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MicroRNA-137 promoter methylation in oral rinses from patients with squamous cell carcinoma of the head and neck is associated with gender and body mass index

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Head and neck cancer represents 3.3% of all new malignancies and 2.0% of cancer deaths in the USA, the majority of which are squamous in origin. The overall 5 year survival is 60% and worsens with increasing stage at diagnosis. Thus, novel biomarkers for early detection of squamous cell carcinoma of the head and neck (SCCHN) are needed. MicroRNA-137 (miR-137) plays a role in cell cycle control and seems to undergo promoter methylation in oral squamous cell carcinoma tissue. The main objectives of this study were to ascertain whether miR-137 promoter methylation is detectable in oral rinse samples, assess its association with SCCHN and identify potential risk factors for its occurrence. Oral rinse samples were collected from 99 SCCHN patients with no prior history of cancer and 99 cancer-free controls, frequency matched on gender; tumor tissue for 64 patients was also tested. Methylation of the miR-137 promoter, assessed using methylation-specific polymerase chain reaction, was detected in 21.2% oral rinses from SCCHN patients and 3.0% from controls [odds ratio (OR) = 4.80, 95% confidence interval (CI): 1.23–18.82]. Among cases, promoter methylation of miR-137 was associated with female gender (OR = 5.30, 95% CI: 1.20–23.44) and inversely associated with body mass index (BMI) (OR = 0.88, 95% CI: 0.77–0.99). Promoter methylation of miR-137 appears to be a relatively frequently detected event in oral rinse of SCCHN patients and may have future utility as a biomarker in DNA methylation panels. The observed associations with gender and BMI help to shed light on potential risk factors for an altered methylation state in SCCHN.

Introduction

In 2008, head and neck cancer accounted for an estimated 47 560 new cases in the USA, representing 3.3% of all malignancies and 11 260 deaths (1). The majority of these (93%) are squamous cell carcinoma of the head and neck (SCCHN) (2). Use of tobacco and alcohol is each independently causally associated with development of this disease and when combined have a synergistic multiplicative effect (3,4). The 5 year survival in SCCHN (∼60%) has remained essentially unchanged over the past three decades despite therapeutic advances (3). The majority of patients present with advanced stage disease (stage III or IV) (3) and survival drastically declines with increasing stage at diagnosis. There is therefore a need to assess novel biomarkers that could aid in the early detection of SCCHN.

DNA promoter methylation, also known as hypermethylation, is an epigenetic change that often occurs as an early event in carcinogenesis (5), resulting in reduced or lost expression of the methylated gene. Aberrant promoter methylation is considered to be at least as common as DNA mutation in the inactivation of tumor suppressor genes. Altered microRNA expression also often manifests early in carcinogenesis (6). An estimated 10% of microRNAs are regulated epigenetically through DNA methylation (7).

MicroRNA-137 (miR-137) is associated with a large CpG island and has been reported to undergo promoter methylation in oral squamous cell carcinoma (OSCC) (8), gastric cancer cell lines (9) and colon cancer (10). Prior evidence suggests that promoter methylation correlates with downregulation of miR-137 in OSCC relative to non-cancerous oral tissue (8). miR-137 appears to play a role in cellular differentiation and cell cycle control, at least in part through negative regulation of Cdk6 expression (8,11). It is hypothesized that overexpression of Cdk6 may result in accelerated progression through the G_1/S-phase checkpoint of the cell cycle, thus leading to increased proliferation and reduction in DNA repair capacity (12).

Oral rinse is a simple, non-invasive mode of DNA collection from the upper aerodigestive tract and can be used to detect promoter methylation for SCCHN (13–17). The goal of this study was to evaluate *miR-137* promoter methylation as a potential biomarker of SCCHN with a case–control design using oral rinse samples as a non-invasive, non-differential mode of DNA collection from case and control subjects. Additionally, this study sought to investigate the association of miR-137 promoter methylation with smoking, alcohol consumption and other potential risk factors for SCCHN.

Abbreviations: BMI, body mass index; CI, confidence interval; FFPE, formalin-fixed paraffin embedded; miR-137, microRNA-137; NPV, negative predictive value; OR, odds ratio; OSCC, oral squamous cell carcinoma; PPV, positive predictive value; SCCHN, squamous cell carcinoma of the head and neck.

