Clinical Cancer Research

Salivary Biomarkers for Detection of Oral Squamous Cell Carcinoma in a Taiwanese Population

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Abstract

Purpose: This study evaluated the discriminatory power of salivary transcriptomic and proteomic biomarkers in distinguishing oral squamous cell carcinoma cases from controls and potentially malignant oral disorders (PMOD).

Experimental Design: A total of 180 samples (60 OSCC patients, 60 controls, and 60 PMOD patients) were used in the study. Seven transcriptomic markers (*IL8*, *IL1β*, *SAT1*, *OAZ1*, *DUSP1*, *S100P*, and *H3F3A*) were measured using qPCR, and two proteomic markers (IL8 and IL1β) were evaluated by ELISA.

Results: Among 7 transcriptomic markers, transcript level of *DUSP1* was significantly lower in OSCC patients than in controls and PMOD patients. Between the proteomic markers, the protein concentration of IL8 and IL1β was significantly higher in OSCC patients than controls and dysplasia patients. Univariate fractional polynomial (FP) models revealed that salivary IL8 protein (IL8p) has the highest AUC value between OSCC patients and controls (0.74) and between OSCC and PMOD patients (0.72). Applying a 2-marker FP model, salivary IL8p combined with IL1β gave the best AUC value for discrimination between OSCC patients and controls, as well as the IL8p combined with *H3F3A* mRNA, which gave the best AUC value for discrimination between OSCC and PMOD patients. Multivariate models analysis combining salivary analytes and risk factor exposure related to oral carcinogenesis formed the best combinatory variables for differentiation between OSCC versus PMOL (AUC = 0.80), OSCC versus controls (AUC = 0.87), and PMOD versus controls (AUC = 0.78).

Conclusions: The combination of transcriptomic and proteomic salivary markers is of great value for oral cancer detection and differentiation from PMOD patients and controls. *Clin Cancer Res; 22(13); 3340–7. ©2016 AACR.*

Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck region. High morbidity and mortality is associated with this disease, but little improvement has been observed in the 5-year survival rate for patients with OSCC along the years (1). One of the main prognostic factors for OSCC patients is advanced disease (2, 3). Considering that, early diagnosis of oral cancer is an important approach to decrease morbidity and mortality rate.

A multistep carcinogenesis process characterizes OSCC development. Cumulative mutational events occur in the mucosal epithelial stem cells, and the cellular proliferation promotes the expansion of a field of DNA-altered cells in the epithelial lining (4–6). Clinical and histopathologic signs of altered epithelium can, sometimes, be observed in a form of leukoplakia/erythroplakia [also called potentially malignant oral disorders (PMOD)] and cellular dysplasia, respectively (7). Under the effect of new mutational events, malignant transformation can occur in these areas, leading to development of an infiltrative disease (4).

Although the rate of malignant transformation of clinically altered epithelium is low (about 0.13%–17.5%; ref. 7), the detection and close follow-up of these lesions are the best approaches to early diagnosis of oral cancer. Nowadays, the unique available method for detection of altered epithelium and oral cancer is clinical examination, but it does not allow a reliable differentiation between PMOD and lesions, with no risk to cancer progression (8). The rate of detection of early stage oral cancer is low. This can be explained by the asymptomatic characteristic of early stage disease and the lack of an adequate routine mucosal exam by health care practitioners (9, 10). The development of a reliable detection method of OSCC and PMOD would be of importance to improve early diagnosis of oral cancer. Consequently, this would favor early treatment, changing survival rates and avoiding the devastating consequences of advanced tumor treatment.

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Translational Relevance

Early diagnosis is a need to decrease morbidity and increase survival of oral squamous cell carcinoma (OSCC) patients. Salivary diagnostics is emerging as an important tool for human cancer detection. In our study, we showed that salivary biomarkers are useful for OSCC diagnosis in a Taiwanese population. Considering previously published results, we can infer that these salivary biomarkers are useful for oral cancer detection regardless of ethnicity. Our results support the idea that salivary diagnostics might be used in the clinical practice for OSCC detection and works as a differential diagnosis test with other oral potentially malignant disorders. In this way, it permits that large-scale screening tests are implemented for oral cancer and potentially malignant disorders detection in high incident areas.

Saliva has been considered an important source of biologic information for the detection of human diseases. Beyond the obvious relationship with the oral mucosa surface, several studies have demonstrated synergism between the expression of molecular markers in saliva and systemic or distant sites diseases. Metabolites, proteins, coding and noncoding RNAs, and DNA have been detected in saliva of diseased patients, showing important value in disease detection (11–18).

