MICRORNA-137 PROMOTER METHYLATION AS AN ETIOLOGIC AND PROGNOSTIC BIOMARKER FOR SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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ABSTRACT

Head and neck cancer accounted for 3.3% of incident malignancies and 2.0% of cancer-deaths in the US in 2009, the majority of which are squamous in origin. Thus, there is a need for novel biomarkers for early detection and prognosis of squamous cell carcinoma of the head and neck (SCCHN). MicroRNA-137 plays a role in cell cycle control through negative regulation of Cdk6, and has been reported to undergo promoter methylation in oral squamous cell carcinoma. Oral rinse is a non-invasive mode of DNA collection, which may have some utility in detection of promoter methylation. The primary goals of this research were to determine if *miR-137* promoter methylation occurs in all SCCHN, including pharyngeal and laryngeal tumors, and whether it is detectable in oral rinse samples; and to assess *miR-137* promoter methylation as an etiologic and prognostic biomarker for SCCHN. DNA was extracted from oral rinses from 99 SCCHN patients and 99 cancer-free control subjects and from tumor tissue of 67 SCCHN patients; paired oral rinses and tumor tissue was available for 64 of the SCCHN patients. Promoter methylation status of *miR-137* was determined by methylation-specific PCR. We identified a strong association between *miR-137* promoter methylation detected in oral rinses and SCCHN (OR = 4.80, 95% CI: 1.23-18.82). There was a strong positive association between female gender and *miR-137* promoter methylation in oral rinse from SCCHN patients (OR = 5.30, 95% CI: 1.20-

23.44) and an inverse association with body mass index (OR = 0.88, 95% CI: 0.77-0.99). Promoter methylation of *miR-137* in tumor tissue was associated with poorer overall survival (HR = 3.68, 95% CI: 1.01-13.38). In spite of its low sensitivity (21.2%), *miR-137* methylation detected in oral rinse may have future value in methylation panels for early diagnosis of SCCHN due to its high specificity (97.0%) and occurrence in early stage disease; and its detection in tumor tissue has promise as a prognostic marker. The identification of novel diagnostic and prognostic biomarkers for SCCHN such as *miR-137* promoter methylation will significantly impact public health through the reduction of morbidity and mortality that occurs as a result of this disease.

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PREFACE

I would like to give thanks and gratitude to my mentors, family and friends, all of who have been integral in the completion of this dissertation. I would like to thank my committee members, Dr. Emanuela Taioli, Dr. Roslyn Stone, Dr. Clareann Bunker and Dr. Robert Sobol, for their mentorship and guidance throughout the dissertation process. Special thanks to Dr. Emanuela Taioli for her role as my academic advisor and for providing advise, encouragement and guidance over the course of my doctoral studies. I would also like to thank Stacy Eckstein for her helpful assistance with DNA extraction from the oral rinse samples; Ashley Brown for her advice and assistance with day-to-day laboratory operations; and Kerry Trent and Jennifer Hetrick for consistent prompt provision of patient data. Lastly, I would like to thank my loving wife Erin for providing constant patience and emotional support throughout the dissertation process, whose love and encouragement made completion of this possible, and it is to her that I dedicate my dissertation.

This research was supported by grants from the Clinical and Translational Science Institute Multidisciplinary Predoctoral Fellowship program awarded through the Institute for Clinical Research Education at the University of Pittsburgh [5TL1RR024155-03] to Scott M. Langevin; American Cancer Society [RSG-05-246-01-GMC] to Robert W. Sobol; National Institutes of Health [1P20-CA132385-03] to Emanuela Taioli and Robert W. Sobol; and

University of Pittsburgh Head and Neck Cancer SPORE [P50CA097190-01A1] to Jennifer R. Grandis.

1.0 INTRODUCTION

Head and neck cancer is a broad classification for a heterogeneous group of malignancies arising in the upper aerodigestive tract. It is estimated that head and neck cancer resulted in 48,010 cases and 11,260 deaths in the United States in 2009 [1], and more than 500,000 annual cases and 300,000 deaths globally [2]. Squamous cell carcinoma of the head and neck (SCCHN) makes up the majority, representing 93% of all head and neck cancers [3]. Although head and neck cancer only accounts for 2% of US and 4.5% of global cancer deaths, it bears substantial morbidity and remains a very preventable cancer, due to its strong association with alcohol and tobacco use.

Epigenetic changes are heritable but reversible genetic modifications that affect gene expression without altering the DNA sequence [4]. DNA methylation is a crucial epigenetic process in controlling gene transcription. Methylation occurs at dinucleotides in which cytosine is upstream and adjacent to guanine, termed CpGs. These CpG dinucleotides are not randomly distributed throughout the genome, but rather are concentrated in CpG rich regions referred to as CpG islands that occur disproportionately in the 5' promoter region of genes. When methyl groups are covalently attached to CpGs in the promoter regions of genes (termed hypermethylation) it generally results in transcriptional silencing. Promoter methylation is at least as common as mutations in tumor suppressor gene inactivation and is considered to be a major event in carcinogenesis, including SCCHN. It occurs at various stages of cancer

development, and is often an early event. Epigenetic alterations can arise as a result of environmental exposures, such as tobacco and alcohol, although the precise mechanisms remain poorly understood [5].

MicroRNAs are small, non-coding RNA molecules, averaging 22 nucleotides in length, that negatively regulate gene expression post-transcriptionally by binding target mRNA and inducing degradation or translational silencing. MicroRNAs affix to their targets with varying degrees of complementarity, and as a result, a single microRNA can affect the expression of many genes [6]. MicroRNA expression can be controlled through epigenetic mechanisms, with an estimated 10% regulated by DNA methylation [7]. As with protein coding genes, they can function as either tumor suppressors or oncogenes: those that regulate expression of proto-oncogenes act as tumor suppressors, while those that target tumor suppressor genes can act as oncogenes if overexpressed. Dysregulation of individual microRNA expression has been correlated with various solid tumors, including SCCHN, and with poorer prognosis for several cancers [8-19].

Early diagnosis of SCCHN is crucial in treatment of the disease, since 5-year survival declines with increasing stage and two-thirds of SCCHN patients are diagnosed with advanced stage disease [20]. Currently, no proven screening method exists aside from visual inspection. Due to early occurrence during carcinogenesis and the relative stability of DNA, methylated tumor suppressor genes have good potential as cancer biomarkers. MicroRNA expression shows more tumor-specificity than mRNA expression, suggesting that alterations affecting microRNA transcription, such as promoter methylation, may be more specific for tumor type than those affecting protein-coding genes. Moreover, dysregulated expression of microRNAs has been reported to have prognostic implications. Promoter methylation can be detected by extracting

DNA from tumor tissue. Additionally, use of oral rinse (mouthwash) for DNA collection is a non-invasive method that may have utility in detection of promoter methylation in upper aerodigestive tract tumors [21-26] and therefore has potential as a screening tool for SCCHN.

MicroRNA-137 (*miR-137*) is embedded in a large CpG island and is located on chromosome 1p21.3 [17, 27]. A recent report observed hypermethylation and corresponding downregulation of miR-137 in a small clinical sample of oral squamous cell carcinomas (OSCC) [27]. One of the known targets of miR-137 is Cdk6, a key protein in cell cycle progression. Cdk6 forms a complex with Cyclin D1 that phosphorylates pRb, releasing E2F transcription factor, inducing transcription of proteins necessary for progression from G1- to S-phase of the cell cycle. Downregulation of miR-137 results in overexpression of Cdk6, leading to increased cellular proliferation [17, 27]; and expression of miR-137 has been inversely correlated with cellular differentiation in gliomas [17], although it is presently unknown whether this involvement with differentiation extends to squamous epithelium of the head and neck. Furthermore, overexpression of Cdk6 has been reported in SCCHN [28-30]. It is because of its involvement in cell cycle control and its potential role in cellular differentiation, as well as its promoter methylation in OSCC, that leads us to study *miR-137* promoter methylation in depth as a biomarker of SCCHN. Exploration of new biomarkers such as this will aid in the search for better diagnostic and prognostic tools for early detection and treatment of SCCHN.

The hypothesis driving this research, illustrated in Figure 1, is that alcohol and tobacco exposures can lead to epigenetic dysregulation and aberrant promoter methylation of *miR-137*, repressing its expression and resulting in increased cellular proliferation and loss of differentiation, thus contributing to the development of SCCHN. The overarching objective of this research is to contribute to the vast, ever-growing body of biomedical science literature

through the evaluation of *miR-137* promoter methylation as a biomarker for SCCHN. Specifically, this research seeks to 1) assess *miR-137* promoter methylation in oral rinse samples as a diagnostic biomarker for SCCHN and identify risk factors involved in its occurrence; 2) evaluate *miR-137* promoter methylation as a prognostic biomarker of SCCHN; and 3) determine the predictive value of oral rinse for the detection of *miR-137* promoter methylation in SCCHN tumor tissue using methylation-specific PCR.

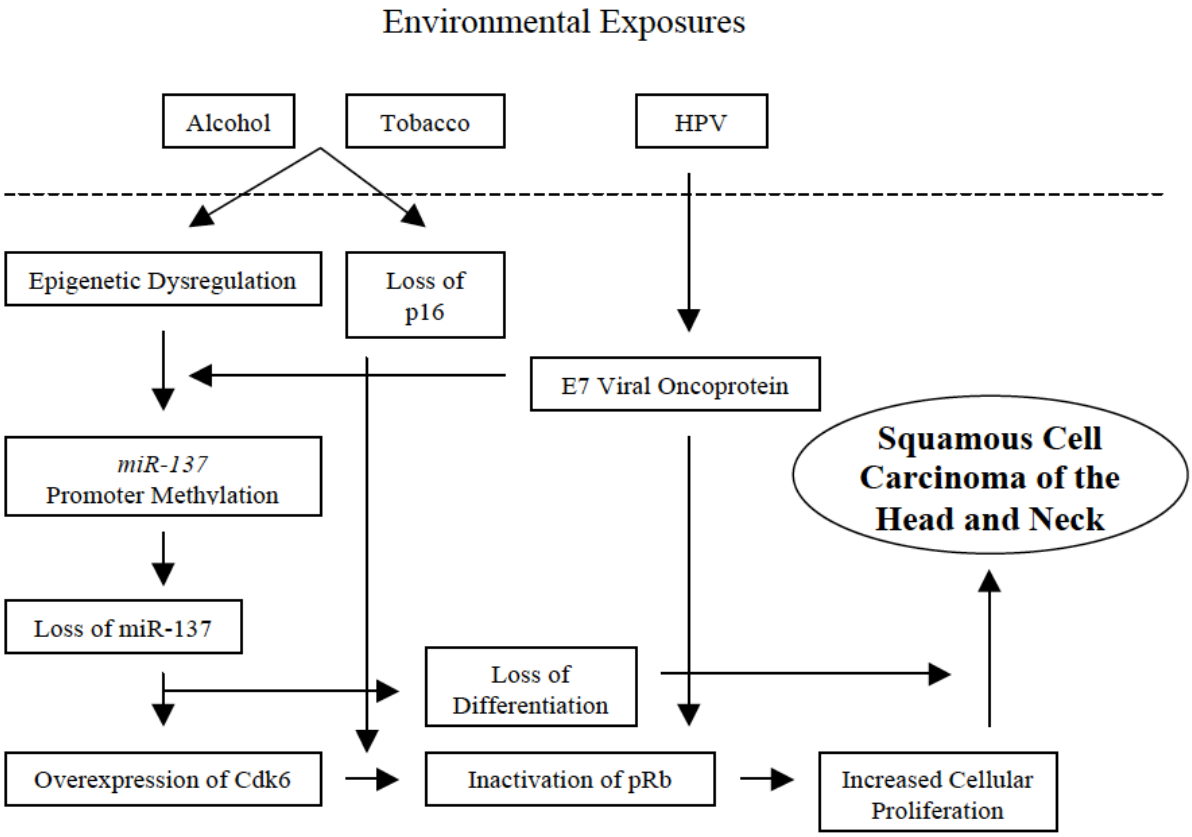


Figure 1. Biological model for hypothesized involvement of *miR-137* promoter methylation in SCCHN oncogenesis

2.0 HEAD AND NECK SQUAMOUS CELL CARCINOMA

Head and neck cancer is a broad term applied to a heterogeneous group of malignancies arising in the upper aerodigestive tract. This encompasses cancers of the oral cavity, oropharynx, hypopharynx, nasopharynx, nasal cavity, paranasal sinuses, and larynx (Figure 2). It is estimated that approximately 93% of these are squamous cell carcinoma of the head and neck (SCCHN) [3]. The focus of this report will be on SCCHN of the oral cavity, pharynx (oropharynx and hypopharynx), and larynx; nasopharyngeal, nasal cavity and paranasal sinus cancers will not be discussed, since they are generally considered to be of a different etiology and are typically evaluated as a separate clinicopathological entity.

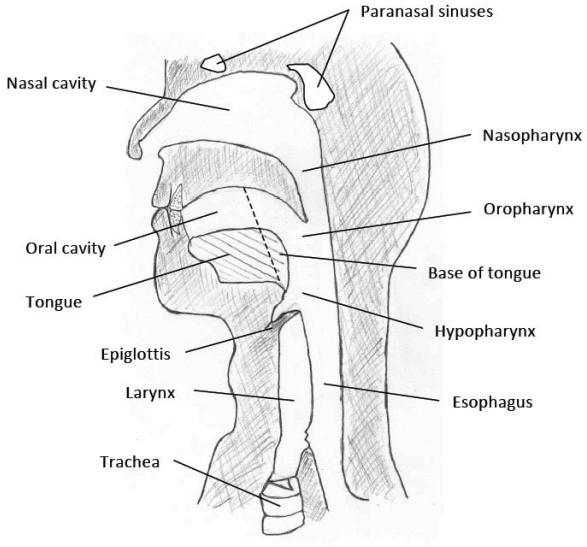


Figure 2. Anatomy of the upper aerodigestive tract

2.1 EPIDEMIOLOGY OF HEAD AND NECK CANCER

2.1.1 Incidence and Mortality

In 2009, it is estimated that head and neck cancer accounted for 48,010 new cases and 11,260 deaths in the United States alone [1]. This represents 3.2% of all incident cancers and 2.0% of all cancer-deaths annually in the US, excluding basal and squamous cell carcinomas of the skin [1]. Considered together, cancers of the oral cavity, pharynx and larynx comprise the 9th most common site for cancer incidence overall and are ranked 15th in cancer related deaths [1]. Cancer of the oral cavity and pharynx accounted for 35,720 (74.4%) head and neck cancer cases and 7,600 (67.5%) deaths in the US in 2009 (excluding nasopharyngeal), while laryngeal cancer accounted for 12,290 (25.6%) of the cases and 3,660 (32.5%) of the deaths [1]. As a whole, head and neck cancer incidence and mortality have been declining over the past 2 decades, although this trend is not consistent across all ages and sites [31]. While rates of oral cavity and laryngeal cancers have gone down, they have remained stable for pharyngeal cancer. As a result, pharyngeal cancer accounts for an increasing proportion of head and neck cancers. Among adults less than 45 years of age, incidence of pharyngeal cancer has actually increased. This is particularly true for cancers of the base of tongue and tonsils, which have risen an average of 2% and 4% per year, respectively [32].

Head and neck cancer is more common in men than women. In the US, males are 2.74 times as likely as females to develop head and neck cancer, and 2.61 times as likely to die from it [1]. When considering site specific incidence, men are 2.41 times as likely as females to be diagnosed with cancer of the oral cavity and pharynx and 2.22 times more likely to die from it [1]. Laryngeal cancer is even more common in males: men are 4.19 times as likely to develop

laryngeal cancer and 3.82 times as likely to die from it [1]. In 2009, head and neck cancer ranked 8th among US men in cancer incidence, representing 4.6% of newly diagnosed cancers per year, and 11th overall in cancer mortality, accounting for 2.8% of US male cancer deaths [1]. Among US women, it is the 13th most common cancer, representing 1.8% of annual incident cancer diagnoses, and ranks 17th in cancer deaths, accounting for 1.2% of US female cancer mortality [1].

The incidence rate for oral cavity and pharyngeal cancer in the US is similar in African-American and Caucasian females, whereas the incidence rate is 30% higher among African-American males compared to Caucasian males [33]. With regard to laryngeal cancer, incidence rates among African-American males and females are double those of their respective Caucasian counterparts. Oral cavity and pharyngeal cancer incidence rates are lowest among Hispanics, while Asians have the lowest incidence rates of laryngeal cancer [33].

Head and neck squamous cell carcinoma tends to occur in older adults. The median age at diagnosis in the US is 62 years, although as previously discussed, incidence in adults younger than 45 years is increasing, particularly for cancer of the tonsil and base of tongue [20, 32-34].

Globally, head and neck cancer is more common than it is in the United States. In 2002 there were an estimated 563,826 new cases of head and neck cancer (excluding nasopharyngeal), representing 5.2% of all incident cancers, and 301,408 deaths, representing 4.5% of the world cancer mortality [2]. Cancer of the oral cavity accounted for 274,289 (48.6%) global head and neck cases and 127,459 (42.3%) deaths; pharyngeal cancer accounted for 130,296 (23.1%) cases and 83,723 (27.8%) deaths; and laryngeal cancer accounted for 159,241 (28.2%) cases and 89,956 (29.9%) deaths.

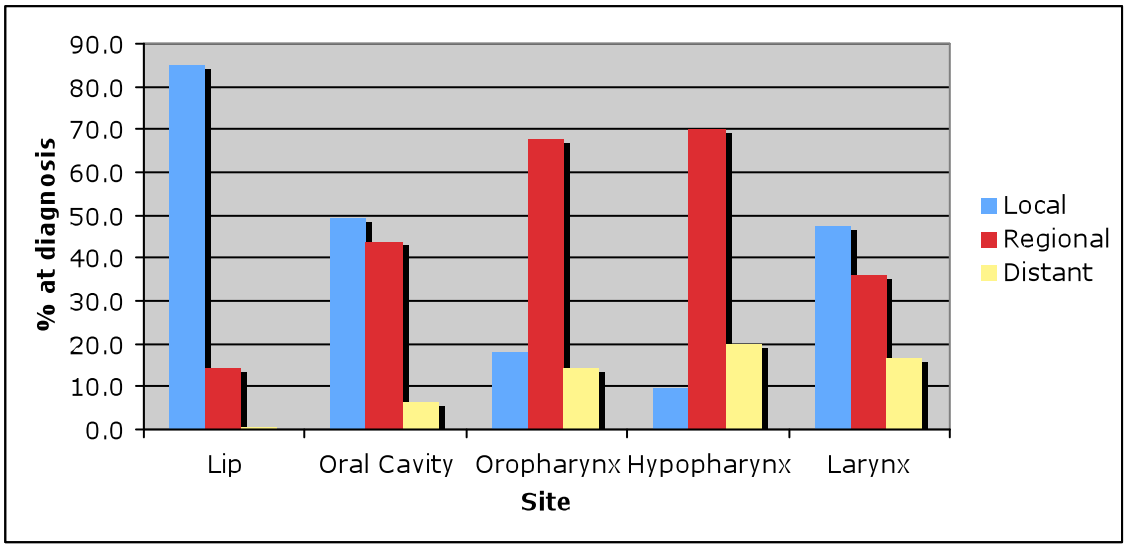
2.1.2 Staging and Survival

Overall 5-year survival for head and neck cancer in the United States is approximately 60% and has remained virtually unchanged over the past 3 decades [20]. Despite therapeutic advances, there have been no significant improvements in survival for laryngeal cancer compared with the 1970s, and only a slight significant gain for cancers of the oral cavity and pharynx [3]. However, there is substantial variability in survival by site. Overall 5-year survival rates by site range from 85.6% for cancer of the lip to 33.3% for hypopharyngeal cancers (Table 1) [3]. Distribution of stage at diagnosis also varies by site, with lip cancers being most likely to be diagnosed at an early stage while cancers of the hypopharynx are the most frequently diagnosed at later stages (Figure 3) [3], although this does not sufficiently explain the survival discrepancies. Variation in adjacent structures at each site may offer further explanation for the prognostic differences between tumor sites.

Table 1. US head and neck cancer 5-year survival by site and stage, for year of diagnosis 1998-1999

	Percent 5-Year Survival			
Site	**Overall**	**Local**	**Regional**	**Distant**
Lip	85.6	89.6	82.7	40.0
Oral Cavity	56.7	72.0	43.8	35.2
Oropharynx	50.6	61.0	50.6	30.2
Hypopharynx	33.3	56.0	34.6	12.9
Larynx	62.0	74.3	53.2	38.3

Data source: Carvalho AL (2005) [3]



Based on SEER summary stages:
Local: invasive neoplasm confined entirely to organ of origin
Regional: extending beyond the limits organ of origin or regional lymph node metastasis
Distant: remote spread of the neoplasm to distant parts of the body

Figure 3. Head and neck cancer stage at diagnosis by site in the US, 1998-1999

Data source: Carvalho AL (2005) [3]

In the United States, sociodemographic disparities exist with respect to SCCHN survival. For cancers of the oral cavity and pharynx, the 5-year survival for African-Americans is 40.6% compared to 62.0% for Caucasians [35]. Likewise, 5-year survival for laryngeal cancer among African-Americans is 50.1% versus 66.0% for Caucasians [35]. Also, men have poorer survival rates than with women. Contrasted with women, the 5-year survival for men is 5.1% and 6.3% lower for cancers of the oral cavity/pharynx and larynx, respectively [35].

Clinical stage and treatment are independent predictors of prognosis for SCCHN patients. Compared with treatment by surgery or combination surgery and radiotherapy, primary radiotherapy is associated with lower survival rates [3]. Higher TNM stage at diagnosis is associated with poorer outcome [3]. Approximately two-thirds of head and neck cancer patients present with regional lymph node involvement and about 10% with distant metastases [20]. The

presence of cervical lymph node metastasis is one of the most powerful predictors of prognosis [36, 37], with these patients experiencing a 50% reduction in 5-year survival [38]. Additionally, when extra-capsular spread (ECS) of lymph node metastasis occurs, prognosis worsens, with increased rates of local and distant metastasis and further declines in survival [38]. ECS occurs in 23% of positive lymph nodes < 1 cm, 39%-59% of nodes < 3 cm and 60%-100% of nodes > 3 cm [39-43]. Perineural spread occurs in 2.5%-5% of cases and has been associated with poorer prognosis [44]. Histologic tumor grade [45], extent of necrosis [45], lymph node burden (number of positive nodes) [45] and positive surgical tumor margins are also correlated with outcome [46-48].

At least 50% of patients with locally advanced SCCHN experience recurrence, generally within 2 years of treatment [20]. Additionally, second primary head and neck tumors commonly occur following an initial head and neck primary, developing in approximately 15% of primary head and neck cancer patients at a rate of 3-5% per year [34, 49]. Tumor recurrence or development of a second primary is a major reason for treatment failure and adversely impacts long-term survival [50, 51].

2.1.3 Risk Factors

2.1.3.1 Tobacco

Cigarette smoking has been identified as the chief avoidable cause of death [52] and is considered to be the major risk factor for SCCHN in the United States. It was first linked to oral squamous cell carcinoma (OSCC) in the late 1950s by Ernst Wynder and colleagues in a landmark case-control study [53]. These findings were validated 1 year later by a large cohort of 187,783 men reporting increased risk of mortality from SCCHN for cigarette smokers compared

to non-smokers [54]. Since then, numerous studies have replicated these findings, establishing the relationship between smoking and SCCHN [55-62]. This is recognized by the International Agency for Research on Cancer (IARC), which considers smoking to be a causal factor for cancer of the oral cavity, pharynx and larynx [63]. Other forms of tobacco consumption have also been associated with SCCHN, including cigar and pipe smoking [53, 55, 59, 61, 64-66] and smokeless tobacco [67-70].

There is a linear dose-response relationship between cigarette smoking and SCCHN, for which duration of use is more important than the exposure intensity, although both matter [33]. However, dose-response alone does not sufficiently explain global variation in SCCHN rates, suggesting that other risk factors may interact with tobacco use [71]. There is about a 3- to 10-fold increase in risk of developing SCCHN among smokers compared with never-smokers [71], and an estimated 5- to 25-fold greater risk for heavy smokers [72]. For former smokers, there is an inverse relationship between time since quitting smoking and risk of SCCHN [55, 57-59, 61, 62, 66], although their level of risk will never return to that of a never-smoker. In addition to initial primary tumors, tobacco use is associated with development of second primary tumors of the head and neck [73, 74].

More than 60 carcinogens have been identified in cigarette smoke, including polycyclic aromatic hydrocarbons (PAH), nitrosamines, aromatic amines, aldehydes, volatile organic compounds, metals and others [75]. The majority of the carcinogenic effect contained in cigarette smoke is derived from the particulate fraction of the product [33]. Of the pro-carcinogens, PAHs such as benzo[α]pyrene are the most potent in burnt tobacco, while nitrosamine metabolites, particularly nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3 pyridyl)-1-butane (NNK), are the strongest in smokeless tobacco, although both are present in

each form [76, 77]. Procarcinogens require metabolic activation by phase I xenometabolic enzymes in order to exert their carcinogenic effect, primarily through the formation of DNA adducts resulting in accumulation of genetic insults [71, 76, 78]. In addition, cigarette smoke contains co-carcinogens and tumor promoters, which do not directly act on DNA but can cause diffuse hyperplasia or act through epigenetic mechanisms such as aberrant DNA methylation [76, 78].

Furthermore, cigarette smoke inhibits the metabolism and storage of folate [79]. Nitrates, nitrous oxide, cyanates and isocyanates found in tobacco smoke have been shown to transform folate into a biologically inactive compound [80, 81]. Studies have observed reduced serum folate levels in smokers relative to non-smokers [82, 83]. Folate deficiency can result in chromosomal damage through impaired nucleotide synthesis and aberrant DNA methylation [84, 85].

2.1.3.2 Alcohol

Globally, an estimated 389,100 cases of cancer per year are attributable to chronic alcohol consumption, accounting for 3.2% of all incident cases (5.2% of all cases in men and 1.7% in women), with a corresponding 232,900 deaths [86]. Consumption of alcoholic beverages has been classified as a causal risk factor for cancer of the oral cavity, pharynx and larynx by IARC [87] and is considered to be a contributive factor in an estimated 75% of all cases [33].

As with smoking, there appears to be a dose-response relationship between alcohol consumption and SCCHN [71, 88], with the highest risk found among the heavy drinkers ($\geq$ 100 g/day), having approximately 4- to 6.5-fold risk relative to non-drinkers [88]. Daily consumption of 50 g, the approximate equivalent of a half-bottle of wine, or more of ethanol increases the risk for cancers of the oral cavity, pharynx or larynx 2- to 3-fold compared to non-

drinkers [87, 88]. Consumption of 3 or more alcoholic beverages per day has been associated with SCCHN in non-smokers [53, 55, 57-59, 61, 62, 89, 90], indicating that alcohol acts as a risk factor independent of tobacco. Additionally, alcohol is a risk factor for development of second primary tumors [73, 74]. Associations have been found with all types of alcoholic beverages, suggesting that ethanol or its metabolites are the primary carcinogen. Theories regarding impurities or contaminants have been proposed but studies attempting to correlate specific beverage types with cancer risk have thus far been inconclusive due to inconsistent findings [33, 71].

Alcohol may exert some of its effect by acting as a solvent for tobacco carcinogens and facilitating the uptake of these compounds into the mucosa [71, 91]. However, this alone is not sufficient to explain its carcinogenicity. As previously discussed, heavy alcohol consumption is still associated with SCCHN in non-smokers. Ethanol is classified as a human carcinogen by IARC [87]. Its carcinogenic effect likely stems from its primary metabolite, acetaldehyde, which has been recognized as a carcinogen in laboratory animals [78]. Acetaldehyde has been shown to interfere with DNA synthesis and repair. It can bind proteins resulting in structural dysfunction, including enzymes involved with DNA repair (O6 MGMT), DNA cytosine methylation and anti-oxidation (glutathione) [92, 93]; and has also been demonstrated to form DNA adducts with human cells *in vitro* and in chronically exposed lab rats [94]. The level of DNA adducts in lymphocytes among alcohol abusers is reported to be 7-fold that of non-drinkers [95]. Furthermore, experimental models have shown that inhalation of acetaldehyde can cause upper aerodigestive squamous cell carcinoma and adenocarcinoma in laboratory animals [71].

The major alcohol metabolizing enzymes are alcohol dehydrogenases (ADH), which oxidize ethanol to acetaldehyde, and aldehyde dehydrogenases (ALDH), which detoxify

acetaldehyde into acetate [87]. A variant allele of ALDH (ALDH2*2) encodes an inactive subunit of the enzyme and has an allele frequency of 28-45% in East Asian populations. Individuals homozygous for the variant rarely consume alcohol due to discomfort and toxic effects stemming from the enzyme deficiency, and therefore are difficult to study epidemiologically. However, heterozygotes have 10% ALDH2 activity, and heterozygous drinkers have been shown to have 3-fold higher concentrations of salivary acetaldehyde compared with wild-type individuals [96] and an increased risk of upper aerodigestive tract cancers [87], further supporting the role of acetaldehyde in carcinogenesis. Studies on ADH1 have been contradictory and therefore inconclusive [93].

Acetaldehyde concentrations found in saliva are high enough to enable it to act as a carcinogen [96-98]. In saliva, ethanol is metabolized to acetaldehyde by bacterial enzymes [93]. Poor oral health further potentiates the risk associated with smoking and alcohol consumption [78], resulting in an increase of oral bacteria, and therefore elevated salivary acetaldehyde levels [99]. Smoking rapidly shifts the oral flora from gram-negative to gram-positive bacteria, which convert ethanol to acetaldehyde in higher quantities, leading to a 50-60% rise in acetaldehyde concentrations compared with non-smokers [100]. Additionally, *Candida albicans*, commonly found in the oral flora of smokers, is also able to convert ethanol to acetaldehyde [100]. Use of antiseptic mouthwash has been shown to decrease salivary acetaldehyde levels by 30-50% [97]. Alcoholics with oropharyngeal cancer have been reported to have very high concentrations of acetaldehyde in their saliva [101].

