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Evaluation of the mRNA expression levels of integrins α3, α5, β1 and β6 as tumor biomarkers of oral squamous cell carcinoma

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Abstract. Integrin signaling may modulate several different functions involved in cell migration, invasion, proliferation and motility, and is a potential candidate biomarker for oral cancer. In the present study, a total of four integrin genes were evaluated as potential biomarkers of oral squamous cell carcinoma (OSCC). Gene expression was determined using the reverse transcription-quantitative polymerase chain reaction in 55 OSCC and 55 matched normal oral tissues. The performance of individual and combined biomarkers was analyzed by receiver operating characteristic (ROC) analysis based on the relative mRNA expression (OSCC vs. matched oral tissue from the tumor-free margin), which was calculated using the ΔΔCq value (ΔCq of OSCC-ΔCq of oral tissue from the tumor-free margin of the same patient). In the individual ROC analysis, the areas under the ROC curve (AUCs) of relative mRNA expression (ΔΔCq) of integrin subunit α3 (*ITGA3*), integrin subunit α5 (*ITGA5*), integrin subunit β1 (*ITGB1*) and integrin subunit β6 (*ITGB6*) in all tumor locations were 0.724, 0.698, 0.640 and 0.657, respectively. For locations 2 (tongue/mouth part) and 3 (edentulous ridge), their individual AUC values were 0.840, 0.765, 0.725 and 0.763, respectively. In the cumulative ROC analysis, *ITGA3*, *ITGA5* and *ITGB1* genes exhibited the highest combined AUC values (0.809 and 0.871 for all locations and locations 2 and 3 combined, respectively) compared with other biomarker combinations. In conclusion, the results of the present study identified that higher mRNA expressions of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes are suitable for OSCC diagnosis biomarkers. Cumulative ROC analysis indicated an improved overall performance compared with the best individual integrin biomarker of OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) represents the sixth most common type of cancer worldwide (1). Several biomarkers for OSCC have been demonstrated previously (2-7) and may be useful for the diagnosis and prognosis of oral cancer. As carcinogenesis is a multistep process, a number of genes involved in the diagnosis of oral cancer have not been identified.

Integrins are a family of transmembrane-type receptor proteins on the cell surface, composed of heterodimeric complexes of 1α chain and 1β chain. The 18α and 8β subunits comprise ~24 different integrin receptors, each of which is capable of binding to a specific type of cell surface and extracellular matrix (ECM) protein ligand (8). They function in specific signal transduction and adhesive interactions between cells, and between cells and the ECM. Integrin signaling may

modulate several different functions involved in cell motility, invasion, proliferation and migration (9,10). Furthermore, integrin signaling may also affect vascular neogenesis (11). Therefore, integrins serve an important role in tumor growth and metastasis (12-14).

Integrin subunit α3 (ITGA3), which combines with integrin subunit β1 (ITGB1) to form integrin α3β1, is the receptor for ECM molecules including fibronectin, laminin and collagen (8,12). Integrin α3β1 has been demonstrated to function in cell proliferation, migration and motility, and in the maintenance of basement membrane integrity (15-18). Several previous studies identified that integrin α3β1 was overexpressed and associated with tumor invasion and metastasis in the majority of types of cancer, including lung (19) and breast (20) cancer cell lines. Integrin subunit α5 (ITGA5) often combines with ITGB1 to form integrin α5β1, and serves as a receptor for fibronectin and fibrinogen to participate in cell differentiation, cell development and wound healing (8,12). It was identified that the emergence of integrin α5β1 expression is associated with tumor progression in lung cancer (21). ITGA5 may also promote tumor metastasis in oral cancer cell lines (22). Integrin subunit β6 (ITGB6) is the β subunit of integrin αvβ6, which is a receptor for fibronectin and cytotactin, and regulates cell invasion, inhibits apoptosis, modulates matrix metalloproteases and activates transforming growth factor β1 (8,23). Overexpression of integrin αvβ6 promotes epithelial-to-mesenchymal transition and is associated with cell invasion in oral cancer cell lines (24,25). However, the majority of these previous studies were focused on OSCC cell line models or non-quantitative immunohistochemical analyses of OSCC tissues. These studies did not use receiver-operating characteristic (ROC) curve analyses, which provide sensitivity and specificity data for the evaluation of OSCC biomarker performances.

In the present study, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was used to determine the mRNA expression levels of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes in OSCC tissues from different tumor locations for ROC curve analyses, in order to identify suitable biomarker genes for the diagnosis of early-stage OSCC. The mRNA expression of *ITGA3*, *ITGA5*, *ITGB1*, and *ITGB6* genes were identified as potential OSCC biomarkers. Furthermore, cumulative ROC analysis of integrin OSCC biomarkers had an improved diagnostic performance compared with individual ROC analysis.

