Glutathione S-Transferase P1 and NADPH Quinone Oxidoreductase Polymorphisms Are Associated with Aberrant Promoter Methylation of P16\(^{\text{INK4a}}\) and O\(^6\)-Methylguanine-DNA Methyltransferase in Sputum\(^1\)

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Abstract

Inactivation of the p16\(^{\text{INK4a}}\) tumor suppressor gene and O\(^6\)-methylguanine-DNA methyltransferase (MGMT) DNA repair gene by aberrant promoter methylation appears to be an important step in respiratory carcinogenesis after exposure to tobacco smoke and radon progeny. The determinants of aberrant promoter methylation are not well characterized. Polymorphic variants of genes of which the products are involved in pathways that modulate and repair DNA damage after carcinogen exposure may affect the occurrence of de novo promoter methylation. On the basis of their associations with risk of lung cancer, we hypothesized that functional polymorphic variants of the NADPH quinone oxidoreductase, glutathione S-transferase P1 and M1, myeloperoxidase, and XRCC1 genes are associated with p16 and/or MGMT promoter methylation in sputum from cancer-free subjects at high risk for developing lung cancer. This hypothesis was tested by conducting a cross-sectional study of 70 former uranium miners from the Uranium Epidemiological Study cohort who were at high risk for lung cancer. The polymorphic variant genotypes were characterized through PCR-RFLP on DNA isolated from peripheral lymphocytes, and the methylation status of the p16 and MGMT promoters was determined by methylation-specific PCR on DNA isolated from sputum. Subjects who had at least one GSTP1 polymorphic allele (A-to-G at bp 104) had an increased risk for MGMT methylation (odds ratio [OR], 4.8; 95% confidence interval [CI], 1.2–18.6) or for either p16 or MGMT methylation (OR, 4.4; 95% CI, 1.3–14.2). Lack of a wild-type NADPH quinone oxidoreductase allele (C at bp 609) was also associated with methylation of either p16 or MGMT (OR, 3.1; 95% CI, 1.0–9.2). These results provide the first link between germ-line functional deficits in pathways that modulate and repair DNA damage after carcinogen exposure and the development of aberrant promoter methylation of the p16 and MGMT genes in the respiratory epithelium of individuals at high risk for lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related death for both men and women in the United States. A substantial body of scientific evidence shows that inhalation of tobacco smoke and/or radon progeny is the leading cause of lung cancer in the United States (1, 2). Although the decreasing mortality rates from lung cancer in men indicate that a substantial population of exposed individuals will remain at high risk for lung cancer for the near future. Furthermore, exposure to residential radon progeny is common, and a large population is at elevated risk of lung cancer from lifelong exposure. Improved secondary preventive methods, including early detection and risk-reduction strategies such as chemoprevention are urgently needed. The development of a molecular-based marker approach would greatly advance the early detection of lung cancer.

Emerging in the field of cancer biology is the recognition that epigenetically mediated gene silencing through promoter hypermethylation is a common event critical to the initiation and progression of cancer (1). Inactivation of the p16\(^{\text{INK4a}}\) tumor suppressor gene and MGMT DNA repair gene by aberrant promoter methylation occurs frequently in non-small cell lung cancer. A recent study by our group has demonstrated that methylation of these genes can also be detected in DNA from sputum in 100% of squamous cell carcinomas up to 3 years before clinical diagnosis (2). These methylation changes can also be detected in cancer-free smokers and former uranium miners exposed to radon progeny at a prevalence that approximates lifetime risk for lung cancer (3). Progress in developing aberrant gene methylation as a molecular marker system for lung cancer risk would be facilitated by the identification of the determinants for methylation. Epidemiological studies continue to support the premise that genetic variation in individual response to carcinogens in tobacco smoke is important for defining inherent susceptibility to this disease (4). Polygenic variants of genes of which the products are involved in pathways that modulate and repair DNA damage after carcinogen exposure may affect the occurrence of de novo promoter methylation. On the basis of their associations with risk for lung cancer, we hypothesized that functional polymorphic variants of the NQO1, GSTP1, GSTM1, MPO, and XRCC1 genes are associated with p16 and/or MGMT promoter methylation in sputum from cancer-free subjects at high risk for developing lung cancer. This hypothesis was tested by conducting a cross-sectional genetic epidemiological study of 70 former uranium miners from the UES cohort who were at high risk for lung cancer.

