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Pretreatment dietary intake is associated with tumor suppressor DNA methylation in head and neck squamous cell carcinomas

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Keywords: DNA methylation, diet, tumor suppressor, folate, vitamin B12

Abbreviations: HNC, head and neck cancer; FFQ, food frequency questionnaire; HPV, human papillomavirus; SPOR, specialized program of research excellence; FFPE, formalin fixed paraffin embedded; AUDIT, alcohol use disorders identification test; NCBI, national center for biotechnology information; GSEA, gene set enrichment analysis; KEGG, kyoto encyclopedia of genes and genomes; HDAC, histone deacetylase; ATRA, all-trans-retinoic acid; GNMT, glycine *N*-methyltransferase; SAM, S-adenosyl methionine; SAH, S-adenosylhomocysteine

Diet is associated with cancer prognosis, including head and neck cancer (HNC), and has been hypothesized to influence epigenetic state by determining the availability of functional groups involved in the modification of DNA and histone proteins. The goal of this study was to describe the association between pretreatment diet and HNC tumor DNA methylation. Information on usual pretreatment food and nutrient intake was estimated via food frequency questionnaire (FFQ) on 49 HNC cases. Tumor DNA methylation patterns were assessed using the Illumina Goldengate Methylation Cancer Panel. First, a methylation score, the sum of individual hypermethylated tumor suppressor associated CpG sites, was calculated and associated with dietary intake of micronutrients involved in one-carbon metabolism and antioxidant activity, and food groups abundant in these nutrients. Second, gene specific analyses using linear modeling with empirical Bayesian variance estimation were conducted to identify if methylation at individual CpG sites was associated with diet. All models were controlled for age, sex, smoking, alcohol and HPV status. Individuals reporting in the highest quartile of folate, vitamin B12 and vitamin A intake, compared with those in the lowest quartile, showed significantly less tumor suppressor gene methylation, as did patients reporting the highest cruciferous vegetable intake. Gene specific analyses identified differential associations between DNA methylation and vitamin B12 and vitamin A intake when stratifying by HPV status. These preliminary results suggest that intake of folate, vitamin A and vitamin B12 may be associated with the tumor DNA methylation profile in HNC and enhance tumor suppression.

Introduction

Head and neck cancer (HNC) is the sixth most common type of cancer and accounts for approximately 5% of all new cancer diagnoses worldwide each year.^{1,2} These malignancies develop from the epithelial tissue of the oral cavity, nasopharynx, oropharynx or larynx, and more than 90% are of squamous cell histologic type. HNC is most often associated with extensive lifetime exposure to tobacco and alcohol consumption, although

high-risk human papillomavirus (HPV) has recently emerged as the primary etiologic factor responsible for an increasing number of oropharyngeal tumors.^{3,4}

Epidemiologic evidence also supports the hypothesis that diet modulates risk, progression and prognosis of head and neck cancer.⁵⁻⁷ However, the molecular mechanisms by which dietary compounds exert their effects are not entirely understood. Epigenetic dysregulation is a key mechanism in tumorigenesis that may be influenced by dietary intake. Aberrant DNA methylation in cancer is typically characterized by gene promoter

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Table 1. Clinical characteristics of the study participants (n = 49)

Patient characteristic		N (%)	Mean (SD), median (range)
Age			57.3 (8.6), 55.0 (41–75)
Gender	Male	39 (80%)	
	Female	10 (20%)	
Stage	1	1 (2%)	
	2	6 (12%)	
	3	12 (24%)	
	4	30 (61%)	
Cancer site of first primary	Oral cavity	9 (18%)	
	Oropharynx	26 (53%)	
	Hypopharynx	2 (4%)	
	Larynx	10 (20%)	
Other		2 (4%)	
Tumor tissue HPV (+) status		21 (43%)	
Smoking status	Never	9 (18%)	
	Past	32 (65%)	
	Current	8 (16%)	
Alcohol problem	AUDIT \geq 8 and drank within 1 year	16 (32%)	

specific hypermethylation, leading to gene silencing and hypomethylation of intergenic regions, leading to overall genomic instability.^{8,9}

