

Line Region Hypomethylation Is Associated with Lifestyle and Differs by Human Papillomavirus Status in Head and Neck Squamous Cell Carcinomas

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Abstract

Genomic hypomethylation is a hallmark of essentially all cancers, but the degree of this hypomethylation differs among individual tumors. Little work has explored what leads to these differences and or asked whether they are clinically meaningful. In this study of head and neck squamous cell carcinoma, we assessed hypomethylation in tumors using a semiquantitative fragment analysis approach to determine the relative methylation status of the line retroviral element *LRE1* (Line-1.2). Because this is an established marker of genomic methylation status, we examined the relationship between the relative methylation, patient demographics, and other risk factors for head and neck squamous cell carcinoma. We determined relative methylation status for 303 patients, 193 of which had complete data for all variables of interest. Using a generalized linear model, we found that patient body

mass index was significantly positively associated with tumor *LRE1* methylation level. Smoking duration, particularly in tumors lacking human papillomavirus (HPV) DNA, was significantly negatively associated with relative methylation level. Having previously assessed relative methylation in blood-derived DNA, we compared tumor with the blood DNA methylation level and observed these to be independent. Finally, the lower *LRE1* methylation in patients whose tumors were HPV DNA negative was associated with poorer patient survival (hazard ratio, 1.6; 95% confidence interval, 1.0-2.6). These findings suggest that HPV-associated tumors differ molecularly from those arising after heavy tobacco use and that this epigenetic alteration may affect survival in HPV-negative patients already exhibiting a more aggressive disease. (Cancer Epidemiol Biomarkers Prev 2008;17(4):966-71)

Introduction

Each year, ~40,000 new cases of head and neck squamous cell carcinoma are diagnosed in the United States, with ~31,000 cases in the oral cavity and pharynx and ~10,000 new cases in the larynx (1). Known risk factors for head and neck squamous cell carcinoma in the United States include tobacco smoke, chewing tobacco, and alcohol with a well-demonstrated synergistic interaction between smoking and drinking such that an attributable fraction of close to 90% has been estimated for these two exposures (2, 3). Human papillomavirus (HPV), specifically high-risk type 16, is also a risk factor for head and neck squamous cell carcinoma, particularly among younger patients and women, and is detected in ~25% of cases (4-14). Nutritional factors have also been linked to risk of head and neck squamous cell carcinoma,

with a particular risk associated with lower body mass index (BMI; refs. 10, 15-20). It remains unclear if the risk associated with leanness is causal to the disease or is a consequence of the developing tumor or the heavy alcohol and tobacco use associated with the disease.

Global DNA hypomethylation is one hallmark of cancers, and it has been hypothesized that this loss of DNA methylation may lead to alterations in the expression of proto-oncogenes, as well as in facilitating chromosomal instability (21, 22). Vogelstein's model of multistep carcinogenesis places a genome-wide demethylation step (23) that mostly affects transposon regions (24) as an early event in carcinogenesis. Investigators have used the methylation status of repeat elements as a biomarker for global methylation status including the Line-1 retrotransposon (*LRE1*). Various types of cancer, including urothelial bladder carcinoma (25), malignant testicular tumors (26), hepatocellular carcinoma (27), chronic lymphocytic leukemia (28), prostate carcinomas (29), and head and neck squamous cell carcinoma (25), show hypomethylation of *LRE1*. Head and neck cancers studied to date have been observed to have significantly greater degree of *LRE1* hypomethylation compared with their normal tissue counterparts (30), and a lower level of *LRE1* methylation in peripheral blood-derived DNA has been associated with risk for head and neck squamous cell carcinoma (31).

