Discovery and pre-validation of salivary extracellular microRNA biomarkers panel for the non-invasive detection of benign and malignant parotid gland tumors.

Johannes H, Matse 1,2, Janice Yoshizawa 3, Xiaoyan Wang 4, David Elashoff 4, Jan G.M. Bolscher 2, Enno C.I. Veerman 2, Elisabeth Bloemena 1, David T.W. Wong 3

Running title: miRNA biomarkers for the detection of parotid gland tumors.

1. Department of Oral and Maxillofacial Surgery and Oral Pathology Academic Centre for Dentistry Amsterdam (ACTA)/ VU University medical center, Amsterdam, the Netherlands.
2. Department of Oral Biochemistry ACTA, University of Amsterdam and VU University, Amsterdam, the Netherlands.
3. School of Dentistry and Dental Research Institute, University of California Los Angeles, Los Angeles, CA, U.S.A.
4. Statistics Core, UCLA David Geffen School of Medicine Division of General Internal Medicine and Health Services Research University of California Los Angeles, Los Angeles, CA, U.S.A.

Corresponding Author:
David T.W. Wong DMD, DMSc
Address: 10833 Le Conte Avenue
73-017 CHS
Los Angeles, CA 90095
U.S.A.
E-mail: dtww@ucla.edu
Phone#: 310-206-3048
Fax#: 310-825-7609
Johannes H. Matse  
Academic Centre for Dentistry Amsterdam (ACTA)/ VU University medical center  
Department of Oral Biochemistry and Department of Oral and Maxillofacial Surgery and Oral Pathology  
Room 12N-13  
Gustav Mahlerlaan 3004  
1081 LA Amsterdam  
The Netherlands  
Email: j.matse@acta.nl

Janice Yoshizawa  
School of Dentistry  
10833 Le Conte Avenue  
73-017 CHS  
Los Angeles, CA 90095  
U.S.A.  
Email: jyoshizawa@ucla.edu

Xiaoyang Wang  
UCLA Department of Medicine Statistics Core  
911 Broxton Ave, Room 314  
Los Angeles, CA 90024  
U.S.A.  
Email: xywang@ucla.edu

David Elashoff  
UCLA School of Public Health  
Department of Biostatistics  
Room 21-254C CHS  
Los Angeles, CA 90095-1772  
U.S.A.  
E-mail: dae@ucla.edu
Translational Relevance

This study can help in the further development of a salivary test for salivary gland tumor diagnosis. The differential expression of 4 miRNAs can distinguish between saliva from patients with a malignant parotid gland tumor and saliva from patients with a benign parotid gland tumor (specificity 95%, sensitivity 69% and AUC 0.90). The results also show that saliva is a good medium to be used as a diagnostic tool. The final validation and clinical implementation of this test will have an impact in the early detection of salivary gland tumors in a clinical context.
Abstract

Purpose:
This study is to explore differences in salivary microRNA (miRNA) profiles between patients with malignant or benign parotid gland tumors as a potential pre-operative diagnostic tool of tumors in the salivary glands.

Experimental Design:
Whole saliva samples from patients with malignant (n=38) or benign (n=29) parotid gland tumors were obtained from the Salivary Gland Tumor Biorepository (SGTB). After total RNA isolation, human miRNA cards were used for miRNA-profiling. The differential miRNA expression was analyzed using 2-sided Wilcoxon test.

Real Time-quantitative PCR (RT-qPCR) was used to validate selected miRNAs in an independent sample set. Receiver-operating-characteristics (ROC) curve and probability of malignancy was exploited to evaluate the diagnostic power of the validated miRNAs.

Results:
With miRNA-profiling, 57 of 750 investigated miRNAs were differently expressed, of which 54 showed higher miRNA expression in samples from patients with malignant tumors than those from patients with benign tumors. Validating the expression in an independent sample set of 9 miRNAs revealed indeed higher expression of miRNAs in malignant samples compared to benign samples. The expression of 6 validated miRNAs was statistically significantly different between the two groups (P <0.05).

A 4-miRNA-combination was able to discriminate between saliva samples from patients with malignant tumors from those of patients with benign parotid gland tumors (sensitivity 69%, specificity 95%).