Materials and methods

Study population

This study was conducted as part of an epidemiology study in the University of Pittsburgh Head and Neck Specialized Program of Research Excellence (SPORE). Subjects included 99 consecutive adult patients (≥18 years of age) with primary squamous cell carcinoma of the oral cavity, pharynx or larynx, diagnosed at the University of Pittsburgh Medical Center (Pittsburgh, PA) between September 2007 and April 2009, with no prior history of cancer, and 99 control subjects, frequency matched on gender. Control subjects were randomly selected from a pool of cancer-free patients with no prior history of malignancy seeking treatment at the University of Pittsburgh Department of Otolaryngology during the same time frame. Institutional Review Board approval was obtained under the University of Pittsburgh Head and Neck Cancer Specialized Program of Research Excellence for sample collection and use of patient data. All subjects provided written informed consent for participation in this study.

Data sources

Study subjects completed an epidemiologic questionnaire providing detailed demographic, personal and family cancer history and behavioral risk factor information. Clinical data from the cancer patients was collected at the time of diagnosis and entered into the University of Pittsburgh Head and Neck Oncology Registry.

Oral rinse samples

All subjects provided an oral rinse sample, obtained by swishing with 20 ml of saline for 20–30 s. Approximately 10 ml of commercial mouthwash (Scope^{TM}) was added as a DNA preservative, and the samples were subsequently frozen at −20°C until DNA was extracted. All samples (n = 99) from the head and neck cancer patients were collected prior to treatment. DNA was isolated and analyzed for miR-137 promoter methylation as indicated below.

Tumor samples

For 64 of the 99 cases (64.6%), archival formalin-fixed paraffin embedded (FFPE) tumor tissue resected prior to initiation of radiation or chemotherapy was obtained. This subset of paired samples was representative of the overall sample of head and neck cancer patients included in the original study, with an average age of 59.7 years ($P = 0.92$ versus age in total cases); the proportion of females was 23.4% ($P = 0.71$ versus female in total cases) and the distribution of site was 39.1% oral cavity, 34.4% pharynx and 26.6% larynx ($P = 0.94$ versus total cancer cases). DNA was isolated and analyzed for *miR-137* promoter methylation as indicated below. Methylation of the *miR-137* promoter in the DNA isolated from tissue samples was then compared with *miR-137* promoter methylation in the DNA isolated from oral rinse samples.

Methylation-specific polymerase chain reaction

DNA was extracted from buccal cells in oral rinse samples using the Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, MN) and from the FFPE tumor tissue using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). DNA concentrations were quantified with the NanoDrop 1000™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and sodium bisulfite treatment was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA).

Methylation-specific polymerase chain reaction was employed for the analysis of miR-137 promoter methylation, and the polymerase chain reaction products were then analyzed by separation on high-resolution 4% agarose E-Gels® (Invitrogen, Carlsbad, CA). The primer set was designed using Methyl Primer Express® software v 1.0 (Applied Biosystems, Foster City, CA), and primer sites were checked for genetic polymorphisms using the Enable Genome Browser (18). The primer sequences used to assess *miR-137* promoter methylation were as follows: methylated alleles— 5′-GCGGTAGTAGTAGCGGTAGC-3' and 5′-ACCCGTCACCGAAAAAAA-3' with an annealing temperature of 58°C and expected amplicon of 86 bp; and unmethylated alleles— 5′-GGTGGTAGTAGTAGTGGTAGT-3' and 5′-TACCCATCACCAAAAAAAA-3' with an annealing temperature of 51°C and expected amplicon of 86 bp. As a quality control check, each polymerase chain reaction included fully methylated and unmethylated bisulfite-converted human DNA (QIAGEN) as positive and negative controls. Samples yielding faint positive signals were repeated twice more and only consistently positive samples were considered to be methylated.

Statistical analysis

Descriptive statistics of the study population were generated separately by case–control status and by *miR-137* promoter methylation status. Categorical variables were compared using Fisher's exact test. The Skewness–Kurtosis test (19) was used to determine if continuous variables were normally distributed. The Mann–Whitney *U*-test was used to compare non-normally distributed continuous variables; otherwise, a two-sample *t*-test was used. The sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) of miR-137 for distinguishing patients with SCCHN from those with non-cancerous ear, nose and throat conditions were calculated. This was performed overall for SCCHN and then by anatomic site.

Multivariable logistic regression modeling was used to estimate the association between miR-137 promoter methylation and SCCHN. A forward stepwise selection approach was employed to identify the preliminary main effects model, comparing the log likelihood of each model ($P_E = 0.15$; $P_R = 0.20$) (20). All variables with $P \leq 0.25$ in univariate analysis were considered for the model. All biologically plausible interactions were evaluated and considered further if $P \leq 0.15$. Functional form of the continuous variables was assessed using fractional polynomials (21), and any polynomial term providing a significant improvement over the linear term at $P \leq 0.10$ was considered. Global fit of the model was assessed using the Hosmer and Lemeshow goodness-of-fit test (22); lack of fit was considered significant if $P \leq 0.10$. Subsequent subgroup analyses were performed restricted to the cases of a specific tumor site (oral cavity, pharynx or larynx) compared with all controls.