As with smoking, another carcinogenic mechanism of alcohol consumption stems from nutritional deficiencies through interaction with folate and retinoid metabolism [93]. It can inhibit the absorption of folate, a key nutrient in the methylation process, and mediates the

inhibition of S-adenosylmethionine (SAM), the universal donor in methyl-transfer reactions, including DNA methylation [93]. Acetaldehyde has also been shown to hinder key enzymes in the DNA methylation process (DNA methyltransferases) in animal studies [102]. DNA methylation is an important epigenetic mechanism for regulation of gene expression and maintenance of chromosomal stability. Aberrant methylation can result in increased expression of oncogenes (hypomethylation) or inhibition of tumor suppressor genes (hypermethylation). In addition, chronic alcohol consumption depletes retinoids, which are fat-soluble A vitamins. Retinoic acid is of particular importance due to its role in cellular growth and differentiation [93]. In animal studies, reduced retinoic acid levels following chronic exposure to ethanol was associated with increased cellular proliferation and decreased apoptosis [103-105].

Alcohol consumption can also affect clinical outcomes from cancer. It has been associated with accelerated metastasis in colon cancer patients [106]. This is likely due to immunosuppression and induction of angiogenesis through the stimulation of vascular epithelial growth factor (VEGF) production [93, 107]. Furthermore, ethanol can interact with chemotherapeutic drugs, reducing their efficacy and increasing side effects [93].

2.1.3.3 Interaction of Smoking and Alcohol

When alcohol and tobacco are used in conjunction, the effect is multiplicative [20, 108]. Combined use of alcohol and tobacco account for 73% of oral and pharyngeal cancers in non-Hispanic Caucasians and 85% in African-Americans [33]. As previously discussed, alcohol is believed to further potentiate the effects of smoking by acting as a solvent for tobacco carcinogens. Conversely, smoking can enhance the carcinogenicity of alcohol by causing in a shift in oral flora, thus increasing the microbial conversion of ethanol to acetaldehyde.

2.1.3.4 Human Papillomavirus

Human papillomavirus (HPV) is associated with nearly all cervical and most anogenital cancers, and is now recognized as a risk factor for SCCHN [109]. Approximately 25% of SCCHN is associated with HPV infection; and 45%-60% of oropharyngeal carcinomas are HPV-positive, particularly those arising in the lingual and palatine tonsils [110, 111]. In contrast, the estimated prevalence of HPV in normal oral mucosa is 10% (95% CI: 6.1%-14.6%) [111]. HPV16 is the most prevalent subtype, accounting for 90%-95% of these tumors [112]. Individuals with seropositivity for HPV16 viral capsid protein antibodies have a 15-fold higher risk for SCCHN [113]. Furthermore, there is an increased risk of developing a second-primary SCCHN in those diagnosed with a cervical or anogenital primary cancer [114, 115], lending further support to an HPV etiology of a portion of the disease.

HPV-positive SCCHN represents a distinct clinical subset of tumors. In contrast to HPV-negative cases, patients with HPV-positive tumors tend to be younger in age, are more likely to be non-smokers or non-drinkers and are more frequently immunosuppressed [20, 78]. Morphologically, these tumors are more apt to have basaloid features and be poorly differentiated compared to HPV-negative cases [20, 72, 78]. However, patients with HPV-positive SCCHN tend to present at a lower T-stage [72] and have better overall and disease-free survival compared to those with HPV-negative tumors [116].

2.1.3.5 Other Risk Factors

Other risk factors for SCCHN include dietary factors, environmental and occupational exposures, gastroesophageal reflux and infection with the human immunodeficiency virus. These risk factors account for a much smaller attributable risk of SCCHN relative to tobacco, alcohol, and HPV and are discussed in more detail in Appendix A.1.6.

2.2 FIELD CANCERIZATION

Development of SCCHN is a multistep process involving accumulation of genetic and epigenetic alterations resulting in cellular dysregulation. The epithelium of the upper aerodigestive tract is exposed and vulnerable to carcinogenic insult, particularly from tobacco and alcohol, resulting in an increased risk of developing multiple primary tumors. According to the "field cancerization" model proposed by Slaughter and colleagues in 1953 [117], multiple cancers develop from distinct, unrelated clones arising due to accumulation of independent mutations associated with chronic exposure of the epithelium to environmental carcinogens. It has since been demonstrated that second primaries distant from the original primary can be clonally related to it [118]. This theory has lead to the development of the "expanding fields" model, which proposes that a single stem cell in the basal layer of the epithelium undergoes a transformation, clonally expands, and gradually replaces the normal epithelium. As cells within the expanding field acquire new alterations, various subclones develop within the field, which can eventually propagate into distinct but related tumors. These models help to explain, in part, the high rate of local recurrences and development of second primaries associated with SCCHN.

3.0 EPIGENETICS

Epigenetics are heritable but reversible changes in gene expression due to genetic modifications that do not alter the DNA sequence [4]. These alterations include DNA methylation and histone modifications (Appendix A.2.1), and play critical roles in regulation of gene expression, embryonic development and genomic stability.

3.1 DNA METHYLATION

DNA methylation is a crucial element in controlling gene expression, including genomic imprinting, X-inactivation and in maintaining the nuclear architecture of the cell. Additionally, methylation of DNA helps prevent chromosomal instability and aberrant translocations and represses the expression of intragenomic viral elements [4, 119-121]. During early embryonic development, alternating waves of methylation and demethylation program cellular growth and differentiation [122]. DNA methylation patterns are established early in embryogenesis and are under tight control.

DNA methylation occurs at dinucleotides in which cytosine is upstream and adjacent to guanine, called CpGs. This takes place when S-adenosylmethionine (SAM) donates a methyl (CH_3) group that is covalently attached to the 5-carbon of a cytosine pyrimidine ring in a reaction catalyzed by the enzyme DNA methyltransferase (DNMT) [120, 123]. Approximately 50-70%

of CpG dinucleotides are methylated in normal human tissue, termed global methylation [124]. However, most of the human genome is depleted of CpG dinucleotides due to the relative instability of m^5C, which can result in spontaneous hydrolytic deamination of the cytosine base to thymine [120]. CpGs are not randomly distributed throughout the genome but rather are concentrated in CpG enriched regions [4, 120, 125] referred to as CpG islands. Specifically, CpG islands are defined as a sequence greater than 0.5 kb with a G+C content greater than or equal to 55% and an observed:expected CpG ratio greater than 65% [126]. They typically range from 0.5 to 5 kb in length and occur on average every 100 kb in the genome [123]. These CpG islands are often disproportionately concentrated in the 5' promoter regions of genes. Approximately 50% of all human genes have CpG islands in the promoter region [120, 123, 125]. While CpG dinucleotides are frequently methylated in normal tissue, promoter-associated CpG islands are generally not methylated, although methylation of subgroups of CpG islands may occur [4, 120, 125].

During DNA replication, the methylation pattern of the parent strand is transferred onto the new strand by DNMT1 [120]. DNMT1 has an affinity for hemimethylated DNA [127] and towards replication foci [128]. However, during early embryonic development or carcinogenesis, previously unmethylated DNA may be methylated in a process mediated by DNMT3a or DNMT3b, which is termed *de novo* methylation [120, 123]. DNMT3b has a propensity to target pericentromeric satellite regions for methylation, which are prone to loss of stability as a result of hypomethylation, leading to chromosomal breakage [129].

There are several mechanisms guarding against aberrant promoter methylation (hypermethylation), including active transcription, active demethylation, timing of replication and prevention of access to DNMT by local chromatin structure [123]. Enzymes that actively

demethylate DNA are called demethylases. These may include the glycosylases thymine-DNA glycosylase (TDG) and methyl-CpG-binding domain protein 4 (MBD4), which remove the methylated cytosine (5-meC) leaving the deoxyribose intact to be replaced with a new cytosine via DNA repair [130, 131]; methyl-CpG-binding protein 2 (MBD2), which is believed to demethylate by hydrolyzing 5-meC to cytosine and methanol [123]; or thymine removal by glycosylases through the G/T mismatch base excision repair pathway following 5-meC deamination to thymine by DNMT3a or DNMT3b [131].

Generally, methylation of CpG islands in the promoter region is associated with transcriptional silencing of the gene, whereas methylation of downstream gene sequences has no influence on expression [120]. DNA methylation is capable of repressing gene expression in three general ways [123]. One mechanism is through direct interference with transcription factors. Several transcription factors, including AP-2, c-Myc, CREB, E2F, and NFkB, recognize and bind promoter regions containing CpG islands and are inhibited by methylation. A second mechanism involves inhibition of transcription through the direct binding of transcriptional repressors to 5-meC in the promoter region, including MBD1, MBD2, MBD4, Kaiso, MeCP1, and MeCP2. Finally, CpG methylation can guide the deacetylation of histones and subsequently alter chromosome structure to prevent transcription. Methylated cytosines of silenced promoters can bind methyl-CpG-binding-domain proteins (MBD), forming a complex involving histone deacetylase enzymes (HDAC) [120].

3.2 EPIGENETIC INTERACTIONS

3.2.1 Epigenetics and Aging

As people age, global methylation decreases and promoter methylation tends to increase [132]. Loss of genomic imprinting [133] and reactivation of X-chromosome inactivated genes [134] over time has been observed in animal models. Differential patterns of DNA methylation & histone modifications have been reported to occur even in monozygotic twins during the aging process. Differences increase with age and are particularly evident among twins spending longer portions of their lifetimes apart, which suggest an environmental interaction with epigenetics [135].

3.2.2 Epigenetics and Gender

Gender may influence susceptibility to epigenetic alterations, although the mechanisms are not fully understood. 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 [136], $p14^{arf}$ in colorectal cancer [137], and *CDH1* in lung cancer tissue [138]; but protective for others, such as *RASSF1A*, *TSLC1/IGSF4*, and *ESR1* in lung cancer [138-140], and methylation latent trait in bladder cancer based on a 16 gene panel [141]. There is experimental evidence suggesting that risk of promoter methylation for certain genes varies 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 [142]. Additionally, female gender has been associated with lower levels of global methylation in blood [143].

3.2.3 Epigenetics and Environmental Exposures

Environmental exposures can alter epigenetic regulation of the genome, although the precise mechanisms are largely unknown. People are most susceptible to epigenetic dysregulation during prenatal and neonatal development, puberty and old age [144].

Tobacco smoke is known to exert its carcinogenicity via both genetic and epigenetic mechanisms. Smoking has been associated with hypermethylation of several tumor suppressor genes in cancers of the lung, bladder, prostate and head and neck [139, 141, 145-147]; and specific associations of smoking dose and duration with promoter methylation of tumor suppressor genes have been reported [139, 145]. Promoter methylation of *CDKN2A* has been reported in the normal oral mucosa of 9.7% of cigarette smokers [148], supporting the notion that this can be an early event in SCCHN. An association of smoking and global DNA hypomethylation in SCCHN tissue has also been reported [149, 150]. Additionally, benzo(α)pyrene diol epoxide (BPDE), a chemical found in cigarette smoke, preferentially binds to guanines adjacent to methylated CpG dinucleotides, forming adducts that can induce G to T transversions [151, 152]. Similarly, acrolein, an aldehyde found in cigarette smoke, favors binding to 5-meC sites that can induce in C to T transitions [153]. Smoking can also affect epigenetics through inhibition of metabolism and storage of folate [79-81], which is the primary methyl source for the DNA methylation process, as previously discussed.

Alcohol can affect DNA methylation through the reduction of available methyl groups by blocking folate absorption and through the inhibition of S-adenosylmethionine (SAM),

preventing methyl transfer [93]. Furthermore, acetaldehyde, the primary metabolite of ethanol, has been shown to inhibit DNMT activity in mice [102]. Consumption of alcohol has been associated with promoter methylation of *Soluble Frizzled Receptor Protein 1* (*SFRP1)* [154], a gene involved in the WNT signaling pathway; and global hypomethylation [150].

Other environmental exposures have been implicated in epigenetic alterations as well. In experimental models, exposure to arsenic depletes s-adenosylmethionine (SAM), the primary methyl donor in DNA methylation, thus inducing global hypomethylation [155, 156]; but has also been associated with promoter hypermethylation of *p53* [157] and *RASSF1A* [141]. Ultraviolet radiation exposure has been reported to induce global hypomethylation [5], while ionizing radiation has been shown to induce hypermethylation of *CDKN2A* [158]. Nickel can actuate *de novo* methylation of tumor suppressor genes through induction of heterochromatin conformation by suppressing H4 acetylation [159, 160]. Chromium exposure can cause gene silencing via histone acetylation through interactions with HAT and HDAC enzymes [144]. Other metals such as cadmium and zinc also can affect epigenetics, both which have been shown to inhibit DNMT activity [161, 162]. Additionally, HDAC inhibitors bind through zinc at zinc-binding domains, preventing chromatin condensation [163].

3.2.4 Epigenetics and Diet

Although much of the effect of diet on epigenetics is unknown, several dietary factors have been associated with altered epigenetic patterns in humans. Among the most compelling are nutrients involved in one-carbon metabolism because they directly affect the supply of available methyl groups. This includes nutrient cofactors such as vitamin B_6, vitamin B_{12}, and the riboflavin derivative, flavin adenine dinucleotide (FAD); as well as dietary methyl, including folate,

methionine, and choline [160]. In one-carbon metabolism, the intracellular form of folate, 5,10-methylenetetrahydrofolate, is irreversibly converted to the predominant extracellular form, 5-methyltetrahydrofolate, which acts as the primary methyl donor in the conversion of homocysteine to methionine. Subsequently, methionine is converted to S-adenosylmethionine (SAM), which acts as the primary methyl donor in the DNA methylation process, catalyzed by DNMT [160]. Deficiencies in any of these nutrients could potentially result in an altered methylation process.

There is experimental evidence that certain dietary factors, such as zinc [164] and selenium [157] deficiency, may induce global hypomethylation. Conversely, other dietary factors, such as vitamin C deficiency, have been associated with local promoter hypermethylation [160]. Furthermore, there are some dietary factors that may result in both global hypomethylation and localized promoter hypermethylation: folate deficiency has been associated with increased global hypomethylation and cancer risk [160], and but also with hypermethylation of the 5' promoter region of *CDH13* (H-cadherin) [165]; and increased intake of retinoic acid has been associated with both as well [160].

There are additional mechanisms through which diet can affect epigenetics. Soy phytoestrogens have been associated with maintenance of a protective methylation pattern and may reduce the risk of certain cancers [160]. Phenols from green tea (epigallocatechin-3-gallate) are reported to affect methylation by binding and inhibiting DNMT, resulting in reactivation of methylation-silenced genes in cancer cell lines [166]. Additionally, dietary factors can act through histone modification. Butyrate, a short-chain fatty acid, has been shown to promote hyperacetylation of histones associated with the RET proto-oncogene, opening the chromatin conformation and thus resulting in its upregulation [167].

3.2.5 Epigenetics and Viral Infection

Although the mechanistic details are poorly understood, viruses may epigenetically alter host gene expression. DNA methylation and chromosome modifications are known to be involved in regulation of viral gene expression [5]. However, viral-associated methylation is not specific for viral DNA but rather occurs throughout the host genome [168, 169]. Infection with human papillomavirus (HPV), Epstein-Barr virus (EBV), simian virus 40 (SV40), hepatitis B (HBV) and hepatitis C (HCV) are all associated with epigenetic modification of the host DNA. EBV has been associated with promoter methylation and silencing of several genes in gastric [170] and nasopharyngeal cancers [171]; HBV and HCV in hepatocellular carcinoma [172-176]; and SV40 in mesothelioma [177, 178].

Likewise, HPV pathogenesis has been associated with altered CpG methylation and promoter methylation in cervical cancer [179-182], SCCHN [154] and in female non-smoking pulmonary adenocarcinoma patients [183]; although Dong and colleagues report an inverse relationship for HPV-16 and promoter methylation in SCCHN [184]. The E7 viral oncoprotein has been observed *in vitro* to directly associate with and stimulate DNMT1 activity [185], and DNMT1 expression is increased in low-grade cervical intraepithelial neoplasia (a premalignant condition) and further elevated in high-grade cervical neoplasia and invasive cervical cancer [186].

3.3 DNA METHYLATION AND CANCER

Epigenetics play a major role in carcinogenesis. Altered methylation patterns are common findings in cancer. DNA methylation generally affects cancer development in 3 main ways: through global hypomethylation, promoter hypermethylation and induction of point mutations (futher discussed in Appendix A.2.2) [120].

3.3.1 Hypomethylation

Cancer cells generally exhibit global hypomethylation [4, 120], with tumor cells losing between 20%-60% of their genomic 5-methylcytosine relative to normal tissue [187]. Hypomethylation generally is an early event in head and neck carcinogenesis and increases as the tumor progresses [4, 188]. This can result in overexpression of oncogenes or oncogenic microRNAs and can allow the transcription of parasitic sequences integrated into the genome, such as viral DNA. However, the main carcinogenic effect of hypomethylation stems from loss of genomic stability, which increases the risk of chromosomal breaks, translocations or allelic loss [4, 120, 189]. This is particularly true for hypomethylation of pericentric chromosomal regions, characteristic of many cancers, and may further increase the probability of chromosomal breakage [120].

3.3.2 Hypermethylation

Cancer cells frequently exhibit localized methylation of promoter CpG islands (hypermethylation), which are generally unmethylated in normal tissue [4, 120]. Promoter methylation is associated with transcriptional silencing, is at least as common as DNA mutation

in the inactivation of tumor suppressor genes and is considered to be a major event in carcinogenesis. There are approximately 100-400 hypermethylated CpG islands in the promoter regions of most tumors [4]. Some genes are hypermethylated in multiple cancers, such as *RASSF1A* and *CDKN2A*, while others are cancer-specific [123]. Hypermethylation can affect genes involved in cell cycle control, DNA repair, carcinogen metabolism, cell-cell interactions, apoptosis and angiogenesis [4, 160]. It occurs at different stages of cancer development in different genes and interacts with genetic lesions; and often begins in early stages, even in normal-appearing tissue preceding cancer, and then progressively increases during carcinogenesis [4, 125, 160].

Patterns of hypermethylated tumor suppressor genes appear to be cancer-specific [190, 191]. The systematic promoter methylation of multiple tumor suppressor genes within the same cancer type, known as CpG island methylator phenotype (CIMP), has been reported in several cancers, including colorectal, gastric, esophageal, pancreatic, esophageal, liver, ovarian, glioblastoma, acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML) [120]. Promoter methylation of *MINT* (*Methylated In Tumor*) loci has been proposed as an objective criterion for defining CIMP [192]. Tumor-specific methylation profiles have also been suggested for SCCHN, non-small cell lung cancer, bladder cancer and mesothelioma, but were not specific enough to consistently classify the tumors, with misclassification rates of 28%-32% [193]. Nonetheless, an association has been reported between *MINT* promoter methylation and methylation index (number of methylated tumor suppressor genes) in SCCHN [193].

Some CpG islands are located in chromosomal regions prone to large-scale epigenetic dysregulation [4]. Additionally, histone modifications can mark a gene for promoter methylation. Methylation of promoter CpGs is associated with a specific combination of histone

markers: deacetylation of histones H3 and H4, loss of H3K4 trimethylation, gain of H3K9 methylation, and gain of H3K27 trimethylation [194-198].

3.4 METHYLATION AND SCCHN

As with other solid tumors, aberrant promoter methylation is a common event in SCCHN. It is among the earliest events in head and neck carcinogenesis, preceding alterations in protein expression levels [25]. Promoter methylation of *CDKN2A* and *MGMT* has been detected in leukoplakia, a premalignant lesion [199]. There are several genes commonly hypermethylated in SCCHN (Table 2), including genes known to function in pathways involving cell-cycle control, apoptosis, cell-cell adhesion, DNA repair and tumor invasion [21, 25, 26, 112, 154].

Table 2. **Genes commonly hypermethylated in SCCHN**

Gene	Function
APC	cell cycle control; cell-cell adhesion; cell motility
*CDH1 (*E-cadherin*)*	cell-cell adhesion
MGMT	DNA repair
*CDKN2B (*p15*)*	cell cycle control
*CDKN2A (*p16/p14*)*	cell cycle control
RARB	transcription regulator; cell growth; differentiation
ATM	cell cycle control; DNA damage signaling
DCC	cell growth; apoptosis; differentiation
DAPK	apoptosis
CCNA1	cell cycle control
AIM1	cytokinesis
PGP9.5 (UCH-L1)	protein ubiquitylation
Lhx6	differentiation
RASSF1A	cell cycle control; apoptosis
ESR	estrogen receptor
HIC1	transcriptional repressor
TIMP3	degradation of the extracellular matrix
SFRP1,2,4,5	cell growth; cell-cell adhesion; morphogenesis

Sources: [21, 25, 26, 112, 154]

As a result of its anatomic location, DNA from head and neck tumors may be shed into saliva, allowing for identification of genetic or epigenetic aberrations using oral rinse (mouthwash) samples. Promoter methylation has been detected in oral rinses from patients with SCCHN and premalignant tumors [21, 23-26, 199]. Studies have reported variable correlation of promoter methylation by gene in oral rinse or saliva to tumor tissue with fair-to-good overall agreement [24-26]. However, it should be noted that the validation studies have thus far been relatively small and limited in scope.

4.0 MICRORNA

4.1 BIOLOGY AND FUNCTION

MicroRNAs (miRNA) are small, evolutionarily conserved, non-coding ribonucleic acid (RNA) molecules involved in regulation of gene expression in essentially all eukaryotic organisms. They are on average 22 nucleotides in length, ranging from 18-25 nucleotides [6, 200-203]. MicroRNAs are a recent discovery, being first described in 1993 with the identification of Lin-4, a small RNA that was determined not to encode a protein but which repressed expression of Lin-14 protein in the nematode *C. elegans* [204]. Presently, there are 695 human miRNA sequences catalogued in the miRNA registry (miRBase) [205, 206]. It is estimated that up to 30% of human genes are regulated by miRNA expression [207]. MicroRNAs are involved in control of crucial cellular functions, including proliferation, apoptosis, development, differentiation and metabolism [201]. They are tightly regulated and have been observed to show tissue-specific expression patterns during embryogenesis [200], though they are expressed in all tissues and at all stages of development [208].

4.1.1 MicroRNA Transcription

MicroRNA expression is regulated by transcription factors and transcribed by RNA Polymerase II (Pol II), similar to protein-coding genes, although the precise mechanisms of transcriptional

control of miRNAs are not entirely understood. While most miRNAs reside within intergenic non-coding regions [209], they can also be located in introns or exons of coding genes [6]. Many miRNAs are embedded close to other miRNAs in the genome, giving rise to miRNA clusters [209]. Single and clustered miRNAs can be transcribed from their own promoters, generally located within 500 base pairs of the 5' end of the miRNA, as single or polycistron transcriptional units, respectively [208, 209].

4.1.2 MicroRNA-Mediated Gene Regulation

Following transcription, the miRNA undergoes a maturation process, described in detail in Appendix A.3.1. The mature miRNA forms a complex with a member of the Argonaut (Ago) protein family, termed the RNA-induced silencing complex (RISC), and guides it specifically to the target messenger RNA (mRNA) through base pairing interactions generally at the 3' UTR of the target. Nucleotides 2-7 in the 5' region of the miRNA, called the seed region, bind the target mRNA through near-perfect base-pairing [210]. The remainder of the miRNA binds the target mRNA with varying degrees of complementarity [210]. If the miRNA is a perfect or near-perfect complement, cleavage and degradation of the mRNA is induced through de-capping of the 5' m^7G cap or de-adenylation of the poly(A) tail [6, 200-203]. If there is a partial complement, RISC inhibits translation [6, 200-203] through competitive m^7G cap binding by Ago 2 with the translational initiating factor eIF4E [211]. These translationally-silenced mRNA-RISC complexes remain in the cytoplasm and accumulate, forming processing bodies (P-bodies) [208]. P-bodies contain decapping proteins and exoribonuclease, and therefore are capable of degrading the mRNAs. However, there is newly emerging evidence that miRNA translational silencing may be reversible, allowing mRNAs to leave P-bodies and migrate to ribosomes for

translation [212]. Since base pairing with the target does not have to be a perfect compliment, a single miRNA can potentially affect mRNA and protein levels of 200 or more genes [6, 203].

4.1.3 Epigenetic Regulation of MicroRNA

MicroRNA expression can be regulated epigenetically, either through DNA methylation [6] or histone modification [213]. Approximately 10% of miRNAs are regulated by DNA methylation [7] and are more frequently methylated than protein-coding genes [202]. Although it is currently unknown exactly why this is, three general reasons have been suggested [202]: (1) the increased frequency of miRNA methylation could be due to the specific nucleotide sequences surrounding the miRNA-associated CpG islands; (2) miRNA could be embedded in specific chromosomal structures predisposing them to methylation; (3) the predilection for methylation could be related to the tight regulation of miRNA expression.

There are three general mechanisms by which miRNA expression can be controlled through methylation: (1) most commonly, miRNA can be embedded within or near a CpG island, which functions as its promoter [6, 202]; (2) miRNAs can be located within an imprinted region [214, 215], thus preventing transcription; (3) intronic miRNAs can be regulated by CpG island methylation of the promoter of the host gene [216, 217]. Forty-seven percent of miRNAs in the miRNA registry database (miRBase), and all miRNAs currently linked to epigenetic regulation, are associated with CpG islands [202]. Some miRNA promoters are unmethylated in normal tissue, while others are normally methylated [6, 202, 203, 218]. Promoter methylation of miRNA results in reduced expression or transcriptional silencing.

4.2 MICRORNA AND CANCER

The first evidence of the association between aberrant miRNA expression and cancer was the 2002 study by Calin and colleagues reporting the down-regulation and frequent deletion of miR-15a and miR-16-1 in chronic lymphocytic leukemia (CLL) [219]. Shortly after came the first description of altered miRNA expression in solid tumors, reporting down-regulation of miR-143 and miR-145 in colorectal carcinoma [220].

MicroRNAs are differentially expressed in cancers compared to normal tissue. These differences are both tissue- and tumor-specific [201-203]. The majority of miRNAs are down-regulated in cancer, however some are up-regulated [202]. Altered miRNA expression occurs early in carcinogenesis [208]. Abnormal miRNA expression has been identified in premalignant tumors, including colonic and pituitary adenomas [220, 221]. Additionally, miR-221 has been shown to be highly overexpressed in normal thyroid tissue adjacent to cancers, but not in normal thyroid tissue in patients without thyroid cancer [222].

MicroRNAs can function as either tumor suppressors or oncogenes (Table 3). Those that target and regulate proto-oncogenes act as tumor suppressors, so when they are down-regulated or silenced, the target oncogene is overexpressed [201]. Conversely, miRNAs that target tumor suppressor genes can act as oncogenes when overexpressed [201]. Some miRNAs operate as both tumor suppressors and oncogenes, dependent upon the tumor type [223]. This may be due to the notion that since different miRNAs are involved with different cellular pathways in different tissues, the effect on processes such as proliferation, differentiation and apoptosis may vary by form of cancer [223].

Table 3. MicroRNAs with altered expression in multiple solid tumors

miRNA	Function	Cancer/Tumor Types
let-7-a-2	Tumor suppressor	Breast, lung, liver
let-7-a-3	Tumor suppressor	Breast, liver
let-7d	Tumor suppressor	Breast, liver
let-7f	Tumor suppressor	Breast, liver, thyroid
miR-10b	Tumor suppressor	Breast, liver
miR-15b	Tumor suppressor	Thyroid, pituitary adenoma
miR-17-92	Oncogenic	Lung, thyroid, liver, neuroblastoma
miR-21	Oncogenic	Breast, lung, thyroid, liver, ovary, pancreas, glioblastoma, head and neck
miR-24-2	Oncogenic	Lung, thyroid
miR-34a	Tumor suppressor	Breast, bladder, prostate, pancreas, melanoma
miR-101	Tumor suppressor	Breast, lung, pituitary adenoma
miR-102	Oncogenic	Breast, thyroid
miR-124a	Tumor suppressor	Lung, liver, colon, medulloblastoma, pituitary adenoma
miR-124a	Oncogenic	Tongue
miR-125a	Tumor suppressor	Breast, lung
miR-125b-1	Tumor suppressor	Breast, ovary
miR-125b-1	Oncogenic	Thyroid, glioblastoma
miR-125b-2	Tumor suppressor	Breast
miR-125b-2	Oncogenic	Thyroid, glioblastoma
miR-127	Tumor suppressor	Breast, lung, colon, pancreas, bladder, cervix
miR-137	Tumor suppressor	Oral, glioblastoma
miR-140	Tumor suppressor	Breast, lung, thyroid, ovary, pituitary adenoma
miR-141	Tumor suppressor	Liver, pituitary adenoma
miR-142	Tumor suppressor	Thyroid, liver
miR-143	Tumor suppressor	Breast, lung, liver, colon
miR-145	Tumor suppressor	Breast, lung, liver, colon, ovary
miR-146	Oncogenic	Lung, thyroid
miR-150	Oncogenic	Lung, pituitary adenoma
miR-155	Oncogenic	Breast, lung, thyroid, pancreas
miR-181a	Tumor suppressor	Liver
miR-181a	Oncogenic	Thyroid
miR-181b	Tumor suppressor	Glioblastoma, pituitary adenoma
miR-181c	Tumor suppressor	Liver, glioblastoma
miR-181c	Oncogenic	Thyroid
miR-191	Oncogenic	Breast, lung, pituitary adenoma
miR-192	Oncogenic	Lung, pituitary adenoma
miR-193a	Tumor suppressor	Oral, glioblastoma
miR-198	Tumor suppressor	Lung, glioblastoma
miR-199b	Tumor suppressor	Lung, liver
miR-202	Oncogenic	Breast, thyroid
miR-203	Oncogenic	Breast, lung
miR-210	Oncogenic	Breast, lung
miR-212	Oncogenic	Lung, pituitary adenoma
miR-213	Oncogenic	Breast, thyroid
miR-219-1	Tumor suppressor	Lung, thyroid
miR-220	Tumor suppressor	Lung
miR-220	Oncogenic	Thyroid

Sources: [17, 27, 200, 223-235]

4.2.1 MicroRNA and Tumor Classification

Profiling studies have identified multiple dysregulated miRNAs in cancer and have been able to classify cancer by type [236, 237]. These profiling studies report global down-regulation of miRNAs in cancer and have thus far been more tumor-specific compared with mRNA expression profiles, which tend to be more tissue-specific than tumor-specific [208].