Materials and methods

Tissue samples. The present study was approved by the Institutional Review Board at Kaohsiung Medical University (Kaohsiung, Taiwan) (approval no. KMUH-IRB-930104), and all patients provided written informed consent. A total of 55 oral tumors and 55 matched normal oral control tissues (at least 2.5 cm between tumor and control tissues) were collected (December 2004 to December 2009) from the Department of Oral and Maxillofacial Surgery, Kaohsiung Medical University Hospital. The age range of this patient cohort is 30-90 years and the median age is 50 years. All samples were blindly examined by at least three pathologists of the Department of pathology, Kaohsiung Medical University Hospital. All control tissues underwent pathological diagnosis for confirmation as non-tumor. All oral tumors underwent pathological diagnosis for OSCC and tumor stage classification used the Tumor-Node-Metastasis (TNM) system (26). The characteristics of the patients with OSCC are summarized in Table I, this basic patient information has been described previously (6).

RT-qPCR. Tissues were sliced and placed into 1.5 ml microcentrifuge tubes containing TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for homogenization using a disposable tissue grinder pestle. Subsequently, the homogenized mixtures were used for total RNA extraction according to the manufacturer's protocol. RT was performed using an OmniScript RT kit (Qiagen GmbH, Hilden, Germany). qPCR was performed using an iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in an iCycler MyiQ real-time machine (Bio-Rad Laboratories, Inc.). The relative mRNA expressions of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes in OSCCs and controls were examined. The forward and reverse primer sequences, and the lengths of PCR products, for these genes are provided in Table II. The thermocycling conditions are as follows: 94˚C for 1 min; 4 cycles of 94˚C for 15 sec, 64˚C for 15 sec and 70˚C for 15 sec; 4 cycles of 94˚C for 15 sec, 61˚C for 15 sec and 70˚C for 15 sec; 4 cycles of 94˚C for 15 sec, 58˚C for 15 sec and 70˚C for 15 sec; 60 cycles of 94˚C for 15 sec, 55˚C for 15 sec and 70˚C for 15 sec; and finally 94˚C for 1 min and 60˚C for 5 min. The PCR assay was performed in duplicate. The relative mRNA expression levels (OSCC/oral tissue from the tumor-free margin ratio) for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were evaluated using the $2^{-\Delta\Delta Cq}$ method (27). The threshold cycle (Cq) value of each integrin gene was subtracted from the Cq value for the *GAPDH* reference housekeeping gene. Melting curve analyses by qPCR analysis and gel electrophoresis by 1.5% agarose containing 0.5 μg/ml ethidium bromide for visualization under a UV box were used to validate the PCR products as described previously (28).

Statistical analysis. Data were analyzed by the Mann-Whitney test and Kruskal-Wallis test using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). ROC curve analyses were commonly used to evaluate the performance of cancer diagnosis (29-34). Accordingly, ROC curves were used to examine the performance of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes as predictive biomarkers for detecting OSCC. Cut-off values for individual ROC curves were calculated using the $60^{-\Delta Cq}$ value, where 60 represents the total PCR cycle number, and no signal was assigned to the Cq value for 60, as described previously (5,6). For individual ROC analysis, the area under the ROC curve (AUC) of each biomarker was used to evaluate its performance as an OSCC biomarker.

To consider the combined effect of each biomarker, the cumulative ROC analysis of biomarkers was performed for OSCC prediction. At first, the critical (cut-off) point of the ROC curve for each biomarker was identified using JMP version 10 statistic software (SAS Institute Inc., Cary, NC, USA). Subsequently, biomarkers that were more relevant to the OSCC (those with values greater than the cut-off point in AUC of individual ROC results) were assigned a score of 1 (for example, values greater than the cut-off point from the AUC

analysis of the individual ROC results), and the remaining biomarkers were assigned a score of 0. The combined scores for different combinations of biomarkers were calculated using the formula function in JMP 10. Finally, the combined scores were used for cumulative ROC analysis of biomarkers for comparison.

Results

Comparison of clinicopathological features and relative mRNA expressions of ITGA3, ITGA5, ITGB1 and ITGB6 genes in patients with OSCC. To evaluate the performance of the OSCC biomarkers, mRNA expression levels of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes of OSCC samples were evaluated by comparing tumor samples with control samples (oral tissues from the tumor-free margin). In different locations (Table III), the differences in *ITGA3* and *ITGB6* gene expression levels were significant (P=0.05 and 0.005, respectively by Kruskal-Wallis test); however, the differences in the *ITGA5* and *ITGB1* gene expression levels were non-significant. In contrast, the differences in mRNA expression levels of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were non-significant at different stages (I-IV) (P=0.19, 0.58, 0.92, and 0.53, respectively by Kruskal-Wallis test) and for the T parameter (1-4) of the TNM staging system (a measure of tumor diameter/dimension) among patients with OSCC (P=0.36, 0.97, 0.75, and 0.83, respectively by Kruskal-Wallis test).

Comparison of patient habits and relative mRNA expression of ITGA3, ITGA5, ITGB1, and ITGB6 genes in patients with OSCC. As indicated in Table IV, the differences in mRNA expression levels of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were not associated with cancer-causing habits, including alcohol consumption (P=0.83, 0.11, 0.68, and 0.18, respectively), betel nut chewing (P=0.65, 0.55, 0.81, and 0.75, respectively) and cigarette smoking (P=0.84, 0.45, 0.86, and 0.40, respectively by Mann-Whitney test).

