Global DNA Methylation Level in Whole Blood as a Biomarker in Head and Neck Squamous Cell Carcinoma


Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is commonly associated with tobacco and alcohol exposures, although dietary factors, particularly folate, and human papillomavirus, are also risk factors. Epigenetic alterations are increasingly implicated in the initiation and progression of cancer. Genome-wide (global) hypomethylation seems to occur in early neoplasia and is a feature of genomic DNA derived from solid tumor tissues, including HNSCC. This study aimed to determine whether global methylation in DNA derived from whole blood, a proxy tissue, is associated with HNSCC and to assess potential modification of this property by environmental or behavioral risk factors.

Methods: Global DNA methylation levels were assessed using a modified version of the combined bisulfite restriction analysis of the LRE1 sequence in a population-based case-control study of HNSCC from the Boston area. Results: Hypomethylation lead to a significant 1.6-fold increased risk for disease (95% confidence interval, 1.1-2.4), in models controlled for other HNSCC risk factors. Smoking showed a significant differential effect (P < 0.03) on blood relative methylation between cases and controls. Furthermore, in cases, variant genotype in the MTHFR gene and low folate intake showed relationships with decreased global methylation, whereas in controls, antibody response to human papillomavirus 16 was associated with an increased global methylation level.

Discussion: DNA hypomethylation in nontarget tissue was independently associated with HNSCC and had a complex relationship with the known risk factors associated with the genesis of HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the ninth most common type of cancer in the United States. Nearly 40,000 new cases of oral, pharyngeal, and laryngeal cancers are expected in 2006, collectively causing ~11,000 deaths (1). Although survival rates can be as high as 75% to 80% if HNSCC is detected in its early stages, the majority of patients are not diagnosed until the disease has progressed to an advanced stage, reducing the chances of survival after 5 years to 35%, a figure which has improved little in the past 25 years (2-4).

Alcohol and tobacco use are long-established risk factors for head and neck cancers and are known to act synergistically (5-10). Infection with high-risk human papillomavirus (HPV) types, particularly HPV16, has been associated with increased risk for HNSCC, and DNA from this virus has been detected in tumors of HNSCC patients (11-16). Antibodies to the L1 protein of HPV16 reflect the presence of viral DNA and have also been associated with risk for HNSCC. Additionally, dietary factors, including deficiencies in dietary folate, are also hypothesized risk factors for HNSCC (17, 18).

Changes in methylation patterns, particularly promoter-specific hypermethylation and global (genome-wide) hypomethylation, are thought to contribute to neoplasia and tumor growth (19, 20). Gene promoter hypermethylation in tumor tissues is a common event in the development of many types of cancer, including HNSCC (21-23). This is because neoplastic growth is frequently preceded by aberrant promoter methylation of tumor suppressor genes, which leads to a loss of function that promotes cell proliferation (24). Cancer-linked global genomic hypomethylation in tumor tissue is a common characteristic in a wide variety of malignancies, ranging from solid tumors, such as breast, colon, oral, and lung cancers, to cancers of the blood (25-29). Whereas hypermethylation occurs chiefly in gene promoter regions, global hypomethylation occurs not only in transcription control regions, such as promoters, but also in repetitive DNA sequences, such as heterochromatic regions and retrotransposons (25). Hypomethylation is thought to contribute to carcinogenesis by inducing genomic instability (30, 31), thereby causing the formation of abnormal chromosomal structures (25, 32).

Evidence also points to the possibility of this alteration activating or enhancing oncogene expression because (a) hypomethylation of promoter regions of certain genes increases the target gene expression (33) and (b) promoter hypomethylation is linked to global methylation levels (34). Furthermore, the causal role of hypomethylation in carcinogenesis has been established using mouse models with decreased methyltransferase activity (31).

The role of folates in carcinogenesis is also strongly linked to genomic methylation levels. Early studies showed that global hypomethylation occurs in rats with low-folate diets (35). A study comparing global DNA methylation levels of tumor tissues in squamous cell lung cancer with those in adjacent, 4 C.S. Furniss, M.D. McClean, J.F. Smith, et al. Human papillomavirus 16 and head and neck squamous cell carcinoma, International Journal of Cancer, in press.
unaffected tissues concluded that hypomethylation is associated with folate deficiency in both diseased and unaffected tissues, supporting the assertion that folate is necessary for proper DNA methylation (36). This study also suggests that although methylation levels are more pronounced in tumor tissues and are tissue specific, proxy tissues may be indicators of local methylation levels.