4.2.2 MicroRNA and Prognosis

The potential prognostic value of miRNAs is supported by their involvement in proliferation, differentiation and apoptotic pathways. MicroRNA expression profiles have been correlated with clinical outcome [201-203]. Dysregulation of individual miRNAs have also been associated with prognosis. For example, downregulation of miR-106a is associated with decreased survival [11], and miR-320 and miR-428 with decreased disease-free survival [16] in colon cancer patients; and decreased expression of let-7 family members has been associated with poorer survival in lung cancer patients [18] and in SCCHN patients when downregulated in conjunction with miR-205 [10], although promoter methylation of let-7-3 has been associated with better prognosis in ovarian cancer [238]. Reduced expression of miR-205 is also an independent predictor of loco-regional recurrence of SCCHN [10]. Upregulation of miR-21 has been associated with poorer survival in breast [19, 239], pancreatic [12] and non-small cell lung cancers [15]; overexpression of miR-211 is associated with poor prognosis in oral squamous cell carcinoma [9]; overexpression of miR-16 is associated with decreased overall and disease-free survival in pediatric acute lymphocytic leukemia (ALL) patients [13]; upregulation of miR-210 in breast cancer correlates with decreased survival [8]; and higher expression levels of miR-20b

and miR-150 are associated with decreased survival in patients with undifferentiated gastric cancer [14]. Altered miRNA levels have also been associated with tumor stage [11, 239], grade [19], lymph node metastasis [9, 14, 239] and vascular invasion [9, 11].

Low total levels of miRNAs correlate with poor differentiation [18, 237, 240]. In fact, miRNA profiles have been shown to better classify poorly differentiated tumors compared to mRNA profiles [241]. As further evidence, miRNA expression can be triggered through induction of cellular differentiation with all-trans retinoic acid [237].

4.2.3 MicroRNA and Metastasis

Metastases are the primary cause of death for patients with solid tumors [187], and there is accumulating evidence that microRNAs play a role in these events. Expression of miR-10b has been reported to be increased in metastatic breast cancer cells compared to non-metastatic cells [242]. Additionally, several metastasis-related genes are predicted miRNA targets, including *Lysyl Oxidase* (*LOX*), *E-cadherin* (*CDH1*), *Integrin alphaVbeta-3*, *Syndecan-1* (*SDC1*), *Mesenchymal-Epithelial Transition Factor* (*c-Met*), *Hypoxia-Inducible Factor-1*α (*HIF-1*α), *Tissue Inhibitor of Metalloproteinase-3* (*TIMP-3*), *Adamalysin Metalloproteinase-17* (*ADAM-17*), and *Fusin* (*CXCR4*) [200]. Furthermore, the angiogenesis inhibiting genes *Thrombospondin-1* (*Tsp-1*) and *Connective Tissue Growth Factor* (*CTGF*) are downregulated during angiogenesis and are potential targets of the oncogenic miR-17-92 cluster [243].

4.2.4 Epigenetic Dysregulation of MicroRNA in Cancer

Dysregulated expression in at least one type of cancer has been reported in 25.8% of known human miRNAs [244], suggesting that they may be one of the largest classes of gene regulators associated with cancer. Dysregulation of miRNAs can lead to changes in expression of their target genes, such as up-regulation of oncogenes or silencing of tumor suppressor genes, resulting in cellular dysfunction and eventually cancer. Further discussion will focus on epigenetic dysregulation of microRNAs; however, other mechanisms are discussed in more detail in the addendum (Appendix A.3.5).

Approximately 10% of miRNAs are regulated through promoter methylation, as previously discussed in Section 4.1.3. Hypermethylation can lead to reduced expression or silencing of tumor suppressor miRNAs, whereas hypomethylation can lead to activation of oncogenic miRNAs. There have been several reports in the literature of associations between altered methylation status of miRNAs and various cancers (Table 4), including lung, colorectal, breast, ovarian, oral, bladder, pancreatic, gastric, cholangiocarcinoma and several hematopoietic cancers [17, 27, 216, 218, 229, 231, 238, 245-252].

Table 4. MicroRNAs with altered methylation status in human cancer

miRNA	Oncogenic or Tumor Suppressor	Cancer Type(s)	Reference
let-7a-3	Oncogenic	Lung, ovarian	[218, 238]
miR-1	Tumor Suppressor	Hepatocellular	[246]
miR-9	Tumor Suppressor	Breast, colorectal	[248, 253]
miR-34a	Tumor Suppressor	Breast, lung, colorectal, renal, bladder, pancreas, prostate, melanoma	[229]
miR-34b/34c	Tumor Suppressor	Colorectal	[251]
miR-124	Tumor Suppressor	Breast, colorectal, lung, gastric, lymphoma, leukemia	[249, 252]
miR-127	Tumor Suppressor	Bladder, colorectal	[231, 253]
miR-129	Tumor Suppressor	Colorectal	[253]
miR-137	Tumor Suppressor	Oral, gastric, colorectal	[17, 27, 252]
miR-193a	Tumor Suppressor	Oral, gastric	[27, 252, 253]
miR-203	Tumor Suppressor	ALL, CML	[245]
miR-223	Tumor Suppressor	AML	[247]
miR-342	Oncogenic	Colorectal	[216]
miR-370	Oncogenic	Cholangiocarcinoma	[250]

4.3 MICRORNA AND SCCHN

Altered expression of miRNAs has been reported in SCCHN, although the degree of dysregulation varies by study. A recent microarray analysis by Kozaki and colleagues evaluated expression levels in 148 miRNAs in oral squamous cell carcinomas (OSCC) compared with normal oral mucosa, reporting down-regulation (< 0.5-fold) in 36.5% (54/148) and up-regulation (> 1.5-fold) in 7.4% (11/148) of miRNAs [27]. Using a 2-fold expression threshold, Childs reported downregulation in 18.2% (43/236) and upregulation in 2.5% (6/236) of miRNAs in SCCHN [10]. In another miRNA microarray of squamous cell carcinomas of the tongue with matched normal tissue, Wong and colleagues identified 8.3% (13/156) down-regulated and 15.4% (24/156) up-regulated miRNAs, using 3-fold expression level changes [234]. In contrast, a miRNA profiling study by Chang and colleagues found only 9 of about 300 miRNAs to be

differentially expressed in SCCHN compared to normal tissue, with 8 overexpressed and 1 underexpressed [224]. Differences in expression thresholds used in each study may explain much of the variability, although some of the heterogeneity between studies may be attributable to variability by head and neck cancer site. The study by Chang used a mix of primary SCCHN tumors and cell lines, whereas Kozaki used only OSCC cell-lines and Wong used only tongue SCC cell-lines, although Tran and colleagues found no significant difference between sites for miRNA expression in SCCHN [254].

Additionally, tumor-specific miRNA signatures for SCCHN have been reported. In a study by Jiang and colleagues, 5 out of 5 SCCHN cell lines produced unique miRNA signatures compared to other cancer cell lines [255].

To date, there have been a limited number of studies regarding miRNA expression in SCCHN. However, the associations of several differentially expressed individual miRNAs with SCCHN have been validated, including both oncogenic and tumor suppressor miRNAs (Table 5).

Table 5. MicroRNAs with altered expression associated with SCCHN, validated on primary tumor samples

miRNA	Site	Function	Reference
miR-1	Oral, Pharynx, Larynx	Tumor Suppressor	[10]
Let-7d	Oral, Pharynx, Larynx	Tumor Suppressor	[10]
miR-21	Oral, Pharynx, Larynx	Oncogenic	[10, 224]
miR-133	Oral, Pharynx, Larynx	Tumor Suppressor	[10]
miR-137	Oral	Tumor Suppressor	[27]
miR-184	Tongue	Oncogenic	[234]
miR-193a	Oral	Tumor Suppressor	[27]
miR-205	Oral, Pharynx, Larynx	Tumor Suppressor	[10]
miR-211	Oral	Tumor Suppressor	[9]
miR-494	Oral, Pharynx, Larynx	Tumor Suppressor	[224]

4.3.1 Tumor Suppressor microRNAs and SCCHN

Tumor suppressor miRNAs that are reported to be downregulated in SCCHN include miR-1, miR-133, miR-137 (OSCC), miR-193a (OSCC), miR-205, miR-211 (OSCC), miR-494 and let-7d. The exact targets and function of miR-1, miR-133, miR-205, miR-211 and miR-494 are presently unknown [224]. However, miR-494 is located on chromosome 14q32.31, which is a region that is commonly deleted in SCCHN [256]. Furthermore, loss of heterozygosity at 14q is associated with poor prognosis in SCCHN patients, with a 3-fold increased risk of death [256]. Similarly, miR-211 is located in intron 6 of the *TRPM1* gene at 15q13-q14, which is a region frequently lost in cancers, including SCCHN [257, 258]. Let-7d is commonly underexpressed in tumors. Confirmed targets of the let-7 family include the *K-Ras* oncogene [259] and *HMGA2* [260], overexpression of which is associated with poor prognosis and metastases in lung cancer [261]. It has been reported that let-7d expression is repressed by c-myc expression [262], an oncogene frequently overexpressed in SCCHN [10].

Promoter methylation and down-regulation of *miR-193a* and *miR-137* have been reported in oral squamous cell carcinoma (OSCC) [27]. In a study of 11 primary OSCC tumors with matched normal oral tissue, miR-193a was found to be hypermethylated in 72.7% (8/11) of cases [27]. Its targets include the transcription factor E2F6; possibly PTK2/FAK, which is involved in cellular growth and signal transduction; and possibly MCL1, an anti-apoptotic member of the BCL-2 family [27]. Expression of miR-193a correlates with increased apoptotic activity. Similarly, in the same study *miR-137* promoter methylation was detected in 63.6% (7/11) of primary OSCC tumors, which will be described in more detail in the subsequent section (Section 4.4).

4.4 MICRORNA-137

MicroRNA-137 (*miR-137*) is located on chromosome 1p21.3, is associated with a large CpG island [17, 27] and is downregulated in OSCC [27], colon cancer [263], and gliomas [17]. *MicroRNA-137* has been reported to undergo promoter methylation in OSCC [27], which was correlated with decreased expression levels; colon cancer [253]; gastric cancer cell lines [252]; and is also likely hypermethylated in gliomas [17]. Targets of miR-137 include Cyclin-dependent kinase 6 (Cdk6) and possibly the transcription factor E2F6 and NCOA2/TIF2, which plays a role in histone acetylation [27].

Cdk6 is a proto-oncogene involved in cell cycle progression (Figure 4). It shares 71% homology with Cdk4 [264], both of which are ubiquitously expressed and form a complex with Cyclin D1 to phosphorylate pRb, leading to its inactivation. The function of pRb is to repress the transcription of genes required for progression from G1 phase to S phase by binding and repressing the E2F transcription factor [264, 265]. When pRb is inactivated, E2F is released and the cell can progress to S phase. Transfection of miR-137 into cells lacking its expression results in decreased Cdk6 expression and cell cycle arrest at the G1-S checkpoint in OSCC [27] and glioma [17] cell lines. In further support, anti-Cdk4/6 has been shown to inhibit pRb phosphorylation at Cdk4/6 specific sites, arrest tumor growth in G1 phase, and induce tumor regression in lung, breast, colon, glioblastoma, and prostate human tumor xenografts [266].

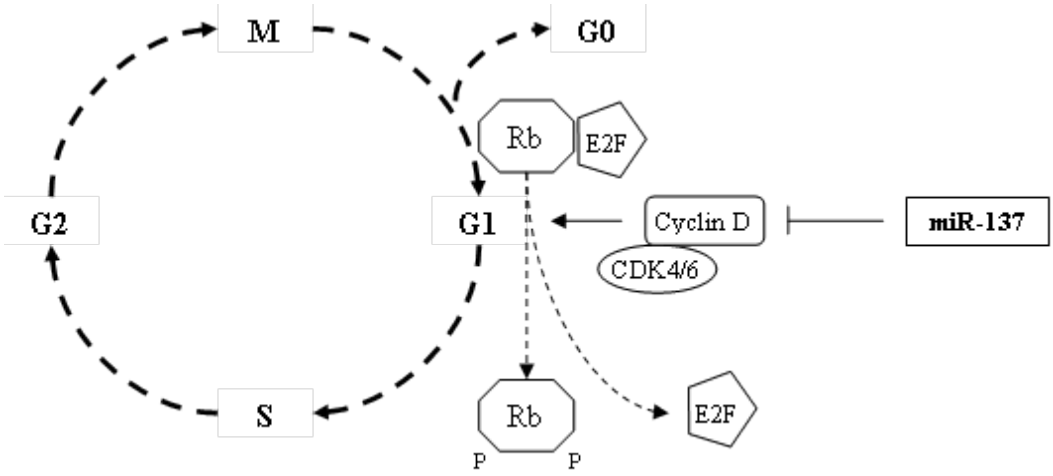


Figure 4. The role of miR-137 in cell cycle control

In addition to its role in cell cycle progression, recent findings suggest that Cdk6 is involved in cellular differentiation [264]. In cultured neural stem cell tissue, miR-137 is upregulated during differentiation but is downregulated in high-grade gliomas [17]. Furthermore, transfection of miR-137 and miR-124 (which also targets Cdk6) was shown to induce neuronal differentiation in glioblastoma multiforme stem cells [17].

Although they are homologues and have overlapping function in the phosphorylation of pRb, Cdk4 and Cdk6 may also have discrete functions [264]. Cdk6 is upregulated in OSCC [28-30] and neuroblastomas [267] without change in Cdk4 expression. In addition, overexpression of Cdk6 has been independently associated with poor prognosis in medulloblastoma patients [268].

Promoter methylation and corresponding downregulation of *miR-137* in OSCC suggests that it may play an important role as a tumor suppressor in head and neck tumorigenesis. This is further substantiated by the reduced expression of miR-137 in other cancers, as well as the biologic function and overexpression of its target, Cdk6, in OSCC.

5.0 BACKGROUND SUMMARY

Head and neck cancer accounts for a considerable amount of preventable morbidity and mortality, both in the US and worldwide. Squamous cell carcinoma (SCCHN) makes up the majority of these cancers, comprising approximately 93% of all cases [3]. The strongest risk factors for SCCHN are tobacco and alcohol use, which have a synergistic multiplicative effect when combined [20, 108].

DNA methylation is an epigenetic event that is crucial in controlling gene expression. Aberrant promoter methylation is at least as common as mutations in tumor suppressor gene inactivation and therefore is considered to be a major event in carcinogenesis, including the development of SCCHN. Alcohol and tobacco are environmental exposures, which may alter the epigenetic machinery, potentially affecting patterns of methylation.

Within the past few years, dysregulated expression of miRNAs, small non-coding regulatory RNA molecules, has been associated with various cancers, including SCCHN, and with poor prognosis. MicroRNA expression profiles show more tumor-specificity than mRNA profiles, suggesting that alterations affecting miRNA transcription, such as promoter methylation, may be more specific for tumor type than those affecting protein-coding genes. Additionally, dysregulated expression of miRNAs has been reported to have prognostic implications. It is estimated that 10% of miRNAs are controlled by DNA methylation [7]. A recent publication reported promoter methylation and downregulation of *miR-137* in oral

squamous cell carcinoma (OSCC) [27]. Additionally, they found that miR-137 directly targets Cdk6, a key protein in cell cycle progression, and that downregulation of miR-137 results in increased cellular proliferation.

Early diagnosis of SCCHN is crucial in treatment of the disease, resulting in a better prognosis for the patient. Presently, visual inspection is the only proven screening method regularly used in clinical settings. Collection of oral rinse (mouthwash) samples is a simple, non-invasive method for obtaining DNA from the upper aerodigestive tract epithelium [269, 270]. Promoter methylation of tumor suppressors can be early events in carcinogenesis that are detectable in oral rinse samples [21-25] and have potential value as biomarkers of SCCHN. Exploration of new biomarkers, such as *miR-137* promoter methylation, will help aid in the search for better diagnostic and prognostic tools for early detection and treatment of SCCHN.

The main objective of this proposed research is to contribute to our understanding of head and neck cancer by evaluating promoter methylation of *miR-137* as a biomarker of SCCHN. Specifically, the 3 aims of this research, each corresponding to the subsequent 3 sections, are as follows:

1a) Evaluation of *miR-137* promoter methylation as a diagnostic biomarker of SCCHN using oral rinse as a non-invasive DNA collection media

1b) Assess potential associations between smoking, alcohol consumption and other personal and behavioral risk factors for *miR-137* promoter methylation in oral rinses from SCCHN patients

2) Evaluate *miR-137* promoter methylation in SCCHN tumor tissue as a prognostic biomarker

3) Determine the predictive value of oral rinse for identifying *miR-137* promoter methylation in SCCHN using methylation-specific PCR

6.0 *MICRORNA-137* PROMOTER METHYLATION IN ORAL RINSES FROM PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Langevin SM, Stone RA, Bunker CH, Grandis JR, Sobol RW, Taioli E. *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.* Carcinogenesis; 2010 Mar:[Epub Ahead of Print].

6.1 ABSTRACT

Head and neck cancer represents 3.3% of all new malignancies and 2.0% of cancer-deaths in the US, 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 PCR, was detected in 21.2% oral rinses from SCCHN patients and 3.0% from controls (OR = 4.80, 95% 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 (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.

6.2 INTRODUCTION

In 2008, head and neck cancer accounted for an estimated 47,560 new cases in the United States, representing 3.3% of all malignancies and 11,260 deaths [271]. The majority of these (93%) are squamous cell carcinoma of the head and neck (SCCHN) [3]. Use of tobacco and alcohol are each independently causally associated with development of this disease, and when combined have a synergistic multiplicative effect [20, 72]. The 5-year survival in SCCHN (approximately 60%) has remained essentially unchanged over the past 3 decades despite therapeutic advances [20]. The majority of patients present with advanced stage disease (stage III or IV) [20], 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 [4], 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 [208]. 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) [27], gastric cancer cell lines [272] and colon cancer [253]. Prior evidence suggests that promoter methylation correlates with down-regulation of miR-137 in OSCC relative to non-cancerous oral tissue [27]. MicroRNA-137 appears to play a role in cellular differentiation and cell cycle control, at least in part through negative regulation of Cdk6 expression, [17, 27]. It is hypothesized that over-expression of Cdk6 may result in accelerated progression through the G1/S-phase checkpoint of the cell cycle, thus leading to increased proliferation and reduction in DNA repair capacity [273].

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 [21, 23-26]. 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.

6.3 MATERIALS AND METHODS

6.3.1 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 ($\geq$ 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. IRB approval was obtained under the University of Pittsburgh Head and Neck Cancer SPORE for sample collection and use of patient data. All subjects provided written informed consent for participation in this study.

6.3.2 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.

6.3.3 Oral Rinse Samples

All subjects provided an oral rinse sample, obtained by swishing with 20 ml of saline for 20-30 seconds. Approximately 10 ml of commercial mouthwash (Scope™) 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.

6.3.4 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 ($p = 0.92$ vs age in total cases); the proportion of females was 23.4% ($p = 0.71$ vs female in total cases); and the distribution of site was 39.1% oral cavity, 34.4% pharynx and 26.6% larynx ($p = 0.94$ vs 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 to *miR-137* promoter methylation in the DNA isolated from oral rinse samples.

6.3.5 Methylation-Specific PCR

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 (ThermoFisher Scientific, Waltham, MA), and sodium bisulfite treatment was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA).

Methylation-specific PCR (MSP) was employed for the analysis of *miR-137* promoter methylation, and the PCR 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 [60]. The primer sequences used to assess *miR-137* promoter methylation were as follows: methylated alleles: GCGGTAGTAGTAGCGGTAGC and ACCCGTCACCGAAAAAAA with an annealing temperature of 58°C and expected amplicon of 86 bp; and unmethylated alleles: GGTGGTAGTAGTAGTGGTAGT and TACCCATCACCAAAAAAAA with an annealing temperature of 51°C and expected amplicon of 86 bp. As a quality control check, each PCR reaction included fully methylated and unmethylated bisulfite converted human DNA (Qiagen, Valencia, CA) as positive and negative controls. Samples yielding faint positive signals were repeated twice more and only consistently positive samples were considered to be methylated.

6.3.6 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 [274] 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 and negative predictive value of *miR-137* promoter methylation for distinguishing patients with SCCHN from those with non-cancerous ENT conditions was 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 ($P_E = 0.15$; $P_R = 0.20$) [275]. 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 [276], 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 [277]; 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 to 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/never smoking term was

included in the model and considered additively with smoking duration ($y = \beta_1 * X_{Ever/Never} + \beta_2 * X_{YearsSmoking}$), where never-smoking = 0 and ever-smoking = 1 [278].

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* (AJCC 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* promoter 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.

6.4 RESULTS

6.4.1 Association of *miR-137* Promoter Methylation with Case-Control Status

A description of the case-control study population is presented in Table 6. SCCHN patients were significantly less educated relative to control subjects ($P < 0.001$), reported eating fewer servings of fruits and vegetables per day ($P < 0.001$) and had a lower median body mass index (BMI; $P = 0.07$). Cases were more likely 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 per day on average ($P < 0.001$). Cases were also more likely 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.

Table 6. 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]
Body mass index (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 per 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 per 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] 1 drink is considered to be 1 beer (12 oz), 1 glass of wine (5 oz) or 1 shot of liquor (1.5 oz)

MicroRNA-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 7. SCCHN patients had nearly 5-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 (OR = 4.80, 95% CI: 1.23-18.82).

Table 7. Association of *miR-137* promoter methylation and SCCHN

		Association with SCCHN	
miR-137 Methylation Status	$n_{cases}/n_{controls}$	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 per day, education and daily servings of fruits and vegetables

The sensitivity of *miR-137* promoter methylation in differentiating patients with SCCHN from those with non-neoplastic ENT conditions was 21.2%, and the specificity was 97.0%. The positive predictive value (PPV) was 87.5%, and the negative predictive value (NPV) was 55.2%.

When considering anatomic site of tumor origin (Table 7), *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 more than 12-times as

high as cancer-free control subjects (OR = 12.18, 95% CI: 2.63-56.36); estimates were not statistically significant for pharyngeal (OR = 3.76, 95% CI: 0.68-20.76) or 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.

6.4.2 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 8 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; while body mass index (P = 0.002) was inversely associated. None of the methylation-positive cases or controls reported being both never-smokers and never-drinkers.

Table 8. 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[c]	54.9 (53.8)	57.7 (57.2)	0.51[a]
Gender, n (%)			0.10[b]			> 0.99[b]
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[a]	2.9 (2.7)	2.9 (3.0)	0.80[a]
Body mass index (BMI; kg/m^2)[d], mean (median)	23.9 (23.0)	28.0 (27.2)	0.002[a]	28.7 (29.0)	28.4 (27.4)	0.83[a]
Cigarette use			0.14[b]			0.57[b]
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[c]	26.5 (26.5)	25.5 (26.0)	0.95[a]
Packs per day, mean (median)	1.1 (1.0)	1.3 (1.0)	0.36[a]	0.8 (0.8)	1.2 (1.0)	0.53[a]
Age started smoking, mean (median)	19.7 (17.0)	17.1 (17.0)	0.51[a]	16.5 (16.5)	16.4 (17.5)	0.68[a]
Alcohol use			0.58[b]			> 0.99[b]
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[c]	27.0 (27.0)	25.9 (28.0)	0.95[a]
Drinks per day, mean (median)	2.4 (2.0)	3.1 (1.7)	0.64[a]	2.9 (2.9)	1.3 (0.3)	0.19[a]
Age started drinking, mean (median)	22.6 (21.0)	20.2 (18.0)	0.18[a]	17.5 (17.5)	23.4 (21.0)	0.12[a]
Combined tobacco/alcohol use			0.64[b]			0.57[b]
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[b]			0.48[b]
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[a]	47.0 (47.0)	18.3 (11.5)	0.12[a]
Mouthwash use, n (%)			> 0.99[b]			> 0.99[b]
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[a]	10.5 (10.5)	7.0 (7.0)	0.21[a]
Years of regular mouthwash use, mean (median)	26.4 (30.0)	19.2 (20.0)	0.06[c]	21.0 (21.0)	15.5 (10.0)	0.89[a]
Tumor site, n (%)			0.009[b]			
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[b]			
Local (stage I, II)	7 (33.3%)	25 (33.3%)		---	---	
Advanced (stage III, IV)	14 (66.7%)	50 (66.7%)		---	---	
Tumor grade, n (%)			0.51[b]			
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] Mann-Whitney U test
[b] Fisher's exact test
[c] Two-sample t-test
[d] Estimated for 1-year prior to study enrollment based on patient reported weight at that time

A multivariable case-case comparison was performed (Table 9); covariates included in the final multivariate model were smoking duration, alcohol intensity, body mass index (BMI), denture use and gender. Women SCCHN cases had 5-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*

promoter methylation with BMI per kg/m^2 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). There was no significant interaction between alcohol consumption and smoking. 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* promoter methylation in SCCHN tissue. Sensitivity of the oral rinse for detection of *miR-137* methylation in tumor tissue was 45.5% and specificity was 81.1%.

Table 9. Case-only analysis for the association of various risk factors with *miR-137* promoter methylation

		Association with *miR-137* promoter methylation			
	$n_{methylated}$/ $n_{unmethylated}$	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)	
Body mass index (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 (≤ 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[g]			0.02		0.09
Never		1.00 (*ref*)		1.00 (*ref*)[e]	
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 per day		1.00 (*ref*)		1.00 (*ref*)[f]	
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*)[f]	
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] Adjusted for gender, BMI, dentures and alcohol use
[f] Adjusted for gender, BMI, dentures, alcohol use and total years smoking
[g] Modeled continuously with an additive binary (ever/never) term as ($y = \beta_1 * X_{Ever/Never} + \beta_2 * X_{YearsSmoking}$), where never-smoking = 0 and ever-smoking = 1 to account for right skewness due to never smokers

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 ($P > 0.99$).

6.5 DISCUSSION

Since 2006, numerous microRNAs have been reported to be hypermethylated in various cancer types [17, 27, 216, 218, 229, 231, 238, 245-251, 253, 272]. MicroRNA-137 is a tumor suppressor that targets Cdk6, an oncogene involved in progression past the G1/S-phase checkpoint, the loss of which has been shown to increase cellular proliferation *in vitro* [17, 27], and is possibly involved in cellular differentiation [17]. Therefore its transcriptional repression via DNA promoter methylation could have consequences with respect to carcinogenesis. Recent studies have identified *miR-137* promoter methylation in several types of solid tumors [27, 253, 272], including oral squamous cell carcinoma [27]. 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 likely 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 and colleagues [27],

where promoter methylation was observed in 7/11 (63.6%) tumor tissue from patients with oral squamous cell carcinoma. 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 2-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 to the Japanese population included in the Kozaki study. Another possible reason for the inter-study variation could be due to the study design and laboratory methods; the present study used MSP analysis of DNA isolated from formalin-fixed paraffin embedded (FFPE) tumor tissue, whereas Kozaki employed combined bisulfite restriction analysis (COBRA) [279] on DNA from fresh-frozen 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 [148, 280, 281], including *CDKN2A*, a tumor suppressor gene also involved control of the G1/S-phase checkpoint, in oral tissue of smokers [148, 281]. *MicroRNA-137* promoter methylation has also been reported in "normal" adjacent colonic tissue of colorectal cancer patients [253]. 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.

Although a small sample-size, all 3 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 oral rinse 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 [136], $p14^{arf}$ in colorectal cancer [137] and *CDH1* in lung cancer tissue [138]; but protective for others, such as *RASSF1A*, *TSLC1/IGSF4* and *ESR1* in lung cancer [138-140] and methylation latent trait in bladder cancer based on a 16 gene panel [141]. Experimental evidence suggests that risk of hypermethylation for certain genes may vary in a tissue-specific, gender-dependent manor, as do the transcriptional targets, based on the effects of sex hormones on epigenetic states and differential distribution of sex hormone receptors [142]. In experimental models, murine studies have demonstrated higher frequencies of methylation of certain genes following estrogen administration [282, 283]. In transgenic breast cancer mouse models, estrogen increases epigenetic inactivation of genes involved in cell cycle control and apoptosis with a dose-response effect [284]. 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 [285, 286], 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 promoter methylation and cigarette smoking in lung [138, 139, 146], prostate [287] and bladder cancer tissue [147]. Others have also found similar dose-response relationships between smoking and promoter methylation [139, 280, 287]. 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 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 [288, 289], wearing of poorly fit or malfunctioning dentures has recently been associated with oral and pharyngeal cancer [290]. Improperly fitting dentures can result in chronic irritation of the surrounding mucosa. Halogenated pyrimidines that stem from damage caused by reactive oxygen species (ROS) as a result of the chronic inflammatory process mimic 5-methylcytosine and have been shown to stimulate DNMT1-mediated CpG methylation [291, 292], 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, while 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 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 oral rinse 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.

7.0 EVALUATION OF *MIR-137* PROMOTER METHYLATION AS A PROGNOSTIC BIOMARKER FOR SCCHN

7.1 ABSTRACT

The overall 5-year survival rate of approximately 60% for head and neck cancer patients has remained essentially unchanged over the past 30 years. MicroRNA-137 (miR-137) plays an essential role in cell cycle control at the G1/S phase checkpoint. However, aberrant *miR-137* promoter methylation observed in squamous cell carcinoma of the head and neck (SCCHN) suggests a tumor-specific molecular defect that may contribute to disease progression. The goal of this study is to assess, in formalin-fixed paraffin-embedded tumor tissue, the association between *miR-137* promoter methylation and survival (both overall and disease-free) and with prognostic factors including stage, tumor size, nodal positivity, tumor grade and surgical tumor margin positivity. Promoter methylation status of *miR-137* was ascertained by methylation-specific PCR and detected in 11/67 SCCHN patients (16.4%), with no significant differences according to site (oral cavity, pharynx, larynx). Methylation of the *miR-137* promoter was significantly associated with overall survival (Hazard Ratio = 3.68, 95% Confidence Interval: 1.01-13.38) but not with disease-free survival or any of the prognostic factors evaluated. This study indicates that *miR-137* is methylated in tumor tissue from pharyngeal and laryngeal

squamous cancers, in addition to oral squamous cell carcinoma; and that *miR-137* promoter methylation has potential utility as a prognostic marker for SCCHN.