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Table 1. Demographic comparison of head and neck squamous cell carcinoma cases from full population and samples included in analysis

Demographic variable	Value in case population (<i>n</i> = 1,069)*	Value in case population included in the model (<i>n</i> = 193)
Patient age (y), median (range)	59 (21-98)	58 (23-91)
Gender, <i>n</i> (%)		
Female	297 (27.8)	41 (21.2)
Male	772 (72.2)	152 (78.8)
Lifetime pack-years smoked, median (range)	31 (0-200) [†]	27 (0-142)
No. years smoking, median (range)	30 (0-70) [†]	30 (0-60)
Lifetime average drinks per week, median (range)	14.0 (0-307) [‡]	13.3 (0-155)
HPV DNA in tumor		
Absent	333 (75.0)	146 (75.7)
Present	111 (25.0) [§]	47 (24.3)
BMI, median (range)	26.7 (15.6-58.4)	26.7 (17.8-53.1)

*Original population described in ref. 32.

[†] *n* = 986 with complete smoking history.

[‡] *n* = 992 with complete alcohol history.

[§] *n* = 444 with HPV tumor DNA complete.

^{||} *n* = 781 with BMI complete.

Although it is clear that genomic hypomethylation is a hallmark of cancer, little work has addressed the etiology of this epigenetic alteration or the variability in the extent of genomic methylation across tumors. Therefore, we sought to determine the possible association of head and neck squamous cell carcinoma risk factors, specifically alcohol, tobacco, HPV, and BMI, with the methylation status of *LRE1*.

Subjects and Methods

Study Population. *LRE1* methylation status was determined in 303 incident cases of head and neck squamous cell carcinoma, drawn from an ongoing population-based case-control study of head and neck squamous cell carcinoma. One hundred ninety-three of these 303 cases had complete data for all variables of interest. The details of the parent study have previously been described in detail (32) and all patients provided informed consent under protocols approved by the appropriate institutional review board. Information on risk factors including age, sex, ethnicity, tobacco and alcohol use, occupation, and diet was collected through patient questionnaires that were explained by study technicians, and clinical and pathologic information on the tumor was collected by chart review. Patient survival was ascertained by interrogating a national vital statistics database.

DNA Extraction and Sodium Bisulfite Modification. Three 20- μ m sections were cut from each fixed, paraffin-embedded head and neck squamous cell carcinoma tumor sample and transferred into microcentrifuge tubes. The paraffin was dissolved with Histochoice Clearing Agent (Sigma-Aldrich) followed by two washes with 100% ethanol and one wash with PBS. The samples were then incubated in SDS lysis solution [50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, 1% SDS] with proteinase K (Qiagen) overnight at 55°C. De-crosslinking was done by adding NaCl (final concentration, 0.7 mol/L) and incubating at 65°C for 4 h. DNA was recovered using the Wizard DNA clean-up kit (Promega) according to the manufacturer's protocols. Sodium bisulfite mod-

ification of the DNA was done using the EZ DNA Methylation Kit (Zymo Research) following the manufacturer's protocol, with the addition of a 5-min initial incubation at 95°C before addition of the denaturation reagent. The de-crosslinking incubation as well as the 95°C incubation ensures complete melting of the DNA and thus complete sodium bisulfite conversion, particularly for the formalin-fixed specimens.

***LRE1* Relative Methylation Assay.** We used a modified version of the combined bisulfite restriction assay of Line-1.2 described by Chalitchagorn et al. (30) as we have used in a previous examination of *LRE1* relative methylation level (31).

Tumor HPV Determination. A 65-bp region of the *L1* gene of HPV16 was amplified using primers previously published (33). SPF1A (forward) and SPF2B (reverse), primers specific to HPV16, were used in the assay. PCR conditions were followed as previously described (14).

Statistical Methods. All statistical analyses were done with SAS software, version 9.1. The relative methylation index was calculated by taking the ratio of *Taq1* positive amplicons to the sum of the *Tas1* and *Taq1* amplicons and fitting this value to a standard curve based on dilutions of methylase treated DNA and *LRE1* PCR product. To estimate predictors of *LRE1* methylation, we modeled *LRE1* relative methylation as a linear function of covariates, using generalized linear models. Because *LRE1* methylation values fall between zero and one inclusive, we used settings appropriate to a beta response: an identity link function, the binomial variance $\text{Var}(\mu) = \mu(1 - \mu)$, and a scale parameter estimated with Pearson residuals. We assessed goodness-of-fit using the cumulative residual method of Lin et al. (34), with 10,000 resampled residual processes. Our model examined the effect of tobacco smoking (examined first as pack-years smoking, but then specifically as years smoking because this variable better captured the response), alcohol consumption as decade-specific lifetime average drinks per week, patient BMI 5 years before diagnosis, tumor HPV DNA status, tumor stage, age, and gender. Alcohol consumption and tumor stage were removed from the