The role of saliva to detect oral cancer has been studied, showing encouraging results (11, 13–15, 19, 20). In 2014 (16), it was determined the salivary transcriptome of oral cancer, showing it contains a set of promising extracellular RNA (exRNA) markers. These salivary exRNA markers were tested in different OSCC populations showing promising performance for disease detection, with sensitivity and specificity higher than 80% (14,19). Inflammatory cytokines have also been investigated as potential biomarkers of oral cancer (13, 14, 21–23). Hoffman and colleagues (2007; ref. 21) verified that serum levels of IL8 were significantly higher in patients with squamous cell carcinoma of the head and neck region when compared with individuals without cancer. Arellano-Garcia and colleagues (2008; ref. 13) also studied the potential of cytokines to predict cancer, but using saliva as a source of biologic information. They observed that IL8 and IL1β were significantly more expressed in saliva of OSCC patients than in healthy controls.

Elashoff and colleagues (2012; ref. 14) demonstrated that the combination of such protein and RNA markers improved the power of predictability of salivary OSCC biomarkers. Although the efficacy of these RNA and protein markers of oral cancer has been demonstrated, race-related variations could occur in biomarker discovery. Considering that an ideal biomarker should have a widespread efficacy regardless of ethnicity, it is important to challenge these biomarkers in a new population.

Bearing in mind the significance of PMOD in the early diagnosis of OSCC, we decided, for the first time, to test the efficacy of these biomarkers in the detection of precursor lesions. The primary objective of this analysis is to assess the discriminatory power of 7 salivary transcriptomic markers (*IL1β*, *IL8*, *SAT1*, *OAZ1*, *DUSP1*, *S100P*, and *H3F3A*) and 2 proteomic markers (IL8 and IL1β) in distinguishing OSCC and PMOD patients from health individuals.

Materials and Methods

Patient selection

Saliva samples were collected, after approval by the Institutional Review Board (IRB; #104-2602C), from patients that agreed to sign an informed consent in the Linko Medical Center of Chang Gung Memorial Hospital at Taoyuan, Taiwan. A total of 180 samples were included in the dataset, including 60 cases of OSCC, 60 cases of PMOD, with histopathologic evidence of dysplasia, and 60 individuals with no clinical sign of malignant or potentially malignant disease in the oral cavity.

Controls were gender and age-matched subjects enrolled during the same period when OSCC subjects were recruited. PMOD and controls received routine physical examination of head and neck regions, and patients with any head and neck disease (exception for PMOD) were excluded. All patients with a history of prior cancer, diabetes, autoimmune disorders, hepatitis, or HIV infection were excluded.

Demographical information was obtained by an IRB-approved questionnaire. Clinical information of OSCC patients was obtained by the pathologic report generated after tumor resection. This information is described in detail in the Supplementary Table S1.

Saliva collection and processing

Unstimulated saliva was collected and processed separately for RNA and protein according to our published protocol (16). For saliva collection, the donors avoided eating, drinking, smoking, and using oral hygiene products for at least 1 hour before the procedure. The collected samples were centrifuged at 3,000 × *g* for 15 minutes at 4°C. The supernatants were immediately treated with a protease inhibitor mixture (Roche; cat. number: 11836145001) and RNase inhibitor (Invitrogen, 10777-019). The samples were aliquoted into smaller volumes and stored at −80°C refrigerator. To avoid protein degradation, thawed saliva samples were used once. Saliva was collected at diagnosis for patients with OSCC and PMOD before any surgical procedure.

All laboratory measurements of salivary biomarkers were performed at the School of Dentistry, Center for Oral/Head & Neck Oncology Research, University of California - Los Angeles (Los Angeles, CA).

Primer design

We used a nested PCR approach for the measurement of RNA molecules in saliva. Outer primers [outer forward (OF) and outer reverse (OR)] were designed for cDNA synthesis and preamplification. Inner primers [inner forward (IF) and inner reverse (IR)] were designed for qPCR measurement of the cDNA targets. Primer pairs were designed using the NCBI/Primer-BLAST software. Three genes were used as saliva internal reference: *GAPDH*, *ACTB*, and *RPS9*. Only samples that exhibited specific qPCR products for these 3 genes were used in the study. C_t values of all target genes were normalized according to RPS9 gene expression. The target RNAs measured in saliva of the studied individuals were *IL1β*, *IL8*, *SAT1*, *OAZ1*, *DUSP1*, *S100P*, and *H3F3A*. Primers sequences are described in the Supplementary Material (Supplementary Table S2).

