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MicroRNA expression in head and neck cancer associates with alcohol consumption and survival

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The contribution of microRNAs (miRNAs) to carcinogenesis in many tumors, including head and neck squamous cell carcinomas (HNSCCs), is clear, but the etiology and clinical significance of their alteration remain important questions. Our previous work has identified four miRNAs as differentially expressed HNSCCs compared with non-diseased epithelia and showed that there is potential diagnostic utility in examining their expression. Here, we used quantitative real-time polymerase chain reaction to determine the relative expression of these miRNAs in a larger independent case series of HNSCC tumors ($n = 169$), examining associations of miRNA expression with exposures and clinical features associated with HNSCC. In multivariate analyses, expression of *miR-375* was shown to increase with alcohol consumption ($P = 0.002$) and showed higher expression in tumors of pharyngeal and laryngeal origin compared with oral tumors ($P < 0.05$ and $P < 0.01$, respectively). Additionally, high *miR-21* expression was associated with significantly decreased 5 year survival in patients (hazard ratio, 1.68; 95% CI: 1.04–2.77) in a model controlled for patient age, gender and tumor stage. Together, these data suggest that alterations in miRNA expression are related to exposures causal in head and neck cancer and may be useful biomarkers of patient outcome.

Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide, arises from various sites in the upper aerodigestive tract (1,2). The intricate anatomy of the primary tumor sites brings about complex patterns of invasion and locoregional spread that have proven difficult to treat (1). These features along with the frequent occurrence of late-stage diagnosis and second primary tumor formation have contributed to a relatively poor 5 year survival rate that has shown only modest improvement over the last three decades (1,3). The major risk factors for HNSCC include tobacco and alcohol, which can act both independently and synergistically, as well as human papilloma virus (HPV) infection, which is an independent risk factor (4). A complete understanding of how these exposures alter cellular functions and the molecular basis for their risk remains elusive. Understanding the molecular nature of HNSCC carcinogenesis is indispensable for improving early diagnosis, predicting prognosis and establishing effective therapeutics. While several attempts have been made at defining genetic biomarkers for HNSCC (5–7), epigenetic biomarkers probably contribute considerable clinical and biological utility to treating and understanding this disease as it is clear that epigenetics plays a pivotal a role in its development and progression.

The field of epigenetic regulation is being intensely researched and in recent years, our group and others have revealed important roles for epigenetic alterations in HNSCC carcinogenesis (8–10). MicroRNAs (miRNAs), a class of non-protein-coding RNAs, are now recognized as critical products of the epigenome, orchestrating events ranging from organogenesis to immunity and they are known to be critical in the development of many diseases, including cancer (11,12). By binding to partially complementary sites in the 3′-untranslated regions of their messenger RNA targets, miRNAs interfere with messenger RNA translation or cause messenger RNA degradation thereby repressing gene expression posttranscriptionally (13).

Dysregulation of miRNAs in cancer has been shown to associate with various tumor characteristics and prognosis in a variety of tumor types (14–17). Though aberrations in miRNA expression in primary HNSCC tumors have recently been defined in several reports (18–21), little is known about how these differences associate with clinical features and disease risk factors. *In vitro* studies and animal models have suggested that the expression of miRNAs is altered in response to various toxicant exposures (22–24). Determining such associations in human populations may be vital in better understanding the molecular mechanism through which exposures and the environment contribute to head and neck carcinogenesis. We have studied the expression of four miRNAs, previously found to be differentially expressed in HNSCC tumors compared with normal tissues (18), and examined associations with clinicopathological features of tumors to determine if these miRNA alterations are useful as prognostic biomarkers. Likewise, we investigated associations of expression of these miRNAs with patient carcinogen exposure in order to better understand if these exposures act via alterations to miRNA.

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; miRNA, microRNA.

Materials and methods

Study population

The cases examined in this analysis were drawn from a population-based case–control study of incident HNSCC, which has been previously described (25,26). Briefly, incident cases of histologically confirmed HNSCC were identified from nine medical facilities in the Boston, MA metropolitan area from 1999 to 2003. All participants provided written informed consent following institutional review board approval at participating hospitals. Questionnaires were administered to collect data on smoking, alcohol use, diet, family history, medication history and demographics. The 169 HNSCC tumor samples used in these analyses represent the fresh-frozen tumor tissue that was available as part of this study collection. A study pathologist confirmed >75% tumor content in each of the HNSCC samples used in these analyses. HPV-16 DNA status was previously determined (27).