model because they showed no significant association with *LRE1* methylation level and their removal did not effect the estimates of the other predictors. Age, gender, and tumor HPV status, although insignificant, were retained in the model to control for residual confounding. To examine the modification of the effect of smoking duration by tumor HPV DNA status, a multiplicative interaction term was added to the model to determine if the interaction was statistically significant.

To examine the effect of *LRE1* methylation status on patient survival, *LRE1* methylation level was dichotomized at its median (0.45) and a Kaplan-Meier survival probability curve was constructed for the groups using the log-rank test to examine the difference in survival by *LRE1* methylation level. To control for additional confounders of patient survival, including tumor stage and patient age and gender, Cox proportional hazards models were used. Survival examinations were done overall and in models stratified by tumor HPV DNA status. All statistical tests were two sided, with $P < 0.05$ considered statistically significant.

Results

The demographics of the study population are shown in Table 1, which compares the full case population to the subjects included in the final model of *LRE1* methylation (those subjects with complete data for all variables of interest, $n = 193$). The subjects included in the model did not differ significantly from the complete case population in terms of demographics or in the distribution of the risk factors of the disease. The *LRE1* methylation level ranged from 0.01 to 0.95, with a median value of 0.45.

We observed in a model of *LRE1* methylation level that the patient's BMI 5 years before diagnosis was significantly associated with an increased *LRE1* methylation level ($P < 0.003$; Table 2). At the same time, lifetime smoking duration was significantly associated with a reduced *LRE1* methylation level ($P < 0.02$; Table 2) or a greater degree of tumor hypomethylation. Tumor HPV16 DNA status was not significantly associated with *LRE1* relative methylation level or with patient age or gender. Further, because we have previously measured *LRE1* relative methylation level in the blood from these patients (31), we compared the levels in blood to those in the tumors and found no association (data not shown). As it has been hypothesized that distinct molecular phenotypes may exist in tumor arising from HPV infection (4, 35), we examined how tumor HPV DNA status may modify the effect of exposure on *LRE1* relative methylation level. Although the interaction between smoking duration and tumor HPV status was of borderline significance [$P < 0.1$ (not significant)], the model suggests that smoking duration in HPV-negative individuals has an enhanced negative effect on *LRE1* methylation status (estimate of effect, -0.0025 ; $P < 0.006$), and that HPV status may also negatively effect *LRE1* methylation level in nonsmoking individuals [estimate of effect, -0.11 ; $P < 0.06$ (not significant)], in models controlled for age, gender, and BMI.

LRE1 relative methylation level also had a borderline statistically significant effect on overall patient survival, with patients who have *LRE1* relative methylation below the median (<0.45) showing reduced survival

time [$P < 0.06$ (not significant); Fig. 1A]. In proportional hazards models, the low *LRE1* methylation level showed a statistically significant ($P < 0.04$) 70% increased instantaneous risk of death (Table 3) in a model controlled