A second multivariable logistic regression model restricted to SCCHN patients was developed to identify potential risk factors for *miR-137* promoter methylation. A modified forward stepwise model selection approach was employed ($P_E = 0.15$; $P_R = 0.20$), starting with smoking duration and alcohol dose already in the model. Otherwise, model checks were similar to those in the first model. An interaction term for smoking and alcohol consumption was tested, considered for the model at $P \leq 0.15$. To account for the right skewness in smoking duration due to never-smokers (0 years) when modeled continuously, a binary ever-smoking/never-smoking term was included in the model and considered additively with smoking duration ($y = \beta_1 \times X_{\text{ever/never}} + \beta_2 \times X_{\text{years smoking}}$), where never smoking = 0 and ever smoking = 1 (23).

Two additional models were created using the same methodology described above, including fruit and vegetable consumption and stage at diagnosis, respectively, to generate adjusted estimates for these covariates. Fruit and vegetable consumption was modeled continuously as log average daily servings of fruits and vegetables, and stage at diagnosis was modeled dichotomously as 'local' (American Joint Committee on Cancer stage grouping I or II) or 'advanced' (stage III or IV).

Sensitivity and specificity was calculated for the ability of oral rinse to detect *miR-137* methylation in tumor tissue. Crude and multivariate logistic regression models were created restricted to the subgroup with FFPE data to assess gender-specific methylation patterns.

Results

Association of miR-137 promoter methylation with case–control status

A description of the case–control study population is presented in Table I. SCCHN patients were significantly less educated relative to control subjects ($P < 0.001$), reported eating fewer servings of fruits and vegetables/day ($P < 0.001$) and had a lower median body mass index [(BMI); $P = 0.07$]. Cases were more probably than controls to report as ever-smokers ($P < 0.001$) and among the ever-smokers, cases had a longer median smoking duration ($P < 0.001$). Although there was no difference by case–control status in those identifying as ever-drinkers, cases reported longer median years of alcohol use ($P = 0.003$) and consumed more drinks/day on average ($P < 0.001$). Cases were also more probably to wear dentures ($P < 0.001$). With respect to tumor site for the SCCHN patients, 37.4% ($n = 37$) had tumors originating in the oral cavity, 37.4% ($n = 37$) in the oropharynx and 25.3% ($n = 25$) in the larynx.

miR-137 promoter methylation was detected in 21/99 (21.2%) of oral rinses from SCCHN patients and 3/99 (3.0%) from cancer-free control subjects. The final multivariate model for the association of miR-137 promoter methylation is presented in Table II. SCCHN patients had nearly five times the odds of having miR-137 promoter methylation relative to normal oral mucosa of cancer-free control subjects, adjusting for smoking duration, alcohol intensity, daily servings of fruits and vegetables and education level [odds ratio (OR) = 4.80, 95% confidence interval (CI): 1.23–18.82].

The sensitivity of *miR-137* promoter methylation in differentiating patients with SCCHN from those with non-malignant ear, nose and throat conditions was 21.2%, and the specificity was 97.0%. The PPV was 87.5%, and the NPV was 55.2%.

When considering anatomic site of tumor origin (Table II), *miR-137* promoter methylation was detected in 14/37 (37.8%) of oral rinse samples from patients with SCCHN of the oral cavity, 5/37 (13.5%) with pharyngeal tumors and 2/25 (8.0%) of laryngeal tumors. The odds of oral cancer patients having *miR-137* promoter methylation were >12 times as high as cancer-free control subjects (OR = 12.18, 95% CI: 2.63–56.36); estimates were not statistically significant for neither pharyngeal (OR = 3.76, 95% CI: 0.68–20.76) nor laryngeal cancer patients (OR = 1.33, 95% CI: 0.12–14.93).

The PPV of *miR-137* promoter methylation was 82.4% and the NPV was 80.7% for tumors of the oral cavity, PPV was 62.5% and NPV was 75.0% for pharyngeal tumors and PPV was 40.0% and NPV was 80.7% for laryngeal tumors.

Evaluation of potential risk factors for miR-137 promoter methylation

Univariate associations between *miR-137* promoter methylation and various risk factors are summarized in Table III by case–control status. While none of the factors considered were significantly associated with *miR-137* methylation status in oral rinse in controls, among cases, age ($P = 0.02$), female gender ($P = 0.007$), denture use ($P = 0.01$) and total years smoking ($P = 0.01$) and drinking ($P = 0.05$) were positively associated with *miR-137* promoter methylation, whereas BMI ($P = 0.002$) was inversely associated. There was no significant interaction between alcohol consumption and smoking. None of the methylation-positive cases or controls reported being both never-smokers and never-drinkers.

