

miR-486-3p, miR-139-5p, and miR-21 as Biomarkers for the Detection of Oral Tongue Squamous Cell Carcinoma

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ABSTRACT: Oral tongue squamous cell carcinoma (TSCC) is a complex disease with extensive genetic and epigenetic defects, including microRNA deregulation. The aims of the present study were to test the feasibility of performing the microRNA profiling analysis on archived TSCC specimens and to assess the potential diagnostic utility of the identified microRNA biomarkers for the detection of TSCC. TaqMan array-based microRNA profiling analysis was performed on 10 archived TSCC samples and their matching normal tissues. A panel of 12 differentially expressed microRNAs was identified. Eight of these differentially expressed microRNAs were validated in an independent sample set. A random forest (RF) classification model was built with miR-486-3p, miR-139-5p, and miR-21, and it was able to detect TSCC with a sensitivity of 100% and a specificity of 86.7% (overall error rate = 6.7%). As such, this study demonstrated the utility of the archived clinical specimens for microRNA biomarker discovery. The feasibility of using microRNA biomarkers (miR-486-3p, miR-139-5p, and miR-21) for the detection of TSCC was confirmed.

KEYWORDS: microRNA, biomarker, miR-486-3p, miR-139-5p, miR-21, formalin-fixed paraffin-embedded, tongue squamous cell carcinoma

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Introduction

Head and neck/oral cancer (HNOc) is the sixth most common cancer worldwide, accounting for 4% of cancers in men and 2% of cancers in women,¹ with an incidence of approximately 600,000 cases per year and a mortality rate of approximately 50%.² In some parts of the world, including Southern China and the Indian subcontinent, head and neck squamous cell carcinoma (HNSCC) is still a major cancer problem. Tongue squamous cell carcinoma (TSCC) is significantly more aggressive compared to other HNOcs, with a propensity for rapid spreading and local invasion³ with a distinct pattern of lymph nodal metastasis,^{4,5} and high recurrence rate.⁶ Despite recent advances in clinical strategies for treating TSCCs, the overall survival has improved only marginally. This is because TSCCs are often detected at later stages. Advances in cancer screening strategies and early detection methods are required for improvement in the prognosis of TSCC patients.

Like most of the other human cancers, TSCC is a disease involving multistep dynamic changes in the genome. While many recent studies have attempted to identify molecular biomarkers for the screening and early diagnosis of TSCC, most of these studies are based on coding genes. The potential value of utilizing microRNAs as biomarkers for TSCC detection is still not entirely clear. MicroRNAs are endogenous noncoding single-stranded small RNA molecules (18–25 nt). A number of microRNA genes have recently been characterized as oncogenes or tumor-suppressor genes, and their deregulations have been detected in many cancers, including HNOcs.^{7–11} MicroRNAs are important gene expression regulators, and they control the expression of their target genes by posttranscriptional mechanisms. Deregulation of these *cancerous* microRNA genes (eg, overexpression or deletion) contributes to tumorigenesis by promoting proliferation, survival, and invasion.^{12,13} MicroRNA deregulation is a frequent event in HNSCC. A number of



recent reports demonstrated the feasibility of utilizing microRNAs as biomarkers to detect cancer cases from noncancerous specimens, with varying degrees of success.^{14,15} This type of microRNA biomarker-based approach can enhance the standard diagnostic technique of histopathologic examination.

In this study, we identified a panel of differentially expressed microRNAs based on the TaqMan array microRNA profiling analysis of archived TSCC samples and normal matched tissue samples. Using a statistical model based on three microRNA biomarkers (miR-486-3p, miR-139-5p, and miR-21), we were able to identify TSCC cases from an independent validation set, with a sensitivity of 100% (15/15), a specificity of 86.7% (13/15), and an overall error rate of 6.7%.

Patients and Methods

Patient cohorts. The following two TSCC patient cohorts were used in this study: (1) the training set: specimens from 10 TSCC cases and the adjacent matched normal tissue samples were used for microRNA differential expression profiling analysis and (2) the validation set: microRNA expression data (deep sequencing-based profiling results) and the associated demographic and clinical information of 15 TSCC cases that have matched normal samples were obtained from The Cancer Genome Atlas repository (TCGA; <https://tcga-data.nci.nih.gov>). Demographic and clinical information of the patients is presented in Table 1. This investigation was approved by the Institutional Review Board (IRB) of the University of Illinois at Chicago.

