A role for microRNAs in the etiology of head and neck squamous cell carcinoma

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The sum of the work presented in this Ph.D. thesis, including experiments, analysis, and discussion as presented herein, have been executed by me. This work is the product of critical collaborations which have been acknowledged appropriately in Chapters 2 and 3.

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Table of Contents

Page

List of Tables

Chapter 1

Chapter 2

Chapter 3

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abstract of A Role for MicroRNAs in the Etiology of Head and Neck Squamous Cell Carcinoma, by Michele Avissar-Whiting, Ph.D., Brown University, May 2010

Head and neck squamous cell carcinomas form a heterogeneous group of cancers of the upper aerodigestive tract, arising from the surface epithelium of the oral cavity, pharynx and larynx. Worldwide, there are about 650,000 new cases annually, which are predominantly associated with behavioral risk factors such as smoking and drinking as well as human papilloma virus infection. Despite advances in multimodality therapy of HNSCC, survival has remained among the worst of the major cancer types. This thesis aimed to determine the role of microRNAs (miRNAs), endogenous small non-coding RNAs, in HNSCC carcinogenesis. MiRNA profiles were determined using a microarray in primary human HNSCC samples compared to non-diseased head and neck epithelial tissues. MiRNA identified as significantly differentially expressed from these profiles were then examined for their ability to distinguish HNSCC from normal epithelia. The associations between the expression of these specific miRNA and clinical, demographic, and exposure histories were examined in a larger case series study of HNSCC. Four miRNAs were found to be significantly differentially expressed in HNSCC tumors compared to normal epithelium ($P < 0.0001$). A miRNA ratio based on *miR-221:miR-375* expression was found to distinguish disease from normal tissue with high specificity and sensitivity (0.93 and 0.92, respectively). Expression of *miR-375* was found to increase significantly with increased exposure to alcohol and was differentially expressed in tumors of laryngeal vs oral and pharyngeal origin. Additionally, high *miR-21* expression was found to associate significantly with poor survival in HNSCC patients. To better

understand the mechanism by which these miRNA elicit their observed effects on this disease we examined potential in-vitro techniques for miRNA overexpression, which suggested that transfection with synthetic mature miRNA mimics may not be an appropriate analog for upregulation of miRNA expression and that vector based systems, which allow for processing by endogenous miRNA machinery may be better suited for functional analysis of miRNA activity. This work has demonstrated that miRNA alterations are important epigenetic determinants of HNSCC carcinogenesis, can be modulated by environmental factors, and ultimately influence the molecular character of the disease

Chapter 1

Thesis Overview and Introduction

Thesis Overview

Head and neck squamous cell carcinoma (HNSCC) is the eighth most common cancer worldwide. While its incidence in the United States is relatively low, it remains a clinically challenging disease for which therapy has met with high failure rates and survival is generally poor. The scientific community has been working toward defining the molecular character of HNSCC with a new appreciation for the discrete subtypes associated with risk factors, pathogenesis and therapeutic response. The aims of this thesis were to define a microRNA (miRNA) signature of HNSCC, identify associations of miRNA aberrations with various risk factors, clinical characteristics and outcomes, and elucidate the roles of specific miRNAs in HNSCC carcinogenesis or progression. These goals were accomplished using complementary molecular epidemiologic and in-vitro approaches.

This thesis begins with a broad introduction to head and neck cancer and to the biology of miRNA, and introduces the population and systems employed in this work. Chapter 2 details the results of a miRNA microarray study and quantitative real-time PCR validation resulting in the identification of aberrantly expressed miRNAs in HNSCC and the determination of a miRNA ratio capable of accurately distinguishing normal epithelia from diseased tissue. The following chapter (Chapter 3) reports on several correlations drawn between the expression of four individual miRNAs and clinical features and exposures associated with HNSCC carcinogenesis. Finally, Chapter 4 describes the results of functional analyses carried out using transfection of HNSCC cell lines with miRNA mimics and expression constructs containing the sequence of a miRNA of interest, in order to determine the best method for examining the specific role

of miRNA in HNSCC carcinogenesis. Chapter 5 summarizes the conclusions of the previous chapters, highlighting the relevance of these findings and discussing future directions for this work.

HNSCC: presentation, diagnosis and treatment

HNSCCs are a heterogeneous class of neoplasms representing more than 90% of cancers of the head and neck (1,2). The tumors arise from the epithelial lining throughout the upper aerodigestive tract including the oral cavity, pharynx and larynx and show microscopic squamous differentiation. Oral cavity and pharyngeal cancers are composed of the following tumor sites and associated International Classification of Disease codes: base of tongue (C01), unspecified or other areas of tongue (C02), gum (C03), floor of mouth (C04), palate (C05), unspecified and other areas of mouth (C06), tonsil (C09), oropharynx (C10), pyriform sinus (C12), hypopharynx (C13), and other and poorly-defined sites on lip, or in the oral cavity and pharynx (C14) (3). Malignant neoplasms of the larynx are classified as follows: glottis (C32.0), supraglottis (C32.1), subglottis (C32.2), laryngeal cartilages (C32.3), and unspecified and other sites of the larynx (C32.9, 33) (3).

Cases can present as premalignant lesions, termed oral leukoplakias or laryngeal keratoses, which are categorized according to indices of dysplasia, keratinization, and differentiation. HNSCC patients may experience swelling, hoarseness or textural changes in the mouth or throat accompanying these lesions. However the frequency of smoking and alcohol use in these populations may cause such symptoms to be obscured by pre-existing problems attributed to the consequences of heavy smoking and alcohol

consumption (4). Several studies have indicated that patients often seek medical help only once persistent symptoms have progressed, and the tendency to seek help at all once symptoms have worsened varies across individuals (5,6). As such, only about half of patients present with early lesions, a fact that has significantly contributed to the overall poor survival associated with the disease (7).

A determination of malignancy is followed by an assessment of the primary tumor size (T), the presence of nodal metastasis (N), and the presence of distant metastasis (M). Though TNM staging is limited in its utility as a prognostic indicator, it is helpful for the determination of an appropriate therapeutic approach (8,9). While early stage disease can often be successfully addressed by single-modality radiotherapy and/or surgery, late stage metastatic disease is difficult to treat and platinum-based chemotherapy or surgeries generally serve only as palliative treatments (9).

Like many other solid tumors, HNSCC is caused by an accumulation of somatic genetic and epigenetic alterations acquired over a long latency period (1). After initiation, the disease seems to progress through a number of clinical and histopathological changes governed by specific genetic and epigenetic events including large chromosomal aberrations, gross genomic alterations, genome-wide cytosine hypomethylation and gene-promoter specific hypermethylation causing inactivation of tumor suppressor genes or activation of protooncogenes (10-13). The most common genetic alterations associated with HNSCC, as determined by meta-analysis of differentially expressed genes reported in the literature (14), have been listed in Table I.

Treatment of HNSCC is often complicated by late stage diagnosis and the frequent incidence of local recurrence and second primary tumors. These phenomena

may be explained by the presence of “field cancerization”, in which a “field” of genetically abnormal cells grows by clonal expansion to surround the tumor, effectively obscuring the boundaries of pre-neoplastic and neoplastic tissue (15). Molecular analysis of macroscopically normal surgical margins using markers such as loss of heterozygosity (16), microsatellite alterations and *TP53* mutations have become an important consideration in HNSCC diagnosis and treatment (17).

HNSCC: epidemiology and survival

Each year approximately 650,000 people are diagnosed with HNSCC worldwide, making it the eighth most common cancer. Remarkably, the incidence in men is more than twice that in women (18). In the United States, HNSCCs represent 3.1% of all incident malignancies with 35,720 new cases and 7,600 deaths expected in 2009 (19). The overall 5-year survival rate for HNSCC is 60% (19). The significantly lower than average survival of 41% in African Americans is thought to be attributed to later stage of detection in this population, though other factors are being explored (20,21).

The fact that survival rates for HNSCC have improved only modestly over the last few decades has been attributed to several factors. HNSCCs are associated with a uniquely high incidence of second primary tumor formation, thought to be related to the phenomenon of field cancerization, in that prolonged exposure to carcinogens can lead to the simultaneous preconditioning of cells to malignant transformation (15). As a result multiple tumors, having genetic alterations identical to the original tumor, can form in distinct locations at distinct times, making patients difficult to monitor. Additionally, early metastatic spread to lymph nodes, high tumor invasiveness, and the difficulty of

determining the bounds of the neoplastic field for tumor resection, all contribute to the frustration of physicians in trying to control the disease (2).

Risk factors for HNSCC: Tobacco and Alcohol

In developed countries, 90% of HNSCC cases can be attributed to tobacco and alcohol use (22) and though these can act as independent risk factors for the disease, consumption of both produces a synergistic effect on risk (23). Cigarette smokers are at 2-10 fold increased risk and heavy smokers as high as 15-fold compared to nonsmokers (24). Smokers are particularly predisposed to cancers of the larynx, for which risk is substantially increased with tobacco smoking (24). Tobacco use in the form of cigars (25), bidis (26), and chewing tobacco (25) has also been linked to increased risk of HNSCC. There are several proposed mechanisms for tobacco-associated carcinogenesis, many of which are concerned with the carcinogenic effects of polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), and tobacco specific nitrosamines (TSNA) like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), potent carcinogens abundant in cigarette smoke (27).

Alcohol has been classified as a carcinogen in humans and chronic alcohol consumption is clearly associated with increased risk of HNSCCs, independent of tobacco smoke (28-30). While in non-smokers there is a dose-response effect of alcohol consumption on risk, exposure to both smoking and drinking confers a higher risk than either exposure alone, increasing risk in a multiplicative rather than additive manner (31). Several mechanisms of alcohol carcinogenesis have been proposed involving the effects of acetaldehyde, interactions with nutritional factors, and physical effects on tissue.

In the oral cavity, ethanol is metabolized first by alcohol dehydrogenase (ADH) of buccal microorganisms into acetaldehyde, its first breakdown product (32). Acetaldehyde is highly unstable and exerts its toxic effects by the rapid formation of free radicals, induction of chromosomal aberrations, and formation of DNA adducts (33,34). The second step of alcohol metabolism is the oxidation of acetaldehyde to acetate, catalyzed by aldehyde dehydrogenase (ALDH). Genetic variants of ALDH in some populations lead to hypoactive or inactive ALDH enzymes and subsequent inefficient conversion of alcohol to acetate and consequent potential modification of cancer risk (35).

Alcohol also has a negative effect on the absorption, bioavailability, and metabolism of folates, members of the B-class vitamins that are required for the synthesis of DNA bases and for proper methylation of macromolecules (36-38). Folate insufficiency has been shown to alter DNA methylation, producing global DNA hypomethylation with simultaneous promoter hypermethylation of specific genes (39-41). For example in HNSCC dietary folate deficiency associated with hypermethylation of tumor suppressor *p16* (42) and alteration of microRNA profiles perhaps as a consequence of methylation changes (43).

The physical properties of alcohol and their effects on tissues have particularly important consequences for cancers of the upper aerodigestive tract like HNSCC. It has been proposed that alcohol's solvent properties can exacerbate the hazards of other carcinogens such as those in tobacco smoke. Though this has not been demonstrated experimentally, it may contribute to the synergy observed with these two risk factors. Local exposure to alcohol may cause membrane and cell injury inducing cellular

hyperproliferation, which may also increase vulnerability to carcinogens (44). Importantly, alcohol intake is often accompanied by poor oral health, another known HNSCC risk factor.

Risk factors for HNSCC: HPV

The increasing risk with intensity and duration of smoking as well as the synergistic interaction with alcohol consumption justify the long standing consideration of this cancer as a disease of "old men"(45,46). In recent years, though, the demographics of the disease have shifted to include non-smoking younger men and women, indicating a novel etiologic factor.

Human papillomavirus (HPV), particularly type 16, previously recognized as the oncogenic virus responsible for the majority of cervical cancers, has emerged as the cause of a distinct form of HNSCC, generally occurring in the oral cavity and oropharynx (47). HPV is a circular DNA virus that can exist as an episome or incorporate into the host genome (48). Host genome integration leads to disruption of the E1 and E2 gene sequences and overexpression of oncogenic E6 and E7 proteins which inactivate tumor suppressors p53 and pRB, respectively.

The clinical and molecular characteristics of HPV-associated HNSCCs are distinct from those with alcohol and tobacco-associated etiologies as they do not display the same genetic and epigenetic alterations characteristic of HPV-negative tumors (49). Interestingly HPV-positive HNSCC patients respond more favorably to treatment with cisplatin and radiation and display overall improved survival compared to their HPV-

negative counterparts (50,51), a fact that may be attributed to different epigenetic profiles in this subset (52).

Risk factors for HNSCC: Other Exposures

It was previously mentioned that dietary folate deficiency modifies risk for HNSCC. Other dietary factors have been shown to play a role in HNSCC etiology. Most notably, an inverse correlation of fruit and vegetable intake and HNSCC risk has been consistently demonstrated (53,54), though there is evidence to show that this risk is further modified by HPV (55). There are many studies that show increased risk of HNSCC with meat consumption (56-58) and recently, it has been suggested that this association also exists with high intake of dairy and animal fat (59).

In a study of HPV-16 positive and negative individuals, several measures of poor oral health and hygiene were found to associate significantly with risk of HNSCC. Patients who reported never attending dental visits and those who used mouthwash more than once daily both showed increased risk of oral cancers compared to those who had regular dental visits or never used mouthwash (60). Less than daily tooth-brushing and sores due to ill-fitting denture use were both associated with increased risk of oral and pharyngeal cancers (61).

Though less significant overall, other exposures have been found to be direct etiological factors or modify risk for HNSCC (2). Increased rates of head and neck cancers, particularly laryngeal cancers, have been observed in other industrial settings including occupational exposure to mustard gas (62,63), hair dye (64), nickel (65,66), wood dust (67,68), rubber products (69), gasoline fumes (68), organic solvents (70),

mineral oil (71), formaldehyde (67,70) and coal dust (67) although many of these studies due not control for appropriate confounders. Though there is considerable data supporting an association between laryngeal cancer and asbestos exposure (72-75), a causal relationship remains controversial. Marijuana was suspected to be a risk factor for HNSCC for some time. While several groups demonstrated a positive correlation (76,77), much of the data on the topic is inconsistent and a recent population-based case control study on the topic even demonstrated that marijuana use may be associated with reduced risk for the disease (77).

Risk factors for HNSCC: Familial Aggregation

Patients with HNSCC are likely to have other family members with the disease (78-80). While this observation may be due to similarities in high-risk behaviors, such as smoking and drinking, amongst families, it may also be caused by genetic susceptibility. Since not all people who consume tobacco or alcohol will develop head and neck cancer, it is likely that genetic variables play a role in predisposition for the disease. For example, some research has been done with the objective of clarifying the contribution of genetic predisposition to alcohol and tobacco addiction and alcoholism to the pattern of familial aggregates seen in this disease (81,82).

Inherited genotypes involving alcohol and tobacco carcinogen metabolism, as described above in the example of ALDH mutations, have been shown to modify risk of HNSCC. Deletion mutations in glutathione S-transferase genes, whose products facilitate the detoxification of cigarette smoke carcinogens such as B[a]P, are associated with a higher risk of HNSCC (83). Similarly, alterations in genes associated with DNA repair

pathways have also been implicated in HNSCC susceptibility. A positive association was found with a variant genotype of the base excision repair gene *hOGG1* (84) and both positive and negative associations have been seen with variants of a gene in the same family, *XRCC1* (85,86). Additionally, several polymorphisms in genes coding for cytochrome P450 (CYP) enzymes and N-Acetyltransferases (NATs), major xenobiotic-metabolizers, have also been identified and associate with increased risk for HNSCC, as reviewed by Ho et al (87).

A well studied example of genetic predisposition to HNSCC is that of Fanconi Anemia (FA), a rare autosomal recessive disease characterized by mutations in one or more of the FA complementation groups (88). Since FA proteins interact with the DNA damage repair-associated BRCA pathway, FA mutations lead to increased cellular sensitivity to DNA damaging agents (89). The reason for the specific association of FA with HNSCCs is still poorly understood.

Epigenetics

The field of epigenetics is concerned with heritable control of the genome that does not involve changes to the underlying DNA sequence (90). Growing interest in the field has been aided by technological advancements in molecular biology and genomics and the development of assays that allow for detection of DNA methylation, histone modifications, and small RNAs— the three major modes of epigenetic regulation. Epigenetic processes are crucial for the transcriptional control of individual genes as well as for the establishment and maintenance of stability in the genome overall but when dysregulated, they can lead to major adverse health effects.

Both DNA methylation and histone acetylation involve modification of the chromatin structure resulting in dynamic control over transcriptionally active and inactive areas of the genome (90,91). DNA methylation occurs in the cytosines preceding guanines (CpG)s, which occur at less than expected frequency in the genome overall but are enriched in gene promoters. Enrichment of CpGs in regulatory regions allows for transcriptional control by methylation, which leads to the recruitment of chromatin remodeling proteins that act to block transcription. Histone modification works in concert with DNA methylation to regulate transcription through various post-translational modifications on histones which alter their affinity for DNA, thereby forming either compact inactive chromatin or relaxed transcriptionally active chromatin. While these mechanisms act at the level of transcription, small regulatory RNA molecules act at the level of translation. Several of these small noncoding RNA species have been identified including microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA) (92). Though they arise by different mechanisms and function in slightly different ways, their regulatory capacity involves interaction with proteins in the Argonaute superfamily to inhibit the translation of protein-coding mRNA.

In cancer, epigenetic dysregulation accounts for one-third to one-half of all known genetic alterations (93). One of the first of these alterations to be identified was the phenomenon of DNA methylation loss in tumors compared to normal tissues (94). This hypomethylation is seen in repetitive DNA sequences and introns, creating a state of genomic instability characterized by mitotic recombination, deletions and translocations, chromosomal rearrangements, and activation of transposons such as LINE and SINE

elements (95). In addition, hypermethylation at gene promoter of tumor suppressor genes are extremely common in cancer and can characterize cancer types (96,97).

MicroRNA

MiRNAs comprise a class of endogenous small non-coding RNAs that have only been discovered within the last couple of decades. Since their discovery, a great deal has been learned about their biogenesis, mode of action in the genome, and their involvement in both normal and pathological processes. The mature miRNA, a 21-24 bp duplex, is processed step-wise from a primary transcript of 100s-1000s of nucleotides in length (98). Greater than half of these primary miRNA transcripts (pri-miRNA) are found within introns of protein-coding genes or non-coding genes, while others exist as monocistronic or polycistronic units in the exons of long nonprotein-coding transcripts (99,100). MiRNA transcripts found in intergenic regions are thought to have their own promoters and like intronic miRNAs, are likely transcribed by RNA Polymerase II (101,102). There is evidence that a subset of miRNAs hidden in Alu repeat regions in the genome are transcribed by RNA Polymerase III (103).

Once transcribed, pri-miRNAs form long hairpin structures containing multiple imperfectly paired stems and are post-transcriptionally modified by addition of a 5' m^7G cap and 3' poly-(A) tail (101). Pri-miRNA processing is catalyzed in two steps by the RNase II type endonucleases Drosha and Dicer, classically involved in the RNA interference pathway (Fig. 1). Drosha, in complex with pasha (DGCR8), processes the pri-miRNA into an imperfect stem-loop of ~70 nt, the premature miRNA (pre-miRNA), in the nucleus (104,105). After being transported to the cytoplasm by Exportin5, a

RanGTP-dependent dsRNA-binding protein, pre-miRNAs are further cleaved by Dicer and its cofactor TAR-RNA binding protein (TRBP) into a ~22-bp miRNA molecule (105,106). The active strand of this mature duplex binds the RNA-induced silencing complex (RISC) and guides it to the 3' untranslated region (UTR) of the target messenger RNA while the complementary strand (*) is usually rapidly degraded (98,107,108).

The mode of mRNA interference by miRNAs is determined by the degree of complementarity of miRNA and target (105). Complete complementarity of miRNA to target is exceedingly rare in metazoans and results in endonucleolytic cleavage of target mRNA. As most miRNAs show only imperfect complementarity to their target message, they will usually function by translational repression. Though homology of the miRNA seed sequence, a 5-7 nt sequence at the 5' end, to the 3' UTR on the target mRNA is a major determinant of stability, other factors are also influential and sometimes necessary for effective repression (109,110). As the flexibility in targeting allows for a huge range of regulatory potential, miRNAs are thought to mediate post-transcriptional silencing of at least 30% of protein coding genes (111).

There is evidence to support translational repression as interfering with various steps in translational initiation, ribosomal subunit assembly, or post-initiation steps (112-114). It seems clear however that a 7-methylguanosine (m^7G) terminal cap, a structure that is recognized by eukaryotic translational initiation factors (eIFs) prior to assembly of the ribosome initiation complex, is necessary for translational repression to occur. Accumulating data has suggested that after miRNA silencing, mRNAs are sequestered in cytoplasmic domains called processing bodies (P-bodies), which contain mRNA deadenylases and enzymes involved in mRNA decapping and degradation (115,116).

Though this would seem to suggest that P-body localization leads to mRNA destruction, some work has indicated that inactive mRNAs are instead stored in P-bodies for a period, only to return to the cytoplasm for translation (117).

MicroRNA aberrations in human cancer

Given that each miRNA has the potential to target a large number of mRNAs, any variations in miRNA expression level, disturbances in the processing of its precursors, or changes in the target sequence may deregulate normal cell processes (118). MiRNA expression can be altered by the same mechanisms that affect gene expression, including gross genomic alterations, epigenetic changes and viral element insertions. Such miRNA aberrations have now been noted as being directly involved in the initiation or progression of many human cancers. The first connection between miRNAs and cancer was linked to the discovery of two miRNAs, *miR-15* and *miR-16*, normally located in the human chromosomal region 13q14, which were found to be deleted in more than half of chronic lymphocytic leukemia cases (119). Subsequent studies determined an inverse correlation between the levels of both *miR-15* and *miR-16*, and anti-apoptotic Bcl2 expression, indicating that these miRNAs target *BCL2* mRNA for degradation ultimately inducing apoptosis (120).

