	48.5	27	38.6	0.09, 0.96
XPA G23A	*GA*	28	41.2	33	47.1	
	AA	7	10.3	10	14.3	
	GA+AA	35	51.5	43	61.4	
		64	100	69	100	
	CC	37	57.8	36	52.2	0.13, 0.94
XPC Ala499Val	*CT*	24	37.5	24	34.8	
	TT	3	4.7	9	13.0	
	CT+TT	27	42.2	33	47.8	
		68	100	70	100	
	AA	27	39.7	22	31.4	3.02, 0.22
XPC Lys939Gln	*AC*	26	38.2	35	50	
	CC	15	22.1	13	18.6	
	AC+CC	41	60.3	48	68.6	
		65	100	70	100	
	AA	15	23.1	32	45.7	0.76, 0.68
XPD Lys751Gln	*AC*	36	55.4	26	37.1	
	CC	14	21.5	12	17.1	
	AC+CC	50	76.9	38	54.3	
		68	100	70	100	
	GG	39	57.4	43	61.4	0.24, 0.89
XPG Asn1104His	*GC*	24	35.3	26	37.1	
	CC	5	7.4	1	1.4	
	GC+CC	29	42.6	27	38.6	

[a] X^2 and P-values for the deviation of observed and the numbers expected from the Hardy-Weinberg equilibrium in the controls.

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

Manuscript V

Slyskova J, Korenkova V, Collins A, Prochazka P, Vodickova L, Svec J, Lipska L, Levy M, Schneiderova M, Liska V, Holubec L, Soucek P, Naccarati A, Vodicka P

Functional, genetic and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas

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Functional, genetic and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas

Jana Slyskova[1,2,3,*], Vlasta Korenkova[4], Andrew R. Collins[5], Pavel Prochazka[1], Ludmila Vodickova[1,3], Jiri Svec[1,6], Ludmila Lipska[7], Miroslav Levy[7], Michaela Schneiderova[8], Vaclav Liska[9], Lubos Holubec[9], Pavel Soucek[10], Alessio Naccarati[1,11], Pavel Vodicka[1,3,*]

[1]*Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Science of the Czech Republic, Prague*

[2]*Faculty of Science, Charles University, Prague, Czech Republic*

[3]*Institute of Biology and Medical Genetics, First Medical Faculty of Charles University, Prague, Czech Republic*

[4]*Laboratory of Gene Expression, Institute of Biotechnology, Prague, Czech Republic*

[5]*Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway*

[6]*Second Department of Internal Medicine, Third Faculty of Medicine, Charles University, Prague, Czech Republic*

[7]*Department of Surgery of Thomayer Hospital and First Medical Faculty of Charles University, Prague, Czech Republic*

[8]*Department of Surgery, General University Hospital in Prague, Czech Republic*

[9]*Teaching Hospital and Medical School Pilsen, Charles University, Prague, Czech Republic*

[10]*Toxicogenomics Unit, National Institute of Public Health, Prague, Czech Republic*

[11]*Molecular and Genetic Epidemiology Unit, Human Genetics Foundation, Torino, Italy*

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***Corresponding authors:**

Department of Molecular Biology of Cancer, IEM, ASCR,

Videnska 1083, 14220 Prague, Czech Republic

Tel.: +420 241 062 694; fax: +420 241 062 782.

E-mail addresses: pvodicka@biomed.cas.cz, jana.slyskova@biomed.cas.cz

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Conflicts of interest:

No conflicts of interest were disclosed.

Translational relevance:

DNA repair influences cancer development and sensitivity to treatment. Categorization of tumours according to their DNA repair characteristics can be relevant for personalized therapy, but functional assays to define DNA repair status of target tissue are needed. In this respect, we optimised BER and NER-specific assays and showed their reliability and applicability to high-throughput screening of human solid tissues. The complexity of multi-gene DNA repair processes is comprehensively reflected by functional analysis of overall DNA repair capacity and should be recommended for DNA repair investigations.

Analysis of BER and NER by functional, genetic and epigenetic approach confirmed that colorectal carcinomas are only moderately altered in these repair pathways as compared to adjacent healthy tissue. Consistency of our and previously reported observations suggests that excision repair is not a factor contributing to the malignant transformation, but might rather contribute to chemoresistency and growth advantage of tumour cells.

Abstract

Purpose: DNA repair capacity (DRC) is a determinant of not only cancer development but also of individual response to therapy. Previously, altered base and nucleotide excision repair (BER and NER) have been described in lymphocytes of sporadic colorectal cancer patients. We, for the first time, evaluate both excision repair capacities in human colon biopsies.

Experimental design: Seventy pairs of tumour and adjacent healthy tissues were analysed for BER and NER-specific DRC by a comet repair assay. Tissue pairs were further compared for expression levels of a panel of 25 BER and NER genes and completed by their promoter methylation status.

Results: We observed a moderate increase of NER-DRC ($p=0.019$), but not BER-DRC in tumours. There was a strong correlation between both tissues for all investigated parameters ($p<0.001$). However four NER (*CSB, CCNH*, *XPA*, *XPD*) and BER *(NEIL1, APEX1*, *OGG1*, *PARP1*) genes showed 1.08-1.28 fold change difference in expression in tumours ($p<0.05$). Individual gene expression levels did not correlate with overall DRC and we did not detect any aberrant methylation of the investigated genes.

Conclusions: Our complex analysis showed that tumour cells are not deficient in BER and NER, but rather follow patterns characteristic for each individual and are comparable with adjacent tissue. Alteration of excision repair pathways is not a pronounced event in colorectal carcinogenesis. This study further shows the feasibility of DRC evaluation in human solid tissues, representing a complex marker of multi-gene DNA repair processes.

Keywords: DNA repair capacity, base excision repair, nucleotide excision repair, colorectal cancer, *in vitro* repair assay

1. Introduction

Despite long and intensive research, colorectal cancer (CRC) has still one of the highest rates of incidence and mortality worldwide (1). With the exception of *KRAS* mutational status for selection of biological treatment, no predictive or prognostic biomarker has yet been validated (2, 3). Keeping in view the importance of DNA repair in the disease development and treatment response, it seems reasonable to contemplate a categorization of tumours based on DNA repair characteristics. Such an approach would require a panel of functional biomarkers that can define the DNA repair status of target tissue (4).