AUC performances of individual OSCC biomarkers of ITGA3, ITGA5, ITGB1, and ITGB6 genes. To test the predictive performance of each of the test biomarkers in OSCC diagnosis, individual ROC curves for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* mRNA expression were constructed by comparing the mRNA expression level between OSCC tumor tissues and their controls (oral tissue from the tumor-free margin). The individual AUCs of the relative mRNA expression for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were 0.724 [95% confidence interval (CI), 0.630-0.819], 0.698 (95% CI, 0.600-0.796), 0.640 (95% CI, 0.536-0.743) and 0.657 (95% CI, 0.555-0.759), respectively (Fig. 1A). At a specificity of 50.91% for all locations (Table V, part A), sensitivities of relative mRNA expression for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* were 90.91, 83.64, 70.91 and 74.55%, respectively. At a specificity of 61.82%, sensitivities of relative mRNA expression for *ITGA3* and *ITGA5* genes were decreased slightly to 85.45 and 78.18%, respectively. However, *ITGB1* and *ITGB6* genes were decreased to 50.91 and 61.82%, respectively. At a specificity of 67.27%, sensitivities of relative mRNA expression for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were markedly decreased, particularly for the *ITGB1* gene (45.45%). Therefore, analysis of different sensitivities suggested that the *ITGA3* and *ITGA5* genes exhibited an improved specificity performance compared with *ITGB1* and *ITGB6* genes for all locations.

As the differences in *ITGA3* and *ITGB6* gene expression by location were significant (Table III), whether the different locations of OSCC may affect the mRNA expression levels of these four integrin genes was additionally investigated. In the example of locations 2 and 3 combined (locations 2/3), i.e., the tongue/mouth floor and edentulous ridge (Fig. 1B), it was demonstrated that the AUC values of relative mRNA expression for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes in the tongue/mouth floor and edentulous ridge were 0.840 (95% CI, 0.728-0.952), 0.765 (95% CI, 0.632-0.898), 0.725 (95% CI, 0.584-0.866) and 0.762 (95% CI, 0.630-0.896), respectively. Notably, the AUC values of

Table I. Basic characteristics of patients and tissue samples.

	(OSCC/matched control oral tissue)	
Characteristics	n (total=55)	(%)
Age, years		
≤40	8	14.55
41-50	21	38.18
51-60	21	38.18
≥61	5	9.09
Sex		
Male	50	90.91
Female	5	9.09
TNM stage		
I	10	18.18
II	9	16.36
III	16	29.09
IV	20	36.37
Lesion location		
Buccal mucosa/retromolar area	23	41.82
Tongue/mouth floor	17	30.91
Edentulous ridge	8	14.55
Others	7	12.72
Carcinogenic factors		
Alcohol		
(+)	46	83.64
(-)	9	16.36
Betel quid		
(+)	50	90.91
(-)	5	9.09
Cigarette smoking		
(+)	48	87.27
(-)	7	12.73

This basic patient information has been described previously (6). TNM, Tumor-Node-Metastasis; Others, lower lip/vestibule/soft palate; OSCC, oral squamous cell carcinoma; +, yes; -, no.

Table II. Forward and reverse primer sequences and the lengths of PCR products for *ITGA3*, *ITGA5*, *ITGB1*, *ITGB6*, and *GAPDH* genes.

Gene names	Primer sequences	Length of PCR products, bp
ITGA3	Forward: 5'-TGCTGTGGAAGTGCGGCT-3' Reverse: 5'- GCGTGGTACTTGGGCATGAT-3'	206
ITGA5	Forward: 5'-TCATCTACATCCTCTACAAGCTTGG-3' Reverse: 5'-GCCGTCAGCACCTTCAAGA-3'	204
ITGB1	Forward: 5'-CGTATTCAGTGAATGGGAACAAC-3' Reverse: 5'-GATTTTCACCCGTGTCCCAT-3'	231
ITGB6	Forward: 5'-ACATGAAAGTGGGAGACACAGC-3' Reverse: 5'-ACACACCCCACACTGGAAAGA-3'	215
GAPDH	Forward: 5'-GCATCCTGGGCTACACTGA-3' Reverse: 5'-CCACCACCCTGTTGCTGTA-3'	162

ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6.

Table III. Comparison of clinicopathological features and relative mRNA expression levels of *ITGA3, ITGA5, ITGB1* and *ITGB6* genes in oral squamous cell carcinoma tumor samples with control samples (oral tissue from the tumor-free margin).

Clinical data	n (total=55)	*ITGA3*		*ITGA5*		*ITGB1*		*ITGB6*	
		Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
Location[b]			0.01		0.33		0.16		0.05
1	23	1.95±5.37		1.29±4.87		2.58±11.30		0.71±6.19	
2	17	4.32±4.74		4.04±4.99		2.41±3.23		0.91±11.08	
3	8	6.25±5.20		1.82±8.40		2.94±3.03		5.07±5.30	
4	7	-6.87±14.91		-1.74±12.27		2.15±3.58		2.66±5.95	
TNM			0.19		0.58		0.92		0.53
T1	14	1.52±12.93		2.88±5.64		1.20±4.29		-2.12±13.83	
T2	21	1.04±4.84		0.65±7.58		1.29±3.66		1.67±4.95	
T3 and T4	20	3.86±5.48		2.34±6.82		4.74±11.37		2.66±3.69	
Stage			0.36		0.97		0.75		0.83
I	10	8.26±15.43		2.53±5.56		0.87±5.03		0.16±9.95	
II	9	0.30±5.13		2.10±4.09		0.83±4.47		0.83±5.86	
III and IV	36	3.03±4.99		1.57±7.07		3.41±8.70		1.37±8.02	