Recently, numerous similar associations were made between genes involved in cell cycle and cell survival and the miRNAs that regulate them, dubbed “oncomiRs” and “tumor suppressor miRs”(121). OncomiRs are miRNAs that are generally upregulated in cancer and whose overexpression is either associated with anti-apoptotic or proliferative activity by targeting tumor suppressor genes for degradation. Alternatively, tumor

suppressor-miRs are often downregulated in cancer and tend to target oncogenes for degradation, leading to decreased proliferation and increased apoptotic activity. Due to the broad range of targets for any single miRNA, it is conceivable that some miRNA function as either oncomiRs or tumor suppressor miRs depending on the cellular context (118).

The development of miRNA-specific microarrays has allowed for high throughput analysis of miRNAs in different tumors and has facilitated the characterization of global miRNA profiles in cancer that may lead to more effective diagnostic and prognostic tools. Further, the direct regulatory activity of miRNAs may make their expression profiles more valuable as biomarkers than conventional mRNA expression profiles. MiRNA signatures of disease have now been defined in a variety of cancers including breast (122), stomach (123), ovarian (124), liver (125) and lung (126) and have been shown to differ across cancer types (127,128). Additionally, miRNA expression profiles have been shown to distinguish not only different tumor types but also stages of differentiation within a tumor type (122,129), consistent with the role miRNAs play in normal processes of cell differentiation and development (130).

MicroRNA and exposures

Though there is a clear link between the alteration of miRNA expression and the development of cancer or other disease states, the cause of these alterations is still not clear. Several studies have been performed exploring the potential of disease-associated exposures in modulating miRNA expression. *In vitro* experiments have shown miRNA profiles to be altered in human lymphoblast cell lines following arsenic exposure and

folate deficiency (43). Also, exposure of breast epithelial cells to the xenoestrogen diethylstilbestrol was shown to induce changes in a set of miRNAs, at least one of which had been repressed via DNA methylation at its promoter (131). Work in mice has demonstrated that treatment with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) leads to aberrant expression of miRNAs in the brain and liver (132) and that exposure to alcohol can alter miRNA levels in rat neurons and fetal mouse brains (133,134). Additionally, molecular epidemiology studies have shown that miRNA expression of exposed tissues varies according to smoking status and alcohol consumption (125,135).

MicroRNA in Head and Neck Cancer

There are a small number of studies investigating a role of miRNA in HNSCC. One of the first was a miRNA microarray performed on nine individual HNSCC cell lines (136). The expression of 33 miRNAs was determined to be high, and 22 low relative to the expression of other miRNAs. Notably, one of the highly expressed miRNAs was *miR-21*, a miRNA commonly upregulated in cancer (137-139). Another study involving microarray of four normal mucosa, four primary HNSCC tissues and four HNSCC cell lines revealed differential expression of nine miRNAs, including overexpression of *miR-21*, in tumor tissue (140). Additionally, this study found that *miR-21* overexpression in HNSCC cells induced increased proliferation and that inhibition of *miR-21* increased apoptosis and cytochrome c release. A third study also sought to determine miRNA profiles of HNSCC, concluding that combined expression of miRNAs *let-7d* and *miR-205* was a predictor of prognosis. Interestingly, though this study also found *miR-21* to be expressed at consistently higher levels in HNSCC tumors compared to normal tissues,

one of the findings on which its conclusion is based, namely that *miR-205* is down-regulated in tumor, contradicts with the previous miRNA study which found *miR-205* to be significantly overexpressed.

References

1. Harrison, L.B., Sessions, R.B. and Hong, W.K. (2004) *Head and neck cancer : a multidisciplinary approach*. Lippincott Williams & Wilkins, Philadelphia.
2. Pai, S.I. and Westra, W.H. (2009) Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. *Annu Rev Pathol*, **4**, 49-70.
3. Fritz, A.G. (2000) *International classification of diseases for oncology : ICD-O*. World Health Organization, Geneva.
4. Brouha, X., Tromp, D., Hordijk, G.J., Winnubst, J. and De Leeuw, R. (2005) Role of alcohol and smoking in diagnostic delay of head and neck cancer patients. *Acta Otolaryngol*, **125**, 552-6.
5. Brouha, X.D., Tromp, D.M., Koole, R., Hordijk, G.J., Winnubst, J.A. and de Leeuw, J.R. (2007) Professional delay in head and neck cancer patients: analysis of the diagnostic pathway. *Oral Oncol*, **43**, 551-6.
6. Dolan, R.W., Vaughan, C.W. and Fuleihan, N. (1998) Symptoms in early head and neck cancer: an inadequate indicator. *Otolaryngol Head Neck Surg*, **119**, 463-7.
7. Scott, S.E., Grunfeld, E.A. and McGurk, M. (2005) The idiosyncratic relationship between diagnostic delay and stage of oral squamous cell carcinoma. *Oral Oncol*, **41**, 396-403.
8. Salesiotis, A.N. and Cullen, K.J. (2000) Molecular markers predictive of response and prognosis in the patient with advanced squamous cell carcinoma of the head and neck: evolution of a model beyond TNM staging. *Curr Opin Oncol*, **12**, 229-39.

9. Gold, K.A., Lee, H.Y. and Kim, E.S. (2009) Targeted therapies in squamous cell carcinoma of the head and neck. *Cancer*, **115**, 922-35.

10. Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S., Corio, R., Lee, D., Greenberg, B., Koch, W. and Sidransky, D. (1996) Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res*, **56**, 2488-92.

11. Carey, T.E., Van Dyke, D.L. and Worsham, M.J. (1993) Nonrandom chromosome aberrations and clonal populations in head and neck cancer. *Anticancer Res*, **13**, 2561-7.

12. Speicher, M.R., Howe, C., Crotty, P., du Manoir, S., Costa, J. and Ward, D.C. (1995) Comparative genomic hybridization detects novel deletions and amplifications in head and neck squamous cell carcinomas. *Cancer Res*, **55**, 1010-3.

13. Hasegawa, M., Nelson, H.H., Peters, E., Ringstrom, E., Posner, M. and Kelsey, K.T. (2002) Patterns of gene promoter methylation in squamous cell cancer of the head and neck. *Oncogene*, **21**, 4231-6.

14. Slaughter, D.P., Southwick, H.W. and Smejkal, W. (1953) Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*, **6**, 963-8.

15. Chen, C., Mendez, E., Houck, J., Fan, W., Lohavanichbutr, P., Doody, D., Yueh, B., Futran, N.D., Upton, M., Farwell, D.G., Schwartz, S.M. and Zhao, L.P. (2008) Gene expression profiling identifies genes predictive of oral squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 2152-62.

16. Braakhuis, B.J., Tabor, M.P., Kummer, J.A., Leemans, C.R. and Brakenhoff, R.H. (2003) A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res*, **63**, 1727-30.

17. Parkin, D.M., Bray, F., Ferlay, J. and Pisani, P. (2005) Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

18. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. and Thun, M.J. (2009) Cancer statistics, 2009. *CA Cancer J Clin*, **59**, 225-49.

19. Carroll, W.R., Kohler, C.L., Carter, V.L., Hannon, L., Skipper, J.B. and Rosenthal, E.L. (2009) Barriers to early detection and treatment of head and neck squamous cell carcinoma in African American men. *Head Neck*.

20. Settle, K., Posner, M.R., Schumaker, L.M., Tan, M., Suntharalingam, M., Goloubeva, O., Strome, S.E., Haddad, R.I., Patel, S.S., Cambell, E.V., 3rd, Sarlis, N., Lorch, J. and Cullen, K.J. (2009) Racial Survival Disparity in Head and Neck Cancer Results from Low Prevalence of Human Papillomavirus Infection in Black Oropharyngeal Cancer Patients. *Cancer Prev Res (Phila Pa)*.

21. Sankaranarayanan, R., Masuyer, E., Swaminathan, R., Ferlay, J. and Whelan, S. (1998) Head and neck cancer: a global perspective on epidemiology and prognosis. *Anticancer Res*, **18**, 4779-86.

22. Lewin, F., Norell, S.E., Johansson, H., Gustavsson, P., Wennerberg, J., Biorklund, A. and Rutqvist, L.E. (1998) Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck: a population-based case-referent study in Sweden. *Cancer*, **82**, 1367-75.

23. Schottenfeld, D. and Fraumeni, J.F. (2006) *Cancer epidemiology and prevention*. Oxford University Press, Oxford ; New York.

24. Stockwell, H.G. and Lyman, G.H. (1986) Impact of smoking and smokeless tobacco on the risk of cancer of the head and neck. *Head Neck Surg*, **9**, 104-10.

25. Sankaranarayanan, R., Duffy, S.W., Nair, M.K., Padmakumary, G. and Day, N.E. (1990) Tobacco and alcohol as risk factors in cancer of the larynx in Kerala, India. *Int J Cancer*, **45**, 879-82.

26. International Agency for Research on Cancer. and IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (1988) IARC monographs on the evaluation of carcinogenic risks to humans. IARC : Distributed for the International Agency for Research on Cancer by the Secretariat of the World Health Organization, Lyon, France, pp. v.

27. Elwood, J.M., Pearson, J.C., Skippen, D.H. and Jackson, S.M. (1984) Alcohol, smoking, social and occupational factors in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Cancer*, **34**, 603-12.

28. Maier, H., Weidauer, H., Zoller, J., Seitz, H.K., Flentje, M., Mall, G. and Born, I.A. (1994) Effect of chronic alcohol consumption on the morphology of the oral mucosa. *Alcohol Clin Exp Res*, **18**, 387-91.

29. Talamini, R., La Vecchia, C., Levi, F., Conti, E., Favero, A. and Franceschi, S. (1998) Cancer of the oral cavity and pharynx in nonsmokers who drink alcohol and in nondrinkers who smoke tobacco. *J Natl Cancer Inst*, **90**, 1901-3.

30. Maier, H., Dietz, A., Gewelke, U., Heller, W.D. and Weidauer, H. (1992) Tobacco and alcohol and the risk of head and neck cancer. *Clin Investig*, **70**, 320-7.

31. Jokelainen, K., Heikkonen, E., Roine, R., Lehtonen, H. and Salaspuro, M. (1996) Increased acetaldehyde production by mouthwashings from patients with oral cavity, laryngeal, or pharyngeal cancer. *Alcohol Clin Exp Res*, **20**, 1206-10.

32. Dellarco, V.L. (1988) A mutagenicity assessment of acetaldehyde. *Mutat Res*, **195**, 1-20.

33. Fang, J.L. and Vaca, C.E. (1997) Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers. *Carcinogenesis*, **18**, 627-32.

34. Druesne-Pecollo, N., Tehard, B., Mallet, Y., Gerber, M., Norat, T., Hercberg, S. and Latino-Martel, P. (2009) Alcohol and genetic polymorphisms: effect on risk of alcohol-related cancer. *Lancet Oncol*, **10**, 173-80.

35. Eisenga, B.H., Collins, T.D. and McMartin, K.E. (1989) Effects of acute ethanol on urinary excretion of 5-methyltetrahydrofolic acid and folate derivatives in the rat. *J Nutr*, **119**, 1498-505.

36. Halsted, C.H., Robles, E.A. and Mezey, E. (1971) Decreased jejunal uptake of labeled folic acid (3 H-PGA) in alcoholic patients: roles of alcohol and nutrition. *N Engl J Med*, **285**, 701-6.

37. Hamid, A. and Kaur, J. (2007) Long-term alcohol ingestion alters the folate-binding kinetics in intestinal brush border membrane in experimental alcoholism. *Alcohol*, **41**, 441-6.

38. Stickel, F., Choi, S.W., Kim, Y.I., Bagley, P.J., Seitz, H.K., Russell, R.M., Selhub, J. and Mason, J.B. (2000) Effect of chronic alcohol consumption on total plasma homocysteine level in rats. *Alcohol Clin Exp Res*, **24**, 259-64.

39. Rampersaud, G.C., Kauwell, G.P., Hutson, A.D., Cerda, J.J. and Bailey, L.B. (2000) Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. *Am J Clin Nutr*, **72**, 998-1003.

40. Duthie, S.J., Narayanan, S., Blum, S., Pirie, L. and Brand, G.M. (2000) Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. *Nutr Cancer*, **37**, 245-51.

41. Kraunz, K.S., Hsiung, D., McClean, M.D., Liu, M., Osanyingbemi, J., Nelson, H.H. and Kelsey, K.T. (2006) Dietary folate is associated with p16(INK4A) methylation in head and neck squamous cell carcinoma. *Int J Cancer*, **119**, 1553-7.

42. Marsit, C.J., Eddy, K. and Kelsey, K.T. (2006) MicroRNA responses to cellular stress. *Cancer Res*, **66**, 10843-8.

43. Seitz, H.K., Poschl, G. and Simanowski, U.A. (1998) Alcohol and cancer. *Recent Dev Alcohol*, **14**, 67-95.

44. Choi, S.Y. and Kahyo, H. (1991) Effect of cigarette smoking and alcohol consumption in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Epidemiol*, **20**, 878-85.

45. Macfarlane, G.J., Zheng, T., Marshall, J.R., Boffetta, P., Niu, S., Brasure, J., Merletti, F. and Boyle, P. (1995) Alcohol, tobacco, diet and the risk of oral

cancer: a pooled analysis of three case-control studies. *Eur J Cancer B Oral Oncol*, **31B**, 181-7.

46. Mork, J., Lie, A.K., Glattre, E., Hallmans, G., Jellum, E., Koskela, P., Moller, B., Pukkala, E., Schiller, J.T., Youngman, L., Lehtinen, M. and Dillner, J. (2001) Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *N Engl J Med*, **344**, 1125-31.

47. Furniss, C.S., McClean, M.D., Smith, J.F., Bryan, J., Nelson, H.H., Peters, E.S., Posner, M.R., Clark, J.R., Eisen, E.A. and Kelsey, K.T. (2007) Human papillomavirus 16 and head and neck squamous cell carcinoma. *Int J Cancer*, **120**, 2386-92.

48. Gillison, M.L. (2004) Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. *Semin Oncol*, **31**, 744-54.

49. Fakhry, C., Westra, W.H., Li, S., Cmelak, A., Ridge, J.A., Pinto, H., Forastiere, A. and Gillison, M.L. (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst*, **100**, 261-9.

50. Licitra, L., Perrone, F., Bossi, P., Suardi, S., Mariani, L., Artusi, R., Oggionni, M., Rossini, C., Cantu, G., Squadrelli, M., Quattrone, P., Locati, L.D., Bergamini, C., Olmi, P., Pierotti, M.A. and Pilotti, S. (2006) High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol*, **24**, 5630-6.

51. Furniss, C.S., Marsit, C.J., Houseman, E.A., Eddy, K. and Kelsey, K.T. (2008) Line region hypomethylation is associated with lifestyle and differs by human

papillomavirus status in head and neck squamous cell carcinomas. *Cancer Epidemiol Biomarkers Prev*, **17**, 966-71.

52. Levi, F., Pasche, C., La Vecchia, C., Lucchini, F., Franceschi, S. and Monnier, P. (1998) Food groups and risk of oral and pharyngeal cancer. *Int J Cancer*, **77**, 705-9.

53. McLaughlin, J.K., Gridley, G., Block, G., Winn, D.M., Preston-Martin, S., Schoenberg, J.B., Greenberg, R.S., Stemhagen, A., Austin, D.F., Ershow, A.G. and et al. (1988) Dietary factors in oral and pharyngeal cancer. *J Natl Cancer Inst*, **80**, 1237-43.

54. Meyer, M.S., Applebaum, K.M., Furniss, C.S., Peters, E.S., Luckett, B.G., Smith, J.F., Bryan, J., McClean, M.D., Marsit, C. and Kelsey, K.T. (2008) Human papillomavirus-16 modifies the association between fruit consumption and head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 3419-26.

55. Garrote, L.F., Herrero, R., Reyes, R.M., Vaccarella, S., Anta, J.L., Ferbeye, L., Munoz, N. and Franceschi, S. (2001) Risk factors for cancer of the oral cavity and oro-pharynx in Cuba. *Br J Cancer*, **85**, 46-54.

56. Petridou, E., Zavras, A.I., Lefatzis, D., Dessypris, N., Laskaris, G., Dokianakis, G., Segas, J., Douglas, C.W., Diehl, S.R. and Trichopoulos, D. (2002) The role of diet and specific micronutrients in the etiology of oral carcinoma. *Cancer*, **94**, 2981-8.

57. Uzcudun, A.E., Retolaza, I.R., Fernandez, P.B., Sanchez Hernandez, J.J., Grande, A.G., Garcia, A.G., Olivar, L.M., De Diego Sastre, I., Baron, M.G. and Bouzas,

J.G. (2002) Nutrition and pharyngeal cancer: results from a case-control study in Spain. *Head Neck*, **24**, 830-40.

58. Peters, E.S., Luckett, B.G., Applebaum, K.M., Marsit, C.J., McClean, M.D. and Kelsey, K.T. (2008) Dairy products, leanness, and head and neck squamous cell carcinoma. *Head Neck*, **30**, 1193-205.

59. Marques, L.A., Eluf-Neto, J., Figueiredo, R.A., Gois-Filho, J.F., Kowalski, L.P., Carvalho, M.B., Abrahao, M. and Wunsch-Filho, V. (2008) Oral health, hygiene practices and oral cancer. *Rev Saude Publica*, **42**, 471-9.

60. Velly, A.M., Franco, E.L., Schlecht, N., Pintos, J., Kowalski, L.P., Oliveira, B.V. and Curado, M.P. (1998) Relationship between dental factors and risk of upper aerodigestive tract cancer. *Oral Oncol*, **34**, 284-91.

61. Easton, D.F., Peto, J. and Doll, R. (1988) Cancers of the respiratory tract in mustard gas workers. *Br J Ind Med*, **45**, 652-9.

62. Wada, S., Miyanishi, M., Nishimoto, Y., Kambe, S. and Miller, R.W. (1968) Mustard gas as a cause of respiratory neoplasia in man. *Lancet*, **1**, 1161-3.

63. Alderson, M. (1980) Cancer mortality in male hairdressers. *J Epidemiol Community Health*, **34**, 182-5.

64. Peto, J., Cuckle, H., Doll, R., Hermon, C. and Morgan, L.G. (1984) Respiratory cancer mortality of Welsh nickel refinery workers. *IARC Sci Publ*, 37-46.

65. Sorahan, T. and Williams, S.P. (2006) Respiratory cancer in nickel carbonyl refinery workers. *Occup Environ Med*, **63**, 856; author reply 856.

66. Laforest, L., Luce, D., Goldberg, P., Begin, D., Gerin, M., Demers, P.A., Brugere, J. and Leclerc, A. (2000) Laryngeal and hypopharyngeal cancers and occupational

exposure to formaldehyde and various dusts: a case-control study in France. *Occup Environ Med*, **57**, 767-73.

67. Brown, L.M., Mason, T.J., Pickle, L.W., Stewart, P.A., Buffler, P.A., Burau, K., Ziegler, R.G. and Fraumeni, J.F., Jr. (1988) Occupational risk factors for laryngeal cancer on the Texas Gulf Coast. *Cancer Res*, **48**, 1960-4.

68. Kogevinas, M., Sala, M., Boffetta, P., Kazerouni, N., Kromhout, H. and Hoar-Zahm, S. (1998) Cancer risk in the rubber industry: a review of the recent epidemiological evidence. *Occup Environ Med*, **55**, 1-12.

69. Berrino, F., Richiardi, L., Boffetta, P., Esteve, J., Belletti, I., Raymond, L., Troschel, L., Pisani, P., Zubiri, L., Ascunce, N., Guberan, E., Tuyns, A., Terracini, B. and Merletti, F. (2003) Occupation and larynx and hypopharynx cancer: a job-exposure matrix approach in an international case-control study in France, Italy, Spain and Switzerland. *Cancer Causes Control*, **14**, 213-23.

70. Ahrens, W., Jockel, K.H., Patzak, W. and Elsner, G. (1991) Alcohol, smoking, and occupational factors in cancer of the larynx: a case-control study. *Am J Ind Med*, **20**, 477-93.

71. Burch, J.D., Howe, G.R., Miller, A.B. and Semenciw, R. (1981) Tobacco, alcohol, asbestos, and nickel in the etiology of cancer of the larynx: a case-control study. *J Natl Cancer Inst*, **67**, 1219-24.

72. Rothman, K.J., Cann, C.I., Flanders, D. and Fried, M.P. (1980) Epidemiology of laryngeal cancer. *Epidemiol Rev*, **2**, 195-209.

73. Hillerdal, G. and Lindholm, C.E. (1980) Laryngeal cancer and asbestos. *ORL J Otorhinolaryngol Relat Spec*, **42**, 233-41.

74. Parnes, S.M. (1990) Asbestos and cancer of the larynx: is there a relationship? *Laryngoscope*, **100**, 254-61.

75. Donald, P.J. (1986) Marijuana smoking--possible cause of head and neck carcinoma in young patients. *Otolaryngol Head Neck Surg*, **94**, 517-21.

76. Zhang, Z.F., Morgenstern, H., Spitz, M.R., Tashkin, D.P., Yu, G.P., Marshall, J.R., Hsu, T.C. and Schantz, S.P. (1999) Marijuana use and increased risk of squamous cell carcinoma of the head and neck. *Cancer Epidemiol Biomarkers Prev*, **8**, 1071-8.

