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Polymorphisms of phase II xenobiotic-metabolizing and DNA repair genes and *in vitro N*-ethyl-*N*-nitrosourea–induced O^6-ethylguanine levels in human lymphocytes

Li Jiao[a], **Ping Chang**[a], **Pervez F. Firozi**[a], **Dejian Lai**[b], **James L Abbruzzese**[a], and **Donghui Li**[a,*]

[a]*Department of Gastrointestinal Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX USA and* [b]*Biostatistics Division, The University of Texas Health Science Center at Houston, School of Public Health, Houston, TX USA*

Abstract

This study tested the hypothesis that genetic variants of phase II detoxification enzymes and DNA repair proteins affect individual response to DNA damage from alkylating agents. In 171 healthy individuals, an immunoslot blot assay was used to measure O^6-ethylguanosine (O^6-EtGua) adduct levels in peripheral blood lymphocytes treated with *N*-ethyl-*N*-nitrosourea (ENU) *in vitro*. The genotypes of *GSTM1*, *GSTT1*, *GSTP1* $I^{105}V$ and $A^{114}V$, *MGMT* $L^{84}F$ and $I^{143}V$, *XPD* $D^{312}N$ and $K^{751}Q$, and *XRCC3* $T^{241}M$ were determined. Demographic and exposure information was collected by in-person interview. Student's *t* test, analysis of (co)variance, and multiple linear regression models were used in statistical analyses. The mean and median (range) O^6-EtGua levels were 94.6 and 84.8 (3.2–508.1) fmol/g DNA, respectively. The adduct level was significantly lower in people who smoked ≥ 25 years than that in never-smokers (square-root transformed mean values 8.20 versus 9.37, $P = 0.03$). Multiple linear regression models revealed that *GSTT1* ($\beta = -2.36$, $P = 0.009$) polymorphism was a significant predictor of the level of adducts in 82 never-smokers, whereas the number of years smoked ($\beta = -0.08$, $P = 0.005$) and *XRCC3* $T^{241}M$ ($\beta = 2.22$, $P = 0.007$) in 89 ever-smokers. The association between *GSTP1* $I^{105}V$, *MGMT* $I^{143}V$, and *XPD* $D^{312}N$ with the level of adducts was not conclusive. Each polymorphism could explain 2% to 10% of the variation of the adduct level. These observations suggest that *GSTT1* null and *XRCC3* $T^{241}M$ polymorphism may have some functional significance in modulating the level of ENU-induced DNA damage and these effects are smoking-dependent. Results from this exploratory study need to be confirmed in other experimental systems.

Keywords

single nucleotide polymorphism (SNP); phase II xenobiotic-metabolizing enzyme; DNA Repair protein; *N*-ethyl-*N*-nitrosourea (ENU); O^6-ethylguanosine (O^6-EtGua); human lymphocytes

*Corresponding author at: Department of Gastrointestinal Medical Oncology, Unit 426, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA. Tel +1 713 834 6690; fax: +1 713 834 6153, *E-mail address:* dli@mdanderson.org (D. Li).

1. Introduction

Exogenous and endogenous exposure to *N*-nitroso compounds is extensive in human beings [1]. *N*-nitroso compounds and their metabolites can react with cellular DNA to form a broad spectrum of alkylating DNA damage, which can be a critical event in the induction of cancer. O^6-alkylguanine is a major premutagenic lesion in cells exposed to *N*-nitroso compounds and constitutes a significant genotoxic burden for the general population [2]. In view of the constant alkylation pressure imposed on cells, the protective mechanisms against alkylating agents deserve special attention. A growing number of studies have shown that subtle differences in carcinogen metabolism and DNA repair capacity (DRC) conferred by the inheritance of polymorphisms may predispose individuals to cancer [3]. However, molecular epidemiological findings on gene polymorphisms and cancer risk have often been inconsistent. Lack of knowledge on the functional significance of the polymorphisms makes the data interpretation more difficult. Understanding the functional significance of the polymorphisms may help to better define the study population and the data interpretation in molecular epidemiology study.

In vitro genotoxicity studies with peripheral blood lymphocytes offer an experimental tool to investigate genotypic and phenotypic associations because they allow assessment of the toxicological response to xenobiotics under controlled experimental conditions [4]. By using different challenging agents and endpoints in a limited cell system, such assays can be applied to examine genotype and exposure interactions and to predict individual response to carcinogens. We have previously established an assay for measuring benzo(a)pyrene diol epoxide (BPDE)-induced DNA adducts in cultured peripheral lymphocytes as a marker for DRC [5] and hence a predictor for the risk of developing smoking-related human cancers [6]. In the current study, the *N*-nitroso carcinogen *N*-ethyl-*N*-nitrosourea (ENU) was used as the challenging agent and O^6-ethylguanosine (O^6-EtGua) level was evaluated as the endpoint biomarker. ENU is a potent monofunctional ethylating agent that is mutagenic in a wide variety of test systems and carcinogenic in various mammalian organs [7]. O^6-EtGua is one of a dozen ethylation products formed in DNA upon exposure to ENU [8]. However, factors that influencing the formation and repair of O^6-EtGua adduct have not been fully elucidated.

The aim of the present study was to examine the association between several polymorphisms in genes encoding phase II xenobiotic-metabolizing enzymes and DNA repair proteins and the level of *in vitro*-induced O^6-EtGua in cultured human lymphocytes. Because ENU is a direct alkylating agent, we focused on genes that are involved in the phase II detoxification (*GSTM1, GSTT1,* and *GSTP1*) and DNA repair pathways (*MGMT, XPD and XRCC3*). Information on the selected single nucleotide polymorphisms (SNPs) is summarized in Table 1. Homozygous deletion of the *GSTM1* and *GSTT1* genes (null genotype) has been associated with the loss or lack of enzyme activity and increased vulnerability to cytogenetic damage [9]. The *GSTP1* $Ile^{105}Val$ and $Ala^{114}Val$ variant alleles have been associated with altered enzyme activities towards different drug or carcinogens [10,11]. The selected DNA repair SNPs all reside in the coding regions of the genes and result in amino acid changes. To our knowledge, this study is the first to examine the association between these genotypes and *in vitro*-induced DNA adduct levels in human lymphocytes. We found that genetic polymorphisms of *GSTT1* contribute to a subtle proportion of the variance in adduct levels in cultured lymphocytes of never-smokers, whereas the number of years smoked and *XRCC3* $T^{241}M$ polymorphism in ever-smokers.