Data are presented as mean ΔΔCq values (ΔCq of OSCC-ΔCq of its matched oral tissue from the tumor-free margin). TNM, Tumor-Node-Metastasis; T in TMN, tumor size and invasiveness in TMN classification. [a]Kruskal-Wallis test. [b]Location 1, buccal mucosa/retromolar area; 2, tongue/mouth floor; 3, edentulous ridge; 4, others (lower lip/vestibule/soft palate). SD, standard deviation; ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6.

locations 2/3 combined (Fig. 1B) for these four integrin genes were increased compared with those of all locations together (Fig. 1A). Analysis of different sensitivities suggested that *ITGA3* and *ITGA5* genes exhibited an improved specificity performance compared with that of *ITGB1* and *ITGB6* genes for locations 2/3 (Table V, part B).

AUC performances of cumulative ROC analyses for different combinations of ITGA3, ITGA5, ITGB1, and ITGB6 biomarkers. To evaluate the diagnostic power of different combinations of these biomarkers, their cumulative ROC curves between OSCC and controls (oral tissue from the tumor-free margin) were calculated. The AUC values for different combinations (two, three and four) of the biomarkers are summarized in Table VI. The combination of *ITGA3*, *ITGA5* and *ITGB1* genes demonstrated the highest AUC values of 0.809 (CI, 0.728-0.890) and 0.871 (CI, 0.770-0.972), for the two types of locations (all locations and locations 2/3, respectively). The combination of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes provided similar AUC values for the two locations.

Table IV. Comparison of patient habits and relative mRNA expression levels of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes in oral squamous cell carcinoma samples with control samples (oral tissue from the tumor-free margin).

		ITGA3		*ITGA5*		*ITGB1*		*ITGB6*	
Habits	n (total=55)	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
Alcohol			0.83		0.11		0.68		0.18
No	9	3.02±3.60		4.66±3.38		1.91±2.26		4.53±5.73	
Yes	46	2.02±8.42		1.27±7.18		2.65±8.27		0.39±8.22	
Betel quid chewing			0.65		0.33		0.81		0.75
No	5	3.59±5.84		4.25±5.21		0.73±3.33		3.74±7.03	
Yes	50	2.05±8.02		1.59±6.93		2.70±7.90		0.80±8.08	
Cigarette smoking			0.84		0.45		0.86		0.40
No	5	3.51±4.90		3.53±6.61		1.13±2.31		4.56±6.98	
Yes	50	2.05±8.07		1.66±6.86		2.66±7.94		0.71±8.05	

Data are presented as mean ΔΔCq values (ΔCq of OSCC-ΔCq of its matched oral tissue from the tumor-free margin). [a]Mann-Whitney U test. SD, standard deviation; ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6.

Table V. Different cut-offs and their relative sensitivity and specificity for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes in patients with oral squamous cell carcinoma.

A, All locations

	ITGA3		*ITGA5*		*ITGB1*		*ITGB6*	
Sensitivity, %	Specificity, %	Cut-off	Specificity, %	Cut-off	Specificity, %	Cut-off	Specificity, %	Cut-off
50.91	90.91	63.20	83.64	56.26	70.91	61.93	74.55	57.90
61.82	85.45	62.87	78.18	55.00	50.91	61.17	61.82	57.21
67.27	67.27	61.94	63.64	54.35	45.45	60.96	58.18	57.06

B, Locations 2/3

	ITGA3		*ITGA5*		*ITGB1*		*ITGB6*	
Sensitivity, %	Specificity, %	Cut-off	Specificity, %	Cut-off	Specificity, %	Cut-off	Specificity, %	Cut-off
64.00	92.00	62.97	84.00	55.00	64.00	61.07	84.00	57.90
68.00	88.00	62.67	84.00	55.00	64.00	60.99	80.00	57.65
76.00	76.00	61.73	76.00	53.94	60.00	60.78	60.00	56.61

All locations including the locations 1, 2, 3 and 4. Location 1, buccal mucosa/retromolar area; location 2, tongue/mouth floor; location 3, edentulous ridge; location 4, others (lower lip/vestibule/soft palate); ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6.

Discussion

The overexpression of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes has been demonstrated in several types of cancer (19-25); however, the suitability for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes as OSCC biomarkers has rarely been considered.

