Journal of Dental Sciences 18 (2023) 382–391

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Overexpression of transient receptor potential melastatin 6 during human oral squamous cell carcinogenesis

Yen-Yun Wang [a,b†], Wen-Chen Wang [a,c,d†], Chiang-Wei Su [e], Ching-Wei Hsu [e], Shyng-Shiou Yuan [b,f,g,h**], Yuk-Kwan Chen [a,c,d*]

[a] School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Division of Oral Pathology & Maxillofacial Radiology, Department of Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[d] Oral & Maxillofacial Imaging Center, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Division of Oral & Maxillofacial Surgery, Department of Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[f] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[g] Translational Research Center, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[h] Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

Received 30 October 2022; Final revision received 30 October 2022
Available online 14 November 2022

KEYWORDS
TRPM6;
Oral squamous cell carcinoma;
Oral potentially malignant disorders;

Abstract *Background/purpose:* Transient receptor potential melastatin (TRPM) channel is involved in cell proliferation and cell survival. Eight members (TRPM1–8) are within the TRPM subfamily. The current study is aimed to investigate TRPM6 expression in human oral carcinogenesis.
Materials and methods: Sixty-six oral squamous cell carcinomas (OSCCs), 47 oral potentially malignant disorders (OPMD) with moderate-severe epithelial dysplasia (ED), 28 OPMD with mild ED, and 33 normal oral mucosa (NOM) samples were subjected to immunohistochemical

* Corresponding author. School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan.
** Corresponding author. Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan.
E-mail addresses: yuanssf@ms33.hinet.net (S.-S. Yuan), k0285@ms22.hinet.net (Y.-K. Chen).
† These two authors contributed equally to this work.

https://doi.org/10.1016/j.jds.2022.11.002

Calcium channel

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 analysis. OCCLs were evaluated for proliferation, migration, invasion assays, and intracellular calcium concentration.
Results: TRPM6 protein expression in OSCC was significantly increased as compared with normal samples. Protein expression of TRPM6 in OCCLs was significantly higher as compared with HOK. Significant decreases in degrees of proliferation, migration, invasion, and intracellular calcium concentration were noted in OCCLs with *TRPM6* siRNA transfection as compared with those without transfection. Significantly increased TRPM6 protein level was noted in OPMD with moderate-severe ED as compared with those with mild ED.
Conclusion: Our results implicate that TRPM6 overexpression is potentially related to human oral carcinogenesis.

Introduction

Human oral squamous cell carcinoma (OSCC) is the fourth most common malignancy in Taiwan and is ranked as the fifth cancer death in males.[1] Some OSCCs are developed from human oral potentially malignant disorder (OPMD), especially with epithelial dysplasia (ED). Therefore, it is important to have early recognition of human OPMD.[2] When dysplastic alterations limited to the lower-one third of the oral epithelium, it is categorized as mild ED whilst moderate ED is regarded as dysplastic alterations were found up to the lower middle of the thickness of the oral epithelium; and severe ED as dysplastic cells occurred within lower-two thirds of the oral epithelium.[3]

Intracellular calcium, the most abundant and ubiquitous second messenger in human, modulates an extensive range of cellular functions, for instance, cell proliferation, cell migration, autophagy, and apoptosis.[4–9] There are four calcium channels or transporters controlling intracellular calcium hemostasis (Table 1).[10] Briefly, as summarized in Table 1, the channel (1) contributes to the release of intracellular calcium via inositol 1,4,5-trisphosphate receptor from endoplasmic reticulum (ER), and the other three channels (2), (3), and (4) contribute to the uptake of calcium back to ER and calcium influx across plasma membrane from ER.

Among the various calcium channels/transporters (Table 1), transient receptor potential (TRP) channels have not been comprehensively studied in human oral squamous cell carcinogenesis. Mammalian TRP channels are classified into six subfamilies: C (canonical), TRPC; V (vanilloid receptor) TRPVR; M (melastatin), TRPM; A (ANKTM), TRPA; P (polycystin), TRPP; and ML (mucolipin) TRPML with respect to homology and partially on channel function. For the six subfamilies of TRP channels, TRPM subfamily is proved to be involved in cell proliferation and cell survival.[11] To date, TRPM subfamily is identified to have a total of eight members (TRPM1 to TRPM8).[11] Reviewing English literature, no study has been performed for the sixth member (TRPM6) in human OPMD and OSCC; hence, the current study is aimed to evaluate TRPM6 expression during human oral squamous cell carcinogenesis.

