Genetic Determinants for Promoter Hypermethylation in the Lungs of Smokers: A Candidate Gene-Based Study

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Abstract

The detection of tumor suppressor gene promoter methylation in sputum-derived exfoliated cells predicts early lung cancer. Here, we identified genetic determinants for this epigenetic process and examined their biologic effects on gene regulation. A two-stage approach involving discovery and replication was used to assess the association between promoter hypermethylation of a 12-gene panel and common variation in 40 genes involved in carcinogen metabolism, regulation of methylation, and DNA damage response in members of the Lovelace Smokers Cohort (N = 1,434). Molecular validation of three identified variants was conducted using primary bronchial epithelial cells. Association of study-wide significance (P < 8.2 × 10−5) was identified for rs1641511, rs3730859, and rs1883264 in TP53, LIG1, and BIK, respectively. These single-nucleotide polymorphisms (SNP) were significantly associated with altered expression of the corresponding genes in primary bronchial epithelial cells. In addition, rs3730859 in LIG1 was also moderately associated with increased risk for lung cancer among Caucasian smokers. Together, our findings suggest that genetic variation in DNA replication and apoptosis pathways impacts the propensity for gene promoter hypermethylation in the aerodigestive tract of smokers. The incorporation of genetic biomarkers for gene promoter hypermethylation with clinical and somatic markers may improve risk assessment models for lung cancer. Cancer Res; 72(3); 707–15. ©2011 AACR.

Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women in the United States and occurs largely from chronic exposure to tobacco carcinogens (1). The development of this disease for more than 30 to 40 years involves field carcinization, characterized as the acquisition of genetic and epigenetic changes in oncogenes and tumor suppressor genes (TSG) throughout the airway epithelium (2–4). The silencing of TSGs through promoter methylation is now recognized as a major and causal epigenetic event that occurs during lung cancer initiation and progression (4). Genes involved in all aspects of normal cell function that include cell-cycle regulation, differentiation, adhesion, and death are silenced by promoter methylation in lung tumors (4). On the basis of the silencing of key TSGs in the lungs of smokers, we hypothesized that the detection of promoter methylation of TSGs in exfoliated cells in sputum would provide an assessment of the extent of field carcinization that in turn may predict early lung cancer. This hypothesis has been validated in several studies (5–7), suggesting that gene methylation in sputum could be used as a molecular marker for identifying people at high risk for cancer incidence.

The fact that promoter methylation of TSGs is a promising marker for early detection of lung cancer makes understanding of factors that influence the individual susceptibility for this epigenetic process throughout the respiratory epithelium a high priority. The precise mechanism by which carcinogens disrupt the capacity of cells to maintain the epigenetic code during DNA replication and repair is largely unknown. Carcinogens within tobacco induce single- and double-strand DNA breaks which, if left unrepaird or if misrepaired, lead to mutagenic and/or carcinogenic changes in cells (8). Accumulating evidence from our group and others suggests that extensive DNA damage could also be responsible for acquisition of promoter methylation of TSGs during lung carcinogenesis. Support for this supposition was provided through several recent studies from our group (9–12). A highly significant association was observed between DNA repair capacity...
(DRC) for double-strand breaks measured in lymphocytes and the propensity for gene methylation detected in sputum from cancer-free smokers from the Lovelace Smokers Cohort (LSC; ref. 9). A subsequent study in the same cohort identified the dietary factors, including folate, leafy green vegetables, andmultivitamin use, as protective against the acquisition of gene methylation possibly through the modulation of DRC and/or the reduction of DNA damage induced by tobacco-derived carcinogens due to their antioxidant effect (10). Chronic exposure of human bronchial epithelial cells to noncytotoxic levels of tobacco-derived carcinogens induced de novo methylation of TSGs, epitelial-to-mesenchymal transition, and eventually transformation (11). Cuozzo and colleagues (12) provide a mechanistic link between DNA damage and methylation by showing activation of homologous recombination following introduction of a double-strand break and subsequent methylation of the recombinant gene. Together, these studies suggest that chronic DNA damage and reduced DRC could be important determinants for inducing gene methylation.