DNA repair is a defensive mechanism to cope with ubiquitous DNA damage, which occurs as a consequence of cellular metabolism or through exogenous exposure. Moreover, a large number of antineoplastic drugs impart their effect by DNA disruption. Therefore, an effective DNA damage response is essential for the maintenance of genome stability in normal cells while in malignant cells, suppression of DNA repair, most likely, would increase the effectiveness of chemotherapy through damage accumulation and consequent apoptosis. Based on the evidence so far, mismatch repair (MMR) defines the strongest link between DNA repair and CRC. A subset of hereditary and sporadic CRC shows genetic or epigenetic defects in MMR that are manifested by microsatellite instability. The phenomenon may also be accompanied by epigenetic instability, characterized by a high degree of aberrant methylation of CpG islands. Germ-line, and not somatic inactivation of base excision repair (BER) gene *MUTYH* causes polyposis, which transforms almost always into carcinoma (5). No study has so far reported any defect in nucleotide excision repair (NER) in any form of CRC.

A potential role of both BER and NER in the pathogenesis of sporadic CRC is plausible. Colon epithelium is one of the most constantly regenerated tissues in the body. It therefore has increased vulnerability to a variety of mutagens present in the bowel contents or in the blood. Cigarette smoke, alcohol, over-cooked red meat or processed saturated fat are established CRC risk factors through generation of strong DNA-reactive compounds (6). Beside others, benzo[a]pyrene, aromatic amines, alkylating agents or reactive oxygen species represent substrates for excision repair. Even detoxified carcinogens can interact with mucosal DNA, as carcinogen-activating enzymes have been detected in colon

epithelium and in colonic bacteria (7-9). Further, the well-known role of chronic inflammation in colon carcinogenesis is explained by enhanced epithelial cell turnover, accompanied by sustainable oxidative stress contributing to neoplastic transformation (10). Moreover, both pathways are reasonably expected to influence the effectiveness of anticancer therapy. The mainstays of CRC treatment are regimes based on 5-fluorouracil and/or oxaliplatin. The NER pathway is known to be essential for removal of platinum adducts, BER, on the other hand, is involved in response to 5-fluorouracil (11, 12).

In general, there is limited information available on the mechanisms of BER and NER in sporadic CRC. No somatic genetic alteration of genes involved in either of the excision repair pathways has been identified. The investigations failed to prove any clear relationship between common genetic variants and the risk of sporadic CRC (13, 14). Aberrant promoter methylation of BER and NER genes have already been reported in other types of cancer, but not studied in CRC (15). Investigation of expression profiles of some BER and NER genes in tumour tissue did provide preliminary characterizations (16). Previous studies carried out in peripheral blood mononuclear cells (PBMC) showed supressed BER and NER capacities in CRC patients compared to healthy individuals (17-19). However, validity of blood as a surrogate for cancer tissue to estimate DRC remains disputable.

The aim of the present study was to investigate BER- and NER-DRC in tumour biopsies from a group of newly diagnosed CRC patients. DRCs of tumour tissues were compared to those in adjacent healthy tissues and, in a subgroup of patients, measurements were also carried out on PBMCs. Furthermore, we compared expression levels of panel of 8 BER and 17 NER genes between studied tissues and investigated the involvement of promoter methylation of excision repair genes in sporadic CRC.

2. Materials and methods

Study patients and collection of biological specimen

The study included seventy sporadic CRC patients who underwent surgical resection. Patients were recruited between 2009 and 2011 in Thomayer Hospital (Prague), General University Hospital (Prague) and Teaching Hospital and Medical School of Charles University (Pilsen). All patients signed informed consent. Ethics approval was granted by appropriate committees of the three hospitals. The group of patients included 53 men and 17 women with a mean age of 66.2 (±10.6). Clinical stage of patients at diagnosis was classified according to the tumour-node-metastasis (TNM) system. Seven patients were diagnosed with pathologic stage I (10%), 29 as stage II (41.4%), 15 as stage III (21.4%) and 19 as stage IV (27.2%). All patients had adenocarcinomas; 44 patients had tumour localized in colon (62.9%) and 26 in rectum (37.1%). In 12 (17.2%) patients tumours were of well differentiated grade, in 47 (67.1%) moderately differentiated and in 11 patients (15.7%) poorly differentiated. Eleven rectal cancer patients (15.7%) received neoadjuvant therapy prior to surgery. Tumour tissue and adjacent healthy colon/rectal tissues (5-10 cm distant from tumour) were resected from all patients. Samples were deep frozen immediately after removal. A day before surgery, peripheral blood was also drawn from a sub-set of patients and was stored at 4°C no longer than 3 hours before being processed. Due to various logistic reasons, not all patients could be analysed for the studied parameters. Therefore, each particular analysis is further specified for actual number of cases for whose analysis was carried out.

Isolation of nucleic acids and protein extracts from blood and tissues

Extraction of nucleic acids: DNA from blood was isolated by standard phenol/chloroform method. Prior to tissue processing, histological analysis was carried out to assess proportion of tumour cells in tumour tissues and to rule out presence of neoplastic cells in the normal mucosal tissues. Briefly, samples were embedded in optimal cutting temperature compound (Sakura Finetek), and cut with a Leica CM 1850 cryostat. Five µm thick serial sections were fixed in 90% ethanol on microscope slides and stained with 1% cresyl violet acetate (Sigma-Aldrich), dehydrated with ethanol, dried and inspected using a Leica DM6000 microscope (Leica). Tissue samples were subsequently homogenized in the MagNA Lyser (Hoffmann-La

Roche). AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) was used to isolate nucleic acids.

Extraction of proteins: PBMC were separated on Histopaque-1077 (Sigma-Aldrich), counted and evaluated by trypan blue exclusion. Cells were suspended in freezing medium (RPMI 1640, 20% FBS, 0.2% ATB, 10% DMSO, Sigma-Aldrich) and frozen at -80°C. Tissues were weighed and ground while frozen. Further, 50µL of buffer A (45mM HEPES, 0.4M KCl, 1mM EDTA, 0.1mM DTT, 10% glycerol, pH 7.8) was added to every 50mg of ground tissue or $5x10^6$ of PBMC. Samples were vortexed, snap frozen and 15µL of 1% Triton X-100 in buffer A was added per each 50µL. Protein concentration was measured by a fluorescamine assay (Sigma-Aldrich), with a NanoDrop 3300 (Thermo Scientific).

