Comprehensive MicroRNA Profiling for Head and Neck Squamous Cell Carcinomas

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Abstract

Purpose: The objective of this study is to investigate the significance of microRNAs (miRNA) in patients with locally advanced head and neck squamous cell carcinoma (HNSCC).

Experimental Design: A global miRNA profiling was done on 51 formalin-fixed archival HNSCC samples using quantitative reverse transcription-PCR approach, correlated with patients’ clinical parameters. Functional characterization of HNSCC-associated miRNAs was conducted on three HNSCC cell lines. Cell viability and proliferation were investigated using MTS and clonogenic assays, respectively; cell cycle analyses were assessed using flow cytometry.

Results: Thirty-eight of the 117 (33%) consistently detected miRNAs were significantly differentially expressed between malignant versus normal tissues. Concordant with previous reports, overexpression of miR-21, miR-155, let-7i, and miR-142-3p and underexpression of miR-125b and miR-375 were detected. Upregulation of miR-423, miR-106b, miR-20a, and miR-16 as well as downregulation of miR-10a were newly observed. Exogenous overexpression of miR-375 in HNSCC cell lines reduced proliferation and clonogenicity and increased cells in sub-G1. Similar cellular effects were observed in knockdown studies of the miR-106b-25 cluster but with accumulation of cells in G1 arrest. No major difference was detected in miRNA profiles among laryngeal, oropharyngeal, or hypopharyngeal cancers. miR-451 was found to be the only significantly overexpressed miRNA by 4.7-fold between nonrelapsed and relapsed patients.

Conclusion: We have identified a group of aberrantly expressed miRNAs in HNSCC and showed that underexpression of miR-375 and overexpression of miR-106b-25 cluster might play oncogenic roles in this disease. Further detailed examinations of miRNAs will provide opportunities to dissect the complex molecular abnormalities driving HNSCC progression.

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Head and neck squamous cell carcinomas (HNSCC) constitute the fifth most common malignancy worldwide (1). Patients with locally advanced HNSCC have 5-year overall survival (OS) rates hovering ∼30%, underscoring significant opportunities for improving outcome (2, 3). Hence, there is a need to acquire deeper understanding of HNSCC biology and to develop predictive molecular signatures, which could improve patient selection for appropriate treatment and guide the development and evaluation of new therapies. MicroRNAs (miRNA) have been recently recognized to play important roles in human cancers and are regarded as key regulators of gene expression in all biological systems (4). Increasing data support the value of miRNA expression profiling to distinguish different types of human malignancies and to categorize various cancer subtypes (5–7).

To date, several reports have described miRNAs associated with HNSCC, evaluated in human cancer cell lines or frozen tissue samples (8–12). With the exception of miR-21, there is no overlap in the identified miRNAs among any of these studies. In addition, a rate-limiting step in the broad application of expression profiling to clinical practice is the paucity of frozen tissues linked to clinical outcome. Archives of formalin-fixed, paraffin-embedded (FFPE) specimens are much more commonly available, providing greater opportunities to acquire biological insights. Archival specimens are amenable to miRNA evaluation due to their short lengths (∼22 nucleotides in length; ref. 13), and we have recently successfully conducted such a global miRNA profiling study for breast cancer FFPE samples (14). In this current study, a comprehensive miRNA evaluation was conducted on diagnostic biopsies from
patients with locally advanced HNSCC, showing miRNA dysregulation and potentially oncogenic roles for miR-106b-25 and miR-375.

Materials and Methods

Patient information. Patient samples were collected from a phase III randomized study (331 participants) of hyperfractionated radiotherapy conducted in 1988 to 1995 (15), with approval from the Institutional Research Ethics Board. Only fifty-four tissue samples out of the collected samples had follow-up data of >4 y and sufficient RNA for miRNA profiling. All patients presented with locally advanced (stage III or IV) HNSCC; the clinical characteristics of these 54 patients are shown in Supplementary Table S1. The predominant subsite was oropharynx (24 of 54, 44%); the majority of patients were males (44 of 54, 82%). With a minimum follow-up of 4 y, 44 patients had no relapse. Four of 54 (82%). With a minimum follow-up of 4 y, 44 patients had no relapse. Four FFPE blocks of normal laryngeal squamous epithelial tissue were used as controls (commercially purchased from Asterand).

