CLINICAL INVESTIGATIONS

Genomewide Study of Salivary MicroRNAs for Detection of Oral Cancer

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Abstract: MicroRNAs (miRNAs) in human saliva have recently demonstrated to be potential biomarkers for diagnosis purposes. However, lack of well-characterized/matched clinical groups and lack of suitable endogenous control (EC) for salivary extracellular miRNA detection and normalization are among the restrictions of applying salivary-based miRNA biomarker discovery. In the present study, we examined the differential expression pattern of miRNAs among 4 groups of subjects—including patients with oral squamous cell carcinoma (OSCC), patients with OSCC in remission (OSCC-R), patients with oral lichen planus, and healthy controls (HCs)—using a genomewide high-throughput miRNA microarray. First, we systematically screened 10 pooling samples and 34 individual samples of different groups to find a proper EC miRNA. We then investigated the genomewide expression patterns of differentially expressed miRNAs in saliva of different groups using NanoString nCounter miRNA expression assay and real-time quantitative polymerase chain reaction, followed by construction of receiver operating characteristic curves to determine the sensitivity and specificity of the assay. We identified miRNA-191 as a suitable EC

miRNA with minimal intergroup and intragroup variability, and we used it for normalization. Of more than 700 miRNAs tested, 13 were identified as being significantly deregulated in saliva of OSCC patients compared to HCs: 11 miRNAs were underexpressed (miRNA-136, miRNA-147, miRNA-1250, miRNA-148a, miRNA-632, miRNA-646, miRNA-668, miRNA-877, miRNA-503, miRNA-220a, miRNA-323-5p), and 2 miRNAs were overexpressed (miRNA-24, miRNA-27b). MiRNA-136 was underexpressed in both OSCC vs. HCs and OSCC vs. OSCC-R. MiRNA-27b levels were significantly higher in OSCC patients compared to those found in HCs, patients with OSCC-R, and patients with oral lichen planus and served as a characteristic biomarker of OSCC. Receiver operating characteristic curve analyses showed that miRNA-27b could be a valuable biomarker for distinguishing OSCC patients from the other groups. Our novel findings established a reliable EC miRNA for salivary-based diagnostic and indicate that the salivary miRNA profiles are discriminatory in OSCC patients.

Key Words: saliva, microRNA-27a, human, biomarker, squamous cell carcinoma, microRNA-191.

Introduction

Oral cancer—of which more than 90% is oral squamous cell carcinoma (OSCC)—is one of the most prevalent cancers worldwide (Chi, 2009). OSCC represents the malignant transformation of squamous epithelium that lines the oral cavity. Over the years, diagnosis and management of OSCC patients have improved through introduction of new treatment modalities in surgery, radiotherapy, and chemotherapy, but the overall 5-year survival rate remains around 62% (Siegel *et al.*, 2013). Late diagnosis, recurrences, and frequent regional lymph node metastatic are the major causes for the poor prognosis. Currently, OSCC detection depends on a clinical examination of the oral cavity, followed by histologic evaluation of suspected lesions, likely undetectable in hidden sites (Brinkmann *et al.*, 2011). Accordingly, clinically effective biomarkers for early detection of OSCC could greatly improve survival rate and prognosis.

Recently, detection of microRNA (miRNA) biomarkers in different biofluids, including plasma, serum, and saliva, has introduced a new paradigm in biomarker discovery (Yoshizawa and Wong, 2013). The miRNAs are endogenous, small, noncoding,

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regulatory RNAs (18-25 nucleotides long) that negatively regulate gene expression at the translational level by base pairing to the 3'-untranslated region of target messenger RNA (Chen, 2005; Lu *et al.*, 2012; Yoshizawa and Wong, 2013). It is estimated that nearly one-third to one-half of human genes are regulated by miRNAs, and each miRNA is predicted to target several hundred transcripts, thus making miRNAs one of the extensive classes of gene regulators (He and Hannon, 2004; Yoshizawa and Wong, 2013).