Conclusions:
Salivary miRNA profiles differ in saliva from patients with malignant from saliva from patients with a benign parotid gland tumor. These preliminary results are promising to develop a non-invasive diagnostic tool for diagnosing tumors in the salivary glands.
Introduction

Tumors of the salivary glands comprise a diverse group of benign and malignant neoplastic lesions. The World Health Organization distinguishes 37 subtypes of salivary gland tumors: 13 benign and 24 malignant salivary gland tumors which constitute approximately 1-7% of all head and neck malignancies and 0.3% of all human tumors. Classification of a tumor in the salivary glands depends upon its histopathological characteristics. Tumors in the salivary glands are challenging to diagnose due to their rarity (0.4-13.5/100,000 cases), the many tumor subtypes and because the tumor subtypes have (partly) overlapping histopathology. Clinical examination, ultrasound scanning with or without fine needle aspiration cytology, and pre-operative CT-scan are the most commonly used methods to diagnose tumors of the salivary glands. Treatment of these tumors involves surgical resection (1).

In recent years, the study of salivary biomarkers has been taking flight and has evolved from the field of oral diseases (2, 3) to the field of systemic diseases (4-7). The development of transcriptomic and proteomic approaches to study such diseases has provided the means to globally profile disease-associated molecules such as metabolites, proteins, DNA, mRNA and microRNA.

MicroRNA (miRNA) is a group of small non-coding RNAs, consisting of 19-25 nucleotides, which are involved in various cellular processes such as cell differentiation, proliferation and survival. They exert their effect by inhibiting translation through binding to complementary sequences in the 3' UTR of multiple target mRNAs, usually resulting in their silencing. MiRNAs are stably expressed in serum, plasma, urine, saliva, and other body fluids (8, 9). MiRNAs are frequently deregulated in cancer and have shown great promise as tissue-based markers for the classification of malignancies (10, 11).

Thus far, few papers have been published regarding the expression of miRNAs in salivary gland tumors tissue. It was reported that 22 miRNAs were expressed differently in pleomorphic adenomas when compared to the matched normal controls (12). Another study described the expression of miRNA indirectly by studying the expression of Dicer in mucoepidermoid carcinoma (13). Dicer is an Rnase III related enzyme required for miRNA maturation that was differently expressed in mucoepidermoid carcinoma compared to the surrounding normal tissue. From these studies, it can be concluded that the miRNAs machinery is deregulated,
resulting in differences between miRNA profiles of salivary gland tumors and of normal tissue.

In this study, we investigated miRNA profiles in saliva from patients with malignant tumors and from patients with benign parotid gland tumors. Differences in miRNA profiles may be helpful to assist the clinical diagnosis of tumors in salivary glands. We present preliminary data that shows significant differences in miRNA profiles between saliva from patients with a malignant and saliva from patients with a benign parotid gland tumor. The discovered salivary biomarkers possess inherent discriminatory potential for a non-invasive diagnostic tool for malignant and benign parotid gland tumors.
Materials and Methods

Patients

Whole saliva samples from patients with malignant (n=38) or benign parotid gland tumors (n=29) were obtained from the Salivary Gland Tumor Biorepository at the MD Anderson Cancer Clinic in Houston, TX. Samples were immediately stored at -80°C until ready for use. Before the samples were used, they were defrosted on ice and centrifuged for 10 min, 5000 rpm at 4°C. The cell-free supernatant was collected from the pellet and used immediately in the next step.

In the discovery phase, samples of whole saliva from 10 patients with malignant and samples of whole saliva from 10 patients with benign parotid gland tumor were matched (gender and ethnicity) and analyzed. Both benign and malignant groups consisted of: 7 men and 3 women. The mean age was 53 (33-82) and 60 (49-74) of the benign and the malignant tumor group, respectively. Both groups consisted of the same ethnic background (1 Hispanic, 1 Black, 8 Caucasians). An independent sample set was used for validation study (19 benign and 28 malignant).

Smoking/drinking habits or clinicopathological data were neither recorded nor provided by the SGTB and were irretrievable. Patient’s characteristics of the discovery and validation sample set are given in Table 1.