77. Copper, M.P., Jovanovic, A., Nauta, J.J., Braakhuis, B.J., de Vries, N., van der Waal, I. and Snow, G.B. (1995) Role of genetic factors in the etiology of squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg*, **121**, 157-60.

78. Foulkes, W.D., Brunet, J.S., Kowalski, L.P., Narod, S.A. and Franco, E.L. (1995) Family history of cancer is a risk factor for squamous cell carcinoma of the head and neck in Brazil: a case-control study. *Int J Cancer*, **63**, 769-73.

79. Gencik, A., Wey, W. and Muller, H. (1986) High incidence of laryngeal carcinoma in a Swiss family. *ORL J Otorhinolaryngol Relat Spec*, **48**, 162-6.

80. Cheng, L.S., Swan, G.E. and Carmelli, D. (2000) A genetic analysis of smoking behavior in family members of older adult males. *Addiction*, **95**, 427-35.

81. Swan, G.E., Carmelli, D. and Cardon, L.R. (1997) Heavy consumption of cigarettes, alcohol and coffee in male twins. *J Stud Alcohol*, **58**, 182-90.

82. Geisler, S.A. and Olshan, A.F. (2001) GSTM1, GSTT1, and the risk of squamous cell carcinoma of the head and neck: a mini-HuGE review. *Am J Epidemiol*, **154**, 95-105.

83. Goode, E.L., Ulrich, C.M. and Potter, J.D. (2002) Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol Biomarkers Prev*, **11**, 1513-30.

84. Hsieh, L.L., Chien, H.T., Chen, I.H., Liao, C.T., Wang, H.M., Jung, S.M., Wang, P.F., Chang, J.T., Chen, M.C. and Cheng, A.J. (2003) The XRCC1 399Gln polymorphism and the frequency of p53 mutations in Taiwanese oral squamous cell carcinomas. *Cancer Epidemiol Biomarkers Prev*, **12**, 439-43.

85. Olshan, A.F., Watson, M.A., Weissler, M.C. and Bell, D.A. (2002) XRCC1 polymorphisms and head and neck cancer. *Cancer Lett*, **178**, 181-6.

86. Ho, T., Wei, Q. and Sturgis, E.M. (2007) Epidemiology of carcinogen metabolism genes and risk of squamous cell carcinoma of the head and neck. *Head Neck*, **29**, 682-99.

87. Alter, B.P. (2003) Cancer in Fanconi anemia, 1927-2001. *Cancer*, **97**, 425-40.

88. Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*, **7**, 249-62.

89. Bird, A. (2007) Perceptions of epigenetics. *Nature*, **447**, 396-8.

90. Turner, B.M. (2002) Cellular memory and the histone code. *Cell*, **111**, 285-91.

91. Matera, A.G., Terns, R.M. and Terns, M.P. (2007) Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev Mol Cell Biol*, **8**, 209-20.

92. Weinhold, B. (2006) Epigenetics: the science of change. *Environ Health Perspect*, **114**, A160-7.

93. Holliday, R. (1987) The inheritance of epigenetic defects. *Science*, **238**, 163-70.

94. Eden, A., Gaudet, F., Waghmare, A. and Jaenisch, R. (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. *Science*, **300**, 455.

95. Costello, J.F., Fruhwald, M.C., Smiraglia, D.J., Rush, L.J., Robertson, G.P., Gao, X., Wright, F.A., Feramisco, J.D., Peltomaki, P., Lang, J.C., Schuller, D.E., Yu, L., Bloomfield, C.D., Caligiuri, M.A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N.J., Zhang, X., O'Dorisio, M.S., Held, W.A., Cavenee, W.K. and Plass, C. (2000) Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet*, **24**, 132-8.

96. Esteller, M., Corn, P.G., Baylin, S.B. and Herman, J.G. (2001) A gene hypermethylation profile of human cancer. *Cancer Res*, **61**, 3225-9.

97. Ambros, V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350-5.

98. Erdmann, V.A., Szymanski, M., Hochberg, A., Groot, N. and Barciszewski, J. (2000) Non-coding, mRNA-like RNAs database Y2K. *Nucleic Acids Res*, **28**, 197-200.

99. Kim, Y.K. and Kim, V.N. (2007) Processing of intronic microRNAs. *EMBO J*, **26**, 775-83.

100. Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H. and Kim, V.N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*, **23**, 4051-60.

101. Zhou, X., Ruan, J., Wang, G. and Zhang, W. (2007) Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol*, **3**, e37.

102. Borchert, G.M., Lanier, W. and Davidson, B.L. (2006) RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol*, **13**, 1097-101.

103. Du, T. and Zamore, P.D. (2005) microPrimer: the biogenesis and function of microRNA. *Development*, **132**, 4645-52.

104. Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102-14.

105. Yi, R., Qin, Y., Macara, I.G. and Cullen, B.R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, **17**, 3011-6.

106. Rand, T.A., Petersen, S., Du, F. and Wang, X. (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell*, **123**, 621-9.

107. Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, **115**, 199-208.

108. Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P. and Bartel, D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*, **27**, 91-105.

109. Rehmsmeier, M. (2006) Prediction of microRNA targets. *Methods Mol Biol*, **342**, 87-99.

110. Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15-20.

111. Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E. and Shiekhattar, R. (2007) MicroRNA silencing through RISC recruitment of eIF6. *Nature*, **447**, 823-8.

112. Humphreys, D.T., Westman, B.J., Martin, D.I. and Preiss, T. (2005) MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A*, **102**, 16961-6.

113. Maroney, P.A., Yu, Y., Fisher, J. and Nilsen, T.W. (2006) Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat Struct Mol Biol*, **13**, 1102-7.

114. Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. and Parker, R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol*, **7**, 719-23.

115. Sen, G.L. and Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, **7**, 633-6.

116. Brengues, M., Teixeira, D. and Parker, R. (2005) Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science*, **310**, 486-9.

117. Cowland, J.B., Hother, C. and Gronbaek, K. (2007) MicroRNAs and cancer. *Apmis*, **115**, 1090-106.

118. Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. and Croce, C.M. (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*, **99**, 15524-9.

119. Cimmino, A., Calin, G.A., Fabbri, M., Iorio, M.V., Ferracin, M., Shimizu, M., Wojcik, S.E., Aqeilan, R.I., Zupo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M. and Croce, C.M. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*, **102**, 13944-9.

120. Cho, W.C. (2007) OncomiRs: the discovery and progress of microRNAs in cancers. *Mol Cancer*, **6**, 60.

121. Blenkiron, C., Goldstein, L.D., Thorne, N.P., Spiteri, I., Chin, S.F., Dunning, M.J., Barbosa-Morais, N.L., Teschendorff, A.E., Green, A.R., Ellis, I.O., Tavare, S., Caldas, C. and Miska, E.A. (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol*, **8**, R214.

122. Guo, J., Miao, Y., Xiao, B., Huan, R., Jiang, Z., Meng, D. and Wang, Y. (2009) Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. *J Gastroenterol Hepatol*, **24**, 652-7.

123. Iorio, M.V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C.G., Alder, H., Calin, G.A., Menard, S. and Croce,

C.M. (2007) MicroRNA signatures in human ovarian cancer. *Cancer Res*, **67**, 8699-707.

124. Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S. and Zucman-Rossi, J. (2008) MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology*, **47**, 1955-63.

125. Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., Stephens, R.M., Okamoto, A., Yokota, J., Tanaka, T., Calin, G.A., Liu, C.G., Croce, C.M. and Harris, C.C. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*, **9**, 189-98.

126. Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-8.

127. Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., Prueitt, R.L., Yanaihara, N., Lanza, G., Scarpa, A., Vecchione, A., Negrini, M., Harris, C.C. and Croce, C.M. (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*, **103**, 2257-61.

128. Nam, E.J., Yoon, H., Kim, S.W., Kim, H., Kim, Y.T., Kim, J.H., Kim, J.W. and Kim, S. (2008) MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res*, **14**, 2690-5.

129. Ambros, V. (2003) MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*, **113**, 673-6.

130. Hsu, P.Y., Deatherage, D.E., Rodriguez, B.A., Liyanarachchi, S., Weng, Y.I., Zuo, T., Liu, J., Cheng, A.S. and Huang, T.H. (2009) Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. *Cancer Res*, **69**, 5936-45.

131. Zhang, B. and Pan, X. (2009) RDX induces aberrant expression of microRNAs in mouse brain and liver. *Environ Health Perspect*, **117**, 231-40.

132. Pietrzykowski, A.Z., Friesen, R.M., Martin, G.E., Puig, S.I., Nowak, C.L., Wynne, P.M., Siegelmann, H.T. and Treistman, S.N. (2008) Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron*, **59**, 274-87.

133. Wang, L.L., Zhang, Z., Li, Q., Yang, R., Pei, X., Xu, Y., Wang, J., Zhou, S.F. and Li, Y. (2009) Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Hum Reprod*, **24**, 562-79.

134. Schembri, F., Sridhar, S., Perdomo, C., Gustafson, A.M., Zhang, X., Ergun, A., Lu, J., Liu, G., Bowers, J., Vaziri, C., Ott, K., Sensinger, K., Collins, J.J., Brody, J.S., Getts, R., Lenburg, M.E. and Spira, A. (2009) MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci U S A*, **106**, 2319-24.

135. Tran, N., McLean, T., Zhang, X., Zhao, C.J., Thomson, J.M., O'Brien, C. and Rose, B. (2007) MicroRNA expression profiles in head and neck cancer cell lines. *Biochem Biophys Res Commun*, **358**, 12-7.

136. Dillhoff, M., Liu, J., Frankel, W., Croce, C. and Bloomston, M. (2008) MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg*, **12**, 2171-6.

137. Qian, B., Katsaros, D., Lu, L., Preti, M., Durando, A., Arisio, R., Mu, L. and Yu, H. (2008) High miR-21 expression in breast cancer associated with poor disease-free survival in early stage disease and high TGF-beta1. *Breast Cancer Res Treat*.

138. Yan, L.X., Huang, X.F., Shao, Q., Huang, M.Y., Deng, L., Wu, Q.L., Zeng, Y.X. and Shao, J.Y. (2008) MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*, **14**, 2348-60.

139. Chang, S.S., Jiang, W.W., Smith, I., Poeta, L.M., Begum, S., Glazer, C., Shan, S., Westra, W., Sidransky, D. and Califano, J.A. (2008) MicroRNA alterations in head and neck squamous cell carcinoma. *Int J Cancer*, **123**, 2791-2797.

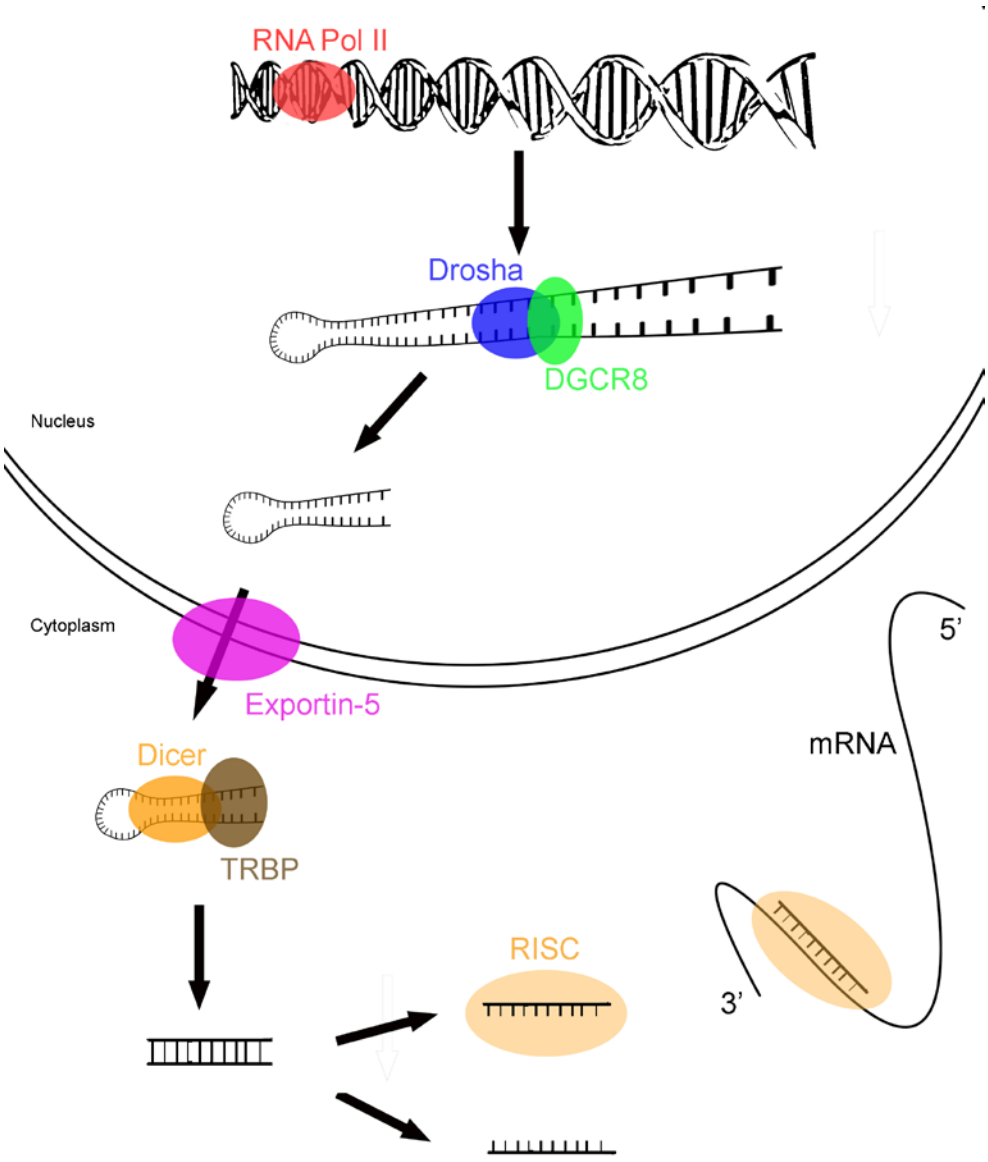

Figure 1. MicroRNA biosynthesis. Primary miRNAs (pri-miRNAs) are usually transcribed by RNA Polymerase II (RNA Pol II) and are processed in two major steps involving RNase III endonucleases. First, Drosha and its cofactor DGCR8 process the 100s-1000s nt pri-miRNA transcript into a premature miRNA (pre-miRNA) of ~70 nt. After the pre-miRNA is exported out of the nucleus via Exportin-5, it is further cleaved by Dicer and partner TAR-RNA binding protein (TRBP) into a mature miRNA duplex of ~22 bp. The active strand of the duplex is loaded onto the RNA-induced silencing complex and directs it to its complementary sequence in the 3' untranslated region of its mRNA target.

Table 1: Ten most commonly differentially expressed genes in HNSCC primary tumor v. normal tissue

gene	refs	chr	fold change
KRT4	18	12q12	-0.73
KRT5	16	12q12	-0.72
PLAU	15	10q24	0.48
FN1	14	2q34	0.45
MAL	14	2cen-q13	-0.96
MMP1	13	11q22.3	1
COL1A2	13	7q22.1	0.4
SPARC	13	5q31.3	0.43
POSTN	12	13q13.3	0.61
IFI6	12	1p35	0.33

Abbreviations: refs, the number of papers reporting gene as differentially expressed; chr, the chromosomal location of the gene

Chapter 2

A MicroRNA Expression Ratio is Predictive of Head and Neck Squamous Cell Carcinoma

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A MicroRNA Expression Ratio is Predictive of Head and Neck Squamous Cell Carcinoma

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Running Title: miRNA Expression Ratio Predicts HNSCC

Keywords: Head and Neck Cancer, microRNA, microarray, carcinogenesis

Statement of Translational Relevance

This work has demonstrated that a microRNA expression ratio can distinguish between non-diseased tissue and tumor tissue with great accuracy in the context of head and neck squamous cell carcinoma (HNSCC), an important public health concern worldwide. The ratio of *miR-221:miR-375* showed high discriminatory potential, with a sensitivity of 92% and specificity of 93% in distinguishing tumor from normal tissue, and suggests that this simple molecular marker may hold significant clinical potential as a diagnostic tool.

Abstract

Purpose: The involvement of microRNAs (miRNAs) in cancer and their potential as biomarkers of diagnosis and prognosis are becoming increasingly appreciated. We sought to identify miRNAs altered in head and neck squamous cell carcinoma (HNSCC) and to determine whether miRNA expression is predictive of disease.

Experimental Design: RNA isolated from fresh frozen primary tumors, fresh frozen non-diseased head and neck epithelial tissues, and HNSCC cell lines was profiled for the expression of 662 miRNAs by microarray. The miRNAs that were both differentially expressed on the array and by qRT-PCR were subsequently validated by qRT-PCR using a total of 99 HNSCC samples and 14 normal epithelia.

Results: A marked difference in miRNA expression pattern was observed between tumors and cell lines. Eighteen miRNAs were significantly altered in their expression between normal tissues and tumors. Four of these miRNAs were validated in the larger sample series, and each showed significant differential expression ($P < 0.0001$). Further, an expression ratio of miR-221:miR-375 demonstrated a high sensitivity (0.92) and specificity (0.93) for disease prediction.

Conclusions: These data suggest that cultured tumor cell lines are inappropriate for miRNA biomarker identification, and that the pattern of miRNA expression in primary head and neck tissues is reflective of disease status, with certain miRNAs exhibiting strong predictive potential. These results indicate that miR-221 and miR-375 should be evaluated further as diagnostic biomarkers, as they may hold utility in defining broadly responsive prevention and treatment strategies for HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) includes carcinomas arising from the epithelium of the oral cavity, pharynx, and larynx, and is the sixth most common malignancy worldwide (1). The major risk factors for the disease are tobacco and alcohol use, and human papillomavirus (HPV) infection (2-4). Despite advances in detection, as well as surgical and chemotherapeutic treatments over recent decades, the five year survival rate for HNSCC has remained around 50%, one of the lowest of the major cancers (5). Frequent late stage diagnosis, formation of additional primary tumors and regional and distant metastases all contribute to this poor survival rate (2).

A better understanding of the molecular pathways that give rise to HNSCC is essential in the identification of novel molecular biomarkers that have clinical utility in predicting prognosis and therapeutic efficacy, as well as in designing targeted therapy for this disease. In recent years, gene expression profiling technologies have become increasingly sophisticated, allowing investigators to explore their diagnostic and therapeutic potential as biomarkers in HNSCC and other cancers (6-8). These biomarkers, however, have had limited success in the clinical setting and, to date, limited utility in further elucidating mechanisms of HNSCC carcinogenesis.

The discovery of microRNAs (miRNAs), ~22 nucleotide long, non-coding RNA molecules, has revolutionized our understanding of the modulation of gene expression. Nearly 700 miRNAs have been identified in humans (miRBase: http://microrna.sanger.ac.uk/), a number that is rapidly growing and expected to reach 1,000 or higher (9). Highly ubiquitous and largely conserved across species, miRNAs regulate gene expression post-transcriptionally by base-pairing, usually imperfectly, to

the 3'-untranslated region (10) of a cognate messenger RNA (11). The interaction of a miRNA with a target mRNA transcript results either in translational repression of the mRNA or in its direct degradation (11). Due to the partial complementarity between miRNAs and their target transcripts, a single miRNA is capable of simultaneously regulating up to hundreds of genes, giving rise to an enormous modulatory potential (12).

Through their targets, miRNAs are known to play important roles in cell differentiation, proliferation, and apoptosis (13). As these processes are known to be deregulated in cancer, it is not surprising that many studies have now identified a role for miRNAs in carcinogenesis (14-16), including head and neck carcinogenesis (17, 18). Indeed, recent work on different cancer types has illustrated the existence of distinct miRNA expression profiles between tumor tissues and their corresponding normal tissue (19-21). Moreover, some studies have identified miRNA expression profiles that can distinguish different tumor subtypes or developmental lineages, findings that may have clinical applications in diagnostics and tumor staging (16, 22).

Here we have compared the miRNA expression of normal head and neck epithelia with primary HNSCC tumors and cultured HNSCC cell lines in order to define those miRNA most capable of differentiating disease, and thus those with the greatest potential as biomarkers. Upon identifying miRNAs specifically altered in head and neck cancer, we sought to validate a subset of these in a larger population of tumors with the aim of identifying a clinically-applicable diagnostic tool.

Materials and Methods

HNSCC samples and cell lines. All fresh-frozen HNSCC samples were obtained, with informed consent after Institutional Review Board approval at participating hospitals, as part of a population-based case-control study of HNSCC spanning December 1999 to December 2003 in the Greater Boston Metropolitan area. The fresh-frozen tumors originated from uvula, larynx, floor of mouth, and tongue resections. Details of this study have been described previously (23). Study pathologists confirmed >75% tumor in all HNSCC samples tested. FaDu and Cal27, HNSCC cell lines, were obtained from American Type Culture Collection (ATCC) and maintained in Eagle's Minimum Essential Medium and Dulbecco's Modified Eagle's Medium, respectively, both supplemented with fetal bovine serum to a final concentration of 10%.

RNA isolation and microarray profiling. Total RNA was isolated from normal tissues, tumors, and cell lines using the mirVANA RNA Isolation Kit (Ambion, Inc., 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 needed. 5 μg of total RNA was sent for miRNA profiling studies at Asuragen Services using the mirVANA miRNA Bioarrays platform v2 (Ambion, Inc) as single channel format according to the standard operating procedures of the company, including pre-array qualitative Bioanalyzer (Agilent, Santa Clara, CA) RNA analysis, as previously described (24). The Bioarrays platform v2 contains probes specific to miRNA identified in human, mouse, and rat, as well as a number of proprietary miRNAs identified through cloning at Ambion, Inc. The Cy5 fluorescence on the arrays was scanned at an excitation

wavelength of 635 nm using a GenePix 4200AL scanner (Molecular Devices, Union City, CA). The fluorescent signal associated with the probes and local background was extracted using GenePix Pro (version 6.0, Molecular Devices). Raw signal data were normalized by first log2 transformation of signal intensity followed by global Variance Stabilization Normalization (25) of all the arrays within the project. Normalized data were submitted to the GEO archive (accession #GSE11163).