The results of the present study identified that *ITGA3* and *ITGB1* mRNA were overexpressed in patients with OSCC. Similarly, it was demonstrated previously that ITGA3 and ITGB1 proteins were overexpressed in prostate tumor tissues, and knockdown of *ITGA3* and *ITGB1* genes by small interfering RNAs inhibited cell migration and invasion in prostate cancer cells (35). In the present study, *ITGA5* mRNA was overexpressed in patients with OSCC. Similarly, *ITGA5* and *ITGB1* genes have been suggested to be potential biomarkers for non-small cell lung cancer (36). In addition, the *ITGB6* gene may be a prognostic biomarker for invasive breast

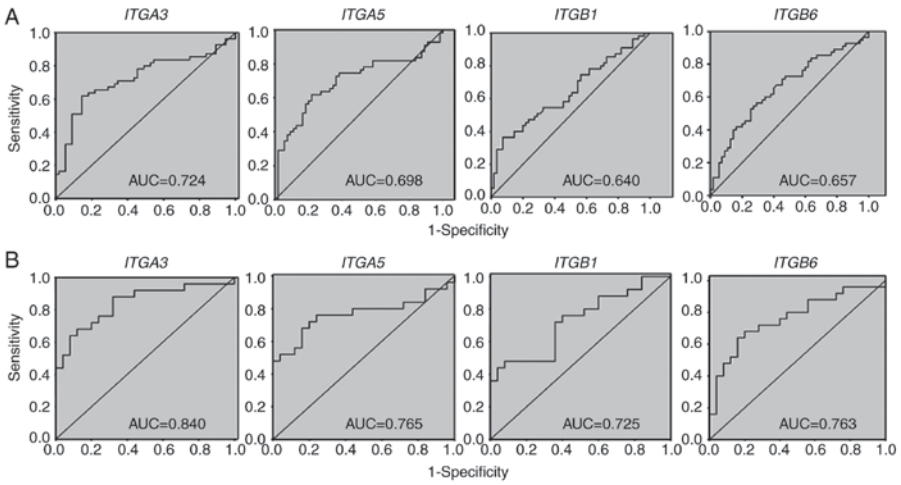

Figure 1. AUC values of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes as individual oral squamous cell carcinoma biomarkers. (A) All tumor locations (locations 1-4). (B) Only tumor locations 2/3. Location 1, buccal mucosa/retromolar area; location 2, tongue/mouth floor; location 3, edentulous ridge; location 4, others (lower lip/vestibule/soft palate); ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6; AUC, area under the curve.

cancer (37). However, to the best of our knowledge, these integrin biomarkers have rarely been investigated in OSCC.

In the present study, individual ROC analyses for all locations identified *ITGA3* and *ITGA5* genes as good biomarkers for OSCC (AUC=0.724 and 0.698, respectively), but *ITGB1* and *ITGB6* genes performed poorly for OSCC prediction (AUC <0.66). Compared with all locations, improved AUC values for *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes were observed in combined locations 2 and 3 (the tongue/mouth part and edentulous ridge).

Previous studies have indicated that a combination of several variants generally provides an improved diagnostic power for ROC analysis compared with ROC analysis of individual markers (38-41). A combination of several biomarkers may therefore improve the diagnostic results for tumors, as has been demonstrated in thyroid cancer (42). Similarly, with the exception of the combination of *ITGB1* and *ITGB6* (AUC=0.686), the present study identified that the majority of the biomarker combinations, including a combination of two (*ITGA3*/*ITGA5*, *ITGA3*/*ITGB1*, *ITGA3*/*ITGB6*, *ITGA5*/*ITGB1* and *ITGA5*/*ITGB6*), three (*ITGA3*/*ITGA5*/*ITGB1*, *ITGA3*/*ITGA5*/*ITGB6* and *ITGA5*/*IGB1*/*ITGB6*) and four (*ITGA3*/*ITGA5*/*ITGB1*/*ITGB6*) genes, exhibited improved AUC values for all locations (ranging between 0.750 and 0.809) compared with the individual AUC values of individual integrin genes (ranging between 0.640 and 0.724). With the exception of the combinations of *ITGA3*/*ITGB1*, *ITGA5*/*ITGB1*, *ITGA5*/*ITGB6*, *ITGB1*/*ITGB6* and *ITGA5*/*ITGB1*/*ITGB6* genes (AUC values ranging between 0.788 and 0.829), it was identified that other combined biomarkers, including two, three and four genes, demonstrated improved AUC values for locations 2 and 3 (ranging between 0.847 and 0.871) compared with that of the individual AUC values of these integrin genes (ranging between 0.725 and 0.840). Among them, *ITGA3*, *ITGA5* and *ITGB1* genes exhibited the highest AUC values for all locations and locations 2 and 3 combined. Therefore, the cumulative ROC analysis, as a method of sensitive and specific evaluation, suggested a combination of multiple biomarkers for the diagnosis of OSCC.

mRNA has been repeatedly demonstrated to be a reliable material for the diagnosis of oral cancer (5,6,43,44). For example, tissue and salivary mRNA biomarkers are suggested to be associated with clinicopathological parameters for the diagnosis of OSCC (44). In clinical practice, the data from the present study of combined integrin biomarkers may be applied for non-invasive diagnosis of OSCC using saliva material for OSCC in the future.