RNA purification from FFPE samples. For each sample, a representative section was stained with H&E and reviewed by a head and neck cancer pathologist (B.P-O.) to identify regions containing >70% malignant epithelial cells for macrodissection. All blocks were processed randomly, with clinical outcome unknown, to avoid experimental bias. Total RNA enriched for small RNA species was isolated using the RecoverAll Total Nucleic Acid Isolation kit for FFPE (Ambion) according to the manufacturer's instructions.

Real-time quantification of miRNAs. The quality of the 54 HNSCC and 4 normal laryngeal tissue samples was assessed by reverse transcription-PCR (RT-PCR) analysis of the endogenous control RNU44 using Applied Biosystems Taqman MicroRNA Assay. This assay includes a RT step using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems), wherein a stem-loop RT primer specifically hybridizes to a miRNA molecule and is then reverse transcribed with a MultiScribe reverse transcriptase (14).

Global miRNA profiling of the 54 FFPE samples was done using an in-house prepared RT and PCR plates. Using Taqman MicroRNA Assays Human Panel (Applied Biosystems), a panel of 312 human miRNAs plus 10 endogenous control miRNAs was simultaneously assayed. With the Biomek FX Laboratory Automation Workstation provided by the Samuel Lunenfeld Research Institute High-Throughput Screening Robotics Facility (Toronto, Ontario, Canada), 322 Taqman human miRNA stem-loop RT primers were transferred to 384-well plates, sealed, and kept at −20°C for storage. PCR plates were prepared similarly, except that each Taqman miRNA probe was pipetted in triplicate. Similar to the individual Taqman miRNA assay, total RNA was first reverse transcribed with the RT plates. For each sample, 1 to 3 μg of total RNA were mixed with the RT reagent and then added to the 384-well RT plates. This was followed by transfer of corresponding cDNA to the PCR plates and then analyzed using the Applied Biosystems 7900HT Real-Time PCR System.

Cell lines and reagents. The human hypopharyngeal FaDu HNSCC cell line was obtained from the American Type Culture Collection and cultured according to specifications. The human laryngeal squamous UTSCC-8 and UTSCC-42a (kind gifts from R. Grénman, Turku University Hospital, Turku, Finland) were maintained with DMEM supplemented with 10% fetal bovine serum (Wisent, Inc.) and 100 mg/L penicillin/streptomycin. The commercially purchased normal epithelial cells were cultured in the recommended medium (from Celprogen). All cells were maintained in a 37°C incubator with humidified 5% CO2.

Functional analysis of differentially expressed miRNAs. The biological effects of overexpressed miRNAs were investigated by knocking down these miRNAs in HNSCC cell lines using locked nucleic acid (LNA) probes containing sequence-specific antisense oligonucleotides targeting miR-25, miR-27a, miR-93, miR-106b, or miR-423 (miRCURY LNA miRNA knockdown probes, Exiqon). FaDu, UTSCC-8, or UTSCC-42a cells were reverse transfected using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Invitrogen) and 40 nmol/L of LNA miRNA knockdown probes seeded in 12-well plates. A scrambled miRNA probe (sequence: GTGTAAACAGTCTATACGCC-CA) served as a negative control (Exiqon).

