Correction

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Correction for "Saliva protein biomarkers to detect oral squamous cell carcinoma in a high-risk population in Taiwan," by Jau-Song Yu, Yi-Ting Chen, Wei-Fan Chiang, Yung-Chin Hsiao, Lichieh Julie Chu, Lai-Chu See, Chi-Sheng Wu, Hui-Tzu Tu, Hsiao-Wei Chen, Chia-Chun Chen, Wei-Chao Liao, Ya-Ting Chang, Chih-Ching Wu, Che-Yi Lin, Shyun-Yeu Liu, Shu-Ti Chiou, Shu-Li Chia, Kai-Ping Chang, Chih-Yen Chien, Su-Wei Chang, Chee-Jen Chang, John D. Young, Chia C. Pao, Yu-Sun Chang, and Leland H. Hartwell, which appeared in issue 41, October 11, 2016, of *Proc Natl Acad Sci USA* (113:11549–11554; first published September 23, 2016; 10.1073/pnas.1612368113).

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Saliva protein biomarkers to detect oral squamous cell carcinoma in a high-risk population in Taiwan

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Most cases of oral squamous cell carcinoma (OSCC) develop from visible oral potentially malignant disorders (OPMDs). The latter exhibit heterogeneous subtypes with different transformation potentials, complicating the early detection of OSCC during routine visual oral cancer screenings. To develop clinically applicable biomarkers, we collected saliva samples from 96 healthy controls, 103 low-risk OPMDs, 130 high-risk OPMDs, and 131 OSCC subjects. These individuals were enrolled in Taiwan's Oral Cancer Screening Program. We identified 302 protein biomarkers reported in the literature and/or through in-house studies and prioritized 49 proteins for quantification in the saliva samples using multiple reaction monitoring-MS. Twenty-eight proteins were successfully quantified with high confidence. The quantification data from non-OSCC subjects (healthy controls + low-risk OPMDs) and OSCC subjects in the training set were subjected to classification and regression tree analyses, through which we generated a four-protein panel consisting of MMP1, KNG1, ANXA2, and HSPA5. A risk-score scheme was established, and the panel showed high sensitivity (87.5%) and specificity (80.5%) in the test set to distinguish OSCC samples from non-OSCC samples. The risk score >0.4 detected 84% (42/50) of the stage I OSCCs and a significant portion (42%) of the high-risk OPMDs. Moreover, among 88 high-risk OPMD patients with available follow-up results, 18 developed OSCC within 5 y; of them, 77.8% (14/18) had risk scores >0.4. Our four-protein panel may therefore offer a clinically effective tool for detecting OSCC and monitoring high-risk OPMDs through a readily available biofluid.

biomarkers | oral cancer | early detection

Oral cavity cancer is a common cancer worldwide that represents a serious and growing problem in many parts of the globe (1, 2). Oral squamous cell carcinoma (OSCC) accounts for more than 90% of oral cancer cases. The major risk factors for OSCC include smoking, alcohol misuse, smokeless tobacco use, and betel quid chewing (3–6). The 5-y survival rate for OSCC is ∼50% in most countries (1). Survival rates approach 80% for patients with stage I disease, but more than 60% of patients present with stage III and IV disease (7). Thus, survival and morbidity would be dramatically improved if we could detect the disease earlier.

Most cases of OSCC develop from visible lesions in the oral cavity. These lesions, which exhibit oral epithelial dysplasia, are called oral potentially malignant disorders (OPMDs) (8, 9). Taiwan's Oral Cancer Screening Program offers members of the at-risk population (individuals 30 y or older with habits of betel nut chewing or cigarette smoking) a free visual examination every other year by a physician or dentist, referrals for pathological confirmation, and subsequent treatment (Oral Cancer Screening Clinical Pathway; Fig. 1*A*). However, the screening program in 2011 and 2012 increased the detection of early-stage

Significance

Oral squamous cell carcinoma (OSCC) accounts for 90% of oral cancers, and earlier detection efficiently increases the survival rate. Here, we used a comprehensive literature review to select candidates and used LC-multiple reaction monitoring-MS to qualitatively and quantitatively measure target proteins in saliva samples from individuals of the at-risk population in Taiwan. Statistical analysis of the results establishes a four-protein panel sufficient to detect 88.6% of early-stage patients and 91.6% of all patients with 80.4% specificity. This panel can also be used to evaluate the risk of malignant progression from high-risk oral potential malignant disorder. Our study offers a practical foundation for clinical trials examining the ability of this panel to enable early detection of OSCC, risk assessment for cancer development, and treatment monitoring.