An advantage of the proposed methods of the present study is a potential improvement of the AUC performance by using accumulative ROC analysis for the mRNA expression of a combination of integrin biomarkers. A disadvantage of this method is that it is based on tissue samples for mRNA evaluation, which are susceptible to degradation by RNase contamination (45). Additionally, expression at mRNA transcriptome levels will not always be consistent with those at protein levels in cases of posttranslational modification (46). Additionally, the present study did not provide information concerning depth of invasion, which is important for clinicians. Additional protein evaluation for ITGA3, ITGA5, ITGB1 and ITGB6 using immunohistochemistry is warranted. In addition, 5/55 the patients included in the present study were female, which may provide a gender bias for these biomarker predictions for oral cancer. Therefore, it is suggested that a larger cohort of female and male patients should be studied in the future to provide an unbiased prediction using these integrins. In the present study, the association between integrin expression and prognosis, was not analyzed which would have been beneficial to improve the reliability of the proposed integrin mRNA biomarker combination for oral cancer prediction.

Alcohol drinking, betel quid chewing and cigarette smoking are well-known risk factors for oral cancer (47), although non-smoking and betel quid non-chewing individuals may also suffer from oral cancer (48). It is possible that these habits may affect the initiation and progression of oral carcinogenesis, but do not directly affect the overexpression of certain genes in patients with OSCC. For example, no significant differences in the expression levels

Table VI. Performances of cumulative ROC analyses for different combinations of *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* biomarkers in patients with OSCC.

	AUC of combined biomarkers for OSCC[a]							
Locations	*ITGA3/ ITGA5*	*ITGA3/ ITGB1*	*ITGA3/ ITGB6*	*ITGA5/ ITGB1*	*ITGA5/ ITGB6*	*ITGB1/ ITGB6*	*ITGA3/ITGA5/ ITGB1*	*ITGA3/ITGA5/ ITGB6*	*ITGA5/ITGB1/ ITGB6*	*ITGA3/ITGA5/ ITGB1/ITGB6*
All[b]	0.777	0.775	0.758	0.762	0.720	0.686	0.809c	0.780	0.750	0.797
2/3	0.847	0.829	0.850	0.823	0.792	0.788	0.871c	0.855	0.825	0.870

[a]Biomarkers that were more relevant to the OSCC (those with values greater than the cut-off point in AUC of individual ROC results, as demonstrated in Fig. 1), were assigned a score of 1, and the others were assigned a score of 0. The combined scores for different combinations of biomarkers were used for cumulative ROC analysis. [b]All locations including the locations 1, 2, 3, and 4. [c]The best AUC performance among different combinations of biomarkers for OSCC. Location 1, buccal mucosa/retromolar area; location 2, tongue/mouth floor; location 3, edentulous ridge; location 4, others (lower lip/vestibule/soft palate); AUC, area under the curve; ROC, receiver operating characteristics; ITGA3, integrin subunit α3; ITGA5, integrin subunit α5; ITGB1, integrin subunit β1; ITGB6, integrin subunit β6; OSCC, oral squamous cell carcinoma.

of the *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* biomarkers were observed between the OSCC and control tissues for each of these variables.

In conclusion, individual ROC analysis provides a sensitive and specific evaluation of biomarkers for OSCC diagnosis and prognosis. The results of the present study demonstrated that *ITGA3* and *ITGA5* genes were improved prognostic OSCC biomarkers for all locations compared with *ITGB1* and *ITGB6* genes, based on individual ROC analysis. However, *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes became more promising OSCC biomarkers when considering specific tumor locations (2 and 3; tongue/mouth part and edentulous ridge, respectively). Additionally, the cumulative ROC analyses indicated that the combination of *ITGA3*, *ITGA5* and *ITGB1* genes exhibited the highest AUC values for locations 2/3 and all locations. Therefore, *ITGA3*, *ITGA5*, *ITGB1* and *ITGB6* genes are suggested to be good OSCC biomarkers, and cumulative ROC analysis is hypothesized to provide an improved strategy for cancer biomarker identification.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

HWC and CYY contributed significantly in writing the manuscript and data discussion/interpretation. CHC, JHT, and YHK collected the samples and analyzed pathological diagnosis of OSCC. HWC instructed JHT and YTC to extract RNA for qPCR analysis. JYT, YYW, and SSY assisted in clinical data collection and performed statistical analysis. SYL was responsible for study conception and overview. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board at Kaohsiung Medical University (Kaohsiung, Taiwan)

(KMUH-IRB-930104), and patients provided written informed consent.

Patient consent for publication

Patient provided written informed consent for the publication of associated data.

Competing interests

The authors declare that they have no competing interests.