Laser-capture microdissection and RNA isolation. Laser-capture microdissection (LCM) procedure was

performed as described previously.^{16,17} In brief, 7 μ m sections were cut with a microtome and mounted onto Leica RNase-free PEN slides (Leica). The paraffin sections were deparaffinized and lightly stained with toluidine blue. The tumor and noncancerous epithelial cells were selectively procured using a Leica LMD7000 Laser Microdissection System. The LCM-captured cells were collected into Eppendorf caps containing 50 μ L of digestion buffer (from RecoverAll kit).

Total RNA was extracted using RecoverAll (Thermo Fisher Scientific), following the manufacturer's protocol with the exception of increased DNase digestion for 60 minutes at 37°C. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

microRNA expression analysis by TaqMan low-density array and by TaqMan assay. MicroRNA profiling analysis was performed using the TaqMan low-density array (TLDA; Applied Biosystems), following the manufacturer's protocol with minor modifications as previously described.¹⁸ In brief, 20 ng of RNA was used as the input for cDNA generation. Eight distinct pools of RT primers were used for analysis of 370 distinct microRNAs. Following dilution, 14 cycles of preamplification with the Megaplex pool protocol for the array were performed on the cDNA. Following dilution, the cDNAs were loaded onto the arrays (Human miRNA Array v1.0; Applied Biosystems). This facilitated analysis of 386 wells; 370 distinct microRNAs were analyzed in singlicate and two housekeeping snoRNAs with eight replicates for each. Individual TaqMan assays were also performed for selected microRNAs in triplicates for the validation study. To control for potential variations in RNA samples isolated from each case,¹⁹ we also assessed U6 snRNA with TaqMan assay (Thermo Fisher Scientific). The polymerase chain reaction (PCR) was performed on an ABI 7900HT real-time PCR system (Thermo Fisher Scientific). Ct (crosses threshold) values were determined for all samples and genes, and delta Ct (Δ Ct) was computed using U6 snRNA as an internal control.²⁰

Data analysis and statistical methods. MicroRNA differential expression analysis was performed using CyberT,^{21,22} and hierarchical clustering and principal component (PC) analysis were performed using ClustVis.²³ Other statistical analyses were performed using the S-Plus 6.0 and R 3.2.2. The differences between groups were evaluated by Wilcoxon signed-rank test. Receiver-operating characteristic (ROC) curve analysis was performed, and the area under the ROC (AUROC) was computed for assessing the predictive power of the selected biomarkers. To select the combination of biomarkers that provides the best prediction, the random forest (RF) classification model was utilized. The Mean Decrease Gini was computed to assess the relative importance of microRNA biomarkers toward an RF classification model. Since the structure of the data for the training set and validation set are different (Δ Ct value for the quantitative PCR measurement, and reads per million miRNA mapped for the deep sequencing-based quantification, respectively), a simple transformation was performed on all data before the statistical

Table 1. Clinical characterization of the TSCC cohorts.

	TRAINING SET (n = 10 PAIRS)	VALIDATION SET ^a (n = 15 PAIRS)
Age		
Median (range)	47 (43–81)	61 (32–87)
Gender		
Male (%)	50.0	53.3
Female (%)	50.0	46.7
Anatomic site		
Tongue (%)	90.0	86.7
Base of tongue (%)	0.0	13.3
Tongue, tonsil (%)	10.0	0.0
pT		
pT3–4 (%)	30.0	46.7
pT1–2 (%)	70.0	53.3
pN		
Positive (%)	50.0	53.3
Negative (%)	50.0	33.3
Data not available (%)	0.0	13.3

Note: ^aThe data for the validation set were extracted from The Cancer Genome Atlas (TCGA) Data Portal.