Direct saliva transcriptome analysis

A multiplex cDNA synthesis and preamplification approach was performed directly in 4 μL of saliva for each sample. This technique was developed by our group and is called direct saliva transcriptome analysis (DSTA; ref. 24). The reactions were performed in 10 μL volume, with a pool of outer primers at 50 μmol/L

Table 1. Clinical characteristics of subjects enrolled in the study

		OSCC Subjects (%)	PMOD Subjects (%)	Controls Subjects (%)	*P*
Gender	Male	57 (95)	57 (95)	55 (91.7)	0.678
	Female	3 (5)	3 (5)	5 (8.3)	
Age	<39	6 (10)	12 (20)	18 (30)	0.923*
	40–49	21 (35)	15 (25)	11 (18.3)	
	50–59	23 (38.3)	21 (35)	14 (23.3)	
	60–69	7 (11.7)	9 (15)	10 (16.7)	
	>70	3 (5)	3 (5)	7 (11.7)	
Ethanol consumption	Yes	41 (68.33)	38 (63.3)	25 (41.7)	0.007
	No	19 (31.67)	22 (36.7)	35 (58.3)	
Betel nut chewing	Yes	52 (86.67)	48 (80)	28 (46.7)	<0.001
	No	8 (13.33)	12 (20)	32 (53.3)	
Tobacco consumption	Yes	50 (83.33)	58 (96.7)	49 (81.7)	0.02
	No	10 (16.67)	2 (3.3)	11 (18.3)	
Tumor size	T1	20 (33.33)	—	—	—
	T2	19 (31.67)	—	—	
	T3	5 (8.33)	—	—	
	T4a	14 (23.33)	—	—	
	T4b	2 (3.33)	—	—	
Lymph node status	N0	34 (56.67)	—	—	—
	N1	8 (13.33)	—	—	
	N2a	1 (1.67)	—	—	
	N2b	15 (25)	—	—	
	N2c	2 (3.33)	—	—	
Distant metastasis	M0	60 (100)	—	—	—
Clinical stage	I	18 (30)	—	—	—
	II	10 (16.67)	—	—	
	III	6 (10)	—	—	
	IVa	24 (40)	—	—	
	IVb	2 (3.33)	—	—	

*ANOVA test.

each and the SuperScript III Taq (Life Technologies) and 2× Reaction Mix. The thermocycler program used is described as following: 2 minutes at 60°C, 30 minutes at 50°C, 2 minutes at 95°C, and 15 cycles of 15 seconds at 95°C, 30 seconds at 50°C, 10 seconds at 60°C and 10 seconds at 72°C, and cooling at 4°C. The PCR products were treated with 4 μL of ExoSAP-IT (Affymetrix) for 15 minutes at 37°C and then heated to 80°C for 15 minutes.

The target transcripts were quantified from 2 μL of preamplified cDNA via singleplex qPCR using the Roche LightCycler 480 Instrument (Roche). The qPCR reactions were done in a 10 μL volume containing 50 μmol/L of each inner primer pair and 2× SYBR Green qPCR Mix. The qPCR was carried out under the following conditions: 95°C for 5 minutes and 40 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C. All of the qPCR reactions were performed in duplicate.

Salivary protein detection

Salivary IL8 and IL1β proteins (IL8p and IL1βp) were measured using specific ELISA Kits (Thermo Fisher Scientific) according to the manufacturer's instructions. For measurement, saliva samples were diluted in PBS according to the recommendation of a previous study (14). For IL8, saliva was diluted 1:8 and for IL1β it was diluted 1:3. For IL values correction, total salivary protein was measured using Bradford method (Bio-Rad) using saliva dilutions of 1:3. All samples were assayed in duplicates using a microplate reader, and the results were expressed in pg/mL.

