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Evaluating the effects of genetic variants of DNA repair genes using cytogenetic mutagen sensitivity approaches

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Abstract

Mutagen sensitivity, measured in short-term cultures of peripheral blood lymphocytes by cytogenetic endpoints, is an indirect measure for DNA repair capacity and has been used for many years as a biomarker for intrinsic susceptibility for cancer. In this article, we briefly give an overview of the different cytogenetic mutagen sensitivity approaches that have been used successfully to evaluate the biological effects of polymorphisms in DNA repair genes based on a current review of the literature and based on the need for biomarkers that would allow the characterization of the biological and functional significance of such polymorphisms. We also address some of the future challenges facing this emerging area of research.

Keywords

DNA repair; polymorphisms; haplotype; biomarkers; mutagen sensitivity; cancer; environmental exposure

Introduction

Cancer is a multistage process that results largely from genomic instability. This concept is supported by cancer prone syndromes such as ataxia telangiectasia and xeroderma pigmentosum, which are associated with *in vivo* and *in vitro* chromosomal instability and defective DNA repair capacity (Maher et al., 1976; Paterson and Smith, 1979). It is well-documented that maintaining genomic integrity is crucial for normal cellular functions, and that genomic instability could lead to cancer development (Hiom, 2010; Cazau 2010; Shibata 2011). DNA repair is a major player in maintaining genomic integrity. The association of mismatch repair deficiency with colon cancer is one of many examples of the critical role of DNA repair in maintaining genomic stability and in cancer prevention (Kolodner and Marsischky, 1999; Hsieh, 2001).

Interindividual variability in DNA repair capacity (DRC) has been reported starting in the 1980s (Setlow, 1985; Oesch et al., 1987; Takano et al., 1991; Spitz and Bondy, 1993; Wei et al., 1996a) and associations between reduced DRC and susceptibility to cancer have since

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Declarations of Interest

The authors report no declaration of interest

been well-documented (Spitz and Bondy, 1993; Wei et al., 1996b; Cheng et al., 1998). A higher prevalence of individuals with reduced DRC has consistently been detected in cancer cohorts compared to healthy cohorts (Spitz et al., 1997; Grossman, 1997; Hulla et al., 1999; Shen et al., 2003; Wu et al., 2007a). These studies, and others, have also demonstrated that a reduced DRC phenotype is associated with an increased risk (odd ratios of 2–10) of developing malignant tumors at several sites, including breast, lung, skin, liver, or head/neck (reviewed by Berwick and Vineis, 2000). In order to evaluate DRC at the population level, several phenotypic assays have been developed and successfully used in many epidemiological studies. These assays included approaches based on the removal of DNA strand breaks or adducts, repair replication (e.g. unscheduled DNA repair synthesis), and cytogenetic approaches based on the induction of chromosome damage by mutagenic agents. Detailed description and discussion of the advantages and disadvantages of these approaches can be found in a recent review by Decordier et al. (2010).

To date, a full explanation for interindividual variability in DRC has not yet been formulated, but it is well-accepted that both genetic and environmental factors are involved (Wu et al., 2006). A plausible explanation for interindividual variability in DRC was spurred by the discovery of single nucleotide polymorphisms (SNPs) in DNA repair genes (reviewed by Ronen and Glickman, 2001). SNPs in DNA repair genes were first described by Shen et al. (1998) and, since then, molecular epidemiological studies have documented significant associations between several SNPs in DNA repair genes and cancer risk at different sites (Goode et al., 2002). Evidence from transgenic and gene targeting studies further supported the potential role for such SNPs in interindividual variability in DRC by showing that disruption of the function of DNA repair genes is associated with increased sensitivity to DNA damaging agents and cancer development (Ishikawa et al., 2001). The mechanism(s) by which SNPs in DNA repair genes can alter DRC remains to be elucidated, and this gap in knowledge constitutes an important barrier towards understanding the role of these SNPs in disease susceptibility and other health effects.

Among the phenotypic assays that have been successfully used in epidemiological studies to evaluate DRC in different populations are cytogenetic approaches involving the induction of chromosome breakage by mutagenic agents in short-term cultures of peripheral blood lymphocytes (PBLs). These approaches have been used successfully to determine genetic predisposition to cancer and for biomonitoring populations exposed to mutagenic and carcinogenic agents (Wu et al., 2007a; Au et al. 2010). These approaches were also used in recent years to evaluate the effects of SNPs in DNA repair genes on DRC (i.e. the genotype-phenotype relationship) by our group and others. In this report we briefly discuss the use of cytogenetic approaches as intermediate biomarkers for cancer risk and in biomonitoring studies. We also describe their usefulness for evaluating the genotype-phenotype relationship of DNA repair polymorphisms based on a current review of the literature and based on the need for biomarkers that would allow the functional characterization of such polymorphisms. Polymorphisms associated with diseases are being discovered daily, yet to justify further in-depth investigations of these variants, one of the biggest challenges is establishing that such particular variants are indeed contributing to a phenotype or causing a disease.