Table 2. Receiver-operating characteristic (ROC) curve analysis of TSCC-associated microRNAs.^a

	TRAINING SAMPLE SET		VALIDATION SAMPLE SET ^b	
	WILCOXON (P VALUE)	ROC (AUC)	WILCOXON (P VALUE)	ROC (AUC)
miR-486-3p	0.0006	0.9333	0.0619	0.7022
miR-21	0.0021	0.9000	<0.0001	0.9911
miR-486-5p	0.0015	0.9111	0.0086	0.7778
miR-139-5p	0.0006	0.9333	0.0002	0.8756
miR-204	0.0133	0.8333	<0.0001	1.0000
miR-489	0.0041	0.8778	0.8985	0.4844
miR-223	0.0057	0.8667	0.1064	0.6756
miR-196b	0.0057	0.8667	<0.0001	0.9822
miR-31	0.0435	0.7778	0.0050	0.7956
miR-422a	0.0042	0.8778	0.0384	0.6333
miR-328	0.2428	0.6667	0.2854	0.6178
miR-146b-5p	0.0279	0.8000	0.0003	0.8667

Notes: ^aThe microRNA data for the training sample set and validation sample set were assessed with different platforms (TaqMan assay and deep sequencing, respectively). To enable the comparison between these datasets, transformation was performed as described in "Patients and methods" section. ^bThe miRSeq dataset for 15 TSCC and paired normal tissue samples was downloaded from TCGA data portal. The levels of microRNAs were extracted as reads per million miRNA mapped.

modeling $[(x - \mu_{(normal)})/\sigma_{(normal)}]$ where $\mu_{(normal)}$ is the mean of the normal group and $\sigma_{(normal)}$ is the standard deviation of the normal group].

Results

LCM was performed on 10 archived TSCC samples and matched noncancerous tissue samples, and microRNA

profiling analysis was performed using TaqMan arrays. As shown in Table 2 and Supplementary Table 1, we identified a panel of 12 differentially expressed microRNAs. Hierarchical clustering (Fig. 1A) and PC analysis (Fig. 1B) suggested that this panel of microRNAs could provide classification values distinguishing TSCC samples from normal tissue samples.

Of the 12 identified microRNAs, the differential expression was validated for 11 microRNAs (miR-486-3p, miR-21, miR-486-5p, miR-139-5p, miR-204, miR-489, miR-223, miR-196b, miR-31, miR-422a, and miR-146b-5p) using the individual TaqMan assays on the same sample set (Fig. 2). Differential expression of miR-328 was not validated ($P = 0.238$; Fig. 2K). To further validate the differential expression of the identified microRNAs, microRNA expression profiling results on 15 TSCC cases and the paired normal tissue samples were obtained from TCGA. Of the 12 microRNAs tested, the differential expression was validated for 8 microRNAs (miR-21, miR-486-5p, miR-139-5p, miR-204, miR-196b, miR-31, miR-422a, and miR-146b-5p) based on the TCGA dataset (Table 2 and Supplementary Table 2). Differential expression of miR-489, miR-223, and miR-328 was not validated. Apparent differential expression of miR-486-3p was also observed, but the difference is not statistically significant ($P = 0.0619$).

The ROC analysis was performed to assess the predictive powers of the identified microRNA biomarkers using both sample sets. The AUROC for miR-486-3p, miR-21, miR-486-5p, miR-139-5p, miR-204, miR-489, miR-223, miR-196b, miR-31, miR-422a, miR-328, and miR-146b-5p were 0.9333, 0.9000, 0.9111, 0.9333, 0.8333, 0.8778, 0.8667, 0.8667, 0.7778, 0.8778, 0.6667, and 0.8000 for the training samples set, and 0.7022, 0.9911, 0.7778, 0.8756, 1.0000,