Several sequence patterns within gene promoters that contain CpG islands and embryonic targets of polycomb-repressive complex 2 are predictive for gene predisposition for methylation in cancer but cannot discriminate the interindividual susceptibility for gene silencing (13–17). Sequence variants in promoters associated with reduced gene transcription lead to allele-specific methylation (ASM) and silencing in glutathione S-transferase pi (GSTP1) and O6-methylguanine-DNA methyltransferase (MGMT) in tumors and premalignant tissues (18, 19). Mechanisms independent of effects on gene transcription were also identified for ASM of the reversion-induced LIM gene (20). Several studies conducting chromosome-wide or genome-wide surveys on nonimprinted, autosomal regions in human lymphocytes suggest that the majority of TSGs are not silenced by sequence variant-dependent ASM (21, 22). On the basis of the likelihood that DNA damage induced by tobacco carcinogens is an important step in the acquisition of de novo methylation and that reduced carcinogen detoxification and DRC have been associated with lung cancer (9–12, 23), we tested the hypothesis that genetic variation in some genes involved in these pathways are associated with susceptibility for smokers to acquire gene-specific promoter methylation detected in sputum that contains exfoliated lung cells. A 2-stage approach involving discovery and replication was used to assess the association between promoter methylation of a 12-gene panel in members of the LSC and common variation in 40 genes involved in carcinogen metabolism, regulation of methylation, and DNA damage response, the latter including DNA damage repair, cell-cycle regulation, and apoptosis. Molecular validation of significant variants was conducted using primary bronchial epithelial cell cultures.

Materials and Methods

Study cohort and sample collection

The LSC was established in 2001 to conduct longitudinal studies on molecular markers of respiratory carcinogenesis in biologic fluids such as sputum from people at risk for lung cancer (9). The enrollment initially focused on female smokers and was expanded to include male smokers in 2004. Enrollment was restricted to current and former smokers aged 40 to 74 years with a minimum of 20 pack-years of smoking. Detailed information regarding sample collection is described in Supplementary Materials and Methods. All participants signed a consent form, and the Western Institutional Review Board approved this project.

Methylation of a 12-gene panel was successfully assessed in cytologic adequate sputum samples from 1,434 cohort members who are either Caucasian or Hispanic and for whom the genotyping call rate was 75% or more. Members with other ethnicities were not included in this study because of their low representation in the LSC (overall <6%). Cohort members were split into 2 populations for the discovery ($n = 713$) and replication ($n = 721$) based on their methylation index and several nongenetic risk factors for gene methylation including gender, ethnicity, current smoking status, and age at enrollment (10). The demographic characteristics for the cohort members in the discovery and replication stages are shown in Table 1.

Sputum processing and gene promoter methylation

A detailed procedure for sputum collection and processing has been described (10). Briefly, sputum samples were stored in Saccomanno’s fixative. Sputum adequacy, defined as the

<table>
<thead>
<tr>
<th>Variables</th>
<th>Discovery ($N = 713$)</th>
<th>Replication ($N = 721$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y, mean ± SD)</td>
<td>56.5 ± 9.5</td>
<td>56.4 ± 9.6</td>
<td>0.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender (male %)</td>
<td>20.9</td>
<td>21.4</td>
<td>0.83&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethnicity (Hispanics %)</td>
<td>19.1</td>
<td>19.4</td>
<td>0.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>58.2</td>
<td>58.1</td>
<td>0.97&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pack-years (mean ± SD)</td>
<td>40.4 ± 20.7</td>
<td>38.7 ± 19.5</td>
<td>0.14&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>5–28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>30.6</td>
<td>33.0</td>
<td>0.35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>28–40</td>
<td>30.3</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>40–166</td>
<td>39.1</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>Methylation index (median, min-max)</td>
<td>2 (0–10)</td>
<td>2 (0–10)</td>
<td>0.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The overall cohort was split into the discovery and replication groups by a method that kept the 2 groups balanced with respect to all of the variables included in this table.

<sup>b</sup>Two-sided 2-sample $t$ test between discovery and replication.

<sup>c</sup>$x^2$ test for differences between discovery and replication.

<sup>d</sup>Two-sided Wilcoxon rank-sum test between discovery and replication.

<sup>e</sup>Moderate smokers ($n = 108$) with pack-years less than 20 were included in the data analysis. The average pack-years for the 108 moderate smokers are 16.6 ± 3.3.
presence of deep lung macrophages or Curschmann’s spiral (7), was assessed by a pathologist. Twelve genes were selected for analysis of methylation in sputum based on our previous studies establishing their association with risk for lung cancer and their specificity to methylation in epithelial cells (5–7). These 12 genes include p16, MGMT, DAPK, RASSF1A, PAX5a, PAX5b, GATA4, GATA5, SULF2, PCDH20, DAI1, and JPH3. Our ongoing study to identify the best gene panel for early lung cancer detection has screened more than 40 genes and suggests that a panel of approximately 12 genes (inclusive of those described above) may provide the best sensitivity and specificity with additional genes not conferring additional risk (Leng, unpublished data). Genomic DNA isolated from the sputum samples was bisulfitenmodified. Given the low percentage (<3%) of lung epithelial cells in sputum samples that also varied significantly between individuals, a 2-stage nested MSP was used to detect methylated alleles (5–7). Our assay can reproducibly detect 1 methylated allele in a background of 20,000 unmethylated alleles (4). Genomic DNA isolated from cell lines that were obtained from and authenticated by the American Type Culture Collection with methylated or unmethylated genes from the 12-gene panel was included in each batch of the MSP assays for quality control. Experiments were carried out in cell lines passes for a maximum of 6-month postresuscitation.