Salivary miRNA biomarkers have recently become an emerging field for monitoring oral diseases and systemic diseases (Liu *et al.*, 2012; Lau *et al.*, 2013; Yoshizawa *et al.*, 2013). In carcinogenesis, overexpression of certain miRNAs could result in downregulation of tumor suppressor genes, while underexpression of certain miRNAs could cause oncogene upregulation (Chen, 2005). This fact indicates that miRNAs may play a role as either tumor suppressors or oncogenes. Moreover, a high fraction of discovered miRNAs are found to be located at fragile sites or in cancer-associated genomic regions, including minimal regions of loss of heterozygosity or minimal amplicons (Calin *et al.*, 2004). Therefore, salivary miRNA screening emerges as a novel diagnostic method for detection of human cancers, especially at early stages. However, lack of well-characterized/matched clinical groups and lack of suitable endogenous controls (ECs) for salivary miRNA detection and normalization are among the restrictions of applying salivary-based miRNA biomarker discovery.

In this study, we first identified a reliable EC miRNA that showed minimal changes in expression levels throughout cohorts of different patient groups and healthy controls (HCs). Second, using proper ECs for normalization, we compared the miRNA expression signatures in OSCC patients, patients with OSCC in remission (OSCC-R), patients with oral lichen planus (OLP), and HCs to determine discriminatory miRNAs for each condition. OLP is a chronic inflammatory disease. Patients with OLP

have been found to have a higher risk for developing OSCC by case reports and some clinical studies, although whether OLP is premalignant is still a controversial issue (Lodi *et al.*, 2005). Our novel findings suggest that salivary miRNA expression profiles could be used as a noninvasive method for detecting OSCC and perhaps for monitoring OSCC development in OLP patients.

Materials & Methods

Patients

This study was approved by the Institutional Review Board of the Texas A&M University–Baylor College of Dentistry, and written informed consent was obtained from all participants. All patients were recruited from April 1, 2010, to September 30, 2011, from the Stomatology Center, Texas A&M University–Baylor College of Dentistry, and from referrals by local surgeons. The saliva samples were collected from 34 subjects: 9 OSCC patients before treatment, 8 patients with OSCC-R, 8 patients with OLP, and 9 HCs.

The inclusion and exclusion criteria of each group are summarized in the Appendix Table 1. The rationale for the inclusion/exclusion criteria has been delineated in our previous works (Cheng *et al.*, 2011; Gorugantula *et al.*, 2012). We employed a convenient sampling method for patient recruitment, in which we recruited those who fit the inclusion/exclusion criteria and were willing to participate in the study.

Saliva Collection

Unstimulated whole saliva was collected from the participants between 6 and 11 AM, using previously published protocols (Cheng *et al.*, 2011; Gorugantula *et al.*, 2012). Briefly, participants were asked to refrain from eating, drinking, or applying oral hygiene procedures on the day of saliva collection. Subjects rinsed their mouth with water before saliva collection to minimize contamination of the samples. Five minutes after the oral rinsing, the participant was asked to sit upright and spit into a 50-mL Falcon tube kept on ice. A maximum of 8 mL

of saliva was collected from each subject within 30 min.

Sample Processing

Saliva samples were processed immediately after collection according to a previously published protocol (Cheng *et al.*, 2013). They were centrifuged at 2,600 g for 15 min at 4°C. Saliva supernatant was then separated from the cellular phase. The RNase inhibitor (SUPERase-In, Ambion Inc., Austin, TX, USA) was added to supernatant, 5 µL of SUPERase-In per milliliter of supernatant, as described previously (Hu *et al.*, 2008). All samples were aliquoted in 440 µL and stored at –80°C until further use.

Total RNA Isolation

Total RNA was extracted by RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol from the same amount of collected saliva for each subject (440 µL). NanoDrop spectrophotometer (ND2000; Wilmington, DE, USA) was used for quantification of the extracted RNA. The quality of isolated RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA ratios (260/230 and 260/280) of NanoDrop measurements.

