

MicroRNA Expression Ratio Is Predictive of Head and Neck Squamous Cell Carcinoma

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

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

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

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

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 infection (2–4). Despite advances in detection, as well as surgical and chemotherapeutic treatments over recent decades, the 5-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, ~22-nucleotide-long non-coding RNA molecules, has revolutionized our understanding of the modulation of gene expression. Nearly 700 microRNAs have been identified in humans (miRBase: <http://microrna.sanger.ac.uk/>), a number that is rapidly growing and expected to reach $\geq 1,000$ (9). Highly ubiquitous and largely conserved across species, microRNAs regulate gene expression posttranscriptionally by base pairing, usually imperfectly, to the 3'-untranslated region (10) of a cognate mRNA (11). The interaction of a microRNA with a target mRNA transcript results either in translational repression of the mRNA or in its direct degradation (11). Because of the partial complementarity between microRNAs and their target transcripts, a single microRNA is capable of simultaneously regulating up to

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Translational Relevance

This work has showed that a microRNA expression ratio can distinguish between nondiseased tissue and tumor tissue with great accuracy in the context of head and neck squamous cell carcinoma, 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.

hundreds of genes, giving rise to an enormous modulatory potential (12).

Through their targets, microRNAs are known to play important roles in cell differentiation, proliferation, and apoptosis (13). Because these processes are known to be deregulated in cancer, it is not surprising that many studies have now identified a role for microRNAs in carcinogenesis (14–16), including head and neck carcinogenesis (17, 18). Indeed, recent work on different cancer types has illustrated the existence of distinct microRNA expression profiles between tumor tissues and their corresponding normal tissue (19–21). Moreover, some studies have identified microRNA 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 microRNA expression of normal head and neck epithelia with primary HNSCC tumors and cultured HNSCC cell lines to define those microRNA most capable of differentiating disease and, thus, those with the greatest potential as biomarkers. Upon identifying microRNAs 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. Nondiseased head and neck epithelial tissue were obtained from the National Disease Research Interchange and consisted of fresh-frozen tongue, larynx and uvula samples. 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 on 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 and maintained in Eagle's MEM and DMEM, respectively,