Dietary intake has been widely hypothesized to influence epigenetic state by determining the availability of functional groups involved in the covalent modification of DNA and histone proteins.^{10,11} Specifically, nutrients that act as methyl donors, such as methionine and folate, or nutrients that act as cofactors in one carbon metabolism pathway, such as betaine, vitamin B12 and choline, are required for establishment and maintenance of methylation marks both on DNA and histone protein tails.^{12–16} A deficiency or excess of any of the nutrients involved in one-carbon metabolism can potentially alter the availability of S-adenosylmethionine (SAM), the universal donor involved in DNA methylation, and ultimately alter epigenetic state.⁸ In addition to diet, it has been shown that in smokers, chronic DNA damage coupled with reduced DNA repair capacity may induce gene promoter methylation.¹⁷ Given these findings, we hypothesize that, in addition to micronutrients involved in one-carbon metabolism, micronutrients involved in cell growth and differentiation, such as vitamin A, or with antioxidant activity such as vitamin C, vitamin E and β -carotene may indirectly influence DNA methylation by reducing DNA damage.¹⁸

The objective of this study was to test the hypothesis that pretreatment dietary intake of methyl donors, antioxidants and foods abundant in these micronutrients is associated with tumor DNA methylation in head and neck cancer. A better understanding of

how dietary intake is associated with tumor DNA methylation in head and neck cancer will enhance our knowledge of disease pathogenesis and may allow for the development of individualized medical nutrition therapy regimens.

Results

Descriptive statistics: study sample. The mean age of the 49 study participants with both dietary intake and methylation data was 57.3 y (range 41–75 y); 80% of the participants were male. The majority of the cancer sites were oropharynx (53%), evidenced by the relatively high proportion of HPV(+) cases (43%). Approximately 85% of the study participants reported being current or former smokers, with an estimated one-third reporting an alcohol problem. Clinical characteristics are displayed in Table 1.

Dietary intake associations with tumor suppressor methylation. Reported average daily intakes of micronutrients and food group servings reported by study participants are presented in Table 2. The distribution of the tumor suppressor methylation score is shown in Figure S1. Table 3 shows associations across quartiles of micronutrient and food group intake and tumor suppressor DNA methylation. Higher antioxidant, methyl donor and food group intake tended to be negatively associated with tumor suppressor score, the sum of individual hypermethylated tumor suppressor associated CpG sites, suggesting an inverse relationship between DNA methylation at promoter regions of these genes and micronutrient intake. The regression coefficients in Table 3 can be interpreted as the number of CpG sites of genes associated with tumor suppression activity that are methylated more than 50% compared with individuals in the lowest quartile of intake. Specifically, individuals in the lowest quartiles of dietary folate, vitamin B12 and vitamin A intake were found to have significantly higher tumor suppressor methylation scores compared with individuals in the highest quartiles of intake. Additionally, tests for trend across quartiles of intake for dietary folate, vitamin B12 and vitamin A intake indicated statistically significantly higher methylation score with each decreasing quartile (Table 3). Individuals in the lowest quartile of cruciferous vegetable intake had a significantly higher tumor suppressor methylation score than individuals in the highest quartile of intake, and the test for trend across quartiles was also statistically significant ($p = 0.013$). Interestingly, individuals consuming the highest servings per day of refined grains had significantly higher tumor suppressor methylation score than individuals consuming the lowest servings per day of refined grains. However the test for trend across quartiles of intake for refined grains was not statistically significant.

In a sensitivity analysis, in which we categorized micronutrient intake into tertiles as opposed to quartiles and adjusted for the same covariates, our findings were similar to the findings reported above in that individuals in the lowest tertile of vitamin B12, vitamin A and cruciferous vegetable intake had significantly higher tumor suppressor methylation score than those in the highest tertile of intake ($\beta = 18.4$, $\beta = 18.2$ and $\beta = 18.2$, respectively; data not shown). The tests for trend across tertiles of intake for the relationship between vitamin B12, vitamin A

Table 2. Summary of mean daily micronutrient and food group servings by quartile of consumption

Dietary component	Quartile 1	Quartile 2	Quartile 3	Quartile 4
	n = 12	n = 12	n = 13	n = 12
Micronutrients (energy-adjusted)				
Total folate (μg/d)	325	441	706	1294
Food folate (μg/d)	202	269	330	422
Vitamin A (IU/d)	3869	6705	10594	222111
Vitamin B6 (mg/d)	1.8	2.5	3.8	10.5
Vitamin B12 (μg/d)	4.7	8.5	13.1	32.2
Vitamin C mg/day	64	114	243	1086
Vitamin D (IU/d)	77	183	462	835
Vitamin E (mg/d)	5.4	8.9	24.6	288
Beta-carotene (μg/d)	1022	1913	3353	9178
Methionine (g/d)	1.4	1.8	2.3	2.8
Food groups (servings/d)				
Cruciferous vegetables	0.02	0.1	0.22	0.62
Green leafy vegetables	0.07	0.15	0.3	0.94
Dark yellow & orange vegetables	0.03	0.09	0.19	0.45
Refined grains	0.32	0.67	1.17	2.9
Whole grains	0.07	0.36	0.86	2.11
Red meat	0.18	0.4	0.66	1.43
Legumes	0.07	0.27	0.27	0.71

and cruciferous vegetables and tumor suppressor methylation score were also significant ($P_{\text{trend}} = 0.015$, $P_{\text{trend}} = 0.034$ and $P_{\text{trend}} = 0.022$, respectively). The inverse association between tumor suppressor methylation score and food folate was no longer statistically significant ($\beta = -14.7$ for tertile 3 vs. tertile 1, $P_{\text{trend}} = 0.074$).

