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Mature miRNA sequence polymorphism in *MIR196A2* is associated with risk and prognosis of head and neck cancer

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Abstract

Purpose—The central role of microRNAs (miRNA) as regulators of translation has been well established, while the relationships between genetic variation in miRNAs and disease risk is only beginning to be explored. A polymorphism in the *MIR196A2* locus has demonstrated associations with lung, breast, esophageal, and gastric tumors, but has not been examined in head and neck cancers, which share similar pathology and etiology to these diseases.

Experimental Design—We studied a polymorphism in the mature sequence of *MIR196A2* (rs11614913, C/T) in a population-based case-control study (total n=1039) of head and neck squamous cell carcinoma (HNSCC) to determine if *MIR196A2* genotype was associated with disease occurrence and patient survival.

Results—Presence of any variant allele was associated with a significantly reduced risk of HNSCC (OR, 0.8; 95% CI, 0.56 – 0.99). Homozygous variant allele carriers with pharyngeal tumors had significantly reduced survival compared to wild-type and heterozygous cases (HR, 7.4; 95% CI, 1.9 – 28.2). Expression analysis in a subset of tumors (n=83) revealed no significant difference in relative expression of either miR-196a or miR-196a* by *MIR196A2* genotype.

Conclusion—These data demonstrate a role for *MIR196A2* genotype in susceptibility and prognosis of head and neck squamous cell carcinoma.

Keywords

MIR196A2; polymorphism; head and neck; susceptibility; prognosis

Introduction

With approximately 45,000 new cases diagnosed and over 11,000 deaths each year, head and neck squamous cell carcinoma (HNSCC) is a relatively common cancer in the United States (1). Three major etiologic factors contribute to risk and prognosis of HNSCC: tobacco

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use, alcohol consumption, and human papillomavirus (HPV) infection (2,3). Recently, examinations of miRNA expression profiles in HNSCC have highlighted the importance of miRNA expression alterations in HNSCC tumorigenesis, disease etiology, and survival (4-10).

Micro-RNAs (miRNAs), short non-coding RNAs which prevent translation of their target mRNAs are critical regulators of the transcriptome (11). MiRNAs are known to play a crucial role in gene regulation, and altered expression of miRNAs in human cancers has been well documented (12). Single nucleotide polymorphisms (SNPs) occurring in microRNAs (miRNAs) or in their binding sites are novel sources of genetic variation that may contribute to cancer susceptibility and prognosis (13,14).

A genetic variant in the mature miRNA sequence of *MIR196A2* (rs11614913) has been associated with reduced risk of breast cancer (15,16), gastric cancer (17), and non-small cell lung cancer (18). In addition, this polymorphism has been associated with prognosis in non-small cell lung cancer (19). The *MIR196A2* gene encodes two mature miRNAs; miR-196a, which is regarded as the main mature product of the hairpin, and miR-196a*, which contains the SNP rs11614913. Prior investigations have suggested that this SNP effects the processing of the pre-microRNA into its mature, regulatory form (15).

We hypothesize that *MIR196A2* genotype is associated with susceptibility to, and or prognosis of HNSCC and examined this hypothesis in a population-based case-control study.

Materials and Methods

Study population

The study population has been previously described (20,21). Briefly, incident cases of HNSCC were identified from nine medical facilities in the Boston metropolitan area, with histologic classification of malignancy reported by pathology of the participating hospitals and confirmed by an independent study pathologist. Population-based controls were drawn from the same greater Boston population and matched to cases by gender, age (+/- 3 years), and town of residence using the Massachusetts town lists. All cases and controls enrolled in the study provided written informed consent as approved by the institutional review boards of the participating institutions. Survival time was determined for cases using publicly available databases.

DNA isolation and genotyping

DNA was extracted from whole blood or buccal cells with the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Genotyping of the *MIR196A2* mirSNP (rs11614913, C/T) was done using Taqman® allelic discrimination (Applied Biosystems, Foster City, CA) with a commercially available primer probe set (assay ID C_31185852_10). Genotyping was performed in a blinded fashion, appropriate controls were included in each run, and approximately 10% of samples were duplicated in a coded fashion as quality assurance with >95% concordance observed between replicates.