Mutagen sensitivity: historical overview

DRC is an important determinant for individual sensitivity to environmental and occupational mutagenic agents. DRC could also largely affect the response of patients to treatments involving radio- and/or chemotherapeutic agents. Therefore, it has become clear in recent years that developing approaches for assessing interindividual variability in DRC is not only important for disease prevention, but would also help efforts aiming at designing

new therapeutic modalities and personalized medicine approaches. Mutagenic sensitivity assays determined by cytogenetic endpoints has been used for over three decades to investigate DRC and to assess human cancer risk. The premise of the mutagen sensitivity assay, originally introduced by T.C. Hsu, is that genetic damage induced by mutagenic exposures varies among the general population with higher levels observed in individuals with an inherent susceptibility to DNA damage (Hsu, 1983). In 1989, Hsu et al. developed the mutagen sensitivity assay to detect potential variations in susceptibility to effects of mutagenic agents among individuals. The assay measured the frequency of induced chromatid breaks at metaphase in short-term cultured human peripheral blood lymphocytes (PBLs) after exposure to mutagens in S-G₂ phase of the cell cycle (Hsu et al., 1989). The original mutagen sensitivity testing by Hsu utilized the radiomimetic agent bleomycin as the test mutagen. Bleomycin is a glycopeptide which produces DNA strand breaks. However, many modifications to the assay were introduced since its development, and several physical and chemical mutagenic agents are now being used. Such mutagenic agents include γ -rays, UV radiation, benzo[a]pyrene diol epoxide (BPDE), hydrogen peroxide (H₂O₂), heterocyclic amines (e.g. PhIP), tobacco-specific nitrosamines (e.g. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK), and others (Roberts et al., 1999; Wu et al., 2000; Abdel-Rahman and El-Zein, 2000; Affatato et al., 2004; Wang et al., 2005a; Hill et al., 2005a,b; El-Zein et al., 2006a). At present, sensitivity to mutagenic agents determined by cytogenetic end points is considered an integrated biomarker that reflects the sensitivity to the tested mutagen, as an indirect measure for DRC, and as an intermediate phenotype for cancer risk (Hsu et al., 1991; Spitz et al., 1995).

Many laboratories have successfully used this approach to identify individuals at high risk of developing cancer through comparing mutagen-induced DNA damage in circulating PBLs of cancer patients and corresponding controls (reviewed by Wu et al., 2007a). It has also been suggested that mutagen sensitivity could be used as a biomarker for predicting prognosis and treatment outcome in cancer patients (López de Mesa et al., 2002). In biomonitoring studies, this approach was successfully used to evaluate the effect of industrial and environmental exposures on DRC in exposed populations (Au and Salama, 2005). Studies have also shown that first degree relatives of mutagen sensitive individuals were also mutagen sensitive, suggesting an element of heritability and genetic susceptibility to certain mutagens (Roberts et al., 1999; Wu et al., 2007a).

Mutagen sensitivity testing using cytogenetic endpoints as an intermediate biomarker for cancer risk

Mutagen sensitivity, measured by quantifying chromosomal aberrations resulting from exposure of PBLs in short-term cultures to a mutagenic agent has been used as an indirect measure of DRC (Hsu et al., 1989). The theory is that in response to a mutagen, genetic damage accumulates more in individuals with reduced DRC compared to individuals with efficient DRC, and as such the level of chromatid breaks scored provides a measure of an individual's DRC. Because of the known relationship between reduced DRC and increased cancer risk, mutagen sensitivity was used to evaluate cancer risk in population studies. Retrospective and prospective epidemiological investigations have consistently linked mutagen sensitivity to cancer risk at different sites (reviewed by Wu et al., 2007a). In a large retrospective epidemiological study of mutagen sensitivity that included nearly 1000 lung cancer cases and controls, the risk for lung cancer associated with mutagen sensitivity was 1.63 (95% Confidence interval (95% CI), 1.36–1.97) for bleomycin sensitivity and 1.85 (95% CI, 1.42–2.42) for BPDE sensitivity (Wu et al., 2007b). The association between mutagen sensitivity and cancer risk was found to be stronger if other cancer risk factors are also present. For example, in a multicenter case-control study of head and neck cancer, while bleomycin sensitivity was associated with a 2.6-fold increased risk of the disease in

presence of smoking, the risk was increased to 44.5-fold (95% CL, 17.4–114.0) and to 57.5-fold (95% CL, 17.5–188.0) with alcohol consumption (Cloos et al., 1996). Prospective mutagen sensitivity studies for large cohorts of subjects followed for cancer outcomes are prohibitively expensive, and therefore are not common. In one small mutagen sensitivity study, Chao et al. (2006) followed cancer-free individuals with Barrett's esophagus and reported a non-significant 1.6-fold increased risk of esophageal carcinoma. Recently, Sigurdson et al. (2011) used Epstein Barr virus-transformed lymphoblastoid cell lines established from prospectively collected PBLs to evaluate lung cancer risk in relation to different DNA repair assays, including the bleomycin cytogenetic mutagen sensitivity assay. Cases (n=117) were diagnosed with lung cancer between 0.3 and 6 years after blood collection and controls (n=117) were frequency matched to cases on calendar year and age at blood collection, gender, and smoking history. Among the DNA repair assays evaluated, only statistically significant increased lung cancer risk was observed for bleomycin mutagen sensitivity (as quartiles of chromatid breaks/cell relative to the lowest quartile, OR=1.2, 95% CI: 0.5–2.5, OR=1.4, 95% CI: 0.7–3.1, OR=2.1, 95% CI: 1.0–4.4), respectively, p trend = 0.04). Notably, the magnitude of the association between the bleomycin cytogenetic mutagen sensitivity assay and lung cancer risk was modest compared to those reported in previous lung cancer studies conducted with freshly collected PBLs, but was strengthened when only incident cases diagnosed more than a year after blood collection (p trend = 0.02) were included.