Candidate gene and tag SNP selection
Genes (n = 40) were selected on the basis of the literature-derived citations establishing the relevance of genes to the regulation of gene methylation, lung cancer, and pulmonary function (Supplementary Table S1, available online). Tag SNPs (n = 718) were selected using pairwise r² algorithm based on the phase 2 HapMap CEU database, linkage disequilibrium (LD) database for DNA repair and carcinogen metabolism genes from the University of Southern California, Los Angeles, CA (ref. 24; Gilliland, unpublished data), or the LD database for methylation regulatory and apoptotic genes from LRRI (Leng, unpublished data) for Caucasian and Hispanic populations. Fifty ancestry informative markers (AIM) were chosen to assess ancestry admixture (25). The description of the 2 LD databases, strategy for tag SNP selection, efficiency of selected single-nucleotide polymorphisms (SNP) for tagging unmeasured SNPs, and the selection of AIMS are described in detail in Supplementary Materials and Methods.

SNP genotyping and quality control
A 768-plex oligo pool assay was designed to genotype lymphocyte DNA from the LSC using the Illuma GoldenGate Technology. Detailed information about genotyping and quality control is provided in Supplementary Materials and Methods.

Real-time PCR for measuring gene expression in cell lines
Normal human bronchial epithelial cells (NHBEC) that were obtained by bronchoscopy from current or former smokers (n = 58) were used in this study. Expression of genes was assessed in cells at passage 1 or 2. Cells were harvested in TRI reagent (Sigma-Aldrich) at approximately 80% confluence and mRNA was isolated following TRI reagent instructions. TaqMan real-time PCR was conducted to measure the expression of candidate genes in cDNA using the ΔCt method with both proliferating cell nuclear antigen (PCNA) and β-actin as the endogenous controls. PCNA and β-actin were selected as the endogenous controls in NHBECs because they show minimal variation in expression across the NHBEC samples and are highly correlated (Pearson correlation coefficient = 0.83, P < 0.0001).

Ascertainment of population admixture
Population admixture was analyzed using the model-based, Bayesian Markov Monte Carlo algorithm implemented in STRUCTURE 2.3.3 (26). A detailed description of ascertaining population admixture in the LSC members is described in the Supplementary Materials and Methods and in reference 27.

Statistical analysis
Genetic association analysis was first conducted for all 600 SNPs that successfully passed the quality check in the 713 members in the discovery stage (Supplementary Table S2, available online). Only SNPs associated with methylation index with the values of P ≤ 0.10 in the discovery stage were then analyzed in the replication stage (n = 721). SNPs had to pass the replication stage with P < 0.05. The SNPs that satisfied the criteria for both the discovery and replication stages also were analyzed with the combined data set. A Bonferroni adjustment over all SNPs included in the analysis (n = 600) was used (P = 8.2 × 10⁻5).

The methylation index based on a 12-gene panel is a discrete count variable defined as the number of genes in the panel that are methylated and ranges from 0 to 10 in the LSC (Fig. 1). Poisson regression was conducted to examine the association between the independent variables, the SNPs/haplotypes, and the outcome variable, the methylation index, with adjustment for covariates selected a priori that include age, gender, ethnicity, current smoking status, and pack-years. The additive model was tested for each SNP, and common homozygotes, heterozygotes, and rare homozygotes were coded as 0, 1, and 2, respectively. The Poisson regression model is a generalized linear model that can be used to estimate the mean frequency ratio (FR) and its 95% confidence interval (CI). The FR was
calculated from the regression coefficient for an explanatory variable in the Poisson regression model, β, through the equation \( FR = e^β \). An FR value more than 1 suggests that a larger value of a covariate is associated with a higher methylation index, whereas an FR value less than 1 suggests that a larger value of a covariate is associated with a lower methylation index.

The PHASE program was used to reconstruct the haplotypes and to calculate their estimated probabilities from the haplotype tagging SNPs (htSNP) data in a block in Caucasians and Hispanics separately (28, 29). The probabilities of the common haplotypes for each individual were used as explanatory variables in the Poisson regression model with adjustment for nongenetic factors to assess the association between the haplotypes and methylation index. Haplotypes with frequency less than 5% were combined into 1 group.

