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Original Article

Expression of Orai1 and STIM1 in human oral squamous cell carcinogenesis

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KEYWORDS
Orai1;
STIM1;
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Calcium channel

Abstract *Background/purpose:* Return of Ca^{2+} to endoplasmic reticulum is mediated by Orai/STIM-mediated store-operated Ca^{2+} entry (SOCE) channel. We aimed to investigate Orai1 and STIM1 expressions in human oral carcinogenesis.
Materials and methods: Sixty-six oral squamous cell carcinomas (OSCCs), 14 oral potentially malignant disorders (OPMD) with moderate-severe oral epithelial dysplasia (OED), 19 OPMD with mild OED, and 14 normal oral mucosa (NOM) samples were subjected to immunohistochemical staining. Two human oral cancer cell lines (OCCLs), an oral premalignant cell line (DOK), and a normal oral keratinocyte culture (HOK) were used for Western blot and real-time quantitative reverse transcription-polymerase chain reaction. OCCLs were evaluated for proliferation, migration, and invasion assays.

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Results: Orai1 and STIM1 protein and mRNA expressions in OSCC were significantly enhanced as compared with normal samples. Protein expressions of Orai1 and STIM1 in OCCLs were significantly enhanced as compared with HOK. Significant decreases in degrees of proliferation, migration and invasion were noted in OCCLs with *Orai1* and *STIM1* siRNA transfection as compared with those without transfection. Significantly increased Orai1 and STIM1 protein levels were noted in OPMD with moderate-severe OED as compared with those with mild OED. *Conclusion:* Our results indicate that Orai1 and STIM1 overexpression is associated with human oral carcinogenesis.

Introduction

Oral squamous cell carcinoma (OSCC) is the 4th most frequently-occurring malignancy in Taiwan and is the 5th top cancer death in males.[1] A portion of OSCC is preceded by human oral potentially malignant disorders (OPMDs), especially with oral epithelial dysplasias (OEDs) indicating early detection of human OPMDs is essential.[2] Lesion with dysplastic changes confined to the lower-one-third of the oral epithelium is classified as mild OED whereas moderate OED is defined when dysplastic changes noted for up to the lower-middle of the thickness of the oral epithelium; and severe OED as dysplastic cells observed within lower-two-third of the oral epithelium.[3]

Intracellular Ca^{2+}modulates a wide spectrum of cellular processes such as gene transcription, cell proliferation, cell migration, autophagy and apoptosis.[4–9] Reviewing literature, network controlling intracellular Ca^{2+} hemostasis essentially consists of four different Ca^{2+} channels or transporters:[10] (1) Inositol 1,4,5-trisphosphate receptor mediating Ca^{2+} release from endoplasmic reticulum; (2) Ca^{2+}-ATPase pumping the return of Ca^{2+} to endoplasmic reticulum from cytosol; (3) Ca^{2+} channels/transporters permitting Ca^{2+} influx via plasma membrane from an extracellular Ca^{2+} reservoir, which includes (i) voltage-gated Ca^{2+} channel; (ii) transient receptor potential channel; (iii) store-operated Ca^{2+} entry (SOCE) channel; (iv) Na^{+}/Ca^{2+} exchange and purinergic receptors; (4) Mitochondrial Ca^{2+} uniporter modulating influx of mitochondrial Ca^{2+}.

Additionally, the uptake of Ca^{2+} back into endoplasmic reticulum is chiefly mediated by the SOCE channel.[4] In brief, the influx of Ca^{2+} to the endoplasmic reticulum mediated by the SOCE pathway is executed through the following steps:[11] (1) Decrease of intracellular Ca^{2+} in the endoplasmic reticulum [via the above-mentioned channel (1)]; (2) The reduced endoplasmic reticulum Ca^{2+} store releases a signal to an endoplasmic reticulum-localized Ca^{2+} sensor [called stromal interaction molecule 1 (STIM1)], then inducing translocation of STIM1 to endoplasmic reticulum-plasma membrane junctions; (3) The clustering of STIM1 molecules at the endoplasmic reticulum-plasma membrane releases retrograde signals to the cell surface pore-forming unit called Orai1 to open the Ca^{2+} channels and allow Ca^{2+} influx.

To the best of our knowledge, Orai/STIM-mediated SOCE channel has not been studied in human oral carcinogenesis. The current study is aimed to evaluate Orai1/STIM1-mediated SOCE channel expression in human oral squamous cell carcinogenesis.

Materials and methods

Immunohistochemistry

Tissue specimens from 66 primary OSCC patients, 14 OPMD with moderate to severe OED patients, 19 OPMD with mild OED patients with the habits of drinking alcohol, chewing betel quid, and smoking cigarettes as well as normal oral mucosal tissues from 14 healthy persons without the oral risk factors for oral malignancy were obtained under the approval of the Ethics Committee for Scientific Research on Human Beings of the institution (KMUHIRB-E(II)-20180,225). The characteristics of the OSCC patients, including age, gender, differentiation, tumor size, histopathological lymph-node involvement, and stage were listed in Table 1. All the tissue specimens were fixed in 10% neutral buffered formalin solution, dehydrated in graded alcohols, cleaned

Table 1 Characteristics of the oral squamous cell carcinoma patients.