2. Materials and methods

2.1. Study subjects

This study was conducted with healthy individuals who were enrolled in a hospital-based case-control study of pancreatic cancer at The University of Texas M. D. Anderson Cancer Center between October 2000 and November 2003. They were companions (non-blood relatives or

friends) of patients who were diagnosed with various cancers. Individuals were considered eligible if they had no previous history of cancer (except for non-melanoma skin cancer) and donated a blood sample of at least 20 ml. There were no age and sex restrictions, and all study subjects were non-Hispanic whites. Each study participant provided written informed consent to an in-person interview and donation of a blood sample. The study was approved by the Institutional Review Board of M. D. Anderson Cancer Center.

During the study period, a total of 184 individuals met the eligibility criteria and adduct levels were measured. Nine subjects were excluded because of missing exposure information; four other subjects were excluded because of large variations (> 50%) in repeated measurements. Therefore, the final results were based on data from 171 study subjects consisting of 89 ever-smokers and 82 never-smokers. The distributions of age and sex were comparable between individuals who were included or not included in the study. None of the study subjects reported a history of occupational exposure to alkylating agents.

2.2. Data collection

A trained study coordinator administered a structured risk factor questionnaire to collect information on demographics, occupation, smoking and alcohol consumption. The definitions of exposure parameters (never, ever, former, and current smokers) have been published elsewhere [12]. Briefly, subjects were classified as ever-smokers if they had smoked more than 100 cigarettes in their lifetime. Former smokers were defined as those who had quit smoking for more than 1 year. The information on duration (the number of years smoked) and intensity of smoking (the number of cigarettes smoked per day) was also collected. Information on alcohol consumption was collected to evaluate its potential confounding effect on the association between polymorphism and adduct levels. The subject was asked whether he/she had consumed at least four alcoholic beverages a month, for six months or more. The answer of ‘no’, ‘yes, but quit’, or ‘yes’ to this question distinguishes the “never”, “former” and “current” drinker, respectively.

2.3. Isolation of lymphocytes and ENU treatment in cell culture

Blood samples were collected in heparinized Vacutainers (BD Biosciences, Franklin Lakes, NJ), and each specimen was labeled with a unique study identification number. Peripheral blood lymphocytes were isolated on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradients. All cell cultures were set up within 2 hours after blood collection. Cell concentrations were adjusted to 1–10 × 10^7/ml with 1 ml serum-free RPMI 1640 medium (GIBCO Invitrogen Corporation, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). ENU (Sigma, St. Louis, MO, CAS# 759-73-9, lot# 79H1011) was dissolved in H_2O-free dimethyl sulfoxide (200 mM, stock solution) and stored at −80°C in the dark. That ENU stock solution was then added to 1 ml cell culture, with a final concentration of 0.64 mM (75 μg/ml). After incubation at 37°C with 5% CO_2 for 1 hour, cells were harvested by centrifugation. Cell pellets were washed with serum-free RPMI 1640 medium twice to remove ENU and then resuspended in 400 μl Tris-EDTA buffer (pH = 8.0) and immediately stored at −80°C until DNA isolation.

2.4. Immunoslot blots, standard curves, and dose response

DNA was extracted by using a FlexiGene DNA kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). DNA was dissolved in 10 mM Tris-HCl (pH = 8.0), quantified by UV spectrophotometry (Amersham Pharmacia Biotech), and stored at −80°C until assay. Semi-quantification of ENU-induced O^6-EtGua was carried out by an immunoslot blot method [13]. Briefly, 5 μg DNA was sonicated and then denatured at 100°C for 10 minutes. The heat-denatured DNA was quickly chilled on ice and immediately slot-blotted onto Hybond-N+

Nylon membranes (Amersham Pharmacia Biotech) by using a Convertible Filtration Manifold System (GibcoBRL, Carlsbad, CA). All 48 slots were rinsed with 200 μl 1 M ammonium acetate and the membranes were baked for 2 hours at 80°C. After single-stranded DNA was immobilized onto the nitrocellular membranes, the membranes were blocked with 5% milk −1×TBS −0.05% Tween 20 and then hybridized with the (O^6-EtGua)–specific mouse monoclonal antibody EM-2-3 (1:5000 diluted, kindly provided by Dr. Menfred F. Rajewsky's laboratory in University of Essen, Germany) for 150 min at room temperature with shaking [14]. The membrane was then hybridized with the secondary antibody goat anti-mouse horseradish peroxidase–labeled IgG (1:1000 diluted) (Chemicon, Temecula, CA) by shaking for 1 hour at room temperature. Enzymatic activity was visualized by chemiluminescence reaction with an ECL^{TM} western blotting detection kit (Amersham Pharmacia Biotech). The intensity of the bands was analyzed with Kodak 1D Image Analysis software (Kodak, New Haven, CT).

A standard for DNA measurement was generated by the *in vitro* reaction of calf thymus DNA with ^{3}H-labeled *N*-methyl-*N*-nitrosourea (NCI Chemical Carcinogen Repository), and the DNA adduct levels were determined by scintillation counting. The labeled DNA was then diluted with naïve calf thymus DNA to a final concentration of 25, 50, 100, 250, 500, 1000, or 2000 fmol adduct per μg DNA. Standard curves were established from this series of DNA concentrations by using the immunoslot blot assay (Figure 1, A). The intensity of the band in each slot was compared with the standard curve to semiquantify the adduct levels. The arithmetic mean was computed from the parallel samples to represent the actual value of each sample.