The family of LINE1 (long interspersed nuclear elements) retrotransposons is reportedly hypomethylated in many cancers and reflects global methylation status in the genome (28, 37), thus examination of methylation at LINE1 regions has served as a proxy for measuring global methylation levels. One long interspersed nuclear element repeat region, LRE1, located on 22q11-q12, has been a consistent indicator of global methylation status (28, 37, 38).

It is increasingly clear that epigenetics plays a causal role in cancer development. HNSCC is a useful disease for studying global hypomethylation because the mechanisms of epigenetic maintenance are related to the risk factors linked to this disease. Examining hypomethylation in this context may shed light on the means by which these factors contribute to carcinogenesis, deepening the general understanding of the complex interaction between epigenetics and environmental exposures in cancer development. Therefore, we have examined, in a population-based case-control study of HNSCC, the association between global genomic methylation, measured as LRE1 methylation status, and HNSCC as well as have examined the associations between risk factors associated with HNSCC and LRE1 methylation status.

Materials and Methods

Study Design. We conducted a case-control study from December 1999 to December 2003 in the Greater Boston Metropolitan area. Details of the case-control ascertainment have been presented elsewhere (39). This region of Massachusetts includes a population of ~3.5 million people in 249 cities and towns within a 1-h drive of Boston. The institutional review boards at all participating institutions approved this study, and all volunteer participants provided informed consent. Briefly, incident cases of HNSCC were identified through multidisciplinary head and neck clinics, otolaryngology, and radiation oncology departments at nine medical facilities located in Boston, Massachusetts. We defined HNSCC as including International Classification of Disease Ninth Revision codes 141, 143-6, 148, 149, and 161. All patients with carcinoma in situ, lip, salivary gland, or nasopharyngeal cancer or recurrent cancer of the head and neck region were excluded. Histologic classification of malignancy was based on that reported by pathology at the participating hospitals. Population-based controls were drawn from the specified greater Boston population. The controls were frequency matched (1:1 to cases by age (<3 years), gender, and town of residence. These controls were identified through random selection from the resident lists for the 249 cities and towns within the study area using the address of the cases as reference.

Participating cases and controls were given a self-administered questionnaire to collect medical history, demographic information, as well as information on tobacco and alcohol consumption. Each questionnaire was reviewed with each participant by a trained research coordinator. Smoking history was ascertained with a standardized instrument that assesses the number of years smoked, the number of cigarettes smoked daily, age at which an individual started smoking, number of years since quitting, and the duration of smoking in a decade-specific manner. Similar information was obtained about lifetime consumption of beer, wine, and liquor. The Willett food frequency questionnaire, a standardized and validated instrument, was used to assess diet history (40, 41). Subjects are asked to recall their usual diet over a 1-year time period 5 years previous to their diagnosis (in a fashion consistent with the validation study of diet recall using this instrument). Because many tumors of the oral cavity and pharynx may affect food intake before being diagnosed, moving the recall period well in advance of tumor development eliminates this possible bias. Questionnaires were given to case participants during an initial clinical visit and subsequently retrieved in-person. Control participants received their questionnaires in the mail and returned them in person to the research assistant.

Eight hundred and twenty-three eligible cases were invited to participate, of these 57 refused to participate. Among the 766 consented subjects, another 44 did not complete the questionnaire. Based on these screening refusal and noncompletion data, including food frequency questionnaire and blood samples were available on 278 cases. Similarly, 1,623 subjects were identified and eligible for participation as controls. Eight hundred twenty-eight refused to participate, 815 subjects were consented, and 771 finally enrolled in the study. Six of the controls were withdrawn as they were matched to a case that became ineligible, such that 765 controls were enrolled and completed. Complete questionnaire data and blood samples were available on 526 people. The characteristics of the final study population are described in Table 1.