Statistical analysis

Missing values were replaced by the half of minimum of the variable. Using the raw data (Supplementary Table S3), nonparametric ANOVA (Kruskal–Wallis test) was performed to test the overall difference in biomarkers among three groups. If the overall difference was significant, Wilcoxon rank sum test was used to perform two specific comparisons: OSCC versus controls and OSCC versus PMOD. Nonparametric tests were used after Shapiro–Wilk test showed that data were not normally distributed. The mRNA markers were normalized subtracting RPS9 gene expression values. Protein markers were normalized dividing its expression levels to total protein values. This was followed by standardization or *z*-score scaled subtracting the mean and dividing by SD. ROC analysis was performed after running univariate fractional polynomial (FP) model (25, 26). The marker with highest AUC value was used as the anchor marker. The relationships between the anchor marker and other markers were checked by Spearman correlation analysis and visualized by 2-D plots. Whether adding a second marker will increase the discriminatory power of the anchor marker was checked by two methods: ROC analysis after running the FP models and logistic regression models. The AUC values and Akaike information criterion (AIC) values were compared for different 2-marker models. In addition, multivariate analyses, including all salivary markers and risk factors exposure, were carried out after running FP and logistic regression models. Statistical analysis was carried out using R version 3.0.2 (R Foundation).

Results

Clinical and demographical data from patients included in this study are described in Table 1. No difference was observed among groups considering gender and age. Ethanol intake as well as betel nut chewing was higher in OSCC and PMOD patients than in

Table 2. Expression values for salivary mRNAs and proteins according to the studied groups

	OSCC Patients			Controls			PMOD Patients			Kruskal–Wallis test	Wilcoxon two-sample test	
Variable	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max	*P*	*P* OSCC vs. Control	*P* OSCC vs. PMOD
IL1β	28.80	4.63	21.10–41.00	27.57	4.29	20.80–41.00	28.73	4.70	21.20–41.00	0.28		
IL8	27.34	3.88	20.10–38.40	27.19	3.97	20.40–41.00	28.09	4.35	20.70–41.00	0.50		
SAT1	27.87	3.23	21.10–36.90	27.55	3.65	21.20–38.3	27.97	2.92	21.40–35.00	0.61		
OAZ1	23.77	3.71	15.90–32.30	24.15	3.92	17.80–33.30	24.11	3.94	17.30–33.10	0.96		
DUSP1	34.99	5.91	22.60–41.00	32.36	6.10	23.40–41.00	32.81	5.95	23.10–41.00	0.03	0.0123	0.0422
S100P	35.38	4.44	25.70–41.00	34.19	4.49	25.50–41.00	35.66	4.30	25.00–41.00	0.19		
H3F3A	20.60	4.21	12.20–33.30	21.00	4.76	13.80–33.00	20.60	3.85	13.00–32.90	0.99		
GAPDH	17.56	3.45	9.80–28.10	17.64	3.20	11.70–26.90	18.11	3.40	11.50–28.50	0.60		
ACTB	23.02	3.77	13.50–32.70	23.25	3.79	16.50–32.70	23.64	4.26	15.40–34.70	0.88		
RPS9	22.62	3.87	15.30–33.00	23.10	3.66	15.20–31.90	23.77	4.10	17.30–36.20	0.40		
IL8_pg/mL	283.75	262.33	0.00–1048.72	127.79	110.84	0.00–654.89	140.35	155.13	0.00–1048.72	<.0001[a]	<.0001[a]	<.0001[a]
IL1β_pg/mL	101.03	112.96	0.00–419.49	48.07	42.01	0.00–161.99	39.66	28.00	1.61–145.83	0.0061[a]	0.01[a]	0.004[a]
Total_protein_mg/mL	1.21	0.64	0.07–2.94	1.08	0.52	0.17–2.61	0.96	0.44	0.28–2.14	0.11		

[a]*P* values obtained from comparisons after normalization to total protein and standardization.

controls. Tobacco use was higher in the PMOD group than among OSCC patients and controls.

The expression values for the analyzed salivary mRNAs and proteins are described in Table 2. Significant differences were observed in the expression level of *DUSP1* between OSCC patients and controls ($P = 0.0123$), as well as between OSCC patients and PMOD patients ($P = 0.0422$). The concentration of IL8p was significantly higher in OSCC patients when compared with controls ($P < 0.0001$) and PMOD patients ($P < 0.0001$; Fig. 1A). Similarly, salivary IL1βp concentration was significantly higher in OSCC patients than in controls ($P < 0.01$) and PMOD patients ($P = 0.004$).

Using univariate FP models, the IL8p gave the highest AUC (AUC= 0.749) in distinguishing OSCC patients from controls after running the model on each biomarker. The univariate model with IL8p as predictor also had the lowest AIC (AIC = 150.99). Similarly, the protein marker IL8p gave the highest AUC (AUC= 0.721) and the lowest AIC (AIC = 150.88) in distinguishing OSCC from PMOD group (Table 3; Fig. 1B).