Mutagen sensitivity testing using cytogenetic endpoints in biomonitoring studies

Mutagen sensitivity assays using cytogenetic endpoints were also used in biomonitoring investigations to evaluate the effect of environmental or industrial exposures on DRC. In these studies, PBLs from workers exposed to industrial chemicals and matched controls were usually exposed to radiation (X- or γ -rays) or UV-light as the mutagenic challenging agent at the G₁ phase of the cell cycle and the levels of mutagen-induced chromosome aberrations were determined at the metaphase stage of the cell cycle (Au et al., 1995a,b). By exposure in G₁ phase, the induced damage would have been subjected to the DNA repair machinery throughout the G₁, S and G₂ phases of the cell cycle, and as such, an increased frequency of chromosome aberrations in exposed subjects compared to non-exposed controls is indicative of exposure-induced DNA deficiency. Using this protocol, cigarette smokers, workers exposed to industrial chemicals, farmers exposed to pesticides, and residents exposed to uranium mining and milling waste were found to have higher frequencies of mutagen-induced chromosome aberrations than their corresponding matched controls (Au et al., 1995 a,b; Au et al., 1999; Oberheitmann et al., 2001). The increase in mutagen-induced chromosome aberrations observed in exposed subjects compared to corresponding controls reflected the effect of exposure on DRC. Details about these studies are summarized in a recent review by Au et al. (2010). This approach was used earlier by El-Zein et al. (1995), who showed that epidermodysplasia verruciformis patients, who are known to be at an increased risk of sunlight-induced skin cancer, were deficient in the repair of chromosome aberrations induced by UV-light. Using this approach, Tuntawiroon et al. (2007) in a study of school children in a high-density traffic area in Bangkok, reported that exposure was associated with a significant reduction in DRC as reflected by an increase in radiation-induced dicentric chromosomes and chromosome deletions per metaphase in exposed children compared to children attending school in a provincial area. Similarly, Navasumrit et al. (2008) reported reduced DRC of temple workers in Thailand who were occupationally exposed to incense smoke containing high concentrations of polycyclic aromatic hydrocarbons, benzene and 1,3-butadiene. In workers exposed to benzene or workers exposed to very low concentrations of styrene, the assay was also able to detect

DNA repair deficiency in exposed subjects (Chanvaivit et al., 2007; Wongvijitsuk et al., 2010).

Mutagen sensitivity testing using the cytokinesis-block micronucleus (CBMN) assay

One of the most commonly used cytogenetic methods for measuring DNA damage is the CBMN assay (Fenech, 2007). Micronuclei (MN) originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. Micronuclei represent therefore a measure of both chromosome breakage and chromosome loss (Kirsch-Volders et al., 1997; Kirsch-Volders et al., 2002; Mateuca et al., 2006; Decordier et al., 2011). In a classical *in vitro* CBMN test, human PBLs are cultured in the presence of phytohaemagglutinin (PHA) followed by addition of cytochalasin B at 44 hours after initiation, to allow nuclear division but block the cytoplasm from dividing. This approach allows the detection of other DNA damage endpoints such as nucleoplasmic bridges (NPB), which represent chromosome rearrangement, and nuclear buds (NBUD), a marker of gene amplification (Kimura et al., 2004). Identification of cells that have completed only one nuclear division, prevents confounding effects caused by differences in cell division kinetics because expression of the genetic damage endpoints is dependent on completion of nuclear division (Fenech, 2000).

The CBMN test is slowly replacing the analysis of chromosome aberrations in lymphocytes because the damage endpoints are easy to recognize, does not require metaphase cells and therefore scoring is easier and results can be obtained in a shorter time (Fenech, 2007). In addition to being a tool to detect clastogenic and aneugenic events, this test can provide additional cellular events that measure genotoxicity and cytotoxicity such as cellular proliferation rate and cell death by apoptosis or necrosis.

The use of micronuclei (MN) as a measure of early genotoxic effects has become a standard assay in human biomonitoring studies. In addition to being used for exposure assessment, the assay also allows for a better understanding of the underlying mechanisms involved in the generation of the damage observed. For example, the assay can be used to measure DNA repair capacity. In such a case, fluorescence in situ hybridization (FISH) is used in combination with the CBMN assay to allow for differentiation between MN containing whole chromosomes and MN resulting from chromosome breaks (Decordier et al., 2011). Only MN resulting from chromosome breaks would be counted since MN containing whole chromosomes are not eliminated by repair processes. MN harboring chromosomal fragments may result from direct double-strand DNA breakage, conversion of single strand breaks (SSBs) into double strand breaks (DSBs) after cell replication, or inhibition of DNA synthesis. Since structural chromosomal damage leading to the formation of MN involves acentric fragments, MN frequency could be linked with the level of unrepaired DNA DSBs at the time of mitosis (Decordier et al., 2010).