Translational Relevance

We have conducted a global microRNA (miRNA) profiling of 51 archival formalin-fixed, paraffin-embedded samples of locally advanced stage head and neck squamous cell carcinoma (HNSCC). Our results show that approximately one-third (38 of 117) of consistently detected miRNAs were aberrantly expressed in this cancer. These included both previously reported HNSCC-associated miRNAs and novel miRNAs of potential biological significance. Many of the dysregulated miRNAs map to fragile sites of the chromosome, indicating that genomic gains or losses account for one mechanism of this aberration. One of the novel miRNAs is miR-106b-25, which seems to play an oncogenic role in HNSCC development and progression, as well as miR-375. Global miRNA profiles were similar between SCCs arising from the larynx, oropharynx, or hypopharynx. miR-451 was the only miRNA that was significantly downregulated in relapsed compared with nonrelapsed patients. These data illustrate the usefulness of miRNA profiling when archival formalin-fixed, paraffin-embedded samples are used for macrodissection.
Downregulated miRNAs were evaluated for biological effect using pre-miR miRNA precursor molecules (30 nmol/L pre-miR-10a or 10 nmol/L pre-miR-375; Ambion) transfected into each of the three HNSCC cell lines. A Pre-miR Negative Control (Ambion) with random sequence pre-miR molecules, which has been documented to be biologically inert, was used. To examine the change in miRNA expression, cells were removed for RNA extraction 72 h after transfection and analyzed using quantitative RT-PCR.

Viability or clonogenic assays on HNSCC cell lines transfected with pre-miR-375 or LNA miR-106b. Cell proliferation effects of pre-miR-375 or LNA miR-106b were examined on HNSCC cells using the MTS cell proliferation assay according to the manufacturer's protocol (Promega). The cellular effects of pre-miR-375 or LNA miR-106b on HNSCC cells were further investigated using clonogenic assays. On the third day after transfection, HNSCC cells were reseeded in six-well plates in triplicate and incubated at 37°C under 5% CO2. After 10 to 12 d of incubation, plates were washed, fixed, and stained with 0.1% crystal violet in 50% methanol, and the number of colonies was then counted. The fraction of surviving cells was calculated by comparison with cells treated with scrambled LNA or pre-miR negative.

Apoptosis and cell cycle effects. Cell cycle analysis of pre-miR-375–transfected or LNA miR-106b– transfected HNSCC cells was done as previously described (16). Briefly, cells were harvested and washed with fluorescence-activated cell sorting buffer (PBS/0.5% bovine serum albumin) and then resuspended and fixed with ice-cold 70% ethanol. After washing, cells were resuspended in fluorescence-activated cell sorting buffer and incubated in the dark before analysis in the BD FACSCalibur using the FL-2A and FL-2W channels. The flow cytometry data were analyzed using FlowJo software (TreeStar).

Data pre-processing. Expression values were calculated using the ΔΔCt method of Pfaffl (17), with a PCR amplification efficiency of E = 2 for all primers, and using the mean Ct of the four normal samples as reference. Ten candidate endogenous control genes were measured (RNU16B, RNU19, RNU24, RNU38B, RNU43, RNU44, RNU48, RNU49, and Z30), from which the mean Ct of the six (RNU6B, RNU24, RNU43, RNU44, RNU48, and RNU49) with two or fewer undetermined values was used as the endogenous control. Additionally, all target miRNAs with undetermined Ct values in three of four normal samples, or 80% of tumor samples, were removed, leaving 117 miRNAs for analysis. Ct values that were undetermined or >36 were imputed to 36. Normalization and all statistical analyses and plotting were done in the R language and environment for statistical computing (R Development Core Team, v2.8.1).

Analysis of miRNA as a function of tumor site, tumor-normal, or disease status. After imputing missing values to 36, expression values for each miRNA were clearly not normally distributed; hence, nonparametric tests were applied for all inferences. To search for miRNAs differentially expressed in tumors from the three different anatomic subsites, each miRNA was examined using the Kruskal-Wallis rank sum test, with the Benjamini-Hochberg adjustment for false discovery rate (FDR) as implemented in the multtest package (version 1.2.0; ref. 18). miRNAs differentially expressed between tumor and normal or between relapsed and nonrelapsed tumors were identified using the Mann-Whitney U test with FDR adjustment for multiple testing as described above. Hierarchical clustering for the tumor versus normal heat map was done with the hclust function in the R base package v2.8.1 (19), with default options and 1 minus Spearman rank correlation as the distance measure, whereas the heat map was produced with the heatmap.2 function in the package gplots v2.6.0 (20).