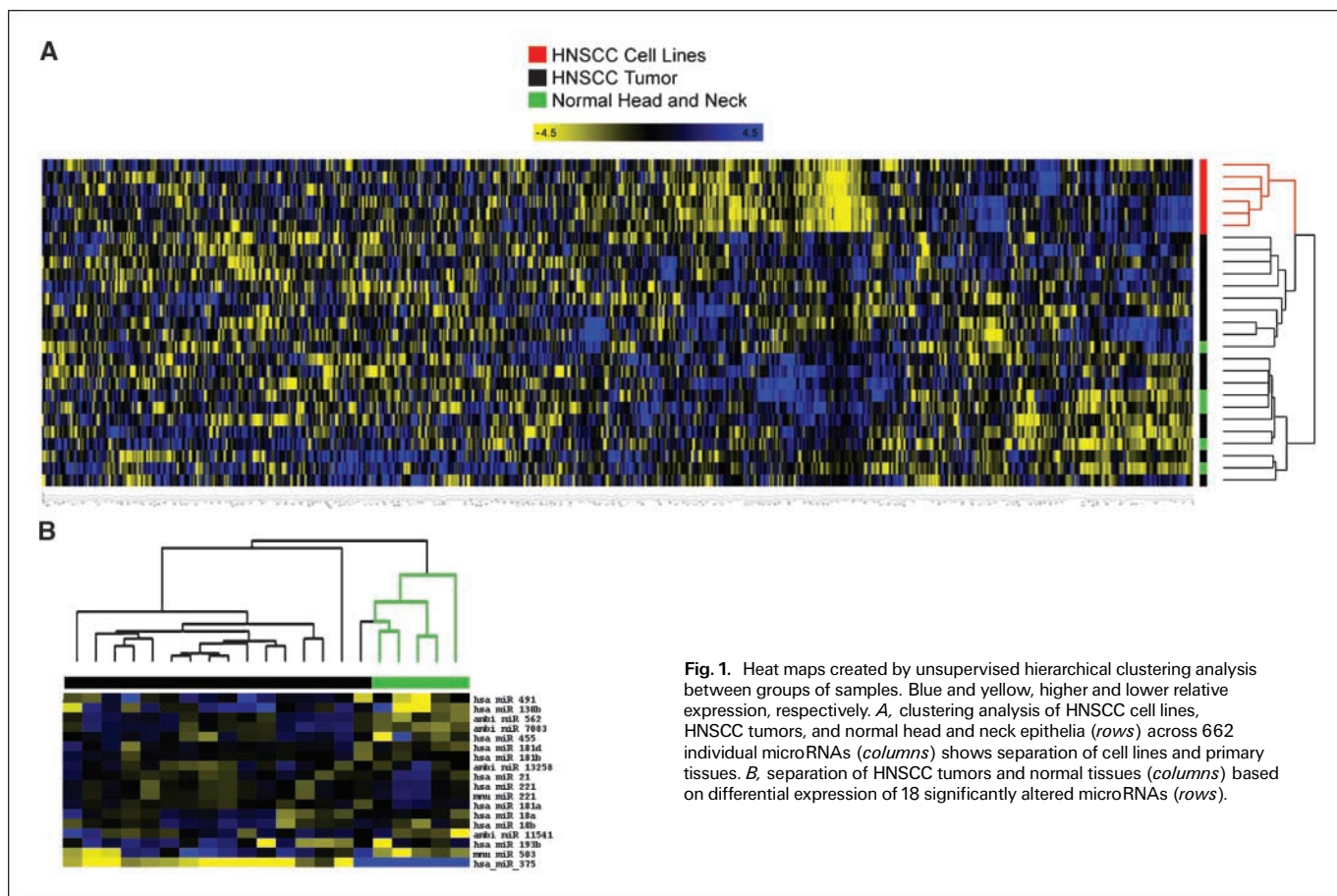


Fig. 1. Heat maps created by unsupervised hierarchical clustering analysis between groups of samples. Blue and yellow, higher and lower relative expression, respectively. **A**, clustering analysis of HNSCC cell lines, HNSCC tumors, and normal head and neck epithelia (rows) across 662 individual microRNAs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

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.

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.) according to the manufacturer's protocol. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop), aliquoted, and stored at -80°C briefly until needed. Five micrograms of total RNA was sent for microRNA profiling studies at Asuragen Services using the mirVANA microRNA Bioarrays platform v2 (Ambion, Inc.) as single-channel format according to the standard

operating procedures of the company, including pre-array qualitative Bioanalyzer (Agilent) RNA analysis, as previously described (24). The Bioarrays platform v2 contains probes specific to microRNA identified in human, mouse, and rat, as well as a number of proprietary microRNAs 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). 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 \log_2 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 microRNA Assays (Applied Biosystems) were used to quantify mature microRNA. cDNA was synthesized by priming with a pool of gene-specific looped primers, including the primers of the microRNAs of interest and RNU48 as a universally expressed endogenous control (Applied Biosystems). Ten microliters of total RNA diluted to a final concentration of 5 ng/ μL was used for each reverse transcription reaction, along with other reverse transcription components, per manufacturer's specifications. Forty-microliter reactions were incubated in an Applied Biosystems GeneAmp PCR system 9700 for 30 mins at 16°C , 30 mins at 42°C , and 5 mins at 85°C , and held at 4°C . Quantitative reverse transcription-PCR was done 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 microRNAs by microarray was determined with Significance Analysis of Microarray