In vitro DNA repair assay

In vitro repair assays, adopted from Langie et al. (20, 21), were implemented using a 12-gel slide format (22). Briefly, protein extracts were incubated with two types of substrate DNA, containing artificially induced lesions known to be repaired specifically by BER or NER pathway. Levels of DNA strand breaks, generated during removal of lesions, reflect the repair activity of the extract.

Substrate DNA: For BER, human PBMC were treated with 2µM Ro 19-8022 (Hoffmann-La Roche) for 5 min, and irradiated by a 500W halogen lamp at a 33 cm distance to induce 8-oxoguanines. For NER, TK6 cells were irradiated with 5 Jm^{-2} of UVC (50 sec at 0.1 $Jm^{-2}s^{-1}$) to generate cyclobutane pyrimidine dimers and 6-4 photoproducts. Untreated PBMC and TK6 cells were prepared in parallel. Cells were aliquoted at $0.5x10^6$ in 1mL of freezing medium (see above) and frozen. Before each experiment, cells were thawed by adding 1mL of cold PBS, spun at 400g, 5 min, 4°C and resuspended in 1mL of PBS. Eighty µL of the cell suspension was mixed with 260µL of 1% LMP agarose to reach the desired concentration of cells. Using a multi-dispensing pipettor, 12 gels per 5µL agarose were placed on each microscope slide. Cells embedded in agarose underwent lysis for 1 hour in 2.5M NaCl, 100mM EDTA, 10mM Tris, 250mM NaOH, 1% Triton X-100, pH 10. Before incubation with protein extracts, slides were washed twice for 5 min with buffer B (45mM HEPES, 0.25mM EDTA, 0.3mg/mL BSA, 2% glycerol, pH 7.8) and placed in incubation chambers (Severn Biotech) (22).

Protein extracts: Extracts were diluted into protein concentration of 3mg/mL in buffer A in the final volume of 50 µL and mixed with 4-volumes of buffer B. For the NER-specific assay, 2.5mM of adenosine-5'-triphosphate was added. Thirty µL of extract was pipetted per agarose gel.

BER-specific assay: Each extract was incubated with Ro-treated and untreated PBMC to determine nonspecific endonuclease activity of the extract. This was used for background correction for each sample. Incubation time was 20 min, at 37°C in a humid environment. Formamidopyrimidine DNA glycosylase was used as a positive control and for a negative control, substrate gels were incubated with 1:4 buffer A + buffer B. Each experimental point was performed in duplicates. Five µM of PARP inhibitor ABT-888 (Selleckchem) was added to the extract to test the effect of inhibition of post-incision phase of BER. Reproducibility of the assay was tested by independent repeat of measurement for randomly chosen 25 samples.

NER-specific assay: Each extract was in parallel incubated for 30 minutes with UV-treated and untreated TK6 cells and used for background subtraction. UV substrate incubated with Endonuclease V was used as positive control and 1:4 buffer A + buffer B as negative control. Aphidicolin (DNA polymerase delta inhibitor; Sigma-Aldrich) in a concentration of 2.5 µM was added to the extract to test the effect of DNA resynthesis inhibition. Reproducibility of the assay was tested by independently repeated measurement of randomly chosen 25 samples.

After the incubation, previously described protocol for single cell gel electrophoresis was followed (23). DRC data were evaluated in tail DNA% (%T).

Gene expression profiling

Gene selection: A panel of BER and NER genes (**supplementary Table 1**) was selected from the list of all DNA repair genes organized according to pathways, which are available online (http://sciencepark.mdanderson.org/labs/wood/DNA_Repair_Genes.html#NER).

Sample preparation: Total RNA was measured on ASP-3700 Spectrophotometer (Avans-Biotechnology) for quantity and $OD_{260/280}$ ratio, which was between 1.8 and 2.0. RNA integrity number (RIN) was checked using Agilent Bioanalyzer 2100, with RNA 6000 Nano Assay (Agilent Technologies). Each pair of tumour/healthy tissue did not differ by more than ±2 RIN units. cDNA was synthesized from 1 µg of RNA using a RevertAid^{TM} First strand cDNA

synthesis kit (MBI Fermentas) using random hexamers and following manufacturer´s instructions. All samples were tested to exclude possible inhibition in qPCR reaction by spiking with DNA from an extraction control kit (Primer Design). cDNA was diluted to 10 ng/µL and preamplified for 18 cycles on a Bio-Rad CFX96 Real Time PCR Instrument (Biorad) according to the manufacturer protocol.

Real-time qPCR: qPCR was performed using the high-throughput platform BioMark™ HD System (Fluidigm). Ten µL of reaction mix contained 1 µL of 20x diluted preamplified cDNA, 2.5 µL of Taqman universal mastermix II without UNG (Applied Biosystems, CA, USA), 5 µL of primer/probe assays with PerfectProbe™ (Primer Design) at final concentration of 300 nM, 2.5 µL of 2x Assay loading reagent and 0.25 µL of 20x GE sample loading reagent (Fluidigm) and 1.25 µL of water. Thermal conditions for qPCR were: 95°C for 10 min, 45 cycles of 95°C for 15 s and 50°C for 60 s. *TOP1* and *18S rRNA* were reference genes selected from a geNorm™ reference genes selection kit (Primer Design) by Normfinder (GenEx Enterprise).

qPCR data processing: Data were collected from two 96x96 arrays. Inter-plate calibration was performed and the technical replicates were averaged. Cut off for cq was set up to 25 and values higher than that were replaced by the value of 25. Data were normalized to reference genes. Assays and samples with more than 12% of missing data were removed from the dataset. Due to this selection, 6 repair genes (*CSA, MMS19L, POLB, UNG, XPG* and *XRCC1*) were excluded from analyses. The rest of missing data were replaced through intrapolation of values from the group. Data were recalculated to relative quantities, the lowest expression was set to 1 and transformed to log2 scale.

Promoter CpG islands methylation profiling

Methylation specific PCR (MSP): Prediction of CpG island sites within promoter region of target genes were screened by CpG Islands Searcher (http://cpgislands.usc.edu/). Primers specific to methylated and unmethylated BC DNA for *OGG1*, *ERCC1* and *XRCC1* genes were designed applying MethPrimer algorithm (24) and produced by Sigma-Aldrich. Previously described primers were used for *XPA*, *XPC*, *XPD* and *XPG* genes (25) (**supplementary Table 2**). Genomic DNA was treated with sodium bisulfite using the Epitect Whole Bisulfitome Kit (Qiagen). MSP analysis of bisulfite converted (BC) DNA was performed using the Epitect MSP kit, following producer´s instructions.