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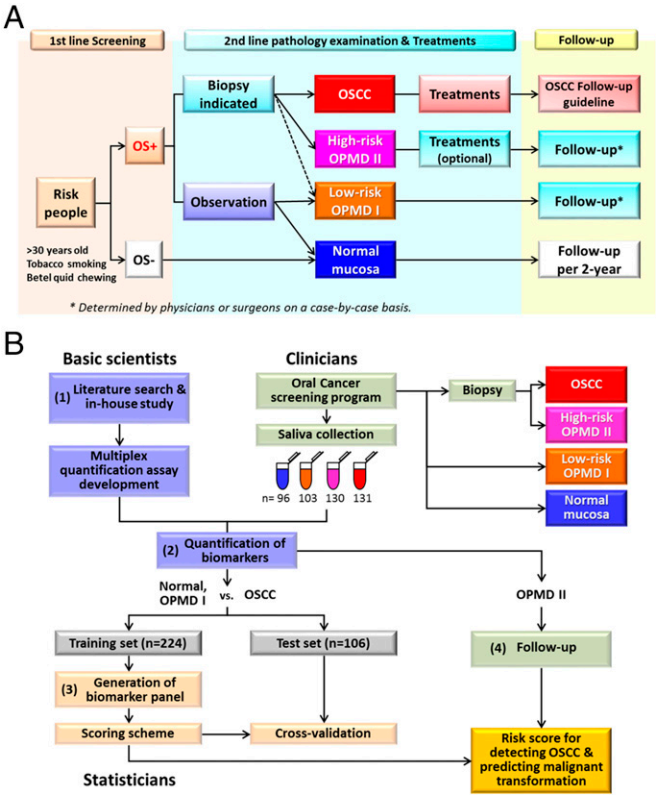

Fig. 1. The clinical protocol of Taiwan's Oral Cancer Screening Program and our study design for identifying OSCC salivary biomarker panels. (*A*) Taiwan's Oral Cancer Screening Program, which was established by the Ministry of Health and Welfare, Taiwan. (*B*) A workflow delineating our research strategy for selecting and testing previously identified protein OSCC biomarkers in saliva samples collected from the Taiwan's Oral Cancer Screening Program.

(i.e., stage I) OSCC by only 3% compared with the detection rate of regular clinics (*SI Appendix*, Table S1).

Here, we set out to identify a panel of biomarkers that are present in oral saliva and could potentially be used to increase the detection of early-stage oral cancer, complementing the effort of the national screening program of Taiwan. We collected saliva samples from individuals during their biannual screenings, such that the cases and controls were all high-risk individuals, and the collection protocols were identical. From the literature, we identified 49 protein biomarkers reported to be diagnostic for oral cancer. We developed highly sensitive and quantitative assays for 28 of these potential biomarkers using newly developed multiple reaction monitoring (MRM) technology (10), and assayed these biomarkers in cases and controls. Finally, we used statistical analysis to identify a four-protein panel that showed the highest sensitivity and specificity in our training set, and validated this panel in a test set.

Results

Study Population and Biosignature Study Design. We recruited 485 at-risk subjects and removed 25 subjects who did not match the inclusion criteria, including 18 normal controls (no smoking or betel nut chewing behavior), 3 high-risk OPMD (2 without smoking or betel nut chewing behavior and 1 with saliva sample collected after the oral lesion removal), and 4 OSCC cases (3 without smoking or betel nut chewing behavior and 1 with saliva sample collected after the oral tumor removal). The remaining 460 at-risk subjects were 96 healthy controls (normal mucosa) and 233 OPMD patients, including 103 OPMD I subjects (low-risk lesions with an expected <5% malignant transformation rate), 130 OPMD II subjects (high-risk lesions with an expected 5–30% malignant transformation rate) (11–13), and 131 OSCC patients and were used for the study. All individuals were enrolled in Taiwan's Oral Cancer Screening Program at a single medical center. Their demographic characteristics (e.g., sex, age, cigarette smoking, and betel nut chewing) are shown in Table 1. Most of the subjects were male, which is consistent with the incidence of OSCC in the Taiwanese population (14).