Materials and Methods

Study Population. The study population consisted of 70 former underground uranium miners who were recruited for a cancer surveillance study from the most highly exposed miners in the UES cohort of 3469 Grants, New Mexico, Mineral Belt miners established by Samet et al. (5). Cumulative exposure to radon progeny in WLMs was estimated as part of the UES (6, 7).

\(^1\) The abbreviations used are: p16, p16\(^{\text{INK4a}}\); MGMT, O\(^6\)-methylguanine-DNA methyltransferase; MSP, methylation-specific PCR; MPO, myeloperoxidase; NQO1, NADPH quinone oxidoreductase; GSTP1, glutathione S-transferase P1; GSTM1, glutathione S-transferase M1; GST, glutathione S-transferase; UES, Uranium Epidemiological Study; WLM, working level month; OR, odds ratio; CI, confidence interval.

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The smoking histories were collected from interviews between 1993 and 1995 from mining company records, and from records of New Mexico uranium miners who had mining-related physical examinations during 1957–1976 (7). Selection of participants was based on cumulative exposure to radon through uranium mining or a combined history of tobacco smoking and uranium mining. All of the patients gave written informed consent for this study.

**Human Tissue Samples.** Induced sputum and venous blood samples were collected during clinic visits. Sputum was induced by nebulized normal saline solution. Expectorated sputum was collected and stored in Saccamanno’s solution until DNA was extracted.

**Nucleic Acid Isolation.** Leukocytes from venous blood samples were isolated by standard procedures. DNA was then extracted from both sputum and leukocyte samples by digestion with Pronase in 1% SDS followed by standard phenol-chloroform extraction and ethanol precipitation. DNA was stored at -4°C until additional analysis.

**MSP.** The methylation status of the p16 and MGMT gene promoters in sputum samples was determined by a nested, two-stage MSP assay (2). Genomic DNA isolated from sputum was modified by treatment with sodium bisulfite, which converts only unmethylated cytosines to uracil. PCR primers specific to both methylated and unmethylated template were used. The conditions for the nested, two-stage PCR approach have been described (2). Products were visualized on 2% agarose gels. Normal human tissue collected at autopsy of never-smokers, and cell lines positive for p16 (Calu6) and MGMT (SkuLu1) methylation served as negative and positive controls, respectively. The two-stage MSP assay requires only nanogram quantities of DNA and can detect methylated alleles in the presence of unmethylated alleles (as is the case with sputum) at a sensitivity of 1 in >50,000 copies.

**Polymorphism Assays.** The presence of polymorphic variants of the NQO1, GSTP1, XRCC1, and MPO genes was determined by PCR-RFLP analysis of genomic DNA isolated from leukocytes. Briefly, a region surrounding the polymorphic site of each gene was generated through PCR amplification. Primer sequences for each polymorphism have been described (8–12). Each PCR was performed with ~200 ng of genomic DNA in a 50-μl reaction volume using Taq Gold polymerase (Perkin-Elmer). Each polymorphism either destroys or creates a specific restriction site within its amplification fragment. Subsequent digestion of the PCR products with appropriate restriction enzymes specific for each variant yields distinct banding patterns that correspond either to the wild-type or polymorphic allele of each gene.

**PCR conditions for analysis of the MPO gene are as follows:** 95°C for 11.5 min, denaturation at 95°C for 60 s, anneal at 58°C for 60 s, and extension at 72°C for 60 s for 40 cycles, followed by a 5-min final extension. The 176-bp PCR product was then digested with AscI. The presence of the polymorphic variant (G to A at position 463 in the promoter region of the MPO gene) destroys an AscI restriction site. An invariant AscI restriction site exists within the amplification fragment. The three possible genotypes are defined by the following banding patterns: G/G (169-, 120-, and 61-bp fragments), A/G (289-, 169-, and 61-bp fragments), and G/A (211-, 120-, and 61-bp fragments).

**Product was then digested with** restriction enzymes specific for each variant yields distinct banding patterns that correspond either to the wild-type or polymorphic allele of each gene.