The genotype-expression correlation in NHBECS was examined for the SNPs associated with the methylation index. The expression data were presented by setting the level of gene expression with wild homozygote genotypes at 100%. The expression level with heterozygote and variant homozygote was calculated as the ratio relative to that of the wild homozygote. The genotype-expression correlation was then analyzed using logarithm-transformed values of the relative quantification of gene expression because this transformation satisfies the normality and homoscedasticity assumption. Logarithm-transformed gene expression with high minor allelic frequency (MAF) was compared across formation satis assumption. Logarithm-trans formed gene expression with 100%. The expression level with heterozygote and variant

Results

Association between individual SNP and methylation index

Individual SNP association analysis identified 109 SNPs associated with the methylation index with \( P < 0.10 \) in the discovery stage (Table 2; not shown). These 109 SNPs were then analyzed in the replication stage. Eleven SNPs in LIG1, TP53, BIK, BMF, and BAK1 were associated with methylation index with \( P < 0.05 \) in the replication stage (Table 2). Seven of these 11 SNPs were tag SNPs in LIG1. Combined analysis showed that the overall \( P \) value for 3 SNPs including rs1641511, rs3730859, and rs1883264 reached study-wide significance (Bonferroni corrected \( P = 8.2 \times 10^{-3} \)). All other SNPs genotyped in the replication stage failed to replicate the associations observed in the discovery stage at the significance level of 0.05. Relaxing the \( P \) value from 0.10 to 0.15 for identifying SNPs to be carried over into the replication stage did not identify any additional SNPs reaching study-wide significance.

Association between haplotype alleles in LIG1 and methylation index

Because multiple tag SNPs were associated with methylation index in LIG1, a haplotype-based analysis was conducted to integrate the genetic variation in each individual genomic locus. LIG1 resides in an 84-kb haplotype block (chr19: 53296285–53381205, NCBI B36) that contains 3 genes: PL2G4C, LIG1, and LOC374920 (Supplementary Fig. S1A, available online). Six htSNP including rs11564609, rs251692, rs3730859, rs3730895, rs4802436, and rs972800 allowed the construction of the 4 common haplotype alleles with minimal \( R^2 \) over 0.82 in both Caucasians and Hispanics (24). These 6 htSNPs accounted for more than 84% of the haplotype variation in the LIG1 block in Caucasians and Hispanics in the LSC

| Table 2. SNPs with the most significant association with gene methylation based on a 12-gene panel in the LSCa |
|---|---|---|---|
| Genes | SNPs | Discovery (\( N = 713 \)) | | Replication (\( N = 721 \)) | | Combined analysis (\( N = 1,434 \)) |
| | FR (95% CI) | \( P \) | FR (95% CI) | \( P \) | FR (95% CI) | \( P \) |
| TP53 | rs1641511 | 1.09 (1.01–1.18) | 2.8 \times 10^{-2} | 1.17 (1.08–1.27) | 1.3 \times 10^{-4} | 1.13 (1.07–1.19) | 1.8 \times 10^{-3} |
| LIG1 | rs3730859 | 1.10 (1.02–1.18) | 1.2 \times 10^{-2} | 1.14 (1.06–1.22) | 4.9 \times 10^{-4} | 1.11 (1.06–1.17) | 3.0 \times 10^{-5} |
| BIK | rs1883264 | 0.91 (0.82–1.00) | 5.7 \times 10^{-2} | 0.92 (0.74–0.91) | 1.3 \times 10^{-3} | 0.96 (0.80–0.93) | 5.4 \times 10^{-5} |
| LIG1 | rs72458512 | 0.92 (0.85–1.01) | 7.1 \times 10^{-2} | 0.96 (0.78–0.94) | 1.3 \times 10^{-3} | 0.89 (0.84–0.95) | 5.6 \times 10^{-4} |
| LIG1 | rs436943 | 0.88 (0.80–0.97) | 6.9 \times 10^{-3} | 0.90 (0.81–0.99) | 2.6 \times 10^{-2} | 0.89 (0.83–0.95) | 6.4 \times 10^{-4} |
| LIG1 | rs1556641b | 1.09 (1.02–1.17) | 1.0 \times 10^{-2} | 1.08 (1.01–1.15) | 3.5 \times 10^{-2} | 1.08 (1.03–1.14) | 1.3 \times 10^{-3} |
| BMF | rs3922335 | 1.09 (1.01–1.17) | 2.7 \times 10^{-2} | 1.08 (1.01–1.17) | 3.2 \times 10^{-2} | 1.09 (1.03–1.14) | 2.2 \times 10^{-3} |
| LIG1 | rs1556640 | 1.06 (0.99–1.14) | 9.9 \times 10^{-2} | 1.11 (1.03–1.20) | 6.2 \times 10^{-3} | 1.08 (1.03–1.14) | 3.5 \times 10^{-3} |
| LIG1 | rs1556645 | 1.08 (1.01–1.16) | 3.0 \times 10^{-2} | 1.08 (1.01–1.16) | 3.1 \times 10^{-2} | 1.08 (1.02–1.13) | 3.8 \times 10^{-3} |
| BAK1 | rs210134 | 1.065 (0.99–1.15) | 9.7 \times 10^{-2} | 1.09 (1.02–1.17) | 1.6 \times 10^{-2} | 1.08 (1.02–1.13) | 5.8 \times 10^{-3} |
| LIG1 | rs4802436 | 0.93 (0.85–1.01) | 9.2 \times 10^{-2} | 0.91 (0.83–0.99) | 3.3 \times 10^{-2} | 0.92 (0.86–0.98) | 8.9 \times 10^{-3} |