DNA Extraction and Sodium Bisulfite Modification of DNA. DNA was extracted from a 200-μL aliquot of whole blood using the QIAamp DNA Blood Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Extracted DNA samples were eluted with 200 μL TE buffer and stored at -20 °C until needed. DNA extracted from whole blood was modified by treatment with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research, Orange, CA) following the manufacturer's protocol.

LRE1 Relative Methylation Assay. To assess the relative methylation status of LRE1 in DNA derived from whole blood samples, we used a modified version of the previously described combined bisulfite restriction analysis LRE1 assay, allowing for fluorescence-based relative quantification of differentially methylated product (38). Sodium bisulfite-modified DNA was subjected to PCR using the fluorescently labeled primers Line1-F (5'-Hex-CCGTAAAGGTTAGG-GAGTTTTT-3') and Line1-R (5'-Hex-RTAAACCTC-CRAACCAAATATAAA-3'). Amplification conditions were as follows: bisulfite-modified DNA, 1 mmol/L each of forward and reverse primers, 2 mmol/L of each deoxynucleotide triphosphate, 1.5 μmol/L MgCl2, 1× PCR buffer (Applied Biosystems, Foster City, CA), and 1.25 units AmpliTaq polymerase were combined in a final volume of 50 μL. Reactions were incubated for 5 min at 95 °C and then for 40 cycles at 94 °C denaturing for 30 s, 50 °C annealing for 30 s, and 72 °C extension for 30 s followed by a final 7-min extension at 72 °C. To confirm amplification of the 160-bp product, 10 μL of the completed PCRs were resolved using a 3% agarose gel in 1× Tris-borate EDTA and visualized with ethidium bromide.

The remaining reaction volumes were then used for double digestion with 2 units TaqI and 8 units of TasI enzyme in 1× TaqI buffer (Fermentas, Hanover, MD) at 65 °C for 18 to 24 h in the dark. Methylated amplicons are TaqI positive and yield two 80-bp fragments, whereas unmethylated amplicons, in which cytosines are converted to uracils during bisulfite modification, are TasI positive and yield 63- and 97-bp fragments. Fragments were prepared for analysis by mixing 1.5 μL of the digested product with 11.5 μL DI formamide and 0.5 μL Genescan 350 TAMRA size standard (Applied Biosystems) and then denatured at 95 °C for 5 min. Samples were resolved using capillary electrophoresis in the ABI Prism 310 Genetic Analyzer. The accompanying Genescan software was used to determine peak heights for restriction products generated by TasI (63 and 97 bp) from unmethylated...
amplicons and TaqI (80 bp) from methylated amplicons. The degree of relative methylation was assessed by taking the ratio of the methylated peak height (80 bp) to the sum of peak heights from all digestions (63, 80, and 97 bp). Controls were generated by using unmodified primers on unmodified DNA derived from whole blood to amplify a 390-bp region surrounding the 160-bp region of interest in LRE1, thus generating an unmethylated control, as the PCR product does not preserve methylation from the original template. Positive control DNA was generated by treating whole-blood DNA with DNA methylase, effectively methylating all available CpG islands. The methylated and unmethylated products were then modified with sodium bisulfite, subjected to PCR under the conditions described above, and the subsequent products were mixed in varying proportions to subsequent products were mixed in varying proportions to generate a standard curve under which samples were expected to decrease. Other controls used to validate this assay were DNA from the K-562 cell line (highly unmethylated) and positive control DNA was generated by treating whole-blood DNA with DNA methylase, effectively methylating all available CpG islands. The methylated and unmethylated products were then modified with sodium bisulfite, subjected to PCR under the conditions described above, and the subsequent products were mixed in varying proportions to generate a standard curve under which samples were expected to decrease. Other controls used to validate this assay were DNA from the K-562 cell line (highly unmethylated) and sperm DNA (highly methylated).

**Statistical Analysis.** Data were analyzed using SAS statistical software. The Wilcoxon rank-sum test was used as a nonparametric comparison of median methylation levels in cases versus controls. To evaluate the effects of individual variables, including LRE1 methylation levels on case-control status, while controlling for intervariable confounding, unconditional logistic regression was used to determine odds ratios and their associated 95% confidence intervals. In this analysis, the LRE1 relative methylation level was broken into terciles based on the distribution in controls and lifetime average number of alcoholic drinks per week was broken into quartiles based on the distribution in controls. HPV16 serology was considered positive in all subjects whose HPV16 titer was greater than the limit of detection (>12 milliMerck units). Dietary folate was first broken into terciles based on the distribution in controls and then dichotomized to examine the effect of dietary folate among the lowest 33rd percentile of consumption.