Methylation-sensitive high resolution melting (MS-HRM): MS-HRM was conducted to verify MSP-positive samples. Primers specific for BC DNA (**supplementary Table 3**) were designed using Methyl Primer Express Software v1.0 (Applied Biosystems). Real-time PCR followed by HRM was carried out in high-performance Eco Real-Time PCR system (Illumina). The reaction in a final volume of 10 µl consisted of 10 ng of template, 1x EpiTect HRM Master Mix (Qiagen) and 300 nM of each primer. PCR conditions were: 95°C for 5 min, 50 cycles at 95°C for 10 s, 57°C for *ERCC1*, 56°C for *XPC* and 58°C for *OGG1* for 20 s and 72°C for 10 s. HRM thermal profile was set up according to the manufacturer´s recommendations (Qiagen).

Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics 18, GenEx Enterprise and by SAS 9.2 softwares. All investigated parameters showed normal distribution in the study population (Kolmogorov-Smirnov test), with the exception of expression data, which were logarithmically transformed. Principal component analysis of the expression analysis indicated that data from different hospitals needed to be adjusted by the vector error correction model according to the Granger representation theorem. After processing, two-tailed T-test or ANOVA for differences between groups were employed. Correlations were determined by a Pearson test. All statistical tests were performed at a 95% confidence level, for expression data correction for multiple testing analyses according Dunn-Bonferroni was applied (significant p-value after correction was 0.0021).

3. Results

BER- specific DNA repair capacity (BER-DRC)

DRCs were measured in matched pairs of tumour and adjacent healthy tissue of all 70 patients. For 28 individuals, DRC were simultaneously assessed in PBMC.

The BER-specific DRC assay showed a high degree of reproducibility, as repeated analysis in 25 samples showed Pearson's correlation coefficient of R=0.75. The presence of PARP inhibitor in the reaction did not influence the incision activity of the 8 analysed extracts (with or without inhibitor: R=0.92).

The difference in BER-DRCs between tumour and healthy tissues was not statistically significant (mean±SD; 17.7±8.3 vs. 15.7±9.6 %T; p=0.22). However, we observed that PBMC showed significantly decreased ability to repair oxidative damage compared to healthy or tumour tissues (8.4±6.3 vs. 16.2±10.4 and vs. 17.1±8.9 %T respectively, p=0.001). There was a strong correlation in BER-DRCs between tumour and healthy epithelium (R=0.57, p<0.001). Similarly, a significant correlation was observed for BER-DRCs between PBMC and healthy epithelium (R=0.45, p=0.011), but not between PBMC and tumour tissue (R=0.26, p=0.16). Results for BER-DRC are presented in **Figure 1**.

Sex and age did not influence BER-DRC, nor did the localization of tumours, which was 18.4± 9.0 %T for colon and 16.7±9.2 %T for rectum, p=0.45. No statistical significance was observed in BER-DRC based on pathological stage of the tumours. For both TNM 1 and 2, we observed BER-DRC of 16.2±10.2 %T (N=36) and 19.4±7.4 %T (N=34) for diffused stages 3 and 4, p=0.14. Distribution of the BER-DRC suggests moderate, but not significant increase in different grades of the tumours with 16.2±9.3 %T for grade 1, 17.7±9.1 %T for grade 2, and 19.6±8.8 %T for grade 3, p=0.66.

NER-specific DNA repair capacity (NER-DRC)

We also observed high reproducibility in DRC assay for NER (N=25, R=0.62) and incision activity of the extracts did not change due to the presence of a DNA polymerase inhibitor (N=8, R=0.84).

Tumour tissues exhibited significantly higher NER-DRC than healthy epitheliums (20.2±11.6 vs 15.4±10.8 %T, p=0.019). The lowest NER-DRC was detected in PBMC compared to both healthy and tumour tissues (6.1±5.0 vs 17.7±14.2 vs 24.3±13.0 %T, p<0.001). Similar to BER-DRC, a correlation between tumour and healthy tissues in NER-DRC was observed (R=0.58,

p<0.001), and also between PBMC and healthy tissues (R=0.51, p=0.006), and PBMC and tumour tissues (R=0.47, p=0.011). Interestingly, BER-DRC and NER-DRC showed mutual correlation in healthy epithelium (R=0.32, p=0.007). Results for NER-DRC are presented in **Figure 2**.

Sex and age did not significantly influence NER-DRC, which was also very similar irrespective of tumour localisation, with 20.0±12.9 %T in the colon and 20.5±11.4 %T in the rectum, p=0.89. Tumours in non-invasive stages (TNM 1 and 2) exhibited moderately higher NER-DRC (22.0±12.2 %T) than those in more diffused stages TNM 3 and 4 (18.3±12.3 %T), p=0.22. We did not observe an apparent difference when comparison was made in NER-DRC on the basis of tumour differentiation. Well differentiated tumour tissues had NER-DRC of 20.3±12.6 %T, tumours with moderate differentiation 18.9±11.2 %T and those with poor differentiation 25.5±15.9 %T, p=0.29.

Gene expression profiling

Successful expression analyses were performed in 53 pairs of tumour/healthy tissue. In total expression levels were determined for 8 BER and 17 NER genes (listed in **Table 1**). We observed a statistically significant correlation in levels of expression of all genes between paired tumours and healthy tissues (overall p<0.001). We found decreased transcription levels of BER genes *NEIL1* and *OGG1* and NER genes *CSB, CCNH* and *XPA* in tumour tissues compared to controls. In contrast, *APEX1* and *PARP1* (BER) and *XPD* (NER) showed higher expression in tumour tissues than healthy tissues. Although changes in expression of DNA repair genes between the healthy mucosa and tumor tissue were small (1.08-1.28-fold), they were significant (p<0.05). Individual gene p-values and fold changes of transcript levels in tumours relative to healthy tissues are reported in **Table 1.** No correlation was detected between DRC and individual gene expression levels, either for BER or for NER. Expression of studied genes was not influenced by any recorded clinicopathological parameter. The expression pattern of excision repair genes was similar irrespectively of tumour localization in colon or rectum and no modifying effect was exerted by TNM stages and tumour differentiation.

