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Discovery and Prevalidation of Salivary Extracellular microRNA Biomarkers Panel for the Noninvasive Detection of Benign and Malignant Parotid Gland Tumors

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Abstract

Purpose: This study was conducted to explore the differences in salivary microRNA (miRNA) profiles between patients with malignant or benign parotid gland tumors as a potential preoperative 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 two-sided Wilcoxon test. Quantitative real-time PCR (qRT-PCR) was used to validate selected miRNAs in an independent sample set. Receiver-operating characteristics 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 with benign samples. The expression of 6 validated miRNAs was statistically significantly different between the two groups ($P < 0.05$). A four 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 noninvasive diagnostic tool for diagnosing tumors in the salivary glands. *Clin Cancer Res; 19(11); 3032–8. ©2013 AACR.*

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% to 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 histopathologic characteristics. Tumors in the salivary glands are challenging to diagnose due to their rarity (0.4–13.5/100,000 cases), many tumor subtypes, and because the tumor subtypes have (partly) overlapping histopathology. Clinical examination, ultrasound scanning with or without fine-needle aspiration cytology, and preoperative computed tomography 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 (miRNA).

miRNA is a group of small noncoding RNAs, consisting of 19 to 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′ untranslated region of multiple target mRNAs, usually

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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 area under the curve 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.

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 articles 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 with 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 with the surrounding normal tissue. From these studies, it can be concluded that the miRNA 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 noninvasive 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 (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 minutes at 5,000 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 years (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 clinicopathologic 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 preamplified using the TaqMan MicroRNA reverse Transcription Kit, TaqMan PreAmp Master Mix, and Megaplex Primers (Applied Biosystems). The preamplification product was not diluted before conducting 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), which were spun and run on the Applied Biosystems 7900HT Fast Real-Time PCR instrument containing a special card holder (Applied Biosystems). Using default TaqMan low density array setting and 6-carboxyfluorescein as a reporter, the quantitative real-time PCR (qRT-PCR) reaction was run at 95°C for 10 minutes to activate the enzyme and was then followed with 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds.

The cycle threshold (C_t) value is defined as the cycle number in the fluorescence emission, which exceeds that of a fixed threshold. A C_t of 15 to 30 was considered high expression and a C_t of 35 is considered low expression. A C_t value more than 40 was considered as undetectable miRNA. For miRNA quantitative PCR (qPCR) experiments, U6 snRNA was used as the reference gene. We calculated ΔC_t by subtracting the C_t value of the reference gene (RNA polymerase III transcribed U6 snRNA) from the C_t value of each candidate biomarker. Data normalization was conducted using RQ manager 1.2.1 and Data Assist v3.0 from Applied Biosystems. The qPCR-based gene expression values between the 2 groups were compared using the nonparametric 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 qRT-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

Table 1. Patients' characteristics

	Discovery phase			Validation phase	
	Benign	**Malignant**		**Benign**	**Malignant**
Mean age (range)	53 (33–82)	60 (49–74)	Mean age (range):	57 (22–88)	57 (19–85)
Sex (M/F):	6/4	6/4	Sex (M/F):	14/5	19/9
Ethnicity			Ethnicity		
Hispanic	1	1	Hispanic	0	2
Caucasian	8	8	Caucasian	19	22
Black	1	1	Black	0	4
Tumor subtypes			Tumor subtypes		
Pleomorphic adenoma	6		Pleomorphic adenoma	13	
Warthin tumor	3		Warthin tumor	5	
Oncocytoma	1		Basal cell adenoma	1	
Neuroendocrine carcinoma		1	Squamous cell carcinoma		9
Oncocytic carcinoma		1	Adenocarcinoma NOS		2
Acinic cell carcinoma		1	Acinic cell carcinoma		3
Mucoepidermoid carcinoma		1	Mucoepidermoid carcinoma		3
Salivary duct carcinoma		2	Salivary duct carcinoma		3
Myoepithelial carcinoma		1	Undifferentiated carcinoma		1
Carcinoma ex-pleomorphic adenoma		1	Carcinoma ex-pleomorphic adenoma		3
Cystadenocarcinoma		1	Renal cell carcinoma		1
Adenoid cystic carcinoma		1	Adenoid cystic carcinoma		2
			Myoepithelial carcinoma		1
			Basal cell carcinoma		1