Materials and methods

Immunohistochemistry

Tissue specimens were obtained from the Oral Pathology Department of our institution, with the approval of the Ethics Committee for Scientific Research on Human Beings of the institution (KMUHIRB-E(II)-20200038). Demographic data of the patients in the current study was shown in Table 2. The characteristics of the OSCC patients, including age, gender, differentiation, tumor size, histopathological lymph-node involvement, and stage were summarized in Table 3. The tissue specimens were fixed in 10% neutral buffered formalin solution for approximately

Table 1 Summary of the four different calcium channels or transporters[10].

Calcium channels or transporters	Function
(1) Inositol 1,4,5-trisphosphate (IP_3) receptor	Mediating calcium release from endoplasmic reticulum (ER)
(2) Calcium-ATPase	Pumping the return of calcium to ER (or extracellular space) from cytosol
(3) Calcium channels/transporters, which includes:	Permitting calcium influx via plasma membrane from an extracellular calcium reservoir
i. Voltage-gated calcium channel	
ii. Transient receptor potential channel (TRP)	
iii. Store-operated calcium entry (SOCE)/calcium release-activated calcium channel (CRAC)	
iv. Na^+/Ca^{2+} exchange (NCX) and purinergic receptors	
(4) Mitochondrial calcium uniporter (MCU)	Modulating influx of mitochondrial calcium

Table 2 Demographic data of the patients in the current study.

Normal oral mucosa		OPMD with mild epithelial dysplasia		OPMD with moderate to severe epithelial dysplasia		Oral squamous cell carcinoma	
	No. (%)		No. (%)		No. (%)		No. (%)
Male	12	**Male**	25	**Male**	41	**Male**	62
mean age: 51.2 yr	(36.4)	mean age: 55.6 yr	(89.3)	mean age: 56.7 yr	(87.2)	mean age: 60.7 yr	(90)
range: 18—79 yr		range: 39—71 yr		range: 24—76 yr		range: 45—81 yr	
Female	21	**Female**	3	**Female**	6	**Female**	4
mean age: 9.3 yr	(63.6)	mean age: 56.3 yr	(10.7)	mean age: 71.3 yr	(12.8)	mean age: 72.7 yr	(10)
range: 25—81 yr		range: 31—77 yr		range: 77—90 yr		range: 69—75 yr	
Total	33	**Total**	28	**Total**	47	**Total**	66
mean age: 50.0 yr	(100)	mean age: 55.7 yr	(100)	mean age: 58.6 yr	(100)	mean age: 61.9 yr	(100)
range: 18—81 yr		range: 31—77 yr		range: 24—90 yr		range: 45—81 yr	

OPMD: oral potentially malignant disorders; yr: years; No.: Number.

Table 3 Characteristics of oral squamous cell carcinoma patients for the current study.

Patient characteristics		No. (%)	Immunoscores (mean ± standard deviation)	*P* values
Sex	Male mean age: 60.7 years range: 45—81 years	62 (93.9)	9.24 ± 2.28	>0.05
	Female mean age: 72.7 years range: 69—75 years	4 (6.1)	8.67 ± 3.06	>0.05
Age	≤55 years	30 (45.5)	8.55 ± 2.98	>0.05
	>55 years	36 (54.5)	9.43 ± 2.14	
Differentiation	Well	53 (80.3)	8.96 ± 2.29	***< 0.001
	Moderate- to poorly	13 (19.7)	11.36 ± 1.43	
Tumor size	≤2 cm	35 (53.0)	7.92 ± 2.19	***< 0.001
	>2 cm	31 (47.0)	10.14 ± 1.91	
Lymph-node metastasis	Yes	27 (57.5)	10.64 ± 1.91	***< 0.001
	No	20 (42.5)	7.84 ± 2.06	
Pathologic stage	I + II	29 (43.9)	7.32 ± 1.70	***< 0.001
	III + IV	37 (56.1)	10.07 ± 1.98	

***Statistically significance.

24 h, dehydrated in graded alcohols, cleaned in xylene, and embedded in paraffin for subsequent immunohistochemical staining.