A multivariable case–case comparison was performed (Table IV); covariates included in the final multivariate model were smoking

Table I. Description of the study population by case–control status

	Cases, $n = 99$	Controls, $n = 99$	P-value
Age, mean years (median)	59.9 (58.7)	57.6 (57.1)	0.51[a]
Gender, *n* (%)			>0.99[b]
Female	27 (27.3%)	27 (27.3%)	
Male	72 (72.7%)	72 (72.7%)	
Race, *n* (%)			0.10[b]
White	97 (98.0%)	91 (91.9%)	
Non-white	2 (2.0%)	8 (8.1%)	
Highest level of education, *n* (%)			<0.001[b]
<High school	13 (13.1%)	4 (4.0%)	
High school	51 (51.5%)	26 (26.3%)	
Some college or technical school	17 (17.2%)	19 (19.2%)	
College	18 (18.2%)	50 (50.5%)	
Family history cancer, *n* (%)			0.67[b]
Yes	54 (55.1%)	58 (59.2%)	
No	44 (44.9%)	40 (40.8%)	
Daily servings of fruits and vegetables, mean (median)	1.7 (1.3)	2.9 (3.0)	<0.001[a]
BMI (kg/m^2)[c], mean (median)	27.2 (26.5)	28.4 (27.5)	0.07[a]
Cigarette use			<0.001[b]
Never	23 (23.2%)	57 (57.6%)	
Ever	76 (76.8%)	42 (42.4%)	
Years smoking, mean (median)	35.7 (38.0)	25.5 (26.0)	<0.001[a]
Packs/day, mean (median)	1.2 (1.0)	1.2 (1.0)	0.44[a]
Age started smoking, mean (median)	17.8 (17.0)	16.4 (17.0)	0.83[a]
Alcohol Use			0.87[b]
Never	25 (25.3%)	27 (27.3%)	
Ever	74 (74.8%)	72 (72.7%)	
Years drinking, mean (median)	34.2 (35.0)	25.9 (28.0)	0.003[a]
Drinks/day[d], mean (median)	3.0 (1.9)	1.4 (0.3)	<0.001[a]
Age started drinking, mean (median)	20.8 (19.0)	23.3 (20.5)	0.04[a]
Combined tobacco/alcohol use			<0.001[b]
Never/never	7 (7.1%)	17 (17.2%)	
Never/ever	11 (11.1%)	35 (35.4%)	
Ever/never	18 (18.2%)	10 (10.1%)	
Ever/ever	63 (63.6%)	37 (37.4%)	
Wear dentures, *n* (%)			<0.001[b]
No	53 (53.5%)	80 (80.8%)	
Yes	46 (46.5%)	19 (19.2%)	
Years wearing dentures, mean (median)	26.6 (25.0)	19.8 (13.0)	0.20[a]
Stage at diagnosis, *n* (%)			
I	18 (18.8%)	—	
II	14 (14.6%)	—	
III	15 (15.6%)	—	
IV	49 (51.0%)	—	
Tumor site, *n* (%)			
Oral cavity	37 (37.4%)	—	
Pharynx	37 (37.4%)	—	
Larynx	25 (25.3%)	—	

[a]Mann–Whitney *U*-test.
[b]Fisher's exact test.
[c]Estimated for 1 year prior to study enrollment based on patient reported weight at that time.
[d]One drink is considered to be one beer (12 oz), one glass of wine (5 oz) or one shot of liquor (1.5 oz).

duration, alcohol intensity, BMI, denture use and gender. Women SCCHN cases had five times the odds of having miR-137 promoter methylation (OR = 5.30, 95% CI: 1.20–23.44), and an inverse association of *miR-137* methylation with BMI per kilogram per square meter unit increase was observed (OR = 0.88, 95% CI: 0.77–0.99). There was a borderline association with wearing dentures and *miR-137* promoter methylation (OR = 3.36, 95% CI: 0.91–12.36). Although there was no significant association with total years of smoking, there was a borderline positive trend ($P = 0.09$). Methylation in tumor tissue was 33.3% in females and 12.2% in males, yielding a crude OR of 4.48 (95% CI: 1.26–15.90) and an adjusted OR of 12.29 (95% CI: 1.69–89.52) for the association between female gender and *miR-137* tissue methylation. Sensitivity of the oral rinse for detection of *miR-137* methylation in tumor tissue was 45.5% and specificity was 81.1%.

Following the identification of a strong association between female gender and *miR-137* promoter methylation, *post hoc* analysis revealed no significant difference in tumor site distribution for miR-137 promoter methylation by gender ($P > 0.99$).

