Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection

Noh Jin Park,[1] Hui Zhou,[1] David Elashoff,[2] Bradley S. Henson,[1] Dragana A. Kastratovic,[3] Elliot Abemayor,[4] and David T. Wong[1,4,5,6,7]

Abstract

Purpose: We have previously shown that a transcriptome is found in saliva and subpanels of these mRNAs can be used as oral cancer biomarkers. In this study, we measured the presence of microRNAs (miRNA) in saliva and determined their potential as an additional set of oral cancer biomarkers.

Experimental Design: A total of 314 miRNAs were measured using reverse transcriptase-preamplification-quantitative PCR in 12 healthy controls. Degradation pattern of endogenous and exogenous saliva miRNAs were measured at room temperature over time. Selected miRNAs were validated in saliva of 50 oral squamous cell carcinoma patients and 50 healthy matched control subjects.

Results: We detected ~50 miRNAs in both the whole and supernatant saliva. Endogenous saliva miRNA degraded much slower compared with exogenous miRNA. Two miRNAs, miR-125a and miR-200a, were present in significantly lower levels ($P < 0.05$) in the saliva of oral squamous cell carcinoma patients than in control subjects.

Conclusions: Both whole and supernatant saliva of healthy controls contained dozens of miRNAs, and similar to saliva mRNAs, these miRNAs are stable. Saliva miRNAs can be used for oral cancer detection. (Clin Cancer Res 2009;15(17):5473–7)

Oral squamous cell carcinoma (OSCC) constitutes ~90% of oral cancers. In the United States, OSCC is the sixth most common cancer, leading to ~8,000 deaths per year (1). The average 5-year survival rate for OSCC is ~50%. Shockingly, this number has not changed in last three decades (2). Therefore, an early detection method for OSCC is needed to increase long term patient survival.

Saliva has been used as a diagnostic medium for OSCC, and saliva analytes such as proteins and DNA have been used to detect OSCC (1, 3, 4). Our laboratory has recently shown that thousands of mRNAs are present in saliva, and a panel of saliva mRNAs can be used for oral cancer detection (5–7). The salivary mRNAs seem to enter the oral cavity through various sources, including the three major saliva glands, gingival crevice fluid, and desquamated oral epithelial cells (8). The majority of saliva mRNAs seem to be present as partially degraded forms (9). These partially degraded mRNAs maintain their stability in saliva through their association with unidentified macromolecules (8).

MicroRNAs (miRNA) *lin-4* and *let-7* were initially discovered in *C. elegans* as key regulators of animal development (10). Since 2000, miRNAs have been mined in mass (11–13). Since then, the mechanism of miRNA production and mode of action have been well characterized. miRNAs are transcribed by RNA polymerase II or RNA polymerase III as a part of an intron of mRNA or as an independent gene unit (14, 15). Initially transcribed miRNAs can be several hundred to thousands of nucleotides long and have a distinct stem-loop structure, which is cleaved into a <100 nucleotide stem loop structure by a type III RNase named Drosha (16). These pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5. In the cytoplasm, the pre-miRNAs undergo another round of endonucleolytic cleavage by Dicer, another type III RNase (17, 18). Fully processed miRNAs are approximately 18 to 24 nucleotides in length. These mature miRNAs are bound by a protein complex called the RNA-induced silencing complex (RISC), which is composed of four argonaute family proteins Ago1-4 (19). This active miRNA-RISC complex binds to target mRNAs based on sequence homology between the miRNA and the mRNA. Typically, the miRNA blocks mRNA translation and/or leads to mRNA degradation. Because miRNAs bind with slightly imperfect complementary to target mRNAs, it is estimated that one

Authors' Affiliations: [1]Dental Research Institute, [2]School of Public Health, [3]Centre for Clinical Pharmacology in Clinical Centre of Serbia, Serbia, [4]Division of Head and Neck Surgery/Otolaryngology, [5]University of California at Los Angeles School of Dentistry, [6]Jonnson Comprehensive Cancer Center, and [7]Henry Samueli School of Engineering, Los Angeles, California

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Current address for N.J. Park: Quest Diagnostics, Molecular Genetics Department, San Juan Capistrano, CA.