Following the clinical pathway of Taiwan's Oral Cancer Screening Program (Fig. 1*A*), we designed a biosignature study to compare the clinical utility of dozens of biomarkers and establish a marker panel for the early detection of OSCC. As presented in Fig. 1*B*, we first critically reviewed more than 1,400 oral/head and neck cancer-related articles reported in PubMed and other open-access databases and also referred to the results of our in-house studies. From these efforts, we identified a total of ~300 candidate biomarkers. We developed a prioritization process (detailed in *SI Appendix*, Fig. S1 and *SI Results*) and selected 49 protein candidates that we believed were very likely to be detected in saliva (*SI Appendix*, Table S2). The saliva samples collected from the 460 subjects were assorted randomly and subjected to multiplexed LC-MRM-MS assays of these 49 selected targets. To minimize the bias that might result from the potential misclassification of OPMD II cases (i.e., when malignant cells are present in the lesions but not detected by biopsy), we combined the healthy controls (having normal mucosa) and OPMD I patients into a non-OSCC group ($n = 199$) and compared the results obtained from this group vs. those of the OSCC group ($n = 131$). These 330 subjects were then randomly divided into a training set ($n = 224$) and a test set ($n = 106$) that were similar in their demographic characteristics (*SI Appendix*, Table S3). The quantification data obtained from the training set were subjected to multiple statistical analyses, from which we generated an optimized panel of biomarkers and identified cutoffs that yielded the best sensitivities and specificities. A scoring algorithm was also established for the purpose of predicting OSCC. The predictive performance of the selected biomarker panel was then validated in the test set using the established scoring algorithm. All laboratory assays (LC-MRM-MS) were performed blinded to case status before allocation to training and test sets, and the statistical analyses were completed using the training set before performed in the blinded test set. Finally, the generated biomarker panel and scoring algorithm were applied to the subjects of the OPMD II group in an effort to further evaluate the power of the panel to distinguish OSCC from OPMD II and/or predict the malignant transformation of OPMD II (as assessed using follow-up data).

Quantification of Salivary Proteins by Multiplex LC-MRM-MS. To determine which candidate proteins (peptides) could be readily detected in saliva samples, 49 surrogate peptides representing the 49 selected proteins were measured using a multiplexed LC-MRM-MS assay, and a standard saliva sample was prepared by pooling the saliva from two OSCC patients and one control individual. We found that the 49 pairs of endogenous and stable isotope-labeled standard (SIS) peptides (*SI Appendix*, Table S4) could be measured efficiently in a 70-min run of nano-LC coupled with a Qtrap5500 instrument (*SI Appendix*, Fig. S2 and Tables S5 and S6). Of the 49 proteins, 28 yielded five or more high-confidence concentration data points in the corresponding calibration curves (*SI Appendix*, Fig. S3).

We quantified these 28 proteins in saliva samples from the 460 recruited subjects. The quantitative results obtained in the OSCC group vs. the three other groups (healthy, OPMD I, and OPMD II) are detailed in *SI Appendix*, Table S7. The proteins were detected at concentrations ranging from subnanograms per milliliters to micrograms per milliliter, covering more than five orders of magnitude (Fig. 2*A*). Among the 28 tested proteins,

Table 1. Demographic characteristics and use of cigarettes and betel nuts by the enrolled subjects

Characteristics	Total	Healthy control	OPMD I	OPMD II	OSCC	*P**
Case no.	460 (100.0%)	96 (20.9%)	103 (22.4%)	130 (28.3%)	131 (28.5%)	
Sex						0.6763†
Male	456 (99.1%)	96 (100.0%)	102 (99.0%)	129 (99.2%)	129 (98.5%)	
Female	4 (0.9%)	0 (0.0%)	1 (1.0%)	1 (0.8%)	2 (1.5%)	
Age (y)	50.7 ± 10.7	48.8 ± 11.8	49.5 ± 10.7	51.4 ± 10.5	52.5 ± 9.7	0.0320‡
Smoke	26.0 ± 21.1	19.13 ± 11.15	24.59 ± 24.15	31.03 ± 21.48	27.01 ± 22.39	0.0030‡
Packs per day × years						
Betel nut	287.7 ± 430.7	138.1 ± 328.6	172.2 ± 187.9	389.6 ± 524.2	386.9 ± 477.7	<0.0001‡
Nuts per day × years						

**P* value of intersect.
†Fisher's exact test.
‡ANOVA.

16 (57%) showed significantly higher levels (more than twofold) in the OSCC group compared with the other three groups (*SI Appendix*, Table S8). This finding supports the value of prioritizing candidate biomarkers with a complete literature review before beginning the verification process. MMP1 (matrix metalloproteinase 1) and KNG1 (kininogen 1) were the top two most highly increased proteins in the OSCC group; Fig. 2*B* shows representative quantification results for their salivary levels in all 460 subjects.

To examine the ability of these potential salivary biomarkers to detect OSCC, we analyzed the power of each protein to distinguish between the OSCC group (n = 131) and the non-OSCC group (n = 199; healthy controls and OPMD I), as shown in Table 2. Among the 28 tested proteins, 21 were significantly increased in the OSCC group vs. the non-OSCC group (P <0.0001); their fold changes ranged from 1.8- to ~83-fold, and their AUC [area under the receiver operating characteristic (ROC) curve] values ranged from 0.705 to 0.871. These 21 proteins were chosen for assembly into candidate biomarker panels.