Establishing a Reliable Endogenous Normalizer

As having a reliable EC miRNA for normalization is considered to be a prerequisite for accurate data interpretation and reliability/reproducibility of the results, we first screened samples for identifying a stable EC in miRNA profiling data. As miRNA-191, miRNA-16, and miRNA-484 reported to be stable miRNAs (Weber *et al.*, 2010; Hu *et al.*, 2012), we systematically screened for stability of these miRNAs as EC miRNAs by testing 10 pooling samples and 32 individual saliva samples of different study groups, using NormFinder algorithm (version 0.953) (Andersen *et al.*, 2004). With a model-based approach, the NormFinder first estimates the intragroup and intergroup variations. Afterward, based on both estimated variations, it combines the

2 types of variations into a stability value, which intuitively adds the 2 sources of variations and thus represents a practical measure of the systematic error that will be introduced when using the investigated gene. This model generates a stability value of which a lower value represents less intragroup and intergroup variability and increased stability in gene expression. The program ranks genes according to their stability in different groups.

NanoString nCounter miRNA Assay for miRNA Profiling

The multiplexed NanoString nCounter miRNA expression assay (NanoString Technologies, Seattle, WA, USA) was used to profile more than 700 human and human viral miRNAs. Human viral miRNAs are virus encoded and can be expressed in humans as a result of previously integrated viral genome. The assay was performed according to the manufacturer's protocol. Briefly, 100 ng of total RNA was used as input material, with 3 μ L of the threefold-diluted sample. A specific DNA tag was ligated onto the 3' end of each mature miRNA, providing an exclusive identification for each miRNA species in the sample. The tagging is performed in a multiplexed ligation reaction utilizing reverse complementary bridge oligonucleotides to dispose the ligation of each miRNA to its designated tag. All hybridization reactions were incubated at 64°C for 18 hr. Excess tags were then removed, and the resulting material was hybridized with a panel of fluorescently labeled, bar-coded reporter probes specific to the miRNA of interest.

NanoString Data Reading and Analysis

Abundances of miRNAs were quantified with the nCounter Prep Station and Digital Analyzer (NanoString) via counting individual fluorescent barcodes and quantifying target miRNA molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed. Each sample was normalized to the geometric mean of the top 100 most highly expressed miRNAs.

Real-time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to validate top significant candidates obtained by NanoString nCounter miRNA assay. Total RNA (10-50 ng) was reverse transcribed to cDNA via the miRNA reverse transcription kit (Applied Biosystems). RT-qPCR was performed with TaqMan MicroRNA assay. MiRNA-191 was used as a reference internal control. All reactions were run in triplicate. A no-template control was used as a negative control. The RT-qPCR reactions were carried out in an MJ Research PTC-200 Thermal Cycler; ΔC_t and $\Delta\Delta C_t$ were calculated and normalized against miRNA-191.

Statistical Analysis

Probability values of significance of different expressed miRNAs were based on 1-way analysis of variance, followed by a 2-tailed Mann-Whitney *U* test or Student's *t* test. Validation of diagnostic value of candidate miRNAs was carried out by receiver operating characteristic (ROC) curves analysis. Area under curve, diagnostic sensitivity and specificity, and optimal cutoff by high sensitivity plus specificity were determined; *p* values less than .05 were considered statistically significant. Data analysis was done with SPSS 12 and GraphPad Prism 5.

Results

Demographic data of different study groups are summarized in Appendix Table 2. There was no significant difference among the mean ages of the different study groups (*p* > .05).

Regarding the suitable EC, miRNA-191 expression levels showed the lowest intragroup and intergroup variability with the stability value of 0.158 according to NormFinder (Table 1). The stability values were higher for miRNA-16, miRNA-484, and even the combination of miRNA-16 and miRNA-484 when compared to the values of miRNA-191. In RT-qPCR analysis, miRNA-191 consistently showed minimal variability in expression levels among different groups of individuals (Appendix Figure).