This study has not been presented elsewhere.

Requests for reprints: David T. Wong, University of California at Los Angeles School of Dentistry, Dental Research Institute, 73-017 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095. Phone: 310-206-3048; Fax: 310-825-7609; E-mail: dtww@ucla.edu.

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Translational Relevance

Early detection is the key to improve the survival of oral cancer patients. Saliva, a local biofluid for oral cancer, has been shown to harbor clinical discriminatory proteomic and transcriptomic biomarkers. Here, we tested the hypothesis that salivary microRNAs exist and can be used clinically to detect oral cancer. Our results show that healthy saliva contains ~50 microRNAs. Two microRNAs, miR-125a and miR-200a, can discriminate oral cancer patients from control subjects ($P < 0.05$). The presence of microRNA in saliva is now substantiated, and it represents a third diagnostic alphabet in saliva, in addition to proteome and transcriptome.

miRNA is capable of binding >100 different mRNAs with different binding efficiencies. With about 1,000 miRNAs expected to be present in the human genome, it is postulated that ~30% of all mRNAs are posttranscriptionally regulated by miRNAs (20, 21).

The recent discovery of hundreds of miRNAs, from various organisms, and functional assays have determined that miRNAs serve important functions in cell growth, differentiation, apoptosis, stress response, immune response, and glucose secretion (22–26). Many research groups have shown that miRNAs are differentially expressed in various cancer cells compared with normal cells, and it seems that miRNAs more accurately cluster different types of solid tumors than mRNA, suggesting that miRNAs can be used to detect cancer (22). Additionally, the fold change in mRNA between cancer cells and normal cells is relatively small, whereas, the expression level of many miRNAs exhibits fold changes of tens to hundreds (27).

In this work, we profiled 314 of the 708 human miRNAs registered in the most recent release of miRBase version 12.0 in both whole and supernatant saliva of healthy controls and OSCC patients.

Materials and Methods

Saliva samples. Whole saliva samples were preserved with RNAlater (QIAGEN, Inc.), and supernatant saliva samples were preserved with SUPERase·In (Ambion, Inc.), as described previously (28). The supernatant phase of whole saliva was collected as described previously (5). All of the saliva samples were kept at -80°C at all times. All of the volunteers signed the University of California at Los Angeles institutional review board–approved consent for participating in the study. At the time of saliva collection, 10 patients were at tumor stage I, 14 were at stage II, 16 were at stage III, and 10 were at stage IV. The average age of OSCC volunteers was 56, and their demographic breakdown was as follows: 41 Caucasians, 4 Asians, 4 Hispanics, and 1 African American; and 32 males and 18 females. The average age of the control volunteers was 52, and their demographic breakdown was as follows: 39 Caucasians, 3 Asians, 3 Hispanics, and 5 African American; and 29 males and 21 females. The volunteers had no history of immunodeficiency, autoimmune disorders, hepatitis, or HIV infection.

Saliva RNA extraction. Four hundred microliters of the whole saliva mixture (200 μL whole saliva and 200 μL RNAlater) and 400 μL of the supernatant saliva were used for RNA extraction. Saliva samples were extracted using the *mir*Vana miRNA Isolation kit according to the manufacturer's guideline (Ambion, Inc.). For the initial lysis step, we used 1 mL of Lysis/Binding solution per 400 μL saliva sample. After extraction, 100 μL of purified RNA was digested with DNA-*free* (Ambion, Inc.) to completely remove any genomic DNA. The RNA samples were then allowed to dry completely using a Vacufuge (Eppendorf) and resuspended in 20 μL of water.

Reverse transcriptase-preamplification-quantitative PCR of 12 healthy subjects. We analyzed a total of 314 miRNAs (see Supplementary Data Table 1 for the list of miRNAs analyzed), and all of the reagents used for reverse transcriptase-preamplification (preamp)–quantitative PCR (RT-preamp-qPCR) were from Applied Biosystems. Reverse transcription (RT) and preamp were carried out using a PTC-200 thermal cycler from Bio-Rad Laboratories, and qPCR reactions were done using a 7500 and 7900HT Fast Real-Time PCR systems (Applied Biosystems).