At the beginning of the study, a dose-response experiment was performed with lymphocytes from 3 healthy volunteers at ENU doses of 0, 0.21, 0.43, 0.64, 0.85, and 1.60 mM; a dose-response relationship was observed (Figure 1, B). The 0.64 mM concentration was selected as the challenging dose because cellular viability was optimal (> 80%, tested by exclusion of 0.4% trypan blue) and the adduct levels were within the linear range of the standard curve. One hour was used as the treatment time because we were interested in the rapid mode of O^6-EtGua repair.

2.5. Genotyping

Genomic DNA was extracted by using a FlexiGene DNA kit and the aliquots stored at 4°C. The genotyping methods are listed in Table 1 [15–17]. No data were missing for the *GSTM1*, *GSTT1*, *MGMT* I^{143}V, and *XPD* K^{751}Q polymorphisms. The rate of missing data varied from 4.3% to 7.4% for the remaining polymorphisms.

2.6. Quality control measures

The same batch of ENU and monoclonal antibody was used in all experiments. The reagents were aliquoted to a small volume and stored at −80°C. ENU was stored in light-protected vials, and the open stock vial was refreshed every 2 weeks and the pH value maintained below 5.0. A positive control (an adduct standard of 500 fmol/μg DNA) and a negative control (untreated calf thymus DNA) were included in every immunoslot blot assay. The same person conducted all the procedures, from the ENU treatment through the final quantification of adducts, according to a standard protocol. Samples were analyzed in parallel in the same experiment, and those with variation greater than 50% in duplicate experiments were eliminated from the final analysis. The two values from repeated measurements were significantly correlated (Pearson's correlation coefficient $\rho = 0.79$, $P < 0.001$).

In genotyping assays, 10% of the specimens were randomly selected for duplication. For the polymerase chain reaction–restriction fragment length polymorphism assays, two investigators

independently read the genotyping results. In the rare cases (0.03%) in which an inconsistency was noted, genotyping was repeated from the polymerase chain reaction step. For the MassCode assays, positive allele controls were run in every experiment. Only conservative calls were taken and any ambiguous call (owing to poor DNA quality and quantity) was excluded from the final report.

2.7. Statistical analyses

Data analyses were performed with STATA 7.0 (Stata Corporation, College Station, TX) and SPSS (SPSS Inc. Chicago, IL) software. All statistical tests were two-sided. P values ≤ 0.05 for any test indicated statistical significance. The distribution of original values of O^6-EtGua was skewed to the left and was normalized by square-root transformation to ensure the normality assumption for parametric testing and to stabilize the group variance. The allele frequency for each polymorphism was estimated by gene counting. The Hardy-Weinberg equilibrium was tested by using the goodness-of-fit χ^2 test.

Independent one-sample Student's t test with equal variance or one way analysis of variance was used to compare induced adducts level according to demographic factors (age and sex) and exposure factors (smoking and alcohol consumption). An analysis of covariance (ANCOVA) was used to compare the difference in mean adduct levels between genotypes adjusted for age, sex, years of smoking, number of cigarettes smoked per day, and alcohol consumption (never, quit, and ever). Variable selection for the multiple linear regression models used the criterion of P value of less than 0.20 in the univariate analysis by smoking status. Generally, the heterozygous and homozygous variant genotype was combined when both showed the same effect on the level of adducts. In the multiple linear regression analyses, the percentage of the variance of dependent variable (adduct level) explained by each polymorphism and other variable was evaluated by subtracting the r^2 value for the full model from the r^2 for a model that excludes the test variable [18]. The full models contained covariates age (in years), sex (0, women; 1, men), alcohol consumption (0, never, 1, quit; 2, current), and all polymorphism terms selected from the univariate analyses. Separate models were run for the never-smokers and the ever-smokers. Multiplicity adjusted P-value was calculated [19]. Lastly, backward elimination regression analysis with exclusion criteria of $P \geq 0.10$ was used to examine the contributions of the polymorphism to the prediction of the O^6-EtGua level.

3. Results

3.1. DNA adduct phenotype according to study subject characteristics

O^6-EtGua levels ranged from 3.2 to 508.1 fmol/μg DNA, with a mean of 94.6 and a median of 84.8 fmol/μg DNA. The distribution of O^6-EtGua values was skewed to the left ($P < 0.001$ for joint skewness/Kurtosis test) and was approximately normalized by square-root transformation ($P = 0.05$ for joint skewness/Kurtosis test). The transformed values were expressed as mean ± standard error (SE). Table 2 shows mean adduct levels according to selected characteristics of the 171 study subjects. The O^6-EtGua level did not differ significantly according to demographic variables. People who smoked more than 25 years had a significantly lower adduct level than did the never-smokers (8.20 versus 9.37, $P = 0.03$).

3.2. Allele and genotype frequency and ANCOVA analysis of O^6-EtGua level by genotypes

Table 3 describes the genotype or allelic frequency for each polymorphism. The frequencies of *GSTM1*, *GSTT1*, and *GSTP1* $A^{114}V$, *MGMT* $L^{84}F$, and *XPD* $D^{312}N$ and $K^{751}Q$ were within the range of those reported by other studies [3,20,21]. The variant allele frequencies of the *GSTP1* $I^{105}V$ and the *MGMT* $I^{143}V$ were 0.38 and 0.16, respectively, which were higher than those reported in other Caucasian populations [22,23]. However, this would not affect our study purpose in examining genotype–adduct associations. None of the genotype distributions

differed significantly from those expected under Hardy-Weinberg equilibrium (data not shown). Table 3 presents the adjusted mean levels of O^6-EtGua and shows the level of O^6-EtGua did not significantly differ by any polymorphism. The *GSTT1* null genotype carriers had borderline significantly lower adduct levels than did the wild-type carrier (8.20 *versus* 9.42, $P = 0.05$).