Results

Global miRNA profiling of HNSCC samples. Comprehensive miRNA profiles were generated for 51 HNSCC patients using a quantitative RT-PCR approach; three patient samples were excluded from analysis due to poor RNA quality. From a total of 322 miRNAs, 117 were detected in >80% of the samples, thereby serving as the pool of data for further analyses. In comparison with normal tissues, HNSCC samples contained more upregulated than downregulated miRNAs (Supplementary Table S2). Using an adjusted FDR of 0.3, 38 of 117 (33%) miRNAs were significantly differentially expressed between cancer versus normal (Table 1), wherein 23 were upregulated and 15 were downregulated. The expression pattern of these listed miRNAs in Table 1 with >2-fold change is presented as a clustered heat map (Fig. 1), showing a distinct cluster between normal and HNSCC samples. The most significant aberration was a >32-fold downregulation of miR-375, observed in 46 of 51 (91%) HNSCC samples. Other distinct dysregulations included overexpression (>4-fold) of miR-106b, miR-142-3p, and miR-423 as well as downregulation of miR-125b and miR-140. Interestingly, many of these differentially expressed miRNAs were located on the same chromosomal region. For example, chromosome 13q31.3 harbored the largest number of upregulated miRNAs, including miR-20a, miR-92, miR-17-5p, and miR-19b. Both 7q22.1 (miR-93, miR-106b, and miR-25) and Xq26.2 (miR-92, miR-19b, and miR-106a) contained three upregulated miRNAs. On the other hand, several downregulated miRNAs were located on the commonly deleted regions of chromosomes 11q24.1 (miR-125b-1, let-7a, and miR-100) or 21q21.1 (miR-125b-2, miR-99a, and let-7c).

Correlation of miRNA expression with clinical parameters. The 51 HNSCC patient samples investigated in this study were derived from the larynx, oropharynx, or hypopharynx. Euclidean clustering analysis showed that the global miRNA profiles from these three subsites were indistinguishable; the supervised clustering in Fig. 1 also showed no apparent grouping. The expression value of individual miRNAs was then directly compared between each tumor subsite with that of normal (Supplementary Fig. S1).
showing five significantly differentially expressed miRNAs ($P < 0.05$). However, using a FDR cutoff of 0.3, only miR-133b remained significantly differentially expressed across tumor subsites (FDR $P = 0.01$), in that it was significantly overexpressed in laryngeal SCCs compared with tumors arising from either the oropharynx or the hypopharynx.

In terms of miRNA expression as a function of disease status, only miR-451 was significantly differentially expressed (Supplementary Fig. S2), with a 4.7-fold higher expression in the 9 nonrelapsed versus the 42 relapsed patient samples (FDR < 0.3, $P = 0.0009$). There were 13 additional differentially expressed miRNAs (using a FDR cutoff of <0.4): 1 with lower expression in nonrelapsed and 12 with higher expression between nonrelapsed and relapsed patients (Supplementary Table S3). The pattern of the other top six miRNAs with differential expression between patients with or without relapse is shown in Supplementary Fig. S2.

### Table 1. List of 38 differentially expressed miRNAs in HNSCC

<table>
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<tr>
<th>miRNAs</th>
<th>Raw $P$</th>
<th>FDR-adjusted $P$</th>
<th>Mean of log$_2$ T:N ratio</th>
<th>Chromosomal location</th>
<th>% Cases with 2-fold differential expression</th>
<th>Described in previous HNSCC studies</th>
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<td>miR-125b</td>
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<td>10, 11</td>
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<td>0.18</td>
<td>1.23</td>
<td>12q14.1</td>
<td>47</td>
<td>9, 11</td>
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Abbreviations: T, tumor; N, normal.

*Taqman miR primers target the same sequence of mature miRNA that originated from different stem-loop sequences.
Functional characterization of HNSCC-associated miRNAs. To understand the biological roles of these differentially expressed miRNAs in HNSCC, the expression of the top 38 miRNAs (Table 1) was measured in three human HNSCC cell lines: FaDu, UTSCC-8, and UTSCC-42a. From this panel of deregulated miRNAs, consistent upregulation of miR-25, miR-27a, miR-423, miR-93, and miR-106b as well as downregulation of miR-10a were observed in all three cell lines (Supplementary Fig. S3). Hence, these six miRNAs, together with miR-375 (the most consistent aberration detected in the patient samples), were selected for further evaluation.