Patients characteristics	Number (%)
Gender	
Male (mean age: 58.6 years)	62 (93.9)
Female (mean age: 75.6 years)	4 (6.1)
Age	
≤55 years	30 (45.5)
>55 years	36 (54.5)
Differentiation	
Well	53 (80.3)
Moderate- to poorly	13 (19.7)
Tumor size	
≤2 cm	35 (53.0)
>2 cm	31 (47.0)
Lymph-node metastasis	
Yes	27 (57.5)
No	20 (42.5)
Pathological stage	
I-II	29 (43.9)
III-IV	37 (56.1)

in xylene, and embedded in paraffin for immunohistochemical staining for Orai1 and STIM1 proteins with the primary rabbit polyclonal anti-Orai1 (Invitrogen, IL, USA; Cat. No. MA5-15776; 1: 100) and anti-STIM1 (Invitrogen; Cat. No. MA5-19451; 1: 100) antibody respectively using the standard avidin-biotin peroxidase complex method with respect to the manufacturer's guidelines (Vector Laboratories).[12] Each set of experiments contained a specimen known to express Orai1 and STIM1 respectively, which served as positive controls. Negative controls were included following the same procedures, but with omission of the primary antibody. The scores of the percentage of positive staining (P) were classified according to Table 2. Total score (S) was determined as P $\times$ I for each section.[13] Quantification of the immunostained slides were performed by two certified oral pathologists independently using the image analysis software Image J Version 1.51e. When disagreement existed between the two observers, a consensus was reached by mutual discussion.

Cell culture

The human oral cancer cell lines (Ca9-22, and OECM-1) and the normal oral keratinocytes primary cultures (HOK) were cultured in high-glucose DMEM (Hyclone, Logan, UT, USA) with the addition of 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Hyclone) at 37 °C in a humidified 5% CO_2 atmosphere. The DOK was cultured in high-glucose DMEM (Hyclone, Logan, UT, USA) with the addition of 10% fetal bovine serum (Hyclone), 2 mM glutamine (Hyclone), 5 μg/ml hydrocortisone (Hyclone), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was changed on every third day.

Western blot

Cells of the human oral cancer cell lines, DOK, and HOK were rinsed with phosphate buffered-saline (PBS; Sigma—Aldrich, St Louis, MO, USA) and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma—Aldrich). The lysates were subsequently centrifuged at 4 °C, 14,000 rpm, for 15 min. Equal amounts of protein were denatured by adding sodium dodecyl sulfate (SDS) running buffer (Sigma—Aldrich) and β-mercaptoethanol (Sigma—Aldrich). The samples were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma—Aldrich) on 15% gels, and the proteins were transferred onto a poly vinylidene fluoride (PVDF) membrane (Sigma—Aldrich) using Bio-Rad's transblot with the primary rabbit polyclonal primary anti-Orai1 (Invitrogen; 1: 1000) and anti-STIM1 (Invitrogen; 1: 1000) antibody respectively with species specificity for human tissues and observed molecular weight 55 kDa for Orai1 and 75 kDa for STIM1 respectively, and β-actin (Sigma—Aldrich; 1: 500), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma—Aldrich; 1: 5000). The amount of protein was then quantified using a Fuji LAS-4000 lumino image analyzer (Fuji Photo Film Co., Tokyo, Japan). The ratio was normalized by the β-actin signal.

Table 2 Grading of immunoscores on immunochemical staining.

Scores	Intensity	Scores	Percentage
0	No staining	0	<1%
1	Light yellow (weak staining)	1	1—24%
2	Brown (Moderate-strong staining)	2	25—49%
3	Dark brown (Strong staining)	3	50—74%
		4	75—100%

Real-time quantitative reverse transcription-polymerase chain reaction (real time qRT-PCR)

Total RNA samples were prepared from human oral cancer cell lines, DOK, and HOK using TRIzol Reagent (Invitrogen).[14] The procedures of real-time qRT-PCR was then implemented according to our previous study[15] with oligo-primers for *Orai1* (forward: 5′-GCT TCG CCA TGG CAA T-3'; reverse: 3′-GCT GAT CAT GAG CGC AAA CA-5'; accession no. NM_032790.3), *STIM1* (forward: 5′-AGA AAC ACA CTC TTT GGC ACC; reverse: 3′-AAT GCT GCT GTC ACC TCG; accession no. NM_001277962) and *β-actin* (forward: 5′-CAC CAT TGG CAA TGA GCG GTT C-3'; reverse: 5′-AGG TCT TTG CGG ATG TCC ACG T-3′), which were designed with reference to the published cDNA sequences in GenBank. After normalization to the expression level of *β-actin* mRNA, the relative expression levels of mRNA for each of human oral cancer cell line, and DOK, were determined as the percentage change as compared with HOK; and additionally, the relative expression levels of mRNA for each of human oral cancer cell line were determined as the percentage change as compared with DOK.

Establishment of human OSCC cell cultures with *Orai1* and *STIM1* genes knock-down

RNA interference using commercially-synthesized *Orai1* and *STIM1* siRNAs (*Orai1*: forward 5′-GGU GAG CAA CGU GCA CAA Utt-3'; reverse 3′-ttC CAC UCG UUG CAC GUG UUA-5'; *STIM1*: forward 5′-GGA AGU CAU CAG AAG UAU Att; reverse 3′-UAU ACU UCU GAU GAC UUC Cat-5′) to establish human oral cancer cell lines with *Orai1* and *STIM1* genes knock-down was performed with the procedures according to our previous study.[15]

Proliferation assay, migration assay, *in vitro* transwell invasion assay

The cell proliferation assay, migration assay, *in vitro* transwell invasion assay experiments for human oral cancer cell lines were measured for 48 h post-transfection of *Orai1* and *STIM1* siRNA respectively using the procedures according to our previous study.[15]

Statistical analyses

Statistical analyses were performed using SAS Statistical Package (Version 9.1.3, SAS Institute Inc.) with statistical significance when the *P* value < 0.05. Paired t-test was used to compare the immunohistochemical expressions among OSCCs, OPMDs and normal oral mucosa, and along with the differentiation, tumor size, histological lymph-node involvement, and pathological stage of the OSCC patients as well as to evaluate the differences in proliferation rate and degrees of migration and invasion between human oral cancer cells with and without siRNA transfection. Nonparametric Kruskal–Wallis tests were used to analyze the results of western blots and real-time quantitative reverse transcription-polymerase chain reactions.