RNA isolation

Total RNA was isolated from tumors obtained at initial resection or normal human tissues from head and neck sites obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) that were snap-frozen immediately in liquid nitrogen. The mirVANA RNA Isolation Kit (Ambion, Austin, TX) was used according to the manufacturer's protocol.

RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), aliquoted and stored at -80°C briefly until used for laboratory analysis.

Quantitative reverse transcription–polymerase chain reaction

TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) were used to quantify mature miRNAs miR-196a (Custom assay) and miR-196a* (Assay ID 002336). To test whether the assay for miR-196a differentially amplified target sequence based on genotype we measured synthetic oligonucleotide sequences for the mature miRNAs and report absolute Ct values as the synthetic miRNA sequences were the only substrate in this experiment. Complementary DNA was synthesized by priming with gene-specific looped primers including the primers of the miRNAs of interest and RNU44, a universally expressed endogenous control (Applied Biosystems). A 5 μl volume of total RNA diluted to a final concentration of 10 ng/ μl was used for each reverse transcription reaction along with other reverse transcription components, per manufacturer's specifications. Fifteen microliter reactions were incubated in an Applied Biosystems GeneAmp PCR system 9700 for 30 min at 16°C , 30 min at 42°C , 5 min at 85°C and held at 4°C . All reactions, excluding no-template controls and non-reverse transcribed controls, were run in triplicate on an ABI 7500 Fast Real-Time PCR Detection System, according to manufacturer's protocol. All real-time polymerase chain reaction data were normalized to the RNU44 transcript, a non-coding RNA used as a normalization control in the study of miRNA expression (6).

Statistical Analysis

Data were analyzed using the SAS software, and all P values represent two-sided statistical tests. Tests for Hardy-Weinberg equilibrium were conducted. Unconditional logistic regression was used to calculate adjusted ORs and 95% CIs for the association of *MIR196A2* genotype and HNSCC risk adjusting for age, sex, HPV16 status, alcohol consumption and tobacco smoking packyears. Although subjects without HPV16 serology data could have been coded as missing and included, as a more conservative approach these subjects were not included in the model. For analyses by tumor location, cases were grouped according to ICD-9 code, with oral cancer encompassing ICD9 141-145, pharyngeal cancers 146-149, and laryngeal cancers 161. As homozygous variant and heterozygous genotypes had similar ORs and in order to improve power for examination of the rare variant allele, these groups were combined, with homozygous wildtype as the referent (dominant model). Patient survival was examined in a recessive model using Cox proportional hazards modeling to control for variables related to patient survival. These survival probability models included variables representing the combined homozygous variant and controlled for patient age (in decades), tumor stage (1 and 2 vs. 3 and 4), and all other covariates in the table. MicroRNA expression data were subjected to a two-sided, unpaired, student's t-test assuming equal means and unequal variance.

Results

Among 1242 eligible subjects with genotyping data, 203 did not have HPV16 serology data and were excluded. There were no differences in the demographic or exposure characteristics of these individuals compared to the full study population. Characteristics of the remaining 484 cases (269 oral, 123, pharyngeal, and 92 laryngeal cancers) and 555 controls are described in Table 1 (total $n=1039$). Distributions of study participant age, gender, and race between cases and controls were similar. Relationships between disease risk, etiologic factors, and tumor site in this study population have been described previously (3,20-22).

We examined the association of genotype in the mature sequence of *MIR196A2* with case status. Genotypes were within Hardy-Weinberg equilibrium and the prevalence of control subject genotypes (C/C, 35.3%; C/T, 49.2; T/T, 15.5) was nearly equivalent to control subject genotypes reported in another study of subjects predominantly of European decent (C/C, 35.6; C/T, 49.1; T/T, 15.3) (15).

In models controlling for potential confounders, presence of any variant allele was associated with a significantly reduced risk of HNSCC (OR, 0.8; 95% CI, 0.6 – 0.99, Table 2). The association between the variant allele and decreased risk of HNSCC was evident for individual tumor sites, though only reached statistical significance among cases with pharyngeal disease (OR, 0.6; 95% CI, 0.4 – 0.96). Both in overall and tumor-site-specific analyses there was no effect modification of the association between genotype and disease by packyears smoked, average drinks per week or HPV16 seropositive status (data not shown).