For both the 314 and 71-plex RT-preamp, a total of 5 μL of the RT reaction contained the following: 2 μL RNA, 0.5 μL 10× RT primer mix (314 miRNA multiplex), 0.1 μL 25 mmol/L deoxynucleotide triphosphates, 1 μL 50 U/μL MultiScribe Reverse Transcriptase, 0.5 μL 10× RT buffer, 0.6 μL 25 mmol/L $MgCl_2$, 0.06 μL 20 U/μL AB RNase Inhibitor, and 0.24 μL water. The RT reaction was carried out in the thermocycler under the following conditions: (16°C for 2 min, 42°C for 1 min, and 50°C for 1 s) for 40 cycles and 85°C for 5 min. Preamp reactions contained 5 μL of RT, 12.5 μL 5× Preamp Primer Mix, 5 μL 314 multiplex 5× preamp primer mix (250 mmol/L each), and 2.5 μL water. The preamp reaction was carried out in the thermocycler under the following conditions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, and (95°C for 15 s, 60°C for 4 min) for 14 cycles. Then, the preamp product was diluted 4-fold by adding 75 μL of water. Finally the 10-μL qPCR reaction contained 0.025 μL diluted preamp product, 5 μL 2× TaqMan Master Mix no UNG, 2.975 μL water, and 2 μL 5× PCR probe/primer mix. All of the qPCR reactions were preformed in duplicate.

RT-preamp-qPCR in 50 OSCC and 50 control subjects. Four-plex RT-preamp-qPCR amplified the following four miRNAs: miR-142-3p, miR-200a, miR-125a, and miR-93. For RT, instead of using a mega-plex RT protocol, we used the standard ABI RT reaction condition that contained following: total of 7.5 μL reaction of 1 μL RNA, 0.075 μL deoxynucleotide triphosphate mix, 0.5 μL 50 U/μL MultiScribe Reverse Transcriptase, 0.75 μL 10× RT buffer, 0.095 μL AB RNase Inhibitor, 3.58 μL water, and 1.5 μL that contained 0.375 μL of each of the four primers. The RT reaction was carried out at 16°C for 30 min and 42°C for 30 min. Preamp was done as described above. Preamp products were diluted 4-fold with water, and 0.1 μL of cDNA was used for qPCR as described above.

Saliva miRNA stability assay. To 10 mL of pooled supernatant saliva, 5 μL of 100 μmol/L miR-124a was added, and 400 μL of triplicate samples were removed at each time point and immediately incubated with Lysis/Binding Solution, a component of the *mir*Vana miRNA Isolation kit, until the time course was completed. Extracted RNA was digested with DNA-*free*, and concentrated to 30 μL. Two microliters of purified RNA were used for the RT reaction as described above. RT was then diluted 10-fold with water, and 2 μL of the diluted RT product was used for a 10-μL qPCR reaction, as described above. qPCR was done in duplicate.

Statistical Analysis. To normalize for the RNA input amount, we also did RT-qPCR reactions for the RNA polymerase III-transcribed U6 snRNA, which is a commonly used RNA for miRNA normalization in cells. Comparison of raw U6 values between the two groups showed no significant difference ($P = 0.27$, Mann-Whitney *U* test). Raw data were normalized by subtracting the U6 cycle threshold (C_T) values from the marker C_T values and were analyzed with the use of the stats Uitilities packages from R[8] and the ROC package from Bioconductor.[9] Statistical comparisons were made with the use of the Mann-Whitney *U* test with consideration of two different distributions

[8] http://www.r-project.org
[9] http://www.bioconductor.org

Table 1. Salivary miRNA

A. No. of miRNAs in saliva with a C_T of <35 of 314 selected miRNAs

Subject	Whole saliva	Supernatant saliva
1	19	46
2	22	37
3	9	17
4	52	58
5	62	55
6	60	53
7	65	66
8	47	58
9	64	66
10	45	50
11	63	56
12	62	60
Average	47	52