**Conditions for analysis of GSTP1 were as follows:** 95°C for 11.5 min, denaturation at 95°C for 30 s, anneal at 55°C for 30 s, and extension at 72°C for 30 s for 40 cycles, followed by a 5-min final extension. The 176-bp PCR product was then digested with BsmAI. The presence of the polymorphic variant (A to G at bp 1578 of exon 5) creates a BsmAI restriction site. The three possible genotypes are defined by the following banding patterns: A/A (uncut 176-bp fragment), A/G (176-, 91-, and 85-bp fragments), and G/G (91- and 85-bp fragments).

**Because the GSTM1 genotype is either wild type or null, a multiplex PCR was performed to amplify the GSTM1 and β-IFN genes (positive control) as described (10). The PCR amplification of GSTM1 was as follows: 95°C for 10 min, denaturation at 95°C for 1 min, anneal at 60°C for 1 min, and extension at 72°C for 1 min for 40 cycles, followed by a 5-min final extension.**

**The PCR amplification of NQO1 was as follows:** 95°C for 11.5 min, denaturation at 95°C for 30 s, anneal at 56°C for 30 s, and extension at 72°C for 30 s for 40 cycles, followed by a 5-min final extension. The 211-bp PCR product was then digested with HinfI. The presence of the polymorphic variant (C to T at bp 609 of exon 6) creates a HinfI restriction site. The three possible genotypes are defined by the following banding patterns: C/C (uncut 211-bp fragment), C/T (121-, 181-, and 30-bp fragments), and T/T (181- and 30-bp fragments).

**The PCR conditions for amplification of the XRCC1 gene was as follows:** 95°C for 11.5 min, denaturation at 95°C for 30 s, anneal at 62°C for 30 s, and extension at 72°C for 30 s for 40 cycles, followed by a 5-min final extension. The Arg allele at codon 399 contains aMspI site that is lost on conversion to the glutamine amino acid. The 198-bp PCR product was digested overnight with 20 units of MspI (New England BioLabs Inc., Beverly, MA) at 60°C. The homozygous Gln allele produced a single 198-bp product, the homozygous Arg allele produced 145-bp and 53-bp products (the 53-bp product produced was too small to accurately resolve), and the heterozygous Arg/Gln allele produced three products of 198-bp, 145-bp, and 53 bp.

**Data Analysis.** Logistic regression models were used to assess the effect of genotype on the occurrence of methylation in sputum samples using additive and dominant models for MPO, XRCC1, GSTP1, and NQO1. The number of subjects who were homozygous for the variant alleles was insufficient to fit codominant models for alleles of these genes. The GSTM1 genotype was defined as null or present. In the additive model, the number of polymorphic variants was used as an independent variable, i.e., wild-type (0), heterozygous (1), and homozygous (2). The polymorphic variant genotypes for MPO, XRCC1, NQO1, and GSTP1 were compared with the homozygous wild-type genotype (the reference group). Data were analyzed using SAS software version 8e (13).

**Results**

**Demographics.** Demographic information and smoking histories for study subjects are presented in Table 1. The percentage of Hispanics (65.7%) was twice that of Anglo subjects (31.4%). Two subjects were Native American. All of the subjects had a history of radon exposure through uranium mining, and ~76% had a history of smoking (mean pack-years, 15). Radon exposure ranged from 4 to 608 WLMs with a mean of 161 WLMs; 75% of the subjects had >100 WLMs. The median age was 62 (range 39–83 years).

**Distribution of Polymorphisms in the MPO, XRCC1, NQO1, GSTP1, and GSTM1 Genes.** The genotype distribution of the five genes among the study population is shown in Table 2, and an example of genotyping by RFLP is depicted in Fig. 1. The MPO genotype was distributed as 51.4% wild-type (G/G), 42.9% heterozygous (G/A), and 5.7% homozygous (A/A). The frequency for the wild-type MPO risk genotype was 59.1% and 47.8% in Anglo and Hispanic subjects, respectively. Smoking status of subjects with the wild-type genotype was similar (52.9% never-smokers, 51.3% former-smokers, and 50% current-smokers, respectively).