**Results**

DNA derived from whole blood samples of 278 case subjects and 526 control subjects was evaluated to determine relative global methylation levels using the LRE1 assay. The characteristics of the population examined are shown in Table 1. As expected, lifetime smoking history and lifetime average alcoholic drinks per week were both associated with HNSCC as was positive serology for antibodies to the HPV16 virus. We observed no significant association between HNSCC and dietary folate intake or with the MTHFR 677C>T polymorphism. In assessing the association between HNSCC and dietary folate intake, we compared the lowest tercile of intake, <418 µg/d (similar to the U.S. recommended daily allowance level of 400 µg/d) with the upper two terciles.

**Table 1. Demographic characteristics of HNSCC cases and controls**

<table>
<thead>
<tr>
<th>Case (n=278), n (%)</th>
<th>Control (n=526), n (%)</th>
<th>Adjusted OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>60.1 (11.7)</td>
<td>61.0 (11.5)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>149 (28.3)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>377 (71.7)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>480 (91.2)</td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>15 (2.9)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>8 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>21 (4.0)</td>
<td></td>
</tr>
<tr>
<td>Alcohol drinks per week (lifetime average)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-3</td>
<td>45 (16.2)</td>
<td>149 (28.3)</td>
</tr>
<tr>
<td>&gt;3-6</td>
<td>38 (13.7)</td>
<td>119 (22.6)</td>
</tr>
<tr>
<td>&gt;6-14</td>
<td>50 (18.0)</td>
<td>122 (23.2)</td>
</tr>
<tr>
<td>&gt;14</td>
<td>145 (52.1)</td>
<td>136 (25.9)</td>
</tr>
<tr>
<td>Lifetime smoking history (pack-years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50 (18.0)</td>
<td>183 (34.8)</td>
</tr>
<tr>
<td>&gt;0-25</td>
<td>80 (28.8)</td>
<td>172 (32.7)</td>
</tr>
<tr>
<td>&gt;25</td>
<td>148 (53.2)</td>
<td>171 (32.5)</td>
</tr>
<tr>
<td>MTHFR 677 genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt/wt</td>
<td>128 (46.2)</td>
<td>218 (41.6)</td>
</tr>
<tr>
<td>wt/var var/var</td>
<td>149 (53.8)</td>
<td>306 (58.4)</td>
</tr>
<tr>
<td>Dietary folate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate to high</td>
<td>176 (64.2)</td>
<td>343 (65.7)</td>
</tr>
<tr>
<td>Low</td>
<td>98 (35.8)</td>
<td>179 (34.3)</td>
</tr>
<tr>
<td>HPV16 serology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>179 (68.3)</td>
<td>422 (89.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>83 (31.7)</td>
<td>51 (10.8)</td>
</tr>
</tbody>
</table>

*Model adjusted for age, gender, race, smoking history, and/or lifetime average alcoholic drinks.

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Figure 1 depicts the distribution of the LRE1 relative methylation level in cases and controls as box plots of the data. The median methylation level of 0.753 in controls was slightly but significantly higher than the median level of 0.747 in cases (P < 0.03, Wilcoxon rank-sum test).

As we observed a difference in the distribution of LRE1 relative methylation between cases and controls, we did logistic regression to examine the association of LRE1 relative methylation level and case status, controlling for other risk factors and confounders (Table 2). In this model, LRE1 methylation was categorized into three groups divided at the 33rd and 66th percentiles, with the highest tertile of relative methylation serving as the referent. Controlling for the matching factors of age, gender, and race as well as confounders of lifetime smoking, lifetime average alcoholic drinks per week, and HPV16 serology, we observed that those patients in the lowest tertile of LRE1 relative methylation had a significant relative risk of HNSCC (odds ratio, 1.6; 95% confidence interval, 1.1-2.4), whereas those in the medium tertile showed an elevated odds ratio of 1.3 (95% confidence interval, 0.9-2.0). These values represent a significant trend (P < 0.03) for increased HNSCC risk with lower LRE1 relative methylation level. To assure that this effect was not related to treatment of the disease, we examined the relationship between LRE1 relative methylation level and the timing of the blood draw in the cases (presurgical or postsurgical resection) and found no significant correlation (Pearson correlation between LRE1 relative methylation level and time from blood draw to surgery; R² = 0.0001; P = 0.9).