Results

Immunochemistry

Immunohistochemistry for Orai1

Strong positive staining (cytoplasmic and nuclear) of Orai1 protein was observed in OSCC (Fig. 1A). On the other hand, weak positive staining of Orai1 protein was observed in normal oral mucosa samples (chiefly membrane) (Fig. 1B). The mean IS of the OSCC (T1-T2) and OSCC (T3-T4) cases was 5.6 and 8.1 respectively, whilst that of the normal oral mucosa samples was 1.84 (Table 3). A significant increase was noted respectively in the mean IS of the OSCC (T1 & T2) and OSCC (T3 & T4) cases in comparison with that of the normal oral mucosa samples (Table 3). Additionally, a significant difference in the mean IS of Orai1 protein was confirmed in tumor size as comparison of the OSCC (T3-T4) and OSCC (T1-T2) patients (Table 4). There was an increased mean IS of Orai1 protein with lymph node metastasis, and pathological stage; however, no significant correlations have been established for tumor size (Table 4).

Positive staining of Orai1 protein was observed in cases of human OPMD with moderate to severe OED (Fig. 1C) and in cases of human OPMD with mild OED (Fig. 1D). The mean IS of the human OPMDs with moderate to severe OED and with mild OED was 5.73 and 2.42 respectively (Table 3), a significant increase being noted when comparing the mean IS of human OPMDs with moderate to severe OED with those with mild OED (Table 3). Also, a significant increase in the mean IS was noted when comparing cases of human OPMDs with moderate to severe OED with normal mucosa samples (Table 3); no significant increase of mean IS being found when comparing human OPMDs mild OED with normal mucosa samples (Table 3). On the other hand, a significant increase of mean IS being noted as comparing human OPMD with mild OED with samples of OSCC (T1-T2) and OSCC (T3-T4) respectively (Table 3); however, no significant increase of mean IS being observed when comparing human OPMDs with moderate to severe OED with samples of OSCC (T1-T2) and OSCC (T3-T4) respectively (Table 3).

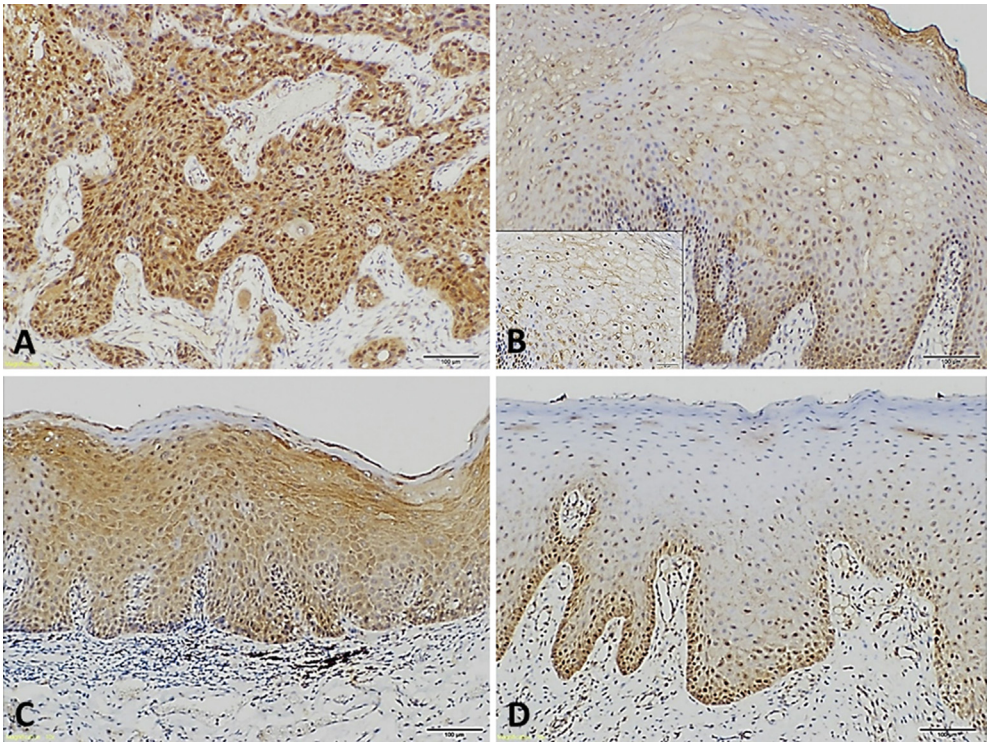

Figure 1 Immunohistochemistry for Orai1 (A) A representative strong immunohistochemical staining of Orai1 protein in human oral squamous cell carcinoma (× 100) (B) A representative weak staining of Orai1 protein in human normal oral mucosa (× 100; × 400 for inset) (C) A representative stronger immunohistochemical staining of Orai1 protein for a human oral potentially malignant disorder with moderate to severe oral epithelial dysplasia (× 100) (D) A representative weaker staining of Orai1 protein for a human oral potentially malignant disorder with mild oral epithelial dysplasia (× 100).

Table 3 Statistical analyses for immunohistochemical expression of Orai1 protein amongst human oral squamous cell carcinomas (OSCCs), human oral potentially malignant disorders (OPMDs) with moderate to severe oral epithelial dysplasia (OED), OPMDs with mild OED, and normal oral mucosa (NOM).

	P values				
	OSCC (T1-T2)	OSCC (T3-T4)	OPMD with moderate to severe OED	OPMD with mild OED	NOM
OSCC (T1-T2) (5.57 ± 2.33)[a]		*<0.05	>0.05	**<0.01	**<0.01
OSCC (T3-T4) (8.14 ± 2.96)	*<0.05		>0.05	*<0.01	**<0.01
OPMD with moderate to severe OED (5.73 ± 1.27)	>0.05	>0.05		*<0.01	**<0.01
OPMD with mild OED (2.42 ± 1.15)	**<0.01	**<0.01	**<0.01		>0.05
NOM (1.84 ± 1.77)	**<0.01	**<0.01	**<0.01	>0.05	

*statistically significance, **statistically significance.
[a] Values within brackets: Immunoscore (mean ± standard deviation)

Immunohistochemistry for STIM1

Strong positive staining of STIM1 protein was observed in OSCC (Fig. 2A). On the other hand, weak positive staining of STIM1 protein was observed in normal oral mucosa samples (Fig. 2B). The mean IS of the OSCC (T1-T2) and OSCC (T3-T4) cases of STIM1 protein was 5.7 and 7.5 respectively, whilst that of the normal oral mucosa samples was 2.6 (Table 5). A significant increase was noted respectively in the mean IS of the OSCC (T1-T2) and OSCC (T3-T4) cases in comparison with that of the normal oral mucosa samples (Table 5). Additionally, a significant difference in the mean IS of STIM1 protein was confirmed in a comparison of the OSCC (T3-T4) with OSCC (T1-T2) patients (Table 6); On the other hand, there were significant increases of the mean IS of STIM1 protein with tumor size, lymph node metastasis, and patholoigcal stage respectively (Table 6).