B. Saliva miRNAs that were detected in at least 11 of 12z examined individuals

Whole saliva	Supernatant saliva	
hsa-miR-16	hsa-miR-16	Let-7b
hsa-miR-19b	hsa-miR-19b	hsa-miR-26a
hsa-miR-24	hsa-miR-24	hsa-miR-30c
hsa-miR-26b	hsa-miR-26b	hsa-miR-30a-3p
hsa-miR-30e-3p	hsa-miR-30e-3p	hsa-miR-30e-5p
hsa-miR-92	hsa-miR-92	hsa-miR-125a
hsa-miR-146a	hsa-miR-146a	hsa-miR-140
hsa-miR-146b	hsa-miR-146b	hsa-miR-155
hsa-miR-150	hsa-miR-150	hsa-miR-181
hsa-miR-191	hsa-miR-191	hsa-miR-195
hsa-miR-200c	hsa-miR-200c	hsa-miR-197
hsa-miR-203	hsa-miR-203	hsa-miR-222
hsa-miR-223	hsa-miR-223	hsa-miR-320
		hsa-miR-342
		hsa-miR-375

NOTE: Two hundred microliters of whole saliva and 400 μL of supernatant saliva were used for miRNA analysis.

for control and OSCC groups. miRNAs that were best differentiated between patient groups ($P < 0.05$) were identified and compared by the area under curve (AUC) value.

Results

miRNAs in saliva. We have previously shown that saliva contains a transcriptome and a subset of the salivary mRNAs can be used as biomarkers for oral cancer detection (5–7). Aiming to discover additional disease biomarkers in saliva, we tested the presence of miRNAs in salivary fluid. Both whole and supernatant saliva from 12 healthy controls were used for miRNA profiling. We extracted total RNA from 200 μL of whole saliva or 400 μL of supernatant saliva. We initially measured 314 miRNAs from six participants using RT-preamp-qPCR. We arbitrarily considered miRNAs with a C_T value lower than 35 as present in saliva. Of the 314 miRNAs initially analyzed, 71 were found to be present in at least two participants. We then further analyzed these 71 miRNAs in the second set of six samples (see Supplementary Table S2 for the list of miRNAs and RT-preamp-qPCR data). Our results from these 12 participants indicated that 47 miRNAs in whole saliva and 52 miRNAs in the saliva supernatant could be detected (Table 1A). In whole saliva, 13 of the 47 miRNAs were present in at least 11 of 12 participants, and in the supernatant saliva, 28 of 52 miRNAs were also present in at least 11 of 12 participants (Table 1B). All 13 miRNAs present in the whole saliva of healthy controls were also present in supernatant saliva (Table 1B). Together the data indicate that both the whole and supernatant saliva contain detectable amount of miRNAs, and there seemed to be a common set of miRNAs present in the saliva of healthy participants.

miRNA stability in saliva. We previously showed that saliva mRNAs are protected from degradation by association with currently unidentified macromolecules (8). This type of mechanism is also observed with plasma and serum mRNAs (8, 29, 30). To test if salivary miRNAs are also protected from degradation, we measured the degradation pattern of endogenous and exogenous miRNAs in saliva. As a model of miRNAs endogenous to saliva, we measured miR-191 because it showed consistently low C_T values across all the saliva samples tested. As a model of exogenous miRNAs, we designed an RNA oligo with sequence homology to miR-124a. All 12 saliva samples from the healthy controls did not contain miR-124a, so this miRNA served as a good representative of RNA exogenous to saliva. At time 0, exogenous miR-124a was added to the saliva, and the time course was carried out at room temperature for 30 min. Aliquots were removed at different time points, and RT-qPCR was done on the purified total RNA for miR-191 and miR-124a. The level of the exogenous miR-124a showed a rapid decrease during the time course, and by 3 minutes, <10% of miR-124a was detected (Fig. 1). These data indicate that miR-124a degrades rapidly in saliva. In contrast, the level of miR-191 decreased much slower, and by 30 minutes, ~30% of miR-191 was still detectable. Together, these data suggest that endogenous miRNAs are degraded at a slower rate than exogenous miRNA.