Validation of the differentially expressed miRNAs on HNSCC cells. Gain-of-function effects of miR-10a were investigated in the three HNSCC cell lines using the targeted pre-miR miRNA precursor molecule. At 72 hours after
transfection with pre-miR-10a (30 nmol/L), miR-10a expression was elevated by >1,000-fold in all three cell lines, particularly for the FaDu cells, compared with Lipofectamine 2000 or pre-miR negative control (30 nmol/L; Fig. 2A). Despite this augmented miR-10a expression, no significant change in cell number was observed in any of the cell lines (data not shown).

The four overexpressed miRNAs (miR-25, miR-27a, miR-93, and miR-423) were then targeted using the LNA approach in the three HNSCC cell lines (Fig. 2B-E). Decreased expression was observed in all cell lines assayed at 72 hours after transfection, with the greatest reduction observed for miR-25 and miR-27a by >100-fold. However, no consistent effect on cell viability or proliferation was observed (data not shown).

As illustrated in Fig. 3C, increased miR-375 expression caused a significant reduction in cell viability observed at 48 hours (13.9%, \( P = 0.008 \)) and 72 hours (14%, \( P = 0.04 \)) after transfection, which translated to a 40% reduction in clonogenic survival (\( P = 0.01 \); Fig. 3D). However, neither cell viability nor colony-forming ability was significantly affected in the UTSCC-8 or UTSCC-42a cells (data not shown).

Cell cycle analysis showed a modest increase (4.4%) in the proportion of FaDu cells at sub-G1, first observed at 48 hours after pre-miR-375 transfection, which increased to 7.5% at 72 hours (\( P = 0.005 \); Supplementary Fig. S4A). UTSCC-8 and UTSCC-42a did not show any significant changes in cell cycle (data not shown).

Effect of miR-106b knockdown on HNSCC cells. The most upregulated miRNA was miR-106b, being 5.56-fold higher in HNSCC versus normal tissues (Table 1). Using a 2-fold cutoff, miR-106b was overexpressed in 42 of 51 (82%) of head and neck tumors. Hence, knockdown of miR-106b by LNA was investigated in FaDu, UTSCC-42a, and UTSCC-8 cells, all showing significant reduction of expression level compared with LNA scramble–treated cells, observed as early as 24 hours, persisting until 72 hours after transfection (Fig. 4). The greatest reduction was observed in the UTSCC-8 cells by >100-fold at 72 hours (Fig. 4C).
The knockdown in miR-106b expression was in turn associated with reduced viability (Fig. 5), particularly for the FaDu and UTSCC-8 cells (Fig. 5A and C). LNA knockdown of miR-106b also reduced clonogenic survival for both FaDu and UTSCC-42a cells, down to 60% and 75%, respectively, compared with scrambled LNA-treated cells ($P = 0.004$ and 0.01, respectively; Fig. 5D). In contrast, colony-forming ability was not significantly affected in the UTSCC-8 cells.

Cell cycle analysis was investigated at 72 hours after miR-106b knockdown. A modest increase in the proportion of cells arrested at G1 was observed for the FaDu, UTSCC-42a, and UTSCC-8 cells, by 4.7%, 4.1%, and 4.3%, respectively (Supplementary Fig. S4B). In turn, this was associated with a slight reduction in the G2-M population; no change in the sub-G1 phase was observed.

**Discussion**

A global miRNA profiling was conducted on 51 archival FFPE samples of locally advanced stage HNSCC arising from the larynx, oropharynx, and hypopharynx. Approximately one third (38 of 117) of consistently detected miRNAs were aberrantly expressed. Some of these miRNAs have been previously described in HNSCC studies, such as miR-21 (8–11). Upregulation of miR-21 is among the commonest miRNA alterations described for human cancers (21), reported to be antiapoptotic (22), cause cell proliferation (23–25), and promote invasion and metastases (24, 26). Several mRNA targets of miR-21 have been identified, including PTEN, RECK, NFIB, TPM-1, PDCD4, and Maspin (24–27). Specific to HNSCC, knocking down miR-21 has been shown to increase cytochrome c release with subsequent apoptosis (9). Identification of miR-21 overexpression (by >3.5-fold) in 78% of our samples further corroborated the importance of miR-21 in HNSCC.