Quantitative reverse transcription-PCR. TaqMan miRNA Assays (Applied Biosystems) were used to quantify mature miRNA. cDNA was synthesized by priming with a pool of gene-specific looped primers including the primers of the miRNAs of interest and RNU48, as a universally-expressed endogenous control (Applied Biosystems). 10 µl of total RNA diluted to a final concentration of 5 ng/µl was used for each reverse transcription (RT) reaction along with other RT components, per manufacturer's specifications. 40µl 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. qRT-PCR was performed as previously described (26) with the following exception: 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 PCR data were analyzed using the comparative C_T method, normalizing against expression of RNU48.

Statistical analysis. Differential expression of miRNAs by microarray was determined with Significance Analysis of Microarray (SAM) software (Stanford University Labs) using 1000 permutations of the data and with delta adjusted to minimize false discovery

rate. All hierarchical clustering analyses were carried out using Cluster 3.0 (Stanford University) with Euclidian distance as the distance metric and centroid linkage between clusters. A two-tailed Student's *t* test was used to compare miRNA expression levels determined by real-time PCR. miRNA expression ratios were calculated following the methods of Gordon et al (27), and receiver operating curve (ROC) analyses were used to assess the predictive power of the miRNA quotients.

Results

miRNA expression patterns differentiate HNSCC cell lines from primary tissues. A microarray platform was used to determine miRNA expression of 662 miRNAs in 16 fresh frozen HNSCC tumors, 5 non-diseased head and neck epithelial tissues, and 2 individual HNSCC cell lines. The normalized data has been deposited in the GEO archive (accession #GSE11163).

Unsupervised hierarchical clustering based on all the miRNAs spotted on the chip revealed a marked, very distinct separation of the cell line miRNA profiles compared to those of primary tissues (Figure 1A). SAM identified 67 significantly differentially expressed miRNAs ($Q < 0.0001$) between cell lines and primary tissues, consistently showing lower expression of miRNAs in cell lines compared to tumors (Supplementary Table 1).

18 miRNAs are differentially expressed in HNSCC tumor tissue compared to normal head and neck epithelia. SAM analysis identified 18 miRNAs to be significantly altered in their expression between non-diseased tissues and primary HNSCC tumors, with 17 being up-modulated and 1 down-modulated in tumors ($Q < 0.0001$) (Supplementary Table 2). Of the 18 differentially expressed miRNAs, 12 were human miRNAs, four were proprietary miRNAs of Ambion Inc. and two were mouse orthologues, both of which have known human counterparts with identical mature sequences. Hierarchical clustering based on this limited set of miRNAs showed clear separation of tumors from normal tissues (Figure 1B).

All human miRNAs identified by SAM method showed greater than two-fold difference between normal and tumor tissue (Table 1). Of the human miRNAs, six (*miR-21, miR-181d, miR-181b, miR-18a, miR-221*, and *miR-375*) were chosen for confirmation of the microarray results using stem-loop based RT-PCR followed by conventional Taqman real-time PCR using miRNA-specific probes, as validated assays were available for these six miRNA and they constituted one primer pool, allowing for examination using a single reverse transcription step. Confirmation assays utilized only the samples screened on the array. Of the six miRNAs tested, four were reliably confirmed (*miR-21, miR-18a, miR-221,* and *miR-375*), showing significant differential expression between tumor and normal ($P < 0.01$, Supplementary Figure 1).

miRNA expression ratio demonstrates high specificity and sensitivity in predicting disease. Validation of the four confirmed miRNAs was performed in nine additional normal tissue samples (total n = 14), and 83 additional tumor samples (total n = 99). Quantification of miRNA expression in this large set of samples showed strong validation of the results seen in the smaller population. *miR-21*, *miR-18a*, and *miR-221* showed significant upregulation in tumors ($P < .0001$; Figure 2a, 2c, and 2d, respectively), whereas *miR-375* was significantly downregulated in tumors ($P < .0001$; Figure 2b).

As *miR-21, miR-18a,* and *miR-221* demonstrated consistent upregulation and *miR-375* consistent downregulation, we next sought to determine if expression ratios constructed between these miRNA could improve their predictive potential for differentiating HNSCC tumor from non-diseased epithelia. Following the methods of Gordon et. al (27), miRNA expression ratios were calculated by dividing the relative

expression value of each of the three miRNAs showing upregulation in tumors by the expression value of the only downregulated miRNA, *miR-375*. ROC analysis was performed to determine which of these ratios demonstrated the greatest predictive power (Figure 3). Table 2 lists the representative sensitivity and specificity of ratios using the cutoff value of 1 for each of the upregulated miRNA to the downregulated *miR-375* in differentiating between non-diseased tissue and HNSCC, using the validation series. *miR-21:miR-375* ratios above 1.0 exhibited high specificity (0.99) but low sensitivity (0.14) whereas the relationship of *miR-18a:miR-375* showed high sensitivity (1.00) but low specificity (0.52) (Table 2). However the ratio of *miR-221:miR-375* exhibited the strongest predictive ability with both high sensitivity and specificity (0.92 and 0.93 respectively; Table 2).

Discussion

The present study revealed a number of miRNAs to be aberrantly expressed in HNSCC tumors, including an extensively validated subset that may hold utility as clinical biomarkers of disease. Microarray profiling of over 600 miRNAs identified 18 miRNAs that were significantly differentially expressed in tumor tissues compared to analogous non-diseased head and neck epithelia. Of the 12 human miRNAs in this group, 4 miRNA which were validated by qRT-PCR were used for in-depth examination of a larger population of fresh frozen HNSCC tumors and normal head and neck tissue.

The permutation-based software SAM was used to identify differentially expressed genes by pair-wise comparisons between groups of interest. It should be noted that SAM was designed (and may be better suited) for identification of important genes from high density microarrays, as it allows the user to control the number of findings based on a desired false discovery rate (FDR) while avoiding parametric assumptions about the data, inherent in tests such as the Analysis of Variance (28) (29).

The microarray data revealed that HNSCC cell lines show a distinct pattern of miRNA expression compared to primary tumors. This is consistent with past reports demonstrating a clear segregation of cell lines away from primary tumors upon high-throughput analysis of their miRNA expression (22, 30), suggesting that cell lines have limited utility as a model system for the identification of clinically relevant miRNA biomarkers. There remains, though, significant utility in utilizing *in-vitro* approaches for defining the biological mechanisms of these miRNAs, with appropriate understanding that the pattern of their expression is markedly different from that observed in the parent primary tissue.

Some of the miRNAs identified as differentially expressed in HNSCC compared to normal tissues have been characterized in past reports, particularly in relation to cancer. One of these, *miR-21*, was shown by quantitative real-time PCR analysis to be significantly upregulated in HNSCC tumors (17). miRNA profiling of breast, cervical, and ovarian tumors, glioblastomas, and head and neck primary tumors and cell lines, amongst others, has shown that *miR-21* is commonly upregulated in cancer (17, 18, 21, 31). In fact, the relative levels of *miR-21* and other miRNAs were reported to have prognostic relevance for predicting patient survival in lung cancer (32). Some known targets of *miR-21* include the tumor suppressor genes tropomyosin 1 (*TPM1*) and programmed cell death 4 (*PDCD4*) (33, 34). Additionally, *miR-221* and *miR-18a*, both shown to be upregulated in HNSCC tumors, have previously been implicated in hepatocellular, prostate, and other cancers (35-38).

miR-375 was the only downregulated miRNA found when comparing tumors and normal tissues, showing a ~22 fold decrease in tumors. Validation experiments also showed it to be sharply and significantly downregulated in tumors relative to normal tissues. *miR-375* has been found to regulate insulin secretion in mice and its downregulation has been implicated in aberrant morphology of pancreatic islet cells in zebrafish (39, 40). Recently, *miR-375* downregulation has also been associated with β-catenin mutation in hepatocellular adenoma (41). The downregulation of *miR-375* in HNSCC tumors, as shown in this work, is consistent with a possible role for *miR-375* in the transcriptional repression of oncogenes, analogous to the regulation of oncogenic *KRAS* by the *let-7* miRNA (42).

In order to improve the predictive potential for individual miRNA alterations, we used expression ratios as described by Gordon et al (27). The ratio of *miR-221:miR-375* demonstrated high discriminatory potential, with a sensitivity of 92% and specificity of 93% in distinguishing tumor from normal tissue. These data suggest that the ratio of these miRNAs may hold significant clinical potential, but further validation is necessary in an independent series of HNSCC tumors.

The identification of an HNSCC-specific miRNA signature indicates a plausible role for miRNAs in the development or progression of this disease. Finding abnormally expressed miRNAs may prove to be an important step in identifying the specific mechanisms of HNSCC carcinogenesis, as these aberrations may constitute early events in initiation or progression of the disease (43). As such, the utilization of a miRNA expression ratio that distinguishes disease tissue from non-diseased tissue holds potential as a simple, early diagnostic for HNSCC. An examination of these miRNA expression ratios in preneoplastic lesions, early stage tumors, and samples obtained for screening such as saliva and mouthwash would clarify the true clinical applicability of these findings in the realm of early diagnostics. Future studies should also examine the potential for associations between miRNA expression and clinical criteria such as tumor stage, metastasis, and prognosis in a larger series of tumors than what has been examined here. Finally, a validation of the miRNAs identified in this study in an independent test population of HNSCCs could be instrumental both in demonstrating that the individual miRNA aberrations are globally specific to HNSCC, and in resolving the true diagnostic potential of the *miR-221:miR-375* ratio.

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References

1. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2(9):533-43.

2. Ragin CC, Modugno F, Gollin SM. The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. J Dent Res 2007;86(2):104-14.

3. Loning T, Ikenberg H, Becker J, Gissmann L, Hoepfer I, zur Hausen H. Analysis of oral papillomas, leukoplakias, and invasive carcinomas for human papillomavirus type related DNA. J Invest Dermatol 1985;84(5):417-20.

4. Furniss CS, McClean MD, Smith JF, *et al.* Human papillomavirus 16 and head and neck squamous cell carcinoma. Int J Cancer 2007;120(11):2386-92.

5. Hardisson D. Molecular pathogenesis of head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol 2003;260(9):502-8.

6. Al Moustafa AE, Alaoui-Jamali MA, Batist G, *et al.* Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. Oncogene 2002;21(17):2634-40.

7. Schlecht NF, Burk RD, Adrien L, *et al.* Gene expression profiles in HPV-infected head and neck cancer. J Pathol 2007;213(3):283-93.

8. Pyeon D, Newton MA, Lambert PF, *et al.* Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. Cancer Res 2007;67(10):4605-19.

9. Blenkiron C, Miska EA. miRNAs in cancer: approaches, aetiology, diagnostics and therapy. Hum Mol Genet 2007;16 Spec No 1:R106-13.

10. Bunz F, Dutriaux A, Lengauer C, *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282(5393):1497-501.

11. Cowland JB, Hother C, Gronbaek K. MicroRNAs and cancer. Apmis 2007;115(10):1090-106.

12. Du T, Zamore PD. Beginning to understand microRNA function. Cell Res 2007;17(8):661-3.

13. Miska EA. How microRNAs control cell division, differentiation and death. Curr Opin Genet Dev 2005;15(5):563-8.

14. He H, Jazdzewski K, Li W, *et al.* The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2005;102(52):19075-80.

15. Murakami Y, Yasuda T, Saigo K, *et al.* Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25(17):2537-45.

16. Lu J, Getz G, Miska EA, *et al.* MicroRNA expression profiles classify human cancers. Nature 2005;435(7043):834-8.

17. Chang SS, Jiang WW, Smith I, *et al.* MicroRNA alterations in head and neck squamous cell carcinoma. Int J Cancer 2008;123(12):2791-7.

18. Tran N, McLean T, Zhang X, *et al.* MicroRNA expression profiles in head and neck cancer cell lines. Biochem Biophys Res Commun 2007;358(1):12-7.

19. Volinia S, Calin GA, Liu CG, *et al.* A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103(7):2257-61.

20. Guo Y, Chen Z, Zhang L, *et al.* Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. Cancer Res 2008;68(1):26-33.

21. Iorio MV, Visone R, Di Leva G, *et al.* MicroRNA signatures in human ovarian cancer. Cancer Res 2007;67(18):8699-707.

22. Blenkiron C, Goldstein LD, Thorne NP, *et al.* MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 2007;8(10):R214.

23. Peters ES, McClean MD, Liu M, Eisen EA, Mueller N, Kelsey KT. The ADH1C polymorphism modifies the risk of squamous cell carcinoma of the head and neck associated with alcohol and tobacco use. Cancer Epidemiol Biomarkers Prev 2005;14(2):476-82.

24. Marsit CJ, McClean MD, Furniss CS, Kelsey KT. Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. Int J Cancer 2006;119(8):1761-6.

25. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 2002;18 Suppl 1:S96-104.

26. Chen C, Ridzon DA, Broomer AJ, *et al.* Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33(20):e179.

27. Gordon GJ, Jensen RV, Hsiao LL, *et al.* Translation of microarray data into clinically relevant cancer diagnostic tests using gene expression ratios in lung cancer and mesothelioma. Cancer Res 2002;62(17):4963-7.

28. Hashibe M, Brennan P, Chuang SC, *et al.* Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Cancer Epidemiol Biomarkers Prev 2009;18(2):541-50.

29. Pan W. A comparative review of statistical methods for discovering differentially expressed genes in replicated microarray experiments. Bioinformatics 2002;18(4):546-54.

30. Szafranska AE, Davison TS, John J, *et al.* MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. Oncogene 2007;26(30):4442-52.

31. Cho WC. OncomiRs: the discovery and progress of microRNAs in cancers. Mol Cancer 2007;6:60.

32. Yanaihara N, Caplen N, Bowman E, *et al.* Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9(3):189-98.

33. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 2008;283(2):1026-33.

34. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007;282(19):14328-36.

35. Fornari F, Gramantieri L, Ferracin M, *et al.* MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 2008;27(43):5651-61.

36. Galardi S, Mercatelli N, Giorda E, *et al.* miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem 2007;282(32):23716-24.

37. Nam EJ, Yoon H, Kim SW, *et al.* MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 2008;14(9):2690-5.

38. Takakura S, Mitsutake N, Nakashima M, *et al.* Oncogenic role of miR-17-92 cluster in anaplastic thyroid cancer cells. Cancer Sci 2008;99(6):1147-54.

39. Poy MN, Eliasson L, Krutzfeldt J, *et al.* A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004;432(7014):226-30.

40. Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol 2007;5(8):e203.

41. Ladeiro Y, Couchy G, Balabaud C, *et al.* MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008;47(6):1955-63.

42. Johnson SM, Grosshans H, Shingara J, *et al.* RAS is regulated by the let-7 microRNA family. Cell 2005;120(5):635-47.

43. Gomes CC, Gomez RS. MicroRNA and oral cancer: future perspectives. Oral Oncol 2008;44(10):910-4.

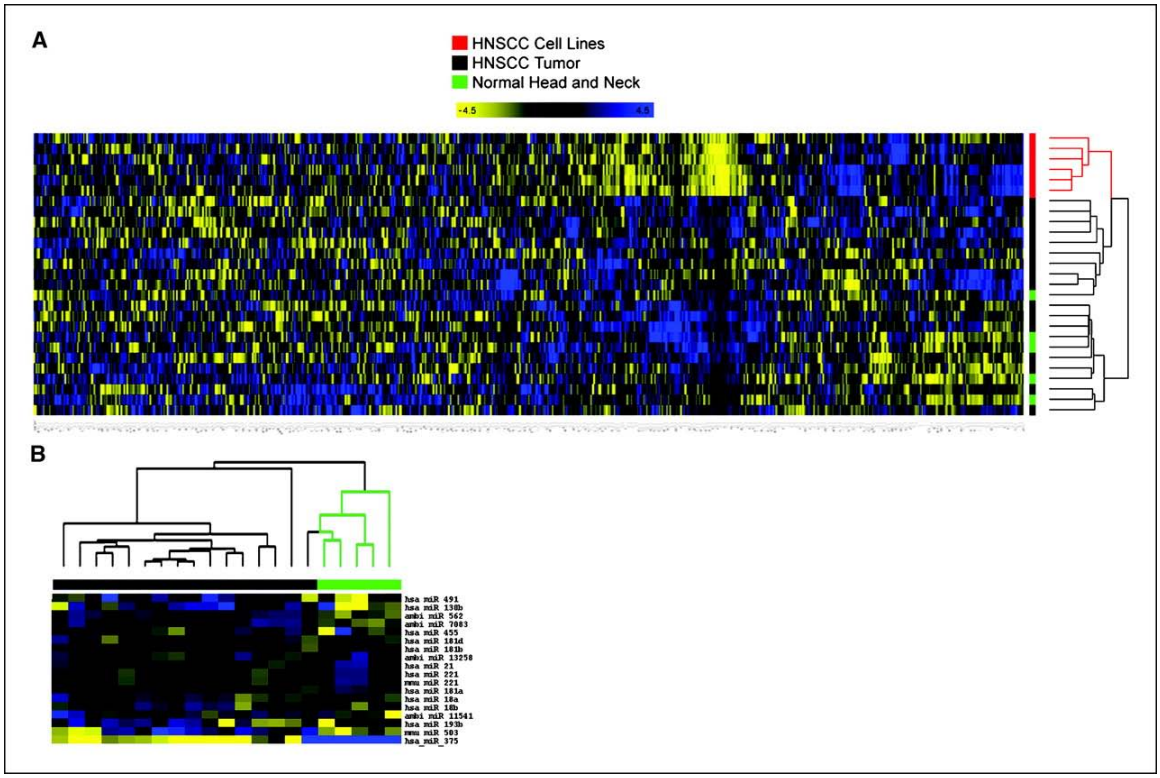


Figure 1. Heat maps created by unsupervised hierarchical clustering analysis between groups of samples. Blue and yellow represent higher and lower relative expression, respectively (A) Clustering analysis of HNSCC cell lines, HNSCC tumors and normal head and neck epithelia across 662 individual miRNAs shows separation of cell lines and primary tissues. (B) Separation of HNSCC tumors and normal tissues based on differential expression of 18 significantly altered miRNAs.

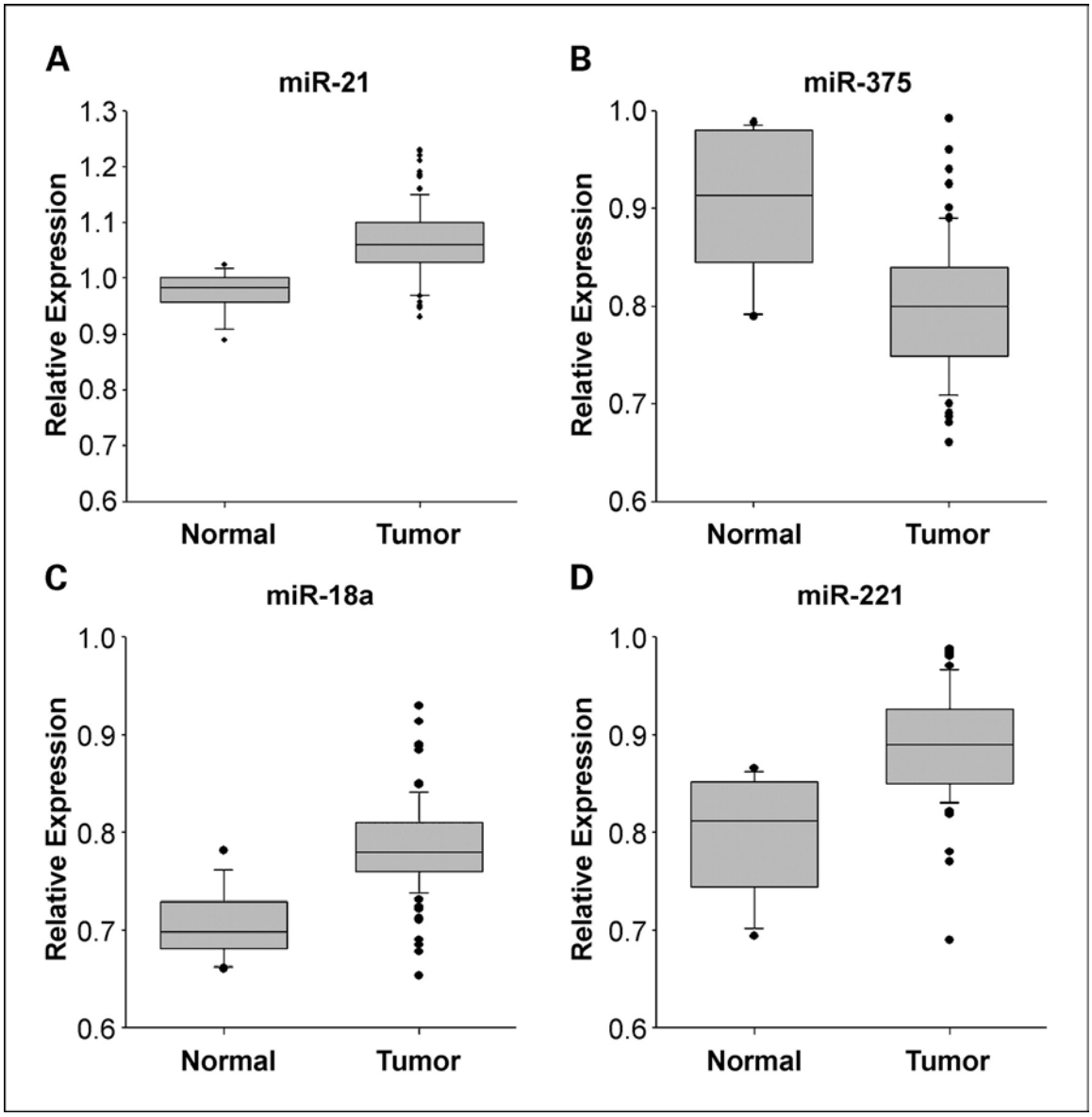


Figure 2. Validation of differential expression of four microRNAs by quantitative real-time PCR in tumor (n=99) and normal (n=14) samples. For each miRNA, boxes denote distribution of expression values from the 25th to 75th percentile. Horizontal lines in boxes represent median values, whiskers represent 5th and 95th percentiles and outliers are denoted by dots. $P < 0.0001$ in all comparisons.