a From Poisson regression models with adjustment for age, gender, ethnicity, current smoking status, and pack-years.
b rs1556641 is a redundant tag SNP for rs156645 \( \chi^2 = 1 \).
The likelihood ratio global tests of association indicate that the genetic variation in the haplotype block where LIG1 resides as a locus is strongly associated with methylation index in the entire LSC ($P = 0.00029$; not shown). Hap1 strongly tagged by rs3730859 is the only risk haplotype allele associated with increased methylation index compared with all other haplotype alleles ($FR = 1.09, P = 0.00036$; Table 3). Hap3 solely tagged by rs4802436 was associated with a reduction of methylation index compared with all other haplotype alleles ($FR = 0.93, P = 0.023$; Table 3). However, when Hap2, Hap4, and others of LIG1 were used as the reference for comparison in a Poisson regression model, only Hap1 was significantly associated with increased methylation index ($P = 0.00032$; Table 3), supporting Hap1 as the most influential haplotype allele defining the global association between this locus and methylation index.

**Genotype–expression correlation for LIG1, TP53, and BIK**

Characterization of the LD for SNPs located 500 kb surrounding each of the top 3 SNPs using the 1,000 Genome Pilot1 CEU population identified 60 SNPs that have high LD ($r^2 > 0.8$) with these 3 SNPs. Interestingly, all 60 SNPs locate within the expanded regions of the 3 candidate genes (Supplementary Fig. S1B–S1D, available online), suggesting that it is unlikely that the association seen for these 3 SNPs is due to long-range LD with functional SNPs located further away from these candidate regions. The functional potential for these SNPs was assessed by searching the FuncPred: Functional SNP Prediction module of SNPinfo Web Server (30). No known nonsynonymous SNPs from the genes in the 4 candidate regions were identified to be in high LD with these SNPs. However, 13 of the 60 SNPs were predicted either to affect the binding of transcription factors or microRNAs or to locate in an exonic splicing enhancer or silencer element (Supplementary Table S3, available online). Specifically, 7 SNPs surrounding the transcription start site of LIG1 that are in high LD with rs3730859 ($r^2 > 0.93$) were predicted to affect the binding of transcription factors.

Thus, we examined whether the top 3 SNPs could *in cis* affect the expression of LIG1, TP53, and BIK in NHBECS. The expression of LIG1, TP53, and BIK was highly correlated in NHBECS with Spearman correlation coefficients from 0.60 to 0.75 ($P < 0.0001$; not shown). As a control, the expression of PLA2G4C which resides in the same haplotype block and directly upstream of LIG1 had no or weak correlation with the expression of LIG1 and the other genes (Spearman correlation coefficients from 0.16–0.42, not shown). Genotype–expression correlation analysis found strong associations between rs3730859 and LIG1, between rs1641511 and TP53, and between rs1883264 and BIK (Table 4). The most striking difference was observed for rs3730859 with the variant homozygotes associated with a 74% reduction of LIG1 expression compared with the wild homozygotes ($P = 0.002$). In addition, as expected, the genotype–expression correlation is more significant for LIG1 using PCNA as the endogenous control compared with $\beta$-actin because LIG1 expression is cell-cycle regulated (31). The genotype-expression correlation for **TP53** has comparable results between PCNA and $\beta$-actin, a gene whose expression during the cell cycle is influenced by the presence of DNA damage. BIK expression is not cell-cycle related and as such the significant genotype–expression correlation was identified using $\beta$-actin as the endogenous control. More importantly, the repressive effect of current smoking status on BIK expression (32) can only be confirmed with $\beta$-actin as the endogenous control (36% reduction in current smokers, $P = 0.048$; Leng, unpublished data), supporting the use of $\beta$-actin for the relative quantification of BIK expression. No association between SNPs and PLA2G4C was identified (not shown). These results suggest that SNPs associated with methylation index could *in cis* affect the expression of LIG1, TP53, and BIK.