Promoter CpG islands methylation profiling

CpG promoter methylation status of *OGG1*, *ERCC1*, *XPA*, *XPC*, *XPD*, *XPG* and *XRCC1* was evaluated in DNA from 70 tumour samples. MSP showed aberrant methylation of *XPC*, *ERCC1* and *OGG1* in 24, 56 and 51% of tumours, respectively. However, the findings were not confirmed by MS-HRM analysis, which clearly showed only non-methylated cytosines in the analysed promoter for all three genes (**Figure 3**).

4. Discussion

In this study, we characterized BER- and NER-specific DRC in matched tumour and healthy tissues from sporadic CRC patients. Both repair pathways are fundamental for the removal of a broad spectrum of DNA lesions and they process DNA damage in a similar way, by cleaving the damaged site and leaving DNA breaks behind. These breaks reflect excision phase of repair process that has been recognized as the rate-limiting step (26), and is measurable by the well-established comet assay technique. In order to assess DRC, diversely modified comet-based assays have been developed (27). Based on recently published methodological reports on BER- (20) and NER-specific assays (21), we in this study employed an *in vitro* repair assay, adapted for the evaluation of DRC in solid tissues. Langie et al. developed *in vitro* assays to measure DRC in animal tissues. We, for the first time, applied that approach to investigate DRC in human colon biopsies after carrying out the necessary optimisation and validation experiments. Reproducibility was tested by replicating assay in 25 samples at different time points. Both NER- and BER-specific DRC assays showed high degrees of reproducibility. We also tested whether results truly reflect the excision process of DNA repair and are not influenced by ongoing resynthesis and ligation. There was no detectable difference between DRCs of extracts with or without an addition of inhibitors of polymerization, ABT-888 specific for BER and aphidicolin for NER. In order to process a large number of samples, we have utilized a medium throughput 12-gel slide format (22), which, in our hands, proved to be efficient, giving consistent and reliable results. The assay has versatile suitability for application in large molecular epidemiological studies.

In a comparison of 70 matched sets of tumour/normal tissues, we found a significant increase of 24% in NER-DRC in tumours. The differences in BER-DRC between tumour and healthy tissues were not significant. Interestingly, we observed significant correlations of both DRCs as well as comparable expression profiles of all analysed genes in healthy and tumour tissues of the investigated CRC patients, indicating distinct individual traits of excision repairs that are not driven by malignant transformation. To the best of our knowledge, there is only one study with design similar to ours that investigated 23 pairs of tumour/adjacent healthy tissues of CRC patients for NER-DRC only. The authors reported in agreement with our findings, a strong correlation between DRCs in two tissues; however, NER activity in tumour tissue was increased ($p=0.015$) (28). Several studies have inferred

higher BER or NER capability in tumours via an indirect approach of measuring the steady state level of DNA damage, assuming that a low damage level reflects a high repair rate. All those studies reported significantly lower level of specific damage in DNA from tumours, presumably explained by up-regulation of repair (29-31). No study reported deficiency of excision repair pathways in tumours. Consistency of listed observations might lead to the conclusion that excision repair is not a factor contributing to the malignant transformation, but most likely it is contributing to the growth advantage of existing tumour mass by decreasing the vulnerability to DNA damage accumulation normally followed by cell death. Sarasin and Kauffmann hypothesized that relative genetic stability given by upregulation of DNA repair might be associated with higher ability of cells to metastasize (32).

In our previous case-control study, we have reported reduced NER-DRC in PBMC of incident CRC patients with no family history of this disease as compared to healthy population (19). This is consistent with many other investigations on various cancers. But are PBMC a valid cell type to study in relation to CRC? There is a belief that PBMC may represent the general condition of the organism, and specifically reflect individual DRC. In the current study we, therefore, attempted to test that hypothesis, by comparing DRC in PBMC, tumour and normal colon epithelium. To ensure the validity of the comparison, we assayed all studied tissues at the equal protein concentration. For both excision repair pathways, PBMC exhibited on average approximately 3-fold lower DRC than either healthy or tumour tissue. This finding is somehow understandable considering that un-stimulated PBMC are terminally differentiated non-dividing cells that do not require the pool of repair proteins in contrast to constantly reproducing cells, such as epithelial colon cells (33). Furthermore, in non-replicating cells, NER is carried out predominantly by transcription coupled repair with supressed global genome repair (34). Interestingly, despite the difference in repair capacities between colorectal epithelial cells and blood cells, there was a clear positive correlation between their repair capacities. This shows that DRC measured in blood cells does indeed reflect the repair potential of the cancer target tissue (35). PBMC, technically easy to obtain, might thus provide a useful index of individual DRC in comparative population studies.

Expression levels of the analysed genes correlated positively between normal and tumour tissues. Although some genes within both pathways were observed to be up or down-regulated in tumours, this difference was relatively modest, never exceeding 1.3-fold. It is questionable whether these nuances in gene expressions might have any fundamental functional consequences. In fact, many potential biomarkers have failed because they showed only a slight change in expression in cancer compared to normal tissue or their cognate protein levels did not correlate with transcript levels (36). In our study, the transcript level of the major BER player, 8-oxoguanine DNA glycosylase (OGG1) was observed to be significantly lower in tumours compared to normal tissues. However, a 1.2-fold change in expression did not cause any difference in its activity (BER-DRC, measured on substrate DNA presenting 8-oxoguanines, reflects predominantly the activity of OGG1 protein). Moreover, no correlation between mRNA level of *OGG1* and its enzymatic activity was observed. On the other hand, overall NER-DRC comprises joint performance of many genes. In this case, the repair process relies on the formation of protein complexes that assemble at the site of the DNA lesions and facilitate their removal in a coordinated fashion. The expression level of none of the NER genes showed any correlation with NER-DRC. Moreover, according to the expression profiling there were several genes up or down-regulated in tumour cells, while the final repair ability was enhanced. Thus, individual gene expression levels did not prove to be sufficiently informative about the overall DRC and measurement of enzymatic activity can presumably give more relevant and interpretable information than can individual transcript measurement. In this respect, several other studies have reported an inconsistency between transcript level and respective protein quantity (37), or actual protein/pathway activity (19, 38-40).