**The XRCC1 genotype was distributed as 48.6% wild-type (A/A), 45.7% heterozygous (G/A), and 5.7% homozygous (G/G). The frequency for the wild-type genotype differed among non-Hispanic white and Hispanic subjects, 63.6% and 39.1%, respectively. The percentages of the wild-type genotype showed little variation by smoking status (47.1% never-smokers, 48.7% former-smokers, and 50% current-smokers, respectively).**

**Analysis of the NQO1 polymorphism revealed a distribution of**
Ethnicity or exposure to tobacco smoke or radon progeny did not modify the relationships between the genotypes and promoter methylation in the sputum.

Discussion

These studies provide the first potential link between germ-line polymorphisms in genes involved in the protection of the cell from heritable DNA damage stemming from tobacco exposure and the epigenetic-mediated silencing of cancer genes by promoter hypermethylation. Although there were small ethnic differences, the overall distribution of genotypes for all five of the genes was similar to those reported previously in cancer-free subjects (11, 12, 14–18). Moreover, the additive model substantiated that the variant alleles for the *NQO1* and *GSTM1* genes were associated with methylation.

The *GSTP1* genotype was significantly associated with *MGMT* methylation alone, and this association was increased when either *p16* or *MGMT* methylation was present in cells in the sputum sample. Although there was not a significant association between the *NQO1* genotype and methylation of the *p16* or *MGMT* promoters individually, the presence of *p16* or *MGMT* methylation was significantly associated. It is plausible that reduced defenses for oxidant damage from radiation and tobacco smoke exposure as a result of variant *NQO1* and *GSTM1* enzymes may work in concert to produce DNA damage severe enough to affect the fidelity for maintenance of CpGs in the *p16* and *MGMT* promoters.

The GSTs form a superfamily of four distinct genes important for defense against oxidative damage to DNA (14, 19). These cytosolic enzymes play an important role in protecting DNA against damage because of adduct formation through the detoxification of reactive oxygen species and electrophiles formed during carcinogen metabolism and absorption of radiation. The major GST protein in the human lung is GSTP1. A polymorphic site at codon 105 of the P1 gene (an A-to-G substitution) leads to an isoleucine-to-valine substitution in the hydrophobic binding region of the protein. This substitution alters the catalytic properties of the enzyme and is associated with a significantly higher level of hydrophobic DNA adducts (12). Lung cancer

Table 2. Distribution of the wild-type and variant alleles of the MPO, XRCC1, NQO1, GSTP1, and GSTM1 polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MPO (G463A)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>36</td>
<td>51.4</td>
</tr>
<tr>
<td>G/A</td>
<td>30</td>
<td>42.9</td>
</tr>
<tr>
<td>A/A</td>
<td>4</td>
<td>5.7</td>
</tr>
<tr>
<td><strong>XRCC1 (A339G)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>34</td>
<td>48.6</td>
</tr>
<tr>
<td>G/G</td>
<td>32</td>
<td>45.7</td>
</tr>
<tr>
<td><strong>NQO1 (C to T)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>36</td>
<td>51.4</td>
</tr>
<tr>
<td>C/T</td>
<td>29</td>
<td>41.4</td>
</tr>
<tr>
<td>T/T</td>
<td>5</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>GSTP1 (A105G)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>25</td>
<td>35.7</td>
</tr>
<tr>
<td>A/G</td>
<td>37</td>
<td>52.9</td>
</tr>
<tr>
<td>G/G</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>GSTM1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Null</td>
<td>36</td>
<td>48.6</td>
</tr>
<tr>
<td>Present</td>
<td>34</td>
<td>51.4</td>
</tr>
</tbody>
</table>

51.4% wild-type (C/C), 41.4% heterozygous (C/T), and 7.1% homozygous (T/T). The frequency of the wild-type *NQO1* genotype among non-Hispanic white subjects was 68.2%, whereas that in Hispanic subjects was 41.3%. The distribution of homozygous wild-type *NQO1* genotype varied by smoking status (58.8% of never-smokers, 56.4% of former-smokers, and 28.6% of current-smokers, respectively).

The distribution of the three *GSTP1* genotypes was 35.7% wild-type (Arg/Arg), 52.9% heterozygous (Arg/Gly), and 11.4% homozygous (Gly/Gly). The frequencies of the wild-type *GSTP1* genotype among non-Hispanic white and Hispanic subjects were 36.4% and 32.6%, respectively. The homozygous wild-type genotype occurred in 35.3% of never-smokers, 43.6% of former-smokers, and 14.3% of current-smokers.