In the present study, the most significantly downregulated miRNA was miR-375, being underexpressed by 32-fold compared with normal tissues (Table 1). miR-375 was first noted in pancreatic islet cells (28) and is involved in pancreatic islet development (29) and insulin secretion (30). In cancers, miR-375 is downregulated in hepatocellular carcinoma, associated with $\beta$-catenin mutations (31).

A previous HNSCC study reported consistent downregulation of miR-375 and suggested that the ratio of high miR-221 to low miR-375 could distinguish cancer from normal
tissues (8). In our current study, miR-221 was not significantly overexpressed; hence, we were unable to corroborate this observation. Functional analyses showed that transfection of miR-375 decreased cell proliferation and clonogenicity of FaDu cells (Fig. 3), along with an increase in the sub-G1 population (Supplementary Fig. S4A). These findings are consistent with a previous report documenting that miR-375–deficient mice are hyperglycemic, with decreased pancreatic α-cell mass suggestive of impaired proliferation (30). Using a combinatorial analysis of putative targets and microarray expression data, these authors showed that candidate miR-375 targets included genes involved in apoptosis, normal development, and regulation of cell growth and proliferation (30). Hence, underexpression of miR-375 in HNSCC would result in dysregulated proliferation and apoptosis, leading to uncontrolled cell growth.

We have also identified a group of novel HNSCC-associated miRNAs, wherein 5 of the top 10 differentially expressed miRNAs are new to HNSCC, such as upregulated miR-423, miR-106b, miR-16, and miR-20a as well as downregulated miR-10a (Table 1). The dysregulation of these novel miRNAs is recapitulated in the HNSCC cell lines, suggesting their potential role in disease progression (Fig. 2). The potential significance of the miR-106b-25 cluster was suggested by our knockdown studies of miR-25, miR-93, and miR-106b, all resulting in significant reduction in their respective miRNA expression levels in the HNSCC cell lines (Figs. 2 and 4). miR-106b is the miRNA with the highest (5.6-fold) overexpression in our cohort of patient samples. Functional analyses further showed that knocking down miR-106b decreased proliferation and clonogenicity of HNSCC cells (Fig. 5), along with G1 arrest (Supplementary Fig. S4B), similar to another study on esophageal cancer (32). The specific downstream targets of miR-106b in HNSCC remain to be dissected, but preliminary results from our in vitro mRNA profiling study suggest that cell cycle and cell proliferation genes are likely relevant.

10 Unpublished data.
miR-106b-25 cluster could be activated by E2F1, in parallel with its host gene Mcm7, which in turn interferes with TGF-β signaling via suppression of p21 mediated by miR-106b and miR-93 as well as silencing of Bim via miR-25 (34). As an added level of complexity, miR-106b and miR-93 can independently regulate E2F1 expression, leading to a negative feedback loop, which is probably important in preventing E2F1 self-activation and apoptosis (34). Similar observations have been reported for the miR-106b-25 cluster in neoplastic transformation of Barrett's esophagus mediated by suppression of p21 and Bim (32).

The role of deregulated TGF-β signaling in HNSCC progression is complex (35, 36), wherein both low levels TGF-β expression and loss of TGF-β RII have been described (37, 38). Similarly, overexpression of E2F1 and MCM7 has been observed (39), correlated with increased invasiveness (40, 41) and more aggressive disease (42), and suggested to be predictive markers for nodal metastases (43). Our current study strongly suggests the biological significance of the miR-106b-25 cluster in HNSCC, indicating that overexpression of this cluster might play an oncogenic role, perhaps mediated by E2F1 activation and impaired TGF-β signaling, ultimately resulting in uncontrolled proliferation, dysregulated cell cycling, and increased invasiveness.

