Smoking and Drinking Can Induce *p15* Methylation in the Upper Aerodigestive Tract of Healthy Individuals and Patients with Head and Neck Squamous Cell Carcinoma

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BACKGROUND. The consumption of tobacco and alcohol has been implicated in the development of head and neck squamous cell carcinoma (HNSCC). Promoter methylation of tumor suppressor genes is common in HNSCC. In this study, the authors evaluated the effects of tobacco and alcohol on *p15* gene methylation of cells in cells from the mouth and throat of physically healthy individuals and patients with HNSCC.

METHODS. The study participants were divided into 3 groups, including a group of 37 healthy nonsmokers and nondrinkers, a group of 22 healthy smokers and/or drinkers and a group of 31 patients with HNSCC.

RESULTS. Methylation of *p15* was detected in cells obtained from mouth and throat (M&T) rinsing fluid samples from 3 of 37 healthy individuals (8%) who did not drink or smoke, from 15 of 22 healthy smokers and/or drinkers (68%), and from 15 of 31 patients (48%) with HNSCC. Among 31 patients with HNSCC, 20 patients (65%) had methylated *p15* gene in their tumor biopsies. With the use of *β-actin* as a reference, the ratio of methylated *p15* to *β-actin* was calculated as an index of the percentage of cells with *p15* methylation. The percentage of exfoliated cells from M&T rinsing fluid samples that had *p15* methylation ranged from 0% to 11% for patients with HNSCC and from 0% to 21% for healthy smokers/drinkers, respectively. The methylation index of tumor cells with *p15* methylation ranged from 0% to 65%.

CONCLUSIONS. The results suggest that *p15* gene methylation can be induced by chronic smoking and drinking and may play a role in the very early stages of carcinogenesis in HNSCC. ***Cancer* 2004;101:125–32.**

KEYWORDS: promoter hypermethylation, *p15* gene, head and neck squamous cell carcinoma, mouth and throat rinsing fluid, methylation index.

Head and neck carcinoma is common worldwide. Both tobacco and alcohol consumption are well documented risk factors with synergistic effects in the pathogenesis of head and neck squamous cell carcinoma (HNSCC).[1] Tobacco-induced genetic changes have been reported in healthy individuals exposed to tobacco.[2,3] The elucidation of genetic markers in the early stage of carcinogenesis is important for evaluating cancer risk, monitoring and evaluating the efficacy of chemopreventive therapy, and screening for premalignancy and early carcinoma. In HNSCC, promoter methylation of tumor suppressor genes is a common and important feature of the disease.[4,5] However, to our knowledge, the role of methylation as an early genetic aberra-

Supported by the Betty and Kadoorie Cancer Research Fund, the Ho Hung Chiu Cancer Research Fund, and a research grant from the University of Hong Kong.

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Received November 13, 2003; revision received March 8, 2004; accepted April 6, 2004.

DOI 10.1002/cncr.20323
Published online 25 May 2004 in Wiley InterScience (www.interscience.wiley.com).

tion in the early stage of carcinogenesis in relation to cigarette or/and alcohol consumption has not been elucidated fully.

The detection of tumor-specific gene methylation in various body fluid samples in patients with carcinoma, and its rare detection in normal healthy individuals, demonstrates the possibility of using methylated promoter DNA as a tumor marker for screening or identifying early genetic aberrations within the high-risk group who have had been exposed repeatedly to carcinogen insult.[6,7] A recent study identified mutation in archived sputum samples before the diagnosis of lung carcinoma, suggesting its potential implication in noninvasive screening for premalignant conditions or early-stage malignancy.[8]

P15 is an effector of transforming growth factor β (TGF-β)-induced cell cycle arrest.[9] Inactivation of *p15* by promoter hypermethylation has been observed in many human malignancies, suggesting that its down-regulation may be essential in neoplastic transformation.[10,11] We also found a high frequency of methylation of both *p15* and *p16* gene in HNSCC tumors, and methylation of the *p15* gene also was found frequently in histologically normal resection margins in the mucosa of patients with HNSCC who were smokers.[12] Therefore, we postulated that methylation of the *p15* gene may play a significant role in the very early stages of carcinogenesis in HNSCC before the clinical or histologic appearance of premalignant lesions or invasive carcinoma. In the current study, our objective was to evaluate the role of *p15* methylation in the early stages of carcinogenesis in HNSCC in relation to smoking and drinking.