Generation of Candidate Biomarker Panels. To generate salivary biomarker panel(s) for OSCC detection, we used logistic regression, discriminant analysis, and classification and regression tree (CART) analysis to process the results obtained from the 21 selected proteins. We first used a training set (n = 224) and then validated our findings in a test set (n = 106); the sets were generated from the 330 subjects in the OSCC (n = 131) and non-OSCC (n = 199) groups, using random assignment at a ratio of 2:1 followed by adjustment for similar demographic characteristics (*SI Appendix*, Table S3).

Our logistic regression (LR) selected four proteins (ANXA2, HSPA5, KNG1, and PRDX2), and our discriminant analysis selected seven proteins (ANXA2, FLNA, HSPA5, KNG1, PRDX2, TIMP1, and YWHAB) (*SI Appendix*, Table S9). Both of these protein panels exhibited high specificity (88.7~96.2%) but poor sensitivity (51.6~75.8%) in the training set. In contrast, our CART analysis selected a four-protein panel (MMP1, KNG1, ANXA2, and HSPA5) that showed high levels of sensitivity and specificity in the training set (sensitivity, 96.7%; specificity, 79.7%) (*SI Appendix*, Table S9). Briefly, the training set samples were subjected to 1,000 simulations followed by CART analysis. The simulation selected seven proteins (MMP1, KNG1, ANXA2, HSPA5, MMP3, SPARC, and CA2) more than 150 times (*SI Appendix*, Fig. S4), indicating that these proteins had relatively good power for distinguishing between non-OSCC and OSCC samples. Notably, the same seven proteins were also selected when all 28 proteins were subjected to this simulation process. These seven proteins were again subjected to CART analysis in the training set, to build a CART decision tree that provided a cutoff value for the saliva concentration of each protein. This analysis yielded a final panel containing four proteins, MMP1, KNG1, ANXA2 (annexin A2), and HSPA5 [heat shock protein family A (Hsp70) Member 5] (Fig. 3*A*; for details, see *SI Appendix, SI Results*), which appeared to be suitable for detecting OSCC cases enrolled in Taiwan's Oral Cancer Screening Program.

Development of the Scoring Scheme. Next, we used logistic regression analysis to calculate the predictive probability as a risk score according to binary results obtained using the four protein markers (i.e., above or below the intrinsic cutoff values). The risk score significantly increased from the healthy control (0.16 ± 0.19) and OPMD I (0.18 ± 0.29) groups to the OSCC group (0.75 ± 0.24) in the training set (P < 0.0001; Fig. 3*B*), and similar results were obtained in the test set (healthy controls, 0.21 ± 0.26; OPMD I, 0.16 ± 0.22; and OSCC, 0.74 ± 0.31; P < 0.0001; Fig. 3*C*). ROC analysis for non-OSCC vs. OSCC samples showed that the AUCs for the training and test sets were 0.926 and 0.91, respectively (Fig. 3*D*). When the cutoff was set at 0.4 (Fig. 3*D*,

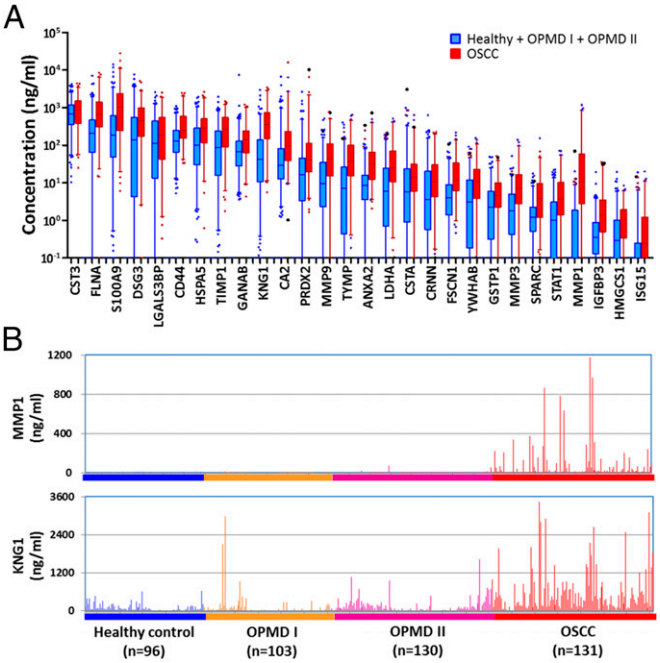

Fig. 2. Quantification of candidate proteins in saliva by LC-MRM/MS. (*A*) Box plot showing the expression levels of the 28 target proteins in the non-OSCC (blue box; healthy control plus OPMD I) vs. OSCC (red box) groups, presented as the upper and lower quartiles and range (box), the median value (horizontal line), and the middle 90% distribution (extended line). (*B*) The salivary levels of MMP1 and KNG1 in the 460 subjects of the four different groups (healthy control, OPMD I, OPMD II, and OSCC).