RNA isolation

Total RNA was isolated from tumors using the mirVANA RNA Isolation Kit (Ambion, Austin, TX) 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 miRNA of *miR-21*, *miR-18a*, *miR-375* and *miR-218*. Complementary DNA was synthesized by priming with a pool of gene-specific looped primers including the primers of the miRNAs of interest and RNU48, a universally expressed endogenous control (Applied Biosystems). A 10 μl aliquot of total RNA diluted to a final concentration of 5 ng/μl was used for each reverse transcription reaction along with other reverse transcription components, per manufacturer's specifications. Forty 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. All real-time polymerase chain reaction data were quantified by calculating fold change using the ΔΔCT method, normalizing miRNA expression of cases to expression data from a pooled set of non-diseased head and neck epithelium samples.

Statistical analysis

All analyses were carried out using SAS 9.1 (SAS Institute, Cary, NC). Fold-change expression values for all miRNAs were log-transformed to create a normal distribution for parametric analysis. Categories of smoking and drinking were created with never-smokers/drinkers as referents and low and high categories dichotomized at the median value for each variable. For univariate analyses, student's *t*-tests or one-way analysis of variance were used for discrete variables of two or greater than two categories, respectively, except in the case of tumor site, where *t*-tests were conducted to compare pharyngeal versus oral and laryngeal versus oral tumors. Spearman's rank correlation was used for continuous variables. Linear regression analysis was used for multivariate testing for associations with exposures and clinicopathological features. Using the Kaplan–Meier method and the logrank test to determine significance, overall 5 year survival rates were compared across strata of miRNA expression using log-transformed miRNA expression values stratified on the top 25th percentile of miRNA expression. A multivariate Cox proportional hazards regression analysis was used to confirm predictors of case fatality. *P*-values of <0.05 were considered significant. For best parsimony in multivariate models, all variables were initially included in models but non-significant variables were removed if their removal did not result in >15% change in the effect estimates of other variables (i.e. the variables were not considered confounders). Models include only subjects with complete data for all variables within the model.

Results

Quantification of miRNA expression

A subset of 169 patients for which fresh-frozen tumor was available and covariate data were well annotated was used for this study (Table I). The population consisted of patients with a mean age of 61.5 ± 11.9, 68% of which were males. Greater than half of the participants had smoked ≥36.75 pack-years and drank ≥18 alcoholic drinks per week (51.1 and 51.8%, respectively). The majority of the patients had oral tumors, high-stage disease and were HPV-16 negative (64, 72 and 90% respectively).

Four miRNAs were selected for analysis based on the results from our previous study identifying these miRNAs as differentially expressed between HNSCC tumor and normal head and neck epithelia.

Table I. Patient demographics and clinicopathological characteristics ($N = 169$)

	n (%)
Gender	
Female	54 (32.0)
Male	115 (68.0)
Age, mean (SD)	61.5 (11.9)
Lifetime pack-years smoked[a]	
None (0)	22 (15.5)
>0–36.75	59 (41.5)
≥36.75	61 (43.0)
Drinks per week[b]	
None (0)	16 (11.5)
>0–18	61 (43.9)
≥18	62 (44.6)
Tumor site[c]	
Oral	94 (64.0)
Pharyngeal	31 (21.1)
Laryngeal	22 (15.0)
Stage[d]	
Low (I, II)	46 (28.0)
High (III, IV)	118 (72.0)
HPV-16 tumor DNA status[e]	
Negative	90 (82.6)
Positive	19 (17.4)

[a]Data missing in 27 samples.
[b]Data missing in 30 samples.
[c]Data missing in 22 samples.
[d]Data missing in five samples.
[e]Data missing in 60 samples.

Quantitative real-time polymerase chain reaction was performed on 169 HNSCC tumors and an expression value of each miRNA was determined by normalizing its expression to a pool of non-diseased samples. The average log-transformed expression values for each of the four miRNAs evaluated are listed in supplementary Table I (available at *Carcinogenesis* Online).

miR-375 expression is associated with tumor site, stage and alcohol consumption

In a univariate comparison, *miR-375* was expressed at a significantly greater level in laryngeal tumors compared with those of the oral cavity ($P = 0.004$; Table II). Tobacco smoking [measured as pack-years smoked, duration (years smoked) or intensity (packs per day)] and HPV status were not associated with *miR-375* or any other miRNA's expression. However, univariate analyses showed that the expression of *miR-375* increased significantly with alcohol consumption ($P = 0.014$; Table II). To control for potential confounders, we employed a multivariate linear regression analysis, which demonstrated that both categories of drinkers, >0–18 and ≥18 drinks per week, were associated with higher *miR-375* expression compared with non-drinkers ($P = 0.039$ and $P = 0.002$, respectively; Table III). A trend test of *miR-375* expression with increasing alcohol consumption was shown to be significant ($P = 0.002$, Table III).