In CRC, epigenetic alteration of gene expression, so called CpG island methylator phenotype, is known to affect several DNA repair genes (*MLH1*, *MSH2*, *MGMT*) (41). DNA hypermethylation is often observed as a targeted event in tumor cells, resulting in loss of gene expression. BER and NER genes have been described to be aberrantly methylated in variety of cancers, such as *OGG1* in thyroid, *XPC* in bladder, *XPG* in ovarian or *ERCC1* in glioma and some CRC cell lines (15). In the present study we have investigated CpG island methylation status of core BER and NER genes in relation to sporadic CRC. Neither BER genes (*OGG1* and *XRCC1*) nor NER genes (*XPA*, *XPC*, *XPD*, *XPG* and *ERCC1*) were hypermethylated in

tumour cells. In the light of gene expression data that showed fairly similar levels of gene transcripts between tumour and control tissues, these findings are coherent.

In conclusion, our complex analysis of BER and NER processes by functional, genetic and epigenetic approach showed that colorectal carcinomas are only moderately altered in these repair pathways. BER-DRC did not differ from adjacent healthy epithelium, while NER-DRC showed moderate up-regulation in tumours. Thus, alterations of excision repair capacities may not be the major driving events in malignant transformation of human colon or rectum, but they might influence chemical sensitivity of the tumour cells to antineoplastic drugs. From methodological point of view, DRC represents a complex marker for functional evaluation of multi-gene DNA repair process. In particular, BER and NER-specific *in vitro* repair assays employed in this study, proved to be highly informative and applicable for high-throughput screening in molecular epidemiology investigations.

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Figure 1. Differences in BER-DRC of tumour and healthy tissue (A); correlation of BER-DRC between tumour and healthy tissue (B); differences in BER-DRC between tissues and PBMC (C); correlation of BER-DRC between healthy tissue and PBMC (D).

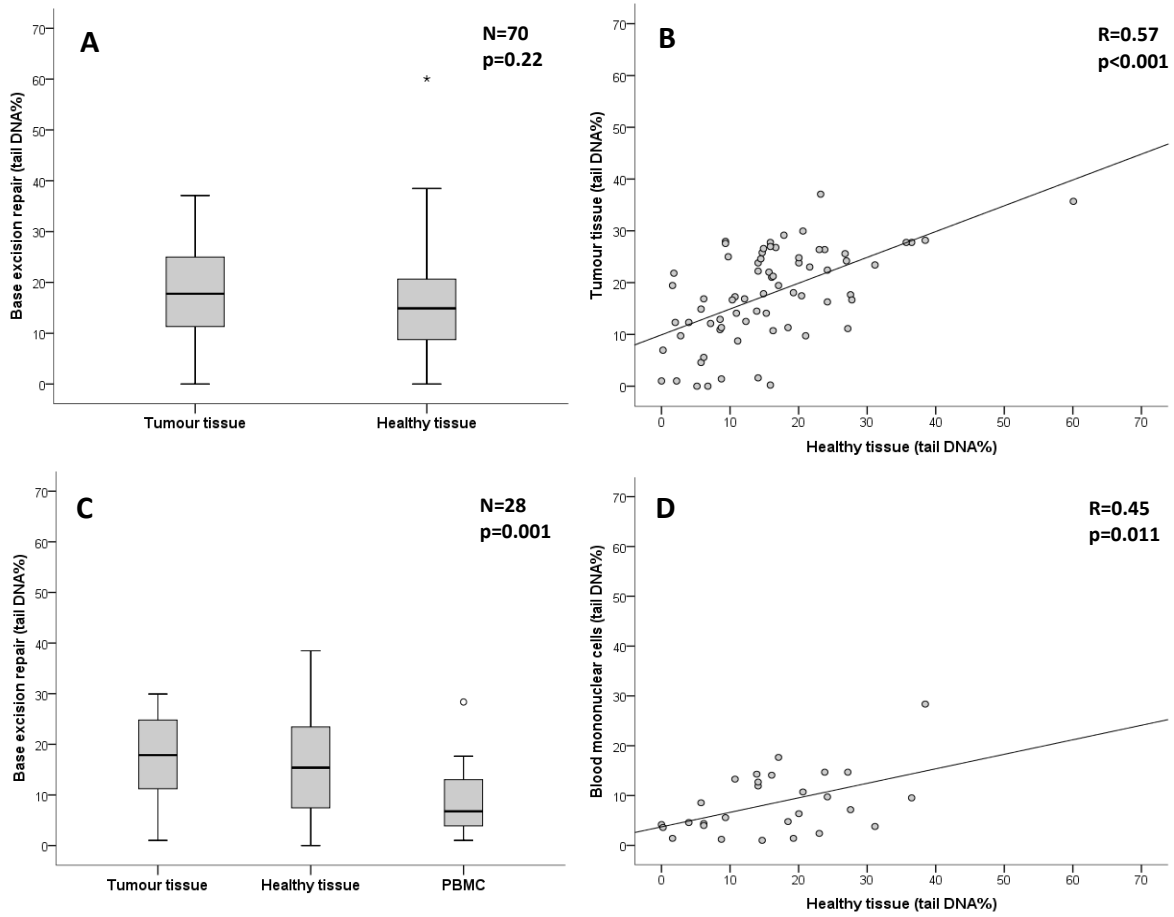


Figure 2. Difference in NER-DRC of tumour and healthy tissue (A); correlation of NER-DRC between tumour and healthy tissue (B); differences in NER-DRC between tissues and PBMC (C); correlation of NER-DRC between healthy tissue and PBMC (D).