Using the salivary expression of IL8p as an anchor marker and applying the 2-marker FP models, the combination of IL8p and IL1β gave the highest AUC (AUC= 0.817) in distinguishing OSCC patients from controls. This 2-marker model also had the lowest AIC (AIC = 138.28; Fig. 1C and D). When the sensitivity of the test was fixed at 0.9 in distinguishing OSCC patients from controls, this 2-marker model gave the highest maximized specificity (MaxSpec = 0.56). In contrast, the combination of IL8p and *H3F3A* gave the highest AUC (AUC= 0.752) and the lowest AIC (AIC = 141.34) in distinguishing OSCC patients from PMOD patients (Table 4; Fig. 1E and F). When the sensitivity of the test was fixed at 0.9 in distinguishing OSCC patients from PMOD patients, this 2-marker model gave the MaxSpec equal to 0.45.

Multivariate models analysis considering the expression of salivary biomarkers and risk factor exposure was carried out using the FP model and the logistic model (Table 5). Using the same marker combination, FP model gave the highest AUC and the lowest AIC values when compared with logistic model (Fig. 1G and H). Areca nut chewing, drinking, and smoking habits associated with salivary expression of *IL1β* transcript was the best variable combination for the distinction between PMOD patients and controls (AUC = 0.785). When sensitivity was fixed at 0.9, the MaxSpec obtained was 0.9. Areca nut chewing associated with salivary expression of DUSP1 transcript, IL1βp, and IL8p revealed the best combinatory effect for differentiation between OSCC patients and controls (AUC = 0.872). After fixing sensitivity at 90%, MaxSpec obtained was 0.63. For separation between OSCC and PMOD patients, smoking associated with salivary *H3F3A* transcript and IL8p expression showed up as the best combinatory markers (AUC=0.802). When sensitivity was set at 0.9, MaxSpec obtained was 0.53.

Table 3. ROC analysis using univariate FP model of salivary transcriptomic and proteomic markers and risk factors exposure

Univariate model	PMOD vs. Controls		OSCC vs. Controls		OSCC vs. PMOD	
Variable	AUC	AIC	AUC	AIC	AUC	AIC
IL8	0.467	166.58	0.449	166.33	0.518	170.13
IL1β	0.542	170.11	0.721	151.49	0.569	168.63
OAZ1	0.508	170.32	0.519	170.33	0.576	168.07
SAT1	0.483	170.23	0.643	162	0.563	168.52
DUSP1	0.563	168.77	0.649	162.8	0.651	162.24
S100P	0.552	169.59	0.597	167.17	0.542	169.48
H3F3A	0.564	166.6	0.524	170.33	0.589	165.18
IL-1βp	0.646	159.09	0.637	159.71	0.655	156.75
IL-8p	0.498	170.16	0.749	150.99	0.721	150.88
Total protein	0.568	168.72	0.551	166.06	0.607	164.39
Smoking	0.575	162.74	0.515	168.76	0.56	163.56
Drinking	0.608	164.66	0.639	159.5	0.531	168.45
Areca nut	0.667	155.6	0.707	144.3	0.541	167.48

Table 4. ROC analysis of 2-marker FP model using the salivary expression of IL8p as an anchor marker

	OSCC vs. Controls		OSCC vs. PMOD	
2-Marker model	**AUC**	**AIC**	**AUC**	**AIC**
IL8p + IL1β	0.817	138.28	0.739	150.7
IL8p + *OAZ1*	0.751	152.97	0.734	148.76
IL8p + *SAT1*	0.794	145.06	0.738	150.98
IL8p + *DUSP1*	0.754	148.62	0.726	153.75
IL8p + *S100P*	0.767	150.54	0.723	152.16
IL8p + *H3F3A*	0.75	152.86	0.752	141.34
IL8p + IL1βp	0.74	152.67	0.685	155.25

Discussion

South and Central Asia has one of the highest incidences and mortality rates of OSCC in the world. The consumption of areca nut (betel) associated or not with tobacco is the main risk factor for OSCC development among these people. Taiwan has one of the highest consumptions of areca nut, explaining why this country has one of the biggest prevalence of PMOD in the world (12.7%) and a high incidence rate of oral cancer (27, 28).