**Ancestry admixture in LSC and its effect on genetic association**

Hispanics in the LSC are highly admixed between Native American ancestry and European ancestry. The component of Native American ancestry in self-reported Hispanics ranges

### Table 3. Haplotype-based association analysis in LIG1 for methylation index in the LSC ($n = 1,434$)$^a$

<table>
<thead>
<tr>
<th>Haplotype alleles</th>
<th>Frequency</th>
<th>Analysis 1$^b$</th>
<th>Analysis 2$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEU</td>
<td>HISP</td>
<td>FR (95% CI)</td>
</tr>
<tr>
<td>Hap1 (CGTGAT)</td>
<td>0.35</td>
<td>0.31</td>
<td>1.09 (1.04–1.15)</td>
</tr>
<tr>
<td>Hap2 (CACTAT)</td>
<td>0.24</td>
<td>0.30</td>
<td>1.03 (0.98–1.09)</td>
</tr>
<tr>
<td>Hap3 (CACTGT)</td>
<td>0.17</td>
<td>0.13</td>
<td>0.93 (0.87–0.99)</td>
</tr>
<tr>
<td>Hap4 (TGCGAT)</td>
<td>0.09</td>
<td>0.10</td>
<td>0.96 (0.88–1.04)</td>
</tr>
<tr>
<td>Others</td>
<td>0.15</td>
<td>0.16</td>
<td>0.90 (0.84–0.97)</td>
</tr>
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</table>

$^a$FR and 95% CI were estimated in a Poisson regression with adjustment for age, gender, ethnicity, current smoking status, and pack-years.

$^b$All other haplotype alleles were used as reference for comparison in a Poisson regression.

$^c$Hap1, Hap4, and others of LIG1 were used as reference for comparison in a Poisson regression to compare the effect of Hap1 and Hap3 in LIG1.
from 18.2% to 56.0% with an average of 33.7%. In contrast, the average component of Native American ancestry in self-reported Caucasians is less than 1.0% with only 10 subjects having Native American ancestry more than 10%. The African ancestry is very low in both Hispanics (2.1%) and Caucasians (0.3%) enrolled in the LSC. In a comparison of methods for adjusting for ancestry, no substantial differences were observed between the results obtained from Poisson regression models that included self-reported ethnicity and those observed between the results obtained from logistic regression adjusting for ancestry, no substantial differences were observed. The criteria for selecting these studies to be summarized include large samples size (>500 pairs of cases and controls), Caucasian ethnicity, pathway or genome-wide association studies, and adequate SNP coverage in LIG1. These criteria were set to minimize the confounding from the publication bias and to ensure inclusion of the sufficiently powered studies. Only association with lung cancer in moderate and heavy smokers (current or former) was presented because more than 90% of cohort members enrolled in the LSC are smokers with more than 20 pack-year smoking history. A case–control study conducted in the Pittsburgh Lung Screening Study cohort included 722 patients with lung cancer and 929 controls who were Caucasian and current or ex-cigarette smokers with more than 10 pack-year smoking history and found that subjects carrying the variant homozygote for rs10500298 that is in high LD with rs3730859 (r^2 = 0.89) in the 1 k genome project CEU population have a 96% increased risk for lung cancer (P < 0.0001; ref. 33). A second case–control study conducted in the Beta-Carotene and Retinol Efficacy Trial cohort also identified rs1566640 and rs1566641 that is in high LD with rs3730859 (r^2 = 0.89) in CEU as a strong risk factor for lung cancer in Caucasian smokers with more than 20 pack-year of smoking history (ORs = 1.20, P < 0.003, 746 cases and 1,477 controls; ref. 34). Two lung cancer genome-wide association studies (GWAS) conducted in U.S. populations were queried as well and subjects with homozygous rs1566641 have a 22% increased risk for lung cancer in subjects with more than 20 pack-year smoking history in MD Anderson GWAS study (P < 0.14, 1,004 cases