Paraffin-embedded 4-μm-thick tissue sections were stained for TRPM6 protein using a primary rabbit polyclonal anti-TRPM6 antibody (Abnova, Walnut, CA, USA; Cat. no. PAB-3252). Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was implemented using graded alcohols. To retrieve the antigenicity, tissue sections were treated three times with microwave radiation in a 10 mM citrate buffer (pH 6.0) for 5 min each. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 45 min to block the endogenous peroxidase activity, and were subsequently incubated in normal goat serum to reduce non-specific binding. Sections were finally incubated for 60 min at room temperature with primary anti-TRPM6 antibody (Abnova; 1: 200) antibody.

The sections were then processed using the standard avidin-biotin peroxidase complex method with respect to the manufacturer's recommendations (Vector Laboratories).[12] Diaminobenzidine (DAB, Merck, Roche, CA, USA; Cat. No. 1718096) was used as a chromogen, and commercial hematoxylin was used for counterstaining. Each set of experiments included a specimen known to express TRPM6, which served as a positive control and ensured the reproducibility of the staining process. Negative controls were included following the same procedure, but with omission of the primary antibody. The scores of the percentage of positive staining (P) were classified as: 0 (<1%); 1 (1—24%); 2 (25—49%); 3 (50—74%); and 4 (75—100%), whereas the scores for the intensity of staining (I) were classified as 0, no staining; 1, light yellow color (weak staining); 2, brown color (moderately strong staining); and 3, dark brown color (strong staining). The total score was then calculated as P × I for each section.[13]

Table 4 Summary of the human oral cancer and precancer cell lines used in the current study.

Cell lines	Tumor source/characteristic	Profile	Ethnicity
Ca9-22[14]	Primary OSCC from gingiva	EGF receptor produced extensively	Japanese
OECM-1[15]	Primary OSCC from gingiva	A history of betel-quid chewing	Taiwanese
DOK[16]	Dysplastic oral keratinocyte from tongue	Mild to moderate ED, with a keratin profile similar to the original dysplasia Non-tumourigenic in athymic nude mice	Caucasian

OSCC: oral squamous cell carcinoma; EGF: epidermal growth receptor; ED: epithelial dysplasia.

Quantification of the immunohistochemical stained sections was implemented by two board certified oral and maxillofacial pathologists independently using the semi-automated image analysis software Image J Version 1.51e. When disagreement was between the two pathologists, aa agreement was attained by mutual discussion.

Cell cultures

The features of the human oral cancer cell lines (Ca9-22,[14] and OECM-1[15]) and oral precancer cell line (DOK)[16] are summarized in Table 4. The human oral cancer cell lines and the normal oral keratinocytes primary culture (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 within a humidified 5% CO_2 atmosphere. The culture medium was changed on every third day. The DOK was cultured in high-glucose DMEM (Hyclone) 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.

Western blot

Cells of human oral cancer cell lines (Ca9-22, and OECM-1), 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. The protein concentrations were measured using a Thermo Pierce Protein Assay Kit. Equal amounts of protein were denatured by adding 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 anti-TRPM6 antibody (Abnova; Cat. no. PAB3252, 1: 500), with species specificity for human tissues and observed molecular weight 171 kDa, and β-actin (Sigma–Aldrich; 1: 5000), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma–Aldrich; 1: 5,000). 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.