We then examined the association of the *MIR196A2* variant genotype with disease survival. Carriers of any variant allele did not have significantly different survival compared to wild-type cases (data not shown). Additionally, homozygous variant cases did not have significantly different survival probability compared to wild-type and heterozygous cases (Log-rank $P = 0.33$, $n=418$; Figure 1A). Stratifying by tumor site, there was no significant survival association for cases with oral cavity tumors ($P = 0.98$, $n=243$; Figure 1B), or laryngeal cancer ($P = 0.55$, $n=74$; Figure 1C). However, cases with pharyngeal cancer and homozygous variant genotype had significantly reduced survival compared to wild-type and heterozygous cases ($P < 0.02$, $n=101$; Figure 1D). Cases with survival data and tumor staging ($n=323$) were entered into a multivariate Cox proportional hazards model to control for potential confounders, and no significant association between genotype and survival was found (Table 3). When stratifying the analysis by tumor site, pharyngeal cancer cases with homozygous variant genotype had significantly reduced survival (HR, 7.4; 95% CI, 1.9 – 28.2; Table 3).

We measured expression levels of mature miRNAs miR-196a and miR-196a* in normal head and neck tissues ($n=14$) and fresh frozen tumor tissues ($n=83$) to determine if *MIR196A2* variant genotype impacts expression. First, in order to validate that presence of the variant allele did not interfere with the detection of mature miRNAs, equivalent quantities of synthetic oligonucleotides corresponding to wild-type and variant miR-196a* sequences (and a 1:1 mix for heterozygous) were measured in triplicate with a stem-loop RT-PCR assay for miR-196a*. This assay did not differentially amplify targets based on genotype (Ct values: wt=15.90, var=16.28, het=16.18). In normal head and neck tissues ($n=14$) we found no significant difference in the relative expression of either miR-196a or miR-196a* by *MIR196A2* genotype (data not shown). Similarly, in tumor tissues ($n=83$) we found no significant difference in relative expression of either miR-196a or miR-196a* by *MIR196A2* genotype, both overall ($n=83$), and when stratified by tumor location (Table 4).

Discussion

Polymorphisms in miRNA genes and their target sites are a novel class of variation in the human genome that are rapidly being identified and investigated in human cancers including HNSCC (13,14,23-25). Motivated by several recent publications linking the *MIR196A2* mature miRNA SNP to cancer susceptibility and prognosis (15-19,26), we tested the hypothesis that this SNP (rs11614913) is associated with susceptibility to, and prognosis of HNSCC; finding a significantly decreased risk of HNSCC, and significantly reduced survival in cases with pharyngeal disease. Notably, the prevalence of control genotypes was completely consistent with another ethnically similar (Caucasian) population-based study

(15). However, unlike some other reports (15,19) when we assessed relative expression of mature miRNAs 196a and 196a* we did not detect significantly different expression by *MIR196A2* genotype in either normal head and neck tissues or tumors.

Comparing expression levels of the mature miR-196a among *MIR196A2* genotypes Hu *et al.* observed significantly lower expression of miR-196a in non-small cell lung tumor samples with C/C genotype (n=6) compared to C/T and T/T individuals (n=17) (19). These authors also measured endogenous levels of pri- and pre-miRNA sequences, but did not observe differential expression of these precursor molecules based on genotype (19). In a separate report, Hoffman *et al.* showed *increased* expression of mature miR-196a in breast cancer cells transfected with pre-miR-196a-C compared to cells transfected with miR-196a-T relative to empty vector control (15), but did not observe differential expression of the precursor miRNA in their transfection experiment, suggesting that *MIR196A2* genotype may result in altered processing of the pre-miR (15). Relative to previous work, our expression results were from a large number of samples (n=83) affording sufficient statistical power to detect even subtle differences, and although the miR-196a expression results appear to be conflicting, cell-type-specific differential expression of miRNAs could explain these results. A recent examination of differential miRNA expression in several cancer types and adjacent normal tissues (including breast and lung, though not head and neck) showed a wide range of miR-196a expression across different normal tissues, tumor types, and between tumor and normal tissues from common sites (GSE14985) (27).