The mechanisms leading to aberrant miRNA expression in human cancers remain unclear. Previous studies have identified that most (53%) of the dysregulated miRNAs are located in cancer-associated genomic regions or fragile sites (44). In this current study, we have also observed nonrandom distributions of aberrant miRNA, which map to chromosomal regions that have been previously described in association with HNSCC. Specifically, miRNAs located on the oncogenic miR-17-92 polycistron were consistently upregulated in our investigated samples (Table 1), including increased expression of miR-20a, miR-92, miR-17-5p, and miR-19b-1, all located in the miR-17-92 cistron at the c13ORF25 of 13q31. Similarly, overexpression of miR-92a, miR-19b, miR-106a, and miR-20b (Supplementary Table S2), which belong to the homologue of miR-106a-92 cluster, located on chromosome Xq26 (Table 1). In addition, upregulation of the aforementioned miR-106b-25 cluster, comprising miR-106b, miR-93, and miR-25, which are all located in intron 13 of the MCM7 gene. These three regions are frequently amplified in HNSCC (42, 45–49). Alternatively, we identified underexpressed miRNAs mapping to two frequently deleted regions in HNSCC (45, 47, 49–51), such as 11q24.1 (miR-125b, let-7a, and miR-100) and 21q21.1 (miR-99a and let-7c). These results collectively show that
one of the common mechanisms of aberrant miRNA expression in HNSCC is a consequence of genomic amplifications or deletions.

In relation to clinical parameters, our current study shows no distinct differences in the global miRNA profiles between squamous cell cancers arising from the larynx, oropharynx, or hypopharynx. The only exception was miR-133b, which was more highly expressed in laryngeal versus the other two subsites; the significance of which remains to be defined. This lack of distinct miRNA expression pattern as a function of subsite would be concordant with previous similar studies wherein no differences in expression profiles were observed among tongue, oropharyngeal, and laryngeal cancer cell lines (12) or differential expression of miR-21 and miR-494 among these subsites (9). Given our aforementioned hypothesis that aberrant miRNA expression is likely arising from genomic aberrations, this would be consistent with reports on similar patterns of chromosomal abnormalities among laryngeal, oropharyngeal, and hypopharyngeal squamous cancers (50).

Disappointingly, we were unable to identify a distinct miRNA pattern between relapsed and nonrelapsed patients in this study, which might be due to the limited sample size and imbalance between relapsed (42 samples) and nonrelapsed (9 samples) patients. It should be mentioned that the treatment delivered in the study in which these patients were enrolled would be considered of lesser intensity as compared with current standards delivering higher doses with or without concurrent chemotherapy. This and the fact that the technical aspects of treatment planning and delivery for these patients were different from current practice may account for the relatively high rates of relapse. Disease relapse due to potential under-treatment by technical or dose intensity factors may overshadow the influence of biological factors and hinder their identification. It remains to be seen therefore if there would exist a correlation between miRNA profiles and outcome in a more contemporary patient population. The only significant differential expression between these groups was downregulation of miR-451 in relapsed patients. miR-451 seems to be a key molecule in normal thyroid differentiation (52) and has also been shown to regulate the multidrug resistance 1 gene in cervix, ovarian, and breast cancer cell lines (53, 54). Underexpression of miR-451 has been associated with worse prognosis in gastric cancer, with macrophage inhibitor factor suggested as its potential mRNA target (55). Hence, downregulation of miR-451 could be a viable candidate marker for HNSCC; we are currently collecting a larger cohort of FFPE samples to determine the potential role of miR-451 in HNSCC.

In conclusion, global miRNA profiling of archival formalin-fixed HNSCC samples has identified that approximately one third of the miRNAs are dysregulated in this disease, with “anatomic” chromosomal aberrations as one mechanism leading to their abnormal expression. The miR-106b-25 cluster and miR-375 likely mediate HNSCC development and progression; miR-451 could be a potential prognostic marker. Further understanding of miRNA biology in HNSCC is a fruitful avenue to derive novel insights into this complex disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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