following thermal cycling conditions: 16°C for 30 minutes, 42°C for the 30 minutes, 85°C for 5 minutes and then cooling to 4°C. Preamplification was conducted with Taq-Man PreAmp Master Mix using the following thermal cycling conditions: 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, 72°C for 2 minutes, 12 cycles at 95°C for 15 seconds and 60°C for 4 minutes, then 99.9°C for 10 minutes 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 uracil-N-glycosylase (UNG). Initial denaturing was conducted for 10 minutes at 95°C and then followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on the Roche Light-Cycler 480 II (Roche). All qRT-PCRs were conducted in duplicate for all candidate miRNA. Samples in which miRNA was omitted were used as negative controls. The C_t was examined and the ΔC_t 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 conducted in duplicate for all candidate miRNA and negative controls (in which RNA was omitted). The C_t was examined and the

ΔC_t was calculated. For miRNA qPCR experiments, U6 snRNA was used as the reference gene.

Statistical analysis

Wilcoxon rank-sum test was conducted to compare the expression of miRNA in samples from patients with malignant versus benign parotid gland tumors 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 ΔC_t 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. Receiver-operating characteristics (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 with patients with a malignant parotid gland tumor ($n = 10$; Wilcoxon test, $P < 0.05$). The expression of 54 of the 57 miRNAs was higher in saliva samples from patients with a malignant tumor compared with 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 than in 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 ($P < 0.05$; Supplementary Table S1 and S2).

qRT-PCR was conducted 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 $P < 0.05$ and fold change (Supplementary 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 ($P < 0.05$) 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).

Table 2. Verified salivary miRNA biomarkers in the original sample set

miRNA	Wilcoxon 2-sided malignant versus benign
mmu-miR-140-5p	0.020[b]
hsa-miR-374	0.021[b]
hsa-miR-222	0.040[b]
hsa-miR-15b	0.046[b]
hsa-let-7g	0.050[b]
hsa-miR-132	0.052[a]
hsa-miR-519b-3p	0.054[a]
hsa-miR-30a-3p	0.070[a]
hsa-miR-223	0.079[a]
hsa-miR-20a	0.2960
hsa-miR-195	0.3840
hsa-miR-194	0.4520
hsa-miR-618	0.5340
dme-miR-7	0.6000
hsa-miR-489	0.7500
hsa-miR-509-5p	0.7890
hsa-miR-381	0.8080
hsa-miR-1285	0.9130

NOTE: 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.
[a]$0.08 > P > 0.05$.
[b]$P < 0.05$.

Table 3. Validated salivary miRNA biomarkers in an independent sample set

miRNA	Wilcoxon 2-sided malignant versus benign
mmu-miR-140-5p	0.0003[a]
hsa-miR-374	0.0022[a]
hsa-miR-222	0.0070[a]
hsa-miR-15b	0.0019[a]
hsa-let-7g	0.0306[a]
hsa-miR-132	0.003[a]
hsa-miR-519b-3p	0.1931
hsa-miR-30a-3p	0.2320
hsa-miR-223	0.0542

NOTE: 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.
[a]$P < 0.05$.