MATERIALS AND METHODS

Patient Selection

This study was approved by the Ethics Committee of the University. All patients and healthy donors involved in the study had signed an agreement form consenting to the donation of their specimen for medical research. Healthy individuals were defined as individuals without any cancer in their past history and at the time of specimen donation. The study participants were divided into three groups: healthy nonsmokers/nondrinkers, healthy smokers/drinkers, and patients with HNSCC.

Details of Smoking and Drinking Habits

The details of smoking and drinking habits were documented by physicians. Details regarding smoking and drinking habits included smoking/drinking status (current, former, or never), duration of consumption, frequency per week, types of alcohol, and quantity (number of cigarettes consumed per day and mL of alcoholic beverages consumed per day). Table 1 summarizes the demographic characteristics of patients with details of cigarette and alcohol consumption. Total cigarettes smoked were calculated by multiplying the average daily consumption of cigarettes (in numbers) and the history of smoking (in days). Total alcohol consumption was calculated by multiplying the average daily consumption of alcohol (in liters) and the history of drinking (in days). Individuals with frequent consumption of quantifiable amounts of cigarette or alcohol were classified as "smokers or/and drinkers". Individuals who never smoked and seldom drank or who drank only small quantities occasionally were classified as "nonsmokers/nondrinkers".

TABLE 1
Demographic Characteristics of Patients with Details of Tobacco and Alcohol Consumption

	No. (%)		
Characteristic	Healthy nonsmokers/ nondrinkers (37) (n = 37)	Healthy smokers and/ or drinkers (n = 22)	Patients with HNSCC (n = 31)
Gender			
Male	15 (41)	20 (91)	23 (74)
Female	22 (59)	2 (9)	8 (26)
Age (yrs)			
Range	24–84	16–81	27–77
Mean	49	43	51
Median	53	52	54
Nondrinking smokers	0 (0)	6 (27)	12 (38)
Nonsmoking drinkers	—	2 (9)	1 (3)
Drinkers and smokers	0 (0)	14 (64)	8 (25)
Nonsmokers/nondrinkers	37 (100)	0 (0)	11 (34)
Smoking history of smokers			
Total cigarettes consumed ($\times 10^3$)			
Range	—	0.4–569.4	1.5–985.5
Median	—	137.8	182.5
Smoking duration (yrs)			
Range	—	1–59	2–54
Median	—	35	30
Drinking history of drinkers			
Total alcohol consumed ($\times 10^3$)			
Range	—	0.07–9.3	0.3–11.0
Median	—	1.1	3.9
Drinking duration (yrs)			
Range	—	2–51	10–47
Median	—	22	33

HNSCC: head and neck squamous cell carcinoma.

Samples of Exfoliated Cells in Mouth and Throat Rinsing Fluid from Healthy Volunteers of Different Ages With or Without a History of Tobacco and Alcohol Consumption and from Patients with HNSCC

Samples of cells from mouth and throat (M&T) rinsing fluid from 37 healthy nonsmokers/nondrinkers, 22

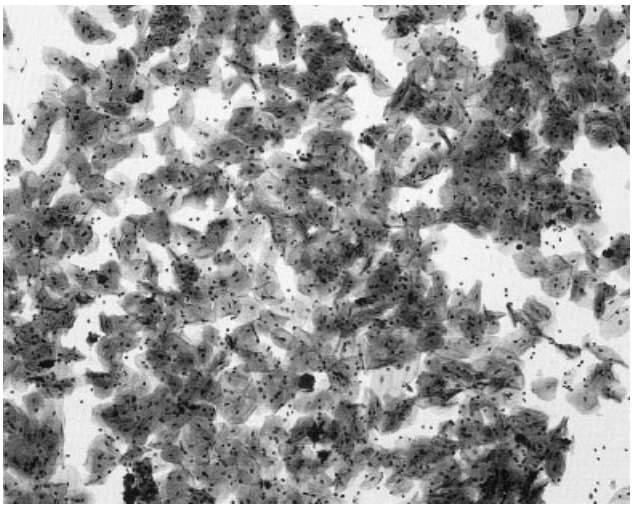

FIGURE 1. Exfoliated cells in mouth and throat rinsing fluid from healthy, normal control individuals (original magnification, × 100).

healthy smokers or/and drinkers, and 31 patients with HNSCC were collected from the Department of Surgery at Queen Mary Hospital, The University of Hong Kong. Thirty-one paired, fresh-frozen primary tumor biopsy specimens (19 from the oral cavity, 6 from the oropharynx, 2 from the hypopharynx, and 4 from the larynx) also were available for the study. Tumors were staged according to the 1997 American Joint Committee on Cancer staging system: There were 7 Stage I tumors, 13 Stage II tumors, 8 Stage III tumors, and 3 Stage IV tumors.