Salivary miRNA profiling and data analysis

Total RNA was isolated from 300 µl saliva supernatant using RNA extraction kits (Ambion mirVana Paris kit). DNase I treatment (DNase I, Qiagen) was used to remove contaminating DNA during RNA extraction. The concentration of total RNA was measured using RiboGreen assay. Extracted RNA (1-10 ng) was reverse transcribed and pre-amplified using the Taqman MicroRNA reverse Transcription Kit, Taqman PreAmp master mix, and Megaplex Primers (Applied Biosystems, Foster City CA). The pre-amplification product was not diluted prior to performing miRNA quantification. For the profiling of 750 miRNAs, a total volume of 105 µl was loaded into each well of the Taqman Human MicroRNA cards (Applied Biosystems, Foster City CA), which were spun and run on the Applied Biosystems 7900HT Fast Real-Time PCR instrument containing a special card holder (Applied Biosystems, Foster City CA). Using default Taqman low density array setting and 6-carboxyfluorescein as a reporter, the RT-qPCR reaction was run at 95°C for 10 min to activate the enzyme and was then followed with 40 cycles at 95°C for 15 s and 60°C for 60 s.
The Ct value is defined as the cycle number in the fluorescence emission which exceeds that of a fixed threshold. A Ct of 15-30 was considered high expression and a Ct of 35 is considered low expression. A Ct value above 40 was considered as undetectable miRNA. For miRNA qPCR experiments, U6 snRNA was used as the reference gene. We calculated ΔCt by subtracting the Ct value of the reference gene (RNA polymerase III transcribed U6 snRNA) from the Ct value of each candidate biomarker. Data normalization was performed using RQ manager 1.2.1 and Data Assist v3.0 from Applied Biosystems. The qPCR based gene expression values between the two groups were compared using the non-parametric Wilcoxon test. Potential miRNA genes were then selected based on $P < 0.05$.

**Verification of salivary miRNA markers**

From the biomarker candidates generated by the Taqman MicroRNA array cards, 19 were verified by real-time quantitative PCR using Taqman MicroRNA assays on the same set of samples used in the discovery. Total RNA were reverse-transcribed with the Taqman MicroRNA Reverse Transcription kit using the following thermal cycling conditions: 16°C for 30 min, 42°C for the 30 min, 85°C for 5 min and then cooling to 4°C. Pre-amplification was performed with Taqman PreAmp master mix using the following thermal cycling conditions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, 72°C for 2 min, 12 cycles at 95°C for 15 s and 60°C for 4 min, then 99.9°C for 10 min to inactivate the enzyme, and then ending at 4°C. qPCR was carried out in 384 well plate in reaction volume of 10 µl using Taqman Universal PCR Master mix with no UNG. Initial denaturing was performed for 10 min at 95°C and then followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min on the Roche LightCycler 480 II (Roche, San Francisco, CA). All RT-qPCRs were performed in duplicate for all candidate miRNA. Samples in which miRNA was omitted were used as negative controls. The Ct was examined and the ΔCt was calculated. For miRNA qPCR experiments, U6 snRNA was used as the reference gene.

**Validation of salivary miRNA markers**

Of the verified biomarker candidates, 9 were validated in an independent sample group of saliva samples from 19 patients with a benign parotid gland tumor and from 28 patients with a malignant parotid gland tumor. Total RNA was isolated as described above. Validation was done using the Taqman microRNA assays as
described above for the verification of miRNAs. All qPCRs were performed in
duplicate for all candidate miRNA and negative controls (in which RNA was omitted).
The Ct was examined and the ΔCt was calculated. For miRNA qPCR experiments,
U6 snRNA was used as the reference gene.