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 2 groups ($P < 0.05$; Table 3; Fig. 1; Supplementary 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 area under the curve 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,
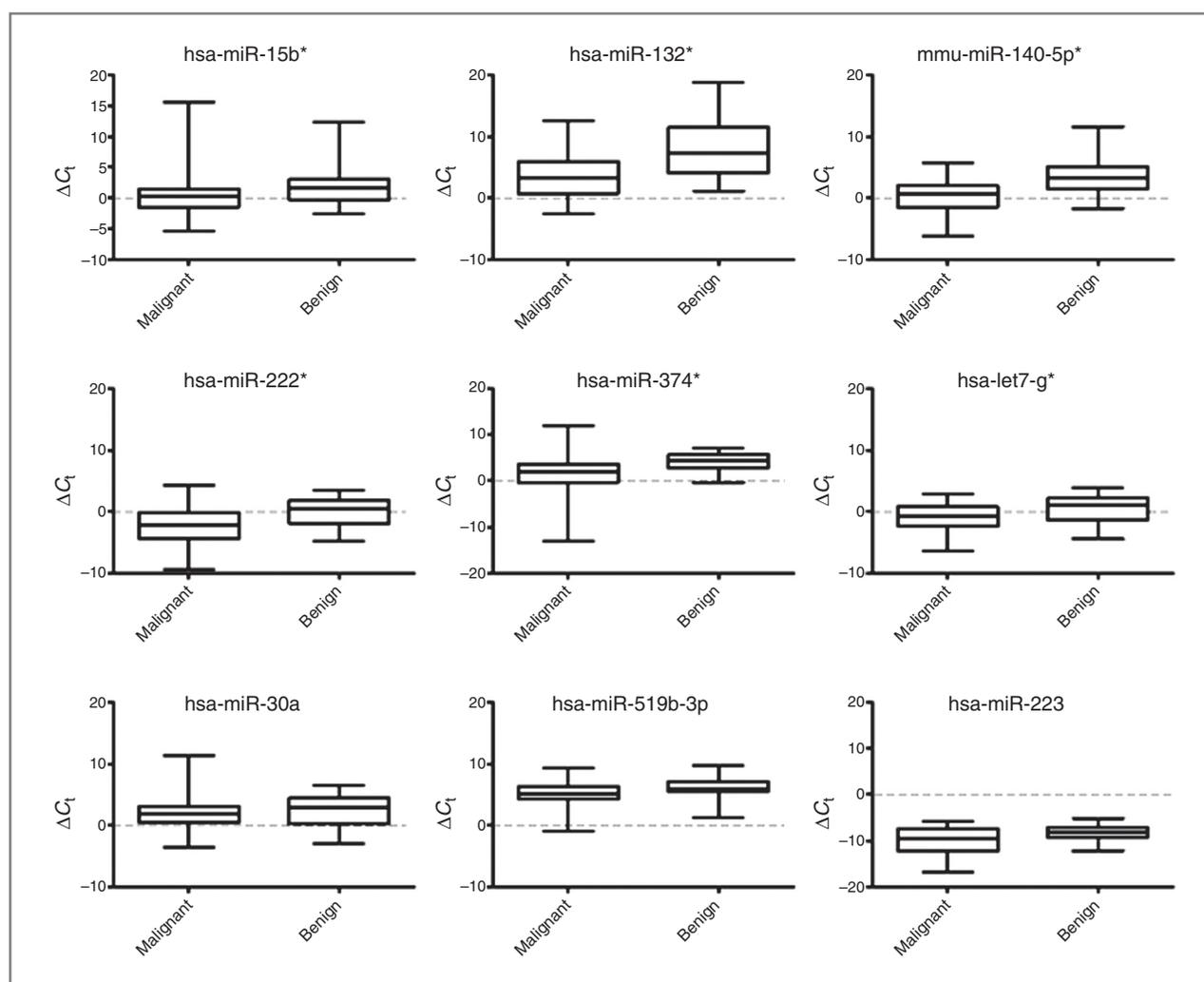
Figure 1. Box and whisker plot of validated miRNA expression profiles in saliva samples from patients with benign ($n = 19$) and malignant ($n = 28$) tumor in the parotid gland. Whiskers represent maximum and minimum ΔC_t. *, $P < 0.05$.

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 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 noninvasive. 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 the measurement of very low levels of analytes in saliva (22–24).