Discussion

Since 2006, numerous microRNAs have been reported to be hypermethylated in various cancer types (8–11,24–35). *miR-137* is a tumor suppressor gene that targets Cdk6, an oncogene involved in progression past the G_1/S-phase checkpoint, the loss of which has been shown to increase cellular proliferation *in vitro* (8,11) and is possibly involved in cellular differentiation (11). Therefore, its transcriptional repression via DNA promoter methylation could have consequences with respect to carcinogenesis. Recent studies have identified *miR-137*

Table II. Association of miR-137 promoter methylation and SCCHN

miR-137 methylation status	$n_{cases}/n_{controls}$	Association with SCCHN	
		Crude OR (95% CI)	Adjusted OR (95% CI)[a]
All sites			
Unmethylated	78/96	1.00 (ref)	1.00 (ref)
Methylated	21/3	8.62 (2.48–29.95)	4.80 (1.23–18.82)
Oral cavity			
Unmethylated	23/96	1.00 (ref)	1.00 (ref)
Methylated	14/3	19.48 (5.17–73.45)	12.18 (2.63–56.36)
Pharynx			
Unmethylated	32/96	1.00 (ref)	1.00 (ref)
Methylated	5/3	5.00 (1.13–22.10)	3.76 (0.68–20.76)
Larynx			
Unmethylated	23/96	1.00 (ref)	1.00 (ref)
Methylated	2/3	2.78 (0.44–17.63)	1.33 (0.12–14.93)

[a]Adjusted for total years smoking, average alcoholic drinks/day, daily servings of fruits and vegetables and education.

promoter methylation in several types of solid tumors (8–10), including OSCC (8). In the present study, we have observed promoter methylation of *miR-137* in 21.2% of oral rinse samples taken from SCCHN patients, a significantly higher proportion than observed in oral rinse samples from controls. At present, this is the only study of *miR-137* methylation status to include patients with cancers of the pharynx and larynx and, although others have detected promoter methylation in oral rinse samples, this marks the first time that microRNA promoter methylation has been evaluated as a biomarker of SCCHN using an oral rinse collection method.

The proportion of samples exhibiting *miR-137* promoter methylation was particularly high among patients with cancers of the oral cavity (37.8%), and lower for pharyngeal (13.5%) and laryngeal (8.0%) cancers. This discrepancy by cancer site could be a result of local variations in quantity and quality of exposure to carcinogens, with the oral cavity more probably to be directly and highly exposed to alcohol and tobacco than the pharynx and larynx. Differences in cancer biology and/or sample collection method could also contribute to the observed differences. The frequency of promoter methylation among cancers of the oral cavity is somewhat lower than that found in a small study conducted by Kozaki *et al.* (8), where promoter methylation was observed in 7/11 (63.6%) tumor tissue from patients with OSCC. In the subset of our patients where tissue from oral cavity cancers was available, we still observed a methylation frequency of 16%. Differences between our results and that of the Kozaki study could be due to random chance ($P = 0.13$ for comparison of frequencies between our study and the Kozaki study using a two-sample test of proportions) or to variations in study populations resulting in genetic, behavioral and exposure diversity leading to differences in frequencies of *miR-137* methylation. The present study includes predominately Caucasian American subjects compared with the Japanese population included in the Kozaki study. Another possible reason for the interstudy variation could be due to the study design and laboratory methods; the present study used methylation-specific polymerase chain reaction analysis of DNA isolated from FFPE tumor tissue, whereas Kozaki employed combined bisulfite restriction analysis (36) on DNA from fresh-frozen tissue.

It is possible that DNA derived from tumor tissue is more frequently methylated than DNA derived from oral rinse as a consequence of the carcinogenetic process. In an attempt to address this issue, we have compared methylation in tumor tissue and in mouthwash in a subset of cases and found that mouthwash has a high specificity (81.1%); therefore, patients with a positive methylation test in the saliva are very likely to have methylation in their cancer tissue.

We observed *miR-137* promoter methylation in 3.0% of oral rinse samples from cancer-free subjects. Others have reported hypermethylation of other genes in benign upper aerodigestive tract mucosa (37–39), including *CDKN2A*, a tumor suppressor gene also involved control of the G_1/S-phase checkpoint, in oral tissue of smokers (38,39). miR-137 promoter methylation has also been reported in 'normal' adjacent colonic tissue of colorectal cancer patients (10). This is the first report to evaluate *miR-137* promoter methylation status in cancer-free subjects and to identify methylation status in benign mucosa of the upper aerodigestive tract.