CpG site-specific associations with vitamin A and vitamin B12 intake. With respect to vitamin A intake, there were no significant associations with site-specific DNA methylation for HPV(+) individuals (all adjusted p values > 0.15). In contrast, a number of CpG sites were found to be differentially methylated for HPV(-) individuals with the highest levels of both vitamin A and vitamin B12 intake compared with those with the lowest levels of intake. Interestingly, vitamin B12 intake was also found to be associated with hypomethylation of a CpG site associated with *RARB* (retinoic acid receptor β) in HPV(+) individuals after adjusting for multiple comparisons (adjusted p value < 0.05). The ten sites identified as the most significantly differentially methylated by both vitamin A and vitamin B12 intake in HPV(-) individuals, as well as the one site differentially methylated by vitamin B12 intake in HPV(+) individuals are shown in **Table 4**. Consistent with the findings of the methylation score analysis, the most differentially methylated CpG sites were, by and large, found to be hypomethylated in tumors from individuals in the highest quartile of vitamin A and vitamin B12 intake compared with tumors from individuals in the lowest quartiles of intake. There was one exception, *CHGA* (chromogranin A), which was found to be hypermethylated in tumors from HPV(-) negative individuals who consumed the largest amounts of vitamin B12. CpG sites associated with

C4B (complement component 4B), *GML* (glycosylphosphatidylinositol anchored molecule like protein), *MLF1* (myeloid leukemia factor 1), and *THPO* (thrombopoietin) were found to be significantly associated with methylation in tumors from HPV(-) individuals based on both vitamin A and vitamin B12 intake.

Table S1 presents the results of the ranked gene set enrichment analysis (GSEA). An analysis of positional enrichment of genes differentially methylated by vitamin B12 intake in HPV(+) cancers revealed a trend toward hypomethylation of chromosome 12p12 with increased vitamin B12 intake. The same chromosomal region was identified as hypomethylated in tumors from HPV(-) individuals with the highest levels of vitamin A intake, as was chromosome 8p21. While there was no consistent enrichment of gene ontology biological processes or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on dietary intake for HPV(-) tumors, for HPV(+) tumors there was a significant enrichment of processes involved in immune function based on vitamin B12 intake.

Discussion

To our knowledge, this is the first study to comprehensively examine the association between dietary intake and promoter methylation of genes related to head and neck cancer. These novel findings suggest diet is significantly associated with epigenetic events that occur in head and neck cancer. While previous studies have indicated deficiencies of folate, vitamin B12 and vitamin A are associated with increased risk of developing head and neck cancer^{5,19-21} as well as with poorer prognosis in patients

Table 3. Results of unadjusted and adjusted linear regression showing associations between tumor suppressor methylation score and quartile of energy adjusted micronutrient intake or intake of food group, setting quartile 1 (lowest intake) as the reference category