Positive staining of STIM1 protein was observed in cases of human OPMD with moderate to severe OED (Fig. 2C) and in cases of human OPMD with mild OED (Fig. 2D). The mean IS of the human OPMD with moderate to severe OED and human OPMD with mild OED was 6.2 and 2.6 respectively (Table 5), a significant increase being noted when comparing the mean IS of human OPMD with moderate to severe OED with human OPMD with mild OED (Table 5). Also, a significant increase in the mean IS was noted when comparing cases of human OPMD with moderate to severe OED with normal mucosa samples (Table 5); no significant increase of IS was found when comparing human OPMD with mild OED with normal mucosa samples (Table 5).

Table 4 Statistical comparison of immunohistochemical expression of Orai1 protein respectively in human oral squamous cell carcinomas with histopathological features.

Patients characteristics	Immunoscores (mean ± standard deviation)	*P* values
Gender		
Male	6.8 ± 3.3	>0.05
Female	10.5 ± 2.1	
Age		
≤55 years	6.9 ± 2.8	>0.05
>55 years	7.0 ± 2.9	
Differentiation		
Well	7.2 ± 3.0	>0.05
Moderate-to poorly	6.5 ± 3.8	
Tumor size		
T1-T2	5.6 ± 2.3	<0.05*
T3-T4	8.1 ± 3.0	
Lymph-node metastasis		
Yes	8.2 ± 2.6	>0.05
No	6.8 ± 3.0	
Pathological stage		
I-II	5.6 ± 2.4	>0.05
III-IV	7.8 ± 3.2	

* Statistically significance.

Western blot

Western blot for Orai1

Western blot analysis of the human oral cancer cell lines (Ca9-22, and OECM-1) indicated overexpression of Orai1 protein as compared with primary cultures of human normal oral keratinocytes (HOK), these results being of statistical significance (Fig. 3A & B). On the other hand, Western blot analysis of human oral premalignant cell line DOK showed overexpression of Orai1 protein as compared with HOK with statistical significance (Fig. 3A & B). Additionally, a significant upregulation of Orai1 protein expression was also noted for human oral cancer cell lines (OECM-1 and Ca9-22) as compared with DOK (Fig. 3A &C).

Western blot for STIM1

Western blot analysis of the human oral cancer cell lines (Ca9-22, and OECM-1) indicated overexpression of STIM1 protein as compared with primary cultures of human normal oral keratinocytes (HOK), these results being of statistical significance (Fig. 4A & B). On the other hand, Western blot analysis of human oral premalignant cell line DOK showed overexpression of STIM1 protein as compared with HOK with statistical significance (Fig. 4A & B). Additionally, a significant upregulation of STIM1 protein

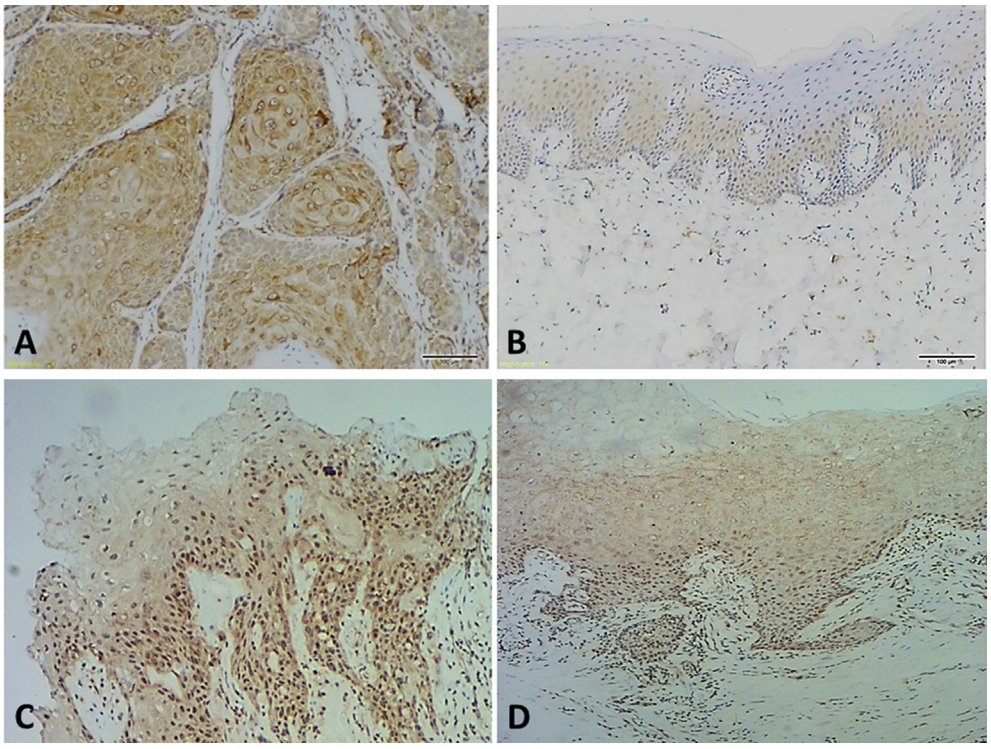

Figure 2 Immunohistochemistry for STIM1 (A) A representative strong immunohistochemical staining of STIM1 protein in human oral squamous cell carcinoma (× 100) (B) A representative weak staining of STIM1 protein in human normal oral mucosa (× 100) (C) A representative stronger immunohistochemical staining of STIM1 protein for a human oral potentially malignant disorder with moderate to severe oral epithelial dysplasia (× 100) (D) A representative weaker staining of STIM1 protein for a human oral potentially malignant disorder with mild oral epithelial dysplasia (× 100).