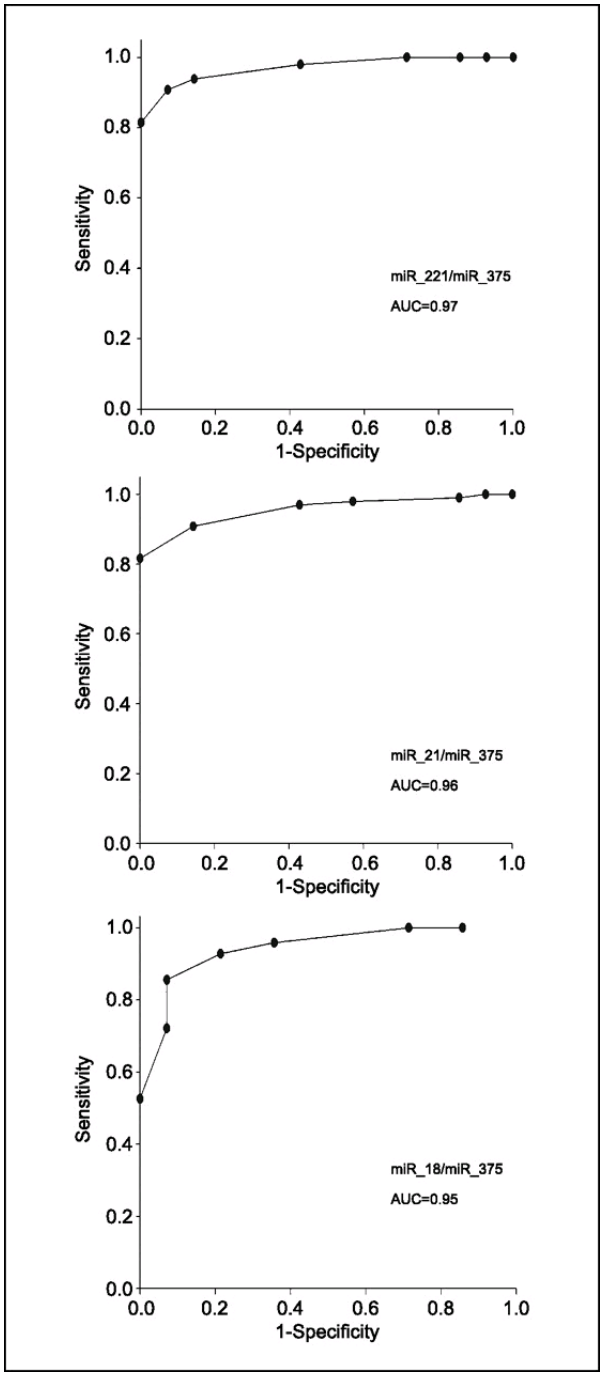


Figure 3. ROC analysis of expression ratios of *miR-221*, *miR-21* and *miR-18a* to *miR-375* for differentiation of HNSCC tumors and normal tissues. Curves were constructed using ratio value cut-offs ranging from 0.75 to 1.25 for each ratio. AUC value is indicated in each panel.

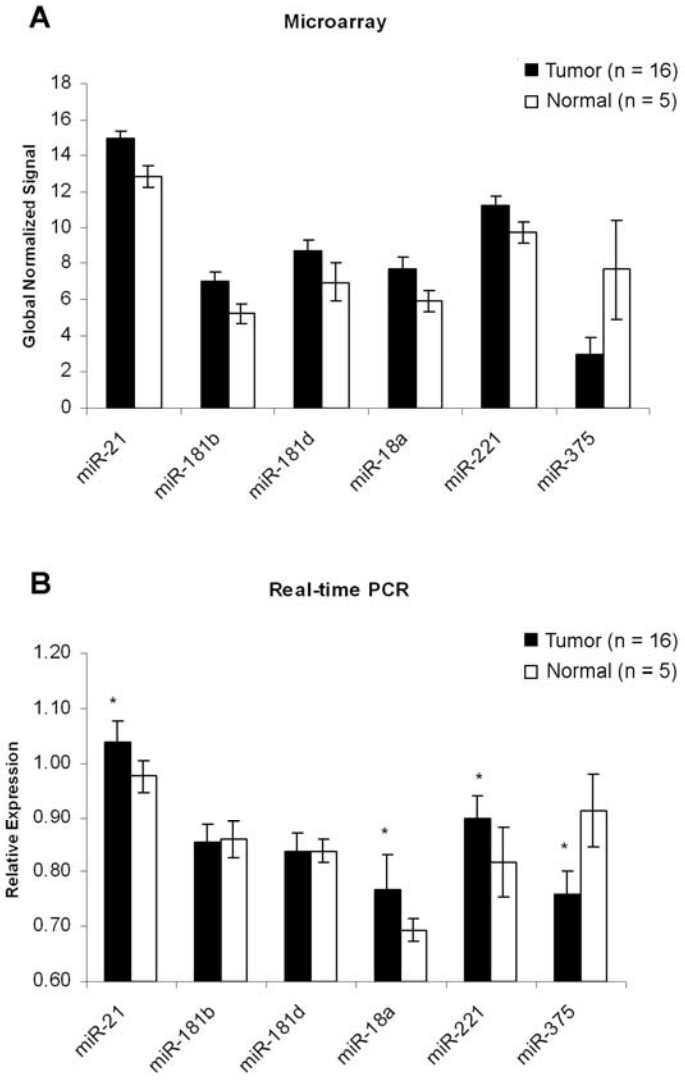
Table 1. Annotated human microRNAs differentially expressed in tumor versus normal by Significance Analysis of Microarray ($Q < 0.001$)

miRNAs	Fold change
miR-21	3.67
miR-181d	2.86
miR-181b	2.97
miR-491	5.08
miR-455	2.58
miR-18a	2.78
miR-130b	4.17
miR-221	2.26
miR-193b	3.44
miR-181a	2.11
miR-18b	2.5
miR-375	-21.88

Abbreviation: miRNAs, microRNAs.

Table 2. Examination of sensitivity and specificity of disease prediction using a microRNA expression ratio > 1.0

Ratio	Sensitivity	Specificity
miR-21/miR-375	0.99	0.14
miR-18a/miR-375	0.52	1
miR-221/miR-375	0.92	0.93



Supplementary Figure 1. Quantitative real-time PCR was used to confirm differential expression of six miRNAs identified as aberrantly expressed in the microarray. (A) Global normalized signal of six miRNAs found to be significantly differentially expressed by SAM method. (B) Relative expression of same six miRNAs determined by real-time PCR. * indicates $P < 0.01$.

Supplementary Table 1. Differentially expressed miRNA in cell lines vs. tumors as determined by SAM method (Q < .001)

miRNA	fold change
hsa_miR_143	-232.76
hsa_miR_145	-244.22
hsa_miR_199a	-88.23
hsa_miR_199a_AS	-127.06
hsa_miR_146b	-38.49
hsa_miR_223	-81.81
hsa_miR_214	-34.36
hsa_miR_126	-37.12
hsa_miR_34a	-11.68
ambi_miR_3046	-29.63
ambi_miR_13258	-9.50
ambi_miR_2660	-9.35
mmu_miR_10b	-11.09
hsa_miR_10b	-9.04
hsa_miR_142_5p	-9.74
ambi_miR_10411	-6.13
mmu_miR_451	-32.32
ambi_miR_7029	-29.44
hsa_miR_152	-5.09
mmu_miR_140_AS	-4.53
hsa_miR_451	-25.01
hsa_miR_199b	-6.69
ambi_miR_13268	-3.28
hsa_miR_34b	-3.30
rno_miR_140_AS	-3.43
mmu_miR_99a	-10.28
hsa_miR_368	-6.22
hsa_miR_99a	-9.55
hsa_miR_24	-2.60
hsa_miR_497	-4.70
hsa_miR_26b	-2.94
hsa_miR_342	-3.37
rno_miR_497	-5.08
hsa_miR_195	-5.03
ambi_miR_2837	-4.39
mmu_miR_199b	-4.20
mmu_miR_203	-22.71
ambi_miR_13205	-2.90
hsa_miR_22	-2.41
hsa_miR_203	-15.66
hsa_miR_150	-7.22
hsa_miR_146a	-9.06
rno_miR_409_3p	-2.92
hsa_miR_27b	-2.45
ambi_miR_444	-2.29
ambi_miR_13232	-2.18

ambi_miR_9651	-2.70
hsa_miR_27a	-2.09
mmu_miR_379	-2.49
hsa_miR_125b	-3.76
hsa_miR_455	-2.02
hsa_miR_452	-3.79
hsa_miR_29c	-2.98
ambi_miR_9451	-2.40
hsa_miR_210	-3.61
ambi_miR_12061	-2.28
hsa_miR_483	-1.87
hsa_miR_126_AS	-2.90
ambi_miR_13260	-2.32
rno_miR_29c_AS	-2.08
mmu_miR_341	-3.14
hsa_miR_139	-2.59
rno_miR_382	-2.24
hsa_miR_189	-2.07
hsa_miR_326	-2.02
mmu_miR_487b	-2.45

Supplementary Table 2. Differentially expressed miRNAs in tumor vs normal as determined by SAM method (Q <.001)

miRNA	fold change
ambi_miR_562	5.24
hsa_miR_21	3.67
ambi_miR_7083	5.45
hsa_miR_181d	2.86
hsa_miR_181b	2.97
hsa_miR_491	5.08
hsa_miR_455	2.58
ambi_miR_13258	2.56
hsa_miR_18a	2.78
ambi_miR_11541	2.28
hsa_miR_130b	4.17
mmu_miR_503	2.63
hsa_miR_221	2.26
hsa_miR_193b	3.44
hsa_miR_181a	2.11
mmu_miR_221	2.17
hsa_miR_18b	2.50
hsa_miR_375	-21.88

Chapter 3

MicroRNA Expression in Head and Neck Cancer Associates with Alcohol Consumption and Survival

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MicroRNA Expression in Head and Neck Cancer Associates with Alcohol Consumption and Survival

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Running Title: miRNA expression associates with clinical features in HNSCC

Keywords: microRNA, Head and Neck Cancer, alcohol

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Abstract

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 in HNSCCs compared to non-diseased epithelia and showed that there is potential diagnostic utility in examining their expression. Here, we used quantitative real-time PCR 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 (HR, 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 bring 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 mRNA targets, miRNAs interfere with mRNA translation or cause mRNA degradation thereby repressing gene expression post-transcriptionally (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 are 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 to normal tissues (18), and examined associations with clinicopathologic 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.

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, Inc., 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-PCR. TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) were used to quantify mature miRNA of *miR-21*, *miR-18a*, *miR-375*, and *miR-218*. cDNA 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, Foster City, CA). A 10 µl aliquot of total RNA diluted to a final concentration of 5 ng/µl was used for each reverse transcription (RT) reaction along with other RT components, per manufacturer's specifications. 40 µl 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 PCR data were quantified by calculating fold change using the $\Delta\Delta C_T$ 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, North Carolina). 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 (28) 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 vs oral and laryngeal vs. oral tumors. Spearman's rank correlation was used for continuous variables. Linear regression analysis was used for multivariate testing for associations with exposures and clinicopathologic features. Using the Kaplan–Meier method and the log-rank 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 greater than 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 more than or equal to 36.75 pack-years and drank more than or equal to 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. Quantitative real-time PCR 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.

***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 to nondrinkers ($P = 0.039$ 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 to 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 (HR = 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 (14,17,29-31). 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 likely 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 clinicopathologic 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 (32-35). *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 (36,37).

miR-21 is one of the best studied miRNAs with a clear role in carcinogenesis and has several confirmed targets (38,39). Though *miR-375* has mainly been studied in the context of diabetes, as it influences beta-cell mass and insulin levels (40,41), its expression has been shown to be decreased in a number of malignancies including pancreatic adenocarcinomas and esophageal squamous cell and adenocarcinomas (42,43). Additionally, the recent identification of a target for *miR-375*, phosphoinosidtide-dependent protein kinase-1 (PDPK1), suggests a feasible role for *miR-375* as a tumor suppressor since PDPK1 is crucial for the activation of anti-apoptotic *AKT* (44).

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 (45-47). 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 (48). The oxidation of ethanol in the saliva by mucosal and microbial alcohol dehydrogenases results in the production of acetaldehyde, which is a known carcinogen in animals and possible carcinogen in humans (49,50). Additionally, it is well established that alcohol interferes with the absorption of folate, a methyl-donor critical for maintenance of normal DNA methylation patterns (51). Though expression of *miR-375* is lower overall in tumors compared to 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 (52,53). 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 (15,16,54). Several targets of *miR-21* have been experimentally validated, many of which are tumor-suppressor genes (55-57). One of these targets, programmed cell death 4 (PDCD4) 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

which shows reduced expression in HNSCC is *PTEN*, a gene whose product inhibits growth and cell survival through antagonism of the AKT/PI3K pathway (58). 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 sites. 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.

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References

1. Chin, D., Boyle, G.M., Porceddu, S., Theile, D.R., Parsons, P.G. and Coman, W.B. (2006) Head and neck cancer: past, present and future. *Expert Rev Anticancer Ther*, **6**, 1111-8.
2. Parkin, D.M., Bray, F., Ferlay, J. and Pisani, P. (2005) Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.
3. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M.J. (2008) Cancer statistics, 2008. *CA Cancer J Clin*, **58**, 71-96.
4. Ragin, C.C., Modugno, F. and Gollin, S.M. (2007) The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. *J Dent Res*, **86**, 104-14.
5. Lothaire, P., de Azambuja, E., Dequanter, D., Lalami, Y., Sotiriou, C., Andry, G., Castro, G., Jr. and Awada, A. (2006) Molecular markers of head and neck squamous cell carcinoma: promising signs in need of prospective evaluation. *Head Neck*, **28**, 256-69.
6. Thomas, G.R., Nadiminti, H. and Regalado, J. (2005) Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol*, **86**, 347-63.
7. Chen, C., Mendez, E., Houck, J., Fan, W., Lohavanichbutr, P., Doody, D., Yueh, B., Futran, N.D., Upton, M., Farwell, D.G., Schwartz, S.M. and Zhao, L.P. (2008) Gene expression profiling identifies genes predictive of oral squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 2152-62.

8. Fan, C.Y. (2004) Epigenetic alterations in head and neck cancer: prevalence, clinical significance, and implications. *Curr Oncol Rep*, **6**, 152-61.

9. Marsit, C.J., Christensen, B.C., Houseman, E.A., Karagas, M.R., Wrensch, M.R., Yeh, R.F., Nelson, H.H., Wiemels, J.L., Zheng, S., Posner, M.R., McClean, M.D., Wiencke, J.K. and Kelsey, K.T. (2009) Epigenetic profiling reveals etiologically distinct patterns of DNA methylation in head and neck squamous cell carcinoma. *Carcinogenesis*, **30**, 416-22.

10. Worsham, M.J., Chen, K.M., Meduri, V., Nygren, A.O., Errami, A., Schouten, J.P. and Benninger, M.S. (2006) Epigenetic events of disease progression in head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg*, **132**, 668-77.

11. Schickel, R., Boyerinas, B., Park, S.M. and Peter, M.E. (2008) MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. *Oncogene*, **27**, 5959-74.

12. Ambros, V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350-5.

13. Cullen, B.R. (2004) Transcription and processing of human microRNA precursors. *Mol Cell*, **16**, 861-5.

14. Calin, G.A., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S.E., Iorio, M.V., Visone, R., Sever, N.I., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M. and Croce, C.M. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*, **353**, 1793-801.

15. Yan, L.X., Huang, X.F., Shao, Q., Huang, M.Y., Deng, L., Wu, Q.L., Zeng, Y.X. and Shao, J.Y. (2008) MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*, **14**, 2348-60.

16. Schetter, A.J., Leung, S.Y., Sohn, J.J., Zanetti, K.A., Bowman, E.D., Yanaihara, N., Yuen, S.T., Chan, T.L., Kwong, D.L., Au, G.K., Liu, C.G., Calin, G.A., Croce, C.M. and Harris, C.C. (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*, **299**, 425-36.

17. Chen, Y. and Stallings, R.L. (2007) Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res*, **67**, 976-83.

18. Avissar, M., Christensen, B.C., Kelsey, K.T. and Marsit, C.J. (2009) MicroRNA Expression Ratio Is Predictive of Head and Neck Squamous Cell Carcinoma. *Clin Cancer Res*, **15**, 2850-5.

19. Chang, S.S., Jiang, W.W., Smith, I., Poeta, L.M., Begum, S., Glazer, C., Shan, S., Westra, W., Sidransky, D. and Califano, J.A. (2008) MicroRNA alterations in head and neck squamous cell carcinoma. *Int J Cancer*, **123**, 2791-2797.

20. Childs, G., Fazzari, M., Kung, G., Kawachi, N., Brandwein-Gensler, M., McLemore, M., Chen, Q., Burk, R.D., Smith, R.V., Prystowsky, M.B., Belbin, T.J. and Schlecht, N.F. (2009) Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. *Am J Pathol*, **174**, 736-45.

21. Ramdas, L., Giri, U., Ashorn, C.L., Coombes, K.R., El-Naggar, A., Ang, K.K. and Story, M.D. (2009) miRNA expression profiles in head and neck squamous cell carcinoma and adjacent normal tissue. *Head Neck*, **31**, 642-54.

22. Chaudhry, M.A. (2009) Real-time PCR analysis of micro-RNA expression in ionizing radiation-treated cells. *Cancer Biother Radiopharm*, **24**, 49-56.

23. Marsit, C.J., McClean, M.D., Furniss, C.S. and Kelsey, K.T. (2006) Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. *Int J Cancer*, **119**, 1761-6.

24. Zhang, B. and Pan, X. (2009) RDX induces aberrant expression of microRNAs in mouse brain and liver. *Environ Health Perspect*, **117**, 231-40.

25. Peters, E.S., McClean, M.D., Liu, M., Eisen, E.A., Mueller, N. and Kelsey, K.T. (2005) The ADH1C polymorphism modifies the risk of squamous cell carcinoma of the head and neck associated with alcohol and tobacco use. *Cancer Epidemiol Biomarkers Prev*, **14**, 476-82.

26. Furniss, C.S., Marsit, C.J., Houseman, E.A., Eddy, K. and Kelsey, K.T. (2008) Line region hypomethylation is associated with lifestyle and differs by human papillomavirus status in head and neck squamous cell carcinomas. *Cancer Epidemiol Biomarkers Prev*, **17**, 966-71.

27. Furniss, C.S., McClean, M.D., Smith, J.F., Bryan, J., Nelson, H.H., Peters, E.S., Posner, M.R., Clark, J.R., Eisen, E.A. and Kelsey, K.T. (2007) Human papillomavirus 16 and head and neck squamous cell carcinoma. *Int J Cancer*, **120**, 2386-92.

28. Hashibe, M., Brennan, P., Chuang, S.C., Boccia, S., Castellsague, X., Chen, C., Curado, M.P., Dal Maso, L., Daudt, A.W., Fabianova, E., Fernandez, L., Wunsch-Filho, V., Franceschi, S., Hayes, R.B., Herrero, R., Kelsey, K., Koifman, S., La Vecchia, C., Lazarus, P., Levi, F., Lence, J.J., Mates, D., Matos, E., Menezes, A., McClean, M.D., Muscat, J., Eluf-Neto, J., Olshan, A.F., Purdue, M., Rudnai, P., Schwartz, S.M., Smith, E., Sturgis, E.M., Szeszenia-Dabrowska, N., Talamini, R., Wei, Q., Winn, D.M., Shangina, O., Pilarska, A., Zhang, Z.F., Ferro, G., Berthiller, J. and Boffetta, P. (2009) Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiol Biomarkers Prev*, **18**, 541-50.

29. Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knoflickova, D., Bednarikova, M., Nenutil, R. and Vyzula, R. (2007) Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology*, **72**, 397-402.

30. Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., Stephens, R.M., Okamoto, A., Yokota, J., Tanaka, T., Calin, G.A., Liu, C.G., Croce, C.M. and Harris, C.C. (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*, **9**, 189-98.

31. Yu, S.L., Chen, H.Y., Chang, G.C., Chen, C.Y., Chen, H.W., Singh, S., Cheng, C.L., Yu, C.J., Lee, Y.C., Chen, H.S., Su, T.J., Chiang, C.C., Li, H.N., Hong, Q.S., Su, H.Y., Chen, C.C., Chen, W.J., Liu, C.C., Chan, W.K., Li, K.C., Chen,

J.J. and Yang, P.C. (2008) MicroRNA signature predicts survival and relapse in lung cancer. *Cancer Cell*, **13**, 48-57.