Of the 734 human miRNAs tested by NanoString nCounter miRNA assay, 221 showed no amplification in more than 60% of the 25 of 32 samples; so, these miRNAs were not included in the analysis. As shown in Table 2, 13 miRNAs were identified as being significantly deregulated in saliva samples of OSCC patients compared to HCs: 11 miRNAs were significantly underexpressed (miRNA-136, miRNA-147, miRNA-1250, miRNA-148a, miRNA-632, miRNA-646, miRNA-668, miRNA-877, miRNA-503, miRNA-220a, miRNA-323-5p) (*p* < .05), while 2 miRNAs were significantly overexpressed (miRNA-24, miRNA-27b) (*p* < .05).

Six miRNAs distinguished OSCC *vs.* OSCC-R, including 4 significantly underexpressed miRNAs (miRNA-136, miRNA-15b, miRNA-99a, miRNA-2054) (*p* < .05) and 2 overexpressed miRNAs (miRNA-720, miRNA-27b) (*p* < .05) (Table 2). Seven miRNAs distinguished OSCC *vs.* OLP, including significantly underexpressed miRNAs: miRNA-146a, kshv-miRNA-K12-6-3p, hcmv-miRNA-US5-2, ebv-miRNA-BART16, miRNA-223, and miRNA-29a (*p* < .05); miRNA-27b was significantly overexpressed (*p* < .05) (Table 2). In this patient cohort, miRNA-27b showed significantly increased levels in OSCC patients compared to the levels found in HCs, patients with OSCC-R, and OLP patients.

ROC curve analyses, performed to evaluate the diagnostic value of miRNA-27b, revealed that miRNA-27b is a valuable biomarker to distinguish OSCC patients from OSCC-R patients, OLP patients, and HCs (Figure 1). At the optimal cutoff value of 14.73 for miRNA-27b in OSCC patients *vs.* HCs where the values of sensitivity and specificity were considered to be maximal for miRNA-27b, the sensitivity and specificity were 85.71% and 100.00%, respectively; at the cutoff value of 16 for miRNA-27b in OSCC patients *vs.* patients with OSCC-R, the sensitivity and specificity were 85.71% and 83.33%, respectively; at the cutoff of 14.44 for miRNA-27b in OSCC patients *vs.* OLP patients, sensitivity and specificity were 85.71% and 100%, respectively. MiRNA-136 was underexpressed in

Table 1.

Intragroup and Intergroup Variations for miRNA-191, miRNA-16, and miRNA-484 Among Different Study Groups

Gene Name	Stability Value	NormFinder Ranking		
miRNA-191	0.158	1		
miRNA-16	0.408	3		
miRNA-484	0.456	4		
miRNA-16 and miRNA-484 ^a	0.261	2		
Group Identifier	OSCC	OSCC-R	HCs	OLP
Intragroup variation				
miRNA-191	0.190	0.077	0.064	0.026
miRNA-16	0.143	0.431	0.363	0.210
miRNA-484	0.551	0.806	0.446	0.888
Intergroup variation				
miRNA-191	0.004	-0.042	0.051	-0.013
miRNA-16	0.191	0.369	-0.362	-0.197
miRNA-484	-0.195	-0.327	0.311	0.210

HCs, healthy controls; OLP, oral lichen planus patients; OSCC, oral squamous cell carcinoma patients; OSCC-R, patients with oral squamous cell carcinoma in remission.

^aBest combination of 2 genes.