Epigenetic silencing of TSGs in lung cells is an intermediate biomarker for lung cancer. Therefore, genetic variation that is strongly associated with epigenetic silencing of TSGs should also impact the risk for lung cancer. Indeed, this premise is supported by studies that systematically assessed the association between tag SNPs in LIG1 and risk for lung cancer (33–36). The criteria for selecting these studies to be summarized include large samples size (>500 pairs of cases and controls), Caucasian ethnicity, pathway or genome-wide association studies, and adequate SNP coverage in LIG1. These criteria were set to minimize the confounding from the publication bias and to ensure inclusion of the sufficiently powered studies. Only association with lung cancer in moderate and heavy smokers (current or former) was presented because more than 90% of cohort members enrolled in the LSC are smokers with more than 20 pack-year smoking history. A case–control study conducted in the Pittsburgh Lung Screening Study cohort included 722 patients with lung cancer and 929 controls who were Caucasian and current or ex-cigarette smokers with more than 10 pack-year smoking history and found that subjects carrying the variant homozygote for rs10500298 that is in high LD with rs3730859 (r^2 = 0.89) in the 1 k genome project CEU population have a 96% increased risk for lung cancer (P < 0.0001; ref. 33). A second case–control study conducted in the Beta-Carotene and Retinol Efficacy Trial cohort also identified rs1566640 and rs1566641 that is in high LD with rs3730859 (r^2 = 0.89) in CEU as a strong risk factor for lung cancer in Caucasian smokers with more than 20 pack-year of smoking history (ORs = 1.20, P < 0.003, 746 cases and 1,477 controls; ref. 34). Two lung cancer genome-wide association studies (GWAS) conducted in U.S. populations were queried as well and subjects with homozygous rs1566641 have a 22% increased risk for lung cancer in subjects with more than 20 pack-year smoking history in MD Anderson GWAS study (P < 0.14, 1,004 cases

| Table 4. Association of genotype and gene expression
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>SNP</td>
<td>Genotype</td>
<td>n</td>
<td>Gene expression 1</td>
<td>Gene expression 2</td>
</tr>
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<td>---</td>
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<td>---</td>
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</tr>
<tr>
<td>LIG1</td>
<td>rs3730859</td>
<td>G/G</td>
<td>17</td>
<td>1.00 ± 0.86</td>
<td>1.00 ± 0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>31</td>
<td>0.70 ± 0.68</td>
<td>0.88 ± 1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>11</td>
<td>0.26 ± 0.30</td>
<td>0.43 ± 0.54</td>
</tr>
<tr>
<td>TP53</td>
<td>rs1641511</td>
<td>A/A</td>
<td>19</td>
<td>1.00 ± 0.81</td>
<td>1.00 ± 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/G + G/G</td>
<td>39 (6)</td>
<td>0.60 ± 0.53</td>
<td>0.61 ± 0.35</td>
</tr>
<tr>
<td>BIK</td>
<td>rs1883264</td>
<td>G/G</td>
<td>42</td>
<td>1.00 ± 0.87</td>
<td>1.00 ± 0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/C + C/C</td>
<td>17 (1)</td>
<td>0.86 ± 0.92</td>
<td>0.62 ± 0.31</td>
</tr>
</tbody>
</table>

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Discussion

This is the first study to comprehensively evaluate the relationship between methylation index in a large population of lung cancer-free smokers and common variation in 40 candidate genes involved in carcinogen metabolism, regulation of methylation, and DNA damage response pathways. Our findings support a role for genetic variation in LIG1, TP53, and BIK as predictors for the acquisition of gene promoter methylation in exfoliated cells in smokers’ sputum. Specifically, molecular validation through a genotype–expression correlation analysis indicates that the 3 most significant SNP changes in LIG1, TP53, and BIK affect the expression of these 3 genes in cis in NHBECS. Thus, genetic variation in genes affecting DNA replication and apoptosis impacts the propensity for gene promoter methylation in the aerodigestive tract of smokers and this effect may be driven by the change in the endogenous expression of LIG1, TP53, and BIK.
and 926 controls; ref. 35). However, no association was identified between LIG1 SNPs and risk for lung cancer in 2 U.S. populations used in the discovery stage in the NCI GWAS study (1,652 cases and 1,212 controls; ref. 36). The SNPs in TP53 and BIK associated with methylation index were also queried in the MD Anderson and NCI GWAS with no significant association to lung cancer identified (not shown). Together, these studies support a moderate association for rs3730859 in LIG1 with risk for the smoking-induced lung cancer and this effect may be mediated by its effect on the predisposition for epigenetic silencing of TSGs.

Among the 3 DNA ligase families (LIG1, LIG3, and LIG4) in vertebrates, LIG1 plays an essential role in the joining of Okazaki fragments during lagging strand synthesis and is also implicated in multiple DNA repair pathways, although the role of LIG1 in DNA repair can be substituted by other DNA ligases (37–40). Deficiency in LIG1 expression results in the delayed joining of Okazaki fragments, genome instability, and increased incidence of epithelial cancers and lymphoma (37–40). Expression of LIG1 in lung tumors (n = 23) is reduced by 34% compared with that in paired normal tissues by which the guidance of the cytosine-DNA methyltransferase to regions that are to be methylated is disrupted during DNA replication (41). Although such a scenario is clearly speculative, an in vitro transformation model or in vivo tumorigenesis model using LIG1 knockout human epithelial cells or animals could be used to directly test this hypothesis.