Univariate analysis also showed *miR-375* expression to be significantly higher in tumors of laryngeal origin compared with those of the oral cavity. In a model controlling for age, gender and tobacco smoking, *miR-375* expression was significantly increased in both pharyngeal and laryngeal tumors compared with oral tumors ($P = 0.049$ and $P = 0.005$, respectively; Table III).

miR-21 expression is associated with poorer patient survival

An unadjusted, Kaplan–Meier survival analysis demonstrated that patients with *miR-21* expression in the highest quartile showed a trend toward worse survival than those with lower *miR-21* expression (Figure 1). In a multivariate Cox proportional hazards model controlling for age, gender and tumor stage, high expression of *miR-21* was shown to be associated with significantly decreased 5 year survival of patients (hazard ratio = 1.68; 95% CI: 1.04–2.77; $P = 0.034$; Table IV).

Discussion

The potential utility in assessing miRNA expression as a useful biomarker in cancer diagnostics, prognostics and therapeutics is becoming increasingly apparent. Many studies have reported significant associations between miRNA profiles and important clinical features of tumors as well as patient survival (14,17,28–30). Here, we analyzed miRNA expression in HNSCC tumors and found a significant correlation with alcohol consumption, a major HNSCC risk factor, as well as associations with tumor characteristics and overall patient survival.

Previously, four miRNAs were validated as significantly differentially expressed between primary HNSCC tumors and analogous normal tissue (18). We hypothesize that these miRNAs are probably to play a role in HNSCC carcinogenesis and that analysis of a large set of tumors would reveal associations between expression of these miRNAs and various clinicopathological and exposure variables amongst tumors. The miRNAs investigated in this study have all been previously implicated in various cancers, yet their functions are largely unknown. Though *miR-18a* and *miR-221* were not found to associate with any covariates in this study, there is good reason to believe that they play a role in HNSCC carcinogenesis. *miR-18a* is a member of the miR-17-92 cluster, found to be overexpressed in gastric, colorectal and ovarian cancers, and is thought to inhibit expression of estrogen receptor-α in hepatocellular carcinoma (31–34). *miR-221* expression is also increased in many cancers and its inhibition, along with that of *miR-21*, has been shown to induce cell cycle arrest and apoptosis as well as sensitizing cells to chemotherapeutic agents (35,36).

miR-21 is one of the best-studied miRNAs with a clear role in carcinogenesis and has several confirmed targets (37,38). Though *miR-375*

Table II. Univariate analysis of miRNA expression with etiological factors and clinicopathological characteristics[a]

	n (%)	Log miR-21		Log miR-375		Log miR-18a		Log miR-221	
		Mean exp (SD)	*P*	Mean exp (SD)	*P*	Mean exp (SD)	*P*	Mean exp (SD)	*P*
Gender									
Female	54 (32.0)	1.76 (1.37)		−2.49 (2.04)		−0.2 (1.14)		1.5 (1.49)	
Male	115 (68.0)	1.64 (1.47)	0.61	−2.20 (2.14)	0.41	−0.33 (0.94)	0.41	1.31 (1.40)	0.42
Age, mean (SD)	61.5 (11.9)	1.68 (1.44)	0.21	−2.3 (2.10)	0.63	−0.29 (1.01)	0.29	1.37 (1.42)	0.12
Lifetime pack-years smoked									
None (0)	22 (15.5)	1.54 (1.69)		−2.47 (2.1)		−0.20 (1.3)		1.66 (1.80)	
>0–36.75	59 (41.5)	1.63 (1.40)		−2.67 (2.07)		−0.20 (0.87)		1.35 (1.29)	
≥36.75	61 (43.0)	1.64 (1.28)	0.96	−1.98 (2.27)	0.21	−0.44 (1.00)	0.37	1.28 (1.39)	0.55
Drinks per week									
None (0)	16 (11.5)	1.62 (1.27)		−3.45 (1.56)		−0.21 (1.00)		1.51 (1.16)	
>0–18	61 (43.9)	1.59 (1.47)		−2.53 (2.38)		−0.26 (1.09)		1.21 (1.62)	
≥18	62 (44.6)	1.68 (1.39)	0.94	−1.81 (1.93)	0.014	−0.32 (0.95)	0.9	1.51 (1.31)	0.49
Tumor site[b]									
Oral	94 (64.0)	1.62 (1.49)		−2.58 (2.06)		−0.32 (1.11)		1.37 (1.54)	
Pharyngeal	31 (21.1)	1.46 (1.36)	0.11	−1.78 (2.52)	0.12	−0.36 (0.82)	0.81	1.14 (1.25)	0.41
Laryngeal	22 (15.0)	2.18 (1.39)	0.56	−1.23 (1.71)	0.004	−0.0006 (0.91)	0.17	1.71 (1.38)	0.31
Stage									
Low (I, II)	46 (28.0)	1.74 (1.36)		−2.46 (1.98)		−0.42 (1.23)		1.02 (1.3)	
High (III, IV)	118 (72.0)	1.60 (1.47)	0.58	−2.36 (2.11)	0.77	−0.38 (0.98)	0.78	1.15 (1.24)	0.31
HPV status									
Negative	90 (82.6)	1.73 (1.33)		−2.27 (2.16)		−0.26 (1.02)		1.53 (1.29)	
Positive	19 (17.4)	1.45 (1.62)	0.43	−2.42 (2.11)	0.79	−0.32 (1.00)	0.9	1.28 (1.48)	0.61