7.2 INTRODUCTION

Head and neck cancer describes a heterogeneous group of malignancies occurring in the upper aerodigestive tract, the majority (93%) of which are squamous cell carcinomas (SCCHN) [3]. The overall 5-year survival of approximately 60% among head and neck cancer patients has been virtually unchanged over the past 3 decades [20]. Despite therapeutic advances in cancer treatment, there has been no significant improvement in laryngeal cancer survival and only a slight gain in survival has been observed for cancers of the oral cavity and pharynx [3]. As treatments improve and become more targeted, there is a need to identify biomarkers that correlate with prognosis and/or prognostic factors in an attempt to understand mechanisms driving aggressive tumor behavior and to identify patients who are at greatest risk for mortality.

MicroRNAs are small, non-coding RNA molecules that negatively regulate target gene expression through degradation and translational inhibition, with potentially hundreds of target mRNAs [201]. Alterations in the expression levels of microRNAs occur in most, if not all, types of cancer [9, 10, 17, 27, 200, 223-235], including SCCHN [9, 10, 27, 224, 234]. The potential prognostic value of microRNAs is suggested by their frequent involvement in proliferation, differentiation and apoptotic pathways. Altered expression of individual microRNAs has been associated with survival [8-10, 12-16, 18, 19, 81, 238, 239, 293], tumor stage [11, 239], grade [19], lymph node metastasis [9, 14, 239] and vascular invasion [9, 11] in multiple tumor types. An estimated 10% of microRNAs are controlled by DNA methylation [7], with aberrant

methylation being one source of microRNA dysregulation. Associations between microRNA promoter methylation and overall [238, 294, 295] and disease-free [294] cancer survival have been reported.

MicroRNA-137 (*miR-137*) is located on chromosome 1p21.3 and lies across a large CpG island [17, 27]. Promoter methylation of *miR-137* has been described in several solid tumors [27, 253, 272], including a report [27] that it is frequently methylated in tumor tissue from oral squamous cell carcinoma (OSCC). A significantly higher frequency of *miR-137* promoter methylation in oral rinse samples from SCCHN patients than from controls was recently reported by our group [296]. Cyclin-dependent kinase 6 (Cdk6) has been identified as a target of miR-137, which interacts with Cyclin D1 to phosphorylate Rb allowing the cell to progress through the G1/S phase checkpoint [17, 27]. Experimental studies have shown that transfection of miR-137 to deficient OSCC cell lines results in a reduction of Cdk6 levels and increased cell cycle arrest at the G1 phase [27]. Loss of control of the G1/S-phase checkpoint could reduce the capacity of the cell to repair DNA damage prior to synthesis, potentially resulting in genomic instability.

Known prognostic factors for SCCHN include stage [3], tumor size and lymph node metastasis [36, 37], tumor grade [45] and surgical tumor margin positivity [46-48]. The primary objective of this study is to evaluate the prognostic value of *miR-137* promoter methylation in SCCHN by assessing its association with overall and disease-free survival, as well as known prognostic factors for the disease.

7.3 MATERIALS AND METHODS

7.3.1 Study Population

This study was conducted as a part of the University of Pittsburgh Head and Neck Cancer Specialized Program of Research Excellence (SPORE). The study population consisted of consecutively diagnosed adult patients ($\geq$ 18 years) at the University of Pittsburgh Medical Center (Pittsburgh, PA) between September 2007 and April 2009 with primary squamous cell carcinoma of the oral cavity, pharynx or larynx and no prior history of cancer. Archival tumor tissue was available for 67/102 (65.7%) patients meeting these criteria. Patients for whom cancer tissue was available did not significantly differ in terms of age, gender, cancer site or stage in comparison to patients without available tissue. IRB approval was obtained under the University of Pittsburgh Head and Neck Cancer SPORE for sample collection and use of patient data. All subjects provided written informed consent for participation in this study.

7.3.2 Data Sources

Upon enrollment, patients completed an epidemiologic questionnaire providing detailed demographic and behavioral information. Clinical data were extracted at the time of diagnosis and recorded in the University of Pittsburgh Head and Neck Oncology Registry. Patients entered into the registry were prospectively followed for the ascertainment of vital status through February 8, 2010. Formalin-fixed paraffin-embedded (FFPE) tumor tissue was obtained and evaluated by a board-certified pathologist to verify that representative sections were used. Tissue samples used in this study were collected prior to administration of radiation or chemotherapy.

7.3.3 Methylation-Specific PCR

DNA was extracted from tumor tissue with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Sodium bisulfite modification of the resultant DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) per the manufacturer's protocol. Methylation status of *miR-137* was determined by methylation-specific PCR (MSP), as previously described [296]. PCR products were analyzed by separation on high-resolution 4% agarose E-gels (Invitrogen, Carlsbad, CA) and visualized and digitally captured with an EDAS 290 high-performance ultraviolet transilluminator using 1D 3.6 software (Kodak, Rochester, NY). Each MSP reaction contained a fully methylated and unmethylated bisulfite converted human DNA (Qiagen, Valencia, CA) as a positive and negative control.

7.3.4 Statistical Analysis

Descriptive statistics for the study population were generated by *miR-137* methylation status for demographic, behavioral and clinical characteristics. Categorical variables were compared using Fisher's exact tests. The Skewness-Kurtosis test was used to evaluate normality of continuous variables [297]. In the case of non-normality, the Mann-Whitney U test was used for comparison; otherwise a two-sample t-test was used.

Exact logistic regression [298] was used to model each prognostic factor of interest, conditioned on select covariates to adjust for potential confounding. Prognostic factors for SCCHN included tumor stage at diagnosis, comparing *advanced* (stage III [T3 N0 M0 or T1-3 N1 M0] or IV [T4 or $\geq$ N2 or M1]) to *local* (AJCC stage group I [T1 N0 M0] or II [T2 N0 M0]); tumor size (T classification), comparing *T3/T4* to *T1/T2*; lymph node positivity (N

classification), comparing *positive* (N1 or greater) to *negative* (N0); tumor grade, comparing *poorly differentiated* to *well/moderately differentiated*; and surgical tumor margins, restricted to patients receiving surgical intervention, comparing patients with *positive* to patients with *negative* margins. The primary exposure of interest was *miR-137* promoter methylation (*methylated*/*unmethylated*). Gender, smoking status (*ever*/*never*), daily fruit and vegetable consumption (above/below median) and tumor site were considered as covariates for each model based on the univariate results and predetermined biological importance. Exact logistic regression results were compared to the results from asymptotic models using bootstrap variance estimators to assess comparability.

Univariate Kaplan-Meier estimates were generated for 2-year overall and disease-free survival by *miR-137* promoter methylation status. Differences in survival by methylation status were assessed using log-rank tests. In the analysis of disease-free survival, patients who died prior to recurrence were considered to be censored at death.

Multivariable Cox proportional hazards models [299] provided estimates of overall and disease-free survival, adjusted for age, stage at diagnosis and tumor site. The proportional hazards assumption was tested for each model using an approach based on the slope of scaled Schoenfeld's residuals as a function of time [300]. *P*-values ≤ 0.05 were considered to be statistically significant throughout.

7.4 RESULTS

SCCHN patients with *miR-137* methylation-positive tumors ate fewer daily servings of fruits and vegetables (P = 0.005) and were more likely to be women (P = 0.13) relative to those with

unmethylated tumors (Table 10). Although not statistically significant, 10 of the 11 patients with *miR-137* methylation (90.9%) used both alcohol and cigarettes compared to 58.9% of unmethylated patients.

Table 10. Demographics of the study population and behavioral risk factors, by *miR-137* methylation status

	miR-137		
	Methylated n = 11	**Unmethylated n = 56**	***P*-value**
Age, mean years (median, σ)	62.4 (65.0, 11.5)	58.9 (57.9, 10.3)	0.33a
Sex, n (%)			0.13b
Male	6 (54.6%)	44 (78.6%)	
Female	5 (45.5%)	12 (21.4%)	
Race, n (%)			> 0.99b
White	11 (100%)	54 (96.4%)	
Non-White	0 (0.0%)	2 (3.6%)	
Body mass index (BMI; kg/m2), mean (median, σ)	27.4 (27.0, 6.9)	27.6 (26.8, 6.4)	0.92c
Daily servings of fruits and vegetables, mean (median, σ)	0.8 (0.6, 0.7)	1.8 (1.3, 1.5)	0.005c
Cigarette dose, n (%)			0.22b
Never	0 (0.0%)	12 (21.4%)	
< 10 pack-years	1 (9.1%)	7 (12.5%)	
≥ 10 pack-years	10 (90.9%)	37 (66.1%)	
Alcohol use, n (%)			0.57b
Never	1 (9.1%)	14 (25.0%)	
≤ 2 drinks/day	6 (54.6%)	27 (48.2%)	
> 2 drinks/day	4 (36.4%)	15 (26.8%)	
Combined cigarette/alcohol use, n (%)			0.37b
Never/never	0 (0.0%)	3 (5.4%)	
Never/ever	0 (0.0%)	9 (16.1%)	
Ever/never	1 (9.1%)	11 (19.6%)	
Ever/ever	10 (90.9%)	33 (58.9%)	

Abbreviations: n = number, σ = standard deviation
Note: all tests are two-sided
[a] T-test
[b] Fisher's exact test
[c] Mann-Whitney U test

Promoter methylation of *miR-137* was detected in tumor tissue from 16.4% (11/67) of SCCHN patients. When considered by tumor site, *miR-137* methylation was detected in 14.8% (4/27) of oral cancers, 22.7% (5/22) of pharyngeal cancers and 11.1% (2/18) of laryngeal cancers (Table 2). The proportions of patients with *miR-137* promoter methylation did not differ

significantly by tumor site (P = 0.71). Univariate comparisons of SCCHN prognostic factors by *miR-137* methylation status showed no other significant differences between patients with *miR-137* methylation-positive and methylation-negative tumors.

Table 11. Clinical Characteristics of the study population by *miR-137* methylation status

	miR-137		
	Methylated n = 11	**Unmethylated n = 56**	***P*-value[a]**
Tumor site, n (%)			0.71
Oral cavity	4 (36.4%)	23 (41.1%)	
Pharynx	5 (45.5%)	17 (30.4%)	
Larynx	2 (18.2%)	16 (28.6%)	
Stage (AJCC)			
T classification, n (%)			> 0.99
T1/T2	6 (54.6%)	29 (51.8%)	
T3/T4	5 (45.5%)	27 (48.2%)	
N classification, n (%)			> 0.99
N0	5 (45.5%)	26 (46.4%)	
N1-3	6 (54.6%)	30 (53.6%)	
Stage group, n (%)			0.71
Local (I/II)	2 (18.2%)	16 (28.6%)	
Advanced (III/IV)	9 (81.8%)	40 (71.4%)	
Grade, n (%)			> 0.99
Well/moderately differentiated	8 (80.0%)	41 (78.9%)	
Poorly differentiated	2 (20.0%)	11 (21.2%)	
Tumor surgical margins[b], n (%)			0.60
Negative	5 (71.4%)	36 (83.7%)	
Positive	2 (28.6%)	7 (16.3%)	

Abbreviations: n = number; AJCC = American Joint Committee on Cancer
Note: all tests are two-sided
[a] Fisher's exact test
[b] Excludes patients not treated with surgery

Neither the crude nor adjusted exact logistic regression models showed any significant associations between *miR-137* promoter methylation and stage at diagnosis, tumor size, nodal positivity, tumor grade or surgical tumor margin positivity for SCCHN, although confidence intervals are wide (Table 12). Similar results were obtained from the asymptotic logistic regression models using a bootstrap variance estimator (not shown).

Table 12. Crude and adjusted odds ratios (ORs) of SCCHN prognostic factors and *miR-137* promoter methylation

	$n_{methylated}$/ $n_{unmethylated}$	Crude OR (95% CI)	Adjusted OR (95% CI)
Stage at diagnosis (AJCC)			
Tumor size (T classification)			
T1/T2	6/29	1.00 (*ref*)	1.00 (*ref*)
T3/T4	5/27	0.90 (0.19-3.99)	0.96 (0.19-4.67)[a]
Nodal positivity (N classification)			
N0 (negative)	5/26	1.00 (*ref*)	1.00 (*ref*)
N1-3 (positive)	6/30	1.04 (0.23-4.85)	1.10 (0.17-7.14)[b]
Stage group			
Local (I/II)	2/16	1.00 (*ref*)	1.00 (*ref*)
Advanced (III/IV)	9/40	1.79 (0.32-18.78)	2.63 (0.35-34.69)[b]
Tumor grade			
Well/moderate	8/41	1.00 (*ref*)	1.00 (*ref*)
Poor	2/11	0.93 (0.08-5.71)	0.77 (0.06-5.20)[c]
Tumor surgical margins[e]			
Negative	5/36	1.00 (*ref*)	1.00 (*ref*)
Positive	2/7	2.02 (0.16-15.94)	1.98 (0.08-45.34)[d]

Abbreviations: OR = odds ratio; CI = confidence interval; AJCC: American Joint Committee on Cancer; n = number
[a] Adjusted for smoking and fruit and vegetable consumption
[b] Adjusted for gender, fruit and vegetable consumption and tumor site
[c] Adjusted for gender and fruit and vegetable consumption
[d] Adjusted for gender and tumor site
[e] Model excludes patients not treated with surgery

The average overall follow-up time was 15.2 months (median = 14.8; 10th percentile = 7.8 months; 90th percentile = 22.9 months), with 14 deaths. The average follow-up for disease-free survival was 13.8 months (median = 13.1; 10th percentile = 4.3 months; 90th percentile = 22.6 months), with 9 recurrences.

In univariate analyses, SCCHN patients with *miR-137* promoter methylation had a significantly lower overall survival rate (P = 0.046) compared to those with unmethylated tumors (Figure 5). No difference was observed for disease-free survival between patients with and without *miR-137* promoter methylation (P = 0.63; Figure 6).

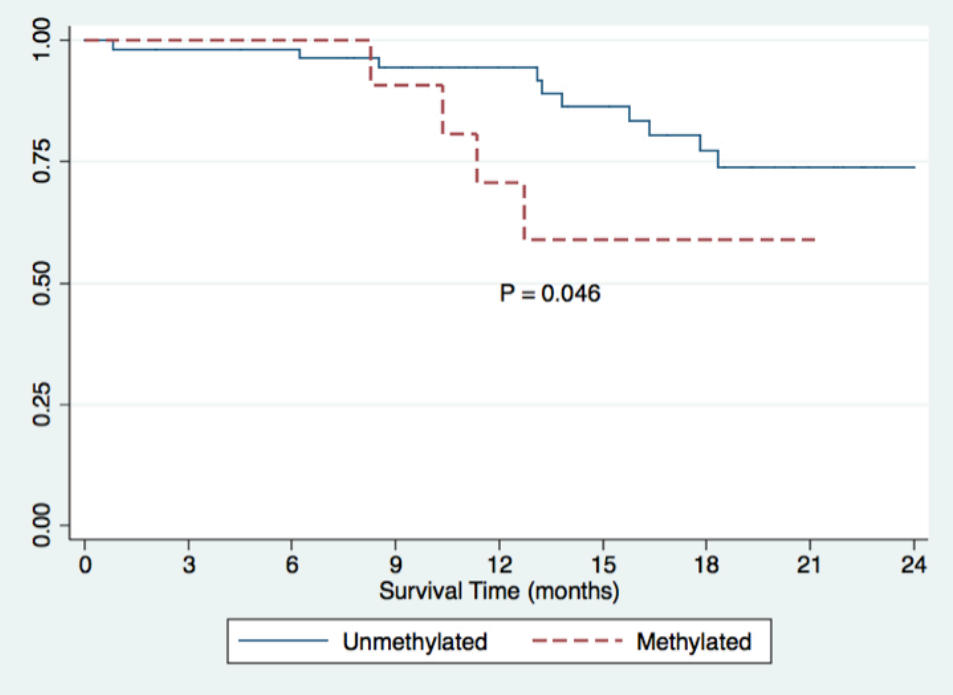


Figure 5. Kaplan-Meier estimate of overall survival for SCCHN patients by *miR-137* methylation status

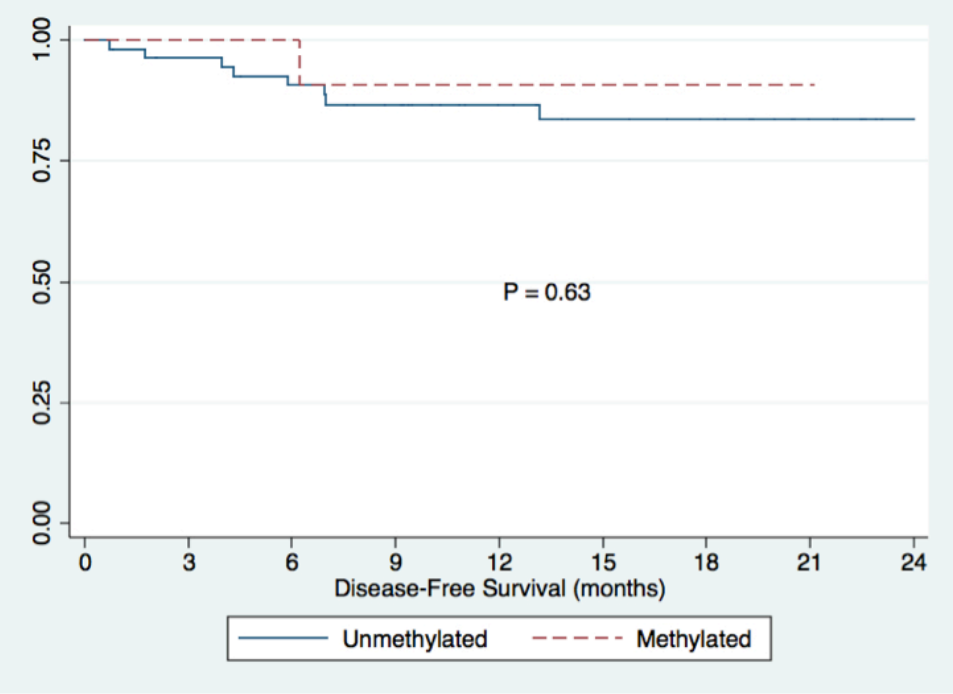


Figure 6. Kaplan-Meier estimate of disease-free survival for SCCHN patients by *miR-137* methylation status

In the multivariable Cox proportional hazards analysis adjusting for age, stage at diagnosis and tumor site (Table 13), SCCHN patients with tumors positive for *miR-137* promoter methylation had a significantly poorer overall survival (HR = 3.68, 95% CI: 1.01-13.38) compared to those with unmethylated tumors. Promoter methylation of *miR-137* was not associated significantly with disease-free survival.

Table 13. Crude and adjusted hazard ratios (HRs) for overall and disease-free survival in SCCHN patients by *miR-137* methylation status

miR-137 Methylation Status	n_{events}/ $n_{at\ risk}$	Crude HR (95% CI)	Adjusted HR[a] (95% CI)
Overall survival			
Unmethylated	10/56	1.00 (*reference*)	1.00 (*reference*)
Methyalted	4/11	3.19 (0.96-10.58)	3.68 (1.01-13.38)
Disease-free survival			
Unmethylated	8/56	1.00 (*reference*)	1.00 (*reference*)
Methyalted	1/11	0.60 (0.07-4.81)	0.53 (0.07-4.35)

Abbreviations: HR = hazard ratio; CI = confidence interval; n = number
[a] Adjusted for age, stage at diagnosis and tumor site

7.5 DISCUSSION

Dysregulation of microRNA expression has been correlated with outcome or prognostic factors for many different cancer types [8-16, 18, 19, 238, 239, 293]. Aberrant promoter methylation is one mechanism through which microRNA expression can be suppressed. There have been recent reports in the literature of associations of promoter methylation of individual microRNAs and cancer prognosis [238, 294, 295], including correlation of *miR-124a*, another microRNA that targets Cdk6, with poorer overall and disease-free survival in acute lymphocytic leukemia patients [294]. Here, we report the detection of *miR-137* promoter methylation in tumor tissue

of 16.4% of SCCHN patients, occurring in oral, pharyngeal and laryngeal cancers; and describe an association of *miR-137* promoter methylation with poorer overall survival among SCCHN patients. To our knowledge, this is the first study to evaluate the potential prognostic value of promoter methylation of a microRNA in SCCHN.

The observed association between *miR-137* promoter methylation and overall survival among SCCHN patients may relate, in part, to the involvement of miR-137 in cell-cycle control through regulation of Cdk6. Loss of miR-137 expression results in reduced ability of the cell to arrest at the G1 phase, increasing proliferation [17, 27], which may lead to accumulation of DNA damage thus enhancing genomic instability. Another potential contributor is the possible role of miR-137 in cellular differentiation. Expression levels of miR-137 are reported to be elevated in neuronal differentiation and decreased in poorly differentiated gliomas [17], although it is presently unknown if this generalizes to other histologies. Contrary to this point, we found no association of *miR-137* promoter methylation with tumor grade, although low statistical power limits our ability to detect such an association. In spite of its purported involvement in cell cycle control, we also found no significant associations of *miR-137* promoter methylation with other SCCHN prognostic factors, including stage, tumor size, nodal involvement or surgical tumor margin positivity.

Strengths of this study include high-quality prospective data collection; employment of methylation-specific PCR, a relatively inexpensive and sensitive method to detect DNA methylation for ascertainment of *miR-137* methylation status; and the use of surgical tumor tissue taken prior to initiation of radiation and/or chemotherapy, which precludes potential bias relating to treatment effects. Another strength is our use of exact logistic regression modeling to obtain more accurate inferences for small sample sizes [298].

This study also has several limitations. The relatively small sample size and proportion of *miR-137* methylation-positive samples limits our power to detect associations with prognostic factors. The present study has power ranging from 10%-18% to detect an association with an OR of 2.0. Another limitation is the relatively short follow-up time; future studies with longer follow-up time are needed to confirm our observed association between *miR-137* promoter methylation and overall survival and further assess the association with disease-free survival. Another aspect that needs to be addressed in future studies is the correlation of promoter methylation with miR-137 expression. Only one previous study with a small sample size has suggested a negative correlation between the two [27]. Despite methylation-specific PCR being a tried-and-true sensitive method for detection of promoter methylation [301], it is not quantitative. *MicroRNA-137* methylation-positive tumors, as detected by methylation-specific PCR, may contain a low percentage of methylated alleles, which may not be sufficient to produce a substantial reduction of miR-137 expression, and this could dilute the magnitude of an association between methylation and prognosis/outcome.

The results of this study suggest that *miR-137* promoter methylation is a relatively common occurrence in SCCHN, occurs across all sites, and may have value as a prognostic biomarker for the disease. However, future research efforts should focus on quantitative methylation analysis; further evaluation of the prognostic value of *miR-137* promoter methylation in expanded cohorts with longer follow-up; and assessment of the combined effect of loss of multiple tumor suppressors involved in regulation of the G1/S phase checkpoint. Continued efforts to identify such novel prognostic biomarkers or biomarker panels are crucial in reaching the greater goal of understanding the biology behind aggressive tumor behavior, and ultimately improving survival and reducing mortality from head and neck cancer.

8.0 PREDICTIVE VALUE OF ORAL RINSES FOR THE DETECTION OF *MIR-137* PROMOTER METHYLATION IN TUMOR TISSUE FROM SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

8.1 ABSTRACT

Head and neck cancer accounted for an estimated 48,010 incident cancers and 11,260 deaths in the US in 2009, the majority of which are squamous cell carcinoma, and inflicts substantial morbidity, including disfigurement and loss or hindrance of basic upper aerodigestive tract functions. Novel diagnostic and prognostic biomarkers and sample collection techniques that facilitate marker ascertainment are needed in order to reduce the mortality and morbidity burden. Oral rinse is a non-invasive DNA collection method that has shown promise in detection of promoter methylation biomarkers for SCCHN. Presently, there are few studies that formally evaluate the ability of oral rinse to predict methylation status in tumor tissue and none regarding promoter methylation of microRNA. Therefore the goal of this study is to assess the predictive value of oral rinse in prediction of *miR-137* methylation status in SCCHN tumor tissue. We have obtained paired oral rinse and tumor tissue samples from 64 patients with incident SCCHN. Methylation status of *miR-137* was determined by methylation-specific PCR for both oral rinse and tumor samples; and sensitivity, specificity, and positive and negative predictive values were calculated. Our results indicate that oral rinse is only a fair predictor of *miR-137* methylation

status in tumor tissue (sensitivity = 45.5%; specificity = 81.1%). Continued efforts at determining the best technique for sample collection via oral rinse and optimal biomarkers to be used in conjunction with it are required in order to establish oral rinse as a valid method for SCCHN screening.

8.2 INTRODUCTION

Head and neck cancer describes a heterogeneous group of malignancies arising in the upper aerodigestive tract, estimated to have resulted in 48,010 cases and 11,260 deaths in the United States in 2009 [1] and more than 500,000 annual cases and 300,000 deaths globally [2]. Squamous cell carcinoma of the head and neck (SCCHN) makes up the majority (93%) of these cancers [3]. Although head and neck cancer only accounts for 2.0% of US and 4.5% of global cancer-deaths, it bears substantial morbidity, including disfigurement and impairment of basic functions, such as talking, swallowing, eating and breathing. Thus, identification of novel biomarkers and practical methods of sample collection that can be used in a clinical setting is desired to reduce the impact of the disease.

Oral rinse is a non-invasive technique for DNA collection from the upper aerodigestive tract. In addition to cells exfoliated directly into the rinse, epithelial cells from the mouth and throat, including tumor cells, may be shed into saliva and picked up by the collection media. Several studies have evaluated the use of oral rinse or saliva as a tool for detection of DNA promoter methylation in subjects with premalignant or malignant head and neck tumors [21, 23-26, 199], although half of these studies have limited their evaluation to lesions of the oral cavity [24, 25, 199], likely due to their direct contact with the collection media. Only 4 of the studies

offer a direct comparison of saliva or oral rinse to tumor tissue [23-26], although to date, no study has formally evaluated the predictive value of oral rinse or saliva for detection of promoter methylation for SCCHN in a site-specific manner (e.g. oral cavity, pharynx, larynx). Furthermore, published reports have been limited to a relatively small subset of tumor suppressor genes, and oral rinse has never before been assessed for promoter methylation of a microRNA.

We recently reported the presence of *miR-137* promoter methylation in 21.2% of oral rinse samples from SCCHN patients [296]. The highest prevalence of *miR-137* methylation occurred in oral rinse samples taken from patients with oral tumors, and the lowest from tumors of the larynx. One could speculate that oral rinse is simply better at detecting oral lesions as a result of anatomy: oral tumors come in direct contact with the collection media and thus are more apt to be exfoliated during the rinsing process, as opposed to laryngeal tumors, which are less likely to come in contact with the rinse. However, as history has often demonstrated, the seemingly obvious logical answer is not always the correct one. Therefore, the primary goal of this study is to formally estimate the predictive value of oral rinse for the detection of *miR-137* promoter methylation in SCCHN tumor tissue, overall and by site.

8.3 MATERIALS AND METHODS

8.3.1 Study Population

This study was conducted as a part of an epidemiology project within the University of Pittsburgh Head and Neck Specialized Program of Research Excellence (SPORE). The study population consisted of 64 patients with primary SCCHN and available paired tumor tissue and

oral rinse samples, diagnosed at the University of Pittsburgh Medical Center (Pittsburgh, PA) from September 2007 to April 2009, with no prior history of non-cutaneous cancer. Primary tumors of the nasopharynx were excluded. IRB approval was obtained under the University of Pittsburgh Head and Neck Cancer SPORE for sample collection and use of patient data. All patients provided written informed consent for participation in this study.

8.3.2 Data Sources and Sample Collection

Patient demographic and behavioral risk factor information was obtained from an epidemiologic questionnaire completed by patients upon study enrollment. Clinical data was obtained from the University of Pittsburgh Head and Neck Oncology Registry. Oral rinse samples were collected prior to treatment by having patients swish for 20-30 seconds with 20 ml of sterile saline. Following the rinse, 10 ml of commercial mouthwash (Scope™) was added to the samples as a DNA preservative, after which the sample was frozen at -20°C until DNA extraction. Tumor DNA was obtained from archival formalin-fixed paraffin-embedded (FFPE) tissue, collected prior to initiation of radiation or chemotherapy. SCCHN tissue samples were reviewed by a board-certified pathologist to ensure that representative sections were captured.

8.3.3 Methylation-Specific PCR

DNA was extracted from oral rinse samples using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN) and from tumor tissue with the DNeasy Blood and Tissue Kit

(Qiagen, Valencia, CA). Following extraction, DNA concentrations were quantified using the NanoDrop 1000™ spectrophotometer (ThermoFisher Scientific, Waltham, MA). Sodium bisulfite modification of the DNA was performed with the EZ DNA Methylation Kit (Zymo Research, Orange, CA) per the manufacturer's protocol, inputting 1 μg of untreated DNA and eluting 10 μl of bisulfite converted DNA per sample. Methylation status of *miR-137* was determined by methylation-specific PCR, as previously described [296], using 2 μl of template bisulfite converted DNA per reaction per sample. PCR products were analyzed by electrophoresis on high-resolution 4% agarose E-gels (Invitrogen, Carlsbad, CA) and visualized and digitally captured with an EDAS 290 high-performance ultraviolet transilluminator using 1D 3.6 software (Kodak, Rochester, NY). Fully methylated and unmethylated bisulfite converted human DNA (Qiagen, Valencia, CA) was included in each PCR batch as positive and negative controls. Any samples producing faint positive bands were rerun twice, and only those that were consistently positive were considered methylated.

8.3.4 Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were estimated for SCCHN, overall and by site, considering *miR-137* methylation in oral rinse to be the diagnostic test and tumor tissue to be the gold standard. Exact binomial 95% confidence intervals [302] were calculated for each of the aforementioned statistics. Youden's Index [303] was calculated as a measure of overall accuracy of oral rinse in predicting *miR-137* methylation status in tumor tissue.