References

1. Shah JP and Gil Z: Current concepts in management of oral cancer-surgery. Oral Oncol 45: 394-401, 2009.
2. Dumache R, Rogobete AF, Andreescu N and Puiu M: Genetic and epigenetic biomarkers of molecular alterations in oral carcinogenesis. Clin Lab 61: 1373-1381, 2015.
3. Nagata M, Noman AA, Suzuki K, Kurita H, Ohnishi M, Ohyama T, Kitamura N, Kobayashi T, Uematsu K, Takahashi K, *et al*: ITGA3 and ITGB4 expression biomarkers estimate the risks of locoregional and hematogenous dissemination of oral squamous cell carcinoma. BMC Cancer 13: 410, 2013.
4. Elashoff D, Zhou H, Reiss J, Wang J, Xiao H, Henson B, Hu S, Arellano M, Sinha U, Le A, *et al*: Prevalidation of salivary biomarkers for oral cancer detection. Cancer Epidemiol Biomarkers Prev 21: 664-672, 2012.
5. Yen CY, Chen CH, Chang CH, Tseng HF, Liu SY, Chuang LY, Wen CH and Chang HW: Matrix metalloproteinases (MMP) 1 and MMP10 but not MMP12 are potential oral cancer markers. Biomarkers 14: 244-249, 2009.
6. Yen CY, Huang CY, Hou MF, Yang YH, Chang CH, Huang HW, Chen CH and Chang HW: Evaluating the performance of fibronectin 1 (FN1), integrin α4β1 (ITGA4), syndecan-2 (SDC2) and glycoprotein CD44 as the potential biomarkers of oral squamous cell carcinoma (OSCC). Biomarkers 18: 63-72, 2013.
7. Wang S, Sun M, Gu C, Wang X, Chen D, Zhao E, Jiao X and Zheng J: Expression of CD163, interleukin-10, and interferon-gamma in oral squamous cell carcinoma: Mutual relationships and prognostic implications. Eur J Oral Sci 122: 202-209, 2014.
8. Barczyk M, Carracedo S and Gullberg D: Integrins. Cell Tissue Res 339: 269-280, 2010.
9. Hood JD and Cheresh DA: Role of integrins in cell invasion and migration. Nat Rev Cancer 2: 91-100, 2002.
10. Desgrosellier JS and Cheresh DA: Integrins in cancer: Biological implications and therapeutic opportunities. Nat Rev Cancer 10: 9-22, 2010.
11. Malinin NL, Pluskota E and Byzova TV: Integrin signaling in vascular function. Curr Opin Hematol 19: 206-211, 2012.
12. Mizejewski GJ: Role of integrins in cancer: Survey of expression patterns. Proc Soc Exp Biol Med 222: 124-138, 1999.
13. Hwang R and Varner J: The role of integrins in tumor angiogenesis. Hematol Oncol Clin North Am 18: 991-1006, vii, 2004.
14. Hynes RO: Integrins: Versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25, 1992.
15. Yamaguchi M, Ebihara N, Shima N, Kimoto M, Funaki T, Yokoo S, Murakami A and Yamagami S: Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. Invest Ophthalmol Vis Sci 52: 679-684, 2011.
16. DiPersio CM, Hodivala-Dilke KM, Jaenisch R, Kreidberg JA and Hynes RO: alpha3beta1 Integrin is required for normal development of the epidermal basement membrane. J Cell Biol 137: 729-742, 1997.
17. Kreidberg JA: Functions of alpha3beta1 integrin. Curr Opin Cell Biol 12: 548-553, 2000.
18. Tsuji T: Physiological and pathological roles of alpha3beta1 integrin. J Membr Biol 200: 115-132, 2004.
19. Takenaka K, Shibuya M, Takeda Y, Hibino S, Gemma A, Ono Y and Kudoh S: Altered expression and function of beta1 integrins in a highly metastatic human lung adenocarcinoma cell line. Int J Oncol 17: 1187-1194, 2000.
20. Coopman PJ, Thomas DM, Gehlsen KR and Mueller SC: Integrin alpha 3 beta 1 participates in the phagocytosis of extracellular matrix molecules by human breast cancer cells. Mol Biol Cell 7: 1789-1804, 1996.
21. Adachi M, Taki T, Higashiyama M, Kohno N, Inufusa H and Miyake M: Significance of integrin alpha5 gene expression as a prognostic factor in node-negative non-small cell lung cancer. Clin Cancer Res 6: 96-101, 2000.
22. Ryu MH, Park HM, Chung J, Lee CH and Park HR: Hypoxia-inducible factor-1alpha mediates oral squamous cell carcinoma invasion via upregulation of alpha5 integrin and fibronectin. Biochem Biophys Res Commun 393: 11-15, 2010.
23. Bandyopadhyay A and Raghavan S: Defining the role of integrin alphavbeta6 in cancer. Curr Drug Targets 10: 645-652, 2009.
24. Ramos DM, Dang D and Sadler S: The role of the integrin alpha v beta6 in regulating the epithelial to mesenchymal transition in oral cancer. Anticancer Res 29: 125-130, 2009.
25. Ramos DM, But M, Regezi J, Schmidt BL, Atakilit A, Dang D, Ellis D, Jordan R and Li X: Expression of integrin beta 6 enhances invasive behavior in oral squamous cell carcinoma. Matrix Biol 21: 297-307, 2002.
26. Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG and Morrow M (eds): AJCC cancer staging manual. Springer-Verlag, New York, 2002.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Chang HW, Cheng CA, Gu DL, Chang CC, Su SH, Wen CH, Chou YC, Chou TC, Yao CT, Tsai CL and Cheng CC: High-throughput avian molecular sexing by SYBR green-based real-time PCR combined with melting curve analysis. BMC Biotechnol 8: 12, 2008.
29. Shih IeM, Salani R, Fiegl M, Wang TL, Soosaipillai A, Marth C, Müller-Holzner E, Gastl G, Zhang Z and Diamandis EP: Ovarian cancer specific kallikrein profile in effusions. Gynecol Oncol 105: 501-507, 2007.
30. Wong HS and Chang WC: Correlation of clinical features and genetic profiles of stromal interaction molecule 1 (STIM1) in colorectal cancers. Oncotarget 6: 42169-42182, 2015.
31. Castilho LS, Cotta FV, Bueno AC, Moreira AN, Ferreira EF and Magalhães CS: Validation of DIAGNOdent laser fluorescence and the international caries detection and assessment system (ICDAS) in diagnosis of occlusal caries in permanent teeth: An in vivo study. Eur J Oral Sci 124: 188-194, 2016.
32. Zhang B, Zhang Z, Zhang X, Gao X, Kernstine KH and Zhong L: Serological antibodies against LY6K as a diagnostic biomarker in esophageal squamous cell carcinoma. Biomarkers 17: 372-378, 2012.
33. Cai C, Shi R, Gao Y, Zeng J, Wei M, Wang H, Zheng W and Ma W: Reduced expression of sushi domain containing 2 is associated with progression of non-small cell lung cancer. Oncol Lett 10: 3619-3624, 2015.
34. Sun L, Xu S, Liang L, Zhao L and Zhang L: Analysis of ROC: The value of HPV16 E6 protein in the diagnosis of early stage cervical carcinoma and precancerous lesions. Oncol Lett 12: 1769-1772, 2016.
35. Kurozumi A, Goto Y, Matsushita R, Fukumoto I, Kato M, Nishikawa R, Sakamoto S, Enokida H, Nakagawa M, Ichikawa T and Seki N: Tumor-suppressive microRNA-223 inhibits cancer cell migration and invasion by targeting ITGA3/ITGB1 signaling in prostate cancer. Cancer Sci 107: 84-94, 2016.
36. Zheng W, Jiang C and Li R: Integrin and gene network analysis reveals that ITGA5 and ITGB1 are prognostic in non-small-cell lung cancer. Onco Targets Ther 9: 2317-2327, 2016.
37. Desai K, Nair MG, Prabhu JS, Vinod A, Korlimarla A, Rajarajan S, Aiyappa R, Kaluve RS, Alexander A, Hari PS, *et al*: High expression of integrin β6 in association with the Rho-Rac pathway identifies a poor prognostic subgroup within HER2 amplified breast cancers. Cancer Med 5: 2000-2011, 2016.
38. He M, Zhao Y, Yi H, Sun H, Liu X and Ma S: The combination of TP53INP1, TP53INP2 and AXIN2: Potential biomarkers in papillary thyroid carcinoma. Endocrine 48: 712-717, 2015.
39. Rozalski R, Gackowski D, Siomek-Gorecka A, Starczak M, Modrzejewska M, Banaszkiewicz Z and Olinski R: Urinary 5-hydroxymethyluracil and 8-oxo-7,8-dihydroguanine as potential biomarkers in patients with colorectal cancer. Biomarkers 20: 287-291, 2015.
40. El-mezayen HA, Metwally FM and Darwish H: A novel discriminant score based on tumor-associated trypsin inhibitor for accurate diagnosis of metastasis in patients with breast cancer. Tumour Biol 35: 2759-2767, 2014.