All three methylation-positive control subjects were identified as an ever-user of either tobacco, alcohol or both. This supports the notion that environmental exposures may play a role in *miR-137* hypermethylation in non-cancerous tissue. These epigenetic field defects could potentially predispose such individuals to SCCHN, and therefore, the relationship between smoking, drinking and *miR-137* promoter methylation in upper aerodigestive tract mucosa of cancer-free individuals should be examined further in future studies with larger sample sizes.

Using detailed information regarding smoking and drinking history, as well as other demographic and behavioral exposures, we were able to examine potential risk factors associated with *miR-137* promoter methylation among SCCHN patients. The most striking finding in this regard is the observed increase in risk for *miR-137* promoter methylation among women. The result was confirmed by the assessment of methylation in tumor tissue, thus making the possibility of a gender bias in mouthwash collection unlikely. Presently, there are mixed reports on the role of sex in DNA promoter methylation. Female gender has been positively associated with DNA methylation for some genes, including *MTAP* in gastric cancer (40), $p14^{arf}$ in colorectal cancer (41) and *CDH1* in lung cancer tissue (42), but protective for others, such as *RASSF1A*, *TSLC1/IGSF4* and *ESR1* in lung cancer (42–44) and methylation latent trait in bladder cancer based on a 16 gene panel (45). Experimental evidence suggests that risk of hypermethylation for certain genes may vary in a tissue-specific gender-dependent manner, as do the transcriptional targets, based on the effects of sex hormones on epigenetic states and differential distribution of sex hormone receptors (46). In experimental models, murine studies have demonstrated higher frequencies of methylation of certain genes following estrogen administration (47,48). In transgenic breast cancer mouse models, estrogen increases epigenetic inactivation of genes involved in cell cycle control and apoptosis with a dose-response effect (49). Previous studies on sex hormone receptor distribution in SCCHN report that 51–79% of tumors are estrogen receptor positive and 42–49% are progesterone receptor positive (50,51), with no apparent variation by site. *Post hoc* analysis of our SCCHN study subjects reveals no significant difference in tumor site distribution for miR-137 promoter methylation by gender.

We also observed an inverse association of BMI with *miR-137* methylation status in cases, although there is presently little to no support for this in the literature. This relationship warrants further examination in future studies to identify the role of BMI in *miR-137* promoter methylation and whether such an effect generalizes to aberrant promoter methylation of other genes.

Recent work in the literature has begun to focus on epigenetic environment interactions, particularly with regard to DNA methylation. Here, we report a borderline positive linear trend between smoking duration and *miR-137* promoter methylation. Although the environmental risk factors for promoter methylation and their corresponding mechanisms are poorly understood at present, several studies have reported an association between hypermethylation and cigarette smoking in lung (42,43,52), prostate (53) and bladder cancer tissue (54). Others have also found similar dose–response relationships between smoking and promoter methylation (37,43,53). This suggests that one mechanism through which chronic cigarette smoking contributes to development of head and neck cancer is through stimulation of aberrant promoter methylation of tumor suppressor genes, possibly including *miR-137*. Although there were no significant associations of *miR-137* promoter methylation with smoking or alcohol, the moderate sample size of this study limits our power to identify small effects. Rather, our finding of a borderline dose–response trend adds support, albeit mild, to the growing body of evidence that smoking is capable of inducing