3.3. Univariate analyses of O^6-EtGua level stratified by smoking status

Since the univariate analysis showed an association between O^6-EtGua level and years of smoking, we further investigated the association between each polymorphism and O^6-EtGua level according to smoking status (Figure 2). Among never-smokers, the *GSTT1* null genotype carriers had significantly lower adduct levels than did the wild-type carriers (7.61 *versus* 9.76, $P = 0.009$) (Figure 2, A). The *GSTP1* ^{105}V allele was associated with a lower adduct level compared with the *GSTP1* $I^{105}I$ genotype (8.80 *versus* 10.29, $P = 0.03$) (Figure 2, B). Among ever-smokers, the *MGMT* ^{143}V allele carriers had higher adduct level than did the $I^{143}I$ genotype carriers (9.80 versus 8.60, $P = 0.13$) (Figure 2, C). Compared with the adduct level of *XRCC3* $T^{241}T$ wild-type carriers, the adduct level of the ^{241}M allele carriers tended to be higher among ever-smokers (8.29 versus 9.50, $P = 0.11$), but lower among never-smokers (10.1 versus 8.97, $P = 0.09$) (Figure 2, D). The adduct level essentially did not differ by other polymorphisms (P for t test ≥ 0.20) (data not shown).

3.4. Multiple linear regression analyses stratified by smoking status

Table 4 summarizes the relationship between O^6-EtGua level and polymorphisms estimated by multiple linear regression models. Among never-smokers, a lower adduct level was related to the presence of the *GSTT1* null genotype ($\beta = -2.36$, $P = 0.009$), the *GSTP1* ^{105}V allele ($\beta = -1.77$, $P = 0.02$), the *XPD* ^{312}N allele ($\beta = -1.46$, $P = 0.05$), and the *XRCC3* ^{241}M allele ($\beta = -1.75$, $P = 0.02$). Among ever-smokers, a higher adduct level was related to the presence of the *MGMT* ^{143}V allele ($\beta = 1.64$, $P = 0.05$) and the *XRCC3* ^{241}M allele ($\beta = 2.22$, $P = 0.007$). These polymorphisms each can explain more than 2% but less than 10% of the variance of the dependent variable among never- or ever-smokers. These five polymorphisms, together with other covariates, can explain 27.3% and 25.3% of the variance of the dependent variable for never-smokers and ever-smokers, respectively. The number of years smoked was a significant determinant for the adduct level among ever-smokers ($\beta = -0.08$, $P = 0.005$). Assuming there were five independent tests in each smoking stratum, the Bonferroni adjusted P value remained ≤ 0.05 for the term *GSTT1* in never smokers, the number of years smoked and *XRCC3* $T^{241}M$ in ever smokers, with the adjusted P value of 0.045, 0.025, and 0.035, respectively. Notable was the differential effect of the *XRCC3* $T^{241}M$ polymorphism on adduct levels for never-smokers and ever-smokers (Table 4).

Backward elimination regression with the exclusion criteria of $P \geq 0.10$ was used to screen the importance of the explanatory variables. The *GSTT1*, *GSTP1* $I^{105}V$, and *XRCC3* $T^{241}M$ polymorphism turned out to be crucial in determining the adduct level among never-smokers. The number of years smoked, the *XRCC3* $T^{241}M$ and *XPD* $D^{312}N$ polymorphisms turned out to be the important determinants for the adduct level among ever-smokers (data not shown).

4. Discussion

By using an *in vitro* experiment system in cultured lymphocytes of non-cancer healthy study subjects, we have observed a significant association between *GSTT1* null genotype in never smokers and the number of years smoked and *XRCC3* $T^{241}M$ in ever-smokers with the level of ENU-induced O^6-EtGua adducts. Inconclusive association (*GSTP1* $I^{105}V$, *XPD* $D^{312}N$, *MGMT* $I^{143}V$) or no association (*GSTM1*, *GSTP1* $A^{114}V$, *MGMT* $L^{84}F$, *XPD* $K^{751}Q$) was observed for the remaining SNPs. We also found that genetic polymorphisms only explain a

small proportion of the variance of adduct level. These observations suggest a potential functional role of *GSTT1* null genotype and *XRCC3* $T^{241}M$ polymorphism in the formation and removal of the *in vitro* ENU-induced O^6-EtGua. It also suggests that the effect of polymorphism may differ according to smoking status.

The *GST* genes encode phase II enzymes that catalyze the conjugation of electrophilic compounds in the detoxification process. Deletion or mutation of these genes is expected to be associated with higher levels of DNA damage [24]. However, previous studies provided inconsistent evidence on the effect of the *GSTM1* and *GSTT1* genotypes on various cytogenetic markers [25]. Polymorphisms of *GSTP1* $I^{105}V$ and $A^{114}V$ are located in the electrophile-binding active site of the GSTP1, producing enzymes with different thermal stability and substrate affinity [26,27]. Decreased enzyme activity towards 1-chloro-2,4-dinitrobenzene [10] and increased binding activity towards benzo[a]pyrene diol epoxide [28] has been associated with the *GSTP1* variant alleles. Very few phenotypic studies have investigated the association between GST enzyme and genotoxic effect of DNA alkylating agents. Salama et al [29] observed that the *GSTM1*, but not *GSTT1* genotype might influence 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanone (NNK)-induced genotoxicity. Lewis et al observed that, based on 44 DNA samples, N7-methylguanine level was higher in bronchial lavage cells of individuals with *GSTM1* null, *GSTT1* null or *GSTP1* Ile/Ile genotypes [30]. Another study suggested that the biological consequences of *GSTT1* genotype are difficult to predict because this enzyme has both detoxifying and activating properties in many environmental pollutants [31]. Although it is not known whether ENU is a substrate of glutathione conjugation catalyzed by any GSTs in human, the current study found a significantly lower level of ENU-induced O^6-EtGua adducts in association with *GSTT1* null and possibly *GSTP1* ^{105}V variant allele among never-smokers, which suggests that these two GSTs may play a role in the metabolism of ENU. Because smoking was associated with the level of O^6-EtGua, the genotype association with the level of adducts was perhaps masked by smoking therefore was not observed in ever-smokers.