Saliva miRNAs as potential oral cancer biomarkers. To test if saliva miRNAs can be used for oral cancer detection, we compared saliva miRNA profiles between OSCC patients and healthy controls of similar age, gender, ethnicity, and smoking history. Saliva supernatant was analyzed to avoid miRNA contamination from cells. In the initial 12 control and 12 OSCC patient data sets, four potential miRNAs were identified as being present at statistically significant levels between the groups ($P < 0.05$). These miRNAs were miR-200a, miR-125a, miR-142-3p, and miR-93 (Table 2A). We then tested the potential significance of these four miRNAs in an additional independent

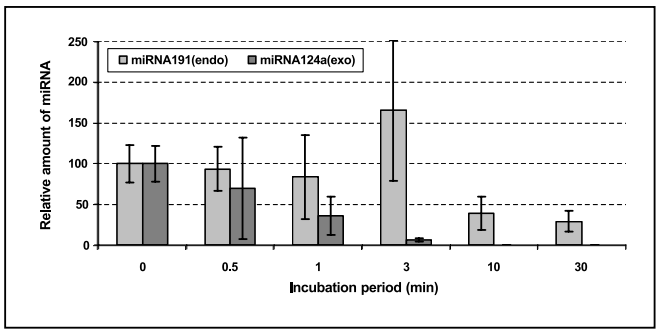

Fig. 1. Stability of endogenous and exogenous miRNA in saliva. At time 0, exogenous miR-124a was added to the supernatant phase of saliva to a final concentration of 50 μmol/L. The saliva was incubated at room temperature for 30 min. At each time point, 400-μL saliva aliquots were removed in triplicate for RT-preamp-qPCR of miR-124a and miR-191. The amount of RNA quantified at each time point was normalized to time 0. *Columns*, mean; *bars*, represent SD.

Table 2. Salivary miRNA in oral cancer and control subjects

A. Summary of potential OSCC miRNA markers in 12 OSCC patients and 12 healthy controls

miRNA	Median C_T in OSCC	Median C_T in control	OSCC SD	Control SD	*P**	AUC*
miR-200a	35.25	34.25	2.08	3.76	0.05	0.54
miR-125a	32.30	30.70	1.83	3.28	0.05	0.53
miR-93	33.30	33.06	4.02	4.19	0.04	0.52
miR-142-3p	37.84	32.62	3.04	2.19	0.02	0.59

B. Summary of potential OSCC miRNA markers in 50 OSCC patients and 50 healthy controls

miR-200a	28.7	27.7	3.94	3.94	0.01	0.65
miR-125a	22.8	22.4	3.28	2.85	0.03	0.62
miR-93	20.2	20.1	3.79	3.29	0.17	0.57
miR-142-3p	19.6	19.2	3.28	3.11	0.18	0.58

*Both the *P* value and AUC were obtained using the U6-normalized values. The Mann-Whitney *U* test was used to obtain the *P* values.

cohort of 38 control and 38 OSCC samples using RT-preamp-qPCR. Because we only wished to measure these four miRNAs, we used a simplified RT reaction in this set of experiment (see Materials and Methods). We also repeated the RT-preamp-qPCR on initial 12 control and 12 OSCC samples using the simplified RT condition (see Materials and Methods). The average C_T, *P*, and AUC values of these miRNAs in the combined data set of 50 controls and 50 OSCC patients is shown in Table 2B. The *P* values for miR-200a and miR-125a were significantly different between the two groups and were 0.01 and 0.03, respectively. However, the *P* values for miR-142-3p and miR-93 were 0.18 and 0.17, respectively, which indicate that these miRNAs are not significantly different between the control and OSCC groups. The AUC for miR-200a and miR-125a were 0.65 and 0.62 respectively, whereas the AUC for miR-142-3p and miR-93 were lower; 0.58 and 0.57, respectively. The combined AUC between miR-200a and miR-125a was 0.66. The SD of RT-preamp-PCR results is also included in Table 2. Together, these data suggest that miRNAs miR-200a and miR-125a are present at significantly lower levels in the saliva of OSCC patients.