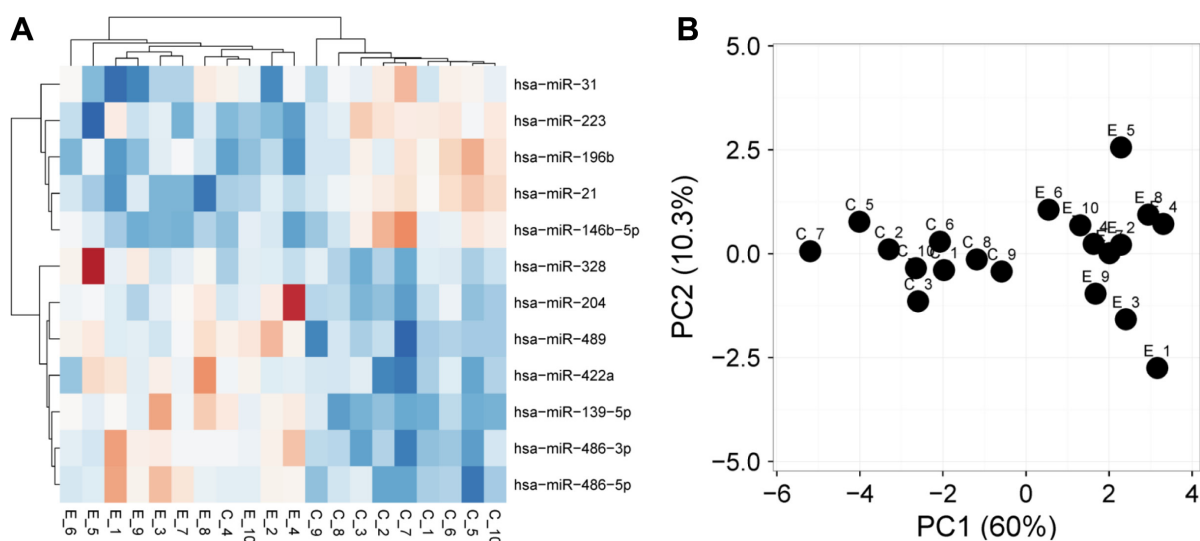


Figure 1. MicroRNAs profiling on TSCC samples. Laser-capture microdissection was performed to acquire tumor cells from 10 cases of archived TSCC samples and matched normal samples. MicroRNA profiling was performed on these samples using TaqMan microRNA arrays. A signature gene set of 12 microRNAs was created as described in the "Patients and methods" section. Hierarchical clustering (A) and principal component (PC) analysis (B) were performed based on this signature set.