Among patients with *miR-137* methylation-negative tumor tissue, key attributes of those with *miR-137* methylation-positive oral rinse were compared to those with methylation-negative oral rinse to determine if this occurrence (*miR-137* methylation-positive oral rinse with negative tumor) was a random event. The Skewness-Kurtosis test was used to assess normality of continuous covariates [304]. Fisher's exact test was used for comparison of categorical covariates; and the Mann-Whitney U test or 2-sample t-test was used for continuous covariates with non-normal or normal distributions, respectively.

8.4 RESULTS

A contingency table (Table 14) is provided to illustrate concordance and discordance for detection of *miR-137* promoter methylation between paired oral rinse and SCCHN tumor tissue (N = 64). There were 48 (75.0%) patients with concordant results between oral rinse and tumor tissue: 5 methylated and 43 unmethylated. There were a total of 10 patients for whom *miR-137* was methylated in DNA obtained from oral rinse but not in the SCCHN tumor tissue; and 6 patients for whom oral rinse failed to detect *miR-137* promoter methylation in positive tumor tissue.

Table 14. Comparison of *miR-137* promoter methylation detected in oral rinse and SCCHN tumor tissue, overall and site-specific

	Tumor Tissue		
Oral Rinse	Methylated	Unmethylated	Total
SCCHN			
Methylated	5 (7.8%)	10 (15.6%)	15 (23.4%)
Unmethylated	6 (9.4%)	43 (67.2%)	49 (76.6%)
Total	11 (17.2%)	53 (82.8%)	64 (100%)
Oral cavity			
Methylated	3 (12.0%)	7 (28.0%)	10 (40.0%)
Unmethylated	1 (4.0%)	14 (56.0%)	15 (60.0%)
Total	4 (16.0%)	21 (84.0%)	25 (100%)
Pharynx			
Methylated	1 (4.5%)	2 (9.1%)	3 (13.6%)
Unmethylated	4 (18.2%)	15 (68.2%)	19 (86.4%)
Total	5 (22.7%)	17 (77.3%)	22 (100%)
Larynx			
Methylated	1 (5.9%)	1 (5.9%)	2 (11.8%)
Unmethylated	1 (5.9%)	14 (82.4%)	15 (88.2%)
Total	2 (11.8%)	15 (88.2%)	17 (100%)

Measures of predictive value for oral rinse in detecting *miR-137* promoter methylation in SCCHN tumor tissue are presented in Table 15. The sensitivity of oral rinse for predicting *miR-137* promoter methylation status in SCCHN was 45.5% (95% CI: 16.8% - 76.6%), specificity was 81.1% (95% CI: 68.0% - 90.6%), PPV was 33.3% (95% CI: 11.8% - 61.6%) and NPV was 87.8% (95% CI: 75.2% - 95.4%). Youden's Index, as a measure of overall accuracy, was 0.266.

Table 15. Measures of predictive value of oral rinse for detection of *miR-137* promoter methylation in SCCHN tumor tissue

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Youden's Index
SCCHN	45.5% (16.8% - 76.6%)	81.1% (68.0% - 90.6%)	33.3% (11.8% - 61.6%)	87.8% (75.2% - 95.4%)	0.266
Oral cavity	75.0% (19.4% - 99.4%)	66.7% (43.0% - 85.4%)	30.0% (6.7% - 65.3%)	93.3% (68.1% - 99.8%)	0.417
Pharynx	20.0% (0.5% - 71.6%)	88.2% (63.6% - 98.5%)	33.3% (0.8% - 90.6%)	78.9% (54.4% - 94.0%)	0.082
Larynx	50.0% (1.3% - 98.7%)	93.3% (68.1% - 99.8%)	50.0% (1.3% - 98.7%)	93.3% (68.1% - 99.8%)	0.433

When restricted to patients with tumors arising in the oral cavity (Table 15), the sensitivity for oral rinse was 75.0% (95% CI: 19.4% - 99.4%), specificity was 66.7% (95% CI: 43.0% - 85.4%), PPV was 30.0% (95% CI: 6.7% - 65.3%) and NPV was 93.3% (95% CI: 68.1% - 99.8%). Youden's Index for oral rinse in subjects with oral cavity tumors was 0.417. For patients with pharyngeal tumors (Table 15), the sensitivity was 20.0% (95% CI: 0.5% - 71.6%), specificity was 88.2% (95% CI: 63.6% - 98.5%), PPV was 33.3% (95% CI: 0.8% - 90.6%) and NPV was 79.0% (95% CI: 54.4% - 94.0%). Youden's Index for oral rinse in patients with pharyngeal tumors was 0.082. For patients with laryngeal tumors (Table 15), the sensitivity was 50.0% (95% CI: 1.3% - 98.7%), specificity was 93.3% (95% CI: 68.1% - 99.8%), PPV was 50.0% (95% CI: 1.3% - 98.7%) and NPV was 93.3% (95% CI: 68.1% - 99.8%). Youden's Index for oral rinse in patients with laryngeal tumors was 0.433.

A comparison of key attributes between patients with *miR-137* methylation-positive oral rinse but negative tumor tissue and those with negative oral rinse and tumor tissue is presented in Table 16. By contrast, those with methylation positive oral rinse samples and negative tumor were on average 8.5 years older (P = 0.009), smoked for 12 more years (P = 0.012) and had lower median body mass index (BMI) by 4.5 kg/m^2 (P = 0.023). There was also a borderline difference in duration of alcohol consumption, where patients with *miR-137* methylation-positive oral rinse and negative tumor drank for an average of 9.6 years longer (P = 0.062).

Table 16. Comparison of patients with *miR-137* promoter methylation in oral rinse but not tumor to those with negative oral rinse and tumor

	+ Oral Rinse/ - Tumor[a]	- Oral Rinse/ - Tumor[b]	*P*-value
Age, mean years (median)	66.8 (68.6)	57.3 (56.5)	0.009[c]
Gender, n (%)			0.38[d]
Female	3 (30.0%)	7 (16.3%)	
Male	7 (70.0%)	36 (83.7%)	
Race, n (%)			> 0.99[d]
White	10 (100%)	41 (95.4%)	
Non-White	0 (0.0%)	2 (4.7%)	
Body mass index (BMI; kg/m^2), mean (median)	23.5 (22.5)	28.0 (27.0)	0.023[e]
Daily servings of fruits and vegetables, mean (median)	1.9 (1.4)	1.7 (1.3)	0.48[e]
Denture use			0.18[d]
Never	3 (30.0%)	24 (55.8%)	
Ever	7 (70.0%)	19 (44.2%)	
Cigarette smoking, n (%)			0.42[d]
Never	1 (10.0%)	11 (25.6%)	
Ever	9 (90.0%)	32 (74.4%)	
Total years smoking[f], mean (median)	46.0 (41.0)	34.0 (38.0)	0.012[c]
Packs per day[f], mean (median)	1.0 (1.0)	1.2 (1.0)	0.29[e]
Alcohol consumption, n (%)			> 0.99[d]
Never	2 (20.0%)	11 (25.6%)	
Ever	8 (80.0%)	32 (74.4%)	
Total years drinking[g], mean (median)	40.8 (37.5)	31.2 (32.5)	0.062[c]
Alcoholic drinks per day[g], mean (median)	2.5 (2.0)	2.3 (1.4)	0.30[e]
Combined smoking and alcohol use, n (%)			0.94[d]
Never/never	0 (0.0%)	3 (7.0%)	
Ever/never	2 (20.0%)	8 (18.6%)	
Never/ever	1 (10.0%)	8 (18.6%)	
Ever/ever	7 (70.0%)	24 (55.8%)	
Tumor site			0.11[d]
Oral cavity	7 (70.0%)	14 (32.6%)	
Pharyngeal	2 (20.0%)	15 (34.9%)	
Laryngeal	1 (10.0%)	14 (32.6%)	
Stage at diagnosis			0.43[d]
Local (stage I/II)	4 (40.0%)	10 (23.3%)	
Advanced (stage III/IV)	6 (60.0%)	33 (76.7%)	

[a] Positive for *miR-137* methylation in oral rinse and negative in tumor tissue
[b] Negative for *miR-137* methylation negative in oral rinse and tumor tissue
[c] 2-sample t-test
[d] Fisher's exact test
[e] Mann-Whitney U test
[f] Restricted to ever smokers
[g] Resticted to ever drinkers

8.5 DISCUSSION

Tumor DNA may be readily shed in to saliva or oral rinse samples, which can be easily collected in a non-invasive fashion in a clinical setting. At present, few studies have evaluated oral rinse or saliva as a mode of DNA collection for detection of promoter methylation [21, 23-26, 199, 296]; and less has been done in comparing results obtained from oral rinse or saliva to those obtained from tumor tissue, with 4 studies taking up this task [23-26] and only 2 reporting measures of validity or reliability [23, 25]. Moreover, only 2 of the studies include pharyngeal and laryngeal tumors in the comparison [23, 26], one of which presents a validity or reliability measure [23], although not in a site-specific manner. All of these publications have evaluated protein-coding genes, with most looking at limited sets of genes. Here we report for the first time an overall and site-specific comparison of oral rinse and tumor DNA for detection of promoter methylation of a microRNA in SCCHN patients.

Our findings indicate that oral rinse is only a fair predictor of *miR-137* promoter methylation in tumor tissue, with an overall sensitivity and specificity of 45.5% and 81.1%, respectively. When evaluated by tumor site, the sensitivity was higher in OSCC but at the cost of specificity, while oral rinse had a low sensitivity for pharyngeal and laryngeal tumors but had good specificity. Righini and colleagues calculated kappa coefficients for detection of methylation for each of 11 genes in SCCHN patients [23] with values ranging from 0.47-0.86, indicating fair to very good concordance. By comparison, our kappa coefficient (calculated post-hoc) for detection of *miR-137* promoter methylation in SCCHN was 0.23. Viet and colleagues present percent positive agreement for 5 genes detected in saliva from OSCC patients, with values ranging from 12.5% - 87.5% [25]. Post-hoc estimation of our percent positive agreement produces a value of 38.5%, which lies within the range of their results.

Some of the discrepancies between studies may be explained by differences in genes analyzed. Even within studies, there is variation in correlation by gene. Moreover, only 11 total genes were evaluated between the two studies, all of which were protein-coding genes, whereas we have evaluated methylation of a microRNA. There is an underlying assumption that regulation of microRNAs via promoter methylation is similar to that of protein-coding genes; however, it is entirely conceivable that this is not the case, as our understanding of epigenetic control of microRNA is still in the nascent stages.

Methodological differences between studies may also account for some of the between-study disparities. We collected oral rinse samples by having our subjects swish with 20 ml of sterile saline for 20-30 seconds; whereas Righini and colleagues, who also employed methylation-specific PCR (MSP) and whose study found the best correlation between oral rinse and tumor tissue, had their study subjects swish and gargle for 3 minutes, increasing total exposure of tumor DNA to the rinse and bringing it in contact with the pharyngeal epithelium. In contrast, Viet and colleagues collected whole saliva for analysis using a methylation-specific quantitative RT-PCR (MethyLight) assay. Additionally, our study and the Viet study extracted DNA from formalin-fixed paraffin-embedded (FFPE) tissue to ascertain tumor methylation status, as opposed to fresh-frozen tumor tissue by Righini and colleagues, who found better agreement between oral rinse and tumor. Compared to fresh-frozen tissue, DNA derived from FFPE tumor tissue is of a lower quality as a result of cross-linking and fragmentation [305]. Thus it is likely that MSP has a lower sensitivity in FFPE tumor tissue relative to fresh-frozen tissue.

We observed 10 instances in which the oral rinse sample was positive for *miR-137* methylation but the tumor tissue was negative. Of the 4 studies comparing oral rinse or saliva to

tumor tissue, 3 report no occurrences of methylation-positive oral rinse or saliva with negative tumor [23, 24, 26]; the fourth study, by Viet and colleagues [25], which is the only one of the 4 studies that used FFPE tumor tissue, also had no such occurrences, per personal communication with the authors. However, a small study that evaluated oral rinse for detection of nasopharyngeal cancer (N = 30) reported one such instance [22]. One possible explanation for this is the development of field defects, arising as a result of chronic exposure of the upper-aerodigestive tract epithelium to carcinogens, such as alcohol and tobacco, resulting in accumulation of molecular insults and giving rise to distinct subclones, which may or may not develop into cancer. With this in mind, it is conceivable that *miR-137* promoter methylation sometimes occur in non-cancerous epithelium away from the tumor, resulting in its detection in oral rinse but not tumor tissue. Adding support to this notion is our post-hoc analysis comparing patients with *miR-137* promoter methylation detected in oral rinse but not tumor tissue to those with both negative oral rinse and tumor tissue. This revealed that this phenomena does not affect subjects at random but rather these patients were on average 8.5 years older, smoked for 12 years longer and had a lower median BMI by 4.5 kg/m^2. It is recognized that as we age, the frequency of aberrant promoter methylation increases [132]. Differential patterns of DNA methylation occur even among monozygotic twins. These differences increase with age and are most apparent between twins spending longer portions of their lifetimes apart, suggesting an environmental interaction [135]. Although there is likely a positive correlation between age and smoking duration, longer exposure time to cigarette smoke may also contribute to *miR-137* promoter methylation in non-cancerous epithelium. Future studies with adequate follow-up time are needed to determine if patients with *miR-137* methylation-positive oral rinse samples and negative tumor tissue are at greater risk of developing tumor recurrences.

Alternatively, the detection of *miR-137* promoter methylation in oral rinse but not tumor may be due to reduced sensitivity of MSP in tumor tissue as a result of using low-quality DNA derived from FFPE tumor tissue, as previously discussed. However, this is rather unlikely given the heterogeneity of DNA collected by oral rinse and that Viet and colleagues, who also used FFPE tumor tissue, did not observe this. Furthermore, we input a fairly high amount of template DNA (~160 ng) per sample per PCR reaction, assuming an 80% DNA recovery rate following bisulfite conversion. False-positive MSP results for oral rinse does not appear to be the issue, as we previously compared oral rinses from 99 SCCNHN patients and 99 cancer-free control subjects [296], blinded to case-control status during MSP, and observed *miR-137* promoter methylation in only 3% of controls compared to 21.2% of cases.

Although it has promise as a non-invasive collection media, there appears to be substantial inter- and intra-study variability in the ability of oral rinse to predict methylation status in tumor tissue, depending upon methodology and the gene of interest. Oral malignancies are the most appealing cancers in which to study oral rinses and saliva collection due to direct contact with the fluid. However, oral cancer by itself may be too rare a disease to warrant population screening and is more amenable to detection via visual examination as compared to pharyngeal and laryngeal cancers. Rather, methods should be sensitive and specific for detection of methylation markers in all SCCHN. Future studies should aim at further determination of the most reliable and valid method for oral rinse collection, while minimizing invasion, and at identification of sensitive and specific biomarker panels in oral rinse that can be used in a clinical setting to aid in the early diagnosis of SCCHN in an effort to reduce morbidity and mortality from this disease.

9.0 CONCLUSIONS

Head and neck cancer describes a heterogeneous class of malignancies that occur in the upper aerodigestive tract, the majority of which are squamous cell carcinoma (SCCHN). It is the 9th most common cancer in the United States, accounting for an estimated 48,010 new cases and 11,260 deaths in 2009 [1]. The 5-year survival for the disease is approximately 60% [20], and despite therapeutic advances there has been little improvement over the past 3 decades. In addition to mortality, SCCHN bears substantial morbidity, including disfigurement and dysfunction, such as difficulty talking, swallowing, eating or breathing. As would be expected, prognosis worsens with increasing stage at diagnosis, which is problematic since approximately two-thirds of patients are diagnosed at an advanced stage (stage III or IV) [20]. Thus, there is a need for identification of biomarkers that can aid in the early detection or prognostication of SCCHN.

Insensitivity to anti-growth signals due to loss of tumor suppressor gene function or expression is considered to be one of the hallmarks of cancer [306]. Epigenetic dysregulation, including promoter methylation, is at least as common as mutation in causing tumor suppressor gene inactivation. Promoter methylation of tumor suppressor genes can occur in the early stages of carcinogenesis [4, 125, 160], including the development of SCCHN [25], making them potentially useful markers for early detection of disease. Additionally, promoter methylation is

detected in DNA samples, which is a relatively stable source that can be readily obtained from patients.

MicroRNAs are involved in negative regulation of their target genes and are capable of controlling up to hundreds of targets. Dysregulation of their expression occurs in many different cancer types [9, 10, 17, 27, 200, 223-235], including SCCHN [9, 10, 27, 224, 234]. It is estimated that 10% of microRNAs are regulated via promoter methylation [7], which generally results in transcriptional silencing. Therefore, aberrant promoter methylation of a microRNA can potentially affect numerous cellular functions, thus making their methylation intriguing for biomarker research.

The goal of this dissertation project was to evaluate *miR-137* promoter methylation as an etiologic and prognostic biomarker for SCCHN. Specifically, it sought to determine if *miR-137* promoter methylation could be detected in tumor tissue and oral rinses taken from SCCHN patients and assess its association with SCCHN; to identify potential risk factors for *miR-137* promoter methylation in oral rinses from SCCHN patients; to assess the prognostic value of *miR-137* promoter methylation in tumor tissue; and to estimate the predictive value for oral rinse in detection of *miR-137* promoter methylation in SCCHN tumor tissue using methylation-specific PCR.

As a result of this effort, *miR-137* promoter methylation was identified in DNA from tumor tissue and oral rinses of SCCHN patients, making this the first report of its occurrence in pharyngeal and laryngeal cancers; and found a strong association between its detection in oral rinse samples and SCCHN, marking the first time that promoter methylation of a microRNA was assessed using oral rinse or saliva samples. Some risk factors for *miR-137* promoter methylation in SCCHN patients were also elucidated, which had never been previously attempted, with

identification of a strong positive association with female gender and an inverse association with body mass index (BMI). This study was also the first assessment of the prognostic value of *miR-137* promoter methylation in SCCHN, finding poorer overall survival among SCCHN patients with *miR-137* methylation-positive tumors. Finally, this was the initial study to evaluate the predictive value of oral rinse for detection of microRNA promoter methylation in tumor tissue. However, there was only fair concordance between oral rinse and tumor tissue for *miR-137* promoter methylation from SCCHN patients, overall and by site; and there were several instances where oral rinse DNA was positive for methylation but SCCHN tumor tissue was negative. This was contrary to reports by other similar studies, although those studies were limited to a small subset of protein-coding tumor suppressor genes. This finding may signify that *miR-137* promoter methylation is acting as a marker for extensive field damage of the upper aerodigestive tract epithelium, possibly due to chronic carcinogen exposure, rather than a true marker of disease.

Overall, this investigation suggests that although *miR-137* promoter methylation detected in oral rinse samples has a low sensitivity for detection of SCCHN, it has potential as a diagnostic biomarker in DNA methylation panels, particularly because of its high specificity. It also indicates that *miR-137* promoter methylation in tumor tissue may have utility as a prognostic marker for SCCHN. Future directions for research include quantitative analysis of *miR-137* promoter methylation; confirmation and additional evaluation of risk factors in studies with larger sample sizes; further assessment of the association of *miR-137* promoter methylation with overall and disease-free survival in expanded cohorts with longer follow-up; optimization of oral rinse sample collection techniques; and evaluation of *miR-137* promoter methylation interaction with other biomarkers, particularly those involved in cell cycle control at the G1/S-phase

checkpoint. Continued efforts at discovery of novel biomarkers for SCCHN, such as *miR-137* promoter methylation, will make positive contributions to overall public health by leading to earlier detection and better prognostic classification, thus reducing the morbidity and mortality burden that ensues from this disease.

APPENDIX A: BACKGROUND SUPPLEMENT

A.1 HEAD AND NECK CANCER SUPPLEMENT

A.1.1 Expanded Global Epidemiology of Head and Neck Cancer

Regional incidence of head and neck cancer differs by site and varies drastically (Figure 7). Highest-risk areas for oral cavity and pharyngeal cancer include France, Switzerland, Italy, Spain, India, the United States (African-Americans), Australia, New Zealand and Melanesia. There is more than 20-fold difference in incidence between the highest-risk regions and the lowest-risk regions, which include China, Scandinavian countries and the United Kingdom [2, 71, 307]. Areas that are high-risk for laryngeal cancers include southern and central Europe, the United States (African-Americans), southern Brazil, Uruguay and Argentina; while Southeast Asia and central Africa have the lowest risk [2, 71, 307]. Mortality rates also exhibit site- and region-specific variability (Figure 8). The highest mortality from oral cavity and pharyngeal cancer occurs in India, Southeast Asia, southern Africa, West Africa (oral cavity), Europe (excluding Scandinavia), Russia, and Melanesia. Regions with the highest mortality from laryngeal cancer include Russia, Spain, Argentina, and Namibia [307].

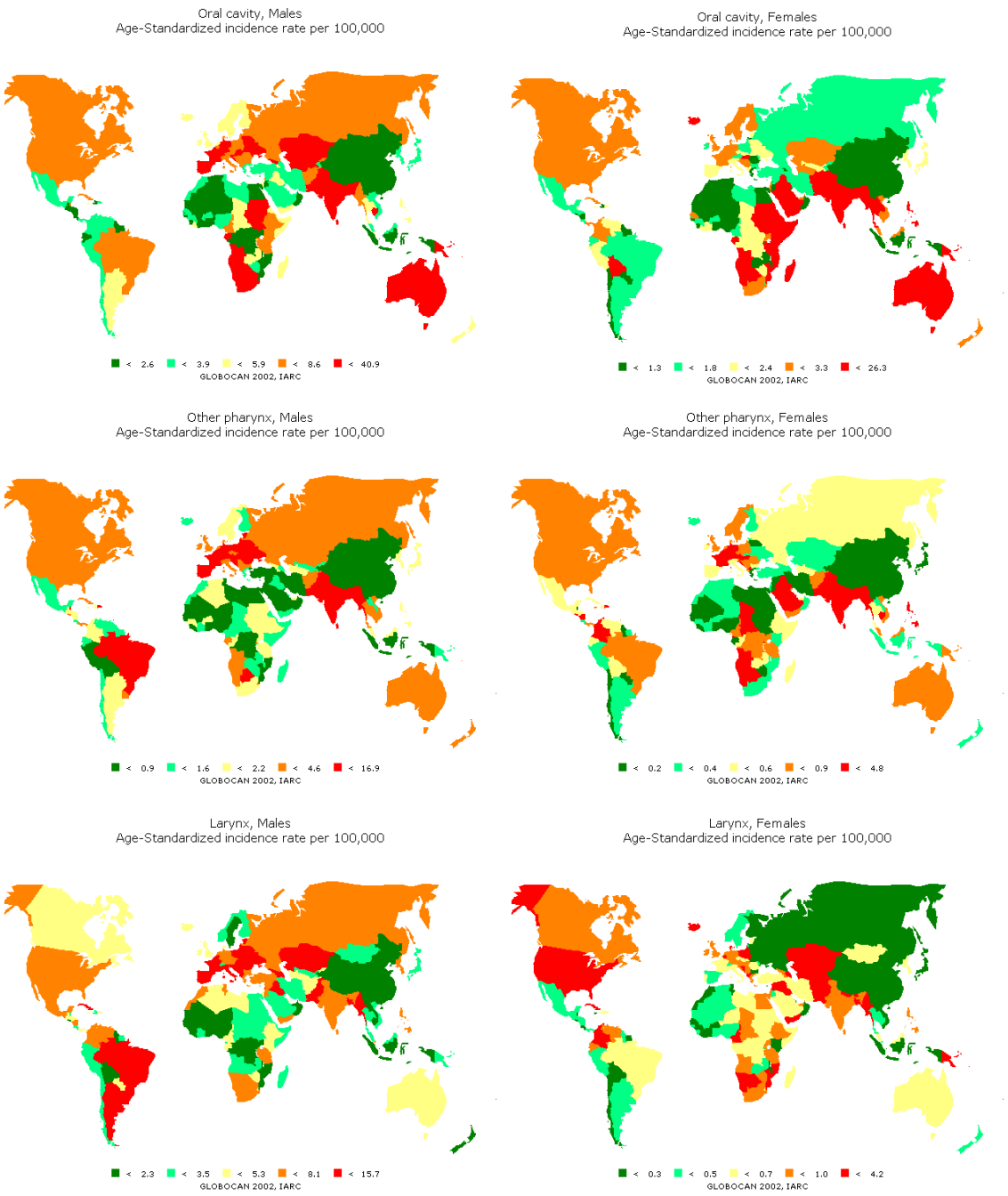


Figure 7. Global incidence of head and neck cancer by region, site and gender; age-adjusted to the WHO World standard population

Source: IARC, Globocan (2002) [307]

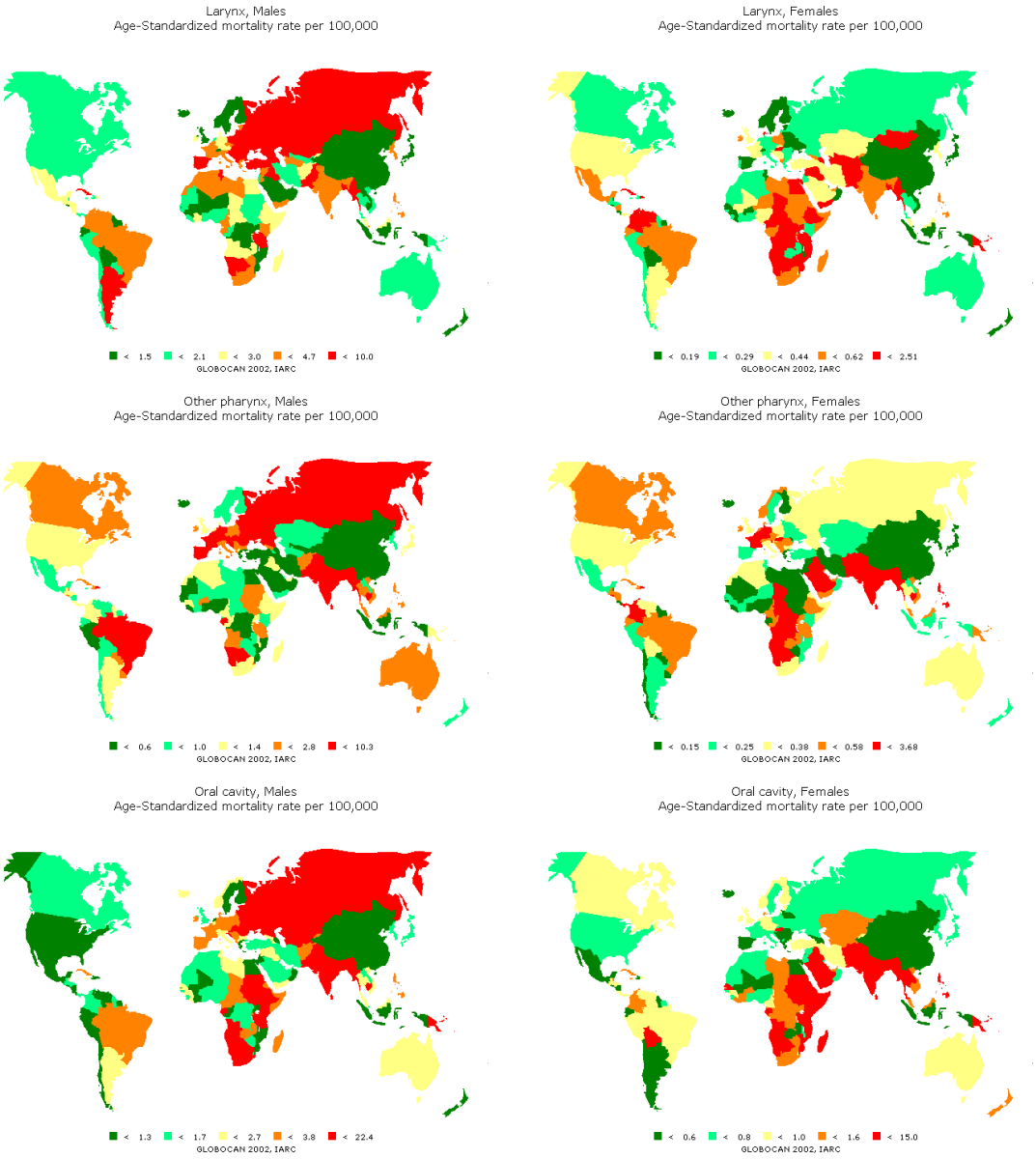


Figure 8. Global mortality of head and neck cancer by region, site and gender; age-adjusted to the WHO World standard population

Source: IARC, Globocan (2002) [307]

Global gender differences are slightly more pronounced than what is observed in the US. Overall, men are 2.96 times more likely than women to be diagnosed with head and neck cancer, excluding nasopharyngeal cancers, and 3.07 times more likely to die from it. It ranks 6th in both incidence and mortality among men and 10th in incidence and 11th in mortality among women

[2]. When considering site-specific cancers, there is still a male predominance but the magnitude of the disparity varies by site. Men are 1.79 times as likely as women to be diagnosed with cancer of the oral cavity, 4.41 times as likely to be diagnosed with pharyngeal cancer and 6.96 times as likely to be diagnosed with laryngeal cancer. The male-to-female ratio of mortality by site mirrors the incidence and is 1.73, 4.24 and 6.94 for oral cavity, pharynx and larynx respectively [307]. In men, incidence rates are highest for cancers of the oral cavity, followed closely by larynx, and are lowest in pharynx [307] (Table 17). However, mortality rates are similar among all 3 sites. For women, the incidence and mortality rates are by far the highest for oral cavity, with relatively low rates in the pharynx and larynx (Table 17).

Table 17. World incidence and mortality rates and 1-year prevalence by gender

	Male			Female		
Site	IR*	MR*	1-Year Prevalence	IR*	MR*	1-Year Prevalence
Oral Cavity	6.3	2.9	133,993	3.2	1.5	75,769
Pharynx	3.8	2.5	71,982	0.8	0.5	16,630
Larynx	5.1	2.9	108,722	0.6	0.4	15,463
Total	15.2	8.3	314,697	4.6	2.4	107,862

* per 100,000, age-standardized to WHO World standard population

Source: IARC, Globocan (2002) [307]

A.1.2 Survival Disparities by Health Insurance Status

There are also prognostic disparities associated with health insurance status of patients with SCCHN. Compared to patients with private insurance, those who are uninsured or have Medicaid coverage are more likely to present with advanced stage disease and lymph node involvement [308, 309] and have 50% poorer survival [309].