DNA Isolation and Morphologic Analysis of Exfoliated Cells in M&T Rinsing Fluid Samples

Collection and preservation of cells from the mouth and throat and DNA isolation were described in our previous report.[6,7] Cells from the mouth and throat were collected by rinsing the mouth and throat with 20 mL of 0.9% normal saline. The exfoliated cells in the M&T rinsing fluid were collected immediately by centrifugation at × 400 *g* for 10 minutes. The cell pellets were resuspended in 400 μL of phosphate-buffered saline for storage at − 80 °C until use. Selected M&T rinsing cell suspensions were processed by cytospin and were examined under the microscope. It was observed that these exfoliated cells were mostly normal appearing, nucleated epithelial cells intermixed with < 1% inflammatory and tumor cells (Fig. 1). DNA from M&T rinsing fluid cells was extracted with a Nucleospin Blood Mini Kit (Clontech, Palo Alto, CA).

Design of Primers and Probes

In this study, all oligonucleotide primers and probes for the target gene, *p15*, or the internal reference gene, *β-actin,* were designed specifically to amplify bisulfite-converted DNA. The amplicon of the internal reference gene, *β-actin*, contains no CpG nucleotides; whereas the amplicon of the target gene, *p15* contains seven CpG nucleotides (two in the probe, three in the antisense primer, and two in other sites). Amplification of the internal reference gene, *β-actin*, is independent of its methylation status; whereas amplification of *p15*, as assessed by the copy numbers generated during polymerase chain reaction (PCR), is dependent on the methylation status within the *p15* promoter.

Cloning and Sequencing

Conventional methylation-specific PCR (MSP) was performed on bisulfite-converted genomic DNA from randomly selected oral rinsing and tumor specimen using the same *p15* primer pair for real-time PCR. Fragments of PCR products were purified by Concert Matrix Gel Extraction System (Invitrogen, Carlsbad, CA) and subsequently ligated to pGEM-T Easy (Promega, Madison, WI). Positive clones were sequenced by a BigDyeTerminator cycle sequencing kit using primer targeted to either T7 or SP6 promoter sequence and analyzed by ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Bisulfite Modification and Real-Time, Quantitative MSP

Bisulfite modification was carried out using a CpGenome™ DNA Modification Kit (Intergen, New York, NY). Sodium bisulfite-treated genomic DNA was amplified by fluorescence-based, real-time MSP, as described previously.[13] The fluorogenic probes and oligonucleotide primers were custom synthesized by PE Applied Biosystems (PerkinElmer Corporation, Oak Brook, IL). The sequences of the primers and probe used to amplify and detect *p15* (GeneBank S75756) were 5′-GAGGGTAATGAAGTTGAGTTTAGGTTTT-3′ (sense primer), 6FAM-5′-AGC GTG GGA AAG AAG GGA AGA GTG TCG-3′-TAMRA (probe), and 5′-CGAATAATCCACCGTTAACCGTAA-3′ (antisense primer). The sequences of the primers and probe used to amplify and detect *β-actin* (GeneBank Y00474) were 5′-GGAGGTTTAGTAAGTTTTTTGGATTGTG-′3 (sense primer), 6FAM-5′-TGTGTTTGTTATTGTGTGTTGGGTGGTGGT-3′-TAMRA (probe), and 5′-AAAACCTACTCCTCCCTTAAAAATTACA-′3 (antisense primer). The copy numbers of *p15* and *β-actin* in each sample were derived from the standard curve of universal methylated DNA (1 copy, 10 copies, 100 copies, or 1000 copies).