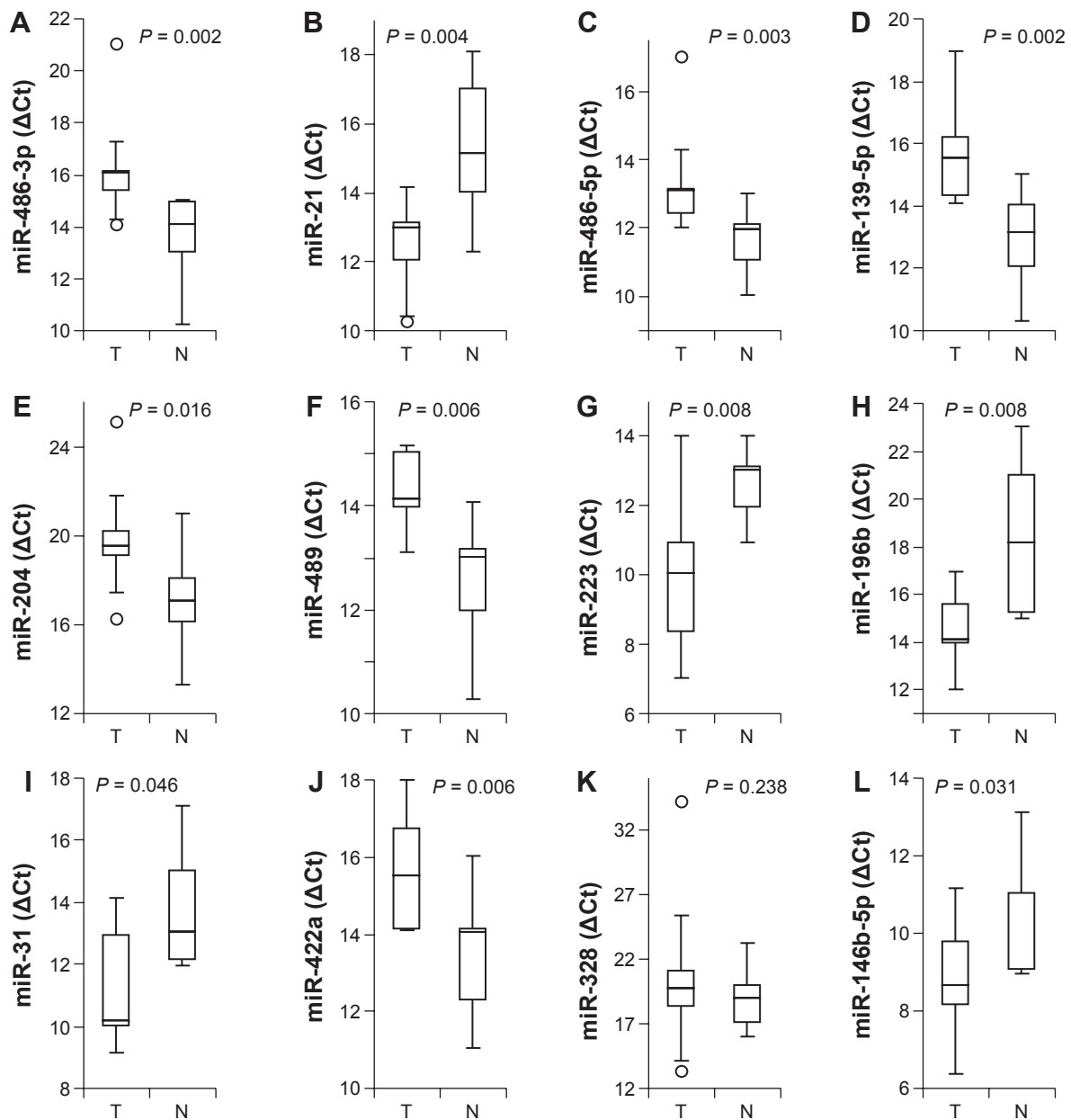


Figure 2. MicroRNAs differential expression on TSCC samples. The TaqMan-based qPCR was performed to assess the levels of miR-486-3p (A), miR-21 (B), miR-486-5p (C), miR-139-5p (D), miR-204 (E), miR-489 (F), miR-223 (G), miR-196b (H), miR-31 (I), miR-422a (J), miR-328 (K), and miR-146b-5p (L) on TSCC samples and normal samples. The boxes represent 25th to 75th percentile of the observations, and the lines in the middle of the box represent the median. The whiskers represent maximum (or minimum) observations below (or above) the 1.5 times of the interquartile range, respectively. Outliers are also indicated in the plots as black circles.

0.4844, 0.6756, 0.9822, 0.7956, 0.6333, 0.6178, and 0.8667 for the validation sample set (TCGA), respectively (Table 2).

The RF model was built for selecting the best biomarker combination that can provide the highest prediction power. The Gini importance values were computed to evaluate the relative importance of the biomarkers, and miR-486-3p, miR-139-5p, and miR-21 have the highest relative importance (Fig. 3). The RF classification model based on these top three microRNA biomarkers was used to classify normal and TSCC cases of the validation sample set ($n = 30$). As shown in Table 3, the overall error rate of this classification

model is 6.7% [sensitivity = 100% (15/15) and specificity = 86.7% (13/15)].

Discussion

While mechanistic studies of the roles of microRNA in tumorigenesis are gaining momentum, translational studies utilizing microRNAs as biomarkers are still in their infancy. Nevertheless, microRNAs are remarkably stable in cells, tissue specimens (archived or fresh), and a number of biofluids, and as such, microRNA-based biomarkers are less prone to minor differences in sample processing, which offers a great

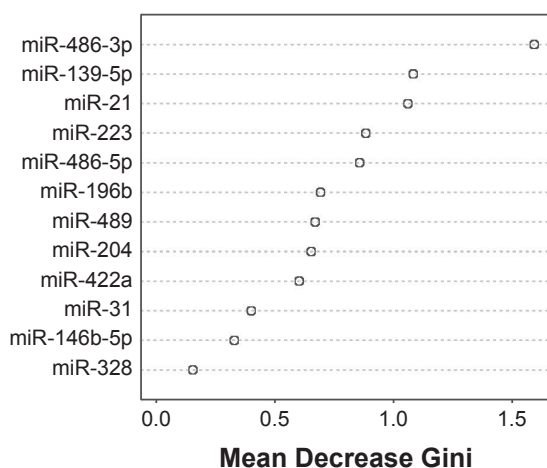


Figure 3. Ranking the microRNA biomarkers by random forest model. The relative importance of microRNA biomarkers toward a random forest classification model was assessed by computing the Gini importance, and the microRNAs were ranked by Mean Decrease Gini.