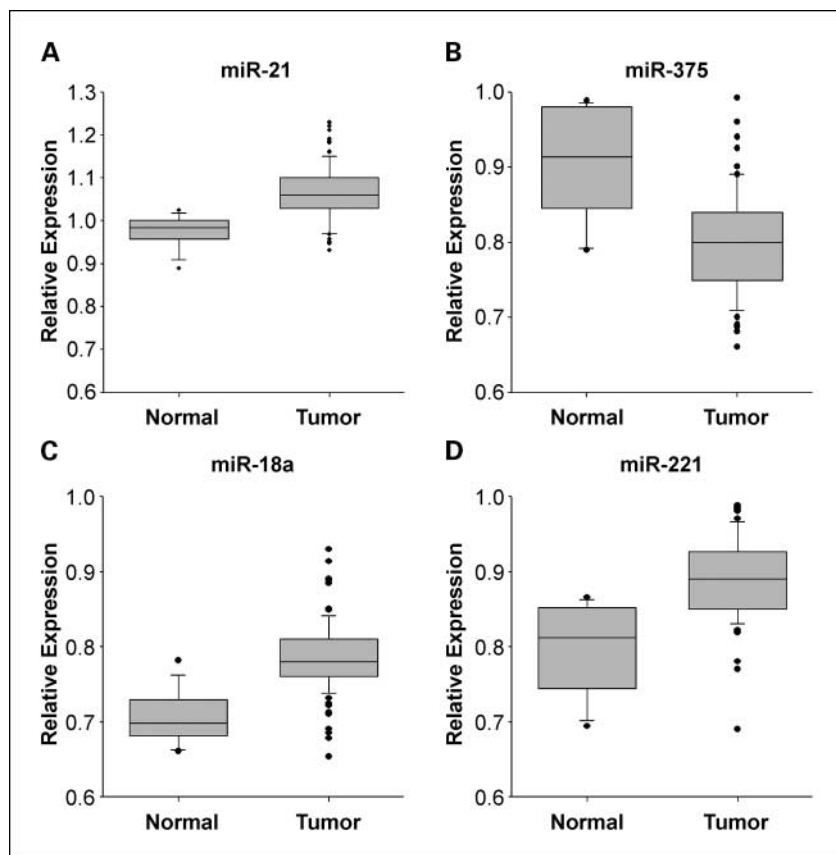


Fig. 2. Validation of differential expression of four microRNAs by quantitative real-time PCR in tumor ($n = 99$) and normal ($n = 14$) samples. Boxes, distribution of expression values from the 25th to 75th percentile for each microRNA. Horizontal lines in boxes, median values; whiskers, 5th and 95th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

and centroid linkage between clusters. A two-tailed Student's *t* test was used to compare microRNA expression levels determined by real-time PCR. MicroRNA expression ratios were calculated following the methods of Gordon et al. (27), and receiver operating curve analyses were used to assess the predictive power of the microRNA quotients.

Results

MicroRNA expression patterns differentiate HNSCC cell lines from primary tissues. A microarray platform was used to determine microRNA expression of 662 microRNAs in 16 fresh-frozen HNSCC tumors, 5 nondiseased 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 microRNAs spotted on the chip revealed a marked, very distinct separation of the cell line microRNA profiles compared with those of primary tissues (Fig. 1A). Significance Analysis of Microarray identified 67 significantly differentially expressed microRNAs ($Q < 0.0001$) between cell lines and primary tissues, consistently showing lower expression of microRNAs in cell lines compared with tumors (Supplementary Table 1).

Eighteen microRNAs are differentially expressed in HNSCC tumor tissue compared with normal head and neck epithelia.

Significance Analysis of Microarray analysis identified 18 microRNAs to be significantly altered in their expression between nondiseased 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 microRNAs, 12 were human microRNAs, 4 were proprietary microRNAs of Ambion, Inc., and 2 were mouse orthologues, both of which have known human counterparts with identical mature sequences. Hierarchical clustering based on this limited set of microRNAs showed clear separation of tumors from normal tissues (Fig. 1B).

All human microRNAs identified by Significance Analysis of Microarray method showed >2-fold difference between normal and tumor tissue (Table 1). Of the human microRNAs, 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 reverse transcription-PCR followed by conventional Taqman real-time PCR using microRNA-specific probes because validated assays were available for these six microRNA and they constituted one primer pool, allowing for examination using a single reverse transcription step. Confirmation assays used only the samples screened on the array. Of the six microRNAs tested, four were reliably confirmed (*miR-21*, *miR-18a*, *miR-221*, and *miR-375*), showing significant