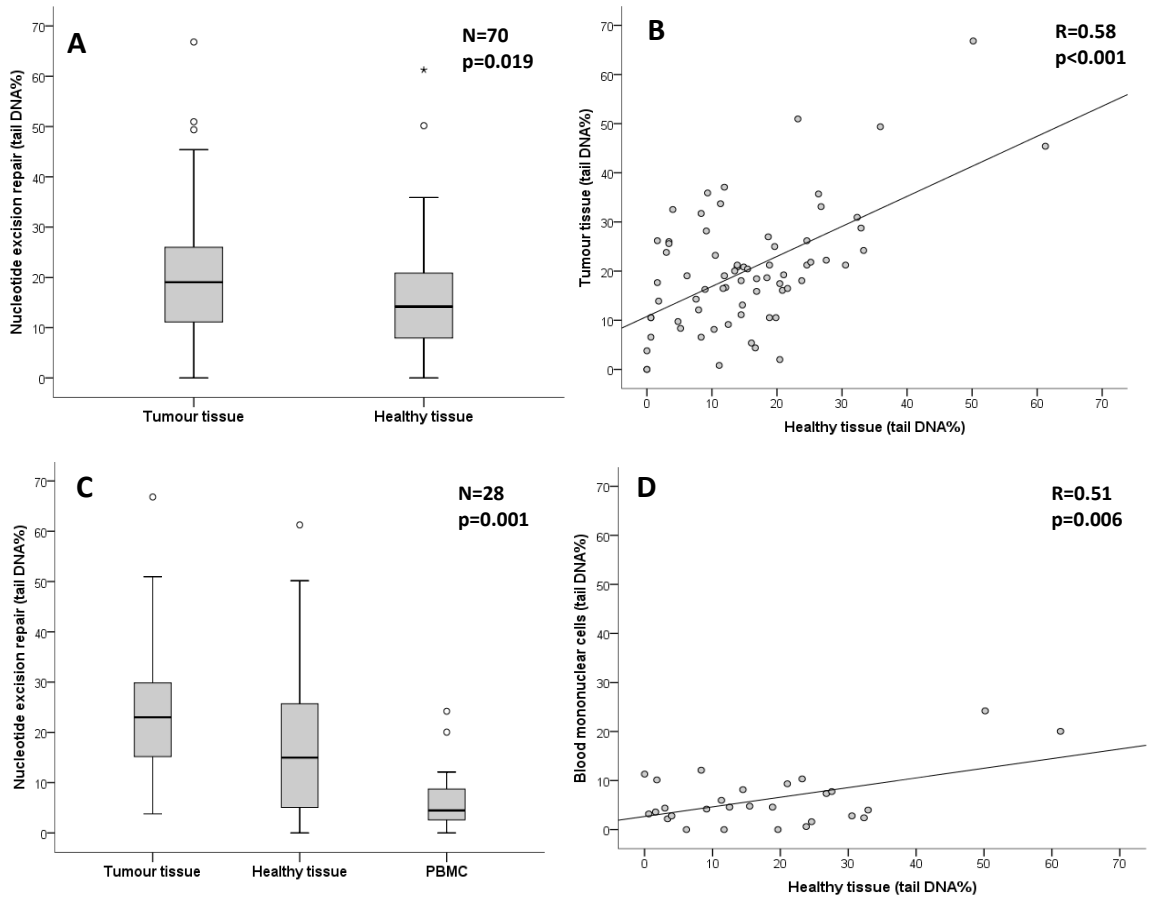
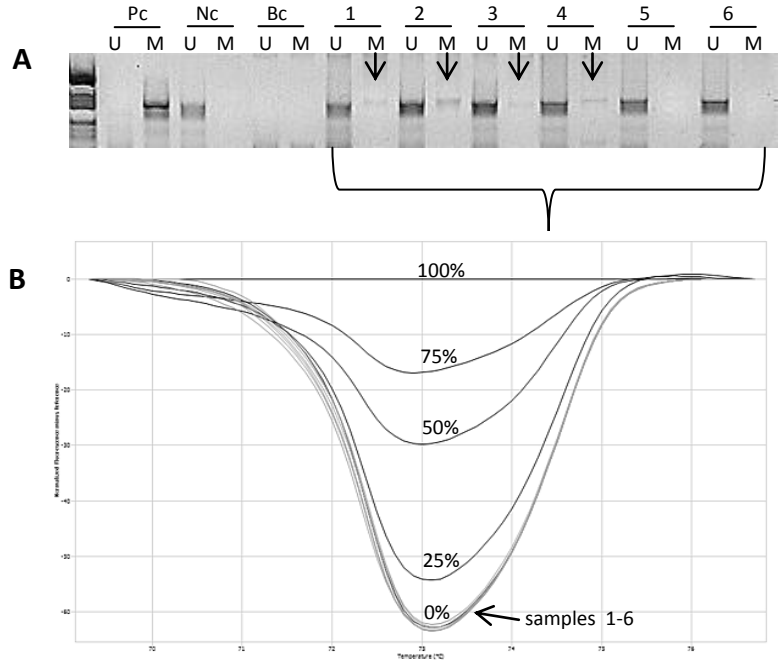


Figure 3. Example of 6 samples analyses for *ERCC1* promoter methylation by MSP (A) and by MS-HRM (B). Positive signals from MSP (arrows) have disappeared in MS-HRM.



NOTE: (A) Pc, positive control of fully methylated DNA; Nc, negative control of fully unmethylated DNA; Bc, nonbisulfite converted DNA; U, primers specific to unmethylated sequence; M, methylation specific primers. (B) Fluorescence of each sample was normalized against 100% methylated DNA control. Percentage shows positive controls of 100, 75, 50, 25 and 0% methylated DNA. For all 6 samples, 0% methylation was detected.

Table 1. Fold-change differences in expression levels of BER and NER genes in tumour compared to healthy tissue.

Gene	Pathway	Fold change	P-value
NEIL1	BER	-1.26	**0.0004**
APEX1	BER	1.15	**0.0138**
OGG1	BER	-1.15	**0.0467**
PARP1	BER	1.12	**0.0210**
LIG3	BER	1.08	0.0922
MUTYH	BER	-1.08	0.4303
MPG	BER	-1.05	0.5807
NTHL1	BER	1.00	0.9551
CSB	NER	-1.28	**0.0002**
CCNH	NER	-1.25	**0.0081**
XPA	NER	-1.15	**0.0190**
XPD	NER	1.08	**0.0408**
LIG1	NER	1.16	0.0708
MNAT1	NER	1.12	0.1128
RPA3	NER	1.08	0.1739
CDK7	NER	1.07	0.4839
XPF	NER	1.06	0.4089
RPA2	NER	1.06	0.4898
XPB	NER	1.05	0.3349
DDB1	NER	1.02	0.6063
ERCC1	NER	1.02	0.6803
DDB2	NER	-1.03	0.6201
RAD23B	NER	-1.03	0.6661
RPA1	NER	-1.03	0.7117
XPC	NER	-1.08	0.2461

NOTE: Significant differences are in bold, those after Dunn-Bonferroni correction ($p<0.0021$) are underlined.

Supplementary Table 1. List of genes involved in BER and NER pathways analyzed in gene expression analysis.