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Table 2. Concentrations of the 28 protein biomarkers in saliva samples from the non-OSCC (healthy control + OPMD I) group and OSCC group

	Healthy control + OPMD I (n = 199)		OSCC (n = 131)							
Protein	Concentration*	$n^{\dagger}$	Concentration*	$n^{\dagger}$	Fold change[‡]	$P^{\S}$	AUC	Youden index	Sensitivity	Specificity
ANXA2	12.4 (15.5)	187	63.7 (98.7)	131	5.1	<0.0001	0.816	48.49	80.2	68.3
CA2	134.6 (398.6)	198	412.4 (1535.7)	131	3.1	<0.0001	0.738	37.14	76.3	60.8
CD44	177.0 (170.3)	199	422.9 (443.8)	131	2.4	<0.0001	0.726	34.12	76.3	57.8
CRNN	23.9 (62.1)	189	27.9 (44.2)	130	1.2	<0.0001	0.640	29.36	77.1	52.3
CST3	853.3 (697.8)	199	1052.9 (850.9)	131	1.2	0.0405	0.567	14.53	36.6	77.9
CSTA	46.5 (233.6)	185	36.5 (79.3)	128	−1.3	<0.0001	0.367	2.68	93.1	9.5
DSG3	469.6 (976)	187	706.5 (822.9)	129	1.5	<0.0001	0.697	37.31	90.1	47.2
FLNA	424.0 (724.7)	191	1211.9 (1533.9)	130	2.9	<0.0001	0.743	39.09	71.8	67.3
FSCN1	6.1 (8.8)	189	25.7 (34.4)	131	4.2	<0.0001	0.798	43.74	61.8	81.9
GANAB	137.5 (539)	199	212.5 (226.6)	131	1.5	<0.0001	0.703	33.67	60.3	73.4
GSTP1	4.8 (7.4)	170	7.4 (8.4)	127	1.5	<0.0001	0.659	29.20	84.0	45.2
HMGCS1	0.8 (1.3)	162	1.5 (1.6)	124	1.9	<0.0001	0.705	35.28	67.9	67.3
HSPA5	227.8 (317.7)	199	404.9 (436.7)	131	1.8	<0.0001	0.680	29.77	90.1	39.7
IGFBP3	0.7 (1.5)	177	3.3 (5.9)	126	4.5	<0.0001	0.751	41.06	68.7	72.4
ISG15	0.5 (1.8)	114	1.3 (2.8)	96	2.4	<0.0001	0.642	27.55	52.7	74.9
KNG1	107.3 (279.9)	197	586.3 (687)	131	5.5	<0.0001	0.870	59.35	84.0	75.4
LDHA	19.6 (35)	186	57.1 (85.9)	129	2.9	<0.0001	0.742	39.86	93.1	46.7
LGALS3BP	253.0 (375.5)	190	410.0 (534.4)	131	1.6	0.0005	0.614	21.75	71.0	50.8
MMP1	0.9 (1.8)	76	76.7 (182.4)	113	83.0	<0.0001	0.871	64.44	69.5	95.0
MMP3	3.6 (5.6)	170	15.9 (24.1)	125	4.4	<0.0001	0.763	39.48	62.6	76.9
MMP9	28.9 (53.9)	180	93.8 (138)	126	3.2	<0.0001	0.726	35.87	75.6	60.3
PRDX2	74.9 (241.2)	195	248.3 (1076.4)	131	3.3	<0.0001	0.710	36.59	92.4	44.2
S100A9	627.7 (1540.2)	197	2006.9 (3385)	131	3.2	<0.0001	0.754	38.81	69.5	69.3
SPARC	1.7 (2.2)	182	8.5 (16.4)	130	4.9	<0.0001	0.751	42.92	56.5	86.4
STAT1	2.5 (3.7)	158	8.9 (13.1)	122	3.6	<0.0001	0.722	35.52	67.2	68.3
TIMP1	162.0 (225.7)	198	360.9 (327.1)	128	2.2	<0.0001	0.726	33.38	77.1	56.3
TYMP	17.1 (30.7)	167	80.9 (111.9)	131	4.7	<0.0001	0.798	45.36	71.0	74.4
YWHAB	9.1 (14.4)	186	19.0 (25)	129	2.1	<0.0001	0.700	37.55	89.3	48.2

*Mean (SD), ng/mL.
[†]Detectable (concentration > 0) case number/total case number.
[‡]Fold change of protein levels in OSCC group to (healthy control + OPMD I) group, and the minus reciprocals indicate decreased protein levels in the OSCC group.
[§]By Mann-Whitney test.

red spot), our four marker-based scoring scheme exhibited high levels of sensitivity (93.4%) and specificity (80.5%) in the training set. For the test set, the sensitivity remained high (87.5%), and the specificity was the same as for the training set (80.5%).