[a]Expression values represent log-transformed values obtained by using the ΔΔCT method to normalize data to a pooled referent of non-diseased head and neck epithelium samples.
[b]*t*-Test results for tumor site compared pharyngeal versus oral and laryngeal versus oral.

Table III. Correlation of *miR-375* expression with etiological factors and tumor site in a multivariate linear regression model (Total $N = 133$)

	n (%)	Reg. coeff.	*P*
Gender			
Female	42 (31.6)	Reference	
Male	91 (68.4)	−0.41	0.33
Age, mean (SD)	61.5 (11.3)	−0.004	0.806
Pack-years smoked			
None (0)	22 (16.5)	Reference	
<36.75	43 (32.3)	−0.87	0.129
≥36.75	68 (51.1)	−0.5	0.375
Drinks per week[a]			
None (0)	13 (9.8)	Reference	
<18	50 (37.6)	1.3	**0.039**
≥18	70 (52.6)	2.26	**0.002**
Tumor site			
Oral	83 (62.4)	Reference	
Pharyngeal	31 (23.3)	0.89	**0.049**
Laryngeal	19 (14.3)	1.7	**0.005**

Reg. coeff., Regression coefficient.
Bold figures represent *P*-values <0.05 in variables of interest.
[a]Trend test $P = 0.002$.

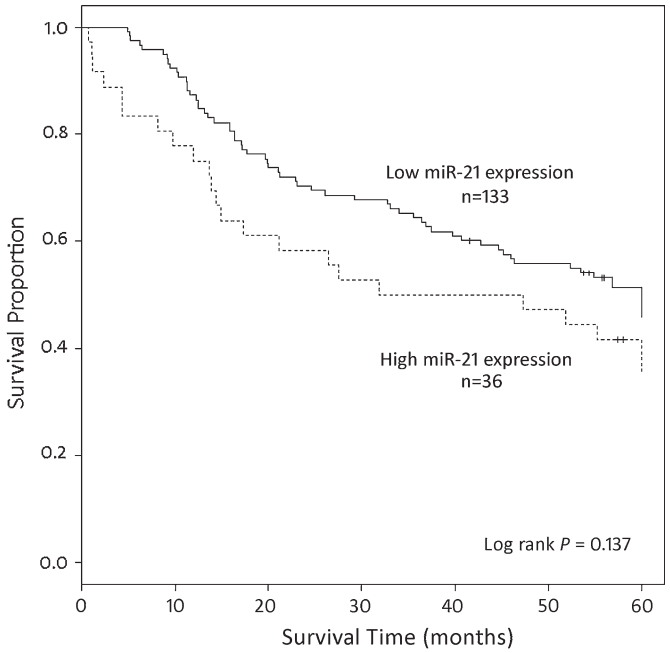

Fig. 1. Kaplan–Meier curves show differences in survival between patients with *miR*-21 expression in the highest 25th percentile (dotted line) compared with all other patients (solid line). Vertical hatch marks represent censored data.

has mainly been studied in the context of diabetes, as it influences beta-cell mass and insulin levels (39,40), its expression has been shown to be decreased in a number of malignancies including pancreatic adenocarcinomas and esophageal squamous cell and adenocarcinomas (41,42). Additionally, the recent identification of a target for *miR-375*, phosphoinositide-dependent protein kinase-1, suggests a feasible role for *miR-375* as a tumor suppressor since phosphoinositide-dependent protein kinase-1 is crucial for the activation of anti-apoptotic *AKT* (43).