The CBMN assay has been used in several studies to measure susceptibility to a number of agents such as ionizing radiation, H₂O₂, and a wide range of other chemicals in order to better estimate disease risk (Scott et al., 1998; Rothfuß et al., 2000; Baeyens et al., 2002; Baeyens et al. 2004). Using the CBMN assay, El-Zein et al. (2006b) showed a differential sensitivity in a relatively large population of lung cancer patients and healthy controls to the tobacco-specific nitrosamine NNK. Lymphocytes from lung cancer patients and controls were challenged *in vitro* in G₁ with NNK and chromosomal damage endpoints, MN, NPB, NBUDs, were scored through the CBMN assay. Both spontaneous and NNK-induced MN frequencies were significantly higher in lung cancer patients as compared with controls. El-Zein et al. (2008) later demonstrated that using the comprehensive CBMN cytome assay

(through including MN in mononucleated cells) improves the positive and negative predictive value for disease status in the same lung cancer case-control study.

In a similar approach with breast cancer patients, Baeyens et al. (2002 and 2005) reported that among sporadic breast cancer patients, 26 percent showed radiosensitivity as compared to 61% of the familial breast cancer patients. In addition, Baeyens et al. (2004) also reported that breast cancer patients with a *BRCA1* or *BRCA2* mutations were on the average more radiosensitive than healthy women, but not different from breast cancer patients without a *BRCA* mutation. Although the importance of the role of *BRCA* genes in DSB repair is well documented (Tutt and Ashworth, 2002; Zhang and Powell, 2005), these results suggest that the mutations in *BRCA1* or *BRCA2* genes do not seem to play a major role in chromosomal radiosensitivity in these patients and that other factors such as low penetrant variations in genes in the processing and break repair pathways may be involved in the genetic predisposition to breast cancer.

Use of cytogenetic mutagen sensitivity approaches to evaluate the biological effects of DNA repair polymorphisms

In the last decades, considerable progress has been made in identifying the numerous gene products that play a role in DNA repair pathways in humans, including base excision repair (BER), nucleotide excision repair (NER) and double strand break/recombinational (DSB/REC) repair (Hannawalt, 1995). It has also become clear that DRC is genetically regulated (Wu et al., 2006; Wu et al., 2007a). A mechanism that may lead to the observed interindividual variability in DRC capacity that has gained attention in recent years is the presence of SNPs in DNA repair genes (reviewed by Ronen and Glickman, 2001). The exact mechanism(s) by which these SNPs can alter DRC are not fully understood, but it is conceivable that these SNPs could alter the levels, structure, and functions of the resulting proteins, and could thus affect DRC through different mechanisms.

In the last few years, several studies reported associations between SNPs in DNA repair genes, mutagen sensitivity, and cancer risk. For example, Lu et al. (2007) reported that the 172G>T variant in the 5' untranslated region of the DNA repair gene *RAD51*, which is involved in homologous recombination repair of DSBs, reduces risk of squamous cell carcinoma of the head and neck (adjusted odds ratio (OR) = 0.66, 95% confidence interval (CI) = 0.50–0.87), compared with carriers of other genotypes. Consistent with a potential protective effect of the 172TT genotype, they reported that significantly fewer γ -rays-induced chromatid breaks per cell (b/c) were present in lymphocytes of 172TT homozygote carriers than in subjects with other genotypes ($p < 0.001$). Liu et al. (2010a) correlated genotype data for tag single-nucleotide polymorphisms (tSNPs) of DNA strand break repair genes with a γ -rays-induced mutagen sensitivity phenotype expressed as mean b/c in samples from 426 glioma patients and found that mutagen sensitivity was modified by a tSNP in the *RAD51L1* gene ($p = 0.025$). These results support earlier studies from the same group, which indicated that sensitivity to γ -rays and the subsequent inability to repair radiation-induced DSBs, as measured by chromatid breaks, may increase the risk for brain tumorigenesis (Bondy et al., 1996; Bondy et al., 2001). Studies have also shown an association between SNPs in DSB repair genes, radiosensitivity and breast cancer risk (Fu et al., 2003; Bau et al., 2007; Willems et al., 2008; Willems et al., 2009). SNP studies in genes involved in non-homologous end joining, the main pathway for radiation-induced DSB repair (Mahaney et al., 2009), provided evidence that the variant alleles of the c.-1310C>G SNP in the *XRCC6/Ku70* and the c.2099–2408G>A in the *XRCC5* gene are risk alleles for breast cancer as well as for chromosomal radiosensitivity (Willems et al., 2008; Willems et al., 2009).