Statistical Methods

Statistical analyses were conducted using the SPSS statistical package (SPSS, Inc, Chicago, IL). Frequency

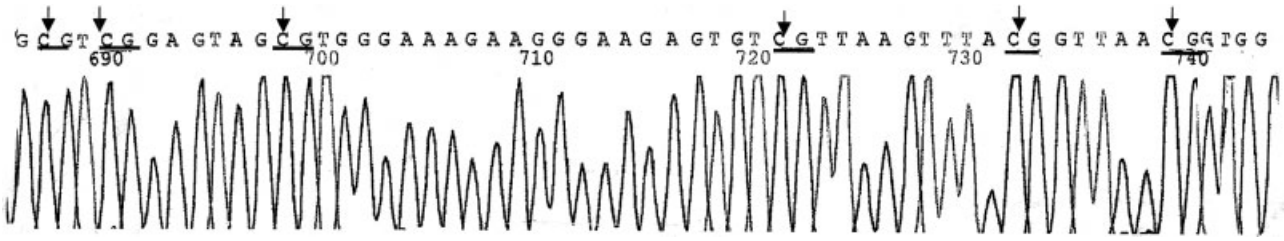

FIGURE 2. Sequencing analysis of *p15* amplicon from a tumor biopsy (Patient 1087). CpG dinucleotides were retained and are indicated by arrows and underlines.

distribution and Mann–Whitney *U* tests were used for ordinal data. All statistical tests were two-tailed and were examined at 95% confidence. To calculate the quantity of tobacco and alcohol consumption in relation to *p15* methylation, two major factors were chosen for statistical analysis: duration and quantity. Correlations between the *p15* M&T rinsing methylation index and continuous variable factors, including age and alcohol/tobacco consumption, were determined by using the Spearman ρ correlation coefficient. The level of significance for analyses of variables was set at $P < 0.05$.

RESULTS

Molecular Cloning and Sequencing of *p15* MSP Products

Sequencing analysis of pGEM clones showed the retention of seven CpG sites in the *p15* amplicons from M&T rinsing exfoliated cells and tumor biopsies (Fig. 2). The results confirmed that, from all samples that contained a heterogeneous mixture of methylated versus unmethylated *p15* gene, methylated *p15* gene was amplified selectively by the primers designed specifically for real-time PCR.

Methylation of *p15* Gene in Healthy Individuals and in Patients with HNSCC

The *β-actin* gene copy number can be used as an indicator of the total amount of cells in the sample. The ratio of *p15* to *β-actin* can be applied as the percentages of cells from the respective samples with *p15* methylation. The *p15* methylation index = (*p15*/*β-actin*) × 100 (%). The plots of the percentage of cells with *p15* methylation from respective samples from HNSCC and normal healthy individuals are summarized in Figure 3.

All 37 nonsmokers and nondrinkers were never smokers, and 4 were occasional social drinkers of small quantities (Table 1). A summary of gene copy numbers, *p15* methylation indices, and frequencies of methylated *p15* gene in the respective groups is provided in Table 2. Aberrant methylated *p15* gene was detected in 3 individuals (8%). The *p15* methylation

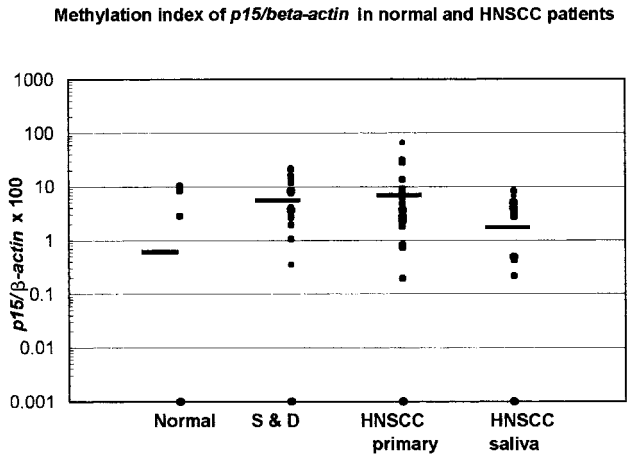

FIGURE 3. Distribution of *p15* methylation levels in samples from normal, healthy individuals and from patients with head and neck squamous cell carcinoma (HNSCC). Each circle represents a different sample. Solid horizontal bars indicate the mean value of the ratios of methylated *p15*/β-actin within a group of patients (original magnification, × 100). Normal: normal, healthy nonsmokers/nondrinkers; S & D: healthy smokers/drinkers; HNSCC primary: primary tumors from patients with HNSCC; HNSCC saliva: exfoliated mouth and throat cells from patients with HNSCC.

indices for these 3 individuals were 2.8%, 9.48%, and 10.3%, respectively.