**Statistical methods**

Wilcoxon rank-sum test was performed to compare the expression of miRNA in samples from patients with malignant versus benign parotid gland tumors in order to determine the level of significance for the miRNA biomarkers. Next we carried out a preclinical validation study on an independent set of 47 saliva samples from 19 patients with a benign and 28 with a malignant parotid gland tumor. The Wilcoxon rank-sum test was used to compare the ΔCt values for miRNAs between malignant and benign. Next, multivariate logistic regression analysis was used to construct a classification model to discriminate between patients with a malignant tumor and patients with a benign tumor. Forward stepwise model selection criterion was used to obtain a final model. ROC curves were constructed to determine the diagnostic/predictive values of individual as well as combined biomarkers from the logistic model. The performance of the model for classification was assessed by identifying the cut-off point of the predicted probability which yielded the largest sum of sensitivity and specificity.
Results

**Discovery and verification of salivary transcriptomic markers**

During biomarker discovery, expression of 750 miRNAs was screened using Taqman Human MicroRNA card. Of the 750 miRNA, 57 miRNAs were differently expressed in saliva from patients with a benign tumor (n=10) compared to patients with a malignant parotid gland tumor (n=10) (Wilcoxon test, $P < 0.05$). The expression of 54 out of the 57 miRNAs was higher in saliva samples from patients with a malignant tumor compared to expression in saliva samples from patients with a benign tumor. The expression of only 3 miRNAs was lower in saliva samples from patients with a malignant tumor compared to saliva samples from patients with a benign parotid gland tumor (hsa-miR-519b-3p, hsa-miR-520C-3p, hsa-miR-520D-3p). These 57 miRNA changes are unlikely due to chance alone ($P < 0.05$; Table S1 and S2).

RT-qPCR was performed on part of the discovery sample set to verify the Taqman Human MicroRNA card results. Eighteen miRNAs were chosen for verification in the original sample set. Selection of miRNAs was based on the $P$-value < 0.05 and fold change (Table S2). Of these eighteen miRNAs, 4 miRNAs (mmu-miR-140-5p, hsa-miR-374, hsa-miR-222, and hsa-miR-15b) differed statistically significant ($p<.05$) and 5 miRNAs (hsa-let-7g, hsa-miR-132, hsa-miR-519b-3p, hsa-miR-223 and hsa-miR-30a-3p) showed a trend ($0.08 > P > 0.05$) (Table 2).

**Validation of salivary miRNA markers**

Next, the expression of 9 miRNAs (mmu-miR-140-5p, hsa-miR-374, hsa-miR-222, hsa-miR-15b, hsa-let-7g, hsa-miR-132, hsa-miR-519b-3p, hsa-miR-223 and hsa-miR-30a-3p) that were statistically significant or showed a trend was determined in a separate independent sample set consisting of saliva from 19 patients with a benign tumor and 28 patients with a malignant parotid gland tumor. The expression of all 9 validated miRNAs was increased in saliva samples from patients with a malignant parotid gland tumor, just as they were in the verification phase. Of the 9 miRNAs validated, the expression of 6 miRNAs (hsa-miR-374, hsa-miR-222, hsa-miR-15b, hsa-let-7g, hsa-miR-132, and mmu-miR-140-5p) was statistically significantly different between the two groups ($P < 0.05$) (Table 3; Fig.1; Table S3).
Evaluation of validated miRNA biomarkers

ROC curves were constructed to determine the diagnostic/predictive values of combined biomarkers from a logistic model. The performance of the model was assessed by identifying the cut-off point of the predicted probability which yielded the largest sum of sensitivity and specificity. A combination of hsa-miR-132, hsa-miR-15b, mmu-miR-140 and hsa-miR-223 yielded the highest values for sensitivity and specificity of 69% and 95%. The AUC for the 4-validated-miRNA combination was 0.90 (Fig. 2). Of the validation data, a box-and-whisker plot predicting the probability for malignancy (Fig. 3) was made based on the 4-validated miRNA combination (hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223). In the malignant tumor group, 14 of the 28 samples scored around the probability of malignancy of 0.95. This group consisted of different subtypes of malignant tumor (6 squamous cell carcinoma, 2 acinic cell carcinoma, 2 carcinoma ex-pleomorphic, 1 mucoepidermoid carcinoma, 1 adenoma not otherwise specified, 1 myoepithelial carcinoma, and 1 renal cell carcinoma). In the benign tumor group 8 from the 19 samples scored around the malignancy probability of 0.02. This group consisted of 8 samples (4 pleomorphic adenoma, 3 Warthin’s tumor and 1 basal cell adenoma).
Discussion

Saliva has been recognized as an emerging diagnostic fluid (14-18). It is readily available, and collection is relatively simple, inexpensive and, non-invasive. Using omics techniques a scientific basis has been laid underpinning its credential as a diagnostic fluid (3, 7, 10, 19-21). The development of new and more sensitive technologies enables measurement of very low levels of analytes in saliva. (22-24).