We also wished to examine whether known demographic and risk factors related to HNSCC play an etiologic role in determining the relative methylation level in subject blood. Observing the significant difference between cases and controls in their relative LRE1 methylation levels, we reasoned that the biology of the global methylation status may be different between diseased and healthy individuals. Thus, we stratified analyses that examined the effect of subject demographic and risk factors on LRE1 relative methylation level, modeling LRE1 relative methylation level as the dependent variable in a generalized linear model. We used a forward selection procedure to arrive at a biologically plausible parsimonious model. The distribution of the predictors used in these stratified models are shown in Table 1, with the results of the stratified models in Table 3.

Table 2: Blood-derived DNA LRE1 relative methylation is associated with HNSCC case status

<table>
<thead>
<tr>
<th>LRE1 relative methylation level* (terciles in controls)</th>
<th>Cases, n (%)</th>
<th>Controls, n (%)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (&gt;0.769)</td>
<td>74 (26.6)</td>
<td>182 (34.6)</td>
<td>1.0 (Referent)</td>
</tr>
<tr>
<td>Middle (0.736-0.769)</td>
<td>97 (34.9)</td>
<td>170 (32.3)</td>
<td>1.3 (0.9-2.0)</td>
</tr>
<tr>
<td>Low (&lt;0.736)</td>
<td>107 (38.5)</td>
<td>174 (33.1)</td>
<td>1.6 (1.2-2.4)</td>
</tr>
</tbody>
</table>

*P<0.03.
†Model is adjusted for age, gender, race, smoking history, lifetime average alcoholic drinks weekly, and HPV16 serology positivity.

In cases (Table 3), in a model controlling for age, gender, race, lifetime average drinks per week, and HPV16 serology, we observed that dietary folate in the lowest tertile, compared with the upper two tertiles, led to a 1% reduction in the relative LRE1 methylation level, although this result was of only borderline significance (P < 0.06). In this same model, cases carrying one or two variant (T) alleles at MTHFR codon 677 showed a significant (P < 0.04) ~1% reduction in LRE1 relative methylation. At the same time, each one pack-year increase in lifetime pack-years of smoking was significantly associated (P < 0.04) with an ~0.02% increase in the LRE1 relative methylation level among cases. In a nonstratified model examining the interaction of each of the covariates, only smoking history, measured as pack-years, showed a statistically significant interaction with case status (P < 0.03).

Controls, on the other hand, showed a different group of significant predictors for LRE1 relative methylation (Table 3). In a model controlled for age, lifetime average alcoholic drinks per week, lifetime pack-years smoked, dietary folate intake, and MTHFR codon 677 genotype, we observed a significant (P < 0.002) 1% increase in the relative LRE1 methylation level in males compared with females and significant ~1% increases in LRE1 relative methylation for subjects with positive HPV16 antibody serology and for subjects of non-Caucasian race compared with Caucasians (P < 0.02 and P < 0.03, respectively).

Discussion

In models controlling for confounders and known risk factors of HNSCC (alcohol, tobacco, and HPV16 serology), we have observed a significant 1.6-fold increased relative risk for HNSCC among subjects with global methylation levels that decrease in the lowest tertile compared with those with levels in the highest tertile, suggesting that hypomethylation is an independent risk factor for HNSCC. We also observed, in this model, a significant trend for increasing relative risk of HNSCC with decreasing LRE1 relative methylation level, indicative of global hypomethylation.