Using the CBMN assay, Cheng et al. (2007) investigated the association between SNPs in NER genes and genetic damage in coke-oven workers. They reported that in coke-oven workers, the *ERCC1* 19007 CC genotype exhibited significantly higher CBMN frequency than the CT or TT genotypes, either independently or in combination. They also reported that the *ERCC6* A3368G SNP was associated with significantly higher CBMN frequency among coke-oven workers. Stratification analysis revealed that the significant associations between *ERCC1* C19007T and *ERCC6* A3368G SNPs, and the CBMN frequencies were only found among older workers. A similar significant association between *ERCC2* G23591A SNP and CBMN frequencies was also found among older coke-oven workers. In a molecular biomonitoring study of nurses exposed to antineoplastic drugs, Cornetta et al. (2008) studied polymorphisms in the BER gene *XRCC1* and reported that exposed nurses who had at least one *XRCC1* variant allele (399Gln) show higher values of MN. Similarly, a recent study of workers exposed to the industrial chemical 1,3-butadiene Liu et al. (2010b) found a significant association between genetic damage in PBLs and the *XRCC4* A245G and the *XRCC4* T1394G polymorphisms. Workers with the *XRCC4* AA genotype exhibited significantly higher NPB frequency than those with the AG or GG genotypes ($p < 0.05$). Younger workers (< 39 year old) with the *XRCC4* TT genotype had significantly higher CBMN frequencies than those with the GG genotype ($p < 0.01$). In a pooled analysis of five biomonitoring studies performed to assess the influence of Ser326Cys SNP in the BER gene *hOGG1*, the Arg399Gln SNP in the BER gene *XRCC1* and the Thr241Met SNP in *XRCC3* gene, which functions in homologous recombination, on MN frequency in human PBLs of workers occupationally exposed to different mutagenic agents (styrene, ionizing radiation, cobalt/hard metal, welding fumes and inorganic arsenite compounds), Mateuca et al. (2008) investigated the effect of genotype, age, exposure to genotoxic agents, and smoking habit on MN induction. The analysis of genotype-genotype, genotype-smoking and genotype-exposure interactions by linear combinations of parameters showed significantly higher MN frequencies in the following subsets: (i) occupationally exposed workers carrying either the Thr/Thr or the Thr/Met *XRCC3*(241) genotypes compared to their referent counterparts ($p < 0.001$) and (ii) carriers of the Met/Met *XRCC3*(241) genotype compared to Thr/Thr *XRCC3*(241) carriers, as far as they are non-exposed and carry the variant (Ser/Cys or Cys/Cys) *hOGG1*(326) genotype ($p < 0.01$). Significantly lower MN frequencies were observed in carriers of the variant *hOGG1*(326) genotype compared to Ser/Ser *hOGG1*(326) carriers in the subgroup of non-smokers with Thr/Thr *XRCC3*(241) genotype ($p < 0.01$). Stratified analysis by occupational exposure showed a significant MN increase with smoking in occupationally exposed carriers of the Arg/Gln *XRCC1*(399) genotype ($p < 0.001$). In contrast, a significant MN decrease with smoking was observed in referents carrying the Ser/Ser *hOGG1*(326) genotype ($p < 0.01$). These findings support the usefulness of CBMN in evaluating the effect of DNA repair polymorphisms and provide evidence that different DNA repair polymorphisms, and their interaction with environmental genotoxic agents, may modulate induction of genetic damage.

Using the CBMN assay and bleomycin as the test mutagen, Angelini et al. (2008) reported a significant association between the Lys751Gln polymorphism in the NER gene *XPB* and both spontaneous (background) and bleomycin-induced MN frequencies. A marginally significant association between the 399Gln polymorphism of the BER gene *XRCC1* and spontaneous MN frequency was also observed, however, no significant differences were observed in bleomycin-induced MN frequencies in individuals with the different *XRCC1* genotypes. Decordier et al. (2007) used the CBMN assay and H_2O_2 as the test mutagen to compare the *in vitro* sensitivity of PBLs of 17 mother-newborn daughter pairs to oxidative stress taking into account genotypes for the DNA repair genes *hOGG1*, *XRCC1*, *XRCC3* and *XPB*. Among the mothers population, subjects carrying the variant allele for *XRCC1* (399) (Arg/Gln or Gln/Gln) or the Thr/Thr wild type allele for *XRCC3* (241) accumulated more MN in binucleated cells after exposure to H_2O_2 than their counterparts. The data for

XRCC1(399) polymorphism is consistent with previous observations indicating that the variant allele is associated with a less efficient DNA repair (Abdel-Rahman and El-Zein, 2000) and a higher frequency of MN (Godderis et al., 2004). Concerning the *XRCC3* (241) SNP, the results are in contrast with previous studies where the variant genotype resulted in a higher frequency of MN (Aka et al., 2004; Godderis et al., 2004; Mateuca et al., 2005). In their studies, however, Decordier et al. (2007) reported that newborn daughters with a variant Met genotype for *XRCC3*(241) showed higher frequencies of H₂O₂-induced MN as compared to their mothers carrying the same genotype, indicating that newborns carrying this genotype might be at risk for increased MN frequencies when exposed to oxidative stress. These findings on the effect of the *XRCC3* 241Met allele are consistent with another report indicating significantly higher MN in PBLs with this variant allele challenged with ethylene oxide as the mutagenic agent (Godderis et al., 2006).