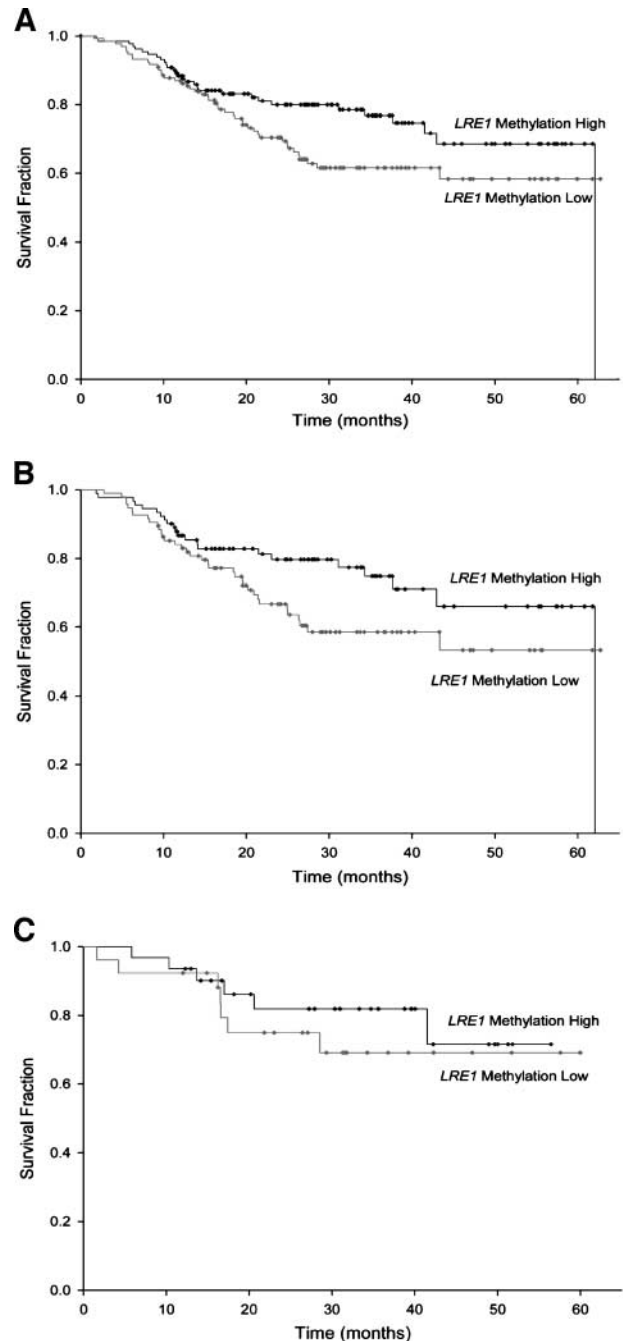


Figure 1. Kaplan-Meier survival probability curves comparing patients whose tumors exhibit *LRE1* methylation level less than the median of 0.45 versus those whose tumor *LRE1* methylation level is ≥ 0.45 , in the overall patient population (A), among patients with HPV-negative tumors (B) and among patients with HPV positive tumors (C). Censored values are represented by hatch marks, and difference in the survival was examined using the log-rank test.

Table 2. Main effect of demographics and exposure on relative *LRE1* methylation in head and neck squamous cell carcinoma tumors

Parameter	Estimate of effect*	SE	P
BMI	0.0088	0.0029	0.003
Lifetime smoking duration (y)	-0.0019	0.0008	0.02
HPV DNA (present vs absent)	-0.0422	0.0354	0.2
Patient age	0.0007	0.0014	0.6
Gender (male vs female)	-0.0498	0.0386	0.2

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

for age, gender, tumor stage, and tumor HPV DNA status, which was previously shown to be associated with better survival (4-6). We also explored whether this association of hypomethylation with survival differed by HPV status. Stratified survival analyses suggested that among HPV16-negative individuals, low *LRE1* relative methylation level is associated with poorer survival [log rank $P < 0.07$ (not significant); Fig. 1B; Table 3], whereas there was no effect on survival by *LRE1* methylation level in patients whose tumors are positive for HPV16 DNA (Fig. 1C; Table 3).

Discussion

Consistent with previous examinations of tumor-specific genomic methylation extent (30), we observed a wide range of *LRE1* relative methylation level in our population of head and neck squamous cell carcinoma tumors. This could suggest that even a relatively small loss of methylation in normally highly methylated DNA among susceptible individuals may be a precipitating factor in carcinogenesis. Of the cancers studied, head and neck squamous cell carcinoma displayed one of the greatest increases in hypomethylation compared with normal tissue (30). The wide range is also strikingly different than what was observed in peripheral blood-derived DNA from this same population, where the range of *LRE1* level was more narrowed and differed only slightly, but significantly, between cases and controls (31).