O^6-EtGua is mainly removed by the DNA repair enzyme MGMT. The *MGMT* codon 143 polymorphism is near the alkyl group acceptor pocket at codon 145. The replacement of Ile by Val has been postulated to affect the acceptance of an alkyl group. However, functional studies of the *MGMT* ^{143}V variant protein have found either no difference from the wild-type or a higher activity [32,33]. The ^{84}F polymorphism has been shown to have similar enzymatic and physicochemical properties to the wild type [34]. Our study showed that the *MGMT* ^{143}V allele tended to be related to a higher adduct level among ever-smokers, but no effect was observed for the *MGMT* $L^{84}F$ polymorphism. The general lack of association between *MGMT* polymorphisms and O^6-EtGua level may be partially explained by the inefficient capacity of the MGMT in repairing the bulky alkyl adduct. Pegg et al. showed that O^6-EtGua was repaired more slowly than was O^6-methylguanine by rat liver MGMT [35]. If this is the case in human beings, the effect of *MGMT* polymorphism may not be seen in our study since lymphocytes were treated with ENU for 1 hour. Moreover, the great variation in both expression and activity of MGMT related to other factors could mask any minor effect conferred by a SNP.

A human lymphoblastoid cell line lacking either MGMT or nucleotide excision repair (NER) capability had similar levels and types of mutation after ENU treatment, which suggested that MGMT and NER are of approximately equal quantitative importance for the repair of ENU-induced damage [36]. The XPD protein has been shown to correlate with resistance to alkylating agents in human tumor cell lines [37]. Previous studies have shown that the ^{312}N allele was associated with an increased level of aromatic DNA adduct among never smoking lung cancer patients [38] and an increased frequency of chromosome aberrations in cells treated with NNK [39] or UV light [40] among healthy individuals. In contrast, Lunn et al. [41] showed that individuals with the *XPD* $D^{321}D$ genotype had insignificantly more X-ray induced

chromosome aberrations than did ^{312}N carriers. Similarly, one group showed that lymphoblastoid cell lines with the homozygous Asn genotype had a significantly higher apoptotic response to UV [42]. In our study, the influence of the *XPD* D^{312}N polymorphism on adduct level was only revealed by multiple linear regression but not by univariate analysis, suggesting that this polymorphism may interact with other polymorphisms in determining the adduct level. Since the association was not stable, its effect is worthy of a further investigation. Many studies have investigated the association between the *XPD* K^{751}Q polymorphism and the *in vivo* level of a variety of bulky DNA adducts in either cancer patients [38,43–45] or healthy individuals [17,46,47]. Most of the studies favored the association between the *XPD* ^{751}Q allele and adduct formation, with a few exceptions [45,47]. However, we failed to observe any statistically significant association for this polymorphism.

The *XRCC3* T^{241}M polymorphism turned out to be one of the important factors in determining the variation of the adduct level in both never- and ever-smokers. Previous study has linked the *XRCC3* T^{241}M polymorphism to higher level of bulky DNA adducts among healthy individual [17]. Our study on *XRCC3* is rather exploratory than hypothesis-driven because XRCC3 has not been directly associated with repair of alkylating DNA damage. Although homologous recombination is the major pathway of processing O^6-methylguanine in replicating cells, the recombination induced by O^6-methylguanine occurs only in the second postexposure replication cycle [48,49]. It is unlikely that this mechanism would occur within 1 hour of exposure to ENU in our experiment. The hamster cell line irs1SF (*XRCC3* mutant) has been shown to be modestly sensitive to DNA alkylating agents [50]. Therefore, the mechanism of *XRCC3* in repair of alkylating DNA adduct should be explored further. Alternatively, the *XRCC3* T^{241}M may be in linkage disequilibrium with other locus of gene involved in processing alkylating DNA damage.

In examining the relationship between O^6-EtGua level and smoking indices, we observed that the number of years smoked was inversely related to the adduct level. It is postulated that higher expression of DNA repair genes among ever-smokers might be attributable to more efficient removal of adduct. We further found that polymorphisms, in particular *XRCC3* T^{241}M, had a different effect on adduct levels in never- or ever-smokers. Cigarette smokers may have altered DRC compared with never-smokers because of long-time exposure to tobacco carcinogens [51]. Wei et al. [52] have shown that the adaptation of DRC to smoking seems to be long-term rather than transient because former smokers and current smokers had similar DRC. This observation supports the argument that the mixture of ever-smokers and never-smokers would mask a true genotype-phenotype relationship when phenotype is related to smoking. Previous studies have suggested that in the examination of relationship between cancer risk, biomarker, and genotype, mixture of individuals with different exposures or different diseases biases the findings and compromises their interpretation [53]. Findings of the current study apparently support this notion.

In view of the complexity and the number of proteins involved in the repair of alkylating DNA damage, the phenotype is perhaps the aggregate of many low-penetrance gene effects. Our study suggests that none of the single polymorphisms can explain more than 10% of the variation of the adduct levels or play a dominant role in this sophisticated process. Furthermore, the effect of polymorphism on phenotypic trait is also likely to be different according to smoking exposure. This would support the gene-smoking interaction effect observed in risk association studies.

This exploratory study has many limitations. The immunoassay itself was plagued by issues of cross-reactivity of the antibody with other DNA alkylation adducts. However, measurement of the class of adducts as a whole, is still a useful marker [54]. The limited detection dynamics of the immunoassay made accurate adduct quantification impossible. The residual variance of

the adduct level was large, suggesting that experimental random error was an important source of the total variance. The multiple comparisons of many factors in this study may increase the chance of false-positive results. Although the findings on *GSTT1* among never-smokers and *XRCC3* $T^{241}M$ among ever-smokers remained statistically significant after the *P* value was adjusted for multiple testing, our study findings still should be interpreted cautiously. The small sample size precluded us to further examine gene-gene interaction and allele-dosage effect in the current analysis.

In summary, the observed associations between *GSTT1/XRCC3* genotypes and the level of ENU-induced DNA adducts should be confirmed in other experimental systems and future study on genotype and phenotype associations should consider the confounding effect of cigarette smoking and other exposure.