Table III. Demographic and exposure variables by methylation status

	Cases ($N = 99$)			Controls ($N = 99$)		
	Methylated, $n = 21$	Unmethylated, $n = 78$	*P*-value	Methylated, $n = 3$	Unmethylated, $n = 96$	*P*-value
Age, mean years (median)	64.7 (61.7)	58.6 (57.9)	0.02[a]	54.9 (53.8)	57.7 (57.2)	0.51[b]
Gender, *n* (%)			0.10[c]			>0.99[c]
Female	9 (42.9%)	18 (23.1%)		1 (33.3%)	26 (27.1%)	
Male	12 (57.1%)	60 (76.9%)		2 (66.7%)	70 (72.9%)	
Daily servings of fruits and vegetables, mean (median)	1.5 (1.1)	1.8 (1.3)	0.51[b]	2.9 (2.7)	2.9 (3.0)	0.80[b]
BMI (kg/m^2)[d], mean (median)	23.9 (23.0)	28.0 (27.2)	0.002[b]	28.7 (29.0)	28.4 (27.4)	0.83[b]
Cigarette use			0.14[c]			0.57[c]
Never	2 (9.5%)	21 (26.9%)		1 (33.3%)	56 (58.3%)	
Ever	19 (90.5%)	57 (73.1%)		2 (66.7%)	40 (41.7%)	
Years smoking, mean (median)	42.0 (41.0)	33.6 (35.0)	0.01[a]	26.5 (26.5)	25.5 (26.0)	0.95[b]
Packs/day, mean (median)	1.1 (1.0)	1.3 (1.0)	0.36[b]	0.8 (0.8)	1.2 (1.0)	0.53[b]
Age started smoking, mean (median)	19.7 (17.0)	17.1 (17.0)	0.51[b]	16.5 (16.5)	16.4 (17.5)	0.68[b]
Alcohol use			0.58[c]			>0.99[c]
Never	4 (19.1%)	21 (26.9%)		1 (33.3%)	26 (27.1%)	
Ever	17 (81.0%)	57 (73.1%)		2 (66.7%)	70 (72.9%)	
Years drinking, mean (median)	39.4 (38.0)	32.6 (33.0)	0.05[a]	27.0 (27.0)	25.9 (28.0)	0.95[b]
Drinks/day, mean (median)	2.4 (2.0)	3.1 (1.7)	0.64[b]	2.9 (2.9)	1.3 (0.3)	0.19[b]
Age started drinking, mean (median)	22.6 (21.0)	20.2 (18.0)	0.18[b]	17.5 (17.5)	23.4 (21.0)	0.12[b]
Combined tobacco/alcohol use			0.64[c]			0.57[c]
Never/never	0 (0.0%)	7 (9.0%)		0 (0.0%)	17 (17.7%)	
Never/ever	2 (9.5%)	9 (11.5%)		1 (33.3%)	34 (35.4%)	
Ever/never	4 (19.1%)	14 (18.0%)		1 (33.3%)	9 (9.4%)	
Ever/ever	15 (71.4%)	48 (61.5%)		1 (33.3%)	36 (37.5%)	
Wear dentures, *n* (%)			0.01[c]			0.48[c]
No	6 (28.6%)	47 (60.3%)		2 (66.7%)	78 (81.3%)	
Yes	15 (71.4%)	31 (39.7%)		1 (33.3%)	18 (18.8%)	
Years wearing dentures, mean (median)	26.1 (22.0)	26.8 (25.0)	0.93[b]	47.0 (47.0)	18.3 (11.5)	0.12[b]
Mouthwash use, *n* (%)			>0.99[c]			>0.99[c]
No	7 (33.3%)	28 (35.9%)		1 (33.3%)	42 (43.8%)	
Yes	14 (66.7%)	50 (64.1%)		2 (66.7%)	54 (56.3%)	
Times per week, mean (median)	7.7 (7.0)	7.4 (7.0)	0.86[b]	10.5 (10.5)	7.0 (7.0)	0.21[b]
Years of regular mouthwash use, mean (median)	26.4 (30.0)	19.2 (20.0)	0.06[a]	21.0 (21.0)	15.5 (10.0)	0.89[b]
Tumor site, *n* (%)			0.009[c]			
Oral cavity	14 (66.7%)	23 (29.5%)		—	—	
Pharynx	5 (23.8%)	32 (41.0%)		—	—	
Larynx	2 (9.5%)	23 (29.5%)		—	—	
Stage at diagnosis, *n* (%)			>0.99[c]			
Local (stages I and II)	7 (33.3%)	25 (33.3%)		—	—	
Advanced (stages III and IV)	14 (66.7%)	50 (66.7%)		—	—	
Tumor grade, *n* (%)			0.51[c]			
Well differentiated	0 (0.0%)	6 (9.4%)		—	—	
Moderately differentiated	16 (80.0%)	44 (68.8%)		—	—	
Poorly differentiated	4 (20.0%)	14 (21.9%)		—	—	

[a]Two-sample *t*-test.
[b]Mann-Whitney *U*-test.
[c]Fisher's exact test.
[d]Estimated for 1 year prior to study enrollment based on patient reported weight at that time.

epigenetic alterations in addition to somatic mutations and chromosomal breaks. At present, no other study has evaluated the effects of environmental exposures specifically on microRNA promoter methylation.

Also, worthy of further discussion is the borderline association with wearing dentures. Although studies have found no apparent relationship between general denture use and oral cancer (55,56), wearing of poorly fit or malfunctioning dentures has recently been associated with oral and pharyngeal cancer (57). Improperly fitting dentures can result in chronic irritation of the surrounding mucosa. Halogenated pyrimidines that stem from damage caused by reactive oxygen species as a result of the chronic inflammatory process mimic 5-methylcytosine and have been shown to stimulate DNA (cytosine-5-)-methyltransferase 1-mediated CpG methylation (58,59), suggesting a potential role of inflammation in the induction of aberrant promoter methylation. Further inquiry into the association with promoter hypermethylation in oral mucosa is required to elucidate the role, if any, of denture use.