We observed an association between smoking duration and tumor genomic hypomethylation, particularly in

HPV16-negative tumors, where smoking is the major etiologic contributor to the disease. We did not observe, however, any association with alcohol consumption, the other major risk factor in non-HPV-associated disease. Previous studies have observed a significant association between global DNA methylation in buccal mucosal cells and malignant tissues of the lung, but not between methylation in peripheral leukocytes and lung tissues (36), consistent with our data. However, this does suggest a possible role for tobacco smoke in the induction of tissue-specific global hypomethylation. The mechanism for tobacco smoke leading to global hypomethylation is unknown, but studies have shown that hydrocarbons such as those present in tobacco smoke are capable of inactivating folic acid and vitamin B12 (37), and thus may alter the availability and enzymatic pathways responsible for methylating DNA appropriately. A study supporting this hypothesis showed that oral mucosal folate levels were much lower in the buccal mucosal cells of smokers than in nonsmokers (38).

We hypothesized that global hypomethylation would be differential based on HPV DNA presence, but found that HPV DNA presence was not associated with global hypomethylation. Although global hypomethylation has not been shown to be associated with HPV presence in tumors, HPV16 itself is targeted by methylation, with hypomethylation correlating with carcinogenic progression (39). The fact that HPV becomes methylated in the host and the fact that it integrates into repeat regions, including transposons (40), led us to hypothesize that global hypomethylation status in the tumor would differ by HPV DNA presence. The lack of association between HPV and global hypomethylation in our study may reflect the fact that HPV is known to integrate randomly throughout the genome (41) and may not induce or alter methylation levels to perform this integration.

Our observation that smoking duration had an association with hypomethylation only in HPV-negative tumors suggests that distinct molecular phenotypes may characterize tumors arising from HPV compared with those arising from exposure to tobacco smoke and alcohol use. These molecular distinctions may also better explain the striking difference in patient response by tumor HPV status, which has been hypothesized to be related to better radiation response of HPV-positive tumors (42-44).

There was, however, a strong positive association between patient BMI 5 years before diagnosis and *LRE1* relative methylation level. The ascertainment of BMI

Table 3. Cox proportional hazards model of survival, overall and stratified by tumor HPV DNA status

	Overall	In tumor HPV DNA-negative patients		In tumor HPV DNA-positive patients
		HR (95% CI)		
Tumor <i>LRE1</i> methylation				
High (≥ 0.45)	1.0 (reference)	1.0 (reference)		1.0 (reference)
Low (< 0.45)	1.7 (1.0-2.7)	1.8 (1.0-3.0)		1.3 (0.5-4.0)
Tumor HPV DNA status				
Negative	1.0 (reference)	—		—
Positive	0.8 (0.4-1.4)	—		—

NOTE: All models are controlled for tumor stage, age, and gender. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

before diagnosis is to prevent potential confounding of patient weight by the development of disease. This finding is of interest, as greater BMI has been linked to protection from risk for head and neck squamous cell carcinoma (15-19), although the mechanism underlying this protective association remains elusive. These results suggest that a higher BMI may play a role in maintaining the DNA methylation status of target tissue, and through this effect a protection from the disease. This is most likely metabolic, perhaps related to better or greater nutrient intake by individuals with higher BMI. It would be of interest to examine if similar trends can be observed in nondiseased tissues based on BMI, or if specific somatic tissues are more susceptible to the effect of nutrition on the epigenetic state.

We observed that tumors with lower *LRE1* relative methylation predict poorer survival, and this is particularly the case in HPV-negative individuals. Those HPV-negative individuals already have a poorer overall survival compared with those individuals whose tumors were HPV16 DNA positive (14, 42-44). This suggests that tumors with reduced *LRE1* methylation have a more aggressive phenotype or have greater resistance to the therapeutic modalities aimed at the disease. These differences may be due to the genomic instability linked to reduced methyl-cytosine content (45-47). This finding could be clinically useful, as patients with reduced methylation levels may benefit from more aggressive therapies.

We have shown an association between smoking and hypomethylation in tumors from head and neck squamous cell carcinoma patients, suggesting an additional mechanism for tobacco smoke in initiating genomic instability. Additionally, we observed that alterations in the extent of genomic methylation may have clinical relevance, a finding that should be further explored.

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