Areca nut usage is an important cause of PMOD, as well as tobacco. However, beyond the development of leukoplakia and erythroplakia, it can also induce a very distinct form of PMOD, called oral submucous fibrosis (OSF; refs. 28, 29). OSF is more prevalent among Southeast Asians, being rare in the Western countries. This may indicate that the pathways involved in the oral cancer development among Southeast Asians might diverge from occidental patients. However, even with these ethnic and behavior variations, the biomarkers developed in the Western population and applied in this study showed a good performance for the discrimination between oral cancer and controls in Taiwanese individuals, revealing high AUC values and high sensitivity. These biomarkers were challenged before in different populations from the United States and Serbia, in which tobacco and alcohol consumption are the main etiologic factors for OSCC (14, 19). In these studies, the performance of the biomarkers was similar to that observed in the current article.

In this study, the proteomic markers had better performance than transcriptomic markers in distinguishing oral cancer cases from PMOD and controls when considered individually. The salivary IL8p alone model performed best among the univariate models, always giving the highest AUC values than other individual marker. In other validation studies in Western populations, IL8p and IL1βp were also potential salivary biomarkers for oral cancer detection, showing sensitivity and specificity ranging from 70% to 80% and an AUC value around 0.7 (14, 19). Although salivary IL1βp was not considered a good marker for OSCC detection in our investigation, IL8 showed AUC value (0.73) similar to other studies, confirming the reproducibility of this marker across different oral cancer populations.

However, as observed by others (14, 16, 19), the combination of proteomic and transcriptomic markers revealed the best discriminatory effect between oral cancer and noncancer individuals. According to Li and colleagues (16), salivary *IL8*, *IL1β*, *SAT*, and *OAZ1* mRNA detection formed the best combinatory markers for OSCC detection. Elashoff and colleagues (14) tested these biomarkers in 5 cohorts of patients and controls. They observed that for some of the cohorts, the combination of biomarkers with the best performance for oral cancer diagnosis would change, giving AUC values varying from 0.75 to 0.86. However, the *IL8* and *SAT* mRNAs were present in all the 5 different combinations, suggesting that these biomarkers were the most consistent ones. For Brinkmann and colleagues (19), the best discriminatory marker for OSCC detection was the combination of IL1βp and the *SAT1* and *DUSP1* mRNAs, revealing an AUC value of 0.86. In our study, we obtained an AUC of 0.817 that is in the range of AUC values obtained in previous studies, but using just two markers, IL8p combined with *IL1β* mRNA.

Beyond univariate and 2-marker analysis, we carried out a multivariate analysis including all possible combinations of salivary analytes and also considering exposure to risk factors related to OSCC and PMOD development. Combining these parameters, we were able to generate a set of predictors of great value for disease detection. The distinction between OSCC and controls, as well as OSCC and PMOD, was greatly dependent on risk factor exposure status. Using such information, we observed a significant increase in test accuracy, achieving an AUC of 0.87 for the differentiation between OSCC and controls and 0.80 for the differentiation between OSCC and PMOD individuals.

One of the most important approaches for improving survival and decreasing morbidity in oral cancer patients is the detection of early stage cancer. The detection of PMOD is of extreme importance, but its discrimination from early stage oral cancer can be challenging. Currently, the most efficient available method of diagnosis of these potentially malignant lesions and discrimination from oral cancer is biopsy, followed by histopathologic examination. Beyond the need of highly trained personal to do such exams, it is costly, time consuming, and is associated with patient distress and risk as it is a surgical intervention. The development of a salivary biomarker with potential of discriminatory diagnosis between these two entities would be of great value. In this study, we showed, for the first time, that salivary biomarkers have discriminatory effect for discrimination between malignant and PMOD. High expression of *IL1β* transcript associated with the consumption of betel, alcohol, and tobacco generated the highest AUC value for the differentiation between PMOD and controls. Importantly, for this combination of parameters, we obtained specificity and sensibility of 90%, indicating a high accuracy test for the detection of PMOD. This finding is of great importance for screening purposes in populations exposed to these risk factors, as the measurement of just one salivary

Table 5. Multivariate model ROC analyses of the optimal models using FP and logistic models

		PMOD vs. Controls	OSCC vs. Controls	OSCC vs. PMOD
	Model	**IL1β + Areca nut + Drinking + Smoking**	***DUSP1* + IL1βp + IL8p + Areca nut**	***H3F3A* + IL8p + Smoking**
FP Model	AUC	0.785	0.872	0.802
	AIC	145.59	120.16	135.32
Logistic model	AUC	0.716	0.81	0.771
	AIC	156.32	137.28	146.07

