Glutathione S-Transferase P1 and NADPH Quinone Oxidoreductase Polymorphisms Are Associated with Abrerrant Promoter Methylation of $p16^{INK4a}$ and $O^{6}$-Methylguanine-DNA Methyltransferase in Sputum

Frank D. Gilliland, Heidi J. Harms, Richard E. Crowell, Yu-Fen Li, Randy Willink, and Steven A. Belinsky

Abstract

Inactivation of the $p16^{INK4a}$ 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-transferases 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 determined by methylation-specific PCR on DNA isolated from sputum. Subjects who had at most 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 protect the cell from tobacco- and radon-induced DNA damage, 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 $p16^{INK4a}$ 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). 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 these 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).

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3 The abbreviations used are: $p16$, $p16^{INK4a}$; 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.
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 Pnaase 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).

**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, denature 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 350-bp PCR product was then digested with \( AciI \). The presence of the polymorphic variant \( (G \to A \text{ at position } 609\text{ of exon } 6) \text{ creates a } AciI \text{ restriction site. The three possible genotypes are defined by the following banding patterns.} \]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Banding Patterns</th>
</tr>
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<tbody>
<tr>
<td>A/A</td>
<td>350-bp fragment</td>
</tr>
<tr>
<td>G/A</td>
<td>211-bp and 145-bp fragments</td>
</tr>
<tr>
<td>G/G</td>
<td>145-bp fragment</td>
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</tbody>
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**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).

**Analysis of the \( NQO1 \) polymorphism revealed a distribution of...**
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. 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 GSTP1 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 GSTP1 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...
patients have also been shown to have a higher frequency of the GG phenotype than controls (12). GSTM1 is involved in Phase II detoxification of activated xenobiotic metabolites and has been weakly associated with lung cancer risk (15, 16). A substantial portion of the population has a null genotype for GSTM1. Our finding of more methylation associated with the G allele of GSTP1 but not the GSTM1 null genotype is consistent with high expression of GSTP1 in the lung and the protective role of wild-type GSTP1 in lung cancer risk.

NQO1 is a cytosolic enzyme that carries out the two-electron reduction of a variety of quinone substrates present in the environment as combustion by-products, including that of automobile exhaust and cigarette smoke, and endogenously as antioxidants (17, 18). In addition, nitroaromatic compounds, heterocyclic amines, and cigarette smoke condensate are metabolized by NQO1. However, NQO1 is also involved in antioxidant defenses through metabolism of quinone antioxidants. A polymorphic variant of the gene (a C-to-T transition at bp 609 of exon 6) is associated with a ~3-fold decrease in NQO1 activity; however, the association of the wild-type allele with lung cancer risk has been inconsistent (9, 20, 21). Interestingly, Lafuente et al. (22) found an association between the NQO1 polymorphic variant and an increased risk of colon cancer. In addition, the polymorphic variant genotype was associated with colon tumors that exhibited mutation in the KRAS gene. The mutated ras gene functions as an oncogene, leading to a prolonged mitogenic signal in affected cells. Consistent with this study, we found that the variant NQO1 allele confers an increased risk of p16 suppression through hypermethylation. However, studies suggest that, depending on the substrate, the wild-type NQO1 allele may confer either an increased or decreased risk for various malignancies. Our results support the hypothesis that NQO1 is an important enzyme involved in modulating lung cancer risk, but the effects of the variant allele may depend on specific exposures.

The connection between DNA methylation and gene expression has been known for almost 20 years. The predominant consequence of gene promoter methylation is transcriptional repression, which can be mediated either directly, by blocking the binding of transcription factors, or indirectly by proteins that specifically bind to methylated DNA (23–25). However, the genetic or environmental factors that underlie the targeting of specific gene promoters for hypermethylation are unknown. DNA damage by tobacco carcinogens could be one 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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