Among 22 healthy individuals who were chronic smokers or/and drinkers, aberrant methylated *p15* gene was observed in 15 individuals (68%). Four of 6 nondrinking smokers (67%) and 9 of 14 individuals who were both smokers and drinkers (64%) demonstrated various copy numbers of methylated *p15* gene (Table 2). There were only 2 nonsmoking drinkers, and their *p15* methylation indices were 8.27% and 10.48%, respectively.

Among 31 patients with HNSCC, methylation of *p15* was found in 20 patients (65%) with primary HNSCC tumors and in 15 of 31 samples (48%) of matched exfoliated cells from M&T rinsing fluid. There were five patients with HNSCC (including three patients with HNSCC of the oral cavity, one patient with HNSCC of the oropharynx, and one patient with

TABLE 2
The *p15* and β-Actin Gene Copies, *p15* Methylation Indices, and Frequencies of *p15* Gene Methylation in Samples from Healthy Nondrinkers and Nonsmokers, Healthy Smokers and/or Drinkers, and Patients with Head and Neck Squamous Cell Carcinoma

	Exfoliated cells from mouth and throat rinsing fluid			
Gene	Healthy nondrinkers and nonsmokers (n = 37)	Healthy smokers and/or drinkers (n = 22)	Patients with HNSCC (n = 31)	Primary tumor cells from 31 patients with HNSCC
No. of *p15* copies (range)	0–33	0–430	0–190	0–1412
No. of methylated *p15* copies[a]				
Range	—	2–430	2–190	2–1412
Median	—	45.52	40.47	158
Mean	—	22.10	11.0	30.0
No. of β-actin copies				
Range	41–10,000	59–5700	17–5600	40–14,424
Mean	607.17	1293.27	1111.1	1871.55
Median	171.0	572.5	390.0	864.0
p15 Methylation index (range)	0.0–10.31%	0.0–21.15%	0.0–11.0%	0.0–65.0%
Positive *p15* methylation index[a]				
Range	—	0.354–21.15%	0.2–11%	0.72–65.31%
Mean	—	5.28%	3.67%	9.9%
Median	—	3.13%	3.03%	3.5%
No. with methylated *p15* genes in each group				
Nondrinking smokers	0	4/6	4/12	6/12
Nonsmoking drinkers	0	2/2	0/1	1/1
Drinkers and smokers	0	9/14	4/7	5/7
Nonsmokers and nondrinkers	3/37	—	7/11	8/11
Total (%)	3/37 (8)	15/22 (68)	15/31 (48)	20/31 (65)

HNSCC: head and neck squamous cell carcinoma.
[a] This representation reflects the true distribution of methylated *p15* gene in tumors and in exfoliated mouth and throat cells.

HNSCC of the hypopharynx) who had methylated *p15* gene in their tumors but not in their oral rinsing samples. The drinking and smoking habits of these five patients included two nondrinking smokers, one nonsmoking drinker, one smoker/drinker, and one nonsmoker/nondrinker. Of the 11 patients with primary HNSCC tumors that showed no methylated *p15* gene, methylation of the *p15* gene was not detected in their matched M&T rinsing fluid samples. Of 31 patients with HNSCC, 20 patients (65%) had regular consumption of alcohol or/and tobacco. Among those 20 patients with HNSCC who were smokers or/and drinkers, aberrant methylated *p15* was detected in 12 primary tumor tissue samples (60%) and in 8 M&T rinsing fluid samples (40%). Of 11 patients with HNSCC who were nonsmokers and nondrinkers, 8 patients (73%) and 7 patients (64%) had methylated *p15* gene in their tumors and in their paired M&T rinsing fluid samples, respectively (Table 2).

Statistical Analysis

The differences in the methylation index for the *p15* gene of various groups were compared (Table 3). In relation to *p15* gene methylation, there seemed to be a significant difference between healthy smokers/drinkers and nonsmokers and nondrinkers ($P < 0.05$). However, no significant difference was found in relation to smoking and drinking status in patients with HNSCC (all $P > 0.05$).