In stratified models examining predictors of LRE1 relative methylation, we observed that gender was significantly related to methylation status, with women being more likely to have reduced methylation level. Although the precise explanation for this is not clear, differences between male and female nutrient intake and loss might explain this distribution. Women may tend to have diets lower in protein or lower caloric intake in general, resulting in comparatively lower levels of nutrients, such as choline, methionine, homocysteine, and folate; these macromolecules are all metabolically related in the process of DNA methylation (43). In addition, folate is essential for erythrocyte formation and development. Because menstruation regularly depletes their supply of erythrocytes, females may tend to have a higher folate requirement, or conversely, lower levels of circulating folate. This may contribute to the difference in global methylation between males and females. In postmenopausal females, who do not menstruate regularly deplete their supply of erythrocytes, females may tend to have a higher folate requirement, or conversely, lower levels of circulating folate. This may contribute to the difference in global methylation between males and females.
have a regular depletion of folate-requiring erythrocytes, other dietary factors and behavioral factors may contribute to low methylation.

The significantly higher levels of relative methylation observed in non-Caucasian controls may also reflect effects of different environmental factors, such as dietary intake, which can contribute to higher global methylation levels, or equally likely, genetic predisposition to constitutively higher global methylation.

Both the association of an increasing \( \text{LRE1} \) methylation level among non-Caucasian controls as well as among men is anomalous when compared with the relationship of these factors with disease risk; non-Caucasians and men are at higher risk for HNSCC. This suggests that these factors, in general, but others as well, do not work in a linear pathway through global hypomethylation to elicit disease. Instead, these data might imply that global methylation functions in a complex pathway to influence individual cancer risk, perhaps interacting with other known and yet-to-be-known factors. Additional studies are needed to more carefully examine how gender and race may influence \( \text{LRE1} \) methylation levels and, within these subgroups, how additional factors, such as diet and lifestyle, influence global methylation.

It is also of interest that, in our data, the relative \( \text{LRE1} \) methylation level was significantly associated with detectable HPV16 antibodies in controls, with those subjects with positive serology having increased relative methylation. It has been observed that the HPV genome itself may become hypermethylated on infection or integration into the host (44, 45) and that the carcinogenicity of HPV16 is then related to hypomethylation of its genome during neoplastic progression (46). There is also evidence, in vitro, that immortalization by HPV leads to specific gene hypermethylation (47). We also observed previously an association between gene-specific hypermethylation of the \( \text{SFRP4} \) gene promoter and HPV16 presence, particularly among nonsmokers. This may reflect a mechanism whereby aberrant promoter methylation results from mis-targeted host defense methylation during viral integration or the genomic instability attributed to HPV16 presence (48). Our observation of greater relative methylation in controls with HPV16 antibodies, which are associated with the presence of viral DNA, also may explain, in part, why these individuals were not susceptible to the carcinogenic effects of the HPV16 virus; increased or more stable global methylation levels may reduce the carcinogenic potential of the HPV16 genome, thus preventing viral neoplastic progression in these individuals. Additionally, higher \( \text{LRE1} \) methylation levels, particularly in lymphocytes, may be related to the ability of the individual to mount an immune response to HPV (measured by HPV serology), thus marking individuals who successfully cleared the HPV infection before carcinogenesis. Therefore, these results suggest that susceptibility to HPV16 carcinogenesis may be an epigenetically modified process.

In contrast to the data in controls, the data from cases show that low daily dietary folate intake and possessing the variant T allele at \( \text{MTHFR} \) codon 677 are correlated with reduced \( \text{LRE1} \) relative methylation. Given the relationship between \( \text{MTHFR} \) function and levels of circulating folate (in the form of 5-methyl-THF), this is to be expected, particularly because various \( \text{MTHFR} \) polymorphisms have been implicated as risk factors for HNSCC (49). Preliminary analysis of our data has suggested that \( \text{MTHFR} \) 677 genotype is not an independent risk factor for HNSCC, but its influence on hypomethylation may suggest the possibility for effect modification by this or other factors. Although low folate has been shown as a risk factor for HNSCC (50, 51), the mechanism of this association has not been fully elucidated. One possible explanation is that a change in the physiology of HNSCC patients, compared with the baseline physiologic characteristics of controls (or a general population), causes methylation levels to become highly folate dependent. A rapidly dividing population of cells, such as a tumor, has a high nutrient requirement. With elevated levels of protein synthesis and DNA replication, requirements for methionine, folate, and homocysteine will increase, upsetting the ratio of cofactors necessary to maintain balance and proper functioning of the methylation process throughout the body and particularly in dividing cells, such as lymphocytes. Essentially, because the folate requirement is increased in cancer patients, but their dietary patterns will not increase to fill that requirement (and may in fact lessen due to the disease), more patients are likely to decrease below that elevated threshold. The methylation status of these individuals becomes even more perturbed as cells struggle to function with lower folate levels. Research has shown that whereas folate supplementation in healthy cells is generally protective against the development of tumors, in epigenetically disrupted cancerous cells, supplementation in fact may increase tumor growth (43, 52, 53), supportive of the results obtained here.