If miRNA SNPs directly affect the expression of mature miRNA species, then the functional relationship between these polymorphisms and disease susceptibility or prognosis may seem clear: altered miRNA expression leads to altered regulation of target mRNAs important in tumorigenesis. Nonetheless, even in the absence of altered mature miRNA expression levels, variation in the mature miR-196a* sequence itself could result in differential regulation of target mRNAs. Mature miR-196a* in variant allele form could have either an increased or decreased affinity for targets of wild-type miR-196a*. A determinant of the impact of miRNA sequence variation is cell-type-specific expression levels of *both* miRNAs and their targets: cell-type-specific transcriptomes. In addition, it is important to note that tumorigenesis-related alterations in a cell's transcriptome may also affect the regulatory capacity of miRNAs to the extent that a miRNA variant allele may have a protective effect for disease as well as predict poor prognosis. Functional characterization of miRNA target transcripts is far from complete and there is currently little data available to speculate on cell-type-specific regulation of mRNAs by miR-196a and miR-196a*.

Though there is conflicting data for the association between *MIR196A2* genotype and miR-196a expression, the variant allele is in the other mature miRNA product of this gene, miR-196a*. Consistent with our data, Hoffman *et al.* did not observe an effect of the variant on expression of mature miR-196a* (15). In addition, studies that report significant associations between the *MIR196A2* polymorphism and cancer risk; breast, gastric, and non-small-cell lung cancers (in both Caucasian and Asian populations) all show the T allele to be associated with decreased risk of disease (15-18). Collectively, these results strongly suggest that reduced risk associated with *MIR196A2* variant genotype is not mediated by differential expression of the gene's mature miRNAs.

In the context of carcinogenesis, the dynamic relationship between a miRNA and its targets may be modified by etiologic exposures. Compounded with cell-type-specific transcriptomes, there are varying etiologic exposures associated with different cancers: in HNSCC etiologic exposures may differ between individuals (within tumor site), and are known to have different magnitudes of effect by tumor site. For instance, HPV16 seropositive individuals have a higher risk of developing pharyngeal disease compared to

other sites (3). Further, HPV16 and other important etiologic exposures in HNSCC have been associated with specific molecular alterations that may have broader implications for a cell's transcriptome, and hence, may differentially impact regulation by miRNAs. Nonetheless, we did not observe any effect modification by exposures for genotype associations in this study, suggesting that the precise mechanism(s) by which genotype confers risk is too dynamic and complex to fully elucidate. In addition to more complete characterization of the miRNA targets, further study of additional cancers with similar risk factors (e.g. cervical cancer and HPV and lung cancer and tobacco smoking) might also be helpful in illuminating precisely how these variant genotypes confer risk.

This work suggests that patients carrying a variant allele in *MIR196A2* have a significantly decreased risk of HNSCC. In addition, homozygous variant cases with pharyngeal disease also had significantly poorer survival, suggesting that the protective effect of the variant allele does not extend to disease severity. Additional studies are warranted to further confirm these findings and explore the relationship between *MIR196A2* genotype and risk of other forms of cancer.

Translational Relevance: We find that carriers of a variant allele in *MIR196A2* have a significantly decreased risk of developing head and neck cancer compared to wildtype individuals. Further, homozygous variant carriers with pharyngeal disease have significantly reduced survival. Importantly, our evaluation of a mature miRNA polymorphism is an example of a directed approach to investigating a growing category of polymorphic variants in miRNAs and miRNA target sites which will likely have tremendous implications for disease risk and prognosis in all types of human cancer.

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Figure 1.

Kaplan-Meier survival probability plots stratified by *MIR196A2* genotype for head and neck tumors with available staging data. Survival time is defined as time from diagnosis to death or last known follow-up, where circles represent censored values. The log-rank method was used to test for a difference between strata. Solid lines represent wild-type and heterozygous *MIR196A2* genotype cases and dotted lines represent homozygous variant allele cases. A) All tumor sites, $n=418$, $P=0.33$. B) Oral cancers, $n=243$, $P=0.98$. C) Laryngeal cancers, $n=74$, $P=0.55$. D) Pharyngeal cancers, $n=101$, $P<0.02$.