Table 2.Differentially Expressed miRNAs in OSCC Patients *vs.* Other Groups

OSCC <i>vs.</i> HCs	Fold Change	<i>p</i>	OSCC <i>vs.</i> OSCC-R	Fold Change	<i>p</i>	OSCC <i>vs.</i> OLP	Fold Change	<i>p</i>
Underexpressed			Underexpressed			Underexpressed		
miRNA-136 ^a	3.68	.004	miRNA-136 ^a	2.34	.004	miRNA-146a ^a	4.04	.021
miRNA-1250 ^a	3.10	.027	miRNA-15b ^a	2.97	.042	Kshv-miRNA-K12-6-3p	42.3	.027
miRNA-147 ^a	21.90	.046	miRNA-99a ^a	5.18	.031	Hcmv-miRNA-US5-2	2.43	.046
miRNA-148a ^a	13	.027	miRNA-2054 ^a	3.78	.016	Ebv-miRNA-BART16	1.28	.046
miRNA-632 ^a	3.67	.027	Overexpressed			miRNA-223 ^a	2.75	.015
miRNA-646 ^a	7.34	.036	miRNA-720	5.18	.016	miRNA-29a ^a	1.63	.027
miRNA-668 ^a	3.07	.008	miRNA-27b ^a	2.89	.038	Overexpressed		
miRNA-877 ^a	7.8	.036				miRNA-27b ^a	2.31	.016
miRNA-503 ^a	13.2	.027						
miRNA-220a ^a	6.9	.008						
miRNA-323-5p ^a	11.41	.027						
Overexpressed								
miRNA-24 ^a	3.67	.046						
miRNA-27b ^a	4.09	.027						

HCs, healthy controls; OLP, oral lichen planus patients; OSCC, oral squamous cell carcinoma patients; OSCC-R, patients with oral squamous cell carcinoma in remission. All microRNAs (miRNAs) validated by real-time quantitative polymerase chain reaction.

^aIndicated the validated data with quantitative polymerase chain reaction.

both OSCC *vs.* HCs and OSCC *vs.* OSCC-R. ROC analyses showed that underexpression of miRNA-136 can distinguish OSCC patients from patients

with OSCC-R and HCs (Figure 2). At the cutoff value of 10.18 for miRNA-136 in OSCC *vs.* OSCC-R group, sensitivity and specificity were 88.89% and 85.71%,

respectively; at the cutoff of 10.44 for miRNA-136 in OSCC patients *vs.* HCs, the sensitivity and specificity were 88.89% and 100%, respectively.

Figure 1.

Curve of receiver operating characteristic and area under the curve. (A, B, C) Receiver operating characteristic curve analysis using differentially expressed microRNA 27b (miRNA-27b) for discriminating oral squamous cell carcinoma (OSCC) patients from OSCC patients in remission (OSCC-R), oral lichen planus (OLP) patients, and healthy controls (HCs). (D) Box presenting the area under the curve, sensitivity, specificity, and 95% confidence interval for every comparison between groups.

Discussion

Salivary-based diagnostic testing was recently introduced as a potential noninvasive approach for making early detection, assessing prognosis, and evaluating treatment response of oral and systemic diseases. In this study, we used a model containing OSCC patients, patients with OSCC-R, and OLP patients—of which both latter groups had no presence of OSCC at the time of saliva collection—to find a reliable salivary biomarker for OSCC detection. This model is different from all previously published studies regarding potential OSCC salivary biomarkers, which included only OSCC patients and non-OSCC normal controls (Liu *et al.*, 2012; Gombos *et al.*, 2013; Soga *et al.*, 2013). We identified miRNA-27b, which showed

significantly higher levels in the OSCC patients when compared to the levels found in the HCs, the OSCC-R patients, and the OLP patients. Our results suggest that a significantly elevated level of miRNA-27b is an indicator of OSCC presence; therefore, miRNA-27b could be a promising candidate for salivary-based OSCC detection. We further investigated the performance and accuracy of using salivary miRNA-27b for OSCC diagnosis by ROC curve analysis. The high levels of area under the curve for OSCC patients *vs.* OSCC-R (0.8810), OSCC patients *vs.* HCs (0.9643), and OSCC patients *vs.* OLP (0.9821), along with high specificity and sensitivity in all the comparisons, strengthen the promising value of using miRNA-27b as a reliable characteristic salivary biomarker for OSCC detection. Moreover, our results

showed that miRNA-136 was significantly underexpressed in both OSCC *vs.* HCs and OSCC *vs.* OSCC-R. In this aspect, our study joins the recent studies (Park *et al.*, 2009; Liu *et al.*, 2012; Soga *et al.*, 2013) in demonstrating that salivary miRNA profiles could be useful in screening for various local and systemic conditions, including OSCC.