32. Liu, W.H., Yeh, S.H., Lu, C.C., Yu, S.L., Chen, H.Y., Lin, C.Y., Chen, D.S. and Chen, P.J. (2009) MicroRNA-18a prevents estrogen receptor-alpha expression, promoting proliferation of hepatocellular carcinoma cells. *Gastroenterology*, **136**, 683-93.

33. Guo, J., Miao, Y., Xiao, B., Huan, R., Jiang, Z., Meng, D. and Wang, Y. (2009) Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. *J Gastroenterol Hepatol*, **24**, 652-7.

34. Motoyama, K., Inoue, H., Takatsuno, Y., Tanaka, F., Mimori, K., Uetake, H., Sugihara, K. and Mori, M. (2009) Over- and under-expressed microRNAs in human colorectal cancer. *Int J Oncol*, **34**, 1069-75.

35. Nam, E.J., Yoon, H., Kim, S.W., Kim, H., Kim, Y.T., Kim, J.H., Kim, J.W. and Kim, S. (2008) MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res*, **14**, 2690-5.

36. Park, J.K., Lee, E.J., Esau, C. and Schmittgen, T.D. (2009) Antisense Inhibition of microRNA-21 or -221 Arrests Cell Cycle, Induces Apoptosis, and Sensitizes the Effects of Gemcitabine in Pancreatic Adenocarcinoma. *Pancreas*.

37. Zhang, C., Kang, C., You, Y., Pu, P., Yang, W., Zhao, P., Wang, G., Zhang, A., Jia, Z., Han, L. and Jiang, H. (2009) Co-suppression of miR-221/222 cluster suppresses human glioma cell growth by targeting p27kip1 in vitro and in vivo. *Int J Oncol*, **34**, 1653-60.

38. Krichevsky, A.M. and Gabriely, G. (2009) miR-21: a small multi-faceted RNA. *J Cell Mol Med*, **13**, 39-53.

39. Selcuklu, S.D., Donoghue, M.T. and Spillane, C. (2009) miR-21 as a key regulator of oncogenic processes. *Biochem Soc Trans*, **37**, 918-25.

40. Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P.E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. and Stoffel, M. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*, **432**, 226-30.

41. Poy, M.N., Hausser, J., Trajkovski, M., Braun, M., Collins, S., Rorsman, P., Zavolan, M. and Stoffel, M. (2009) miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A*, **106**, 5813-8.

42. Mathe, E.A., Nguyen, G.H., Bowman, E.D., Zhao, Y., Budhu, A., Schetter, A.J., Braun, R., Reimers, M., Kumamoto, K., Hughes, D., Altorki, N.K., Casson, A.G., Liu, C.G., Wang, X.W., Yanaihara, N., Hagiwara, N., Dannenberg, A.J., Miyashita, M., Croce, C.M. and Harris, C.C. (2009) MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. *Clin Cancer Res*, **15**, 6192-200.

43. Bloomston, M., Frankel, W.L., Petrocca, F., Volinia, S., Alder, H., Hagan, J.P., Liu, C.G., Bhatt, D., Taccioli, C. and Croce, C.M. (2007) MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA*, **297**, 1901-8.

44. El Ouaamari, A., Baroukh, N., Martens, G.A., Lebrun, P., Pipeleers, D. and van Obberghen, E. (2008) miR-375 targets 3'-phosphoinositide-dependent protein

kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. *Diabetes*, **57**, 2708-17.

45. Pietrzykowski, A.Z., Friesen, R.M., Martin, G.E., Puig, S.I., Nowak, C.L., Wynne, P.M., Siegelmann, H.T. and Treistman, S.N. (2008) Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron*, **59**, 274-87.

46. Wang, L.L., Zhang, Z., Li, Q., Yang, R., Pei, X., Xu, Y., Wang, J., Zhou, S.F. and Li, Y. (2009) Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. *Hum Reprod*, **24**, 562-79.

47. Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S. and Zucman-Rossi, J. (2008) MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology*, **47**, 1955-63.

48. Seitz, H.K. and Stickel, F. (2007) Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer*, **7**, 599-612.

49. Homann, N., Tillonen, J., Meurman, J.H., Rintamaki, H., Lindqvist, C., Rautio, M., Jousimies-Somer, H. and Salaspuro, M. (2000) Increased salivary acetaldehyde levels in heavy drinkers and smokers: a microbiological approach to oral cavity cancer. *Carcinogenesis*, **21**, 663-8.

50. Muto, M., Hitomi, Y., Ohtsu, A., Shimada, H., Kashiwase, Y., Sasaki, H., Yoshida, S. and Esumi, H. (2000) Acetaldehyde production by non-pathogenic

Neisseria in human oral microflora: implications for carcinogenesis in upper aerodigestive tract. *Int J Cancer*, **88**, 342-50.

51. Hamid, A., Wani, N.A. and Kaur, J. (2009) New perspectives on folate transport in relation to alcoholism-induced folate malabsorption--association with epigenome stability and cancer development. *FEBS J*, **276**, 2175-91.

52. Iorio, M.V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C.G., Alder, H., Calin, G.A., Menard, S. and Croce, C.M. (2007) MicroRNA signatures in human ovarian cancer. *Cancer Res*, **67**, 8699-707.

53. Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A., Downing, J.R., Jacks, T., Horvitz, H.R. and Golub, T.R. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-8.

54. Markou, A., Tsaroucha, E.G., Kaklamanis, L., Fotinou, M., Georgoulias, V. and Lianidou, E.S. (2008) Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. *Clin Chem*, **54**, 1696-704.

55. Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A. and Lund, A.H. (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem*, **283**, 1026-33.

56. Li, T., Li, D., Sha, J., Sun, P. and Huang, Y. (2009) MicroRNA-21 directly targets MARCKS and promotes apoptosis resistance and invasion in prostate cancer cells. *Biochem Biophys Res Commun*.

57. Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T. and Patel, T. (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*, **133**, 647-58.

58. Pedrero, J.M., Carracedo, D.G., Pinto, C.M., Zapatero, A.H., Rodrigo, J.P., Nieto, C.S. and Gonzalez, M.V. (2005) Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. *Int J Cancer*, **114**, 242-8.

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]	
0 (none)	22 (15.5)
>0 to <36.75	59 (41.5)
≥36.75	61 (43.0)
Drinks per week[b]	
none(0)	16 (11.5)
>0 to <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 5 samples

[e]Data missing in 60 samples

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

		log miR21		log miR375		log miR18a		log miR221	
	n (%)	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									
0 (none)	22 (15.5)	1.54 (1.69)		-2.47 (2.1)		-0.20 (1.3)		1.66 (1.80)	
>0 to <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 to <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 vs oral and laryngeal vs 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	
>0 to <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	
>0 to <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**

[a] Trend test *P*= 0.002

Table IV. Cox Proportional Hazards Multivariate Model (n = 150)[a]

			95% CI		
	n (%)	Hazard Ratio	Lower	Upper	*p*
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

[a] Survival data missing in 15 samples

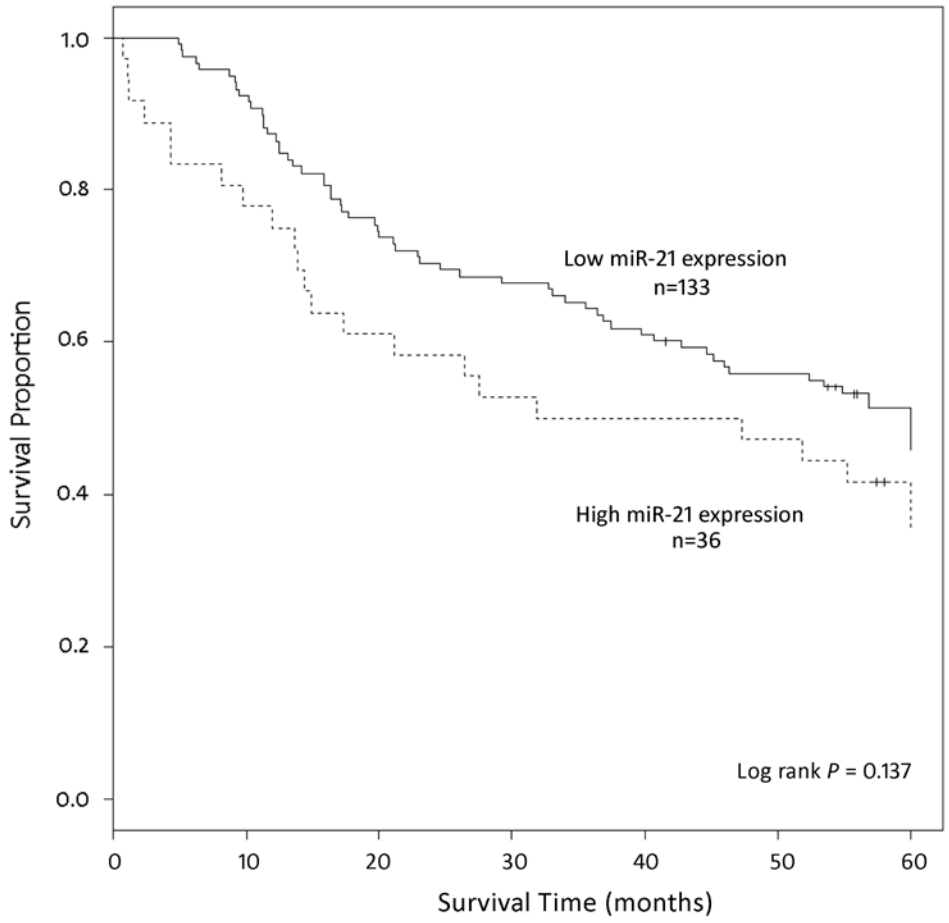

Fig. 1. Kaplan-Meier curves show differences in survival between patients with *miR-21* expression in the highest 25th percentile (dotted line) compared to all other patients (solid line). Vertical hatch marks represent censored data.

Supplementary Table I. Log-transformed normalized fold-change expression of miRNAs

microRNA	Mean expression (range)
miR-21	1.66 (-2.15, 5.74)
miR-375	-2.3 (-7.76, 2.82)
miR-221	1.36 (-2.36, 6.44)
miR-18a	-0.3 (-3.01, 3.53)

Chapter 4

Establishing a system for investigating the role of *miR-375* in head and neck cancer

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Establishing a system for investigating the role of *miR-375* in head and neck cancer

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Abstract

MicroRNAs (miRNA) are small non-coding RNA molecules, which influence biological functions through their interactions with multiple targets. Overexpression studies have been indispensable for the validation of miRNA targets and determination of cellular functions. Our recent study has shown *miR-375* expression to be down-regulated in head and neck squamous cell carcinoma (HNSCC), leading us to hypothesize that this miRNA may function as a tumor suppressor in HNSCC carcinogenesis. We have looked at the effects on proliferation, migration and sensitivity to cisplatin (CDDP), in cells transfected with mature *miR-375* mimic molecules and those transfected with a plasmid vector carrying the premature *miR-375* sequence under the control of a CMV promoter. *miR-375* mimic transfection resulted in >2,000 fold upregulation in *miR-375* levels, faster proliferation and migration and increased resistance to CDDP in *miR-375* transfected cells. On the other hand, the *miR-375* expression vector-based transfection only increased *miR-375* expression by ~4 fold and caused slower proliferation and increased sensitivity to CDDP. Our results have shown that different methods of miRNA overexpression vary considerably in their resultant biological effects perhaps due to disparities in the extent to which they analogize endogenous miRNAs. As such, the caveats of their use in such studies must be carefully considered.

Introduction

MicroRNAs (miRNA) are now known to be important post-transcriptional regulators of gene expression, orchestrating diverse molecular functions through their mRNA targets (1). Primary miRNA transcripts (pri-miRNA) of 100s to 1000s of base pairs in length are transcribed from intergenic regions or intronic sequences into large stem-loop structures which are sequentially processed first generating a ~70 bp hairpin, which is exported out of the nucleus, and then a mature ~22 bp duplex (2). The active strand of the duplex is incorporated into a ribonucleoprotein effector complex known as RNA-induced silencing complex (RISC) in the cytoplasm. The miRNA/RISC can then target a range of partially or fully complementary mRNA transcripts, resulting either in their translational repression or degradation, respectively (2).

An involvement of miRNAs has been identified for almost all major cancers (3). Many of the miRNAs significantly altered in cancer tend to target genes that regulate cell proliferation, differentiation and death, disruptions in which are classically associated with malignancy. Our previous work showed *miR-375*, a miRNA primarily associated with pancreatic islet cells (4), to be down-regulated in head and neck squamous cell carcinoma (HNSCC) compared to non-diseased tissue (5). *miR-375* has been shown to target 3'-phosphoinositide-dependent protein kinase-1 (PDPK-1) in pancreatic beta cells (6). As PDPK-1 is a major activator of anti-apoptotic AKT (7), it is possible that *miR-375* down-regulation in HNSCC potentiates a pro-survival carcinogenic phenotype.

The growing interest in miRNAs' involvement in disease has spurred the development of myriad systems designed to study their expression levels, identify targets, and define their pleiotropic roles in vitro as well as in vivo (8). Given their diminutive

size, and the fact that they do not code for proteins, the detection of miRNAs has required customized technologies including the development of specialized miRNA arrays and adaptation of traditional real-time PCR techniques for their quantification. The examination of miRNA functions in cultured cell lines carries several caveats as their expression is generally lower in cell lines compared to analogous primary tissues (9,10).

Here we have performed several experiments to test changes in cell proliferation, migration and sensitivity to cisplatin, a chemotherapeutic agent commonly used in the treatment of HNSCC. Two different systems were used for overexpression of *miR-375* in the invasive HNSCC cell line FaDu. The first system involves transient transfection with a synthetic double-stranded RNA molecule designed to mimic the mature miRNA. Functioning much like small interfering RNAs (siRNAs), these miRNA precursors are chemically modified to ensure that the appropriate strand becomes incorporated into the RISC. The second method utilizes a vector-based system to allow expression of an engineered miRNA sequence from a Pol II promoter. This expression vector contains the human cytomegalovirus (CMV) immediate early promoter, allowing for constitutive miRNA expression in mammalian cells. Both proprietary systems have advantages caveats to their use and this study aims to investigate which system is better suited for determination of miRNA function and which most closely reproduces miRNAs generated endogenously.

Materials and Methods

Cell lines

FaDu cells were obtained from ATCC (HTB-43) and cultured in Eagles's Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Reverse transfection of miRNA mimics

Pre-miR™ miRNA Precursor Molecules (Ambion) were transfected into cells according to manufacturer's instructions. Briefly, 0.24 µl or 2.4 µl of precursor and .45 or 4.5 µl of NeoFX Transfection Agent (Ambion) were diluted in Opti-MEM Reduced Serum Medium, combined and applied to cells while passaging. Normal growth medium was replaced after 24 hours and cells were used for experiments after another 24 hours.

Creation and transfection of *miR-375* expression constructs

Single stranded oligonucleotides containing the premature *miR-375* sequence were designed as follows: sense, 5′-TGCTGCCCCGCGACGAGCCCC-TCGCACAAACCGGACCTGAGCGTTTTGTTCGTTCGGCTCGCGTGAGGC-3′; and antisense, 5′-CCTGGCCTCACGCGAGCCGAACGAACAAAACGCTCAGGTCCGGTTTGTG CGAGGGGCTCGTCGCGGGGC-3′. The oligos were annealed and the double-stranded oligo was ligated into the Block-iT™ Pol II miR RNAi Expression Vector according to the manufacturer's instructions (Invitrogen). Both *miR-375* and negative control plasmids

were transformed into TOP10 Competent E.coli using One Shot TOP10 Transformation Protocol (Invitrogen). Transformants were analyzed by sequencing and successful clones expanded, and their plasmids purified by maxi-prep (Qiagen). FaDu cells were seeded to 90% confluency in 6-well plates the day before transfection. A vector containing either *miR-375* or the negative control miRNA plasmid was transfected into cells by applying 10 µl Lipofectamine-2000 (Invitrogen) and 4 µg plasmid DNA, each diluted in Opti-MEM Reduced Serum Medium (Invitrogen) and combined. Medium was changed to normal growth medium after 4 hours incubation. Vectors contained the coding sequence of EmGFP (Emerald Green Fluorescent Protein) such that the pre-miRNA insertion site is in the 3' untranslated (3'UTR) region of the fluorescent protein mRNA, allowing for determination of transfection efficiency. Cells were used for experiments 24 hours after transfection.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using mirVana miRNA Isolation Kit (Ambion Inc.) according to manufacturer's protocol. Reverse Transcription PCR using a GeneAmp PCR System 9700 (Applied Biosystems) was performed using 5X primers for *hsa-miR-375* and RNU48 as an endogenous control (Applied Biosystems). An ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) was used for quantitative real-time PCR analysis using 20X probes for *hsa-miR-375* and RNU48. The expression levels of *miR-375* were normalized to levels of RNU48. Technical triplicates were performed.

Wound healing assay

Six-well plates were seeded with 1.2 million cells per well and transfected using the precursor molecules as above. Forty-eight hours after transfection, the wells were scratched lightly with 200 μl pipette tips. The rate of wound closure was recorded at 12-hour intervals for 60 hours using 20X magnified microscope photographs. Wound closure was then measured using a fixed angle ruler tool in Adobe Photoshop CS3. Experimental and control conditions were performed in duplicate.

Proliferation assay

96-well plates were seeded with 5000 cells per well. Cells were stained with 20 μL per well of CellTiter 96 Aqueous One Solution (Promega) and incubated for one hour at 37°C before plates were read using a SpectraMax M2 Microtiter Plate Reader. Plates were then analyzed at 6 hours to establish a baseline reading, followed by readings at 24, 48, and 72 hours. SoftMax Pro software was used to record absorbance at the indicated time points. Experimental and control conditions were performed in 12 technical replicates.

Clonogenic assay and CDDP treatment

For clonogenic survival studies, 10 mm dishes were seeded with 3000 transfected cells and either allowed to grow for 14 days to examine basal growth of transfected cells or allowed to adhere overnight before treating with 0.5 μM cisplatin (CDDP) or dimethyl formamide (DMF) control for 24 hrs before allowing to grow for 14 days. Experimental and control conditions were performed in triplicate.

Results

Transfection of cells with *miR-375* miRNA mimic leads to strong overexpression of *miR-375*, increased colony growth and migration

Precursor molecules mimicking the mature form of *miR-375* or a control sequence with no known gene target were transfected into the invasive HNSCC cell line, FaDu, at increasing concentrations. Real-time PCR analysis showed strong upregulation of *miR-375*, from ~2000 fold to >10,000 fold overexpression compared to control-transfected cells (Figure 1A).

Effects of increasing *miR-375* expression on FaDu cells were assessed by clonogenic assay, which showed an overall increase in colony formation across transfection groups (Figure 1B; $P = 0.002$, ANOVA). A wound healing assay was performed following transfection with 5 nM *miR-375* precursor or negative control. *miR-375*-transfected cells tended to show faster wound closure compared to control (Figure 1C).

Transfection of cells with *miR-375* miRNA mimic leads to increased proliferation and resistance to CDDP

A cell proliferation assay was used to test the effects of *miR-375* mimic-transfection on proliferation of FaDu cells. Results showed that *miR-375*-transfected cells proliferated slightly faster than control-transfected cells (Figure 2A). To test the effect of *miR-375* mimic-transfection on sensitivity to CDDP, a commonly used chemotherapeutic drug used in the treatment of HNSCC (11), a clonogenic assay was performed following 0.5

μM CDDP exposure. *miR-375*-transfected cells showed greater survival following CDDP treatment than negative control-transfected cells (Figure 2B).

Transient transfection of cells with *miR-375* expression constructs leads to modest overexpression of *miR-375* decreased proliferation and increased sensitivity to CDDP

miR-375 expression constructs were created by inserting the premature *miR-375* sequence into a Pol II expression vector and two transformants were selected for purification. Transient transfection efficiency was determined to be ~40%, determined by counting the GFP-positive cells (data not shown). Following transfection of expression plasmids into FaDu cells, *miR-375* expression was assessed using real-time PCR. *miR-375* expression in both constructs showed modest overexpression (~4 fold) compared to cells transfected with even a low concentration (0.5 nM) of *miR-375* precursor molecules (Figure 3). Contrary to the results seen following transfection with the miRNA mimic, cells transfected with *miR-375* expression constructs exhibited slower rates of proliferation and greater sensitivity to CDDP compared to negative control-transfected cells (Figure 4A-B).

Discussion

In vitro miRNA gain-of-function studies are important for identifying targets and determining biological function. This work has demonstrated two different methods of overexpressing *miR-375*, a miRNA that was found to be down-regulated in HNSCC. *miR-375* may act as a tumor-suppressor miRNA, normally targeting genes that are upregulated in cancer, such as its known target, PDPK-1 (6). We hypothesized that increasing *miR-375* expression in cancer cells would rescue some of their malignant characteristics such as increased proliferation, migration and resistance to chemotherapeutic agents. Though both types of transfections tested are commonly used in overexpression studies of miRNA, they produce drastically different results, both in the level of upregulation of the miRNA and the resultant phenotype seen in transfected cells.

The first method used was the more simplistic of the two, involving transfection, via a lipid-based transfection agent, of a small double stranded miRNA molecule, analogous to the miRNA in its mature form. The duplex is chemically modified in such a way as to ensure strand specificity and though the details of this modification are proprietary, they are likely based on the current comprehension of strand bias in small RNAs. In siRNA, the strand incorporated into RISC is the one with the 5' end that is less tightly paired to its complement (12). In human miRNA, it is known that the active strand shows U enrichment while the alternate strand shows C enrichment at its 5' end (13). Additionally the purine/pyrimidine content of the two strands is markedly different. Once inside cells, the active strand, having an identical sequence to the mature miRNA of

interest, binds the RISC in the cytoplasm and carries out its function of targeting its cognate mRNAs.