We previously investigated the relationship between codons 194 (Arg194Trp) and 399 (Arg399Gln) SNPs in the BER gene *XRCC1* and sensitivity to the tobacco-specific nitrosamine NNK. The repair of genetic damage induced by reactive metabolites of NNK involves several DNA repair pathways, including the BER and NER pathways (Cloutier et al., 2001). We reported a significant difference ($p < 0.05$) in NNK-induced sensitivity between individuals with the 399Gln allele (either homozygous or heterozygous) and individuals with the homozygous 399 Arg/Arg genotype. No significant difference in NNK-induced genetic damage was observed between codon 194 Arg/Arg genotype and codon 194 Arg/Trp genotype in our studies (Abdel-Rahman and El-Zein 2000). Tuimala et al. (2002) reported that the *XRCC1* codon 280 variant allele was associated with reduced DRC as reflected by increased frequencies of chromatid breaks in bleomycin-treated PBLs with this allele ($p = 0.002$). Wang et al. (2003a) evaluated the effect of Arg194Trp and Arg399Gln SNPs using both bleomycin and BPDE as test mutagens. They reported that PBLs from individuals with the wild type codon 194 Arg/Arg exhibited significantly higher values of b/c than those with one or two variant Trp alleles ($p = 0.005$ for bleomycin and $p = 0.05$ for BPDE). For codon 399 SNP, PBLs from subjects who were Gln/Gln homozygotes had higher b/c than did those with other genotypes, with evidence of a gene dosage effect. When the two polymorphic sites were combined and codon 194 Arg/Trp and Trp/Trp and codon 399 Arg/Arg genotypes were used as the reference category, these differences were enhanced for bleomycin sensitivity (p for trend = 0.032), but not for BPDE sensitivity (p for trend = 0.821). These data are biologically plausible since damage induced by bleomycin is expected to be repaired by the BER pathway while the damage induced by BPDE would be repaired by the NER pathway. Consistent with this observation, Au et al. (2003) studying the effect of the two *XRCC1* SNPs (Arg194Trp and Arg399Gln) on chromosome aberrations with X-rays as the mutagenic agent reported a significant association between the *XRCC1* 399Gln SNP and increased chromosome deletions. In the same study, when the effect of the Asp312Asn and 751Gln SNPs of the NER gene *XPD* were evaluated following exposure to X-rays, no increase in chromosome aberration frequencies were observed. However, when the effect of both these *XPD* SNPs was evaluated following exposure to UV (which induces genetic damage repaired by the NER pathway), both SNPs were associated with increases in chromatid breaks compared with their corresponding wild-type (Au et al., 2003). In the same study, Au et al. (2003) also reported that the 148Glu SNP of the BER gene *APE* had no influence on the repair of either X-rays or UV light-induced DNA damage. The findings by Au et al. (2003) are in contrast to earlier negative observations by Lunn et al. (2000) who used X-rays as the challenging agent to evaluate the effect of the Asp312Asn SNP in the NER gene *XPD* and reported no association between this SNP and DRC. Lunn et al. findings also contrasted observations from other laboratories, including ours, indicating significant associations between SNPs in the *XPD* gene and mutagen sensitivity (Affatato et al., 2004; Hemminki et al., 2001; Spitz et al., 2001).

We and others have also used cytogenetic mutagen sensitivity assays to evaluate the genotype-phenotype relationship of SNPs in DNA repair genes involved in pathways other than the BER and NER pathways. We evaluated the effects of the L84F and I143V SNPs in the *MGMT* gene, which encodes the direct-reversal DNA repair protein *O*⁶-methylguanine-DNA-methyltransferase that removes DNA adducts formed by alkylating mutagens. Using the alkylating agent NNK as the test mutagen, we found a significant ($p < 0.02$) increase in NNK-induced CA in cells from individuals with the 84F SNP compared to cells from individuals homozygous for the referent L84 allele. A significant positive interaction between this SNP and smoking, gender and age was observed ($p < 0.03$). In subjects with the variant 143V allele, significantly higher levels of NNK-induced chromosome aberrations were also observed. Our data also indicated that Individuals who inherited two SNPs had significantly higher levels of NNK-induced chromosome aberrations compared to individuals with none or with one SNP ($p < 0.002$). These data suggest that the 84F and 143V SNPs may alter the function characteristics of the MGMT protein, resulting in suboptimal repair of genetic damage induced by alkylating agents (Hill et al., 2005a). Because the Thr241Met polymorphism in the DNA-repair gene *XRCC3* was reported to be associated with increased risk of tobacco-related cancers (Shen et al., 2002), especially among women (Stern et al., 2002; Wang et al., 2003b), we tested the hypothesis that individuals who inherit the variant 241Met allele are more sensitive to the genotoxic effects of NNK. We observed that NNK-induced chromosome aberrations was significantly higher in women compared with men ($p = 0.02$). When smoking and gender were considered together, a significant interaction was observed. PBLs from female smokers had significantly higher frequencies of NNK-induced chromosome aberrations, compared with female non-smokers ($p = 0.02$). In view of the close association between NNK exposure and adenocarcinoma of the lung (Hecht et al., 1998), our data may provide an explanation, at least in part, for the increase in the incidence of this cancer observed among women (Payne, 2001).

In studies from our laboratory of PBLs from 129 healthy subjects, using absolute quantitative reverse transcription PCR, we found that the BER gene *NEIL2* transcription varied significantly (up to 63 fold) and that this variability was influenced by certain SNPs located 5' of the start site. We used the mutagen sensitivity assay to characterize the biological significance of these SNPs and observed a significant increase in mutagen-induced genetic damage associated with two SNPs in the promoter region of the *NEIL2* gene. These results guided our efforts to characterize the functional significance of these SNPs. We engineered luciferase-reporter constructs of the *NEIL2* promoter with mutations corresponding to these SNPs. We transfected these constructs into MRC-5 cells and evaluated their impact on *NEIL2* expression levels. Our results indicate that *NEIL2* expression was significantly reduced by over 50% ($p < 0.01$) in the presence of the two SNPs (ss74800505 and rs8191518) located near the *NEIL2* start site, which were in significant linkage disequilibrium ($D' = 73\%$; $p < 0.05$). These data identified SNPs in the *NEIL2* promoter region that do indeed have functional effects (Kinslow et al., 2008).