Unadjusted				
Micronutrient	Quartile 2 β (S.E)	Quartile 3 β (S.E)	Quartile 4 β (S.E)	p value for trend
Dietary folate	-4.3 (8.6)	-7.0 (8.6)	-16.8 (8.4)	0.047
Total folate	1.3 (8.7)	-1.0 (8.7)	-6.2 (8.7)	0.450
Vitamin A	-8.6 (8.6)	-7.9 (8.6)	-13.3 (8.6)	0.146
Vitamin B6	-7.1 (8.6)	-1.0 (8.6)	-11.4 (8.6)	0.301
Vitamin B12	-7.2 (8.0)	-6.6 (8.2)	-23.9 (8.2)	0.008
Vitamin C	0.8 (8.6)	2.0 (8.6)	-8.4 (8.6)	0.389
Vitamin D	-8.8 (8.6)	-11.2 (8.6)	-10.2 (8.6)	0.218
Vitamin E	-1.8 (8.8)	-3.9 (8.8)	-2.4 (8.8)	0.727
Beta-carotene	-3.3 (8.6)	-7.5 (8.6)	-13.1 (8.6)	0.111
Methionine	1.5 (8.5)	11.7 (8.5)	-4.4 (8.5)	0.932
Cruciferous vegetables	-4.7 (8.0)	-16.5 (8.0)	-18.1 (8.5)	0.014
Green leafy vegetables	0.7 (8.5)	-6.1 (8.9)	-6.6 (8.9)	0.343
Dark yellow vegetables	-5.3 (9.3)	-5.3 (9.0)	-10.1 (8.4)	0.245
Refined grains	12.3 (8.5)	9.3 (8.8)	14.3 (8.7)	0.150
Whole grains	9.1 (8.7)	8.0 (9.3)	-1.5 (8.8)	0.808
Red meat	-17.8 (8.2)	-11.3 (8.5)	-12.4 (8.0)	0.185
Legumes	-5.6 (8.3)	-5.4 (9.6)	-9.8 (9.0)	0.307
Adjusted*				
Dietary folate	-3.1 (8.9)	-4.0 (9.5)	-18.7 (8.9)	0.043
Total folate	4.6 (9.1)	-3.2 (9.6)	-9.1 (9.2)	0.258
Vitamin A	-13.3 (9.8)	-15.1 (9.9)	-21.1 (9.7)	0.040
Vitamin B6	-5.5 (9.6)	-2.2 (9.3)	-12.9 (9.2)	0.219
Vitamin B12	-7.6 (8.8)	-11.0 (8.7)	-27.6 (8.8)	0.003
Vitamin C	0.2 (8.9)	-1.9 (9.4)	-16.6 (9.3)	0.089
Vitamin D	-11.4 (9.3)	-15.6 (9.5)	-18.1 (9.8)	0.059
Vitamin E	-4.3 (9.2)	-11.4 (9.8)	-12.2 (9.9)	0.175
Beta-carotene	-3.4 (9.2)	-12.5 (9.0)	-15.6 (9.4)	0.055
Methionine	3.9 (9.1)	9.6 (9.1)	-7.9 (9.7)	0.687
Cruciferous vegetables	-6.8 (8.5)	-16.2 (8.5)	-20.4 (9.2)	0.013
Green Leafy vegetables	4.8 (9.0)	-8.7 (9.4)	-10.9 (9.5)	0.183
Dark Yellow vegetables	-6.8 (9.6)	-15.4 (10.8)	-15.4 (9.8)	0.098
Refined grains	15.3 (9.7)	13.5 (10.3)	22.6 (10.7)	0.069
Whole grains	10.2 (9.7)	0.8 (10.7)	-3.8 (10.9)	0.542
Red meat	-14.8 (10.3)	-12.8 (10.5)	-14.9 (11.8)	0.189
Legumes	-6.2 (8.9)	-4.5 (10.6)	-14.2 (10.6)	0.227

*Adjusted for age, sex, smoking status, HPV status and problem drinking. Food group analyses also adjusted for total calorie consumption. Bold text indicates $p < 0.05$.

diagnosed with the disease,²² our study has shown a decrease in tumor suppressor methylation in the highest quartile of vitamin B12 and vitamin A intake. Epigenetic dysregulation, such as the silencing of tumor suppressor genes via promoter hypermethylation, is a major event in malignant disease.

Though the underlying biological mechanism related to how folate and vitamin B12 deficiencies may be associated with DNA hypermethylation has not been elucidated, we have two suggested

hypotheses. First, folate and vitamin B12 play important roles in the synthesis of DNA and DNA damage repair.²³⁻²⁵ Increases in DNA methylation have been observed in an experimental model inducing in vitro DNA damage, in the form of DNA strand breaks, and homology directed DNA repair.²⁶ An increased rate of DNA strand breaks was also observed in both animal models and cell culture models of folate deficiency,^{27,28} potentially providing a mechanism that links folate deficiency, DNA strand

Table 4. CpG sites with tumor DNA methylation values significantly different between highest (4th quartile) and lowest (1st quartile) of vitamin B12 and vitamin A intake, stratified by HPV status