The second transfection method involved the generation of a *miR-375* expression vector which contains the premature sequence of *miR-375* under the control of a constitutive CMV immediate early promoter. Upon transfection, the plasmid enters the nucleus, the promoter is recognized by Pol II, and the *pre-miR-375* sequence is transcribed. From this point, the premature sequence likely forms a hairpin structure and is exported and further processed alongside endogenous pre-miRNAs.

Overexpression of *miR-375* using the miRNA precursor molecule system resulted in massive upregulation of the mature miRNA, increases in colony formation, proliferation, and migration, as measured by wound healing assay. Consistent with this, *miR-375* mimic-transfected cells were also more resistant CDDP, a chemotherapeutic agent commonly used to treat HNSCC (11). These results seemed to oppose the hypothesis that *miR-375* acts as a tumor-suppressor miRNA. However, the extremely high level of *miR-375* upregulation seen when using this transfection method calls into question the reliability of the results.

In our previous work, we showed that in primary tissues, there was a 22-fold reduction in *miR-375* expression in HNSCC tumors compared to normal epithelia(5). Endogenous miRNAs are a tightly regulated class of molecules which, in their mature form, are quite stable(14). Due to their wide regulatory scope, even small changes in miRNA expression, on the order of 2 or 3 fold-change, have been shown to influence biological function significantly (15,16). In the precursor transfection system, a large number of these mature miRNA analogs are transfected into cells and function at the

level of the mature miRNA. It is possible that oversaturation of the enzymatic effector complex, RISC, with these more stable, nonendogenous mimics may be interfering with the targeting functions of the many other miRNA that depend on RISC, resulting in an artifactual result related to inhibition of the functional pathway.

Using the expression vector system to increase *miR-375* levels resulted in a much more modest ~4 fold upregulation of the miRNA. Experiments also showed results opposite to the previous system, including slower proliferation and increased sensitivity to CDDP in *miR-375*-transfected cells compared to control. This system requires far more steps and is more complex than the previous system, as plasmid DNA must enter the nucleus and be recognized, transcribed and processed by nuclear enzymes. This is likely to result in far fewer mature miRNA being generated, as the processing rate is limited by endogenous cell machinery. Additionally, since a transient transfection only results in ~40% transfection efficiency, the population of cells in which miRNA expression is measured is quite heterogeneous, contributing to a lower expression change and a more muted phenotypic change when examining an entire population of cells.

Several experiments could be performed to confirm or address the potential problems inherent in the systems used to overexpress *miR-375*. A RISC activity assay, as described by Liu et al. (17), may be performed to confirm the hypothesis that miRNA mimics overwhelm the RISC. Additionally, the miRNA/RISC complexes can be isolated to determine the proportion of functional *miR-375* vs other miRNAs bound to RISC(18). Lysates of transfected cells would be subjected to immunoprecipitation using antibodies against Ago-2 proteins which interact with miRNAs in the RISC.

The expression vector system allows for generation of a stable line of cells overexpressing *miR-375*. Stable transfection may yield a more homogeneous population of cells with higher expression of *miR-375* and a more dramatic phenotypic change. Though this may have the desired effect on *miR-37*5 level, the observed phenotype may be influenced by compensatory mechanisms exerted by the cells in response to the vector's integration into the genome and the resultant constitutive expression of the miRNA.

The use of a luciferase reporter assay based on the *mir-37*5 target sequence would help to elucidate the relative levels of *miR-375* function in the two systems used (19). A construct bearing the firefly luciferase mRNA with the 3' UTR sequence of a known target of *miR-375*, PDPK-1, or a perfect complement of miR-375 could be designed and co-transfected with either the miR-375 mimic or expression construct and luciferase activity could be assayed to determine whether the transfected miRNAs are functioning as they do endogenously, inhibiting their target mRNA.

Compared to the miRNA precursor system, the expression vector system for miRNA overexpression seems to be a more relevant method for increasing miRNA expression as it more closely mirrors true miRNA processing and results in expression changes on the order of magnitude of physiologic miRNA alterations. To determine which system is best suited for miRNA functional analysis, additional work must be done to determine which allows for proper miRNA function without disruption of endogenous miRNA machinery.

References

1. Ambros, V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350-5.
2. Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102-14.
3. Croce, C.M. (2009) Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*, **10**, 704-14.
4. Poy, M.N., Hausser, J., Trajkovski, M., Braun, M., Collins, S., Rorsman, P., Zavolan, M. and Stoffel, M. (2009) miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A*, **106**, 5813-8.
5. Avissar, M., Christensen, B.C., Kelsey, K.T. and Marsit, C.J. (2009) MicroRNA Expression Ratio Is Predictive of Head and Neck Squamous Cell Carcinoma. *Clin Cancer Res*, **15**, 2850-5.
6. El Ouaamari, A., Baroukh, N., Martens, G.A., Lebrun, P., Pipeleers, D. and van Obberghen, E. (2008) miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. *Diabetes*, **57**, 2708-17.
7. Mora, A., Komander, D., van Aalten, D.M. and Alessi, D.R. (2004) PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol*, **15**, 161-70.
8. Schmittgen, T.D. (2008) Part B--microRNAs: microRNA methods. *Methods*, **44**, 1-2.

9. Blenkiron, C., Goldstein, L.D., Thorne, N.P., Spiteri, I., Chin, S.F., Dunning, M.J., Barbosa-Morais, N.L., Teschendorff, A.E., Green, A.R., Ellis, I.O., Tavare, S., Caldas, C. and Miska, E.A. (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol*, **8**, R214.

10. Szafranska, A.E., Davison, T.S., John, J., Cannon, T., Sipos, B., Maghnouj, A., Labourier, E. and Hahn, S.A. (2007) MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*, **26**, 4442-52.

11. Matta, A. and Ralhan, R. (2009) Overview of current and future biologically based targeted therapies in head and neck squamous cell carcinoma. *Head Neck Oncol*, **1**, 6.

12. Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, **115**, 199-208.

13. Hu, H.Y., Yan, Z., Xu, Y., Hu, H., Menzel, C., Zhou, Y.H., Chen, W. and Khaitovich, P. (2009) Sequence features associated with microRNA strand selection in humans and flies. *BMC Genomics*, **10**, 413.

14. Schmittgen, T.D., Lee, E.J., Jiang, J., Sarkar, A., Yang, L., Elton, T.S. and Chen, C. (2008) Real-time PCR quantification of precursor and mature microRNA. *Methods*, **44**, 31-8.

15. Calin, G.A. and Croce, C.M. (2006) MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res*, **66**, 7390-4.

16. Peltier, H.J. and Latham, G.J. (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA*, **14**, 844-52.

17. Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P. and Wang, X. (2003) R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science*, **301**, 1921-5.

18. Goff, L.A., Davila, J., Swerdel, M.R., Moore, J.C., Cohen, R.I., Wu, H., Sun, Y.E. and Hart, R.P. (2009) Ago2 immunoprecipitation identifies predicted microRNAs in human embryonic stem cells and neural precursors. *PLoS One*, **4**, e7192.

19. Gibcus, J.H., Tan, L.P., Harms, G., Schakel, R.N., de Jong, D., Blokzijl, T., Moller, P., Poppema, S., Kroesen, B.J. and van den Berg, A. (2009) Hodgkin lymphoma cell lines are characterized by a specific miRNA expression profile. *Neoplasia*, **11**, 167-76.

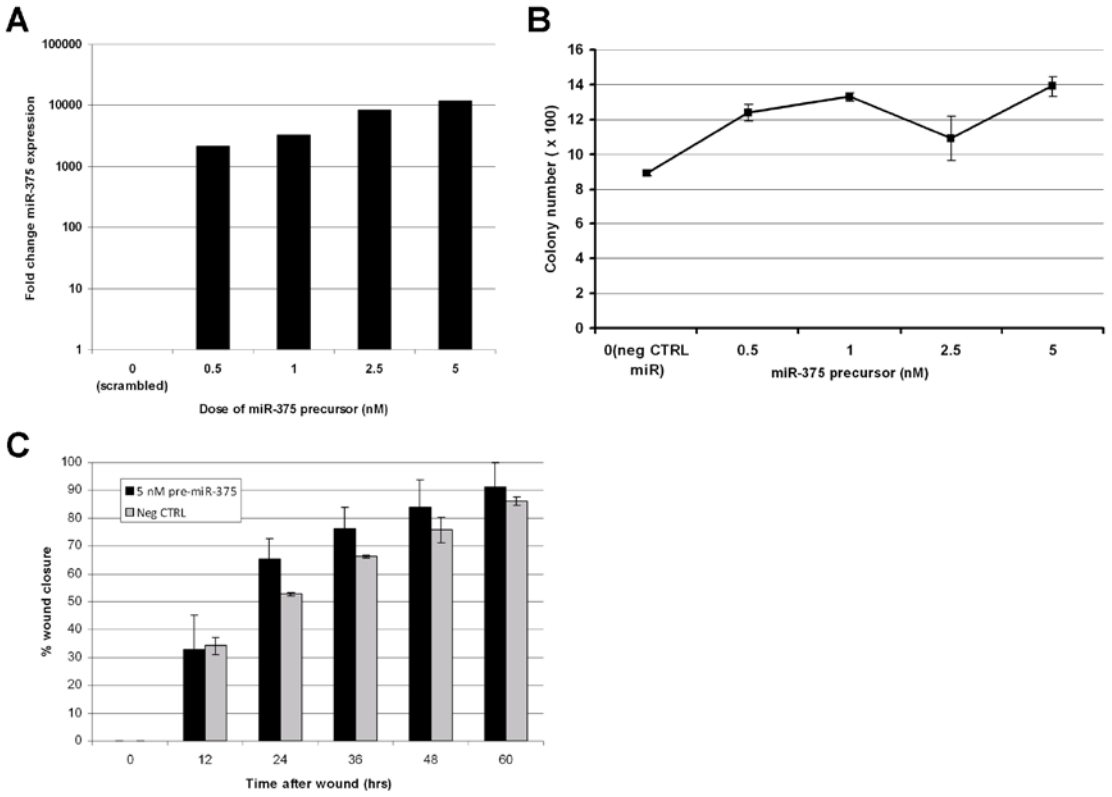

Figure 1. Precursor *miR-375* transfection in FaDu cells. (A) Increase in fold change expression of *miR-375* compared to negative control-transfected cells. Expression was normalized to RNU48 expression and was determined as fold-change above negative-control transfected cells by the $2^{-(\Delta\Delta C_T)}$ calculation.
(B) Increasing colony formation with increasing concentration of *miR-375* precursor. (C) Change in wound healing ability in cells transfected with 5 nM *miR-375* precursor compared to control-transfected cells.

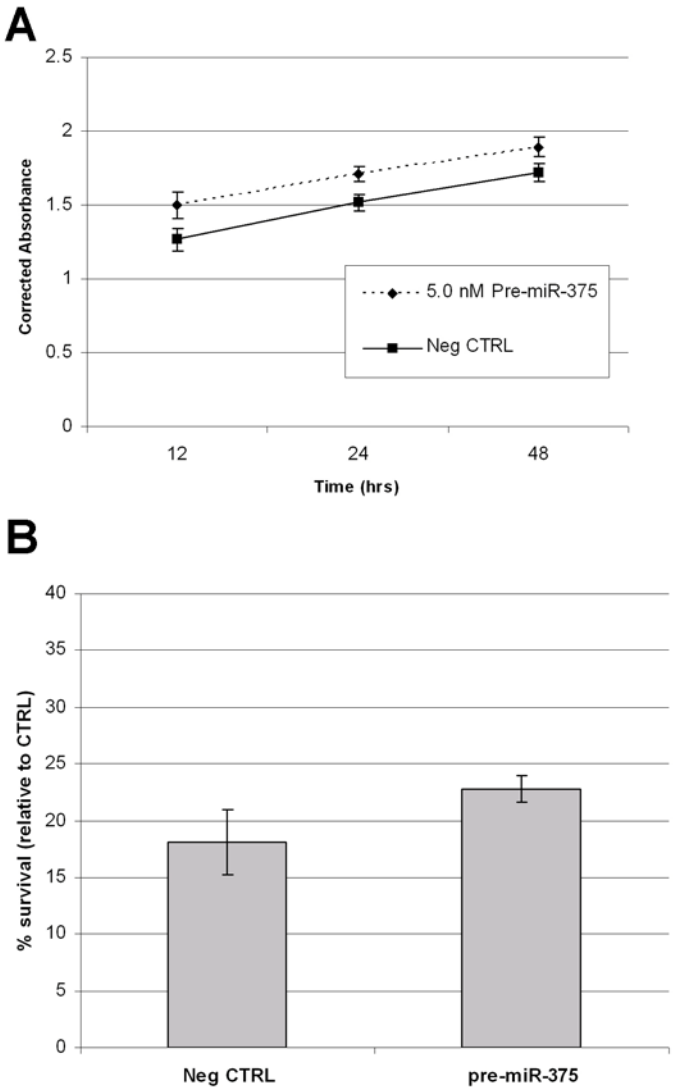

Figure 2. Precursor *miR-375* transfection in FaDu cells. (A) Increased proliferation rate in *miR-375*-transfected cells compared to control as measured by proliferation assay. (B) Increased resistance to 0.5 μM CDDP in *miR-375*-transfected cells compared to negative control as assessed by colony formation assay.

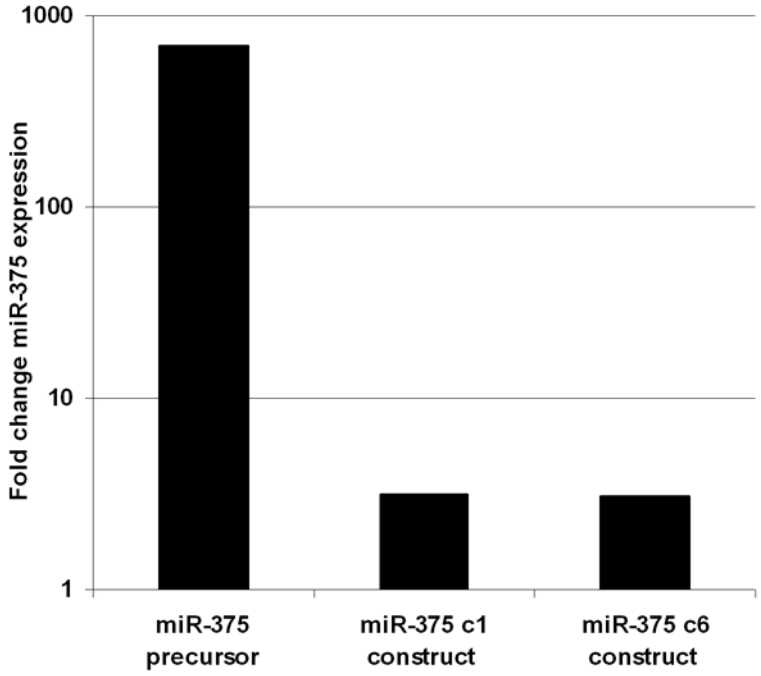

Figure 3. Quantitative real-time PCR showing *miR-375* expression following both methods of overexpression. Expression was normalized to RNU48 expression and was determined as fold-change above negative-control transfected cells by the $2^{-(\Delta\Delta C_T)}$ calculation.

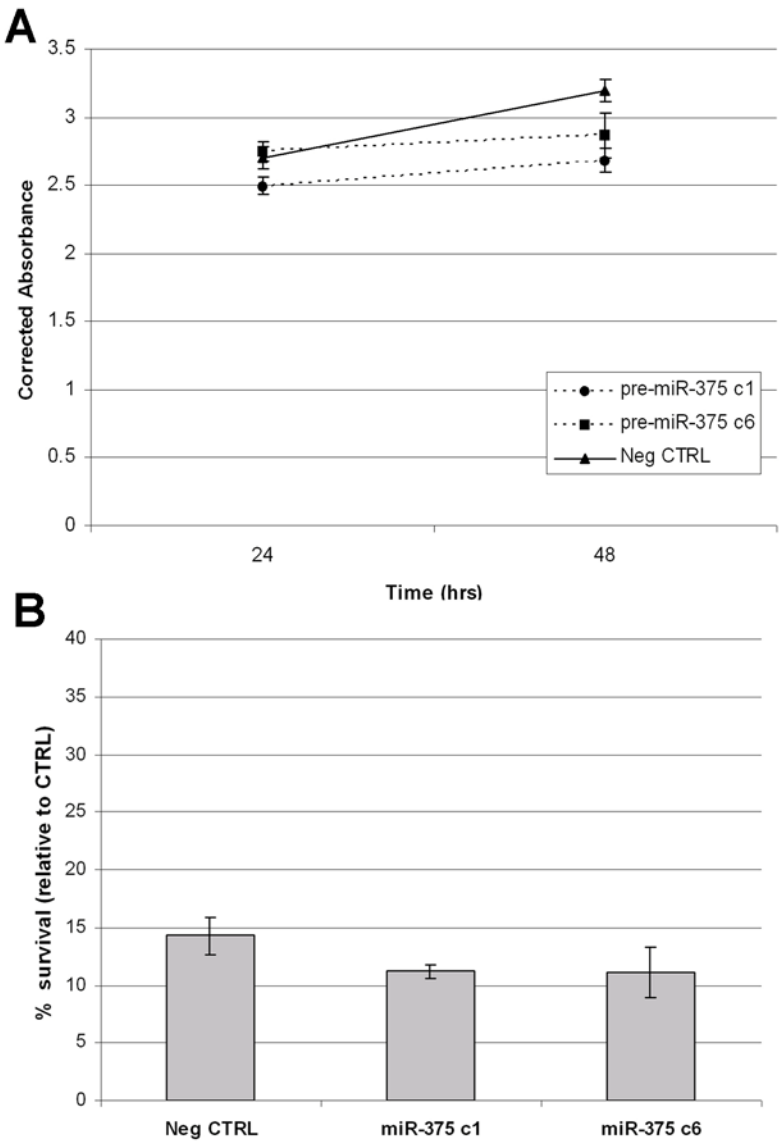

Figure 4. *miR-375* expression vector transfection in FaDu cells. (A) Decreased proliferation rate in both *miR-375*-transfected clones compared to control-transfected cells as measured by proliferation assay. (B) Increased sensitivity to 0.5 μM CDDP in *miR-375*-transfected clones compared to negative control as assessed by colony formation assay.

Chapter 5

Discussion

Head and neck squamous cell carcinoma (HNSCC) is a significant global health concern and its treatment has presented health professionals with a serious challenge. Despite medical advances in diagnostics and chemotherapy, the 5-year survival rate of HNSCC has improved only marginally over the last two decades and remains around 60% (1). Poor survival can be attributed in part to late stage diagnosis and subsequent insensitivity of high stage tumors to chemotherapeutic strategies (2-4). Surgical resection of incident tumors is also limited in its efficacy due 1) to field cancerization, resulting in an indistinct field of early dysplastic cells surrounding the tumor, 2) the frequency of local recurrence and 3) the particular propensity of cancers of the upper aerodigestive tract to the occurrence of second primary tumors (5-7). It should also be noted that even when surgical treatment is successful, there remains significant morbidity to the patient.

The recent addition of human papilloma virus (HPV) infection to the risk factors attributed to HNSCC development explains the altered demographic landscape of the disease, which had been predominated by smokers and heavy drinkers, generally men in their 50s and 60s (2-4). Though tobacco and alcohol exposure remain the most significant etiological factors in HNSCC carcinogenesis, HPV has introduced a discrete form of the disease characterized by a lack of cytogenetic and genetic alterations and consequent improved response to treatment and improved overall survival in patients (11-15). The advent of this molecularly and clinically distinct subtype of HPV-positive HNSCCs highlights the importance of understanding the molecular character of the disease in ultimately establishing more refined tools for its management.

In the past two decades, the genetics of HNSCC have been studied extensively with the aim of developing new targets for therapy or determining specific markers that

can predict prognosis or appropriate treatment regimens. Cytogenetic analyses have revealed frequent loss of heterozygosity (LOH) (2) or allelic loss at 3p, 5q, 6p, 8q, 9p, 9q, 11p, 11q, 13p, 17p, and 18q (3). Though many single genes anomalies have been identified in HNSCC, some are more common and more deleterious than others. As in many cancers, the *TP53* and *CDKN2A (*encoding p16INK4A) tumor suppressors are frequently mutated or subject to LOH in HNSCC, likely due to the actions of carcinogens, like benzo[a]pyrene in tobacco (17,18). Amplification of proto-oncogene *CCND1* (encoding CYCLIN D1) has also been associated with HNSCC, as have amplification of *EGFR* and *STAT3* (5,19,20). Though several of these genetic alterations have been correlated to various clinical parameters of HNSCC such as survival and tumor progression, therapeutic strategies formed on the basis of these findings have met with limited success.

The field of epigenetics has contributed enormously to our understanding of the carcinogenic process as epigenetic changes comprise a large proportion of all the molecular alterations in neoplasias (4,5). One epigenetic mechanism that has been intensely researched is DNA methylation. Due to their hypermutability via spontaneous deamination of cytosine to uracil over the course of evolution, CpG dinucleotides are relatively scarce in the genome overall but have a specific preponderance in promoter regions. Promoter CpG island hypermethylation is now understood to be as important a mechanism as somatic mutation for the loss of tumor suppressor function, as in the case of *CDKN2A* (23-25). Global hypomethylation is also an important early neoplastic event in head and neck cancer and leads to genomic instability, likely due to reversal of transcriptional silencing at LINE and SINE elements which encode ancient

retrotransposable elements (5). The harnessing of epigenetic mechanisms has found utility in cancer treatment, as with the use of 5-azacytidine, a methyltransferase inhibitor, theoretically used to reactivate hypermethylated tumor suppressor genes (6). Much of the mechanistic basis of gene expression control occurs at the level of the histone-DNA interaction, and post-translational modification of histone proteins is considered an additional and critical mode of epigenetic regulation (7). Histone deacetylase inhibitors, have also been utilized for clinical purposes, again as they are believed to lead to the reactivation of silenced genes (8). The focus of this thesis is the involvement of another form of epigenetic alteration, that of post-transcriptional control of gene expression through the actions of small RNA, specifically microRNA (miRNA) dysregulation, in HNSCC carcinogenesis.