41. Yin MZ, Tan S, Li X, Hou Y, Cao G, Li K, Kou J and Lou G: Identification of phosphatidylcholine and lysophosphatidylcholine as novel biomarkers for cervical cancers in a prospective cohort study. Tumour Biol 37: 5485-5492, 2016.
42. Arcolia V, Journe F, Renaud F, Leteurtre E, Gabius HJ, Remmelink M and Saussez S: Combination of galectin-3, CK19 and HBME-1 immunostaining improves the diagnosis of thyroid cancer. Oncol Lett 14: 4183-4189, 2017.
43. Michailidou E, Tzimagiorgis G, Chatzopoulou F, Vahtsevanos K, Antoniadis K, Kouidou S, Markopoulos A and Antoniades D: Salivary mRNA markers having the potential to detect oral squamous cell carcinoma segregated from oral leukoplakia with dysplasia. Cancer Epidemiol 43: 112-118, 2016.
44. Bu J, Bu X, Liu B, Chen F and Chen P: Increased expression of tissue/salivary transgelin mRNA predicts poor prognosis in patients with oral squamous cell carcinoma (OSCC). Med Sci Monit 21: 2275-2281, 2015.
45. Grady LJ, Campbell WP and North AB: A comparison of several procedures for reducing RNase contamination in preparations of DNase and a particularly successful combination of methods. Anal Biochem 101: 118-122, 1980.
46. Febbraio F, Andolfo A, Tanfani F, Briante R, Gentile F, Formisano S, Vaccaro C, Scirè A, Bertoli E, Pucci P and Nucci R: Thermal stability and aggregation of sulfolobus solfataricus beta-glycosidase are dependent upon the N-epsilon-methylation of specific lysyl residues: Critical role of in vivo post-translational modifications. J Biol Chem 279: 10185-10194, 2004.
47. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM and Tsai CC: Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med 24: 450-453, 1995.
48. Petti S, Mohd M and Scully C: Revisiting the association between alcohol drinking and oral cancer in nonsmoking and betel quid non-chewing individuals. Cancer Epidemiol 36: e1-e6, 2012.