Gene symbol	Chromosome	CpG coordinate	Distance to TSS	t-value	p value	Adjusted p value
HPV positive cases: methylation by vitamin B12 intake						
<i>RARB</i>	3	25444872	114	-5.72	3.18E-05	0.0479
HPV negative Ccases: methylation by vitamin B12 intake						
<i>C4B</i>	6	32057984	171	-5.39	1.79E-05	0.0178
<i>GML</i>	8	143912938	-281	-5.23	2.64E-05	0.0178
<i>PTHR1</i>	3	46893982	-258	-5.11	3.55E-05	0.0178
<i>MLF1</i>	3	159771921	243	-4.78	8.14E-05	0.0306
<i>THPO</i>	3	185578143	483	-4.58	0.0001	0.0341
<i>CHGA</i>	14	92459297	52	4.57	0.0001	0.0341
<i>XIST</i>	X	72989344	-31	-4.44	0.0002	0.0350
<i>LCN2</i>	9	129951398	-141	-4.41	0.0002	0.0350
<i>SRC</i>	20	35406338	-164	-4.40	0.0002	0.0350
<i>SFN</i>	1	27062338	118	-4.26	0.0003	0.0442
HPV negative cases: methylation by vitamin A intake						
<i>PTHLH</i>	12	28016940	-757	-6.55	1.11E-06	0.0017
<i>LMO2</i>	11	33870264	148	-5.80	6.64E-06	0.0050
<i>SIN3B</i>	19	16800704	-514	-5.26	2.48E-05	0.0092
<i>TNFSF8</i>	9	116732775	-184	-5.19	2.91E-05	0.0092
<i>C4B</i>	6	32057984	171	-5.17	3.06E-05	0.0092
<i>GML</i>	8	143912938	-281	-4.66	0.0001	0.0241
<i>HGF</i>	7	81238681	-1293	-4.65	0.0001	0.0241
<i>MLF1</i>	3	159771921	243	-4.59	0.0001	0.0244
<i>THPO</i>	3	185578143	483	-4.51	0.0002	0.0244
<i>HOXA11</i>	7	27191320	35	-4.44	0.0002	0.0244

breaks, and DNA methylation. Second, while the DNA methylation changes that occur in cancer have been well characterized, there is also interest in describing the DNA methylation changes that occur with aging, as these changes could reflect what occurs in the early stages of carcinogenesis. A recent study has described that DNA methylation of two genes, *ERα* and *SFRP1*, in colon tissue increases with age and also with folic acid supplementation status.²⁹ In our study, we not only observed a decrease in tumor suppressor methylation in the highest quartile of vitamin A, vitamin B12 and food folate intake, but also a trend toward increased tumor suppressor methylation in the individuals who consumed the highest levels of refined grains. During the refinement process these grains are fortified with folic acid, niacin, riboflavin, thiamin and iron. It is unclear how these nutrients may be associated with increased DNA methylation and more research is necessary. The relative bioavailability of these nutrients could modulate the normal stochastic changes in methylation that occur due to aging and may be reflected in tumor DNA methylation.

Other studies support our finding that increased consumption of folate and folate-rich foods are associated with decreased methylation of genes associated with tumor suppression. Another study conducted in head and neck cancer patients reported increased methylation of *p16^{INK4a}*, a gene involved in cell cycle

regulation that is commonly lost or silenced in head and neck cancer via epigenetic mechanisms, in those consuming the lowest levels of folate compared with the highest levels.³⁰

Based on our findings that folate intake was inversely associated with tumor suppressor DNA methylation, it is not surprising that high cruciferous vegetable consumption was associated with lower methylation levels, given this food group is a rich source of folate. However, it is interesting to note that cruciferous vegetables are also rich sources of glucosinolates such as sulforaphane and indole-3-carbinol, both of which are believed to exhibit anti-cancer activities. Though there is little evidence suggesting involvement of sulforaphane in DNA methylation, this compound has been shown to act as a histone deacetylase (HDAC) inhibitor. HDACs have been shown to be widely overexpressed in a number of cancers and are involved in gene silencing by inducing a more condensed chromatin state, leaving DNA less accessible for transcription.³¹ HDAC inhibitors, such as sulforaphane, have been shown to be promising cancer therapies because of their ability to induce expression of epigenetically silenced genes.³²

The finding that vitamin A intake is significantly associated with decreased DNA methylation in HPV(-) tumors is unexpected, considering vitamin A does not play a direct role in the one-carbon metabolism pathway. However, it is possible

vitamin A can alter DNA methylation by influencing the availability and activity of methyltransferases. Two studies have reported rats treated with all-trans-retinoic acid (ATRA), a vitamin A metabolite, displayed increased levels and activity of glycine *N*-methyltransferase (GNMT), the enzyme involved in the conversion of *S*-adenosyl methionine (SAM) to *S*-adenosylhomocysteine (SAH).^{33,34} Increased GNMT activity led to a reduction of SAM, thus potentially resulting in a reduction of methylation. Similarly, Das et al. reported demethylation of 402 gene promoters in neuroblastoma cells treated with ATRA. These cells also exhibited downregulation of the methyltransferases, *DNMT1* and *DNMT3B*, which the authors proposed as the potential mechanism by which ATRA exerts its effect on DNA methylation.³⁵