Table 1

Participant characteristics of HNSCC cases and controls genotyped for rs11614913 in *MIR196A2*.

Characteristic	All HNSCC sites				Oral (n=269) Unadjusted OR (95% CI)	Laryngeal (n=92) Unadjusted OR (95% CI)	Pharyngeal (n=123) Unadjusted OR (95% CI)
	Cases, n (%) (n = 484)	Controls, n (%) (n = 555)	Unadjusted OR (95% CI)				
Age (y), mean (SD)	59.4 (11.6)	61.0 (11.5)					
Gender							
Male	359 (74.2)	407 (73.3)					
Female	125 (25.8)	148 (26.7)					
Race							
Non-white	41 (8.5)	50 (9.0)					
White	443 (91.5)	505 (91.0)					
Tobacco (lifetime pack-years)*							
≤ 1	98 (20.3)	197 (35.5)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
> 1 to ≤ 8	43 (8.9)	73 (13.1)	1.2 (0.8 - 1.9)	1.2 (0.8 - 1.9)	1.2 (0.7 - 2.0)	3.2 (1.0 - 10.9)	0.8 (0.4 - 1.8)
> 8 to ≤ 34	111 (22.9)	142 (25.6)	1.6 (1.1 - 2.2)	1.6 (1.1 - 2.2)	1.4 (0.9 - 2.1)	5.3 (1.9 - 14.4)	1.3 (0.8 - 2.4)
> 34	232 (47.9)	143 (25.8)	3.3 (2.4 - 4.5)	3.3 (2.4 - 4.5)	2.3 (1.6 - 3.3)	17.1 (6.7 - 43.5)	3.2 (1.9 - 5.2)
Alcohol use (lifetime average drinks/week)*							
≤ 2	111 (22.9)	122 (22.0)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
> 2 to ≤ 6	74 (15.3)	157 (28.3)	0.5 (0.4 - 0.8)	0.5 (0.3 - 0.8)	0.5 (0.3 - 0.8)	0.6 (0.3 - 1.2)	0.5 (0.2 - 0.9)
> 6 to ≤ 15	78 (16.1)	133 (24.0)	0.7 (0.4 - 0.9)	0.7 (0.4 - 0.9)	0.6 (0.4 - 0.9)	0.5 (0.2 - 1.1)	0.9 (0.5 - 1.7)
> 15	221 (45.7)	143 (25.8)	1.7 (1.2 - 2.4)	1.7 (1.2 - 2.4)	1.6 (1.1 - 2.4)	1.6 (0.9 - 2.8)	2.0 (1.2 - 3.4)
HPV16 seropositivity							
No	341 (70.5)	494 (89.0)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
Yes	143 (29.5)	61 (11.0)	3.4 (2.4 - 4.7)	3.4 (2.4 - 4.7)	2.8 (1.9 - 4.2)	1.8 (1.0 - 3.3)	6.8 (4.3 - 10.5)
<i>MIR196A2</i> genotype							
C/C	182 (37.6)	188 (33.9)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)	1.0 (Reference)
C/T + T/T	302 (62.4)	367 (66.1)	0.8 (0.7 - 1.1)	0.8 (0.7 - 1.1)	0.92 (0.7 - 1.3)	0.8 (0.5 - 1.3)	0.8 (0.5 - 1.1)

* Tobacco pack-years and average DPW based on quartile distribution in controls

Table 2

MIR196A2 variant genotype is associated with significantly reduced risk of HNSCC.