To more closely examine if these differences in the effects of specific covariates on \( \text{LRE1} \) relative methylation truly differ in cases and controls, we did a nonstratified analysis, using the same generalized linear model, but included terms for the interactions of each of the covariates with case status. According to this approach, only lifetime smoking history showed a significant interaction at the \( P < 0.05 \) level, suggesting that it is truly acting differentially to predict \( \text{LRE1} \) relative methylation in cases and controls. The nonsignificant

**Table 3. Effects of demographics, exposures, and lifestyle factors on relative \( \text{LRE1} \) methylation in cases and controls**

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>( P ) value for difference between cases and controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.51</td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>(&lt;0.0001)</td>
<td>0.0058</td>
<td>1.0</td>
</tr>
<tr>
<td>Race (non-Caucasian vs Caucasian)</td>
<td>0.0058</td>
<td>0.0099</td>
<td>0.56</td>
</tr>
<tr>
<td>Smoking history (lifetime pack-years)</td>
<td>0.0002</td>
<td>0.0010</td>
<td>0.04</td>
</tr>
<tr>
<td>Lifetime average drinking alcohol per week</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.61</td>
</tr>
<tr>
<td>HPV16 serology (positive vs negative)</td>
<td>0.0025</td>
<td>0.0057</td>
<td>0.66</td>
</tr>
<tr>
<td>Dietary folate (lowest tertile vs two higher tertiles)</td>
<td>0.0101</td>
<td>0.0055</td>
<td>0.09</td>
</tr>
<tr>
<td>( \text{MTHFR} 677 ) genotypes (wt/var vs var wt)</td>
<td>0.0101</td>
<td>0.0049</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Stratified generalized linear models were done for both cases (n = 278) and controls (n = 526) and included all covariates in the table. Interaction P values were obtained from a Wald test for interaction.

* A positive estimate of the correlation between the risk factor and \( \text{LRE1} \) methylation level reflects an increasing \( \text{LRE1} \) response to the risk factor; a negative estimate reflects an decreasing (hypomethylation) \( \text{LRE1} \) response to the risk factor.

*HPV16 serology was not available for 69 individuals. These individuals HPV16 serology status was coded as missing and included in all models.
results among the other predictors which seem to significantly affect the LRE1 relative methylation differentially in the stratified models suggest that we are underpowered to examine these interactions. We also found that race and HPV16 serology were differentially associated with relative LRE1 methylation, having opposite effects on LRE1 relative methylation in cases and controls. These variables predict reduced LRE1 relative methylation in cases but increased LRE1 relative methylation in controls. Male gender, on the other hand, seems to have a stronger positive relationship to LRE1 relative methylation in controls (estimate of effect, 0.012) than in cases (estimate of effect, <0.0001). Similarly, both low dietary folate intake and variant MTHFR C677T genotype show stronger negative relationships with LRE1 relative methylation in cases compared with controls.

Therefore, we believe that our data from the stratified analyses in cases and controls, as well as our overall model of HNSCC risk, suggest that LRE1 relative methylation is an independent epigenetic biomarker of HNSCC. Feinberg, et al. (54) have proposed that cancer may derive from epigenetic progenitors and that epigenetic status, unlike genetic susceptibility, which is defined by inherited polymorphisms, may be more plastic. Our results build on this model, suggesting that epigenetic biomarkers, such as LRE1 relative methylation, can potentially be influenced by a variety of factors and may thus aid in explaining the modification of effect of these factors on risk for disease.

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References


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