Risk Scores in Stage I–IV OSCC Patients. The present study included 50 stage I, 29 stage II, 16 stage III, and 36 stage IV OSCC patients. We used our four-marker-based scoring scheme to calculate the risk scores for these patients. As shown in Fig. 4*A* (*Left*), the risk scores increased gradually from the early to advanced stages (stage I, 0.63 ± 0.29; stage II, 0.78 ± 0.23; stage III, 0.83 ± 0.23; stage IV, 0.85 ± 0.20). More importantly, we obtained a significantly higher risk score for stage I OSCC compared with the non-OSCC group (healthy controls + OPMD I; average score, 0.17 ± 0.24; $P < 0.0001$). Moreover, 84% (42/50), 97% (28/29), 94% (15/16), and 97% (35/36) of the stage I, II, III, and IV OSCC patients, respectively, had risk scores >0.4 (Fig. 4*A*, *Right*), indicating that our four protein panel-based scoring system has a good potential to detect a significant portion (>80%) of stage I OSCC patients.

Risk Scores in OPMD II Patients and Their Follow-Up Results. Given that OPMD II lesions can comprise a mixture of potentially malignant cells, malignant cells, and normal cells (11, 15–17), it can be difficult to distinguish OSCC from OPMD II. However, the average risk score of the OPMD II group (0.32 ± 0.33) was higher than that of the non-OSCC group (healthy controls + OPMD I; 0.17 ± 0.24), but significantly lower than that of OSCC group (0.75 ± 0.26) (Fig. 4*B*, *Left*). Notably, 42% (55/130) of the OPMD II cases had risk scores >0.4 (Fig. 4*B*, *Right*). This observation is consistent with the argument that OPMD II lesions may harbor malignant cells.

In addition to the need to detect OSCC, another important open issue is our lack of a means to predict or monitor malignant transformation in a large population of OPMDs, especially the high-risk OPMD II group. Among the 233 OPMD patients enrolled in this study, the malignant statuses of 153 cases (65 OPMD I and 88 OPMD II) were retrospectively retrieved from follow-up periods ranging from 13.5 to 76.6 mo. No malignant transformation was observed during follow-up in the OPMD I group. On the other hand, 18 cases in the OPMD II group showed malignant transformation to OSCC within 1.2–65.5 mo; these cases included 1 each of erythroleukoplakia, erythroplakia plus submucous fibrosis, submucous fibrosis, and speckle leukoplakia, 4 cases of verrucous hyperplasia, and 10 cases of verrucous hyperplasia plus submucous fibrosis. In this cohort, the malignant transformation rate of the OPMD II patients was about 20.5% (18/88), which was twofold higher than that among the OPMD patients (11.8%, 18/153) (18–20). The clinical characteristics and follow-up data of the 88 OPMD II cases are detailed in *SI Appendix*, Table S10. Among them, 37 showed risk