Conclusions, Future directions and Challenges

Because of their sensitivity in detecting genetic damage resulting from exposure to genotoxic agents, cytogenetic biomarkers have become the most frequently used biomarkers in human population studies. An important aspect that has contributed to their success is their validation as early predictors of human cancer risk (Bonassi et al., 2000; Bonassi et al., 2004; Smerhovsky et al., 2001; El-Zein et al., 2006b; El-Zein et al., 2011). From the data presented above, it can be concluded that there is ample evidence that DNA repair polymorphisms alter DRC and that such alteration can be detected using cytogenetic biomarkers that incorporate mutagen sensitivity approaches. The data generated from the

studies presented above support this conclusion. The data also bring to the attention several issues that need to be discussed.

It is noteworthy that cytogenetic mutagen sensitivity assays utilize physical agents and chemicals to induce chromosome aberrations. These assays are conducted in PBLs, which, compared to other organs such as the liver for example, have a much lower bioactivation capacity. In many instances, this entails the use of high concentrations of the mutagenic chemical for an effect to be observed. Such concentrations are sometimes several orders of magnitude higher than concentrations occurring in exposed populations and care should be taken to insure that the concentrations used are not toxic to the cells. This pitfall is common in mutation assays in general, and in *in vitro* mutation assays with cells or bacteria. In *in vitro* mutation assays, this is often circumvented by addition of a metabolizing system such as liver S9 of rats. It should also be noted that the use of human PBLs poses another challenge when using chemical agents as test mutagens, since their metabolic activation capacity could also be influenced by metabolic polymorphisms not accounted for. However, the current accepted concept is that the mechanisms for the induction of chromosomal damage are similar in different tissues, therefore the extent of chromosomal damage evaluated in lymphocytes and other surrogate tissues is likely to reflect the level of damage in cancer-prone tissues and in turn cancer risk (Norppa et al., 2006).

In studies evaluating the effects of DNA repair variants, it was noted in some cases that the effects of certain variants are observed in conjunction with exposure, but not in the absence of it. For example, in studies from our laboratory evaluating the effect of the Asp312Asn SNP in the NER gene *XPD*, we found a significant association between mutagen-induced chromosome aberrations and the 312Asn allele (OR=3.69 (95% CL=1.29–10.56; $p = 0.02$) when all the population studied was included in the analysis. When smoking was considered, the risk was significantly elevated in smokers (OR=4.62; 95% CL=1.14–18.70; $p = 0.04$) but not in non-smokers (OR= 2.62; 95% CL=0.53–13.1; $p = 0.43$) (Affatato et al., 2004). The fact that the effect of a polymorphism is more pronounced in presence of exposure is not surprising since the biological concept is that a reduced repair capacity would be more important if exposure has occurred and that continuous exposure to carcinogens and mutagens present in tobacco smoke could overwhelm the DNA repair machinery, making the effect of polymorphisms that reduce repair capacity more pronounced. Thus, the inheritance of polymorphisms that result in even a slight decrease in DNA repair could lead to more noticeable genetic damage in smokers compared to non-smokers.

Another topic that merits consideration is the variability in results observed between different studies evaluating the same SNPs. For example, Angelini et al (2008) reported no differences in bleomycin-induced MN frequencies in individuals with the different genotypes for *XRCC1* codon 399 polymorphism, however studies from our laboratory (Abdel-Rahman and El-Zein, 2000) and others (Wang et al., 2003a; Au et al., 2003) indicate an effect for the variant allele on genetic damage. In some cases, opposite effects for the same SNP were observed. For example while the Thr/Thr wild type allele for *XRCC3* (241) was reported to be associated with accumulation of more MN after exposure to H_2O_2 (Decordier et al. 2007), in other studies it was the variant allele that was associated with increased genetic damage (Aka et al., 2004; Godderis et al., 2004; Mateuca et al., 2005). There may be several explanations for the observed conflicting results. One explanation lies in the importance of the choice of the test mutagen, which should be appropriate for the evaluation of the effect of SNPs of the DNA repair gene studied since it may impact the results observed. The study by Lunn et al. (2000) illustrates that example. In their studies, Lunn et al. used X-rays as the challenging agent to evaluate the effects of SNPs in the NER gene *XPD*. X-rays are not ideal for evaluating *XPD* functions since X-rays damage is

repaired by pathways other than the NER pathway. Another explanation for the different results observed could be in the choice of the cytogenetic endpoint that should be appropriate for determining the effect of SNPs in a certain gene. For example, when using X-rays as the test mutagen, chromosome-type aberrations (e.g. deletions, translocations, dicentrics and fragments) should be considered, while with UV light chromatid-type aberrations (e.g. chromatid breaks) should be evaluated (Au et al., 2010).