A.1.3 Economic Costs

The overall cost of SCCHN to society is substantial. In the United States, SCCHN accounts for $2.02 billion in direct, morbidity and mortality costs annually (2001 US dollars) [310]. Additionally, it is estimated that SCCHN is responsible for $2.8 billion per year in lost productivity [311]. Oral and pharyngeal squamous cancer results in 133,900 person-years of life lost per year [35]. The median 1-year cost of treatment from 1995-2000 was $22,658 and $27,655 for patients with early- and late-stage disease, respectively [312]. Medicare SCCHN patients spend an average of 8.7 hospital-days and 13.7 physician visits per year in contrast to Medicare patients without cancer, who average 1.8 hospital-days and 10.9 physician visits [312].

A.1.4 US Smoking Trends

As of 2005, 20.9% of Americans (45 million) were current smokers, down from a high of 42.5% in 1965, with an additional 21.6% (47 million) who were former smokers. This has been accompanied by a sharp reduction in annual per capita cigarette consumption since 1965. Conversely, the number of never-smokers has increased substantially since 1965, climbing to 57.5% of the population (124 million) in 2005, up from a low of 44.0% [313, 314]. However, due to population gains, the absolute number of current smokers has remained relatively stable over the past 4 decades. There is a 10-15 year lag in SCCHN incidence as it relates to reductions in smoking among the population. As such, age-adjusted incidence (2000 US standard) of SCCHN has decreased in the US since the 1980s, reflecting reductions in per capita cigarette

consumption that began in the 1970s [31, 315]. Mortality rates have also declined over the past 2 decades [31], mostly due to decreasing incidence since there have been minimal gains in survival during this time [3]. These declines are across all races and gender, although there are still major disparities, particularly for men and African-Americans.

A.1.5 US Alcohol Consumption Trends

There was a decreasing trend in alcohol consumption in the US from the 1900s to 1940, followed by an increasing trend until 1980, after which consumption steadily declined [316]. In 2002, 51.0% of Americans (120 million) were current drinkers (at least 1 drink in the past 30 days), 22.9% (54 million) were binge drinkers (5 or more drinks on 1 occasion in the past 30 days) and 6.7% (15.9 million) were heavy drinkers (5 or more drinks on the same occasion 5 or more times in the past 30 days) [317]. Males were more likely to be current drinkers compared with females. Adult excessive drinkers, defined as those averaging 2 or more alcoholic drinks per day, account for 46.3% of total US alcohol consumption, and the heaviest drinkers (top 2.5%) consume 27% of the total [318].

A.1.6 Additional Risk Factors for SCCHN

Diet

Dietary factors have also been implicated in SCCHN. Diets high in animal fat and low in fruit and vegetable consumption have been associated with SCCHN [89, 319-323]. Vitamin A and beta-carotenes may have a protective effect, while vitamin A deficiency has been associated with

SCCHN [33, 72]. Some studies have reported an association with folate deficiency and SCCHN, although the literature has been inconsistent [324-326].

Environmental and Occupational Exposures

Occupational exposures are more commonly associated with nasopharyngeal carcinomas but play a minor role in development of squamous cancers of the oral cavity, pharynx or larynx [327]. However, there have been a few reports correlating metalworking with laryngeal carcinoma [328, 329]. Exposure to toxins during mustard gas production has also been associated with increased risk of pharyngeal and laryngeal cancers with approximately 3- and 5-fold increases in risk, respectively [330]. There have been mixed results regarding associations of SCCHN with exposure to asbestos [331, 332] and exposure to wood dust [328, 333], although IARC classifies wood dust as a group 1 carcinogen with a clear association with nasopharyngeal carcinoma [334].

Indoor air pollution may also contribute to the development of SCCHN. Heating and cooking with fossil fuels, including oil, coal, gas and woodstoves, has been associated with SCCHN, with increased risk ranging from 1.6- to 3.6-fold [335-337].

The US Environmental Protection Agency (EPA) classifies environmental tobacco smoke as a human carcinogen [338]. Measurable levels of tobacco metabolites have been found in the urine and blood of non-smokers exposed to second-hand smoke [339, 340]. Chronic exposure to environmental tobacco smoke has been associated with a 1.5- to 5-fold risk for SCCHN, with a dose-response relationship [341-343].

Gastroesophageal Reflux

Several observational studies have found an association between gastroesophageal reflux and laryngeal and pharyngeal cancer [344-346]. This is substantiated by multiple studies reporting a high prevalence of gastric reflux into the laryngopharnyx, based on 24-hour pH probe monitoring [347-349].

Human Immunodeficiency Syndrome

An excess of SCCHN has been reported among HIV-positive individuals [350]. Risk of oropharyngeal high-risk HPV infection was found to be 9.2% higher among HIV-positive adults compared to HIV-negative adults [351]. Moreover, HIV-infected men were found to have 3-fold higher risk of tonsilar carcinoma, which is commonly associated with HPV-16 infection, compared with the general population [352]. Although much of the excess risk is likely attributable to immunosuppression and HPV infection, it should be noted that HIV-positive populations as a whole are more likely to engage in other risk factors for SCCHN, particularly smoking and drinking, relative to the general population.

A.1.1 Common Genetic Alterations

Head and neck squamous cell carcinoma is characterized by complex karyotypes with a non-random pattern of recurrent losses and gains of chromosomal regions [353]. Common genetic alterations in SCCHN involve activation of oncogenes or inactivation of tumor suppressor genes, resulting in tumor progression. A schematic of common events in SCCHN development is shown in Figure 9.

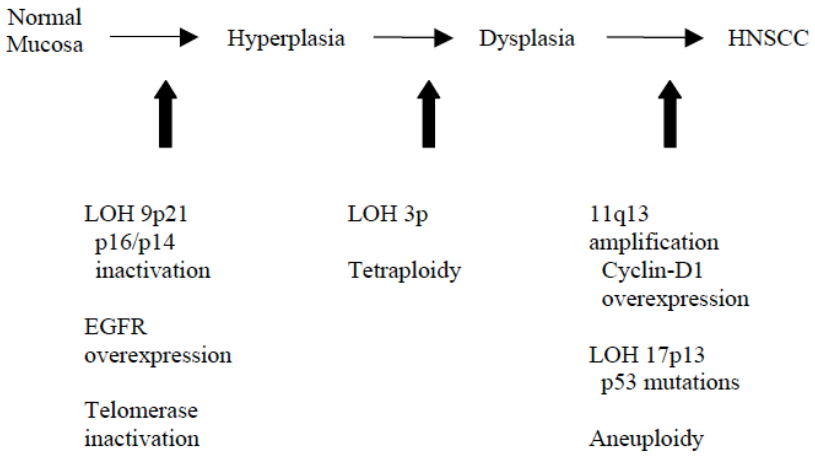


Figure 9. Model of common genetic alterations in the development of SCCHN

Adapted from: Perez-Ordonez B (2006) [354]

Loss of heterozygosity (LOH) is the loss of expression of both normal copies of a gene, which can lead to the inactivation of tumor suppressor genes. Loss of heterozygosity of chromosome region 9p21 is among the most common events in SCCHN development, occurring early in the progression of head and neck squamous tumors. It is found in 70-80% of dysplastic and malignant lesions and is commonly a result of hypermethylation or deletion [353-355]. Embedded in this region is the *CDKN2A* locus, which encodes tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$ [353-355]. The $p16^{INK4A}$ protein is involved with regulation of the G1 phase in the cell cycle through inhibition of the CDK4/6-cyclinD1 complex, preventing it from phosphorylating the retinoblastoma (Rb) protein, resulting in arrest of cell-cycle progression [353-355]. Expression of p16 is lost in 83% of oral squamous cell carcinomas and 60% of premalignant conditions [356-358]. The $p14^{ARF}$ protein interacts with the MDM2 protein to stabilize p53 and prevent its degradation [353-355].

Loss of heterozygosity also frequently occurs at chromosomal region 3p early in SCCHN tumorigenesis and is found in 60-70% of SCCHN [353-355]. The specific 3p locus is presently

uncharacterized, but 4 distinct regions whose loss is associated with SCCHN have been identified: 3p14, 3p21, 3p24, and 3p26 [354]. As with 9p21, loss of these regions is commonly a result of hypermethylation or deletion. Among the genes contained in these loci are tumor suppressors *FHIT* (3p14) and *RASSF1A* (3p21), each of which has been found to be inactivated in a small subset of SCCHN [353, 354]. Thirty percent of benign hyperplasias, an early premalignant change, contain LOH at 3p, 9p21, or both [359, 360].

Another site commonly associated with LOH is chromosomal region 17p, which tends to occur at a later stage of tumorigenesis compared with 9p21 and 3p, and is involved in the progression of dysplasia to invasive SCCHN [353-355]. Loss of heterozygosity of 17p occurs in 50-70% of SCCHN, commonly as a result of mutation or deletion [354]. The p53 gene is included in this region (17p13) [353]. Mutation of p53 has been associated with exposure to tobacco and alcohol, and results in loss of genomic stability leading to increased rates of mutations [355].

Amplification of chromosomal region 11q13 occurs in 30-60% of SCCHN [353-355]. This region contains the oncogene *CCND1*, which encodes cyclin-D1. Amplification results in upregulation of cyclin-D1, a protein involved in the progression of the cell cycle from G1 to S-phase by forming a complex with CDK4/6 and phosphorylating pRb, releasing transcription factor E2F [353-355]. In addition to conferring a growth advantage, overexpression of cyclin-D1 is associated with lymph node metastasis and poor prognosis [354].

One of the best-studied oncogenes associated with SCCHN is the *Epidermal Growth Factor Receptor* (*EGFR*). Expression of *EGFR* is upregulated in 80-90% of SCCHN [112, 353]. EGFR is known to activate pathways related to proliferation, apoptosis, invasion, angiogenesis and metastasis [112]. Overexpression of *EGFR* or its ligand, *Transforming Growth Factor*

Alpha (*TGF-α*), occurs early in tumorigenesis and increases progressively during the transition from dysplasia to SCCHN [361, 362]. Elevated expression has been shown to be predictive of worse overall and disease-free survival [363].

Telomerase has been shown to be reactivated in 90% of SCCHN and premalignant lesions [364]. It is involved in telomere maintenance and is not normally expressed in somatic cells, thus preventing unlimited replication. Activation of telomerase results in cellular immortality, one of the hallmarks of cancer [306].

A.1.8 HPV-Mediated Oncogenesis

As previously discussed, HPV-mediated SCCHN is a distinct pathological entity. High-risk HPV-mediated carcinogenesis operates primarily through the expression of the E6 and E7 viral oncoproteins. These proteins bind to and facilitate ubiquitin-mediated degradation of p53 and pRb tumor suppressors, respectively [109]. By inactivating p53, E6 disrupts the G1/S checkpoint and results in the loss of p53-mediated apoptosis. The inactivation of pRb by E7 releases the E2F transcription factor, allowing transcription of S-phase related genes for cell cycle progression. Additionally, expression of HPV16 E6 and E7 oncoproteins results in cellular immortalization [109] and have been shown to predispose the cell to genomic instability [109]. Furthermore, the E7 oncoprotein of HPV-16 stimulates the activity of DNMT1, potentially leading to aberrant promoter hypermethylation [185]. Compared with HPV-negative SCCHN, HPV-positive tumors are more likely to have wild-type p53, more likely to overexpress p16 and less likely to have amplification at chromosomal region 11q13 [365].

A.2 EPIGENETIC SUPPLEMENT

A.2.1 Histone Modifications

Promoter methylation is not the sole epigenetic mechanism capable of silencing gene expression. Modification of histone proteins can result in the alteration of chromatin structure, directly affecting gene transcription, DNA repair, DNA replication and chromosomal organization [4, 120]. Histones are protein octamers, containing 2 of each of H2A, H2B, H3, and H4, around which approximately 146 bp of DNA is wound, forming a nucleosome [120]. The nucleosome is a recurring structure of eukaryotic DNA that comprises the chromosomes, condensing the DNA so that the entire genome can fit into the nucleus. Most chromatin exists as tightly compacted nucleosomes, called heterochromatin, which is transcriptionally incompetent. This is represented by the dark staining portion of the nucleus on light microscopy. Euchromatin has less compact nucleosomes, forming an open chromatin structure that can be readily transcribed. This appears as the lightly staining portion of the nucleus on light microscopy [120].

Histone modification occurs in different histone proteins, histone variants and histone residues such as lysine, arginine and serine. Modifications typically involve addition or removal of acetyl or methyl groups to the histone proteins at the N-terminal tails protruding from the nucleosomes [4, 120].

Histone acetylation is associated with transcriptional activation. In transcriptionally active promoters with unmethylated cytosines, histones are acetylated by histone acetyl transferases (HAT). These form a complex with transcription activator and coactivator proteins to initiate transcription. Conversely, histone deacetylases (HDAC) form complexes with methyl-CpG-binding-proteins (MBD) and methylated cytosines in the promoter, allowing them to

remove acetyl groups from the N-terminal tails of the histones, causing condensation of the nucleosome, resulting in transcriptional inactivation [4, 120].

Histone methylation can result in either transcriptional activation or repression, depending upon the protein and amino acid type methylated and its position in the histone tail [4, 120]. It can also have different degrees, including mono-, di- and trimethylation. Histone methylation is catalyzed by a class of enzymes called histone methyltransferases, while histone demethylases are responsible for demethylation [120, 366]. Trimethylation of lysine at position 9, 27, or 36 of the N-terminal tail of H3 (H3-K9, H3-K27, or H3-K36) or lysine at position 20 on H4 (H4-K20) results in chromosomal structure alterations (heterochromatin) leading to transcriptional silencing. Trimethylation of lysine at position 4, 36, or 79 on H3 (H3-K4 or H3-K79) is associated with a euchromatin conformation and active transcription [366-368]. Several other covalent methyl histone modifications have been identified, but their precise effects on transcription are presently unknown [120].

A.2.2 CpG Methylation and Point Mutations

DNA point mutations, if left unrepaired, can cause alterations or loss of function of genes, potentially resulting in dysregulation of cellular function. There are 3 general mechanisms through which CpG methylation can induce point mutations: deamination of 5-meC, enhancement of exongenous carcinogens and silencing of DNA repair genes.

The first mechanism is endogenous. Methylated cytosine can undergo hydrolytic deamination causing a C to T transition [120]. The frequency of C to T methylation-associated transitions varies by tissue-type, probably due to tissue-specific differences in mismatch repair [120]. More than 30% of disease-related germline point mutations occur at CpG dinucleotides

[120]. Nearly half of all somatic and one-third of all germline p53 mutations take place at methylated CpGs, and many common p53 mutations that manifest in somatic cells are caused by C to T transitions, including "hot spot" mutations at codons 248, 273, and 282 [369]. The risk of p53 mutation at 5-meC is 10-fold that of unmethylated cytosine, and CpG dinucleotides in these regions have been observed to be methylated in normal tissue [369].

Secondly, DNA methylation can enhance the mutagenic effect of exogenous carcinogens [120]. An example of this is the affinity of benzo(α)pyrene diol epoxide (BPDE) for adduct formation on guanines adjacent to 5-meC, resulting in G to T transversions in aerodigestive tract cancers in smokers [151, 152, 370]. Similarly, acrolein has an affinity for binding 5-meC, leading to C to T transitions [153]. Also, the addition of a methyl group changes the light absorption wavelength for cytosine, favoring formation of pyrimidine dimers in skin DNA upon UV exposure [120].

Finally, promoter methylation can induce gene silencing of DNA repair genes. This results in increased risk of unrepaired mutation accumulation, which can ultimately lead to malignancy [120].

A.3 MICRORNA SUPPLEMENT

A.3.1 Post-transcriptional microRNA Processing

There are several steps involved in the post-transcriptional processing of miRNA. The primary transcript, called pri-miRNA, is typically 3 to 4 kilobases in length with a 5' 7-methylguanosine (m^7G) cap and poly-adenylated (poly-A) tail, similar to mRNA [244]. Following transcription, a

stable hairpin structure of at least 30 bp is necessary to serve as the initiation signal for the processing steps [371]. The pri-miRNAs are cleaved in the nucleus by a multiprotein complex called Microprocessor, composed of the RNase III enzyme Drosha and double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha, producing one or more precursor miRNAs (pre-miRNA) [6, 200-203]. DGCR8/Pasha recognizes the junction of single and double-stranded RNA at the base of the pri-miRNA hairpin, binding Microprocessor to it, allowing Drosha to cleave it [371]. Pri-miRNAs often contain several pre-miRNAs, known as clusters. Microprocessor activity can be inhibited through direct competitive binding of RNA-binding nuclear proteins [371-373], structural alterations of pri-miRNA [372] or direct protein interaction with Microprocessor [371].

Pre-miRNAs are 65-100 nucleotides long with a hairpin structure containing a double-stranded RNA stem [371]. Exportin 5 (Exp5) recognizes the 3' overhang, which is characteristic of pre-miRNA, and a portion of the RNA duplex structure [375, 376] and transports the pre-miRNA from the nucleus to the cytoplasm. Once in the cytoplasm, the pre-miRNA is bound by a protein complex called RISC-loading complex (RLC), which consists of another RNase III, called Dicer, along with Argonaut 2 and TRBP proteins [6, 200-203, 370]. Dicer recognizes the stem of the hairpin structure as double-stranded RNA and cleaves it on the loop side, leaving an 18-25 base pair miRNA duplex (miRNA:miRNA*) [6, 200-203, 208].

The strand of the duplex with its 5' end on the less thermodynamically stable end of the duplex, termed the guide strand, is retained and becomes the mature miRNA [377, 378]. The other strand, denoted as miRNA*, is removed and degraded [6, 200-203]. This is facilitated by Dicer, which also may help stabilize the miRNA and play a role in mRNA target identification [208]. The mature miRNA is then incorporated into a protein complex formed with an Argonaut

family (Ago) protein, called RNA Induced Silencing Complex (RISC) [6, 200-203, 208]. There are 4 Argonaut proteins expressed in humans, of which only Ago 2 possesses endonucleolytic activity [208].

A.3.2 Mirtrons

Mirtrons are a class of miRNAs that originate from short intronic hairpins and bypass the Microprocessor cleavage step [379, 380]. Alternatively, they are expressed in conjunction with their host gene and are processed via the splicing mechanism and lariat-branching enzyme and rejoin miRNA processing at the nuclear export step.

A.3.3 Positive Regulation of Gene Expression by MicroRNA

In addition to negative regulation, there is recent evidence that some miRNAs may also function as positive regulators of gene expression. MicroRNAs, miR-369-3 and let-7, have been reported to induce transcription of Tumor Necrosis Factor-α (TNF-α) by interacting with its AU (adenine and uracil)-rich elements (ARE) in the 3' UTR during cell-cycle arrest [381]. In contrast, during cellular proliferation, let-7 negatively regulates TNF-α expression [381].

A.3.4 MicroRNA Regulation of Epigenetics

Alternatively, miRNAs can regulate epigenetics by targeting transcripts of genes involved in epigenetic regulation [6]. The miR-29 family and miR-148 have been reported to target *DNMT3a* and/or *3b*, leading to reduced expression, global hypomethylation and decreased

methylation of hypermethylated promoter CpG islands [382, 383]. The miR-290 cluster has been shown to target *Rbl-2*, a repressor of *DNMT3a* and *3b*. Therefore loss of miR-290 expression results in increased expression of DNMT3a and 3b [384, 385]. Additionally, miRNAs have been reported to target enzymes involved in histone modification [386-388].

A.3.5 Non-Epigenetic Mechanisms of MicroRNA Dysregulation

Transcriptional Dysregulation

Dysregulation at the transcriptional level is one way that miRNA expression can be altered. For example, transcription of miR-210 is dependent on Hypoxia Inducible Factor [373], miR-34 is a direct target of the p53 transcription factor [229], and miR-17-92 transcription is induced by c-Myc [390, 391]. In contrast, Nuclear Factor I/A (NFI-A) binds to the promoter of miR-223 during human granulocyte differentiation, inhibiting transcription [374]. Changes in levels of such transcriptional promoters/repressors can lead to variation in miRNA expression.

Dysregulation of Processing Pathways

Another mechanism through which miRNA expression can be modified is via functional changes in the processing pathways. Dysregulation of the Drosha or Dicer processing steps results in down-regulation of miRNAs [375]. This is one of the major causes of decreased global miRNA expression in cancer [375]. Furthermore, reduced Dicer expression correlates with decreased survival among lung cancer patients [376]. Additionally, single-nucleotide polymorphisms (SNP) have been identified in pri-, pre- and mature miRNAs in various cancers that have been shown to alter miRNA expression by increasing [395] or decreasing levels of miRNA [396-398]. For example, a SNP in pre-miR-196a2 was associated with an increase in mature miRNA levels

but not pre-miRNA levels, suggesting that the alteration affects Dicer processing of the pre-miRNA to its mature form [395].

Target mRNA Modifications

Modifications of target mRNA altering the miRNA binding-site, such as mutations or translocations, can affect miRNA function [377]. Functional SNPs in miRNA-binding sequences in the 3' UTR of target mRNAs that result in altered protein expression have been described in several cancers [222, 400-402] and have been associated with survival [400] and drug resistance [378].

Reversal of Translational Silencing

RNA-binding proteins can reverse miRNA-mediated translational repression by binding the 3' UTR of mRNA. For instance, HuR binds the 3' UTR of CAT-1 under conditions of stress, signaling the release of miR-122/RISC, allowing CAT-1 to leave the P-body and become translationally active [379]. Aberrant expression of such proteins could result in changes in levels of miRNA targets.

Copy Number Alterations and Mutations

Copy number alterations may also influence miRNA expression. Amplification can increase miRNA levels, while deletions can reduce or eliminate expression. Furthermore, mutations or translocations of miRNA can alter its functionality, such as its ability to bind to its target mRNA. Approximately 50% of known miRNAs are located in or near fragile sites and in regions prone to loss of heterozygosity, amplifications and common breakpoints associated with cancer [380].

A.3.6 Oncogenic microRNAs and SCCHN

Oncogenic miRNAs that are differentially expressed in SCCHN include miR-21 and miR-184. Mir-21 has been reported to be upregulated in oral, pharyngeal and laryngeal squamous cell carcinomas [224], which is supported by the findings of Tran and colleagues that it is highly expressed in SCCHN [254]. Moreover, it is also upregulated in other cancers, including breast, lung, thyroid, liver, ovary, pancreas and glioblastoma [223]. Known targets of mir-21 include *PDCD4* [405] and *PTEN* [406] tumor suppressors, both of which are involved in apoptotic signaling. Furthermore, loss of heterozygosity of *PTEN* is a common event [407, 408] and *PDCD4* is underexpressed [10] in SCCHN. MicroRNA-184 has been reported to be upregulated in squamous cell carcinoma of the tongue [234]. When lingual squamous cancer cell lines were transfected with miR-184 inhibitor, there was a decrease in cellular proliferation, reduced expression of the c-Myc oncogene, and increased apoptosis [234].

APPENDIX B: LABORATORY PROTOCOLS

B.1 DNA EXTRACTION

B.1.1 DNA Extraction from Oral Rinse Samples

DNA was extracted from oral rinse samples using the Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, MN) for DNA extraction from buccal cells in mouthwash. All samples were pelleted by centrifugation for 10 minutes at 3400 rpm. Pellets were then frozen at -20° C until the start of the DNA extraction process.

Cells were lysed via the addition of 1000 µl of cell lysis solution and 5 µl of RNase A solution (4 mg/ml) to the cell pellet directly into the 50 ml conical tube, which was subsequently incubated for 15 minutes at room temperature. After incubation, to complete cell lysis, 10 µl of proteinase K solution (20 mg/ml) was added to the lysate, vortexed vigorously for 20 seconds and incubated for 10 minutes at room temperature. The sample was then divided into two 1.5 ml microfuge tubes (500 µl each).

To precipitate any protein in the lysate, 170 µl of protein precipitate solution was added to each microfuge tube, the samples were vortexed for 20 seconds and placed in an ice bath for 10 minutes. They were then centrifuged for 10 minutes to pellet the protein precipitate. The supernatant from each tube was transferred into a clean 1.5 ml microfuge tube containing 500 µl

of isopropanol and 2.5 µl of glycogen solution (20 mg/ml) and centrifuged for 5 minutes to precipitate the DNA. The supernatant was discarded and the remaining protein pellet washed by adding 500 µl of EtOH and centrifuging for 1 minute. The EtOH was discarded and the DNA pellet allowed to air dry.

DNA was hydrated in 200 µl of DNA hydration solution overnight and then stored at -20°C until ready for use. DNA concentration was determined using the Thermo Scientific NanoDrop 1000™ Spectrophotometer (ThermoFisher Scientific, Waltham, MA).

B.1.2 DNA Extraction from Formalin-Fixed Paraffin-Embedded SCCHN Tissue

DNA was extracted from formalin-fixed paraffin-embedded SCCHN tissue using the DNeasy Kit (Qiagen, Valencia, CA), following a modified version of the manufacturer's protocol, detailed below.

Three 10-micron thick curls from formalin-fixed paraffin-embedded tissue blocks from each subject with available tumor tissue were obtained from the University of Pittsburgh Head and Neck Tissue Bank, provided in 1.5 ml microfuge tubes. A board-certified pathologist reviewed slides cut from the beginning and end of each section of the cut tissue to ensure that tissue samples were representative of the tumor.

The first overarching step in the extraction process was to deparaffinize the tissue. Each sample was incubated for 30 minutes at 60°C in a heat block to melt the paraffin. If necessary, tissue was carefully pushed down into the bottom of the tube using sterile, autoclaved toothpicks. Following incubation, 1 ml of 100% xylene was added to each tube and centrifuged for 5 minutes, after which the supernatant was carefully poured off, and this step was repeated two

more times. Next, three EtOH washes were subsequently performed using 500 µl of EtOH solutions each, where the first used 100% EtOH centrifuged for 3 minutes; the second 75% EtOH centrifuged for 3 minutes; and the final used 50% EtOH centrifuged for 5 minutes. After each spin, the supernatant was carefully poured off into a sterile 1.5 ml microfuge tube, so that the pellet would not be lost if it broke loose with the supernatant. Following the final wash, the tissue was allowed to dry under the hood before proceeding with the protocol.

The next overarching step in the DNA extraction process involved the tissue lysis. After drying the tissue under the hood, 300 µl of Buffer ATL (provided in the DNeasy kit) was added to each tube, followed by the addition of 100 µl of proteinase K (also provided). The samples were thoroughly mixed by vortexing and incubated overnight at 56°C in a shaking incubator set at 150 rpm, in which samples were suspended at a 45° angle.

The final overarching step of the DNA extraction process is the actual DNA extraction from the lysed tissue. Following overnight incubation, each sample was vortexed for 15 seconds. Next, 400 µl of Buffer AL (provided in the DNeasy kit) was added, the samples were vortexed again, and incubated at 70°C for 10 minutes. After incubation, 400 µl of 100% EtOH was added to each sample and then vortexed. Half of the solution was pipetted into DNeasy spin columns, labeled with the respective sample identifier, placed in a 2 ml collection tube, and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded and the process was repeated using the second half of each sample using the same spin column. Each spin column was then placed in a new collection tube and 500 µl of Buffer AW1 (provided in the DNeasy kit) was added. The tubes were centrifuged at 8000 rpm for 1 minute and the collection tube and flow-through were discarded. The spin columns were placed in new collection tubes and 500 µl of Buffer AW2 (provided in the DNeasy kit) was added. The sample was centrifuged at 14,000

rpm for 3 minutes, after which the collection tube and flow-through were discarded. The spin column was then placed in a 1.5 ml microfuge tube, 50 µl of Buffer AE (provided in the DNeasy kit) was added and incubated at room temperature for 1 minute, and then centrifuged at 8,000 rpm for 1 minute to elute the DNA. This step was then repeated, for a final volume of 100 µl of eluted DNA solution. The DNA concentration of each sample was then quantified using the NanoDrop 1000™ Spectrophotometer (ThermoFisher Scientific, Waltham, MA). Samples were stored at -20°C until bisulfite conversion and methylation-specific PCR.

B.2 BISULFITE CONVERSION AND RECOVERY

Sodium bisulfite conversion is a technique used in methylation analysis to discriminate between methylated and unmethylated cytosines. After bisulfite treatment, methylated cytosines are preserved as cytosines, while unmethylated cytosines are deaminated and converted to uracil and subsequently amplified as thymine in the PCR reaction [381]. Following treatment, the forward and reverse strands are no longer complementary as a result of conversion of unmethylated cytosines to thymines, which can no longer pair with the guanine on the opposite strand (Figure 15) [381]. PCR reactions can be designed to amplify either the top (plus) or bottom (minus) strand.

The bisulfite conversion and recovery step was performed using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA).

Prior to starting the protocol, the CT Conversion reagent included in the kit was prepared by adding 750 µl of sterile water and 210 µl of M-Dilution buffer to one tube of CT Conversion

reagent and mixed by vortexing every 1-2 minutes for a total of 10 minutes. CT Conversion reagent is photosensitive, so light exposure should be avoided, and therefore was used immediately following preparation. Additionally, the M-Wash buffer was prepared by adding 24 ml of 100% EtOH to the M-Wash buffer concentrate.

The first step of the bisulfite conversion process was the addition of 5 µl of the M-Dilution buffer to 500 ng of sample DNA, adjusting the volume to 50 µl using sterile water. In cases where the DNA concentration was less than 11.1 ng/µl (< 500 ng in 45 µl) a total of 45 µl of DNA was used.