Table 5 Statistical analyses for immunohistochemical expression of STIM1 protein amongst human oral squamous cell carcinomas (OSCCs), human oral potentially malignant disorders (OPMDs) with moderate to severe oral epithelial dysplasia (OED), OPMDs with mild OED, and normal oral mucosa (NOM).

	P values				
	OSCC (T1-T2)	OSCC (T3-T4)	OPMD with moderate to severe OED	OPMD with mild OED	NOM
OSCC (T1-T2) (5.7 ± 2.6)[a]		**<0.01	>0.05	***<0.001	***<0.001
OSCC (T3-T4) (7.5 ± 1.8)	**<0.01		**<0.01	***<0.001	***<0.001
OPMD with moderate to severe OED (6.2 ± 1.1)	>0.05	**<0.01		***<0.001	***<0.001
OPMD with mild OED (2.6 ± 1.5)	***<0.001	***<0.001	***<0.001		>0.05
NOM (2.6 ± 1.0)	***<0.001	***<0.001	***<0.001	>0.05	

**statistically significance, ***statistically significance.
[a] Values within brackets: Immunoscore (mean ± standard deviation).

expression was also noted for human oral cancer cell lines (Ca9-22, and OECM-1) as compared with DOK (Fig. 4A &C).

Real time qRT-PCR

Real time qRT-PCR for Orai1

Real-time qRT-PCR analyses of the human oral cancer cell lines (Ca9-22, and OECM-1) indicated upregulation of *Orai1* mRNA expression with statistical significance as compared with HOK (Fig. 5). On the other hand, real-time qRT-PCR analyses of the human oral cancer cell lines (Ca9-22, and OECM-1) indicated an upregulation of *Orai1* mRNA expression as compared with DOK with statistical significance (Fig. 5). Additionally, there was an overexpression of *Orai1* mRNA expression of DOK when compared with HOK but without statistical significance (Fig. 5).

Real time qRT-PCR for STIM1

Real-time qRT-PCR analyses of the human oral cancer cell lines (Ca9-22, and OECM-1) indicated upregulation of *STIM1*

Table 6 Statistical comparison of immunohistochemical expression of STIM1 protein in human oral squamous cell carcinomas with histopathological features.

Patients characteristics	Immunoscores (mean ± standard deviation)	*P* values
Gender		
Male	6.5 ± 2.5	>0.05
Female	7.5 ± 0.6	
Age		
≤55	7.0 ± 2.4	>0.05
>55	6.3 ± 2.5	
Differentiation		
Well	6.4 ± 2.5	>0.05
Moderate-to poorly	7.5 ± 2.2	
Tumor size		
T1-T2	5.7 ± 2.6	**<0.01
T3-T4	7.5 ± 1.8	
Lymph-node metastasis		
Yes	7.5 ± 2.0	*<0.05
No	6.1 ± 2.3	
Pathologic stage		
I-II	5.5 ± 2.5	***<0.001
III-IV	7.4 ± 2.0	

*Statistically significance.

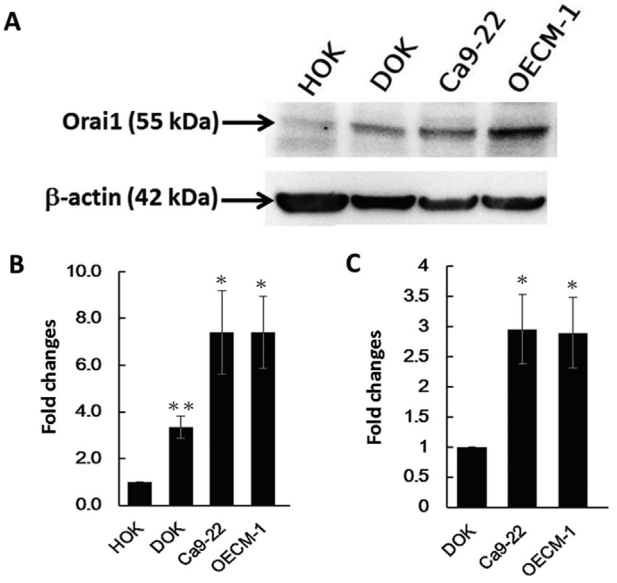

Figure 3 Western blot for Orai1. Upregulation of Orai1 protein expression in human oral cancer cell lines (Ca9-22, and OCEM-1) as compared with the primary culture of human normal oral keratinocytes (HOK) (A, B). Human oral premalignant cell line (DOK) showed overexpression of Orai1 protein as compared with HOK (A, B). Upregulation of Orai1 protein expression of Ca9-22, and OECM-1 was observed as compared with DOK respectively (A, C). Results were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to the HOK. Bars represent means ± standard error of the mean (*$P < 0.05$; **$P < 0.01$). A representative result of three independent experiments is shown.