Establishment of human OSCC cell cultures with *TRPM6* gene knock-down

RNA interference using commercially-synthesized *TRPM6* siRNA (sense: 5'-GCTCCCTATCTGATAACTCAA-3', antisense: 5'-TTGAGTTATCAGATAGGGAGC-3'; accession no. TRCN0000021588) was performed to create human oral cancer cell lines (Ca9-22, and OECM-1) with *TRPM6* gene knock-down according to the procedures described in our previous study.[17]

Proliferation assay, migration assay, in vitro transwell invasion assay

The assays for cell proliferation, migration, in vitro transwell invasion for human oral cancer cell lines (Ca9-22, and OECM-1) were examined for 48 h post-transfection of *TRPM6* siRNA using the procedures according to our previous study.[17]

Intracellular calcium measurement

Cultured cells of the human OSCC cell lines (Ca9-22, and OECM-1) with and without *TRPM6* knock-down genes were loaded with 5 μM Fluo-4 acetoxymethyl ester (Invitrogen, Waltham, MA, USA) for 30 min at 37 °C following the published procedures.[18] It was then replaced with balanced salt solution (BSS) solution (Merck; Cat. no. 21023CM) containing 1.2 mM calcium for 15 min at room temperature. Intracellular calcium concentration was monitored by fluorescence microscopy with a 10 × objective in a single-wavelength spectrofluorometer. The excitation wavelength used was 495 nm; and the emission wavelength was 510 nm. Endoplasmic reticulum calcium store was depleted by treatment with 5 μM thapsigargin (Invitrogen) in BSS solution containing 0.5 mM EGTA (Sigma–Aldrich). Calcium entry was accomplished by addition of 2 mM $CaCl_2$ (Sigma–Aldrich). The activity was presented as Δ intensity, the difference between the basal and maximal values of F495 after addition of 2 mM $CaCl_2$ in BSS solution.

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 of TRPM6 protein among OSCC, OPMD, and normal oral mucosa, and