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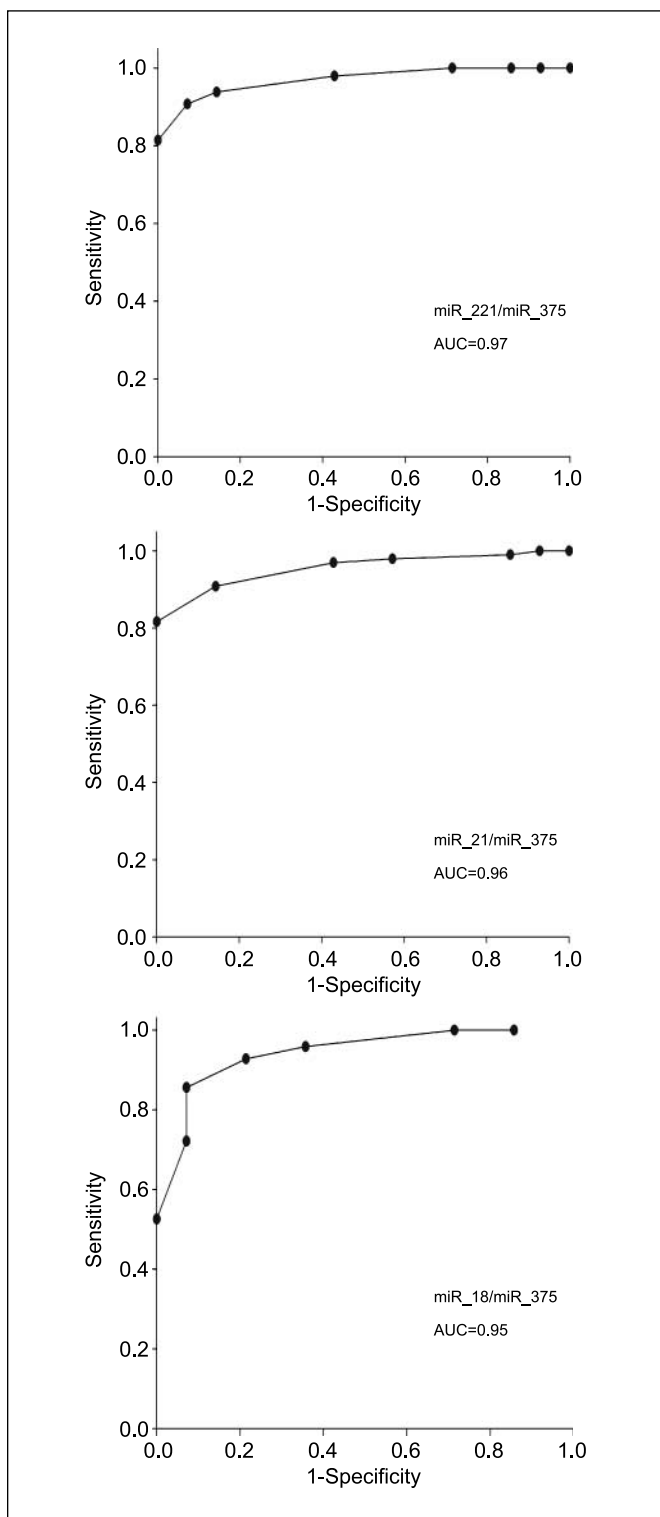


Fig. 3. Receiver operating curve analysis of expression ratios of *miR-221*, *miR-21*, and *miR-18a* to *miR-375* for differentiation of HNSCC tumors and normal tissues. Curves were constructed using ratio value cutoffs ranging from 0.75 to 1.25 for each ratio. Area under the curve value is indicated in each panel.

software (Stanford University Labs) using 1,000 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

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

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

differential expression between tumor and normal ($P < 0.01$; Supplementary Fig. 1).

MicroRNA expression ratio shows high specificity and sensitivity in predicting disease. Validation of the four confirmed microRNAs was done in 9 additional normal tissue samples (total $n = 14$) and 83 additional tumor samples (total $n = 99$). Quantification of microRNA 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 up-regulation in tumors ($P < 0.0001$; Fig. 2A, C, and D, respectively), whereas *miR-375* was significantly down-regulated in tumors ($P < 0.0001$; Fig. 2B).