The GSEA findings of this study confirm the previous work of our group and others describing the differences in tumor biology between HPV(+) and HPV(-) HNCs.^{36,37} In this study, we identified that gene sets involved in immune function, including cytokine production and antigen processing and presentation, were significantly enriched based on DNA methylation differences in HPV(+) tumors from individuals with high and low levels of vitamin B12 intake. There was no consistent KEGG or Gene Ontology Biological Processes enriched in HPV(-) tumors, based on either vitamin A or vitamin B12 intake. A positional enrichment analysis identified the same chromosomal region, chromosome 12p12, as differentially methylated in HPV(+) tumors based on vitamin B12 intake as HPV(-) tumors based on vitamin A intake. This region has previously been identified as a candidate tumor suppressor region in head and neck and other cancers,³⁸ and may represent a region of the genome that is more labile to the epigenetic influences of dietary intake.

Results of this preliminary study should be interpreted in light of several limitations. Our small sample size poses analytic limitations in detecting associations of interest and the complexity of statistical tests used. Although we have adjusted for several covariates, there are no doubt other unmeasured sources of confounding we were not able to consider. The FFQ is susceptible to measurement error and potential unquantified systematic biases that may have led to misclassification of dietary intake. The FFQ was designed to assess usual dietary intake over the course of the year prior to diagnosis with HNC. Thus we cannot make any conclusions about the association between lifetime dietary exposures and tumor DNA methylation. While the Goldengate Cancer Panel provides a comprehensive measurement of methylation across 1,505 CpG sites in promoter regions of genes with known roles in cancer, this technology does not provide an unbiased assessment of the epigenome. Finally, due to these limitations, the findings of this study should be validated in another set of tumors from similar individuals.

In conclusion, dietary intake of micronutrients involved in one-carbon metabolism such as folate and vitamin B12, as well as other micronutrients such as vitamin A, may be significant regulators of epigenetic events occurring in head and neck cancer. These findings have potential clinical implications for the treatment of head and neck cancer. The idea that dietary interventions could potentially reprogram the epigenome in such a

way as to optimize the likelihood of positive disease outcomes is appealing. Further research is necessary to better understand the complex role these micronutrients play in regulating the epigenetic process, as well as how nutrients may differentially regulate this process according to HPV status. Such studies could give rise to highly specific randomized controlled trials and ultimately to the development of individualized medical nutrition therapy regimens that may improve prognosis in the head and neck cancer population.

Methods

Design. This was a cross-sectional study of patients enrolled in the University of Michigan Head and Neck Cancer Specialized Program of Research Excellence (SPORE). The independent variables were micronutrients involved in one-carbon metabolism, antioxidant nutrients and food groups abundant in these micronutrients. Confounding variables included age, sex, smoking, alcohol problem and HPV status. The outcome variable was DNA methylation. Our analyses were limited to foods and nutrients we hypothesized are associated with DNA methylation—namely those involved in one-carbon metabolism or with antioxidant activity. A two-tiered analysis was conducted; first we observed whether dietary intake is associated with methylation at CpG sites in tumor suppressor genes overall and second, we observed whether DNA methylation at specific CpG sites is associated with dietary intake.

Study population. Sixty-eight newly diagnosed cases of head and neck cancer were identified and recruited at two sites participating in the University of Michigan Head and Neck SPORE: the University of Michigan Health System and the Ann Arbor Veterans Administration (VA) Medical Center. Individuals eligible for participation included patients diagnosed with first primary head and neck cancer between January 1, 2003 and December 31, 2005 who signed written, informed consent, completed an epidemiologic questionnaire that includes questions on demographic and behavioral data, and had a paraffin-embedded tumor available for analysis with adequate residual tissue for microdissection. Exclusion criteria included: (1) < 18 y of age, (2) pregnant, (3) non-English speaking, (4) diagnosed as mentally unstable or (5) a diagnosis of another non-upper aerodigestive tract cancers (such as thyroid or skin cancer). For the purposes of the current analysis, patients were excluded if they had not completed a baseline FFQ ($n = 19$), yielding a final sample size of 49 participants. Excluded patients did not differ significantly from included patients in respect to age, sex, tumor site, cancer stage or smoking status. This study was approved as being within the ethical standards of the Institutional Review Boards of the University of Michigan and the Ann Arbor VA Medical Center.