Next, the samples were incubated at 37° C for 15 minutes. Following incubation, 100 µl of CT Conversion reagent was added to each sample and incubated in the dark at 50° C for 12-16 hours. The samples were then placed on ice for 10 minutes. Next, 400 µl of M-Binding buffer was added to each sample and loaded into a Zymo Spin I column, which was placed into a 2 ml collection tube and centrifuged at 10,000 g for 30 seconds. The subsequent flowthrough was discarded following each centrifugation step. Two hundred µl of M-Wash buffer was added to the column and again centrifuged at 10,000 g for 30 seconds. Two hundred µl of M-Desulfination buffer was added to the column, allowed to incubate at room temperature for 15 minutes, and centrifuged at 10,000 g for 30 seconds. Two hundred µl of M-Wash buffer was added and centrifuged for 30 seconds, followed by the addition of another 200 µl of M-Wash buffer, centrifuged for 2 minutes. Finally, 10 µl of M-Elution buffer was added directly to the matrix of each spin column, which was placed into a 1.5 ml microfuge tube and centrifuged for 1 minute to elute the DNA. The bisulfite converted DNA was diluted 1:10 and either used immediately or stored at -80° C.

B.3 METHYLATION-SPECIFIC PCR

Methylation-specific PCR (MSP) is a bisulfite-dependent polymerase chain reaction-based assay that allows for the detection of DNA promoter methylation of a target gene [301]. Due to its use of PCR amplification, the assay has a high sensitivity capable of detecting methylation in as little as 0.1% of alleles in the sample [411].

The assay requires 2 sets of primers: 1 specific for methylated DNA and 1 specific for unmethylated DNA. The primer sets are designed to amplify the same DNA sequence. Following PCR amplification, results are obtained via gel electrophoresis. Detection of bands for either the methylated sequence or both methylated and unmethylated sequences signifies the presence of promoter methylation.

The sequences for the methylation- and unmethylated-specific primers for the *miR-137* promoter region were designed using Methyl Primer Express® software v 1.0 (Applied Biosystems, Foster City, CA). Primer sites were checked for genetic polymorphisms using the Ensemble Genome Browser [382] and were determined to be free of common variants. Each primer set for miR-137 (methylated and unmethylated) has an expected amplicon of 86 bp. The MSP primer sequences are provided in Table 18.

Table 18. Primer sequences for the *miR-137* methylation-specific PCR (MSP) assay

Specificity	Sequence	Annealing Temp	Length (bases)	CpGs
Methylation		58ºC		
Forward primer	5' GCGGTAGTAGTAGCGGTAGC 3'		20	3
Reverse primer	5' ACCCGTCACCGAAAAAAA 3'		18	2
No methylation		51ºC		
Forward primer	5' GGTGGTAGTAGTAGTGGTAGT 3'		21	---
Reverse primer	5' TACCCATCACCAAAAAAAA		19	---

The MSP assay for *miR-137* promoter methylation was performed following bisulfite-conversion of the sample DNA (previously described in section 2.3.2). Separate master mixes are required for the primer sets specific for methylated and unmethylated DNA. The master mix for the assay included 5 μl of 10X PCR buffer II; 5 μl of 25 mM $MgCl_2$; 5 μl of 10X nucleotide mix; 2 μl each of forward and reverse primer (10 pmol/μl); 0.5 μl of TaqGold DNA polymerase; and 28.5 μl of sterile water, for a master mix volume of 48 μl.

Initially, 2 μl of bisulfite converted fully methylated and unmethylated DNA was added to 24 wells each for the methylated and unmethylated reactions, respectively (for a total of 48 wells. A temperature gradient was performed to determine the optimal annealing temperature for the primer sets using a 96-well thermocycler, with annealing temperatures ranging from 47º C to 62º C. Ten μl of amplified product was added to 1 μl of 6x Loading Buffer and 9 μl of sterile water and run on high-resolution 4% agarose E-gels (Invitrogen, Carlsbad, CA) for 30 minutes. The optimal annealing temperature was selected by identifying the temperature with the best resolution for methylated and unmethylated DNA.

For MSP, 48 μl of each respective master mix was added to each well along with 2 μl of sample DNA (for a total reaction volume of 50 μl) for the PCR reaction, which was performed under the cycling conditions provided in Table 19. Each sample occupies 2 wells (1 for the methylated DNA reaction and 1 for the unmethylated reaction). Each PCR reaction contained both positive and negative controls using fully-methylated and unmethylated bisulfite converted DNA (Qiagen, Valencia, CA) and negative controls using 2 μl sterile water in place of template DNA.

Table 19. Cycling conditions for MSP

Methylation-Specific			Unmethylation-Specific		
Step	**Temperature**	**Time**	**Step**	**Temperature**	**Time**
1	94° C	2 min	1	95° C	5 min
2	95° C	30 s	2	95° C	30 s
3	64° C	30 s	3	51° C	30 s
4	72° C	30 s	4	72° C	30 s
Cycle step 4 to step 2, 4 times			Cycle step 4 to step 2, 35 times		
5	95° C	30 s	5	72° C	4 min
6	61° C	30 s	6	4° C	pause
7	72° C	30 s			
Cycle step 7 to step 5, 4 times					
8	95° C	30 s			
9	58° C	30 s			
10	72° C	30 s			
Cycle step 10 to step 8, 26 times					
11	72° C	5 min			
12	4° C	pause			

Following PCR amplification, 1 μl of Loading Buffer and 9 μl of sterile water were added to 10 μl of each sample (for a total of 20 μl). Analysis was performed on high-resolution 4% agarose E-gels (Invitrogen, Carlsbad, CA) using a 100 bp marker (5 μl of Hyperladder V) with 5 samples per gel. Samples were loaded in series, pairing methylated and unmethylated reactions for each subject. Amplicons were visualized via an EDAS 290 high-performance ultraviolet transilluminator using 1D 3.6 software (Kodak, Rochester, NY). Presence of a band was indicative of a positive signal. Samples were considered methylated if there was a positive signal for either the methylation-specific reaction alone or both methylation- and unmethylation-specific reactions.

APPENDIX C: STATISTICAL ANALYSIS SUPPLEMENT

C.1 CHAPTER 6 ANALYTIC SUPPLEMENT

C.1.1 Preliminary Data Checks for Logistic Regression Model Building

Logistic regression model selection for *MicroRNA-137 Promoter Methylation in Oral Rinses from Patients with SCCHN* was based on Hosmer and Lemeshow's strategy for model selection [383].

Association of miR-137 Promoter Methylation with Case-Control Status

Univariate differences in potential personal and behavioral risk factors were assessed between SCCHN patients and cancer-free control subjects, as described in the *Materials and Methods* (section 6.3.4). Sparse or empty cells were collapsed for categorical variables. Kernel density plots were generated for continuous covariates by case-control status to ensure overlap and preliminarily assess functional form (Figure 10).

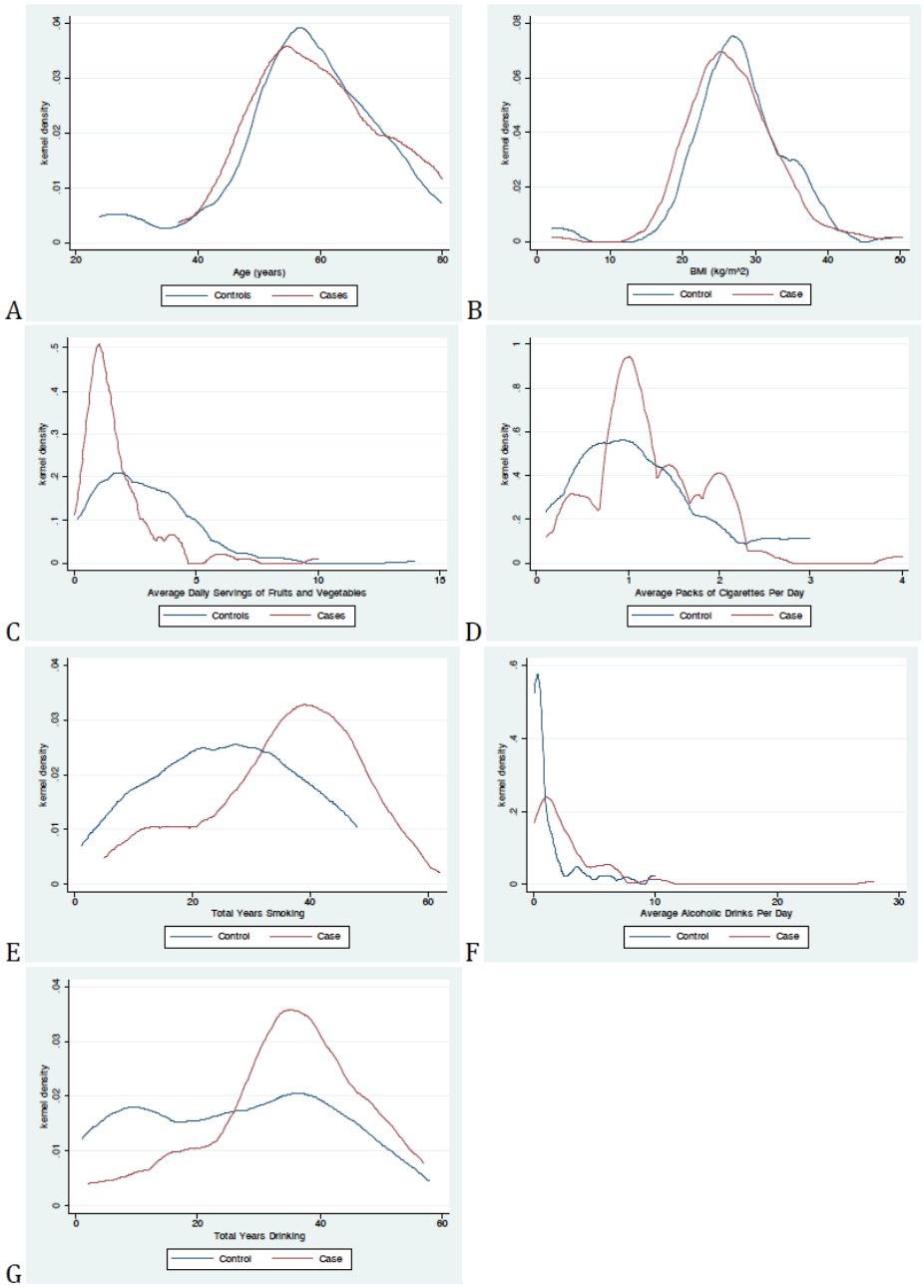


Figure 10. Kernel density plots of continuous covariates by case-control status

Continuous covariates were plotted against the logit function to assess univariate linearity, where case-control status was the outcome of interest (Figure 11).

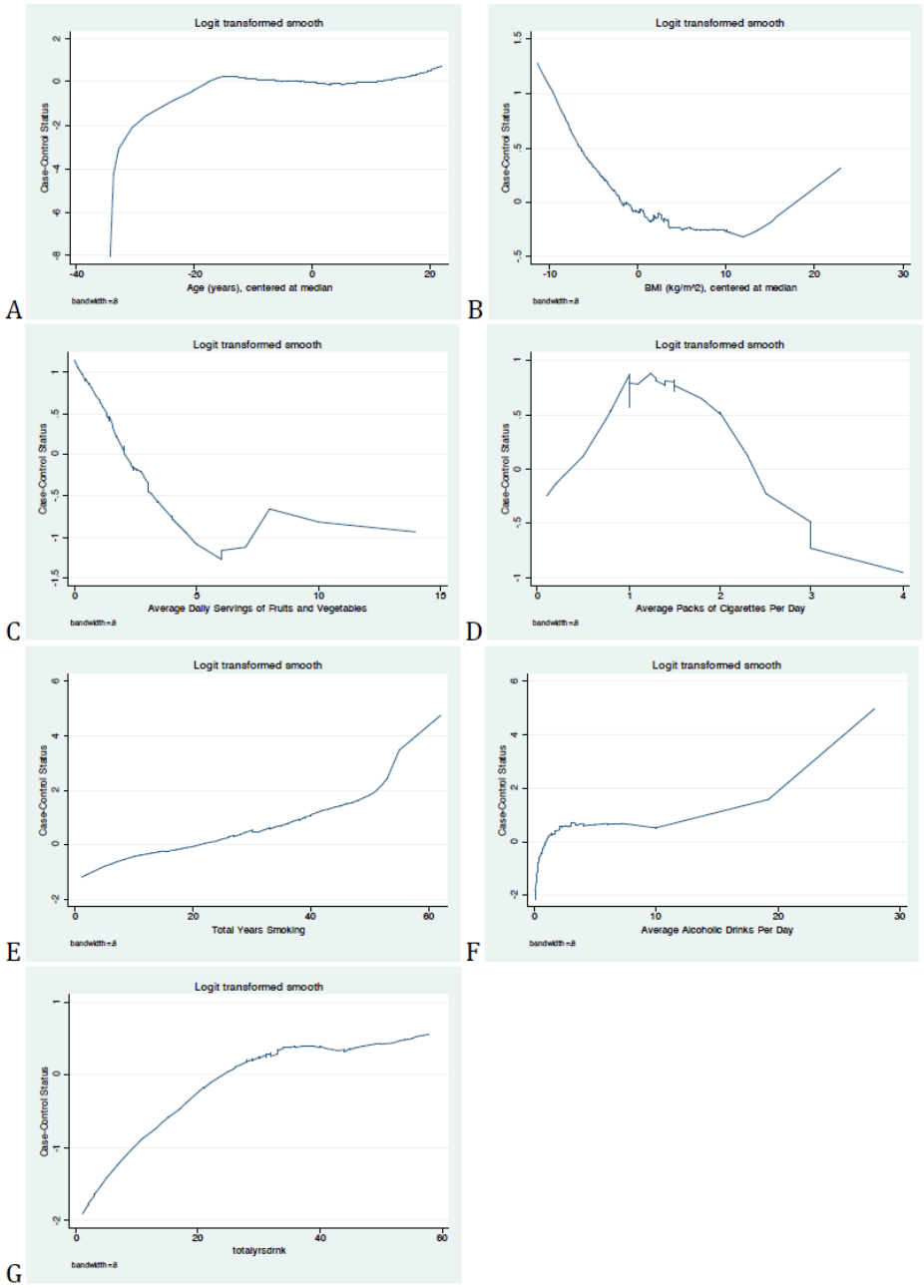


Figure 11. Logit transformed lowess smoother plots of continuous covariates with case-control as the dependent variable

Covariates with non-linear relationships with the logit were either transformed to achieve linearity or treated categorically.

Evaluation of Potential Risk Factors for miR-137 Promoter Methylation

Univariate differences in potential personal and behavioral risk factors were assessed by *miR-137* promoter methylation status, as described in the *Materials and Methods* (section 6.3.4). Sparse or empty cells were collapsed for categorical variables. Kernel density plots were generated for continuous covariates by *miR-137* promoter methylation status to ensure overlap and preliminarily assess functional form (Figure 12).

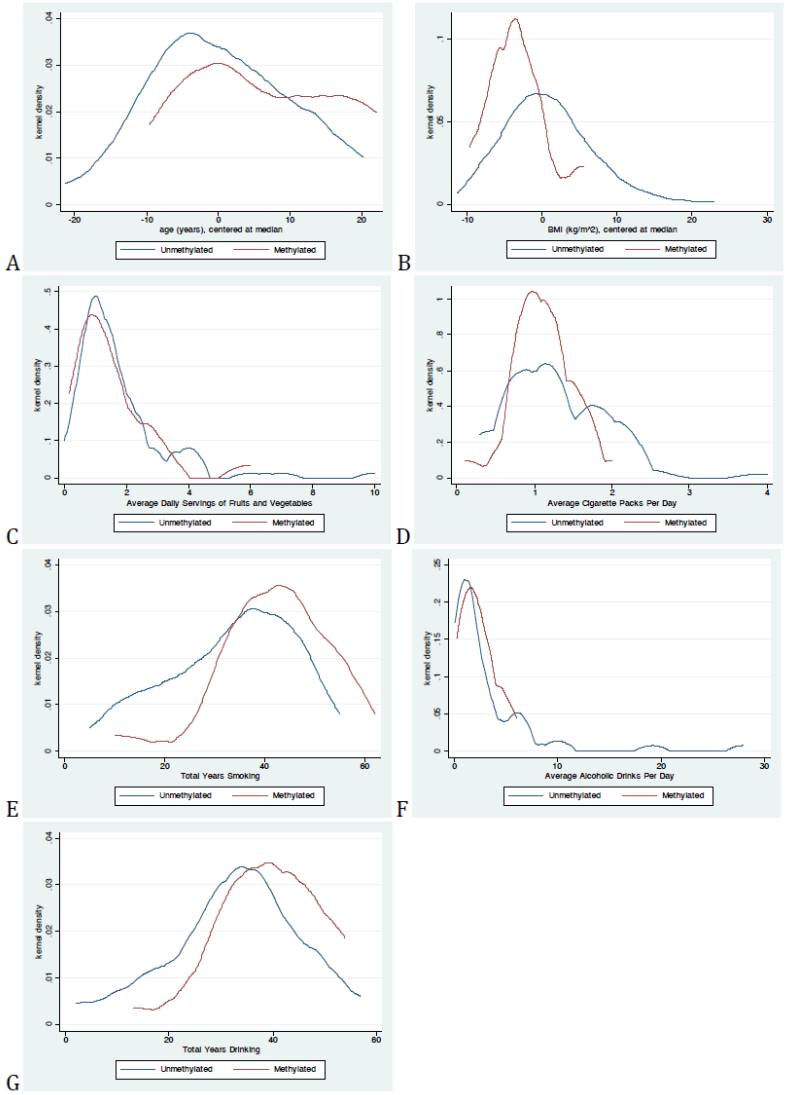


Figure 12. Kernel density plots of continuous covariates by *miR-137* methylation status

Continuous covariates were plotted against the logit function to assess univariate linearity, where *miR-137* methylation status was the outcome of interest (Figure 13).

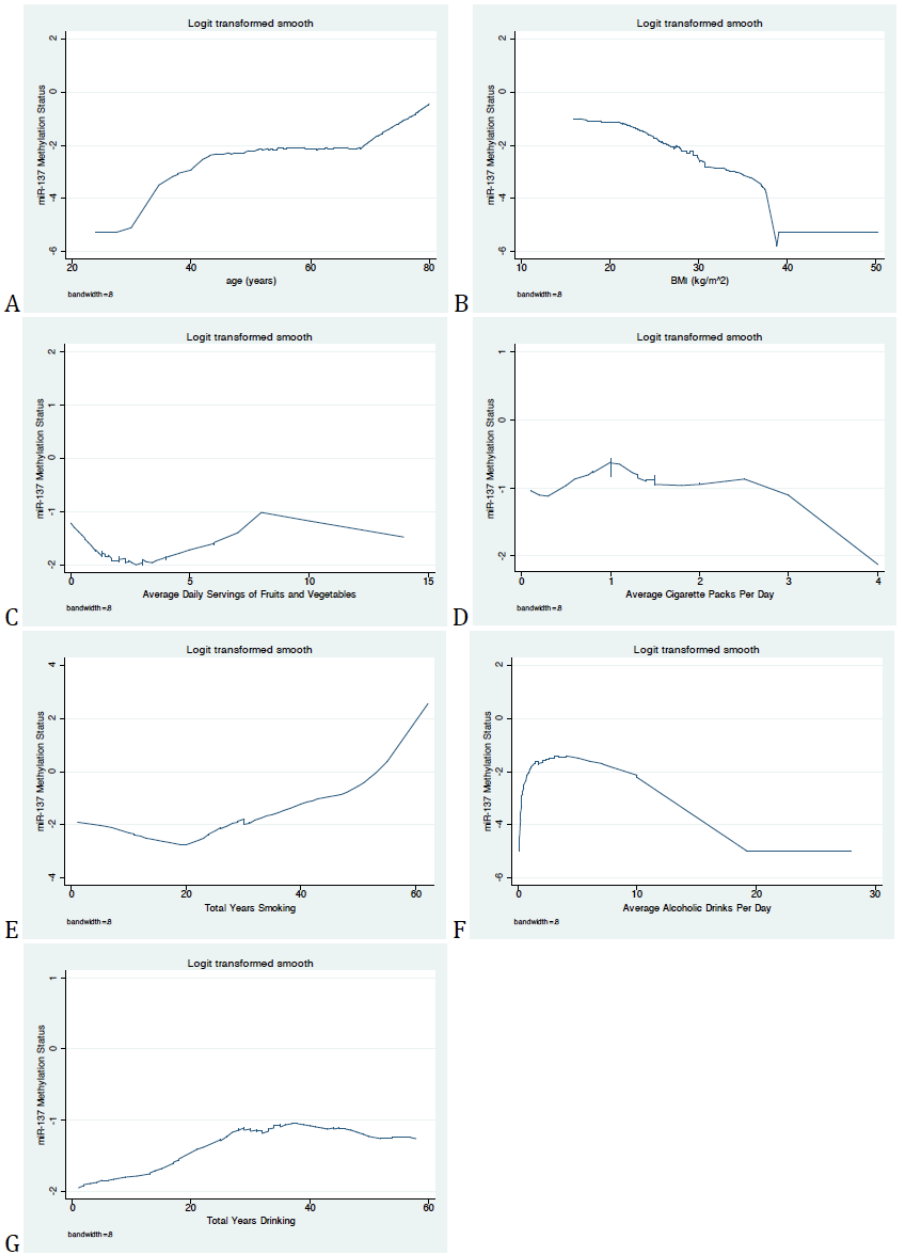


Figure 13. Logit transformed lowess smoother plots with *miR-137* methylation status as the dependent variable of interest

Covariates with non-linear relationships with the logit were either transformed to achieve linearity or treated categorically.

C.1.2 Assessment of Poorly-Fit and Influential Points in Logistic Regression Models

Cook's Distance [384] and deviance residuals [385] were calculated and plotted against each other to assess influential and poorly fit points, respectively, for each logistic regression model (Figures 14-17). Sensitivity analysis was subsequently conducted excluding the most influential points based on the plots.

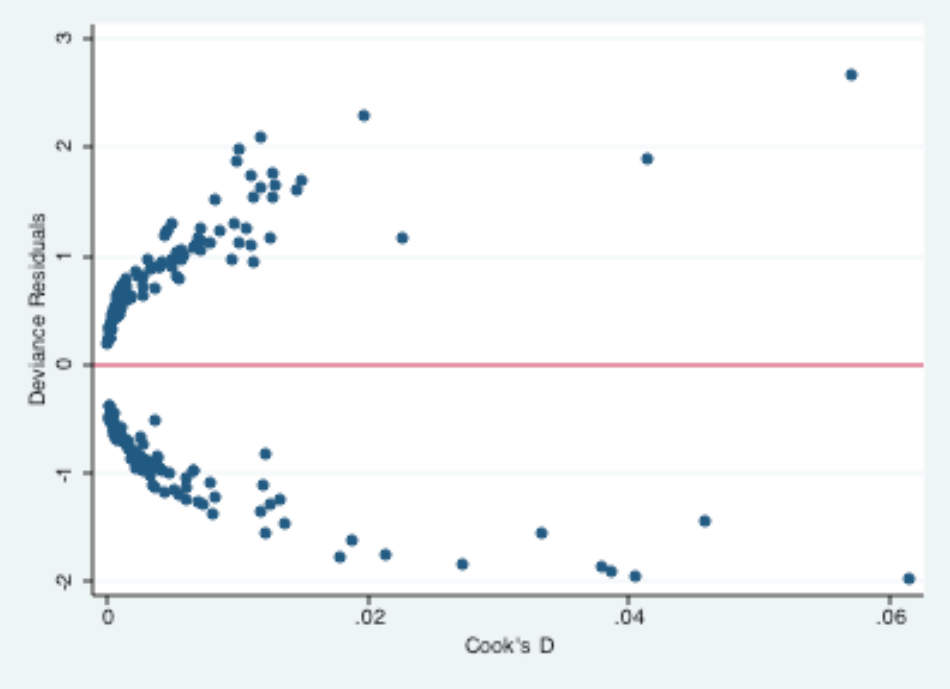


Figure 14. Cook's Distance versus deviance residual plot for the case-control model for the association of *miR-137* promoter methylation and SCCHN

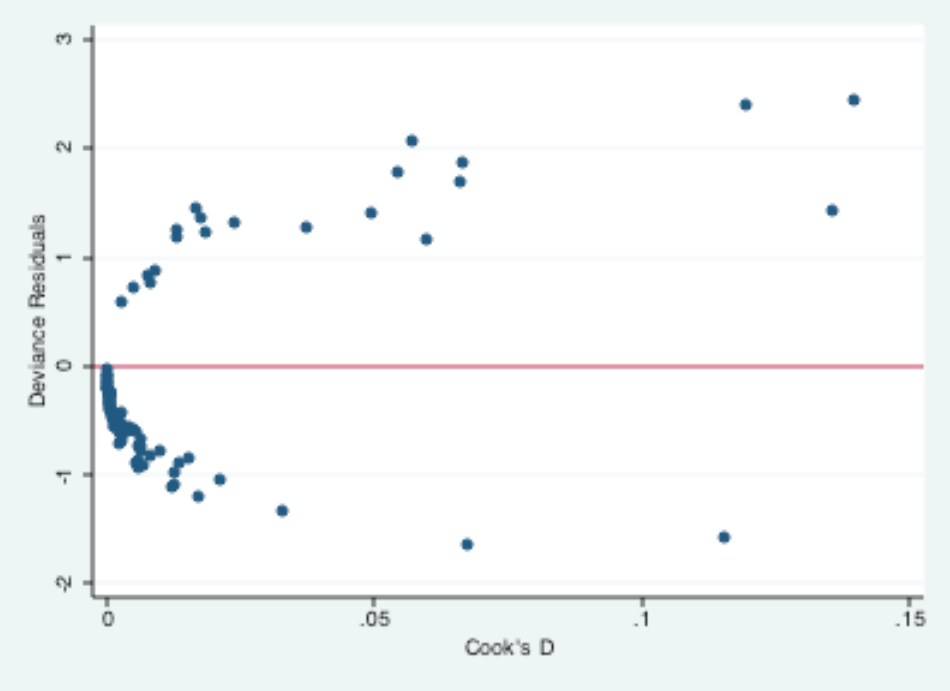


Figure 15. Cook's Distance versus residual deviance plots for the main case-series model for the association of *miR-137* promoter methylation and potential risk factors

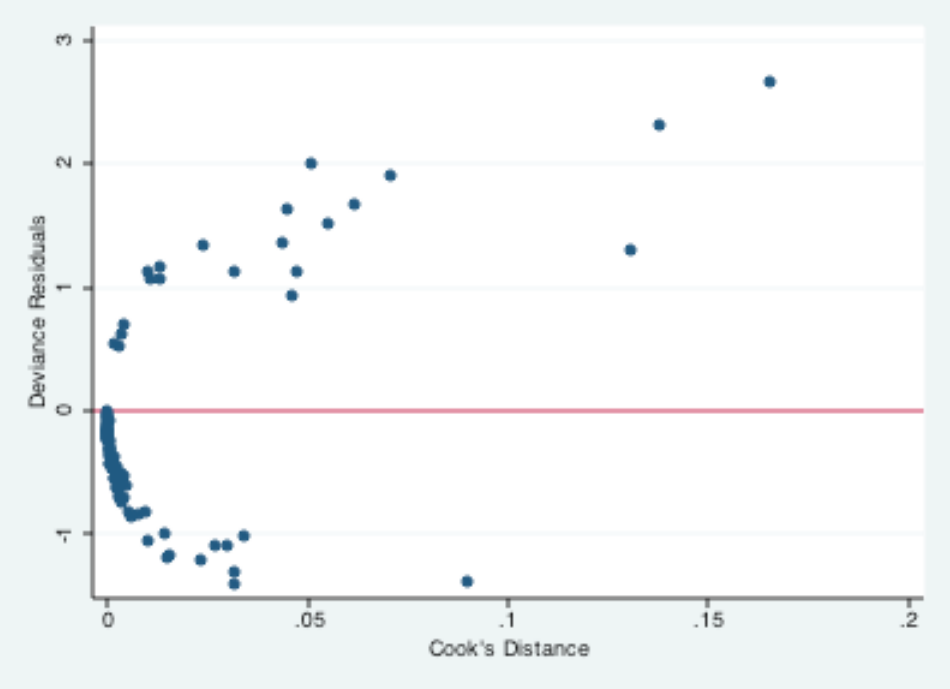


Figure 16. Cook's Distance vs residual deviance plot for case-series model including daily fruit and vegetable consumption

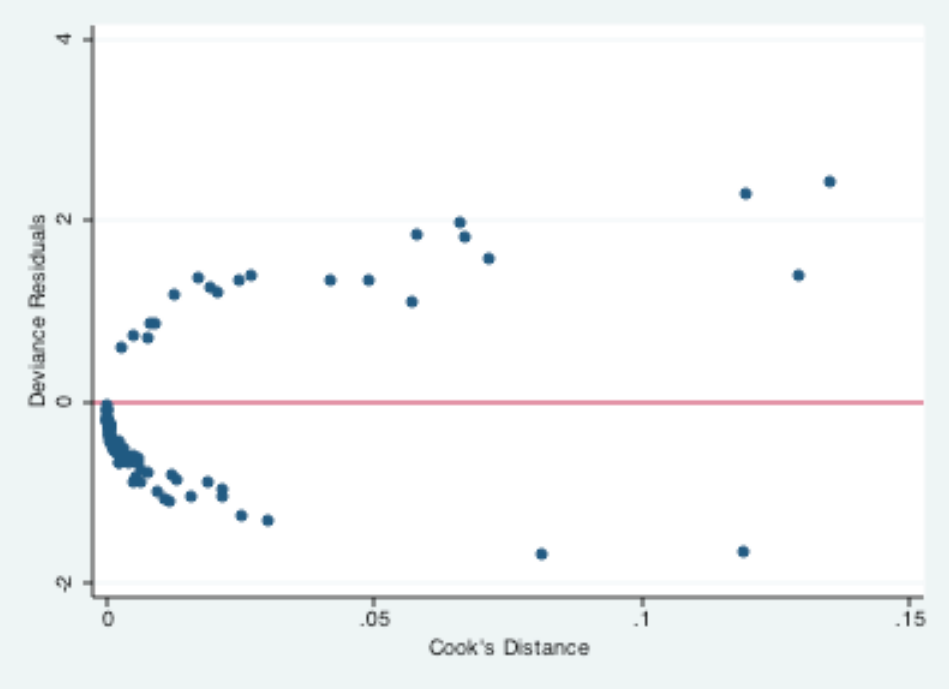


Figure 17. Cook's Distance vs deviance residual plot for case-series model including stage at diagnosis

C.2 CHAPTER 7 ANALYTIC SUPPLEMENT

C.2.1 Preliminary Data Checks for Cox Proportional Hazards Model Building

Categorical covariates considered for use in the multivariable Cox proportional hazards models were tabulated by *miR-137* methylation status and sparse or empty cells were collapsed. A two-way kernel density plot was generated for age (the only continuous covariate considered for the models) by *miR-137* promoter methylation status to ensure overlap (Figure 18).