Strengths of this study include the high quality data collection and exposure details, which allowed us to carefully examine the relationship between potential environmental exposures and *miR-137* promoter methylation. Complete data eliminated some potential biases, whereas the detailed exposure data was conducive for quantitative assessment of smoking and drinking duration and intensity. The use of oral rinse as a collection media allowed for non-differential sample ascertainment from SCCHN patients and cancer-free control subjects, reducing the possibility of sample collection bias. However, statistical power may be an issue due to the moderate sample size of this study. It is therefore conceivable that the null findings of associations with alcohol and tobacco exposures are due to a lack of power rather than a true lack of effect. It is also possible that this study understates the prevalence of *miR-137* promoter methylation as a result of misclassification bias stemming from the collection method, due to differential contact of tumor cells with the oral rinse media among cases. This may be particularly evident in smaller tumors or tumors that are not in

Table IV. Case only analysis for the association of various risk factors with *miR-137* promoter methylation

	$n_{methylated}/n_{unmethylated}$	Association with *miR-137* hypermethylation			
		Crude OR (95% CI)	P_{trend}	Adjusted OR (95% CI)	P_{trend}
Gender					
Male	12/60	1.00 (ref)		1.00 (ref)[a]	
Female	9/18	2.50 (0.91–6.88)		5.30 (1.20–23.44)	
BMI					
Median (BMI = 27.2 kg/m^2)		1.00 (ref)		1.00 (ref)[b]	
Per unit increase		0.85 (0.76–0.95)		0.88 (0.77–0.99)	
Wear dentures					
No	6/47	1.00 (ref)		1.00 (ref)[c]	
Yes	15/31	3.79 (1.33–10.83)		3.36 (0.91–12.36)	
Alcohol use			0.59		0.24
Never	4/21	1.00 (ref)		1.00 (ref)[d]	
Light/moderate ($\leq$2 drinks/day)	10/34	1.54 (0.43–5.56)		2.01 (0.39–10.40)	
Heavy (>2 drinks/day)	7/23	1.60 (0.41–6.25)		3.34 (0.49–22.78)	
Total years smoking[e]			0.02		0.09
Never		1.00 (ref)		1.00 (ref)[f]	
10 year smoker		0.57 (0.06–5.25)		0.63 (0.06–7.08)	
20 year smoker		1.08 (0.16–7.11)		1.04 (0.13–8.34)	
30 year smoker		2.07 (0.40–10.68)		1.73 (0.27–11.12)	
40 year smoker		3.95 (0.84–18.63)		2.88 (0.47–17.57)	
50 year smoker		7.56 (1.48–38.74)		4.80 (0.69–33.36)	
Fruit and vegetable consumption					
0 servings/day		1.00 (ref)		1.00 (ref)[g]	
Per log daily serving increase		1.05 (0.83–1.32)		1.56 (0.72–3.38)	
Stage at diagnosis					
Local (stage I or II)	7/25	1.00 (ref)		1.00 (ref)[g]	
Advanced (stage III or IV)	14/50	1.00 (0.36–2.79)		0.86 (0.25–2.89)	

[a]Adjusted for BMI, dentures, alcohol use and total years smoking.
[b]Adjusted for gender, dentures, alcohol use and total years smoking.
[c]Adjusted for gender, BMI, alcohol use and total years smoking.
[d] Adjusted for gender, BMI, dentures and total years smoking.
[e]Modeled continuously with an additive binary (ever/never) term as ($y = \beta_1 \times X_{ever/never} + \beta_2 \times X_{years\ smoking}$), where never smoking = 0 and ever smoking = 1 to account for right skewness due to never smokers.
[f]Adjusted for gender, BMI, dentures and alcohol use.
[g]Adjusted for gender, BMI, dentures, alcohol use and total years smoking.

or adjacent to the oral cavity, such as some pharyngeal and most laryngeal tumors.

Overall, the results of this study suggest that promoter methylation of *miR-137* is a relatively common event in SCCHN. Although it has a low sensitivity (21.2%), *miR-137* promoter methylation may have utility as a biomarker in DNA methylation panels, particularly given the good specificity (97.0%) and its presence in early stage tumors. Additionally, we have shed some light on environmental and personal risk factors associated with aberrant methylation of *miR-137*, particularly female gender. Due to the involvement of miR-137 in cell cycle control and differentiation pathways, subsequent studies should further evaluate promoter methylation as a prognostic biomarker of SCCHN. Also, future avenues of research should be aimed at correlation of *miR-137* promoter methylation in mouthwash with tissue samples, overall and by specific tumor site, and at quantitative assessment of *miR-137* promoter methylation in tumor tissue, with the final aim of developing etiologic and prognostic markers that could be used on large populations in a public health setting.

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