Discussion

Previous reports from our group showed that saliva mRNAs can be used as biomarkers for oral cancer, and the combined measurement of seven different mRNAs showed a specificity and sensitivity of 0.91 for oral cancer discrimination (5, 7). To enhance the diagnostic power of saliva for oral cancer, we profiled salivary miRNAs to determine if any miRNAs could be used as potential diagnostic markers. We have shown that both whole and supernatant saliva contain miRNAs, and their profiles are highly similar. Comparisons of saliva miRNAs between OSCC patients and healthy controls showed that two miRNAs are present in significantly lesser amounts in the OSCC patient saliva than in the control saliva. Similar to mRNAs in saliva, saliva miRNAs are more stable than exogenous miRNAs. Recently, cell-free maternal and placental-origin miRNAs were identified in the plasma of pregnant females. This study also showed that endogenous plasma miRNAs are more stable than exogenous miRNAs (31).

On average, we detected ~50 miRNAs in both whole and supernatant saliva. However, whole saliva seemed to contain a more heterogeneous population of miRNAs because fewer miRNAs were detected consistently (~13) compared with supernatant saliva (~28). The heterogeneity of the miRNA profile for whole saliva may be due to individual variation of the amount of desquamated oral epithelial cells in saliva. We have previously shown that oral fluids separately collected from each of the three major salivary glands and the gingival crevice contains hundreds of mRNAs (8). Therefore, we believe that although whole saliva contains miRNAs from desquamated oral epithelial cells and miRNAs from different oral fluids, supernatant saliva should contains few or none miRNAs from desquamated oral epithelial cells. The amount of desquamated oral epithelial cells in saliva varies from person to person depending on oral health conditions and saliva flow rate, which leads to a heterogeneous population of miRNAs in whole saliva compared with supernatant saliva between individuals.

Our previous data showed that endogenous saliva mRNAs are more stable than naked exogenous mRNAs due to their association with macromolecules (8). In cells, mature miRNAs are bound by the RISC complex, and this interaction confers stability to miRNAs in cells. It is likely that salivary miRNAs are also bound by RISC, conferring stability to these miRNAs in saliva. Immunoblot analysis of saliva using an antibody specific to Ago 2, a component of the RISC complex, showed that Ago 2 is present in supernatant saliva (data not shown).

We found two salivary miRNAs, miR-200a and miR-125a, which were present at lower levels in OSCC patient saliva than in healthy controls. Through transient transfection studies, miR-125a along with it homologue miR-125b, have been shown to reduce ERBB2 and ERBB3 oncogenic protein levels in SKBR3 cells, a human breast cancer cell line (32). miR-200a has been reported to be differentially expressed in head and neck cancer cell lines and other cancer cells (27, 33–35). Interestingly, in the present study, miR-200a is present at lower levels in OSCC patients compared with healthy controls. In contrast, miR-200a is present at higher levels in various oral squamous cell lines (27, 35). This discrepancy could be due to observing cell-free state of miRNAs compared with the ones in living cells. Because the supernatant saliva is the cell free phase of saliva, some of the miRNAs in supernatant saliva are likely byproducts of cell death. It is possible that cancer-specific miRNAs undergo a more rapid degradation and/or

have a shorter half-life during cell death, similar to the degradation of regulatory mRNAs (36).

In conclusion, we have shown that miRNAs are present in both whole saliva and supernatant saliva. Two of these miRNAs, miR-125a and miR-200a, are differentially expressed in the saliva of the OSCC patients compared with that of healthy controls. These findings suggest that the detection of miRNAs in saliva can be used as a noninvasive and rapid diagnostic tool for the diagnosis of oral cancer. Thus, miRNAs are the third diagnostic alphabet in saliva.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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