One of these analytes is miRNA. These small noncoding 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 article, we have identified and prevalidated 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
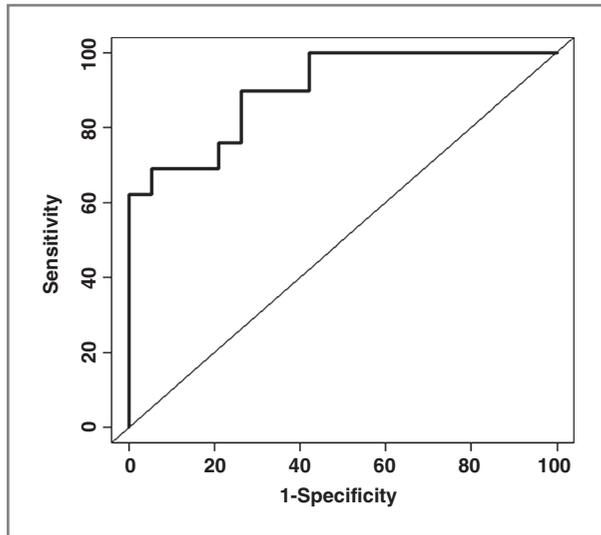
Figure 2. ROC curve computed from final logistic regression model. This model includes hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223, which, in combination, provided the best prediction. The area under the ROC curve was 0.9, the specificity was 95%, and sensitivity was 69%.

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, for example, colorectal (29, 30) and gastric cancer (31). The reduced RECK expression is often correlated with poor prognosis.

Among the validated targets of let-7g are genes that are associated with cancer such as *RAS*, *MYC*, and *CDKN2A*.
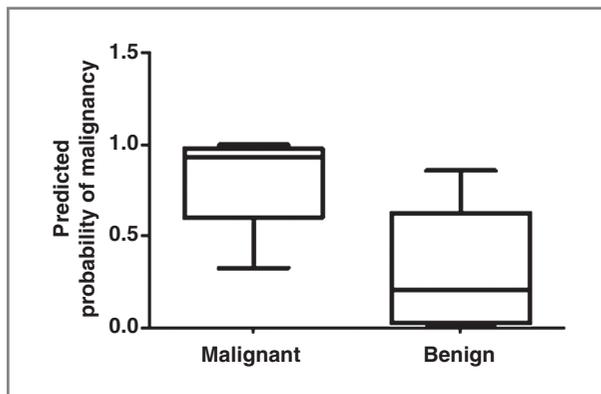
Figure 3. Box and whisker plot showing the "predicting probability for malignancy" based on the validation data of 4 miRNAs (hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223). Saliva samples from patients with a malignant tumor in the parotid gland have a higher "predicting probability for malignancy" than saliva samples from patients with a benign tumor in the parotid gland. Whiskers represent maximum and minimum probability for malignancy.

CDKN2A, also known as p16, is a tumor-suppressor gene that 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, showing 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 prevalidated the differences of miRNA expression in whole saliva from patients with malignant and benign parotid gland tumor. There seems to be a general upregulation of miRNAs in saliva from patients with a malignant parotid gland tumor when compared with miRNA expression in saliva from patients with a benign parotid gland tumor. The area under the curve of the 4 validated biomaker 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 conducted 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 Conflicts of Interest

Authors' Contributions

Conception and design: J.H. Matse, J. Yoshizawa, J.G. Bolsher, E.C. Veerman, E. Bloemena, D.T. Wong
Development of methodology: J.H. Matse, J. Yoshizawa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.H. Matse, J. Yoshizawa, E. Bloemena
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.H. Matse, X. Wang, D. Elashoff
Writing, review, and/or revision of the manuscript: J.H. Matse, J. Yoshizawa, X. Wang, D. Elashoff, J.G. Bolsher, E.C. Veerman, E. Bloemena, D.T. Wong
Study supervision: J.G. Bolsher, E.C. Veerman, E. Bloemena

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