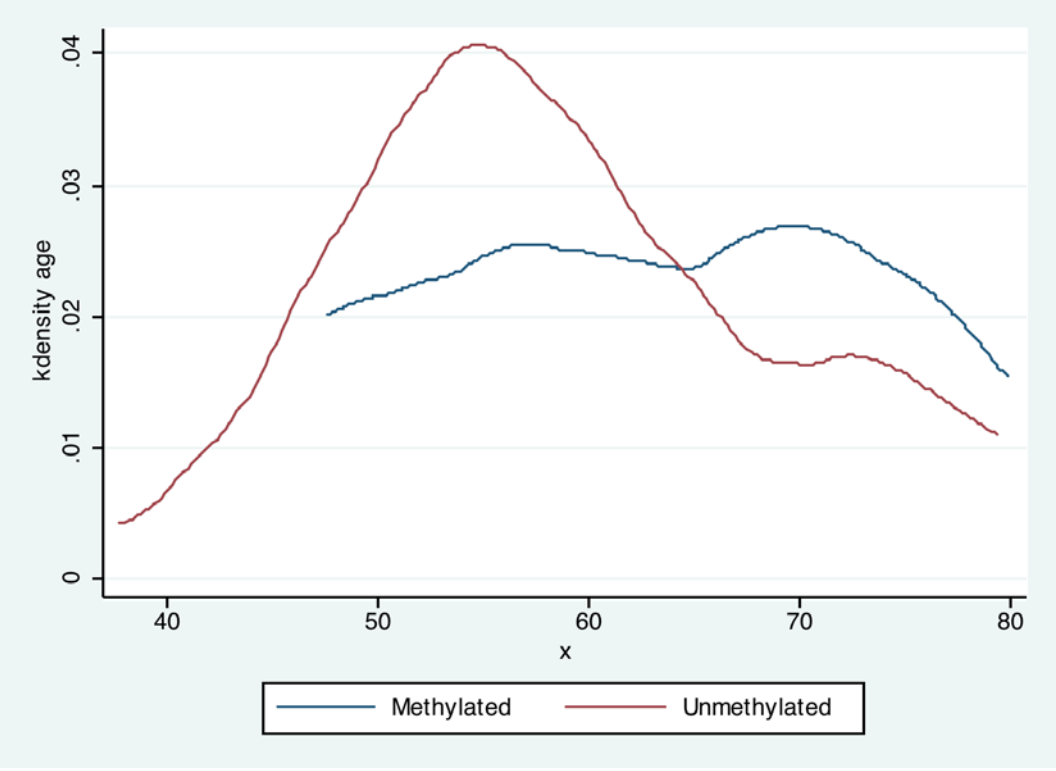


Figure 18. Kernel density of age by *miR-137* methylation status for the multivariable Cox proportional hazards models

C.3 POWER CALCULATIONS

C.3.1 Association of *miR-137* Promoter Methylation and SCCHN

Statistical power for the association of *miR-137* promoter methylation and SCCHN was calculated overall and by site using the logistic regression function of PASS (NCSS, Kaysville, UT) for detection of odds ratios of 1.5, 2.0, 2.5 and 3.0 (Table 20), where alpha = 0.05. The estimates were based on a total sample size of 198 for the overall calculation; and 136, 136 and 124 for oral cavity, pharyngeal and laryngeal, respectively. For SCCHN, the baseline event probability (P0; based on model intercept coefficient) was estimated at 0.65 and the frequency of X = 1 was 24%; for tumors of the oral cavity, P0 = 0.25 and the frequency of X= 1 was 13%; for pharyngeal tumors, P0 = 0.49 and the frequency of X=1 was 6%; and for laryngeal tumors, P0 = 0.23 and the frequency of X=1 was 4%.

Table 20. Power calculations for the association of *miR-137* promoter methylation and SCCHN, overall and site-specific

	Predicted Power			
	OR = 1.5	OR = 2.0	OR = 2.5	OR = 3.0
SCCHN	18.4%	42.2%	62.8%	77.0%
Oral cavity	12.6%	28.0%	44.0%	57.9%
Pharyngeal	7.7%	13.9%	20.3%	26.2%
Laryngeal	7.7%	14.1%	20.6%	26.8%

C.3.2 Assessment of Risk Factors for *miR-137* Promoter Methylation

Statistical power for the association of *miR-137* promoter methylation and potential risk factors in oral rinse from SCCHN patients was calculated using the logistic regression function of PASS (NCSS, Kaysville, UT) for detection of odds ratios of 1.5, 2.0, 2.5 and 3.0 (Table 21), where alpha = 0.05. The baseline probability (P0) was based on the model intercept coefficient or the probability at the mean for binary and continuous covariates, respectively. Power estimates for gender (binary) were based on a total sample size (N) of 99, with a P0 = 0.01 and frequency of X= 1 was 10%. Power estimates for body mass index (BMI; continuous) were assessed per kg/m^2 and based on N = 99 and P0 = 0.01. Power estimates for smoking duration (continuous) were assessed per 10 years of smoking and based on N = 99 and P0 = 0.02. Power calculations for alcohol intensity was determined separately for light/moderate drinkers and heavy drinkers compared to never drinkers. Estimates for light/moderate drinkers (binary) were based on N = 69, P0 = 0.01 and frequency of X=1 was 44%; and for heavy drinkers (binary) were based on N = 55, P0 = 0.01 and frequency of X= 1 was 30%. Power estimates for denture use (binary) were based on N = 99, P0 = 0.01 and frequency of X=1 was 46%. Power estimates for stage at diagnosis (binary) were based on N = 96, P0 = 0.02 and frequency of X=1 was 67%. Power estimates for daily fruit and vegetable consumption (continuous) were assessed per log-daily serving and based on N = 99 and P0 = 0.01.

Table 21. Power calculations for the assessment of *miR-137* promoter methylation risk factors in oral rinses from SCCHN patients

	Predicted Power			
	OR = 1.5	OR = 2.0	OR = 2.5	OR = 3.0
Gender	6.0%	9.9%	13.6%	17.1%
Body Mass Index (per kg/m^2)	6.0%	10.1%	14.6%	19.2%
Smoking Duration (per 10 years)	8.1%	16.0%	24.7%	33.4%
Alcohol Consumption				
Light/Moderate	4.5%	6.8%	9.2%	11.9%
Heavy	5.3%	8.5%	11.8%	15.1%
Denture Use (never vs ever)	4.3%	6.5%	8.8%	11.3%
Fruit and Vegetable Consumption (per log-daily serving)	6.0%	10.1%	14.6%	19.2%
Stage (local vs advanced)	3.6%	5.1%	7.0%	9.2%

C.3.3 Association of *miR-137* Promoter Methylation and SCCHN Prognostic Factors

Statistical power for the association of *miR-137* promoter methylation in tumor tissue and prognostic factors (where the prognostic factors are the outcome) was calculated using the logistic regression function of PASS 2008 (NCSS, Kaysville, UT) for detection of odds ratios of 1.5, 2.0, 2.5 and 3.0 (Table 22), where alpha = 0.05. Frequency of the primary predictor (*miR-137* methylation) = 16%, except for the estimates for tumor margins, where it = 14%. Power estimates for T-stage (binary) were based on total sample size (N) of 67 and baseline probability event probability (P0; based on model intercept coefficient) = 0.48; N = 67 and P0 = 0.54 for nodal positivity (binary); N = 67 and P0 = 0.71 for stage at diagnosis (binary); N = 62 and P0 = 0.21 for tumor grade (binary); and N = 50 and P0 = 0.16 for tumor margins.

Table 22. Power calculations for the association of *miR-137* promoter methylation and SCCHN prognostic factors

	Predicted Power			
	OR = 1.5	OR = 2.0	OR = 2.5	OR = 3.0
Stage at diagnosis (AJCC)				
T classification (tumor size: T1/T2 vs T3/T4)	8.5%	16.2%	24.2%	31.7%
N classification (nodal positivity: N0 vs N1-3)	8.0%	14.7%	21.5%	27.9%
Stage Group (local vs advanced)	6.0%	9.5%	12.7%	15.6%
Tumor Grade (well/moderate vs poor)	8.9%	17.7%	27.1%	36.2%
Tumor Margins (negative vs positive)	7.8%	14.5%	21.5%	28.4%

C.3.4 Association of *miR-137* Promoter Methylation and SCCHN Survival

Statistical power for the multivariable Cox proportional hazards models for the association of *miR-137* promoter methylation and survival were estimated (Table 23) using the *stpower cox* function in Stata 11 (StataCorp, College Station, TX). A total sample size of N = 67 was used to produce power estimates for hazard ratios of 1.5, 2.0, 2.5 and 3.0. For overall survival, the probability of failure (death) = 0.21; and for disease-free survival, the probability of failure (recurrence) = 0.13.

Table 23. Power calculations for the association of *miR-137* promoter methylation and SCCHN survival

	Predicted Power			
	HR = 1.5	HR = 2.0	HR = 2.5	HR = 3.0
Overall Survival	11.5%	25.4%	40.3%	53.8%
Disease-Free Survival	8.8%	17.9%	27.9%	37.7%

APPENDIX D: PROJECT 1 QUESTIONNAIRE

D.1 PROJECT 1 QUESTIONNAIRE VARIABLE LIST

Variable	Format
Sex	Male/Female
Race	Categorical
Race description if "other"	Text
Hispanic origin	Yes/No
Country of birth	Categorical
Country of birth description if "other"	Text
Year move to U.S.	Year
Longest childhood country	Text
Mother's country of birth	Text
Country mother lived longest	Text
Mother's race	Categorical
Mother hispanic	Yes/No
Father's country of birth	Text
Country father lived longest	Text
Father's race	Categorical
Father hispanic	Yes/No
Highest level of school	Categorical
Years of education	Years
Marital status	Categorical
Height (feet)	Measurement
Height (inches)	Measurement
Reference Weight (pounds)	Measurement
Weight in 20's	Measurement
Weight in 40's	Measurement
Weight in 60's	Measurement
Weight in 70's	Measurement
Weight in 90's	Measurement
Personal history of upper aerodigestive tract (UADT) cancer	Yes/No
Upper aerodigestive cancer type	Categorical
Age at diagnosis (if history of UADT cancer)	Age
Prior personal history of other cancer	Yes/No
Other cancer type	Categorical
Age at diagnosis (if history of other cancer)	Age
Any additional cancers	Yes/No
Description of other cancers	Categorical
Ever diagnosed with chronic respiratory disease	Yes/No
Any blood relative with upper aerodigestive cancer	Yes/No
Any blood relative diagnosed with chronic respiratory disease	Yes/No
Any blood relative with other cancer	Yes/No
Ever smoke cigarettes	Yes/No
Years smoke cigarettes	Years
Pack-years cigarettes	Pack-years
Currently smoke	Yes/No
Years quit smoking	Years
Age start smoking	Age
Age stop smoking	Age
Average cigarettes per day	Number
Filtered or non-filtered cigarettes	Categorical
Menthol or non-menthol cigarettes	Categorical

Variable	Format
Ever smoke cigars	Yes/No
Cigar start age	Age
Cigar stop age	Age
Years smoking cigars	Years
Cigars per week	Number
Ever smoke a pipe	Yes/No
Pipe start age	Age
Pipe stop age	Age
Years smoking pipe	Years
Pipe bowl(s) per week	Number
Ever use smokeless tobacco (chew)	Yes/No
Chew start age	Age
Chew stop age	Age
Years using chew	Years
Chew(s) per week	Number
Minutes keep each chew in mouth	Minutes
Ever use alcohol	Yes/No
Alcohol start age	Age
Alcohol stop age	Age
Number of drinks per day	Number
Maximum number of drinks per day	Number
Did any members of your household smoke before you were 18	Yes/No
Servings of fruit per week	Number
Servings of vegetables per week	Number
Normal eating habits reflective of usual adult life	Yes/No
Vegetarian diet	Yes/No
High fiber diet	Yes/No
Low salt diet	Yes/No
Diabetic diet	Yes/No
Low cholestrol diet	Yes/No
Weight gain diet	Yes/No
Weight reduction diet	Yes/No
Other diet	Text
Times per week brush teeth	Number
Ever use mouthwash	Yes/No
Mouthwash use per week	Number
How long did you use mouthwash regularly	Months
Ever wear dentures	Yes/No
Age start wearing dentures	Age
How long did you wear dentures	Years

D.2 PROJECT 1 QUESTIONNAIRE

University of Pittsburgh
Medical Center
*University of Pittsburgh School of Medicine
Department of Otolaryngology
*University of Pittsburgh Cancer Institute

SUBJECT ID_____

PARTICIPANT QUESTIONNAIRE

INTERVIEWER ID: ___ ___ ___

SUBJECT STATUS: _____

1 – Case
2 - Control

TODAY'S DATE: ___ ___ / ___ ___ / ___ ___ ___ ___

DATE OF DIAGNOSIS/ASCERTAINMENT: ___ ___ / ___ ___ / ___ ___ ___ ___

REFERENCE DATE: ___ ___ / ___ ___ / ___ ___ ___ ___

- Cases- Use one year before diagnosis date.
- Controls- Use one year before ascertainment date.

Throughout this interview, all the questions will be about a period of time before (REFERENCE DATE).

TIME INTERVIEW BEGAN: ___ ___ : ___ ___ AM/PM

Section A. Medical History

I'd like to start by asking you some questions about your medical history before (REFERENCE DATE).

REFERENCE DATE: ___ ___ / ___ ___ ___ ___

A1. What is your Birthday? ___ ___ / ___ ___ / ___ ___ ___ ___

REFERENCE AGE ___ ___ ___
(Ref. year – Birth year)

A2. How tall are you? ___ ___ ___
INCHES

___ ___ ___ ___
FEET INCHES

A3. How much did you weigh in (REFERENCE DATE)? ___ ___ ___
POUNDS

___ ___ ___
POUNDS

******Ask A4 for each age through subject's reference age.******

A4. What was your usual adult weight when you were in your:

20s? ___ ___ ___

40s? ___ ___ ___

60s? ___ ___ ___

70s? ___ ___ ___

80s? ___ ___ ___
POUNDS
(D.K. = 998)

A5. Before (REFERENCE DATE), did a doctor ever tell you that you had cancer, including blood cancers such as leukemia, lymphoma, Hodgkin's disease, or multiple myeloma?

1. – Yes
2. – No (A6)
9. – D.K. (A6) _____

A5.1 What type of cancer was it?

_____ ___ ___

A5.2 When were you first told about this cancer? ___ ___ ___
AGE

___ ___ ___ ___ ___ ___
AGE YEAR

A5.3 Where you ever diagnosed with any other cancers? _____

1. – Yes
2. – No (A6)
9. – D.K. (A6)

A5.4 What type of cancer was it?

_____ ___ ___

A5.5 When were you first told about this cancer? ___ ___ ___

___ ___ ___ ___ ___ ___
AGE YEAR

A5.6 Where you ever diagnosed with any other cancers? _____

1. – Yes
2. – No (A6)
9. – D.K. (A6)

A5.4 What type of cancer was it?

_____ ___ ___

A6. Were any of your immediate blood relatives, that is, your natural parents, sisters, brothers, or children, ever diagnosed by a doctor as having <u>HEAD and NECK or ANY TYPE of CANCER?</u>
(DO NOT INCLUDE HALF-SIBLINGS)

1. – Yes (COMPLETE CANCER CHART)
2. – No (SECTION B)
9. – D.K. (SECTION B)

_____

CANCER CHART

Who was the (first/ next) relative who had cancer?	**What type of cancer did your (RELETIVE) have?**	**How old was your (RELETIVE) when diagnosed with cancer?**	**Have any other of your closest relatives had cancer?**
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)

CANCER CHART

Who was the (first/ next) relative who had cancer?	What type of cancer did your (RELETIVE) have?	How old was your (RELETIVE) when diagnosed with cancer?	Have any other of your closest relatives had cancer?
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)
1- Father 2- Mother 3- Sister 4- Brother 5- Child	________________ ___ ___	___ ___ ___ AGE AT DIAGNOSIS	1- Yes 2- No (SECTION B) 9- DK (SECTION B)

Section B. Tobacco Exposure

B1. Up until the age of 18 did your father, mother, or anyone else in your household smoke cigarettes?

1- Yes* _____
Who were they? (LIST ALL)
2- No (B2)
3- DK (B2) _____

*If yes, complete passive smoking chart for each person

CHILDHOOD PASSIVE SMOKING CHART

B1.1 RELETIVES AS LISTED ABOVE	B1.2 How many years did (RELETIVE) smoke while you were living in the same household?	B1.3 On average, how many cigarettes per day did your (RELETIVE) smoke during that time?
1- Father 2- Mother 3- Sibling 4- Other_____ 9- DK	__ __	__ __
1- Father 2- Mother 3- Sibling 4- Other_____ 9- DK	__ __	__ __
1- Father 2- Mother 3- Sibling 4- Other_____ 9- DK	__ __	__ __
1- Father 2- Mother 3- Sibling 4- Other_____ 9- DK	__ __	__ __
1- Father 2- Mother 3- Sibling 4- Other_____ 9- DK	__ __	__ __

The next series of questions is about <u>your</u> use of cigarettes.

B2. Did you ever smoke at least one cigarette a day for six months or longer?

1- Yes (COMPLETE CHART)
2- No (B3)
3- DK (B3) _____

CIGARETTE CHART

B2.1 At what age did you first/next start smoking cigarettes?	___ ___ AGE STARTED	___ ___ AGE STARTED	___ ___ AGE STARTED	___ ___ AGE STARTED
B2.2 Did you ever stop smoking them for one year or longer? (IF NO, RECORD REF. AGE IN B2.2a)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)
B2.2a How old were you when you first/next stopped?	___ ___ AGE STOPPED	___ ___ AGE STOPPED	___ ___ AGE STOPPED	___ ___ AGE STOPPED
B2.3 On average, how many cigarettes did you smoke in a day between the ages of (B2.1) and (B2.2a)?	___ ___ CIGARETTES/DAY	___ ___ CIGARETTES/DAY	___ ___ CIGARETTES/DAY	___ ___ CIGARETTES/DAY
B2.4 During this time, did you usually smoke non-filtered or filtered cigarettes?	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK
B2.5 Were they <u>usually</u> Menthol or Non-Menthol cigarettes?	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK
***IF STOPPED SMOKING, ASK:** **B2.6 Did you ever start regularly smoking cigarettes again after (AGE IN B2.2)?**	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)

CIGARETTE CHART (continued)

B2.1 At what age did you first/next start smoking cigarettes?	____ ____ AGE STARTED	____ ____ AGE STARTED	____ ____ AGE STARTED	____ ____ AGE STARTED
B2.2 Did you ever stop smoking them for one year or longer? (IF NO, RECORD REF. AGE IN B2.2a)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)	1- Yes 2- No* 9- DK (B2.3)
B2.2a How old were you when you first/next stopped?	AGE ___ ___ STOPPED	AGE ___ ___ STOPPED	AGE ___ ___ STOPPED	AGE ___ ___ STOPPED
B2.3 On average, how many cigarettes did you smoke in a day between the ages of (B2.1) and (B2.2a)?	____ ____ CIGARETTES/DAY	____ ____ CIGARETTES/DAY	____ ____ CIGARETTES/DAY	____ ____ CIGARETTES/DAY
B2.4 During this time, did you usually smoke non-filtered or filtered cigarettes?	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK	1- Non-Filter 2- Filter 3- Equal Mix 9- DK
B2.5 Were they <u>usually</u> Menthol or Non-Menthol cigarettes?	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK	1- Menthol 2- Non-Menthol 3- Equal Mix 9- DK
***IF STOPPED SMOKING, ASK: B2.6 Did you ever start regularly smoking cigarettes again after (AGE IN B2.2)?**	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)	1- Yes (B2.1) 2- No (B3) 9- DK (B3)

B3. Did you ever smoke at least one cigar per week for six months or longer?

1- Yes
2- No (B4)
9- DK (B4) ____

B3.1 How old were you when you first started smoking at least one cigar per week?

___ ___ ___ ___
AGE

B3.2 How old were you when you last smoked them?

___ ___ ___ ___
AGE

B3.3 From age ____ to ____, how many years did you smoke cigars? Please do not include any periods in which you did not smoke cigars.

___ ___ ___ ___
YEARS

B3.4 How many cigars did you usually smoke in a week? ___ ___
___ ___ per week

B.4 Did you ever smoke at least one pipe per day for six months or longer?

1- Yes
2- No (B5) ____
9- DK (B5)

B4.1 How old were you when you first started smoking at least one pipe per day?

___ ___ ___ ___
AGE

B4.2 How old were you when you last smoked a pipe?
(CODE REFERENCE AGE IF STILL USING)

___ ___ ___ ___
AGE

B4.3 From ____ to ____, how many years did you smoke a pipe? Please do not include any periods in which you did not smoke a pipe.

___ ___ ___ ___
YEARS

B4.4 How many bowls would you usually smoke in a week?

___ ___ per week ___ ___

B5 Did you ever use smokeless tobacco/chew/snuff at least once a day for 3 months or longer?

1- Yes
2- No (SECTION C) ___ ___
3- DK (SECTION C)

B5.1 How old were you when you first started using smokeless tobacco at least once per day?

___ ___ ___ ___
AGE

B5.2 How old were you when you last used smokeless tobacco?
(CODE REFERENCE AGE IF STILL USING)

___ ___ ___ ___
AGE

B5.3 From ages ____ to ____ how many years did you use smokeless tobacco? Please do not include any periods in which you did not use smokeless tobacco.

___ ___ ___ ___
YEARS

B5.4 How many chews would you usually have in a week?

___ ___ ___ ___
CHEWS

B5.5 How long would you usually leave a chew in your mouth?

___ ___ ___ ___ ___
MINUTES

Section C. Alcohol Exposure

Next I'd like to ask some questions about the alcoholic beverages that you may have drunk during your lifetime. As before, we only want to know about your history before (REFERENCE DATE). Please consider a glass of wine, a can or bottle of beer, or a drink of hard liquor as the same.

C1. Did you ever have one or more drinks per month for a year or longer?

1- Yes
2- No (SECTION D)
9- DK (SECTION D)

_____

ALCOHOL CONSUMPTION CHART

C1.1 At what age did you (first/next) start drinking alcohol 1 or more times per month?	___ ___ AGE STARTED	___ ___ AGE STARTED	___ ___ AGE STARTED	___ ___ AGE STARTED
C1.2 Did you ever stop for one year or longer? (IF NO, RECORD REF. AGE IN C1.2a)	1- Yes (C1.2a) 2- No (C1.3) 9- DK (C1.3)	1- Yes (C1.2a) 2- No (C1.3) 9- DK (C1.3	1- Yes (C1.2a) 2- No (C1.3) 9- DK (C1.3	1- Yes (C1.2a) 2- No (C1.3) 9- DK (C1.3
C1.2 How old were you when you (first/next) stopped?	___ ___ AGE STOPPED	___ ___ AGE STOPPED	___ ___ AGE STOPPED	___ ___ AGE STOPPED
C1.3 On average, how many times in a week or month would you have at least on drink, between the ages of ____ and ____?	___ ___ 1- Week 2- Month	___ ___ 1- Week 2- Month	___ ___ 1- Week 2- Month	___ ___ 1- Week 2- Month
C1.4 On a day that you drank, how many glasses would you usually have? (GLASS=CAN=BOTTLE)	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK
C1.5 What would be the highest number that you might have in a day?	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK	___ ___ NUMBER DRANK
***IF STOPPED DRINKING, ASK C1.6 (OTHERWISE GO TO SECTION D):** **C1.6 Did you ever start drinking one or more drinks per month for one year or longer after (C1.2a)?**	1- Yes (C1.1) 2- No (SECT D) 9- DK (SECT D)	1- Yes (C1.1) 2- No (SECT D) 9- DK (SECT D)	1- Yes (C1.1) 2- No (SECT D) 9- DK (SECT D)	1- Yes (C1.1) 2- No (SECT D) 9- DK (SECT D)

Section D. Diet

Now I'd like to ask you a few questions about your diet three to five years before (REFERENCE DATE).

D1. Not counting salad or potatoes, how many serving s of vegetables did you eat per day or per week?

___ ___ SERVINGS
1- Day
2- Week

___ ___

_____

D2. Not counting juices, how many servings of fruit did you eat per day or per week?

___ ___ SERVINGS
1- Day
2- Week

___ ___

____

D3. Did your eating habits three to five years ago reflect those of your usual adult life?

1- Yes
2- No (SECTION E)
9- DK (SECTION E)

_____

D4. How did your eating habits change? (CIRCLE ALL THAT APPLY)

1- Diabetic Diet ____

2- Low-Cholesterol Diet ____

3- High-Fiber Diet ____

4- Low-Salt Diet ____

5- Vegetarian Diet ____

6- Weight Reduction Diet ____

7- Weight Gain Diet ____

8- Other (Specify) _____ ____

9- DK ____

Section E. Oral Care

E1. Have you ever worn dentures, a partial or a bridge?

1- Yes
2- No (QUESTION E2) ____
9- DK (QUESTION E2)

E1.2 How old were you when you began to wear dentures?

___ ___ ___ ___
AGE

E1.3 How many years did you wear dentures? Please do not include any periods in which you did not wear dentures.

___ ___ ___ ___
YEARS

E2. How often did you usually brush your teeth before(REFERENCE DATE/ILLNESS)?

___ ___ per week ___ ___

E3. Have you ever used mouthwash at least once a week?

1- Yes ____
2- No (SECTION F)
3- DK (SECTION F)

E3.1 How often did you usually use mouthwash before (REFERENCE DATE/ILLNESS)?

___ ___ per week ___ ___

E3.2 For how long did you use mouthwash regularly (at least once each week)?

___ ___ ___ ___

1- Months
2- Years ____

Section F. Background Characteristics

Finally, I'd like to ask some questions about your background.

F1. In what country were you born?

01- United States (Go to F2) ___ ___
02- Canada
03- Mexico
08- Other _____ (SPECIFY)

F1.1 In what year did you fist move to the United States ____ ____ ____ ____

F2. In what country did you live the longest during your childhood, that is, up to age 18?

_____ ___ ___
(USE F1 CODES)

F3. What was your marital status in (REFERENCE DATE)?

1- Married
2- Living as married
3- Widowed
4- Divorced
5- Separated
6- Never married
9- DK

F4. What Race do you consider yourself?

1- White
2- Black or African American
3- Asian
4- American Indian/Eskimo
5- Other _____ ____
(SPECIFY)
6- Refused
9- DK

F5. Do you consider yourself to be of Hispanic origin?

1- Yes
2- No ____

F6. Now I will ask about your relatives' background.

	Mother (01)	Father (02)	Maternal Grandmother (03)	Maternal Grandfather (04)	Paternal Grandmother (05)	Paternal Grandfather (06)
F6.1 In what country was (RELETIVE) born?						
F6.2 In what country did (RELETIVE) live the longest?						
F6.3 What race did/do you consider your (RELETIVE)?						
F6.4 Do you think he/she considered themselves to be of Hispanic origin?	1-Yes 2-No	1-Yes 2-No	1-Yes 2-No	1-Yes 2-No	1-Yes 2-No	1-Yes 2-No

F7. What is the highest level of school that you completed?

LEVEL OF SCHOOL	YEARS	
1- Grade School	1 2 3 4 5 6 7 8	____
2- High school	9 10 11 12	LEVEL
3- Technical/Vocational	1 2+	
4- College	1 2 3 4 5+	____
5- DK	9	YEARS

F8. Did you have a telephone in your household in (REFERENCE DATE)?

1- Yes
2- No
9- DK ____

TIME INTERVIEW BEGAN: ___ ___ : ___ ___ AM/PM

INTERVIEW OUTCOME ____

1- Complete
2- Partial

Section G. Miscellaneous and Interviewer Comments

This concludes this part of the interview.

G1. Are there any comments or questions that you have at this time?

_____
_____
_____
_____
_____

CASES ONLY

G3. Do you have any ideas about what may have caused you to get this disease?

_____
_____
_____
_____
_____

Supplementary Questions

A1. Have you ever had **RADIATION THERAPY** (X-ray or seed treatments) for Cancer or any other condition?

1. YES 2. NO

TYPE OF CANCER and/or BODY PART YEAR OF TREATMENT _____

_____

_____ _____

_____ _____

A2. Have you ever had **CHEMOTHERAPY** or **METHOTREXATE** treatment for cancer or arthritis?

1. YES 2. NO

CONDITION/ BODY PART YEAR/TYPE OF TREATMENT

_____ _____

_____ _____

_____ _____

A3. Have you been treated with **RADIATION or CHEMOTHERAPY** within the last month?

1. YES 2. NO

DATE OF LAST TREATMENT: __ __/ __ __ / __ __

A4. Have you ever worked in a nuclear power plant?

1. YES 2. NO

A4.1 What was your job?

_____

__

(SPECIFY)

A4.2 Were you ever exposed to radiation during that job?

1. YES (Comment Below) 2. NO

THANK YOU FOR YOUR TIME AND HELP WITH THIS STUDY!

Interviewer's Comments:

G4. How reliable do you think the information was on this questionnaire?

1- High quality
2- Generally reliable
3- Questionable
4- Unreliable ____

G5. What is the main reason for the questionable or unreliable quality of this information (INCLUDE COMMUNICATION PROBLEMS)?

_____
_____
_____

G6. Does Respondent want study results?

1- Yes
2- No ____

G7. GENDER OF SUBJECT:

1- Male
2- Female ____

Address: _____

Apt./Suite: _____

City: _____

State: _____

Zip Code: _____

County: _____

Telephone #: _____

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