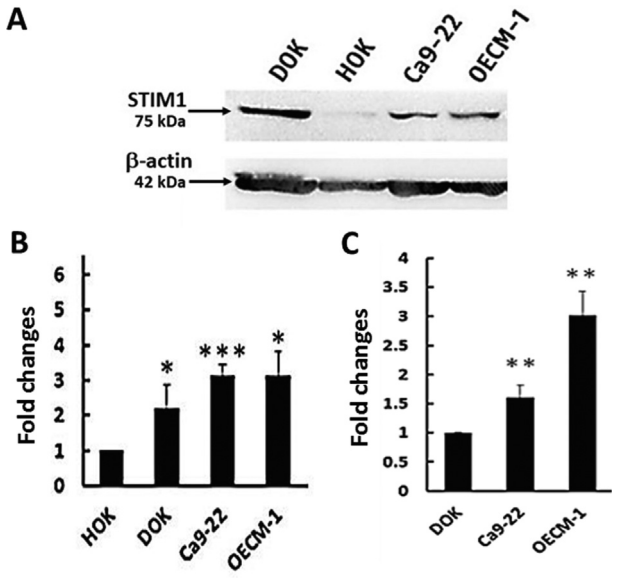

Figure 4 Western blot for STIM1. Upregulation of STIM1 protein expression in human oral cancer cell lines (Ca9-22, and OCEM-1) as compared with the primary culture of human normal oral keratinocytes (HOK) (A, B). Human oral premalignant cell line (DOK) showed overexpression of STIM1 protein as compared with HOK (A, B). Upregulation of STIM1 protein expression of Ca9-22, and OECM-1 was observed as compared with DOK respectively (A, C). Results were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to the HOK. Bars represent means ± standard error of the mean (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). A representative result of three independent experiments is shown.

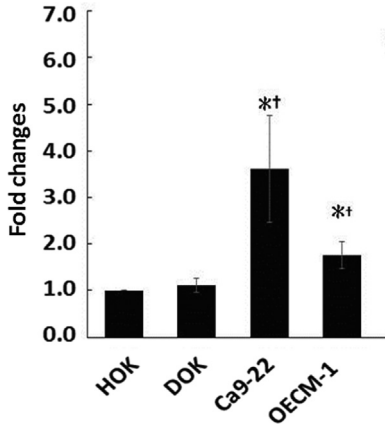

Figure 5 Real-time quantitative reverse transcription-polymerase chain reaction for Orai1. Upregulation of *Orai1* mRNA expression was observed in human oral cancer cell lines (Ca9-22, and OECM-1) as compared with the primary culture of normal human oral keratinocytes (HOK) (*$P < 0.05$). Upregulation of *Orai1* mRNA expression was observed in human oral cancer cell lines (Ca9-22, and OECM-1) as compared with the human oral premalignant cell line (DOK) ($^{\dagger}P < 0.05$). Upregulation of *Orai1* mRNA expression without statistical significance was observed in DOK as compared with HOK. Results were normalized by the level of β-actin & expressed as fold change relative to HOK & DOK respectively. Bars represent mean ± standard error of the mean.

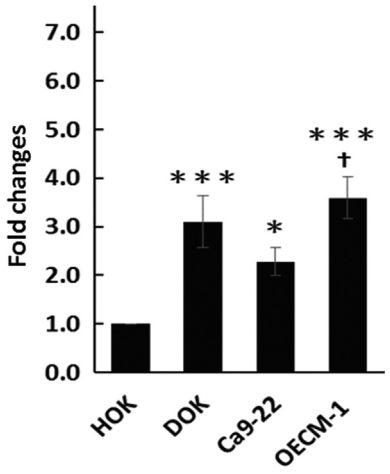

Figure 6 Real-time quantitative reverse transcription-polymerase chain reaction for STIM1. Upregulation of *STIM1* mRNA expression was observed in human oral cancer cell lines (Ca-922, and OECM-1) as compared with the primary culture of normal human oral keratinocytes (HOK) (*$P < 0.05$; ***$P < 0.001$). Upregulation of *STIM1* mRNA expression was observed in the human oral premalignant cell line (DOK) as compared with HOK (***$P < 0.001$); upregulation of *STIM1* mRNA expression was observed in OECM-1 as compared with DOK ($^{\dagger}P < 0.05$). Results were normalized by the level of β-actin and expressed as fold change relative to HOK & DOK respectively. Bars represent means ± standard error of the mean.

mRNA expression with statistical significance as compared with HOK (Fig. 6). On the other hand, real-time qRT-PCR analyses of OECM-1 indicated a significant upregulation of *STIM1* mRNA expression as compared with DOK (Fig. 6). Additionally, there was a significant overexpression of *STIM1* mRNA expression of DOK when compared with HOK (Fig. 6).

Proliferation assay

Proliferation assay for Orai1

The proliferation rates of the oral cancer cell lines (Ca9-22, and OECM-1) with *Orai1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *Orai1* siRNA transfection (Fig. 7).

Proliferation assay for STIM1

The proliferation rates of the human oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *STIM1* siRNA transfection respectively (Fig. 8).

Migration assay

Migration assay for Orai1

The degrees of migration of the human oral cancer cell lines (Ca9-22, and OECM-1) with *Orai1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *Orai1* siRNA transfection (Fig. 9).

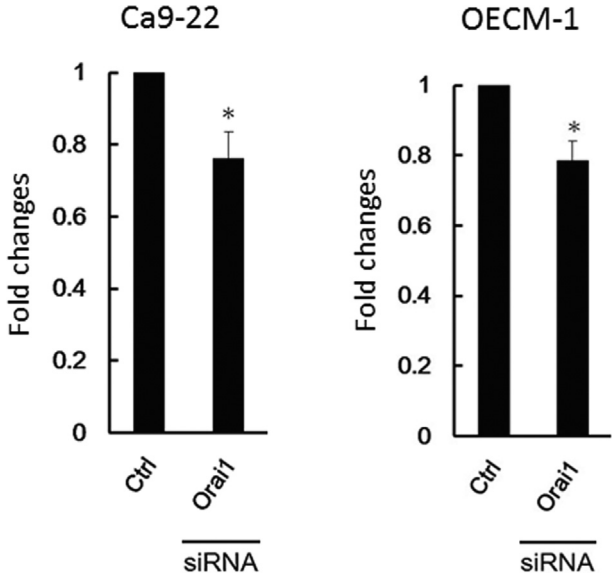

Figure 7 Proliferation assay for Orai1. The proliferation rates of the oral cancer cell lines (Ca9-22, and OECM-1) with *Orai1* siRNA transfection for 48 h were significantly lower than those of the oral cancer cell lines without *Orai1* siRNA transfection (*$P < 0.05$).