The minute but ubiquitous small RNA molecule known as the miRNA was introduced to the scientific community by Victor Ambros and colleagues in 1993 following their serendipitous finding of a gene called *lin-4*, the loss of which interfered with developmental timing in the *C. elegans* larva (9). The product of *lin-4* was found to be a small non-protein coding RNA, one of many that would eventually be cloned and characterized in worms, and for which orthologs would be identified in fruit flies, mice, and humans. Originating from long primary transcripts that are processed stepwise, first into hairpin structures and finally into ~22 bp duplexes, miRNAs interact with the 3' untranslated regions of over 30% of mRNAs, disrupting their translation (10). Through this mechanism, miRNAs regulate diverse fundamental processes such as development, differentiation, cell proliferation and cell death.

Frequent loss of the 13q14 chromosomal region in chronic lymphocytic leukemia (CLL) led to a search for a tumor suppressor gene which did not turn up a protein coding gene but instead resulted in the discovery of two miRNA genes, *miR-15a* and *miR-16-1*, which mapped to the specific area of deletion at 13q14 (28). A translocation breakpoint was found to sever the precursor of the co-cistronic miRNAs, resulting in their loss. Later it was found that *miR-15* and *miR-16-1* expression were lost in 70% of CLLs and this loss was inversely correlated with *Bcl2* expression in this disease (29). The direct repression of *Bcl*2 by these miRNAs could induce apoptosis in leukemia cell lines, suggesting that they act as tumor suppressor miRNAs in CLL. Indeed, *miR-15* and *miR-16-1* were found to be downregulated in other cancers as well (30,31) and their discovery prompted a flood of miRNA-centered cancer studies aiming to identify miRNA profiles and biomarkers of every cancer type and subtype.

The introduction of several commercially available miRNA microarray platforms has made study and high throughput analysis accessible to labs interested in discovering the potential contribution of miRNAs to their cancer or other disease of interest. Along with the usual caveats of high throughput technologies (11), these labs had to contend with the additional problems of detecting molecules of such diminutive size. The lack of amplification combined with the limiting expense of performing replicates diminishes the sensitivity of the array while the challenge of designing specific mature miRNA probes makes specificity a major concern (12). To address these problems, efficient systems for miRNA quantification based on quantitative real-time PCR have been developed (12,13) and though limited in throughput compared to an array, they are now indispensable for sensitive and specific validation of miRNA alterations.

The first microarray-based miRNA study of HNSCC, performed by Tran et al., identified highly expressed miRNA (notably *let-7a, miR-16, miR-21,* and *miR-205*) and several showing low expression amongst HNSCC cultured cell lines (13). Though this marked an important first step in establishing a miRNA profile for HSNCC cell lines, the study did not include a comparative analysis with non-tumorigenic cell lines so the results may not necessarily reflect miRNA alterations specific to disease vs non-diseased cells. Also it has now been shown by several groups that miRNA profiles of cultured cell lines are, in fact, not generally analogous to those of primary tissues (30-32).

Following this study, Chang et al. performed a microarray analysis on a small number of tissues, both HNSCC tumors and normal epithelia, along with HNSCC cell lines (14). They also found *miR-21* and *let-7* overexpressed, in addition to 5 others, and 1 miRNA downregulated, though the study only expanded on two of these, which were validated by real time PCR. *In vitro* studies performed by this group showed *miR-21* to promote growth and inhibit apoptosis in HNSCC cell lines. A study by Fletcher et al, published concomitantly with the Chang study followed up on the results of Tran et al, exploring the potential use of *miR-205* as a biomarker for HNSCC (15). This study concluded that, in fact, *miR-205* was not differentially expressed in tumor vs normal tissues, but the absence of its expression in normal lymph node tissue makes it a candidate biomarker for detection of metastasis.

In early 2009, Childs et al published the results of a miRNA microarray performed on an undisclosed number of tumor tissues and adjacent normal tissues taken from the same field as the surgical resection of the tumor (35). The study did not describe microdissection or pathologist examination ensuring that this adjacent tissue was not

dysplastic, a particular concern given the phenomenon of field cancerization. The study did however find some results that were consistent with the previous research, including upregulation of *miR-21* and *let-7d* but it also found *miR-205* to be down-regulated, in contradiction with the work of Tran et al. and that of Fletcher et al. These studies highlight the importance of relevant controls and thorough validation for miRNA expression studies.

This thesis focused on investigating the involvement of miRNAs in HNSCC, determining the specificity of miRNA alterations in malignant tissue and the interactions of risk factors and clinical variables with miRNA expression in HNSCC. Here we have established a panel of miRNA specific to the disease and a miRNA ratio that could distinguish HNSCC primary tissues from non-diseased epithelial tissue with great specificity and sensitivity. We have applied the examination of these alterations to a large well-characterized population-based study of HNSCC to examine their associations with various clinical and etiological variables. Subsequent to this, and with the ultimate goal of determining the specific function of a miRNA found to be altered in HNSCC, we sought to investigate a relevant method for miRNA overexpression in cultured HNSCC cell lines.

Chapter 2 describes the results of a miRNA microarray performed on 16 tumors of oral, pharyngeal and laryngeal origin and 5 tissues samples taken from normal tongues, larynges and uvulae. Twelve human miRNAs were significantly overexpressed and one underexpressed in tumors compared to normal tissue, all showing greater than 2-fold differential expression. Three overexpressed miRNA (*miR-21*, *miR-221*, and *miR-18a*) and the one underexpressed miRNA (*miR-375*) were validated as differentially

expressed by quantitative real-time PCR in this set and in a larger set of 99 HNSCC samples and 14 normal epithelia. Surprisingly, *miR-375* was not identified as differentially expressed in any of the previous studies of miRNAs in HNSCC, despite the strong (~22-fold) underexpression revealed in our study. Perhaps not surprising was the finding that *miR-21* was upregulated in tumor tissue, which is consistent with many of the previous miRNA studies of HNSCC and indeed with profiling studies of a great many malignancies (17-20). In vitro studies of *miR-21* function have found it to positively regulate cell cycle progression, proliferation, and invasion, to negatively regulate apoptosis, and to contribute to tumor resistance to chemotherapy (18,21,22). Some of the confirmed targets of *miR-21* seem to support these observations, as it is known to target tumor suppressors programmed cell death 4 (*PDCD4*), tropomyosin 1 (*TPM1*) and *PTEN*, as well as the candidate tumor suppressor, *LRRFIP1* (16-19). A study looking at alterations of the PI3-K/AKT/PTEN pathway in HNSCC found that reduced protein levels of PTEN in tumors could not be accounted for by allelic loss, nor point mutations or homozygous deletions, suggesting a potential epigenetic mechanism for *PTEN* disruption (27). Although in some tumors DNA hypermethylation and genetic events have been linked to loss of PTEN protein expression or aberrant activation of downstream oncogenic pathways (1,2), studies in lung and lymphomas have demonstrated that PTEN protein loss may be associated with more complicated regulatory mechanisms, perhaps involving post-transcriptional control (37-39). Moreover, as the targeting activities of miRNAs can vary from one tissue or cell type to another, an important step in ascertaining the specific role of *miR-21* in HNSCC will be to determine which genes it regulates in this context.

After determining relative expression values of the four miRNAs of interest for each sample, we developed a binary classifier of disease status using receiver operator curves based on the ratio of the expression of each upregulated miRNA to the expression of *miR-375* in each sample. Similar methods have been used by Gordon et al. to establish a gene expression ratio to differentiate malignant pleural mesotheliomas and adenocarcinomas (20) and by Neely et al. to create a miRNA ratio distinguishing invasive from noninvasive bladder tumors (21). The ratio of *miR-221* to *miR-375* expression proved to be a highly specific and sensitive predictor of disease in our cohort, though testing this ratio on a separate population of HNSCC tumors will be indispensable for determining its true diagnostic value. Several groups have demonstrated an association of miRNA expression with progression from Barrett esophagus to esophageal adenocarcinomas (22,23). Testing the utility of the *miR-221:miR-375* ratio against other parameters, such as progression from dysplasia to malignancy would be of interest though the feasibility of such a study is dubious considering the potential difficulty of obtaining leukoplakias due to the frequency of delayed diagnosis in this disease.

In Chapter 3 we utilized a case series of 169 HNSCC primary tumor tissues with annotated clinical and exposure-related data on patients. Expression of *miR-21*, *miR-18a*, *miR-221*, and *miR-375* was determined for all samples and statistical analyses were performed to determine whether expression of these miRNA could be correlated with clinical variables such as tumor stage, tumor site, and 5-year survival or exposure variables such as smoking, drinking and HPV status.

One important result of our analyses was the discovery of an association between high levels of *miR-21* and poor 5-year survival in patients. Though previous studies of

miR-21 in HNSCC, which investigated correlations with outcome, failed to find this association, it is consistent with findings in pancreatic cancer, breast cancer, colon adenocarcinomas and esophageal squamous cell carcinoma, the last of which, interestingly, also showed reduced expression of *miR-375* (23-27). A recent study specifically studying squamous cell carcinoma of the tongue also correlated *miR-21* expression with poor prognosis (23).

We also identified a significant correlation between *miR-375* expression and alcohol consumption. Alcohol is known to be an independent risk factor for HNSCC and there are several proposed mechanisms for its carcinogenicity, including the production of acetaldehyde, a known carcinogen, and its solvent properties, which may enhance the effects of carcinogens in tobacco smoke (24). In light of the overall lower *miR-375* expression observed in HNSCC tumors compared to normal tissues, the range of *miR-375* expression seen amongst drinkers may represent subtle regulation occurring at the level of the tumor microenvironment. One way in which this may be controlled is through promoter methylation. Though *miR-375* is an intergenic monocistronic miRNA, its purported regulatory region contains a CpG cluster (30). As alcohol consumption is known to interfere with folate metabolism, resulting in dysregulation of methylation (31,32), it is possible that increased alcohol intake disrupts methylation of the *miR-375* regulatory region, leading to a local increase in *miR-375* expression. Methylation of *miR-375*'s regulatory region should be assessed in these tissues to determine whether it correlates with *miR-375* expression in general and whether this regulation is modified by alcohol consumption.

The association with alcohol consumption may be coincident with the increased expression of *miR-375* observed in tumors of oral and pharyngeal origin compared to those of laryngeal origin. Several studies have reported risk associated with alcohol use to be greater for oral and pharyngeal cancer than for laryngeal cancers (8,9). Taken together, these results may indicate greater alcohol-induced epigenetic regulation taking place in oral and pharyngeal tissues, which may be more heavily affected by the toxicant. This study is the first population-based molecular epidemiological study of miRNA profiles to demonstrate a clear association between etiological exposures and miRNA expression. These results suggest that genetic regulation by epigenetic mechanisms like miRNA interference is dynamic, influenced by environmental factors and continually modulate tumorigenic progression.

The intriguing findings regarding *miR-375* in the previous chapters led us to hypothesize that *miR-375* is likely to play an important and complex role in HNSCC carcinogenesis. Chapter 3 details our experimentation with two very different methods of miRNA overexpression in an invasive HNSCC cell line, with the aim of determining which system is the most relevant for use in the investigation of *miR-375's* role in HNSCC. The use of a synthetic double stranded molecule, referred to as a miRNA mimic, yielded extremely high levels of mature *miR-375*, greater than 2000-fold as determined by real-time PCR analysis. Its upregulation resulted in increased rates of proliferation and migration as well as increased resistance to the chemotherapeutic drug cisplatin (CDDP). An expression vector system placing miRNA expression under the control of a cytomegalovirus promoter was also used to overexpress *miR-375* and yielded

~4-fold upregulation of *miR-375*. In this case, *miR-375* upregulation resulted in slowed proliferation and increased sensitivity to CDDP.

Methods for testing miRNA function have only been developed in the past few years and are constantly being improved. The assays currently available may be somewhat crude, perhaps more useful for determining miRNA targets but less suited for studying the fine control of miRNA expression crucial for determination of overall function. We hypothesized that the massive upregulation of the mature miRNA induced by the mimic system might actually inundate endogenous miRNA machinery, such that the observed phenotype is merely an artifact of aberrant miRNA pathway functioning. In order to determine whether this is the case, the expression of other miRNAs and changes in RISC activity should also be assessed following transfection. If the results seen with the expression vector system more accurately reflect the function of *miR-375* in HNSCC, the generation of a cell line stably expressing *miR-375* may result in more dramatic and significant results than those observed in our study, and in results more consistent with the functional role of this miRNA in epithelial tissues.

Assuming *miR-375* overexpression indeed results in decreased proliferation and increased sensitivity to chemotherapeutic drugs like CDDP, a functional model for the mechanism of *miR-375* action in HNSCC can be proposed (Fig. 1). 3'-phosphoinositide-dependent protein kinase-1 (*PDPK-1*) has been demonstrated to be a direct target of *miR-375* in pancreatic beta-cells. Though the existence of this relationship remains to be confirmed in tissues of the head and neck, it would fit with a model of *miR-375* carcinogenesis as AKT is the major effector of PDPK-1 and influences metabolism, proliferation and apoptosis, ultimately promoting cell growth and survival (25).

Additionally, increased levels of phosphorylated AKT and the overactivity of the AKT pathway in general have both been observed in HNSCC (8).

Several of the miRNA alterations described herein, such as *miR-21*, and *miR-375* may have potential as targets for therapy, though the practical clinical utility of this and other studies investigating the roles of miRNAs in cancer have yet to be determined. However, experiments performed in mice suggest that synthetic miRNAs or miRNA inhibitors could be used for cancer therapy. Calin and colleagues, who originally discovered the link between *miR-15a* and *miR-16-1* and CLL, have recently used a vector expressing *miR-15a/16-1* to suppress tumor growth of leukemic cells (26), a result which offers promise for the exploitation of miRNAs in the treatment of human cancers.

The utilization of a population based study is one of the major strengths of the work presented in this thesis. A population-based study has the advantage of generalizability – the results are considered directly applicable not only to the studied population but to the population at large, though independent validation is always necessary to establish true generalizability. There are still some inherent limitations of such a study, one being the problem of reverse causality, where a given association can only be established as correlative and not causal. Some bias may exist in the process of collecting samples and information. Reporting of exposure data, for example, may depend on the personal biases and mental capacity of the patient or the individual reporting on his or her behalf. Additionally, the tumors available for study were generally those that were fully resected and for which enough tumor content existed for analysis, potentially introducing a bias for tumors of later stage and larger size.

Another advantage of this study is the use of primary tissues. Results of molecular research in primary tissues are directly interpretable to human health and primary tissues turn out to be particularly important in the realm of miRNA profiling, as we and others have concluded that cultured cell lines may be poor models of disease with regard to miRNA expression. Cancer cell lines are often highly genetically dysregulated and may lose expression of miRNAs in successive passaging. Primary cell lines may be better suited for use in miRNA profiling studies though their use in functional studies may not be feasible due to technical limitations.

In summary, the results presented in this thesis have contributed not only to the understanding of the role of miRNAs in head and neck cancer, but also to the current understanding of how miRNAs are influenced by environmental exposures, a factor that, until now, had not been addressed extensively in the literature. Our preliminary results with *miR-375* functional studies, suggest that restoration of *miR-375* in HNSCC cells may lead to the reversal of some malignant phenotypes, and motivated the proposal of a role for *miR-375* in HNSCC. Taken together, our results indicate that miRNA alterations are important considerations in the molecular pathology of HNSCC and may eventually serve as useful therapeutic targets, refining the quality of treatment and improving overall survival for the disease.

References

1. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. and Thun, M.J. (2009) Cancer statistics, 2009. *CA Cancer J Clin*, **59**, 225-49.
2. Chen, C., Mendez, E., Houck, J., Fan, W., Lohavanichbutr, P., Doody, D., Yueh, B., Futran, N.D., Upton, M., Farwell, D.G., Schwartz, S.M. and Zhao, L.P. (2008) Gene expression profiling identifies genes predictive of oral squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 2152-62.
3. Schottenfeld, D. and Fraumeni, J.F. (2006) *Cancer epidemiology and prevention*. Oxford University Press, Oxford ; New York.
4. Sharma, S., Kelly, T.K. and Jones, P.A. (2009) Epigenetics in Cancer. *Carcinogenesis*.
5. Esteller, M. (2008) Epigenetics in cancer. *N Engl J Med*, **358**, 1148-59.
6. Issa, J.P. and Kantarjian, H.M. (2009) Targeting DNA methylation. *Clin Cancer Res*, **15**, 3938-46.
7. Campos, E.I. and Reinberg, D. (2009) Histones: Annotating Chromatin. *Annu Rev Genet*.
8. Lane, A.A. and Chabner, B.A. (2009) Histone deacetylase inhibitors in cancer therapy. *J Clin Oncol*, **27**, 5459-68.
9. Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843-54.
10. Ambros, V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350-5.

11. Somorjai, R.L., Dolenko, B. and Baumgartner, R. (2003) Class prediction and discovery using gene microarray and proteomics mass spectroscopy data: curses, caveats, cautions. *Bioinformatics*, **19**, 1484-91.

12. Yin, J.Q., Zhao, R.C. and Morris, K.V. (2008) Profiling microRNA expression with microarrays. *Trends Biotechnol*, **26**, 70-6.

13. Tran, N., McLean, T., Zhang, X., Zhao, C.J., Thomson, J.M., O'Brien, C. and Rose, B. (2007) MicroRNA expression profiles in head and neck cancer cell lines. *Biochem Biophys Res Commun*, **358**, 12-7.

14. Chang, S.S., Jiang, W.W., Smith, I., Poeta, L.M., Begum, S., Glazer, C., Shan, S., Westra, W., Sidransky, D. and Califano, J.A. (2008) MicroRNA alterations in head and neck squamous cell carcinoma. *Int J Cancer*, **123**, 2791-2797.

15. Fletcher, A.M., Heaford, A.C. and Trask, D.K. (2008) Detection of metastatic head and neck squamous cell carcinoma using the relative expression of tissue-specific mir-205. *Transl Oncol*, **1**, 202-8.

16. Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A. and Lund, A.H. (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem*, **283**, 1026-33.

17. Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T. and Patel, T. (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*, **133**, 647-58.

18. Zhu, S., Si, M.L., Wu, H. and Mo, Y.Y. (2007) MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem*, **282**, 14328-36.

19. Li, Y., Li, W., Yang, Y., Lu, Y., He, C., Hu, G., Liu, H., Chen, J., He, J. and Yu, H. (2009) MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme. *Brain Res*, **1286**, 13-8.

20. Gordon, G.J., Jensen, R.V., Hsiao, L.L., Gullans, S.R., Blumenstock, J.E., Ramaswamy, S., Richards, W.G., Sugarbaker, D.J. and Bueno, R. (2002) Translation of microarray data into clinically relevant cancer diagnostic tests using gene expression ratios in lung cancer and mesothelioma. *Cancer Res*, **62**, 4963-7.

21. Neely, L.A., Rieger-Christ, K.M., Neto, B.S., Eroshkin, A., Garver, J., Patel, S., Phung, N.A., McLaughlin, S., Libertino, J.A., Whitney, D. and Summerhayes, I.C. (2008) A microRNA expression ratio defining the invasive phenotype in bladder tumors. *Urol Oncol*.

22. Feber, A., Xi, L., Luketich, J.D., Pennathur, A., Landreneau, R.J., Wu, M., Swanson, S.J., Godfrey, T.E. and Litle, V.R. (2008) MicroRNA expression profiles of esophageal cancer. *J Thorac Cardiovasc Surg*, **135**, 255-60; discussion 260.

23. Maru, D.M., Singh, R.R., Hannah, C., Albarracin, C.T., Li, Y.X., Abraham, R., Romans, A.M., Yao, H., Luthra, M.G., Anandasabapathy, S., Swisher, S.G., Hofstetter, W.L., Rashid, A. and Luthra, R. (2009) MicroRNA-196a is a potential marker of progression during Barrett's metaplasia-dysplasia-invasive adenocarcinoma sequence in esophagus. *Am J Pathol*, **174**, 1940-8.

24. Seitz, H.K., Poschl, G. and Simanowski, U.A. (1998) Alcohol and cancer. *Recent Dev Alcohol*, **14**, 67-95.

25. Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.

26. Calin, G.A., Cimmino, A., Fabbri, M., Ferracin, M., Wojcik, S.E., Shimizu, M., Taccioli, C., Zanesi, N., Garzon, R., Aqeilan, R.I., Alder, H., Volinia, S., Rassenti, L., Liu, X., Liu, C.G., Kipps, T.J., Negrini, M. and Croce, C.M. (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc Natl Acad Sci U S A*, **105**, 5166-71.

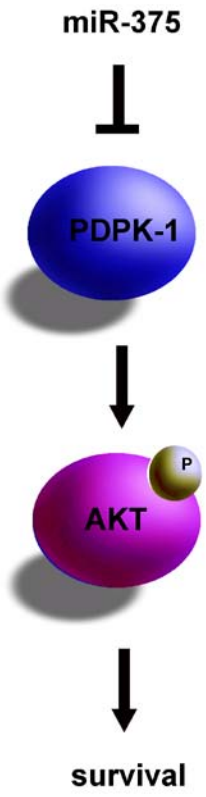

Figure 1. Model for the role of *miR-375* in HNSCC carcinogenesis.