Perhaps one of the most important factors that may explain conflicting results between different studies is the issue of linkage disequilibrium (LD) between SNPs in a given gene. One plausible explanation therefore may be that the SNPs under study are not responsible for the observed associations. Rather, the effects may have been due to other SNPs, existing with various degrees of LD with the evaluated SNPs, which were under-evaluated or not evaluated at all in these studies. Racial/ethnic variability and sampling inconsistency, coupled in some cases with incomplete LD between SNPs, may not always capture SNPs with functional effects.

It is noteworthy that the majority of the studies conducted so far addressed the effect of a few non-synonymous SNPs that result in amino acid changes in the coding regions of DNA repair genes. While it has long been assumed that intronic and synonymous SNPs (i.e., those that do not result in amino acid change) were “silent” (i.e., inconsequential) since the primary sequence of the protein is retained, several mechanisms by which synonymous and noncoding SNPs can alter the expression, structure, and function of encoded proteins have been elucidated in recent years. For example, studies from our laboratory (Wolfe et al., 2007) and others (Wang et al., 2005b; Johnson et al., 2005; Marin, 2008; Hunt et al., 2009; Kimchi-Sarfaty et al., 2007; Siller et al., 2010) demonstrated that synonymous SNPs can alter the protein function by altering mRNA expression, splicing, stability, and structure, as well as protein folding. Kimchi-Sarfaty et al. (2007) showed that synonymous SNPs can result in a protein with altered structure and function, despite the identical protein sequence, as a result of a difference in the rate of translation which affects protein folding.

In addition to non-synonymous SNPs, there are hundreds of synonymous and intronic SNPs in each DNA repair gene that have not been methodically evaluated and their functional and biological effects are currently unknown. These SNPs can act in combination with each other to influence the phenotype, thus creating an obvious challenge for studies aiming at evaluating the effect of SNPs on DRC. One approach to address this challenge is to evaluate the effect of SNPs in DNA repair genes in the context of haplotypes rather than in the context of independent SNPs. The studies by Lin et al. (2007) who observed a significant correlation between an increasing number of variant alleles in the *XPC* gene and increased mutagen sensitivity when studying the K939Q, A499V and PAT polymorphisms support this idea. SNPs in coding as well as non-coding regions that are in linkage disequilibrium (LD) with each other forming specific haplotypes (i.e. SNPs combinations) (Gabriel et al., 2002) may act collectively through different mechanisms to influence the phenotype.

The first step to characterize the effect of haplotypes of a certain gene should therefore be to characterize the haplotype structure of that gene and to determine if different haplotypes have different phenotypic effects. An example of such approach is illustrated in a recent study from our laboratory, in which we constructed a comprehensive haplotype map encompassing all common SNPs of the *XPC* gene and used the mutagen-sensitivity assay to evaluate the haplotype effects on DRC (Rondelli et al., 2010). In this study, we identified 92 SNPs in the *XPC* gene, of which 35 had minor allele frequencies ≥ 0.05 . Bayesian inference and subsequent phylogenetic analysis identified 21 unique haplotypes, which segregated into 6 distinct phylogenetically-grouped haplotypes (PGHs A-F). The relationships between *XPC* haplotypes and mutagen-induced chromosome aberrations were then evaluated in a

population of smokers matched to non-smokers. We observed significant interactions among smoking and PGH-D ($p = 0.023$) and PGH-F ($p = 0.007$) for mutagen-induced CA frequencies. These data illustrates the usefulness of the haplotype approach by indicating that certain *XPC* haplotypes (rather than a few SNPs in the gene) significantly alter DRC in smokers and, thus, can contribute to cancer risk (Rondelli et al., 2010).

A similar approach involving bioinformatics analysis was adopted by Leng et al. (2008) to evaluate the effect of 134 SNPs dispersed over the entire gene and regulatory regions of three major cytosine *DNA methyltransferases* (*DNMT1*, *DNMT3A* and *DNMT3B*). This study evaluated the hypothesis that sequence variants in *DNMT1*, *DNMT3A* and *DNMT3B* are associated with mutagen sensitivity induced by the tobacco carcinogen BPDE in 278 cancer-free smokers. DNA sequence variation in the *DNMT1* and *DNMT3B* loci was globally associated with breaks per cell ($p < 0.04$ for both). No global association between *DNMT3A* and breaks per cell was seen ($p = 0.09$). The association between sequence variations of *DNMT1* and *DNMT3B* and mutagen sensitivity was further evaluated by a haplotype-based approach. Two haplotypes in block I of *DNMT1* (H284) and 3B (H70) were found to be associated with 16 and 24% increase in breaks per cell, respectively. Subjects with three or four adverse haplotypes of both *DNMT1* and 3B had a 50% elevation in mean level of breaks per cell compared with persons without adverse alleles ($p = 0.004$). This study provides another example of possible approaches that could be used to comprehensively evaluate the biological and functional effects of multiple genetic variants concomitantly as they actually exist in a population.

In conclusion, mutagen sensitivity assays have been shown in numerous studies to be highly reliable in assessing sensitivity to a mutagen, as well as in evaluating DRC in human populations. We have documented that such sensitivity is affected by genetic polymorphisms in DNA repair genes as evident from the large body of published literature. Future studies should focus on addressing the controversy associated with SNP studies through focusing the analysis on haplotypes rather than single SNPs.

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