One of these analytes is miRNA. These small non-coding RNAs are stably expressed in all body fluids (9). MiRNAs are also less prone to being degraded, unlike mRNA or proteins. This stable expression of miRNAs and the fact that miRNAs are less prone to be degraded make miRNAs a good choice for biomarkers (25, 26). Cancer associated changes, such as chromosomal alterations, chromosomal losses or gains, mutation in miRNA gene and methylation of miRNA promoter often lead to changes in gene expression patterns. Consistent and validated changes in miRNA expression can be used for diagnostic purposes. (27).

In this paper, we have identified and pre-validated the miRNAs (mmu-miR-140-5p, hsa-miR-374, hsa-miR-222, hsa-miR-15b, hsa-let-7g and, hsa-miR-132) that were differently expressed between saliva samples of patients with a malignant tumor and benign parotid gland tumor. Hsa-miR-15b has caspases 3, 8, 9 as validated targets. These proteins are involved in apoptosis pathways. Activation of caspase 3 by proteolytic cleavage due to activated caspase 8 and caspase 9 leads to irreversible commitment to apoptosis (28). Inhibition of these caspases by hsa-miR-15b will result in blockage of the apoptosis pathway. Another target of hsa-miR-15b is the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene. RECK encodes for a glycosylphosphidylinositol anchor glycoprotein and is an important inhibitor of matrix metalloproteinases. The expression of RECK is frequently reduced in carcinomas, e.g. colorectal (29, 30) and gastric cancer (31). The reduced RECK expression is often correlated with poor prognosis.

Amongst the validated targets of let-7g are genes which are associated with cancer such as RAS, MYC, and CDKN2A. CDKN2A, also known as p16, is a tumor-suppressor-gene which is frequently deleted in a wide variety of cancers. Deletion of this gene can result in increased cell proliferation (32). RAS and MYC are oncogenes and are frequently mutated in cancer. These mutations in the RAS and MYC genes may have a negative effect on the binding of hsa-let-7g to its target mRNA, lowering the inhibiting effect of hsa-let-7g, which may lead to overexpression of RAS and
MYC. This overexpression may result in an increased cell proliferation, demonstrating the double role miRNAs can play in cancer.

Fine-needle aspiration cytology (FNAC) is one of the techniques currently used to diagnose salivary gland neoplasms. A systematic review investigating the performance of FNAC in parotid gland lesions concluded that FNAC had a specificity of 97% and a sensitivity of 80%. However, the performance variability was relatively large (33). Therefore, additional (molecular) markers, such as those identified in the present study, can add to the accuracy of diagnoses on FNAC.

This study investigated and pre-validated the differences of miRNA expression in whole saliva from patients with malignant and benign parotid gland tumor. There appears to be a general up-regulation of miRNAs in saliva from patients with a malignant parotid gland tumor when compared to miRNA expression in saliva from patients with a benign parotid gland tumor. The AUC of the 4-validated-biomarker combination was 0.9 with a high specificity of 95% and a sensitivity of 69%.

There is some overlap in expression values; however, by using a combination of miRNA markers we improved the specificity and therefore the clinical usefulness of the diagnostic test. Furthermore, both the biomarker discovery and biomarker validation were performed using a variety of malignant parotid gland tumor types (18 subtypes). These clinical performances can be improved if we carry out the biomarker development using a specific subtype of salivary gland tumor (e.g. mucoepidermoid carcinoma).

Even though the data presented in this study are preliminary, they are encouraging toward developing a clinical application to distinguish malignant from benign parotid gland tumors.
Disclosure of Potential Conflict of interest

David Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostic company. PeriRx LLC sublicensed intellectual properties pertaining to molecular diagnostics from RNAmeTRIX. David Wong is a consultant to PeriRx.
References


**Figure Legend:**

**Figure 1.** Box-and-whisker plot of validated miRNA expression profiles in saliva samples from patients with benign (n=19) and malignant (n=28) tumor in the parotid gland. Whiskers represent maximum and minimum ∆Ct. * P < 0.05

**Figure 2.** ROC curve computed from final logistic regression model. This model includes hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223, which in combination provided the best prediction. The area under the ROC curve was 0.9, the specificity was 95% and sensitivity was 69%.