Lastly, the distribution of the *GSTM1* genotype was 48.6% null and 51.4% present. The frequencies of the present genotype among non-Hispanic white and Hispanic subjects were 63.6% and 41.3%, respectively. The percentages of the *GSTM1* genotype were 58.8% of never-smokers, 41.3% of former-smokers, and 57.1% of current-smokers, respectively.

Methylation of *p16* and *MGMT* in Sputum. The frequency of *p16* and *MGMT* promoter gene methylation was determined previously in sputum samples (2). Abnormal *p16* and *MGMT* gene methylation was present in sputum from 10 (14.3%) and 21 (30.0%) of 70 subjects, respectively. The frequency of methylation of these two genes was similar in tobacco-plus-radon versus radon exposure alone. Methylation of either *p16* or *MGMT* was detected in 40% of subjects, and of *p16* and *MGMT* in 3 subjects. Methylation among ethnic groups was not statistically different.

Relationship between Genetic Polymorphism and Gene-specific Promoter Methylation. Polymorphic variants of *GSTP1* and *NQO1* were significantly associated with *p16* and/or *MGMT* promoter methylation (Table 3). Interestingly, although there was not a significant association between the variant *NQO1* genotype and methylation of the *p16* or *MGMT* promoters individually, the presence of *p16* or *MGMT* was significantly associated (OR, 3.1; 95% CI, 1.0–9.2). The absence of one or both wild-type alleles for the *GSTP1* gene was significantly associated with *MGMT* methylation alone (OR, 4.8; 95% CI, 1.2–18.6) and for either *p16* or *MGMT* methylation in sputum (OR, 4.4; 95% CI, 1.3–14.2). These significant associations were still observed in the additive model as well (Table 3). No association was apparent among the *MPO*, *XRCC1*, or *GSTM1* polymorphisms and *p16* or *MGMT* promoter methylation. Although ORs were adjusted for ethnicity, no evidence of confounding was found in our data set.

Fig. 1. Ethidium bromide gels show the possible genotypes from the polymorphisms of the *MPO* (G-to-A), *GSTP1* (A-to-G), and *NQO1* (C-to-T) genes. A, *MPO* gene, the 350-bp amplification product contains an invariant *AscI* restriction site yielding a constant 61-bp fragment seen in all lanes. Individuals homozygous for the A allele have no additional recognition sites and show only two bands at 289- and 61-bp. The G allele creates an additional restriction site such that homozygous G individuals have three bands at 169, 120, and 61-bp. Heterozygotes show all four bands. B, *GSTP1* gene, as a result of the A-to-G polymorphism, a *BsmAI* restriction site is created. Homozygous A individuals show an uncut 176-bp fragment, whereas homozygous G individuals have two bands at 91- and 85-bp. Heterozygotes show all three bands. C, *NQO1* gene, as a result of the C-to-T polymorphism, a *HinfI* restriction site is created. The homozygous C individuals show an uncut 211-bp fragment, whereas homozygous T individuals show a 181-bp fragment (the resulting 30-bp fragment is not shown on the gel photo). Heterozygotes show the 211- and 181-bp fragments (again, with the 30-bp fragment not shown).
factor leading to regional disruption of normal chromatin structure. This change in genomic integrity could lead to inappropriate methylation by the cytosine DNA methyltransferases of CpG sites in the normally protected promoter region of genes such as p16 and MGMT.

A limitation of our study was the low power to detect ORs <2.5. Population stratification may be a source of bias in multiethnic populations. We adjusted for ethnicity but did not have information to adjust for admixture within ethnic groups. Furthermore, ethnic differences in genetic effects could not be adequately assessed. In the present study, all of the subjects had substantial radon progeny exposure through uranium mining. The frequency of methylation of the p16 and MGMT genes was similar in tobacco-plus-radon versus radon exposure alone. Additional investigations with an expanded study population will more precisely define the influence of these and other germ-line polymorphisms working together or alone on the propensity to acquire multiple epigenetic changes in sputum. This information could greatly aid in the ultimate development of a genetic and epigenetic risk model for lung cancer.

References


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