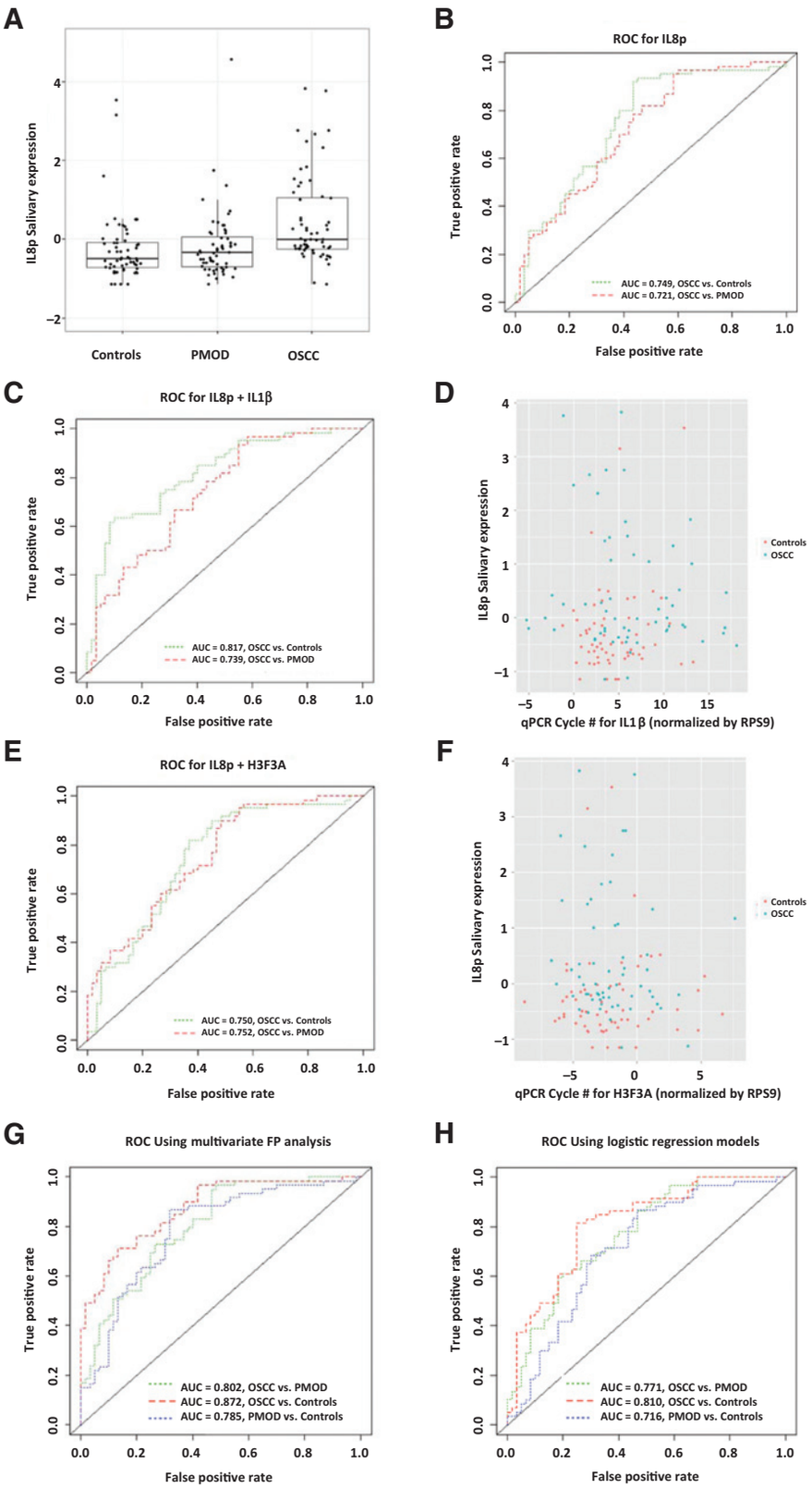

Figure 1.
A, salivary expression of IL8p among the studied groups. IL8 expression was significantly higher in cancer patients compared with controls and PMOD patients. Whiskers represent median. B, ROC curves for salivary IL8p. The AUC value for cancer patients distinguishing from controls and PMOD patients was 0.749 and 0.721, respectively. C, ROC curves for the combination of salivary IL8p and *IL1β* mRNA. The AUC value for cancer patients distinguishing from controls and PMOD patients was 0.81 and 0.73, respectively. D, 2D scatterplot showing the correlation between the expression of salivary IL8p and *IL1β* mRNA in controls and oral cancer patients. E, ROC curves for the combination of salivary IL8p and *H3F3A* mRNA. The AUC value for cancer patients distinguishing from controls and PMOD patients was 0.75 and 0.75, respectively. F, 2D scatterplot showing the correlation between the expression of salivary IL8p and *H3F3A* mRNA in controls and oral cancer patients. G, ROC curves generated by multivariate FP analysis using three different combinations of variables. Blue line: *IL1β* + areca nut + drinking + smoking for differentiation between PMOD and controls (AUC = 0.802). Red line: *DUSP1* + IL1βp + IL8p + areca nut for differentiation between OSCC and controls (AUC = 0.872). Green line: *H3F3A* + IL8p + smoking for differentiation between OSCC and PMOD (AUC = 0.785). H, ROC curves generated by logistic regression models using three different combinations of variables. Blue line: *IL1β* + areca nut + drinking + smoking for differentiation between PMOD and controls (AUC = 0.716). Red line: *DUSP1* + IL1βp + IL8p + areca nut for differentiation between OSCC and controls (AUC = 0.810). Green line: *H3F3A* + IL8p + smoking for differentiation between OSCC and PMOD (AUC = 0.771).

biomarker would give a very accurate indication of PMOD diagnosis.

For some of our analysis, setting high sensitivity values (90%) led to low maximum specificity. This occurred for differentiation between OSCC and controls and OSCC and potentially malignant oral lesions (PMOL). Although low specificity represents a limitation of the proposed biomarker combination, we believe that for a screening approach, high sensitivity is the most important parameter, as it provides a low number of false negative cases and select candidate cases for complementary clinical evaluation.