Alcohol consumption has been associated with altered miRNA expression in hepatocellular tumors and alcohol exposure has been shown to affect miRNA levels in rat neurons and fetal mouse brains (44–46). We found that *miR-375* expression changed with alcohol consumption independent from tobacco smoking, showing a significant trend for higher expression of *miR-375* with increasing alcohol consumption. While it is known that alcohol is an independent risk factor for HNSCC, the mechanism for this association is poorly understood. Though alcohol itself is not directly carcinogenic, it can act as an irritant and inflammation is a well-known contributor to carcinogenesis (47). The oxidation of ethanol in the saliva by mucosal and microbial alcohol dehydrogenases results in the production of

Table IV. Cox proportional hazards multivariate models ($N = 150$)[a]

	n (%)	Hazard ratio	95% CI		*P*
			Lower	Upper	
Gender					
Female	49 (32.7)	1.0 (reference)			
Male	101 (67.3)	1.24	0.76	2.01	0.388
Age, mean (SD)		1.03	1.02	1.05	<0.001
Log miR-21 expression					
<2.58 ($n = 117$)	117	1.0 (reference)			
≥2.58 ($n = 33$)	33	1.68	1.04	2.77	**0.034**
Tumor stage					
I, II ($n = 43$)	43	1.0 (reference)			
III, IV ($n = 107$)	107	1.56	0.94	2.6	0.096

Bold figures represent *P*-values <0.05 in variables of interest.
[a]Survival data missing in 15 samples.

acetaldehyde, which is a known carcinogen in animals and possible carcinogen in humans (48,49). Additionally, it is well established that alcohol interferes with the absorption of folate, a methyl-donor critical for maintenance of normal DNA methylation patterns (50). Though expression of *miR-375* is lower overall in tumors compared with normal tissues, fine-tuning of miRNA expression can occur at the level of the tumor microenvironment and can vary according to exposures and location of the tumor. In this case, alcohol consumption could contribute to altered expression of *miR-375* within HNSCC tumors. The regulation of miRNAs is complex and perturbations of the normal homeostatic mechanisms responsible for overall epigenetic stability could play a crucial role in potentially carcinogenic gene expression.

Higher expression of *miR-375* was also found in pharyngeal and laryngeal tumors compared with tumors of the oral cavity. This observation is consistent with findings indicating that miRNA profiles are tumor and cell-type specific and can even precisely differentiate tumor subtypes (51,52). Moreover, the proclivity for differential expression of *miR-375* in tissues might reflect etiology. The significant association observed between drinking and *miR-375* expression coupled with its tendency for higher expression in pharyngeal and laryngeal tumors may suggest that the dysregulation of miRNA by exposures occurs preferentially in certain tissues.

Though the univariate logrank test for survival was not significant, as it did not consider explanatory factors such as stage and was limited in power by the sample size, an unadjusted Kaplan–Meier curve showed a clear trend for worse survival in patients with high *miR-21* expression. A multivariate model controlling for confounding factors identified a significant association between high *miR-21* expression in tumors and poor patient survival, the same relationship that has been demonstrated in cancers of the breast and colon as well as in non-small-cell lung cancer (15,16,53). Several targets of *miR-21* have been experimentally validated, many of which are tumor suppressor genes (54–56). One of these targets, programmed cell death 4 is known to be down regulated in HNSCC and in two recent studies of miRNA profiles in HNSCC, its expression was shown to be inversely related to *miR-21* in tumors (20,21). Another important target that shows reduced expression in HNSCC is *PTEN*, a gene whose product inhibits growth and cell survival through antagonism of the AKT/PI3K pathway (57). Thus, it is probable that *miR-21* functions through several targets to contribute to HNSCC malignancy, thereby modifying risk associated with the disease.

Our results suggest that miRNAs, such as *miR-375*, may modulate the carcinogenic response associated with exposure to risk factors for the disease. Further, this modulation may be differentially regulated in tumors depending on the tissue, as the expression of *miR-375* was shown to differ amongst tumor site. More in-depth study of *miR-375* may prove invaluable for our understanding of how exposures modify risk or progression of HNSCC. Additionally, high *miR-21* expression correlated with poor prognosis in HNSCC patients. As *miR-21* seems to be a significant indicator of prognosis for this and other cancers, it should be considered as a potential therapeutic target for these diseases. Our results suggest there may be significant prognostic utility in examining these specific miRNA expression signatures.

Supplementary material

Supplementary Table I can be found at http://carcin.oxfordjournals.org/

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