Tumor blocks were recut for uniform histopathologic review and microdissection, with the first and last slides of a series of 12 reviewed by a qualified pathologist (JM and ND) to confirm the original diagnosis and to circle areas for DNA extraction. Percent cellularity was estimated for each tumor, and areas with > 70% cancer cellularity were designated for use in the analyses.

Measures. *FFPE tissue, DNA isolation and bisulfite conversion.* Regions that were identified for DNA extraction were cored from the formalin fixed paraffin embedded (FFPE) tissue blocks using an 18 gauge needle. Isolation of DNA from the cored tissue samples was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen) with a modification to the manufacturer's recommended lysis protocol (incubation overnight at 56°C in lysis buffer). DNA concentration and purity was confirmed via NanoDrop spectrophotometer (Thermo Scientific). We performed sodium bisulfite modification on 500 ng to 1 µg of DNA using the EZ DNA Methylation kit (Zymo Research) following the manufacturer's recommended protocol.

Bead array methods. The commercially available Illumina Goldengate[®] Methylation Cancer Panel was utilized to detect DNA methylation patterns in tumor samples. The Cancer Panel interrogates 1,505 CpG sites located in known CpG islands across 807 genes related to cancer, including oncogenes, tumor suppressor genes, imprinted genes, and genes involved in cell cycle regulation, DNA repair, apoptosis and metastasis. Bead arrays were run at the University of Michigan DNA Sequencing Core Facility according to the manufacturer's protocol. Briefly, bisulfite converted tumor DNA was hybridized to the bead array as described previously,³⁹ and bead arrays were imaged using Illumina Cancer Panel Reader software. Raw bead array fluorescence data was initially analyzed using Illumina BeadStudio Methylation software, which converts fluorescence values of the methylated (Cy5) and unmethylated (Cy3) alleles into an average methylation value at a specific probe using the formula $\beta = [\text{Max}(\text{Cy}5,0)]/[\text{Max}(\text{Cy}5,0) + \text{Max}(\text{Cy}3,0) + 100]$ with $\beta \in (0,1)$.

Methylation at specific CpG probes on the Goldengate Cancer Panel has been shown to be biased by probe thermodynamic properties.⁴⁰ Known biases include probe length and GC content, which can affect the melting temperature of the probes as well as probe fluorescence intensities. Thus, we used the method proposed by Kuan et al. to normalize our average β values based on probe length and GC content.⁴⁰

Detection p values on the Goldengate Cancer Panel are calculated based on fluorescence signal at a probe compared with background fluorescence and represent one minus the probability that a signal is stronger than background fluorescence. The weighted methodology proposed by Kuan et al. was used to develop sample and site weights based on p-values of detection. Both samples and sites with larger detection p-values are generally considered less reliable and were down-weighted in further gene specific analyses.

Dietary intake estimation. Dietary components of interest in this study included antioxidant micronutrients, micronutrients involved in one-carbon metabolism, and food groups serving as rich sources of these micronutrients including cruciferous vegetables, green leafy vegetables, dark yellow vegetables, refined grains, whole grains, red meat and legumes. Pre-treatment dietary intake was collected using a self-administered, semi-quantitative FFQ, designed to assess respondents' usual dietary intake from food and supplements over the year prior to diagnosis. The reproducibility and validity of this 131-item questionnaire has been reported previously.⁴¹⁻⁴³ The FFQ includes given standard portion sizes

for each item (i.e., 1 orange or 3–5 oz chicken) which allowed participants to choose their average frequency of consumption over the past year from a list of 1–9 choices ranging from “almost never” to “≥ 6 times per day.” Daily energy and nutrient intake was estimated by summing intakes from each food based on the given standard portion size, reported frequency of consumption, and nutrient content of each food item.⁴³ Daily food group servings were estimated by summing the frequency weights of each food item based on reported daily frequencies of consumption.

Covariates. Each participant completed a self-administered health questionnaire at baseline. This questionnaire was designed to collect demographic data as well as data on smoking, alcohol use, physical activity, sleep, comorbidities, depression and quality of life. Age, sex, smoking and alcohol data were collected from the health questionnaire. Smoking data permitted analysis by “current-past-never” smoking. Alcohol consumption was measured using the previously validated Alcohol Use Disorders Identification Test (AUDIT).⁴⁴ An AUDIT score ≥ 8 was considered problem drinking. Tumor site and stage was recorded from operative notes and surgical pathology forms at baseline. As we have previously shown HPV status to be associated with both tumor DNA methylation profiles at specific gene promoter regions⁴⁵ and micronutrient intake,³⁶ we considered HPV status as a potential confounder in statistical analyses.