advantage over other classes of biomarkers. In the clinical setting, the storing of formalin-fixed paraffin-embedded (FFPE) tissue blocks is the standard method for pathology departments to archive almost all tissue samples, which can then be linked with clinical databases, including disease diagnoses and patients' follow-up information. As such, reliable biomarkers based on FFPE samples will greatly facilitate large-scale clinical cohort-based studies and provide an extremely powerful tool for cancer research. Hence, it may be the only way to make the integration of new biomarkers into the large-scale and clinical trial studies possible. However, it has been assumed that FFPE samples have insufficient quality of RNA, and, as a result, these samples have not been routinely used in biomarker studies. In recent years, it has been shown that RNA stability varies among different RNA species and that microRNAs are very stable in relation to other RNA species (eg, mRNA). One explanation is that both ends of the mature microRNA are protected by Argonaute family proteins and the entire microRNA molecule is incorporated into the RNA-induced silencing complex (RISC).²⁴ These protein-microRNA complexes may prevent microRNA from degradation, especially during the formalin fixation process and while in long-term storage in paraffin.

Table 3. Random forest classification model for TSCC prediction.^a

Prediction	TRUTH	
	NORMAL	TSCC
Normal	13	0
TSCC	2	15

Notes: ^aRandom forest classification model based on top three microRNA biomarkers (miR-486-3p, miR-139-5p, and miR-21) was used to predict normal and TSCC cases of the validation sample set ($n = 30$). The sensitivity of this classification model is 100% (15/15), and the specificity is 86.7% (13/15).

The potential of utilizing microRNA as biomarkers in FFPE samples has recently been explored by several groups.^{18,25-30} Recent studies indicated that quantitative PCR methods based on small amplicons (such as TaqMan assays) work well with microRNA quantification, even with suboptimal starting materials, eg, samples acquired FFPE tissue blocks.¹⁸ Our current study confirms the feasibility of utilizing microRNAs from archived specimens as biomarkers to discriminate TSCC from noncancerous control tissues. We identified a panel of 12 differentially expressed microRNAs using the FFPE tissue blocks of TSCC specimens, including miR-486 (both -3p and -5p), miR-21, miR-139-5p, miR-204, miR-489, miR-223, miR-196b, miR-31, miR-422a, miR-328, and miR-146b-5p. Aberrant expression of miR-486 is a common event in many cancer types³¹⁻³⁷ and has been suggested to have potential diagnostic value for lung cancer³⁸ and prognostic values for patients with esophageal squamous cell carcinoma (ESCC) or gastric adenocarcinoma (GC).³⁹ MicroRNA-21 is one of the most well-documented oncogenic microRNAs^{40,41} and has been suggested as a biomarker for many types of cancer, including HNOc.⁴²⁻⁴⁶ Downregulation of miR-139 has been observed in HNOc,^{7,8,47} and a recent study suggested that miR-139-5p from saliva samples may be a molecular biomarker for the early diagnosis of TSCC.⁴⁸ While miR-204 has been suggested as a tumor suppressor in HNOc,⁴⁹⁻⁵¹ conflicting results regarding its expression pattern have been reported in various cancer types. The enhanced expression of miR-204 was observed in insulinomas and acute lymphocytic leukemia;^{52,53} downregulation of miR-204 was reported in other cancer types, including HNOc, lung cancer, gastric cancer, endometrial cancer, and renal cancer.⁵⁴⁻⁵⁷ Furthermore, conflicting results regarding the miR-204 expression were reported in some other cancer types including breast cancer⁵⁸⁻⁶⁰ and prostate cancer.^{61,62} Our results here confirmed that miR-204 is downregulated in TSCC, which supports its tumor-suppressor role. Relatively little is known on the role of miR-489 in cancer, and conflicting results regarding the aberrant expression pattern of miR-489 have been reported in HNOc.^{63,64} We observed downregulation of miR-489 in our training set, but no statistical significant change in miR-489 level in the validation set. Thus, the observed differential expression of miR-489 in HNOc remains controversial, and additional studies with large sample sizes will be needed to fully explore this. Upregulation of miR-223 has been consistently observed in both tumor tissues and plasma samples of HNOc patients,^{11,65-67} and the diagnostic value of circulating miR-223 as a biomarker for HNOc has recently been suggested.⁶⁷ MicroRNA-196b (together with miR-196a, the other member of miR-196 family) has been suggested as an OncomiR in HNOc, which promotes invasive phenotype and metastasis,^{68,69} and may serve as a biomarker for early detection and prognosis.^{70,71} The role of miR-31 appears to be cancer type specific; although miR-31 inhibits metastasis in breast cancer,⁷² upregulation