Covariate	All sites			Oral		Laryngeal		Pharyngeal	
	cases n (%) Total n = 484	controls n (%) Total n = 555	Adjusted OR (95% CI)	n (%) Total n=269	Adjusted OR (95% CI)	n (%) Total n=92	Adjusted OR (95% CI)	n (%) Total n=123	Adjusted OR (95% CI)
Age									
≤ 50	105 (21.7)	95 (17.1)	1.0 (Reference)	66 (24.5)	1.0 (Reference)	14 (15.2)	1.0 (Reference)	25 (20.3)	1.0 (Reference)
> 50 to ≤ 60	164 (33.9)	177 (31.9)	0.6 (0.4 - 0.9)	92 (34.2)	0.6 (0.4 - 0.9)	32 (34.8)	0.6 (0.3 - 1.4)	40 (32.5)	0.7 (0.4 - 1.3)
> 60 to ≤ 70	133 (27.5)	161 (29.0)	0.5 (0.3 - 0.7)	65 (24.2)	0.4 (0.3 - 0.7)	27 (29.3)	0.5 (0.2 - 1.0)	41 (33.3)	0.7 (0.3 - 1.3)
> 70	82 (16.9)	122 (22.0)	0.5 (0.3 - 0.7)	46 (17.1)	0.5 (0.3 - 0.8)	19 (20.7)	0.4 (0.2 - 0.96)	17 (13.8)	0.4 (0.2 - 0.8)
Gender									
Female	125 (25.8)	148 (26.7)	1.0 (Reference)	84 (31.2)	1.0 (Reference)	17 (18.5)	1.0 (Reference)	24 (19.5)	1.0 (Reference)
Male	359 (74.2)	407 (73.3)	0.7 (0.5 - 0.98)	185 (68.8)	0.6 (0.4 - 0.8)	75 (81.5)	1.0 (0.5 - 2.0)	99 (80.5)	1.2 (0.7 - 2.2)
Race									
White	443 (91.5)	505 (91.0)	1.0 (Reference)	248 (92.2)	1.0 (Reference)	83 (90.2)	1.0 (Reference)	112 (91.1)	1.0 (Reference)
Non-white	41 (8.5)	50 (9.0)	0.8 (0.5 - 1.2)	21 (7.8)	0.7 (0.4 - 1.2)	9 (9.8)	0.7 (0.3 - 1.6)	11 (8.9)	0.6 (0.3 - 1.3)
Tobacco (lifetime pack-years)*									
≤ 1	98 (20.3)	197 (35.5)	1.0 (Reference)	66 (24.5)	1.0 (Reference)	5 (5.4)	1.0 (Reference)	27 (22.0)	1.0 (Reference)
> 1 to ≤ 8	43 (8.9)	73 (13.1)	1.3 (0.8 - 2.1)	29 (10.8)	1.2 (0.7 - 2.0)	6 (6.5)	3.7 (1.1 - 12.7)	8 (6.5)	0.8 (0.3 - 2.0)
> 8 to ≤ 34	111 (22.9)	142 (25.6)	1.6 (1.1 - 2.4)	66 (24.5)	1.4 (0.9 - 2.2)	19 (20.7)	6.3 (2.2 - 18.1)	26 (21.1)	1.4 (0.7 - 2.8)
> 34	232 (47.9)	143 (25.8)	3.9 (2.7 - 5.7)	108 (40.2)	2.6 (1.6 - 4.0)	62 (67.4)	22.2 (8.1 - 60.7)	62 (50.4)	4.3 (2.3 - 8.0)
Alcohol use (lifetime average drinks/week)*									
≤ 2	111 (22.9)	122 (22.0)	1.0 (Reference)	64 (23.8)	1.0 (Reference)	22 (23.9)	1.0 (Reference)	25 (20.3)	1.0 (Reference)
> 2 to ≤ 6	74 (15.3)	157 (28.3)	0.5 (0.3 - 0.7)	42 (15.6)	0.5 (0.3 - 0.8)	17 (18.5)	0.5 (0.2 - 1.1)	15 (12.2)	0.4 (0.2 - 0.9)
> 6 to ≤ 15	78 (16.1)	133 (24.0)	0.6 (0.4 - 0.9)	41 (15.2)	0.6 (0.4 - 0.99)	12 (13.0)	0.4 (0.2 - 0.8)	25 (20.3)	0.7 (0.4 - 1.5)
> 15	221 (45.7)	143 (25.7)	1.3 (0.9 - 1.9)	122 (45.4)	1.5 (0.9 - 2.3)	41 (44.6)	0.8 (0.4 - 1.6)	58 (47.2)	1.1 (0.6 - 2.2)
HPV16 seropositivity									
No	341 (70.4)	494 (89.0)	1.0 (Reference)	199 (74.0)	1.0 (Reference)	75 (81.5)	1.0 (Reference)	67 (54.5)	1.0 (Reference)
Yes	143 (29.6)	61 (11.0)	4.1 (2.8 - 5.8)	70 (26.0)	3.0 (2.0 - 4.6)	17 (18.5)	2.1 (1.1 - 4.1)	56 (45.5)	8.2 (5.0 - 13.5)
<i>MIR196A2</i> genotype									
C/C	182 (37.6)	188 (33.9)	1.0 (Reference)	96 (35.7)	1.0 (Reference)	36 (39.1)	1.0 (Reference)	50 (40.7)	1.0 (Reference)