Current advances in molecular biology and high-throughput screening techniques enable researchers to characterize the large-scale miRNA patterns in body fluids such as serum, plasma, and saliva. However, the lack of suitable ECs for normalizing (standardizing) salivary miRNAs is a major issue. In contrast to tissue or cellular miRNA in which U6 serves as an EC (Hu *et al.*, 2012), no salivary miRNA was proposed to be an appropriate

Figure 2.

Curve of receiver operating characteristic and area under the curve. (A, B) Receiver operating characteristic curve analysis using differentially expressed microRNA 136 (miRNA-136) for discriminating oral squamous cell carcinoma (OSCC) patients from OSCC patients in remission (OSCC-R) and healthy controls (HCs). (C) Box presenting the area under the curve, sensitivity, specificity, and 95% confidence interval for every comparison between groups.

EC for saliva samples. In our study, we showed that miRNA-191 has minimal intragroup and intergroup variability in the saliva of individuals from different groups, regardless of whether the individual had OSCC, OLP, or not. These findings suggest that miRNA-191 could be considered a suitable EC for salivary miRNA biomarker studies.

In accordance with our observation regarding miRNA-27b overexpression in saliva of OSCC patients, miRNA-27b has been reported to be an oncogenic miRNA and to be upregulated in ectodermal-originated malignancies such as breast cancer (Jin *et al.*, 2013), ovarian cancer (Park *et al.*, 2013), and colorectal cancer (Kjersem *et al.*, 2013). Wang *et al.* (2009) showed that applying antagomir of miRNA-27b suppressed cell invasion in a highly invasive human breast carcinoma cell line, MDA-MB-231, whereas pre-miRNA-27b stimulated invasion in

moderately invasive ZR75 breast carcinoma cells. Analysis of human breast carcinoma tissues showed that miRNA-27b expression increases during cancer progression, paralleling a significant diminish in ST14 expression. Consistent with the role of miRNA-27b in the carcinogenesis, Jin *et al.* (2013) recently reported that miRNA-27b is highly upregulated in a human breast cancer cell line. In the *in vivo* model, they found that engineered knockdown of miRNA-27b substantially repressed breast cancer growth in mice and increased miRNA-27b levels correlate with poor treatment outcomes. Park *et al.* (2013) showed that miRNA-27b is overexpressed in ovarian ALDH1-positive cells and associated with chemoresistance and tumor progression.

Although these studies identified miRNA-27b as an oncogenic miRNA, some studies identified miRNA-27b as a

tumor suppressor (Lo *et al.*, 2012; Wan *et al.*, 2014). Interestingly, in contrast to our finding in saliva of OSCC patients, reduced levels of miRNA-27b have been reported in the cancer tissues and the plasma of OSCC patients (Lo *et al.*, 2012). These different results of miRNA-27b levels in saliva and tissue could be related to the dual activity of miRNA-27b in tumor initiation and progression. Jin *et al.* (2013) highlighted the role of miRNA-27b overexpression mostly in progression of breast cancer rather than initiation. In addition, it has been shown that the profile of miRNAs in cancerous tissues and biofluids (*e.g.*, plasma and serum) could be totally different and that miRNA secretion to the biofluid is a tightly specific controlled process (Boeri *et al.*, 2011; Aushev *et al.*, 2013). Consequently, OSCC cells may selectively release miRNA-27b into saliva, and this phenomenon results in significantly

elevated salivary miRNA-27b levels. This is in accordance with the recent finding of selective sorting and release of miRNAs via extracellular vesicles (Valadi *et al.*, 2007; Aushev *et al.*, 2013). This selection in secretory cargo could lead to a different profile of biomacromolecules in biofluid and cancer tissue. Further studies are needed to elucidate these mechanisms.

In conclusion, we identified miRNA-191 as a stable EC for normalization of salivary miRNA data and found that miRNA profiles in OSCC patients, patients with OSCC-R, OLP patients, and HCs were distinctively different. In addition, overexpression of miRNA-27b appeared to be a promising OSCC salivary biomarker. Limitation of our study was the relatively small sample population. Therefore, further large-scale study is required to confirm the results of our findings.

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