HPV status was determined by an ultra-sensitive method using real-time competitive polymerase chain reaction and matrix-assisted laser desorption/ionization-time of flight mass spectroscopy with separation of products on a matrix-loaded silicon chip array. Multiplex PCR amplification of the E6 region of 15 discrete high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73), and human GAPDH control was run to saturation followed by shrimp alkaline phosphatase quenching. Amplification reactions included a competitor oligo identical to each natural amplicon except for a single nucleotide difference. Probes that identify unique sequences in the oncogenic E6 region of each type were used in multiplex single base extension reactions extending at the single base difference between wild-type and competitor HPV so that each HPV type and its competitor were distinguished by mass when analyzed on the MALDI-TOF mass spectrometer.

Statistical methods. *Methylation score.* To create a measurement of stochastic methylation across genes that could be associated with dietary intake, we developed a novel method of calculating a methylation score based on methylation of genes associated with specific cellular functions. Since the Goldengate Cancer Panel quantifies methylation of cancer-related genes with various functions, we first subsetted genes associated with our main cellular function of interest, tumor suppression. To identify genes associated with tumor suppression, we conducted a simple query of the National Center for Biotechnology Information (NCBI) Gene database for “tumor suppressor” and extracted Gene IDs. A total of 444 individual CpG sites across 237 genes on the Goldengate Cancer Panel were linked to tumor suppression. Next, for each study participant, the methylation score was defined as the number of tumor suppressor gene-associated CpG sites with normalized average β values above 0.5. A β value cutoff

of 0.5 was established as a methylation level likely to result in decreased expression of tumor suppressor genes. Thus, the methylation score ranged from 0 to 444. We infer that a higher methylation score is associated with higher levels of DNA methylation of promoter regions of genes associated with tumor suppression and cell cycle regulation.

Micronutrients were adjusted for total energy intake using the residual method.⁴⁶ Descriptive statistics, including means, medians, maxima and minima were calculated for both the energy adjusted nutrients and the methylation score. Associations between energy-adjusted micronutrient intake, food group intake, and methylation score were assessed with Spearman rank correlation due to the typical non-normal distribution of micronutrient and food group intake. Additionally, we used linear regression models with methylation score as the outcome and energy-adjusted micronutrient or food group intake as the main predictor, categorizing dietary variables into quartiles, and adjusting for age, sex, smoking status, problem alcohol drinking, and HPV status, as well as analyzing for trend. To examine the robustness of our findings, we performed a sensitivity analysis in which we categorized micronutrient intake into tertiles as opposed to quartiles and adjusted for the same covariates. Regression diagnostic plots were examined to ensure that associations observed were not being driven by influential points. All analyses were performed in SAS software, Version 9.2 or R Version 2.13.0.

Site-specific analyses. To determine whether methylation at specific sites of the genome may be labile to effects due to dietary intake, we calculated associations between DNA methylation at specific CpG sites and dietary intake, stratifying by HPV status. To limit the number of multiple comparisons conducted, we restricted our analysis to micronutrients identified as significant in the tumor suppressor gene methylation score analysis following sensitivity analysis, vitamin B12 and vitamin A, and compared site specific DNA methylation between highest and lowest quartile of intake.

The association between micronutrient intakes and individual CpG site DNA methylation at the 1505 CpG sites measured on the Goldengate Cancer Panel were examined using the *Limma* package in R 2.10.1.⁴⁷ Sample weights based on detection p-values across samples were used in the *lmFit* function from the *Limma* package to downweight samples with higher detection p-values. An empirical Bayes method (using the *eBayes* function in *Limma*) was used to shrink standard errors to a common value and to

rank CpG sites in order of differential methylation. Multiple comparisons were accounted for using the q-value method previously described.⁴⁸ Micronutrient intakes were categorized into quartiles, and differences in individual CpG site methylation between the highest and lowest quartile of intake were calculated, adjusting for age, sex, smoking status, and problem drinking.

Based on the site-specific analyses, we used gene set enrichment analysis (GSEA) to identify common pathways and chromosomal locations for genes identified as differentially methylated by micronutrient intake.⁴⁹ All sites were ranked by t-value and input into GSEA as a ranked list. The full list of genes assayed on the Goldengate Cancer Panel was input into GSEA as a chip platform file, which provided the background for the enrichment analysis. Weighted enrichment statistics were calculated by the GSEA software, using a minimum gene set size of 5 to account for the limited number of genes assayed by the Goldengate Cancer Panel. Enrichment at the nominal p value of 0.05 and an adjusted q value of 0.25 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/21038

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