of miR-31 is essential to the TGF-beta-induced invasion and metastasis of colon cancer.⁷³ The increases of miR-31 in tumor tissue, saliva, and plasma samples have all been suggested as potential biomarkers of HNOc.^{74–76} MicroRNA-422a has been suggested as a tumor suppressor, and the reintroduction of miR-422a to cancer cells led to inhibition in cell proliferation, migration, invasion, metastasis, and enhanced chemosensitivity in various cancers.^{77–79} A recent study suggested that miR-422a deregulation may promote locoregional recurrence of HNOc.⁸⁰ The potential of utilizing miR-422a as a biomarker has been suggested for several cancer types, including colorectal cancer and osteosarcoma.^{81–84} Several studies have suggested that miR-328 may serve as a biomarker for glioma, thyroid cancer, and non-small cell lung cancer (NSCLC).^{85–89} However, the differential expression of miR-328 was not validated in our training set, and no statistical significant change of miR-328 was observed in our validation set. The expression of miR-146b-5p appears to be cancer type specific. In glioma, miR-146b-5p expression is downregulated and acts as a tumor suppressor.⁹⁰ However, in thyroid cancer and lung cancer, miR-146b-5p acts as an oncogene and has been suggested as a potential diagnostic marker.^{91,92}

Our statistical analysis indicated that miR-486-3p, miR-139-5p, and miR-21 have the best classification power in terms of discriminating TSCC from normal control samples. We constructed an RF model by combining miR-486-3p, miR-139-5p, and miR-21, and we were able to achieve an excellent classification outcome (sensitivity = 100%, specificity = 86.7%, and overall error rate = 6.7%). These results clearly indicated that microRNA molecules acquired from archived TSCC specimens can be accurately measured and employed as molecular biomarkers for the early detection of TSCC. However, our sample size is relatively small. Additional studies with larger sample sizes are required to fully evaluate these microRNA biomarkers in detecting TSCCs.

MicroRNA deregulation has also been identified in premalignant lesions^{57,75,93,94} and in the field of cancerization.^{95–98} Thus, microRNA biomarker-based approaches can potentially be implemented as a cancer-screening tool for monitoring the oral premalignant lesions and the field of cancerization. In our future study, we anticipate to expend our investigation by utilizing cancer tissue, premalignant lesions, and histologically normal tissue samples adjacent to cancer, as well as other clinical samples (eg, blood, serum, and/or saliva) from the same TSCC patients. This will allow us to fully explore the feasibility of utilizing microRNA biomarkers for cancer screening.

Conclusion

Our study demonstrated that microRNA deregulation can be accurately measured in archived clinical specimens and can be employed as molecular biomarkers for detecting TSCC. While utilizing microRNAs from snap-frozen specimens as biomarkers can produce better overall error rate,¹⁸ in the present study, microRNA biomarkers from archived clinical

specimens also resulted in excellent levels of sensitivity and specificity. A specific combination of microRNA biomarkers (miR-486-3p, miR-139-5p, and miR-21) can achieve optimal outcomes in distinguishing TSCC from noncancerous tissue samples. Advances in in situ hybridization (ISH) probes for microRNA detection present us with potential strategies of integrating microRNA-based analysis with histopathologic examination,^{99–101} which may lead to improvement of early detection and prevention of oral cancer. One of the main limitations of our study is the small sample size. It is possible that there are still other microRNA biomarkers yet to be identified. Additional studies consisting of early-stage TSCC cases with larger sample sizes are required to fully evaluate the feasibility of this microRNA-based approach for early detection.

Author Contributions

Conceived and designed the experiments: RJC, YJ, XL, YD, XZ. Performed experiments: ZC, YJ, IM. Analyzed the data: TY, LH, YD, XZ. Wrote the first draft of the manuscript: TY, RJC, YD, XZ. Contributed to the writing of the manuscript: ZC, YJ, IM, XL, LH. Agree with manuscript results and conclusions: ZC, TY, RJC, YJ, IM, XL, LH, YD, XZ. Made critical revisions and approved final version: TY, RJC, YD. All authors reviewed and approved of the final manuscript.

Supplementary Materials

Supplementary table S1. The levels of 12 microRNAs on 10 cases of OTSCC and paired normal tissues.

Supplementary table S2. The levels of 12 microRNAs on 15 paired OTSCC and normal tissues (TCGA dataset).

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