Methylation of the *p15* gene was evaluated for correlations with various factors, including age, quantity of tobacco, and tobacco and/or alcohol consumption among healthy smokers/drinkers or among smokers/drinkers with HNSCC (Table 4). We had only three nonsmoking drinkers in this study; thus, sole effect of alcohol consumption on *p15* methylation was not analyzed. There were no significant correlations between the *p15* methylation index and the amount of tobacco, the tobacco and alcohol intake, or the duration of tobacco or alcohol consumption among healthy smokers/drinkers and among smokers/drinkers with HNSCC (Spearman ρ, all $P > 0.05$). However, there was a significant correlation between the *p15* methylation index and the amount of tobacco intake among patients who had *p15* gene metylation.

TABLE 3
Statistical Analysis of *p15* Methylation Index in Exfoliated Mouth and Throat Cells

Characteristic	Mann–Whitney *U* test	*P* value (two-tailed)
Healthy smokers vs. healthy nonsmokers	181.0	0.00
Healthy smokers/drinkers vs. healthy nonsmokers/nondrinkers	164.0	0.00
HNSCC smokers vs. HNSCC nonsmokers	88.0	0.247
HNSCC smokers/drinkers vs. HNSCC nonsmokers/nondrinkers	76.0	0.131

HNSCC: head and neck squamous cell carcinoma.

TABLE 4
Correlation between the *p15* Methylation Index in Exfoliated Mouth and Throat Cells and the Quantity of Tobacco and Alcohol Consumption[a]

Characteristic	Spearman ρ	*P* value (two tailed)
Age	0.067	0.529
Quantity of tobacco consumption (healthy nondrinking smokers)		
Duration (yrs)	0.319	0.538
Quantity (no. of cigarettes consumed per day)	0.00	1.00
Total quantity (total cigarettes consumed)	0.203	0.7
Quantity of both tobacco and alcohol consumption (healthy smokers and drinkers)	−0.182	0.533
Quantity of tobacco consumption (HNSCC nondrinking smokers)		
Duration (yrs)	−0.025	0.938
Quantity (no. of cigarettes consumed per day)	0.011	0.974
Total quantity (total cigarettes consumed)	0.006	0.985
Quantity of both tobacco and alcohol consumption (HNSCC smokers and drinkers)	0.334	0.462
Quantity of tobacco consumption (HNSCC patients with methylated *p15* gene)	0.676	0.00

HNSCC: head and neck squamous cell carcinoma.
[a] This analysis did not include nonsmoking drinkers (one healthy individuals and two patients with HNSCC).

DISCUSSION

Methylation of the tumor suppressor gene by carcinogen insult has been implicated as an early event in carcinogenesis.[14] Indeed, a recent study showed that target cells affected by carcinogens exhibited increased DNA-methyltransferase activity and a marked increase in overall DNA methylation during lung carcinoma progression, suggesting epigenetic change in the very early stages of cancer development.[15] The major advantage of using the real-time, PCR-based assay, compared with conventional MSP, is the possible quantification of the degree of tumor-specific gene hypermethylation in the sample.

By using a real-time MSP assay, we observed a significantly higher frequency of aberrant methylated *p15* gene in oral and throat mucosal cells from healthy smokers/drinkers (68%) compared with nonsmokers/nondrinkers (8%). This suggests that smoking and drinking can induce *p15* methylation in upper aerodigestive tract mucosa. The majority of smokers and drinkers in the current study were heavy smokers and drinkers of long duration. The presence of methylated *p15* gene was detected in three nonsmokers/nondrinkers. This observation suggests that other minor etiologic factors (such as pollution, passive smoking, chemicals, food, etc.) also may play a role in *p15* methylation.[16,17]