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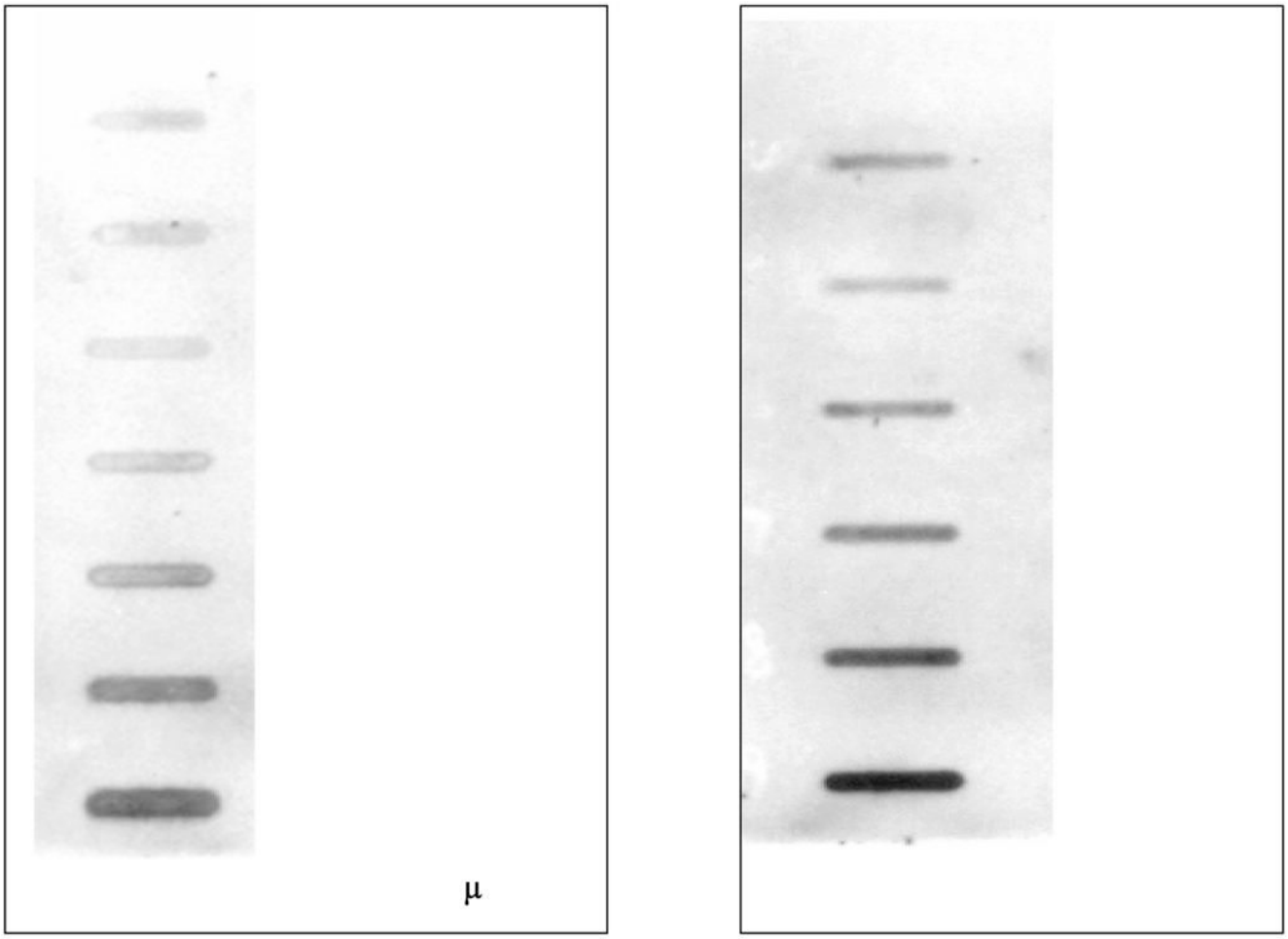

Figure 1.

A. Immunoslot blot assay result for the standard curve. A standard for DNA measurement was generated by the *in vitro* reaction of calf thymus DNA with ^{3}H-labeled *N*-methyl *N*-nitrosourea. The DNA adduct levels were determined by scintillation counting. The labeled DNA was then diluted with naïve calf thymus DNA to a final concentration of 25, 50, 100, 250, 500, 1000, or 2000 fmol adduct per μg DNA.

B. Immunoclot blot assay result for a dose-response experiment. The lymphocytes from 3 healthy volunteers were treated with ENU at doses of 0, 0.21, 0.43, 0.64, 0.85, and 1.60 mM.