Gene	Pathway	Accession number	Gene ID	Sense primer (5'→3')	Antisense primer (3'→5')	bp	Ta
APEX1	BER	NM 001641	328	TGCCTTCAAGAGACCAAATGTT	CGCCACTGTACCCTTCCTT	115	50
LIG3	BER	NM 013975	3980	AGTACGATGGAGAGCGAGTC	GGAAAAGCCTGGGGAATGTAG	127	50
MPG	BER	NM 002434	4350	CGAGTGTGTCAGGGTGTTTG	CAAAACTGCTTTGGTTTCTTCATC	85	50
MUTYH	BER	NM 012222	4595	AATTTCTTTCGGTCTCACATCTC	AAATAAGCACTTTACTAACAACAGGA	115	50
NEIL1	BER	NM 024608	79661	GACTGGCGCTTTCTGATTTC	AGCCAAGCAACAACAACAAC	95	50
NTHL1	BER	NM 002528	4913	TGCACAGAATCGCCAACAG	AGCCCACCAAGAGTCCATT	129	50
OGG1	BER	NM 016821	4968	TGGGGCATCGTACTCTAGC	AGATTGTCCAGAAGGCAGAAC	101	50
PARP1	BER	NM 001618	142	CCACACACAATGCGTATGACT	CCACAGCAATCTTCGGTTATGA	113	50
CCNH	NER	NM 001239	902	GGGGTACGGGTGTTTTACG	GCTTCTGACTACTGTTGTGGTA	107	50
CDK7	NER	NM 001799	1022	CAGTTTGCCACCGTTTACAAG	CTTTAGCTTCTGATCTATGTCCAAG	94	50
CSB	NER	NM 001469	2074	ATGCGTGGATTGTCGTCTTC	TTCTTGTTCTTCCTCTGCTTCTT	129	50
DDB1	NER	NM 001923	1642	TGCTGGGAGACATGGAAGG	CAACACCATTATCAAGGTATGTCAAG	150	50
DDB2	NER	NM 000107	1643	CAGAGGTGGCGATTTGTTAAAG	AGGCAAGTCCAGAGCATTAAC	119	50
ERCC1	NER	NM 202001	2067	CAATCCCGTACTGAAGTTCGT	TGGGTGCAGGTTGTGGTAG	124	50
LIG1	NER	NM 000234	3978	AGAGGCTGAAGTGGCAACA	CTGCTTTGGAGGTCTTTAGGG	86	50
MNAT1	NER	NM 002431	4331	GGTTGCCCTCGGTGTAAGA	CTCTCACAAACAGTAAATCTACACAA	112	50
RAD23B	NER	NM 002874	5887	TTTACGGAATCAGCCTCAGTTT	TGAATAAAATGCTCCTGGTGTTG	144	50
RPA1	NER	NM 002945	6117	GAAAGACTCACTTGTAGACATCATC	ATTCCTCTTGGCAACTTCTCTG	100	50
RPA2	NER	NM 002946	6118	GAAGTGTTCAGAATTGGGAATGTT	GCTGTCATGTCATCTATTTTGTAAAC	113	50
RPA3	NER	NM 002947	6119	AAAAATGTTTATTCTTTCAGATGGAGA	CAACCACTTCCACAATTCCAG	98	50
XPA	NER	NM 000380	7507	GGGGTGATATGAAACTCTACTTAAAG	CCTGTCGGACTTCCTTTGC	102	50
XPB	NER	NM 000122	2071	GAAAGTTGCCACCCTGATGTA	GATTTGCTTGTGAAAGTCTCTGT	122	50
XPC	NER	NM 004628	7508	TCCGAGATGTCACACAGAGG	TCTGGTATGGTCTCAAGGTCTC	102	50
XPD	NER	NM 000400	2068	CCAAAGGCTTCACCATCATCA	ACAGACTGGAAACGCTCAAATA	130	50
XPF	NER	NM 005236	2072	CTGGAACACTGAGAAGAAACATC	GCAGACAGGCAGAAGTATGG	89	50

NOTE: bp, base pairs of the PCR product; Ta, annealing temperature of primers

Supplementary Table 2: Primers and annealing temperatures for methylation-specific PCR

Gene	Primers	Sense primer (5'→3')	Antisense primer(3'→5')	bp	Ta
ERCC1	U	TTAAATGTTGTTTTTTAGAATGTGT	ACTTACCTTTTAAAATTATAATCACATC	200	50
	M	GTTTAAACGTTGTTTTTTAGAATGC	TACCTTTTAAAATTATAATCGCGTC	199	52
OGG1	U	GTGTTTATAGGTTTTGGGGGT	CCTACATACCTCACCCTTTACAA	127	52
	M	CGTTTATAGGTTTTGGGGGC	CTACATACCTCGCCCTTTACGA	127	56
XPA	U	TATTTAGATTTTGTTTAGTGTTTGG	CTCCACAAATTACTCTAAAACCACC	237	53
	M	TATTTAGATTTCGTTTAGCGTTCG	CGCGAATTACTCTAAAACCG	234	55
XPC	U	GAGTAATGTTAATTTTTGGAAATGT	CCTTCATTAAAAACCTAATCACACC	151	53
	M	AAGAGTAACGTTAATTTTCGGAAAC	CCTTCGTTAAAAACCTAATCACG	153	55
XPD	U	TGTATTGTTTTATTTGAGAGTTAGTTGT	AAAACCAAATCATAAACATCCTCA	155	53
	M	CGTATCGTTTTATTCGAGAGTTAGTC	CGAATCGTAAACATCCTCGA	150	55
XPG	U	TGTTTTTAGGATGTTAGTGTGATGG	ACCCATAAAAAATAAACCCACTCAAT	177	56
	M	AACGTTTTTAGGATGTTAGCGTGAC	CATAAAAAATAAACCCGCTCGAT	176	58
XRCC1	U	TTTAGATATTAATTTTGGAGGTTGT	TCCAAAACAAAAAAAACACTACATT	164	52
	M	TTTAGATATTAATTTCGGAGGTCGT	AAAACGAAAAAAACGCTACGTT	161	54

NOTE: bp, base pairs of the PCR product; Ta, annealing temperature of primers; U, unmethylation-specific primers; M, methylation-specific primers

Supplementary Table 3: Primers and annealing temperatures for methylation-sensitive high resolution melting

Gene	Sense primer (5'→3')	Antisense primer(3'→5')	bp	Ta
ERCC1	TGGGGAGAATTTATAGTTTTTG	AACAAAACAACCTTATTCCC	123	57
XPC	AGGTGGGTTTTTAATAGGAAAAG	TCTCCAAATCACCATAACCTAA	100	56
OGG1	GTTTGTTGTGGAAATGTTTGTT	TCTCAAACTCAATCACTCACC	170	58

NOTE: bp, base pairs of the PCR product; Ta, annealing temperature of primers