Migration assay for STIM1

The degrees of migration of the oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *STIM1* siRNA transfection (Fig. 10).

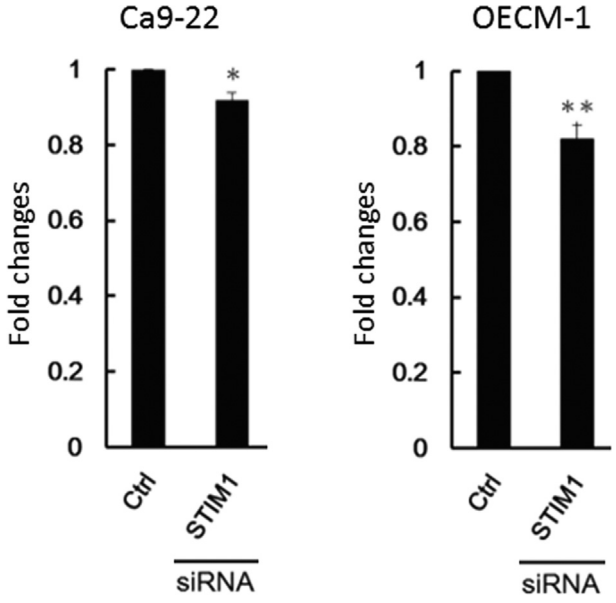

Figure 8 Proliferation assay for STIM1. The proliferation rates of the oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h were significantly lower than those of the oral cancer cell lines without *STIM1* siRNA transfection (*$P < 0.05$, **$P < 0.01$).

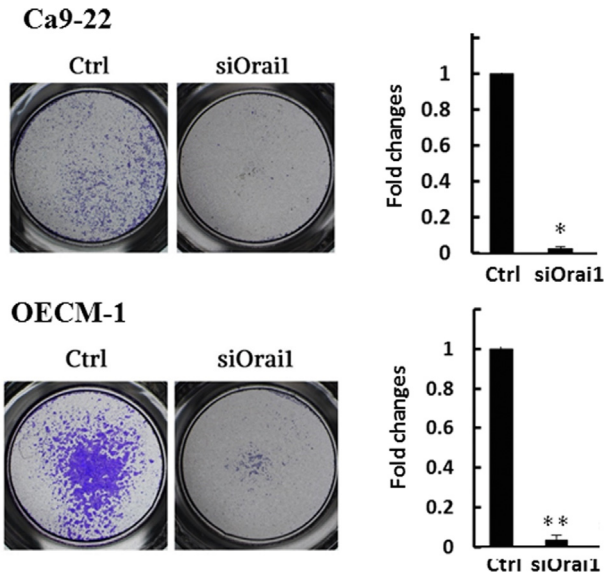

Figure 9 Migration assay for Orai1. Degree of migration of the oral cancer cell lines (Ca9-22, and OECM-1) with *Orai1* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without transfection (*$P < 0.05$, **$P < 0.01$).

In vitro transwell invasion assay

In vitro transwell invasion assay for Orai1

The degrees of invasion of the human oral cancer cell lines (Ca9-22 and OECM-1) with *Orai1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *Orai1* siRNA transfection (Fig. 11).

In vitro transwell invasion assay for STIM1

The degrees of invasion of the oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h were significantly decreased as compared with the oral cancer cell lines without *STIM1* siRNA transfection respectively (Fig. 12).

Discussion

Reviewing English literatures, disruption of normal intracellular Ca^{2+} is reported being associated to the formation of cancer in a number of studies.[16–24] Dysregulated intracellular Ca^{2+} signaling in cancer cells has also been found to induce cancer angiogenesis, cancer progression, and cancer metastasis.[16–24] Therefore, studying the particular genes such as Orai1 and STIM1 that attribute to dysregulated intracellular Ca^{2+} signaling in dysplastic cells is an essential area for oral cancer study for human oral mucosa.

In the current study, we have demonstrated that significantly increased Orai1 protein expression was observed in OSCCs as compared with normal oral mucosa; and Orai1 protein expression also increased significantly between T3-T4 and T1-T2 OSCC patients. An increased Orai1 protein level was noted in OPMDs with moderate to severe OED as compared to those with mild OED, and was also found in OPMDs with moderate to severe OED in comparison with normal mucosa. There is also a shift of membranous staining to cytoplasmic/nuclear staining from normal mucosa to lesions of OPMD and OSCC. Additionally,

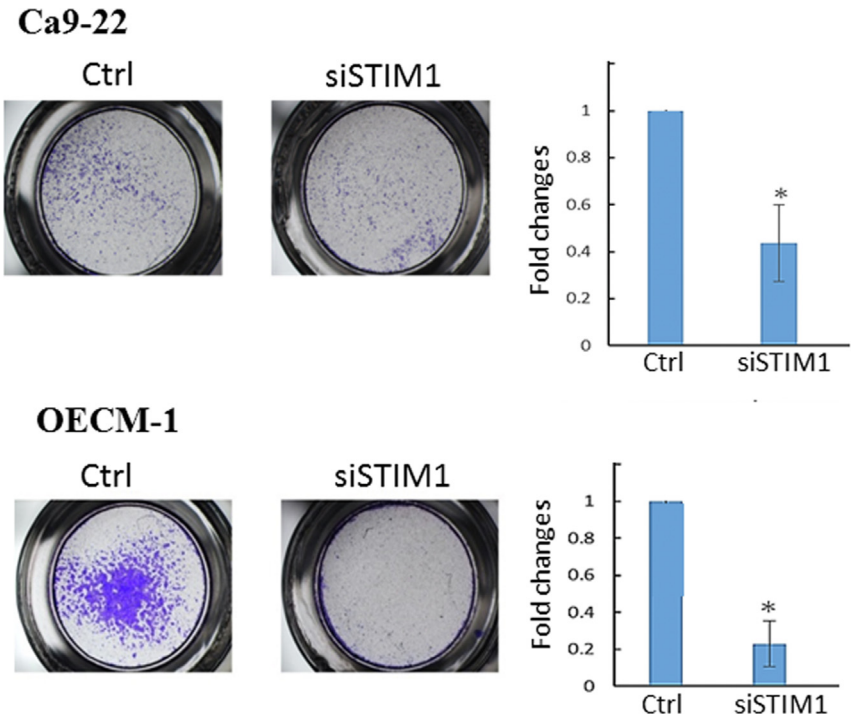

Figure 10 Migration assay for STIM1. Degree of migration of the oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without transfection (*$P < 0.05$).