The frequency of methylation in the M&T rinsing fluid samples from chronic smokers and/or drinkers was 68% and was comparable to the frequency of 65% in primary tumor samples from patients with HNSCC. The slightly lower (48%) *p15* methylation frequency in exfoliated cells from the mouth and throat of patients with HNSCC was due to the few patients with HNSCC who had positive methylation in their primary tumors but negative methylation in their oral exfoliated cells. Those patients also had no *p15* methylation in the rest of their oral and throat mucosa. Random bias of sample selection also may have contributed to the paradoxical percentage. The other possible reason for the discrepancy is that only 65% of patients with HNSCC had smoking or/and drinking habits, compared with 100% of the physically healthy smokers/drinkers. Smoking has been associated with other genetic abnormalities. In an analysis of DNA adducts and H-*ras* mutations in human laryngeal tissue (n = 16 patients) from patients with laryngeal carcinoma who were both smokers and nonsmokers/exsmokers, DNA adducts were evident only in smokers (n = 13 patients), and smoking cessation even for only 10 months resulted in the detection of no DNA adducts (n = 3 patients).[18] In the current study, the finding of *p15* methylation in exfoliated cells in M&T fluid samples from both healthy smokers/drinkers but rarely in healthy nonsmokers/drinkers demonstrated that smoking can induce *p15* methylation of epithelial cells of the upper aerodigestive tract.

The fact that *p15* methylation has been documented in many types of human malignancies that are not associated with smoking and drinking may explain why, despite the tight association of smoking and *p15* methylation, 7 of 11 nonsmokers (63%) with HNSCC

had *p15* gene methylation. Smoking and drinking are not the only factors that induce *p15* methylation in HNSCC carcinogenesis. Many patients HNSCC have no known predisposing factors identified, including a history of smoking or drinking. In addition, *p15* methylation is common in HNSCC and is found frequently in both smoking/drinking-related tumors and nonsmoking/nondrinking-related tumors.[10–12] Thus, there may be multiple pathways leading to *p15* methylation in the process of cancer development, including HNSCC.[19–21] The mechanisms of interaction of carcinogens within tobacco and alcohol with other etiologic factors or genetic aberrations require further elucidation.

It has been well documented that smoking and drinking are risk factors for HNSCC. It also has been well documented that *p15* methylation plays a role in the development of carcinoma, including HNSCC. The missing link we want to establish is whether smoking and drinking are related to *p15* methylation. We have found methylation of the *p15* gene under repeated insult of tobacco or/and alcohol in healthy individuals and in patients with HNSCC, suggesting that smoking and drinking can induce *p15* methylation in the upper aerodigestive tract. Thus, *p15* methylation may be an important feature in the very early stages of HNSCC development. We also previously found *p15* methylation in normal margins from patients with HNSCC who smoked.[12] This implies that *p15* methylation is present before HNSCC is developed fully in the stepwise carcinogenesis process and may be a marker of field cancerization, particularly in relation to smoking. Cancer development in relation to smoking and drinking is a very lengthy process over decades. We have not determined the lifetime risk of cancer development among apparently healthy smokers and drinkers who have *p15* methylation. Apart from smoking and drinking insult, other genetic aberrations are necessary in the multistep development of HNSCC.[19–21] Further studies will be required to evaluate the risk of developing cancer in these healthy smokers and drinkers and to elucidate the effect of *p15* methylation in association with other predisposing factors and genetic aberrations in the carcinogenesis of HNSCC in relation to smoking and drinking.

In the current study, we found that *p15* methylation was related closely to the regular consumption of tobacco and alcohol. Although determining the lifetime risk of developing HNSCC in individuals with *p15* methylation will require further study, there has been a report of chronic smokers who harbored an epigenetic alteration and subsequently developed lung carcinoma.[22] A recent study of the genetic alterations of *p53*, K-*ras*, and *p16* in symptomatic, chronic smokers and in patients with lung carcinoma found *p53* mutations and *p16(INK4a)* promoter hypermethylation in chronic smokers. Three of eight chronic smokers who harbored an epigenetic alteration were diagnosed subsequently with lung carcinoma.[22] Our results and those from other reports have demonstrated clearly that smoking can induce multiple genetic aberrations that can be identified in apparently healthy epithelial cells before a clinical presentation of carcinoma.

The presence of *p15* methylation found in mouth and throat epithelial cells from healthy control smokers/drinkers and patients with HNSCC, but rarely found in healthy control nonsmokers/nondrinkers, demonstrated that *p15* methylation is an early step in the carcinogenesis of HNSCC in relation to chronic smoking and drinking. Determining the lifetime risk in these healthy smokers and drinkers with cellular *p15* methylation in mouth and throat epithelial cells will require further study.

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