Because *miR-21*, *miR-18a*, and *miR-221* showed consistent up-regulation and *miR-375* consistent down-regulation, we next sought to determine if expression ratios constructed between these microRNA could improve their predictive potential for differentiating HNSCC tumor from nondiseased epithelia. Following the methods of Gordon et al. (27), microRNA expression ratios were calculated by dividing the relative expression value of each of the three microRNAs showing up-regulation in tumors by the expression value of the only down-regulated microRNA, *miR-375*. Receiver operating curve analysis was done to determine which of these ratios showed the greatest predictive power (Fig. 3). Table 2 lists the representative sensitivity and specificity of ratios using the cutoff value of 1 for each of the up-regulated microRNA to the down-regulated *miR-375* in differentiating between nondiseased 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 microRNAs to be aberrantly expressed in HNSCC tumors, including an extensively validated subset that may hold utility as clinical biomarkers of disease. Microarray profiling of >600 microRNAs identified 18 microRNAs that were significantly differentially expressed in tumor tissues compared with analogous nondiseased head and neck epithelia. Of the 12 human microRNAs in this group, four microRNA which were validated by quantitative reverse transcription-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 Significance Analysis of Microarray was used to identify differentially expressed genes by pairwise comparisons between groups of interest. It should be noted that Significance Analysis of Microarray was designed (and may be better suited) for identification of important genes from high density microarrays because it allows the user to control the number of findings based on a desired false discovery rate while avoiding parametric assumptions about the data, inherent in tests such as the ANOVA (28).

The microarray data revealed that HNSCC cell lines show a distinct pattern of microRNA expression compared with

primary tumors. This is consistent with past reports showing a clear segregation of cell lines away from primary tumors upon high-throughput analysis of their microRNA expression (22, 29), suggesting that cell lines have limited utility as a model system for the identification of clinically relevant microRNA biomarkers. There remains though significant utility in using *in vitro* approaches for defining the biological mechanisms of these microRNAs, with appropriate understanding that the pattern of their expression is markedly different from that observed in the parent primary tissue.

Some of the microRNAs identified as differentially expressed in HNSCC compared with 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 up-regulated in HNSCC tumors (17). MicroRNA 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 up-regulated in cancer (17, 18, 21, 30). In fact, the relative levels of *miR-21* and other microRNAs were reported to have prognostic relevance for predicting patient survival in lung cancer (31). Some known targets of *miR-21* include the tumor suppressor genes tropomyosin 1 (*TPM1*) and programmed cell death 4 (*PDCD4*; refs. 32, 33). In addition, *miR-221* and *miR-18a*, both shown to be up-regulated in HNSCC tumors, have previously been implicated in hepatocellular, prostate, and other cancers (34–37).

miR-375 was the only down-regulated microRNA found when comparing tumors and normal tissues, showing a ~22-fold decrease in tumors. Validation experiments also showed it to be sharply and significantly down-regulated in tumors relative to normal tissues. *miR-375* has been found to regulate insulin secretion in mice, and its down-regulation has been implicated in aberrant morphology of pancreatic islet cells in zebrafish (38, 39). Recently, *miR-375* down-regulation has also been associated with β -catenin mutation in hepatocellular adenoma (40). The down-regulation 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* microRNA (41).

To improve the predictive potential for individual microRNA alterations, we used expression ratios as described by Gordon et al. (27). 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. These data suggest that the ratio of these microRNAs may hold significant clinical potential, but further validation is necessary in an independent series of HNSCC tumors.

The identification of an HNSCC-specific microRNA signature indicates a plausible role for microRNAs in the development or progression of this disease. Finding abnormally expressed microRNAs may prove to be an important step in identifying the specific mechanisms of HNSCC carcinogenesis because these aberrations may constitute early events in initiation or progression of the disease (42). As such, the use of a microRNA expression ratio that distinguishes disease tissue from nondiseased tissue holds potential as a simple early diagnostic for HNSCC. An examination of these microRNA 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 microRNA 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 microRNAs identified in this study in an independent test population of HNSCCs could be instrumental in showing that the individual microRNA aberrations are globally specific to HNSCC and in resolving the true diagnostic potential of the *miR-221:miR-375* ratio.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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