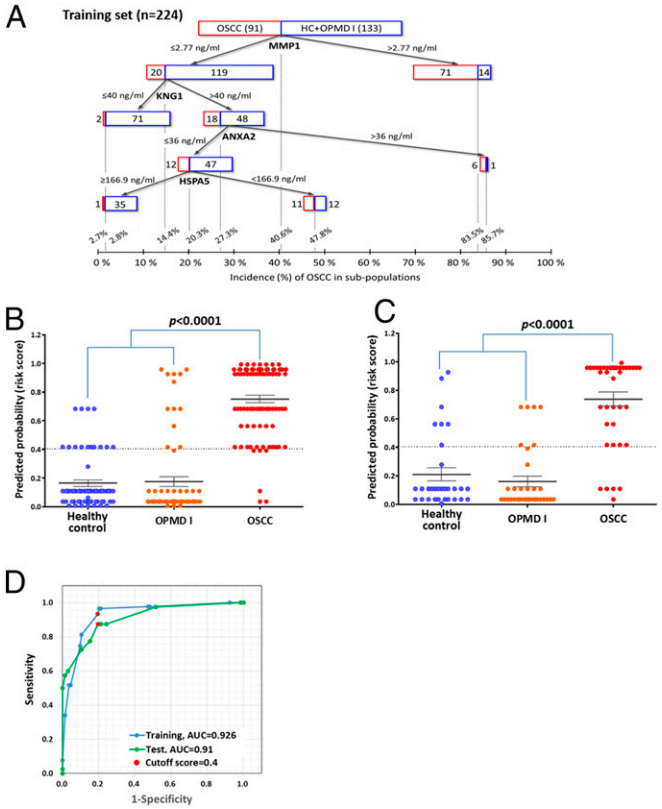


Fig. 3. The four-protein panel generated by the CART analysis and its scoring scheme. (*A*) A classification tree showing the selected four proteins and the cutoff value of concentration (ng/mL) at each split node. (*B* and *C*) A 2D dot plot showing the risk scores for individual subjects in the healthy control, OPMD I, and OSCC groups in the training set (n = 224) (*B*) and the test set (n = 106) (*C*). (*D*) ROC analysis shows the power of risk score for discriminating OSCC from non-OSCC (healthy control + OPMD I) in training set (blue) and test set (green). The cutoff score at 0.4 (red spot) showed the point with the highest Youden index in training set and then applied to test set.

scores >0.4, and of these cases, 37.8% (14/37) transformed to OSCC during follow-up. This transformation rate was much higher than that of the 51 OPMD II cases harboring risk scores <0.4 (7.8%; 4/51) (Fig. 4*C*). Of the 18 OSCC-transformed cases, 77.8% (14/18) had risk scores >0.4.

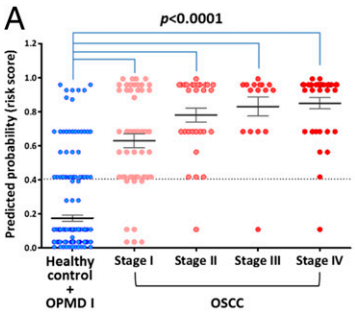
Discussion

The early detection of OSCC could save many lives, reduce the burden of morbidity resulting from surgical resection of late-stage disease, and dramatically reduce the economic burden of disease treatment. However, the current strategy for detecting OSCC, which includes visual inspection of the mouth followed by detection of cancer by biopsy, is ineffective. In a number of countries, it has yielded homogenously high specificity but varied sensitivity (most respondents were unsatisfied) for OSCC detection (21). Some patients are unable to fully open their mouths for inspection. In addition, the biopsy is usually limited to a single sample, which could miss the cancer site, especially in patients with multiple types of lesions. Identification of a biomarker signature with high sensitivity and specificity for detecting oral cancer from saliva could dramatically improve the early detection of OSCC.

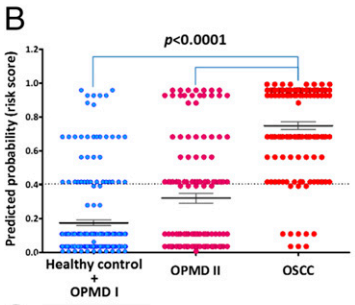
Although more than 1,000 published studies have searched for biomarkers for head and neck cancers, including OSCC, few reported biomarkers have moved into clinical practice. We believe that this failure reflects an insufficient effort to compare the reported candidate biomarkers against one another in adequate case and control samples. Here, we present a solution that overcomes this major barrier by (*i*) using intensive literature reviews to select candidate proteins that have been tested in multiple types of clinical samples by our group and others and (*ii*) comparing case (OSCC) and control (healthy control and OPMD I) samples from a high-risk population that shares similar risk factors (smoking and betel nut chewing).

Our multiple reaction monitoring-based biomarker assays offer reliable reproducibility in saliva samples (*SI Appendix*, Tables S5 and S6) similar to previous reports in plasma samples (10). The protein (peptide) levels in saliva were analyzed by LC-MRM-MS, which is an established technology for performing both qualitative and quantitative protein measurements. We were able to detect the 28 candidate protein markers at concentrations ranging from 1 to 2,000 ng/mL This detection limit is as good as the sensitivity of an antibody (such as that used in ELISA), and the use of LC-MRM-MS avoids the bias that could be introduced by off-target antibody effects.

As the samples in this study are all collected from a high-risk population, we chose a diagnostic adjunct with high sensitivity (selected by CART) rather than high specificity (selected by LR or discriminant). Smoking and betel nut chewing are two of the most important risk factors for the development of OSCC (3–6); thus, it is relevant that we did not observe any significant association between the levels of these four proteins and the risk



OSCC stage	Case No.	Risk score ≥ 0.4	
		Negative case No. (%)	Positive case No. (%)
I	50	8 (16%)	42 (84%)
II	29	1 (3%)	28 (97%)
III	16	1 (6%)	15 (94%)
IV	36	1 (3%)	35 (97%)
Total	131	11 (8.4%)	120 (91.6%)



Group	Case No.	Risk score ≥ 0.4	
		Negative case No. (%)	Positive case No. (%)
Healthy control + OPMD I	199	160 (80%)	39 (20%)
OPMD II	130	75 (58%)	55 (42%)