Covariate	All sites		Oral		Laryngeal		Pharyngeal	
	cases n (%) Total n = 484	controls n (%) Total n = 555	Adjusted OR (95% CI)	n (%) Total n=269	Adjusted OR (95% CI)	n (%) Total n=92	Adjusted OR (95% CI)	n (%) Total n=123
C/T + T/T	302 (62.4)	367 (66.1)	0.8 (0.6 - 0.99)	173 (64.3)	0.8 (0.6 - 1.1)	56 (60.9)	0.6 (0.4 - 1.1)	73 (59.3)

*

Tobacco pack-years and average DPW based on quartile distribution in controls

Models are controlled for all variables in the table

Table 3
Cox proportional hazards models of overall survival by *MIR196A2* genotype controlled for confounders.

Covariate	cases n (%) Total n = 323	All sites		Oral		Laryngeal		Pharyngeal	
		Adjusted HR (95% CI)	n (%) Total n=179	Adjusted HR (95% CI)	n (%) Total n=59	Adjusted HR (95% CI)	n (%) Total n=85	Adjusted HR (95% CI)	n (%) Total n=85
Age									
≤ 50	77 (23.9)	1.0 (Reference)	51 (28.5)	1.0 (Reference)	9 (15.3)	1.0 (Reference)	17 (20.0)	1.0 (Reference)	17 (20.0)
> 50 to ≤ 60	105 (32.5)	1.2 (0.6 - 2.4)	59 (33.0)	1.5 (0.7 - 3.2)	23 (39.0)	0.6 (0.1 - 7.6)	23 (27.1)	0.5 (0.1 - 4.2)	23 (27.1)
> 60 to ≤ 70	97 (30.0)	1.9 (1.0 - 3.6)	42 (23.5)	1.3 (0.6 - 3.0)	18 (30.5)	3.2 (0.3 - 39.5)	37 (43.5)	3.3 (0.6 - 18.2)	37 (43.5)
> 70	44 (13.6)	3.2 (1.6 - 6.5)	27 (15.0)	2.7 (1.2 - 6.2)	9 (15.2)	4.8 (0.4 - 62.1)	9 (9.4)	2.8 (0.4 - 21.1)	9 (9.4)
Gender									
Female	78 (24.2)	1.0 (Reference)	50 (27.9)	1.0 (Reference)	12 (20.3)	1.0 (Reference)	16 (18.8)	1.0 (Reference)	16 (18.8)
Male	245 (75.8)	1.4 (0.8 - 2.5)	129 (72.1)	1.6 (0.8 - 3.5)	47 (79.7)	2.9 (0.5 - 16.3)	69 (81.2)	1.3 (0.4 - 4.5)	69 (81.2)
Tobacco (lifetime pack-years)*									
≤ 1	64 (19.8)	1.0 (Reference)	43 (24.0)	1.0 (Reference)	4 (6.8)	1.0 (Reference)	17 (20.0)	1.0 (Reference)	17 (20.0)
> 1 to ≤ 8	27 (8.4)	0.2 (0.1 - 1.1)	19 (10.6)	0.3 (0.1 - 1.5)	3 (5.1)	-	5 (5.9)	-	5 (5.9)
> 8 to ≤ 34	80 (24.8)	1.0 (0.5 - 1.9)	44 (24.6)	1.0 (0.4 - 2.3)	16 (27.1)	0.7 (0.04 - 12.3)	20 (23.5)	1.3 (0.3 - 5.4)	20 (23.5)
> 34	152 (47.0)	1.1 (0.6 - 2.0)	73 (40.8)	1.5 (0.7 - 3.4)	36 (61.0)	0.7 (0.04 - 10.0)	43 (50.6)	1.0 (0.3 - 3.2)	43 (50.6)
Alcohol use (lifetime average drinks/week)*									
≤ 2	78 (24.1)	1.0 (Reference)	41 (22.9)	1.0 (Reference)	19 (32.2)	1.0 (Reference)	18 (21.2)	1.0 (Reference)	18 (21.2)
> 2 to ≤ 6	51 (15.8)	0.6 (0.2 - 1.3)	32 (17.9)	0.3 (0.1 - 1.0)	10 (17.0)	2.6 (0.4 - 18.7)	9 (10.6)	1.1 (0.1 - 11.0)	9 (10.6)
> 6 to ≤ 15	48 (14.9)	0.7 (0.4 - 1.5)	27 (15.1)	0.6 (0.3 - 1.6)	4 (6.8)	-	17 (20.0)	0.6 (0.1 - 2.8)	17 (20.0)
> 15	146 (45.2)	1.0 (0.6 - 1.7)	79 (44.1)	0.7 (0.3 - 1.4)	26 (44.0)	0.7 (0.2 - 2.3)	41 (48.2)	1.9 (0.6 - 5.9)	41 (48.2)
HPV16 seropositivity									
No	221 (68.4)	1.0 (Reference)	131 (73.2)	1.0 (Reference)	44 (74.6)	1.0 (Reference)	46 (54.1)	1.0 (Reference)	46 (54.1)
Yes	102 (31.6)	0.6 (0.4 - 1.0)	48 (26.8)	0.7 (0.4 - 1.4)	15 (25.4)	0.7 (0.2 - 3.0)	39 (45.9)	0.6 (0.2 - 1.7)	39 (45.9)
Stage									
1 or 2	93 (28.8)	1.0 (Reference)	54 (30.2)	1.0 (Reference)	25 (42.4)	1.0 (Reference)	14 (16.5)	1.0 (Reference)	14 (16.5)
3 or 4	230 (71.2)	2.2 (1.3 - 3.6)	125 (69.8)	2.4 (1.2 - 4.7)	34 (57.6)	7.5 (1.5 - 37.1)	71 (83.5)	0.7 (0.3 - 1.9)	71 (83.5)
MIR196A2 genotype									
C/C + C/T	275 (85.1)	1.0 (Reference)	147 (82.1)	1.0 (Reference)	49 (83.1)	1.0 (Reference)	79 (92.9)	1.0 (Reference)	79 (92.9)