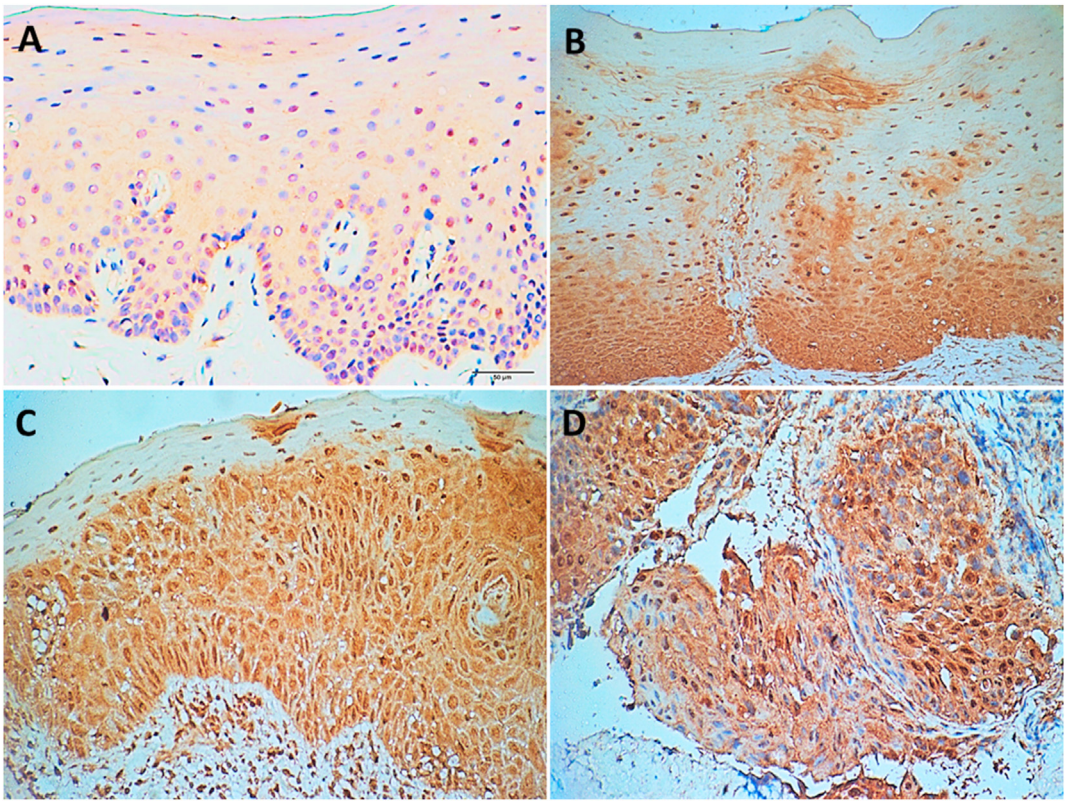

Fig. 1 (A) A representative weak staining of TRPM6 protein in human normal oral mucosa (× 100). (B) A representative moderate staining of TRPM6 protein in human oral potentially disorders with mild epithelial dysplasia (× 100). (C) A representative strong immunohistochemical staining of TRPM6 protein in human oral potentially disorders with moderate to severe epithelial dysplasia (× 100). (D) Representative strong immunohistochemical staining of TRPM6 protein in human oral squamous cell carcinoma (× 100).

to evaluate the differences in proliferation rate and degree of migration, degree of invasion, and intracellular calcium concentration between oral cancer cells with and without siRNA transfection. Chi-square analysis was used to compare the TRPM6 protein expression along with the sex, age, differentiation, tumor size, histological lymph-node involvement, and pathologic stage of the OSCC patients. Nonparametric Kruskal—Wallis tests were used to analyze the results of western blots.

Results

Immunohistochemistry

Weak positive membranous staining of TRPM6 protein was essentially observed in human normal oral mucosa samples (Fig. 1A) whereas moderate positive cytoplasmic staining was predominantly found in OPMD with mild epithelial dysplasia (ED) (Fig. 1B). On the other hand, strong positive cytoplasmic and nuclear staining of TRPM6 protein was also predominantly noted in OPMD with moderate to severe ED (Fig. 1C) and OSCC respectively (Fig. 1D).

Statistical analyses of the mean immunoscores (ISs) of TRPM6 protein for the patients in the current study were summarized in Table 5. There was a significant increase of the mean IS for human OSCC (T1-T4) when compared with human OPMD with moderate to severe ED, OPMD with mild ED, and normal oral mucosa respectively. A significant increase was also noted when comparing the mean IS of human OPMD with moderate to severe ED with OPMD with mild ED. Moreover, a significant increase in the mean IS was noted when comparing cases of human OPMD with moderate to severe ED with normal mucosa samples; a significant increase of mean IS being found when comparing human OPMD with mild ED with normal mucosa samples. Additionally, there was a significant increase of the mean IS of TRPM6 protein with differentiation, tumor size, and lymph-node metastasis respectively (Table 3).

Western blot analysis

Significant upregulation of TRPM6 protein expression in human oral cancer cell lines (Ca9-22, and OCEM-1) was noted as compared with the primary culture of human normal oral keratinocytes (HOK) ($P < 0.01$, Ca9-22; $P < 0.001$, OECM-1; non-parametric Kruskal—Wallis test) (Fig. 2A and B). Human oral precancer cell line (DOK) showed significant overexpression of TRPM6 protein as compared with HOK ($P < 0.01$) (Fig. 2A, D). Significant upregulation of TRPM6 protein expression of Ca9-22, and OECM-1 was also observed as compared with DOK respectively ($P < 0.05$, Ca9-22; $P < 0.01$, OECM-1) (Fig. 2A, C).

Table 5 Statistical analyses for immunohistochemical expression of TRPM6 protein for the patients in the current study.

	P values			
	OSCC (T1-T4)	OPMD moderate to severe OED	OPMD with mild OED	NOM
OSCC (T1-T4) (9.21 ± 2.30)#		*< 0.05	***< 0.001	***< 0.001
OPMD with moderate to severe ED (7.81 ± 2.48)	*< 0.05		**< 0.01	***< 0.001
OPMD with mild ED (5.33 ± 167)	***< 0.001	**< 0.01		***< 0.001
NOM (1.54 ± 1.20)	***< 0.001	***< 0.001	***< 0.001	

#Values within brackets: Immunoscore (mean ± standard deviation); *statistically significance, **statistically significance, ***statistically significance (independent t-test); NOM: normal oral mucosa; OPMD: oral potentially malignant disorders; ED: epithelial dysplasia; OSCC: oral squamous cell carcinoma.