C

Subgroup of OPMD II	Case No.	No. of cases with follow-up data (%)	No. of cases with OSCC transformation (%)
Risk score ≥ 0.4	55	37 (67.3%, 37/55)	14 (37.8%, 14/37)
Risk score < 0.4	75	51 (68.0%, 51/75)	4 (7.8%, 4/51)

Fig. 4. The four protein panel-based risk scores obtained for OSCC patients of different stages and for OPMD II subjects. (*A*) (*Left*) A 2D dot plot analysis of the four protein panel-based risk scores of OSCC patients in stages I–IV (n = 50, 29, 16, and 36, respectively) compared with the non-OSCC group (healthy control + OPMD I; n = 199). (*Right*) The percentage of subjects with risk scores >0.4 in OSCC patients of stages I–IV. (*B*) (*Left*) A 2D dot plot analysis of the four protein panel-based risk scores of the OPMD II group (n = 130) compared with those of the non-OSCC group (healthy controls + OPMD I; n = 199) and the OSCC group (n = 131). (*Right*) The percentage of subjects with risk scores >0.4 in the non-OSCC (healthy controls + OPMD I) and OPMD II groups. (*C*) The OSCC transformation rate during follow-up in OPMD II subjects with four protein panel-based risk scores of >0.4 or <0.4.

habits of the 460 subjects (*SI Appendix*, Table S11). KNG1, ANXA2, and HSPA5 but MMP1 in the CART-selected four-protein panel were selected by LR and discriminant analysis. This discrepancy was likely resulted from the selection principle of these methods (22). CART is a nonparametric and nonlinear method to repeatedly partition sample into subgroups, not only consider the overall sample of patients, but also subsequent subgroups. On the other hand, LR and discriminant analysis aim to find a linear combination of covariates and prefer to select marker showing high sensitivity, and only select one from highly correlated markers. In this context, MMP1 was highly correlated with KNG1 (*SI Appendix*, Table S11), which has high sensitivity.

Screening is useful to detect OSCC at their early stage or to determine the status of potential malignant disorders; both are critical for reducing mortality in high-risk populations. The four-protein panel could be used as a diagnostic adjunct to eliminate diagnosis delay due to patient delay by patients themselves or professional delay of diagnosis by the primary physician (23). The cutoff values of scores at 0.4 and 0.6, which showed high sensitivity (91.6%) and high specificity (90%), respectively, to discriminate OSCC from non-OSCC (*SI Appendix*, Table S12), might be used for OSCC detection in high-risk population. We propose that (*i*) subjects with high-risk score (≥0.6) will need to undergo rebiopsy or to comprehensively detect occult tumor; (*ii*) subjects with medium risk score (≥0.4 and <0.6) will be followed up twice per year; (*iii*) subjects with low risk score (<0.4) can be managed following the current follow-up protocol (once per 2 y); (*iv*) subjects with low risk score (<0.4) and also with normal mucosa might be a meaningful indicator for more regressive management, such as extending the interval of follow-up check.

Our present study offers a practical foundation for clinical trials examining the ability of this four-marker panel to (*i*) detect OSCC in high-risk populations, such as those enrolled in Taiwan's Oral Cancer Screening Program; (*ii*) assess the risk for the presence of malignant cells in clinically suspicious lesions; (*iii*) select OPMD II patients for close follow-up; and (*iv*) monitor treatment response or disease recurrence.

Although our present results are promising, our strategy could be improved in several ways. For example, the specificity of our four-protein panel (about 80%) could be improved by combining it with other types of salivary cancer cell markers, such as tumor-specific microRNAs (24) and/or DNA mutations (25). In addition, the validity of our saliva-based test should be tested with larger numbers of samples collected prospectively from multiple hospitals; the sample used in this study was not large and was from a single institution. With the support of the Ministry of Health and Welfare in Taiwan, we are currently planning a clinical trial in which larger numbers of OSCC and OPMD II patients will be collected from the high-risk populations of two additional hospitals. Finally, the potential impact of our assay on public health will need to be tested in a cohort study to see if biomarker screening can remove the late-stage cancer cases from the population and facilitate the timely application of clinical interventions for those with newly appearing early-stage disease.

Materials and Methods

Detailed descriptions of the following methods may be found in *SI Material and Methods*: literature review and prioritization of candidate biomarkers; study subjects and saliva samples; selection of surrogate peptides for target proteins; preparation of salivary proteins and LC-MRM-MS analysis; and statistical analyses. The saliva samples were enrolled in the Taiwan's Oral Cancer Screening Program at Chi-Mei Medical Center (Liouying, Taiwan) from 2008 to 2013 (Table 1). Each subject signed an informed consent form approved by the Institutional Review Board of Chi-Mei Medical Center, permitting the use of saliva samples for this study.

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