Another potential limitation of our findings is the lack of periodontal evaluation of studied cases. Inflammatory diseases, such as periodontitis, are one of the most common pathologies in oral cavity, representing the most common inflammatory disease in humans (30, 31). Considering that some of our candidate biomarkers are cytokines (IL8 and IL1β), one may suggest that inflammatory diseases in oral cavity may represent a confusion factor in our analysis. Furthermore, OSCC, PMOD, and periodontitis share the same etiologic factors, such as tobacco (32, 33). However, Cheng and colleagues (2014; ref. 34) compared the salivary IL8p expression between OSCC patients and patients with periodontitis. They observed that IL8 salivary levels were significantly higher in OSCC when compared with chronic periodontitis patients ($P < 0.001$) and healthy controls ($P = 0.014$). Also, mean expression in chronic periodontitis patients was lower (0.58 ± 0.26 pg/mL) than in healthy controls (0.80 ± 0.41 pg/mL). This may indicate that inflammatory conditions may have little effect on our results. Moreover, inflammatory reaction elicited in periodontitis and in OSCC is of different nature, as microbes are the main players in the induction of the former (34).

An important aspect of this work is the use of DSTA technique, which allows the measurement of salivary transcripts directly from saliva, with no need of RNA extraction prior to the analysis (24). This approach is of utmost importance for the implementation of such biomarkers in a clinical practice. RNA extraction is involved with higher costs, need for trained personnel, and is time consuming. In this work, we demonstrated that salivary RNA and protein could be measured directly from saliva, with no need of prior treatment. We believe that in the near future, these biomarkers could be measured using portable technologies, permitting their use in an ambulatory environment (27).

We concluded that proteomic and transcriptomic salivary biomarkers are of great value for oral cancer and PMOD detection in Taiwanese population. Salivary analytes and the status of risk factors exposure related to oral carcinogenesis emerged as the best combination of variables for OSCC and PMOL detection. Also, for the first time, we demonstrated that salivary analytes have discriminatory power for PMOD diagnosis, representing a potential tool for early detection of patients in risk of oral cancer development.

Disclosure of Potential Conflicts of Interest

D.T.W. Wong has ownership interest in RNAmeTRIX Inc. and is a consultant for PeriRx LLC. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.O. Gleber-Netto, M. Yakob, F. Li, Z. Feng, J. Dai
Writing, review, and/or revision of the manuscript: F.O. Gleber-Netto, M. Yakob, F. Li, Z. Feng, Y.-L. Chang, K.-P. Chang
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