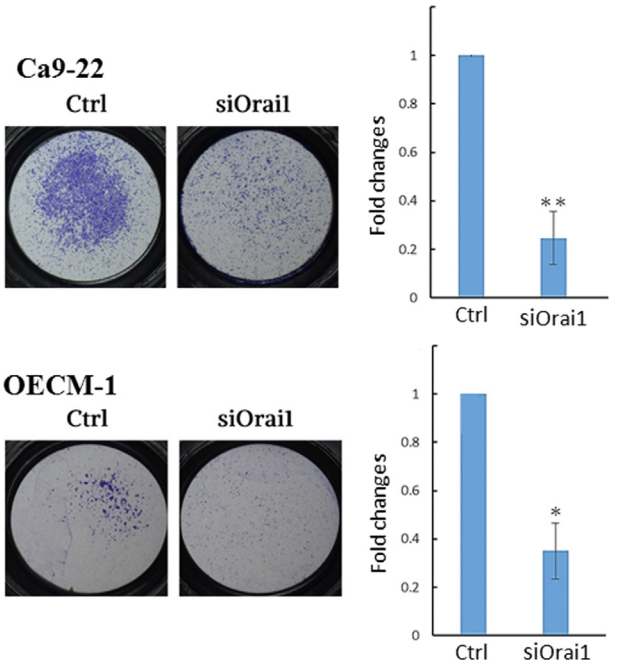

Figure 11 Invasion assay for Orai1. Degree of invasion of the oral cancer cell lines (Ca9-22, and OECM-1) with *Orai1* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without transfection (*$P < 0.05$, **$P < 0.01$).

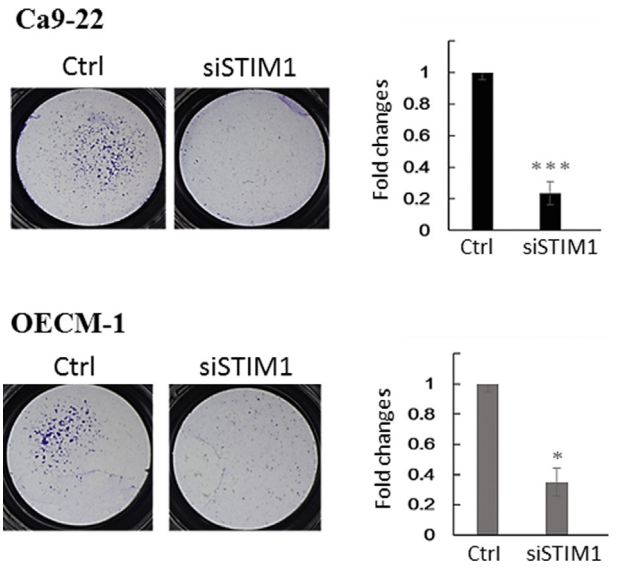

Figure 12 Invasion assay for STIM1. Degree of invasion of the oral cancer cell lines (Ca9-22, and OECM-1) with *STIM1* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without transfection (*$P < 0.05$, ***$P < 0.001$).

the *in vivo* data for Orai1 are largely compatible to the *in vitro* results. Orai1 protein and mRNA expressions were significantly enhanced as compared with the primary culture of normal oral keratinocytes (HOK). Significant decreases in the proliferation rate, degrees of migration, and invasion were noted in oral cancer cell lines with *Orai1* siRNA transfection as compared with those without siRNA transfection.

Furthermore, our findings on Orai1 have been compatible with the other studies on various malignancies including breast carcinoma[20] glioblastoma[21] pancreatic adenocarcinoma[25] melanoma[26,27] renal carcinoma[28] esophageal squamous cell carcinoma[29] non-small-cell lung carcinoma[30] prostate carcinoma.[31]

On the other hand, our study has confirmed a significantly increased STIM1 protein expression in human OSCCs when compared with normal oral mucosa; and STIM1 expression was also increased significantly between OSCC (T3-T4) and OSCC (T1-T2) patients. A significantly increased STIM1 protein level was noted in OSCC (T3-4) as compared with OPMD with moderate to severe OED; and was also noted in lesions of OPMD with moderate to severe OED in comparison with normal oral mucosa. These *in vivo* data for STIM1 are consistent to the *in vitro* results. STIM1 protein and mRNA expressions were significantly enhanced ranging from oral cancer cell lines, oral premalignant cell line (DOK) to HOK. On the other hand, significant reductions in the proliferation rate and degrees of migration, invasion was found in oral cancer cell lines with *STIM1* siRNA transfection as compared with those without siRNA transfection. These *in vitro* findings were compatible to the *in vivo* data of STIM1 protein in which a significant increase was noted in tumor size, lymph-node metastasis and pathologic stage respectively. The aforementioned findings in the current study on STIM1 have been compatible with the other studies on miscellaneous types of cancers including breast carcinoma[32,33] cervical cancer[18,19] hepatocellular carcinoma[24] melanoma[27] pancreatic adenocarcinoma[25] and colorectal cancer.[34]

Taken together for the current findings of Orai1 and STIM1, to our knowledge, the study would be the first to elucidate the expression of Orai1 and STIM1 in human oral mucosa ranging from normal mucosa, premalignant, and malignant lesions implicating that Orai1/STIM1-mediated SOCE channel expression is potentially associated to human oral squamous cell carcinogenesis.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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