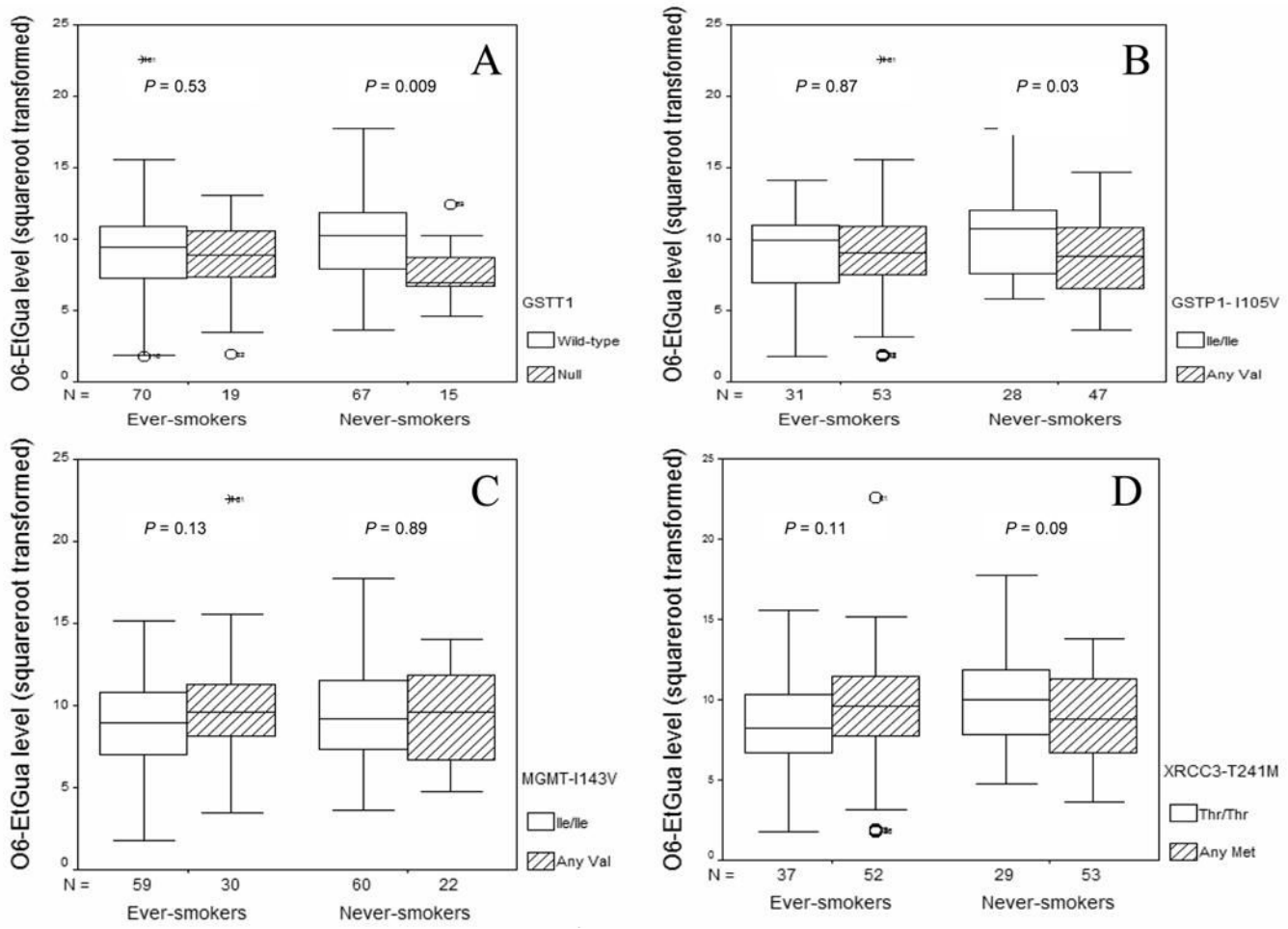

Figure 2.

Box-plot of adduct levels by *GSTT1* (panel A), *GSTP1* $I^{105}V$ (panel B), *MGMT* $I^{143}V$ (panel C), and *XRCC3* $T^{241}M$ (panel D) genotype in ever-smokers and never-smokers. *P* value was from Student's *t* test. The solid line indicates median; the box extents mark the 25th and 75th percentiles of the observed values, and the capped bars indicate the 10th and 90th percentiles. Symbols indicate the outliers of adduct level.

Table 1

Information on the polymorphisms under investigation

Gene	GeneID	Chromosome	Polymorphism	Amino Acid Change	Reference SNP ID	Genotyping assay
GSTM1	2944	1p13.3	Deletion			Multiplex PCR[a]
GSTT1	2952	22q11.23	Deletion			Multiplex PCR[a]
GSTP1	2950	11q13.2	Exon 5–24 A>G	Codon 105 Ile (I) → Val(V)	947894	MassCode[b]
GSTP1	2950	11q13.2	Exon 6+5 C>T	Codon 114 Ala (A) → Val (V)	1799811	MassCode[b]
MGMT	4255	10q26	Exon 2–25 C>T	Codon 84 Leu (L) → Phe (F)	12917	MassCode[b]
MGMT	4255	10q26	Exon 5+13 A>G	Codon 143 Ile (I) → Val (V)	2308321	PCR-SSCP[a]
XPD	2608	19q13.3	Exon10–16 G>A	Codon 312 Asp (D) → Asn (N)	1799793	MassCode[b]
XPD	2608	19q13.3	Exon23+61 A>C	Codon 751 Lys (K) → Gln (Q)	13181	PCR-RFLP[a]
XRCC3	7517	14q32.3	Exon 8–53 C>T	Codon 241 Thr (T) → Met (M)	861539	MassCode[b]

From the SNP500Cancer database of the U.S. National Cancer Institute. PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism. GST, glutathione-S-transferase; MGMT, O^6-methylguanine-DNA-methyltransferase; XPD, xeroderma pigmentosum group D; XRCC3, X-ray repair cross-complementing group 3.

[a]Methods are described in reference 12 (for the *GSTM1* and *GSTT1* polymorphisms), reference 13 (for the *MGMT* $I^{143}V$ polymorphism) and reference 14 (for the *XPD* $K^{751}Q$ polymorphism).

[b]Masscode™ technology (BioServe Biotechnologies, Ltd., Laurel, MD).

Table 2

Characteristics of study subjects and levels of ENU-induced O^6-EtGua

Selected Variables	Study Subjects N	%	O^6-EtGua level[a] Mean	S.E.[b]	P value[c]
	171		9.21	0.25	
Age (years)					
≤ 50	37	21.6	9.32	0.44	
50–60	43	25.1	8.82	0.54	
61–70	61	35.7	9.54	0.46	
> 70	30	17.5	8.76	0.45	0.60
Sex					
Men	87	50.9	8.84	0.35	
Women	84	49.1	9.52	0.35	0.17
Smoking					
Never-smoker	82	47.9	9.37	0.32	
Ever-smoker	89	52.0	9.00	0.37	0.62
Former	63	36.8	9.03	0.49	0.53[d]
Current	26	15.2	8.92	0.46	0.48[d]
No. years smoked[e]					
< 25	43	25.1	9.85	0.58	0.43[d]
≥ 25	46	26.9	8.20	0.45	0.03[d]
No. cigarettes/day[e]					
< 20	27	15.8	9.36	0.90	0.99[d]
≥ 20	62	36.2	8.84	0.37	0.28[d]
Alcohol					
Never	76	44.5	8.91	0.40	
Quit	24	14.0	10.21	0.64	
Current	71	41.5	9.09	0.35	0.22

[a] Square-root transformed value of fmol/μg DNA.