**Figure 3.** Box-and-whisker plot showing the “predicting probability for malignancy” based on the validation data of 4 miRNAs (hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223). Saliva samples from patients with a malignant tumor in the parotid gland have a higher “predicting probability for malignancy” than saliva samples from patients with a benign tumor in the parotid gland. Whiskers represent maximum and minimum probability for malignancy.
**Table 1. Patients’ characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Discovery phase:</th>
<th>Validation phase:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Benign</td>
<td>Malignant</td>
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<tr>
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<td>60</td>
</tr>
<tr>
<td>(range):</td>
<td>(33-82)</td>
<td>(49-74)</td>
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<td>6/4</td>
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<tr>
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<td>1</td>
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<tr>
<td>Caucasian</td>
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<td>8</td>
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<tr>
<td>Black</td>
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<td>1</td>
</tr>
<tr>
<td><strong>Tumor subtypes:</strong></td>
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<td></td>
</tr>
<tr>
<td>Pleomorphic adenoma</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Warthin tumor</td>
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<td></td>
</tr>
<tr>
<td>Oncocytoma</td>
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<td></td>
</tr>
<tr>
<td>Neuroendocrine cancer</td>
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<td></td>
</tr>
<tr>
<td>Oncocytic carcinoma</td>
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<td></td>
</tr>
<tr>
<td>Acinic cell carcinoma</td>
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<td>Mucoepidermoid cancer</td>
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<td></td>
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<tr>
<td>Salivary duct carcinoma</td>
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<td></td>
</tr>
<tr>
<td>Myoepithelial carcinoma</td>
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<td></td>
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<tr>
<td>Carcinoma ex-pleomorphic adenoma</td>
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<td></td>
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<tr>
<td>Cystadenocarcinoma</td>
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<td></td>
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<tr>
<td>Adenoid cystic carcinoma</td>
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Table 2. Verified salivary miRNA biomarkers in the original sample set. This set consisted of 10 whole saliva samples from patients with a benign parotid gland tumor and 10 whole saliva samples from patients with a malignant parotid gland tumor.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Wilcoxon 2-sided malignant vs. benign</th>
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<tbody>
<tr>
<td>mmu-miR-140-5p</td>
<td>0.020**</td>
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<tr>
<td>hsa-miR-374</td>
<td>0.021**</td>
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<tr>
<td>hsa-miR-222</td>
<td>0.040**</td>
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<tr>
<td>hsa-miR-15b</td>
<td>0.046**</td>
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<tr>
<td>hsa-let-7g</td>
<td>0.050**</td>
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<tr>
<td>hsa-miR-519b-3p</td>
<td>0.054*</td>
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<td>hsa-miR-30a-3p</td>
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<tr>
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<tr>
<td>hsa-miR-1285</td>
<td>0.9130</td>
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</table>

* 0.08 > P > 0.05; ** P < 0.05
Table 3. Validated salivary miRNA biomarkers in an independent sample set. This set consisted of 19 whole saliva samples from patients with a benign parotid gland tumor and 28 whole saliva samples from patients with a malignant parotid gland tumor.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Wilcoxon 2-sided malignant vs. benign</th>
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</thead>
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<tr>
<td>mmu-miR-140-5p</td>
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<td>hsa-miR-222</td>
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<td>hsa-miR-15b</td>
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<tr>
<td>hsa-let-7g</td>
<td>0.0306*</td>
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<td>hsa-miR-132</td>
<td>0.003*</td>
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<tr>
<td>hsa-miR-519b-3p</td>
<td>0.1931</td>
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<td>0.2320</td>
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<tr>
<td>hsa-miR-223</td>
<td>0.0542</td>
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</table>

* P < 0.05
Fig. 1.
Fig. 2.
Fig. 3.
Clinical Cancer Research

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Johannes H Matse, Janice Yoshizawa, Xiaoyan Wang, et al.

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