Evasion apoptosis is a hallmark of cancer and occurs during the progression from premalignant lesions to carcinoma in the lung (42). Apoptosis occurs through 2 mechanisms: the death receptor FAS/FAS ligand (also called extrinsic) pathway and the mitochondrial (DNA damage–induced and p53-mediated, also called intrinsic) pathway (43). The intrinsic apoptosis pathway plays a key role in eliminating lung epithelial cells with extensive DNA damage upon carcinogen exposure (43). Thus, the association between functional SNPs in TP53 and BIK and methylation index support the concept that the TP53-mediated apoptosis pathway could prevent the acquisition of heritable genetic and epigenetic changes through eliminating lung cells with extensive DNA damage. The loss of TP53 function by gene mutation is a common genetic alteration found in human cancers and more than 50% of lung cancer cases carry somatic mutation of TP53 (44). TP53 mutation or reduced level of wild-type TP53 protein was associated with apoptosis suppression during progression from premalignant lesions to carcinoma in the lung (42). Thus, rs1641511 that was associated with a 40% reduction of TP53 transcription could render lung cells more resistant to apoptosis upon extensive DNA damage in smokers, which in turn could result in an increased methylation index. Although rs1641511 is geographically closer to ATP1B2, the gene downstream of TP53, LD analysis using the Phase 2 HapMap CEU data and the 1,000 genome project identify one SNP (rs72829457) around 3 kb downstream of TP53 in moderate LD with rs1641511 (D’ = 1, r2 = 0.5). Moreover, the deletion/insertion polymorphisms that usually account for 15% to 20% of the entire genetic variation are not available from the above 2 databases. Thus, it is possible that the missing deletion/insertion polymorphisms in high LD with rs1641511 are causal for the reduced expression of TP53.

BIK (Bcl2-interacting killer) is the founding member of the proapoptotic BH3-only proteins (45). BIK functions as a death sensor to mediate activation of the mitochondrial apoptosis pathway as shown in response to oncogenic stress signals or DNA damage in epithelial cancer cell lines (45). The role of BIK in cancer development may vary by tumor type. Inactivation of BIK in some primary tissues occurs frequently by chromosomal deletion in renal cell carcinomas (40%), colorectal cancers (22%), and gliomas (42%) but only occasionally in head and neck cancers (5%; ref. 45). Somatic mutations that lead to amino acid changes were also detected in 8% of patients with B-cell lymphomas (45). Epigenetic silencing was also proposed as a potential mechanism to silence BIK expression in specific tumor types, although this mechanism was only indirectly tested on the basis of BIK reexpression in tumor cell lines treated with DNA demethylating agent and/or histone deacetylase inhibitor (45). rs1883264 associated with reduced BIK expression in NHBECS is actually protective for gene methylation in smokers. This seems to be in conflict with the proapoptotic role of BIK identified in epithelial cancer cell lines. However, BIK is overexpressed in lung tumors (n = 23) versus paired normal tissues (P = 0.015; Leng, unpublished data). Furthermore, CpG sites in the BIK promoter and exon 1 are not methylated in lung adenocarcinomas interrogated on the Illumina HumanMethylation 27 K BeadChip (Leng, unpublished data). Consistent with these findings in lung cancer, BIK overexpression was also reported in breast tumors (46). In addition, poor prognosis of non–small cell lung cancers correlated with high expression of BIK (47). These results support different mechanisms for BIK regulation in cancer biology by tumor site and carcinogen exposure pattern. Thus, the protective effect of rs1883264 on gene methylation in smokers identified in our studies is consistent with the
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increased expression of BIK in lung tumors. Additional mechano-
inetic studies are required to understand the regulation of BIK
biology in cigarette smoke–induced lung cancer.

These studies identified the DNA replication and apoptosis
pathways as new determinants for acquiring gene methylation
in lung cells from smokers. Lung cancer GWAS studies suc-
cessfully identified several loci in the human genome that are
associated with risk for lung cancer at $10^{-8}$ significance level
(35, 36). However, these validated genetic variants only explain
a small fraction of lung cancer heritability. Some of the missing
heritability likely exists in the genetic variants with less sig-
ificant $P$ values. The detection of promoter methylation of
TSGs in exfoliated cells in sputum as an assessment of the
extent of field cancerization in the lung can be used as a
functional readout to help identify the true association from
the candidate SNPs with GWAS $P > 10^{-8}$. Thus, the genetic
variants in LIG1 provide a proof-of-concept that SNPs strongly
associated with risk for gene methylation in lung cells from
smokers can be moderately associated with risk for lung cancer
and should be incorporated into risk assessment models for
lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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