[b] Standard error.

[c] *P* value for the two-sample independent Student's *t* test or one-way analysis of variance.

[d] Compared with adduct levels of never-smokers.

[e] Cutoff point was the median of the continuous variable.

Table 3

ANCOVA of single polymorphism and O^6-EtGua adduct levels in 171 study subjects

Genotype	N (%)[a]	Adduct level[b] (adjusted mean ± SE)	% Relative increase	*P* value[c]
GSTM1				
Wild-type	88 (51.5)	9.13 ± 0.35	Ref.	
Null	83 (48.5)	9.22 ± 0.36	+0.98	0.88
GSTT1				
Wild-type	137 (80.1)	9.42 ± 0.28	Ref.	
Null	34 (19.9)	8.20 ± 0.55	−12.9	0.05
GSTP1 $I^{105}V$				
I/I	59 (37.1)	9.59 ± 0.43	Ref.	
I/V	78 (49.1)	9.12 ± 0.37	− 4.9	
V/V	22 (13.8)	8.35 ± 0.71	−12.9	0.32
I/V + V/V	100 (62.9)	8.95 ± 0.34	−6.7	0.24
V allele frequency	0.383			
GSTP1 $A^{114}V$				
A/A	133 (81.6)	9.22 ± 0.28	Ref.	
A/V	30 (18.4)	9.26 ± 0.59	+0.43	0.95
V/V	0			
V allele frequency	0.092			
MGMT $I^{143}V$				
I/I	119 (69.6)	8.97 ± 0.30	Ref.	.
I/V	50 (29.2)	9.70 ± 0.46	+8.1	
V/V	2 (1.2)	8.57 ± 0.31	−4.4	0.40
I/V + V/V	52 (30.4)	9.65 ± 0.45	+7.6	0.21
V allele frequency	0.162			
MGMT $L^{84}F$				
L/L	131 (79.4)	9.38 ± 0.28	Ref.	
L/F	34 (20.6)	8.65 ± 0.56	−7.8	0.25
F/F	0			
F allele frequency	0.103			
XPD $D^{312}N$				
D/D	72 (45.3)	9.05 ± 0.38	Ref.	
D/N	66 (41.5)	9.21 ± 0.40	+1.8	
N/N	21 (13.2)	8.62 ± 0.71	−4.7	0.76
D/N + N/N	87 (54.7)	9.07 ± 0.35	+0.2	0.95
N allele frequency	0.347			
XPD $K^{751}Q$				
K/K	70 (40.9)	8.93 ± 0.39	Ref.	
K/Q	91 (53.2)	9.50 ± 0.34	+6.9	
Q/Q	10 (5.9)	7.94 ± 1.02	−11.1	0.26
K/Q + Q/Q	101 (59.1)	9.34 ± 0.32	+4.6	0.42
Q allele frequency	0.325			
XRCC3 $T^{241}M$				
T/T	66 (38.6)	9.12 ± 0.40	Ref.	
T/M	75 (43.9)	9.13 ± 0.38	+0.1	
M/M	30 (17.5)	9.39 ± 0.60	+2.7	0.86
T/M + M/M	105 (61.4)	9.21 ± 0.32	+1.0	0.87
M allele frequency	0.394			

[a] Numbers do not add up to expected totals because of missing genotyping data.

[b] Adduct levels were estimated by ANCOVA for all study subjects, adjusted for age (continuous), sex, smoking status (never-smokers and ever-smokers), and alcohol consumption (never, quit, current).

[c] *P* value for ANCOVA F-test.

Table 4

Multiple linear regression analysis of predictor for O^6-EtGua level[a]

Covariates	Never-smokers Multivariate β[c]	P[d]	P[e]	r^2 variance explained[f]	Ever-smokers Multivariate β[c]	P[d]	P[e]	r^2 variance explained[f]
GSTT1 (0, wild-type; 1, null)	**−2.36**	**0.009**	**0.045**	8.7%	−1.26	0.20		1.8%
GSTP1 $I^{105}V$ (0, I/I; 1, any V)	−1.77	0.02		8.9%	−0.50	0.53		2.1%
MGMT $I^{143}V$ (0, I/I; 1, any V)	−0.69	0.37		1.0%	1.64	0.05		4.3%
XPD $D^{312}N$ (0, D/D; 1, any N)	−1.46	0.05		2.9%	0.91	0.25		12.3%
XRCC3 $T^{241}M$ (0, T/T; 1, any M)	−1.75	0.02		7.0%	**2.22**	**0.007**	**0.035**	8.5%
No. years of smoked	NA[b]			NA[b]	**−0.08**	**0.005**	**0.025**	9.4%
Age	−0.03	0.24		1.7%	−0.04	0.35		0.9%
Sex (0, women; 1, men)	0.68	0.34		1.1%	0.09	0.91		0.0%
Alcohol (0, never, 1, quit, 2 current)	0.22	0.57		0.4%	−0.32	0.47		0.6%
r^2	27.3%[g]				25.3%[g]			

[a] The square-root transformed value is the dependent variable. Age, sex, alcohol consumption are covariates.

[b] For never-smoker stratum, the regression model did not include covariate 'No. years of smoked'.

[c] β coefficient: the unit change of dependent variable caused by 1 unit change of variable. For genotype covariates, β is the difference of the mean of each genotype coded as 0 or 1.

[d] *P* value for the term in the multiple linear regression.

[e] Bonferroni multiplicity adjusted *P* value = (listed *P* value x 5), assuming 5 independent hypothesis testing in each stratum.

Bolded indicates multiplicity adjust *P* value ≤ 0.05.

[f] The difference of the r^2 of the full model and the r^2 of the reduced model without that variable.

[g] r^2, percentage of variation of the dependent variable that the model can explain.