Covariate	cases n (%) Total n = 323	All sites		Oral		Laryngeal		Pharyngeal	
		Adjusted HR (95% CI)	n (%)	Total n=179	Adjusted HR (95% CI)	n (%)	Total n=59	Adjusted HR (95% CI)	n (%)
T/T	48 (14.9)	1.3 (0.8 - 2.2)	32 (17.9)	0.8 (0.4 - 1.6)	10 (16.9)	2.5 (0.5 - 13.2)	6 (7.1)	7.4 (1.9 - 28.2)	

* Tobacco pack-years and average drinks per week based on quartile distribution in controls.
Models are controlled for all variables in table.

MIRN196A genotype is not associated with mature miR-196a or miR-196a* expression in HNSCC.

Table 4

Mature miRNA and site	Relative expression [†] by <i>MIRN196A</i> genotype			n
	C	C/T	TT	
miR196a, mean (SD)				
Overall	0.71 (0.09)	0.72 (0.13)	0.71 (0.05)	83
Oral	0.69 (0.09)	0.70 (0.08)	0.70 (0.06)	55
Laryngeal	0.75 (0.05)	0.68 (0.04)	0.8 (NA)	15
Pharyngeal	0.71 (0.07)	0.94 (0.26)	0.72 (0.04)	13
miR196a*, mean (SD)				
Overall	0.55 (0.06)	0.55 (0.07)	0.56 (0.06)	83
Oral	0.55 (0.07)	0.55 (0.06)	0.56 (0.08)	55
Laryngeal	0.55 (0.05)	0.52 (0.04)	0.56 (NA)	15
Pharyngeal	0.54 (0.07)	0.61 (0.11)	0.54 (0.04)	13

[†] Relative to RNU44 expression