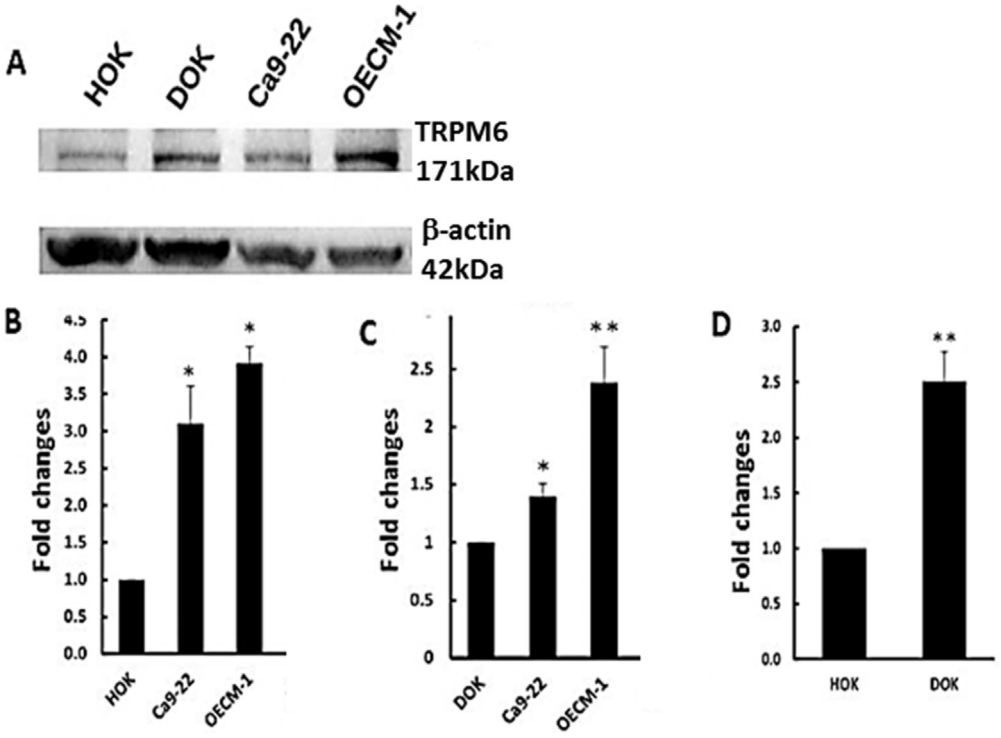

Fig. 2 Upregulation of TRPM6 protein expression in human oral cancer cell lines (Ca9-22, and OCEM-1) as compared with the primary culture of human normal oral keratinocytes (HOK). Human oral precancer cell line (DOK) showed overexpression of TRPM6 protein as compared with HOK. Upregulation of TRPM6 protein expression of Ca9-22, and OECM-1 was observed as compared with DOK respectively (A). Results were quantified using densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to the HOK (B, D) or DOK (C). Bars represent means ± standard error of the mean (*$P < 0.05$; **$P < 0.01$). A representative result of three independent experiments is shown.

Proliferation assay of human OSCC cell cultures with *TRPM6* gene knock-down

The proliferation rates of the human oral cancer cell lines Ca9-22 (Fig. 3A), and OECM-1 (Fig. 3B) with *TRPM6* siRNA transfection for 48 h respectively were significantly decreased as compared with the oral cancer cell lines without *TRPM6* siRNA transfection ($P < 0.05$, Ca9-22; $P < 0.05$, OECM-1).

Migration assay of human OSCC cell cultures with *TRPM6* gene knock-down

The degree of migration of the human oral cancer cell line Ca9-22 (Fig. 4A) and OECM-1 (Fig. 4B) with *TRPM6* siRNA transfection for 48 h was significantly decreased respectively as compared with the oral cancer cell lines without *TRPM6* siRNA transfection ($P < 0.01$, Ca9-22; $P < 0.05$, OECM-1).

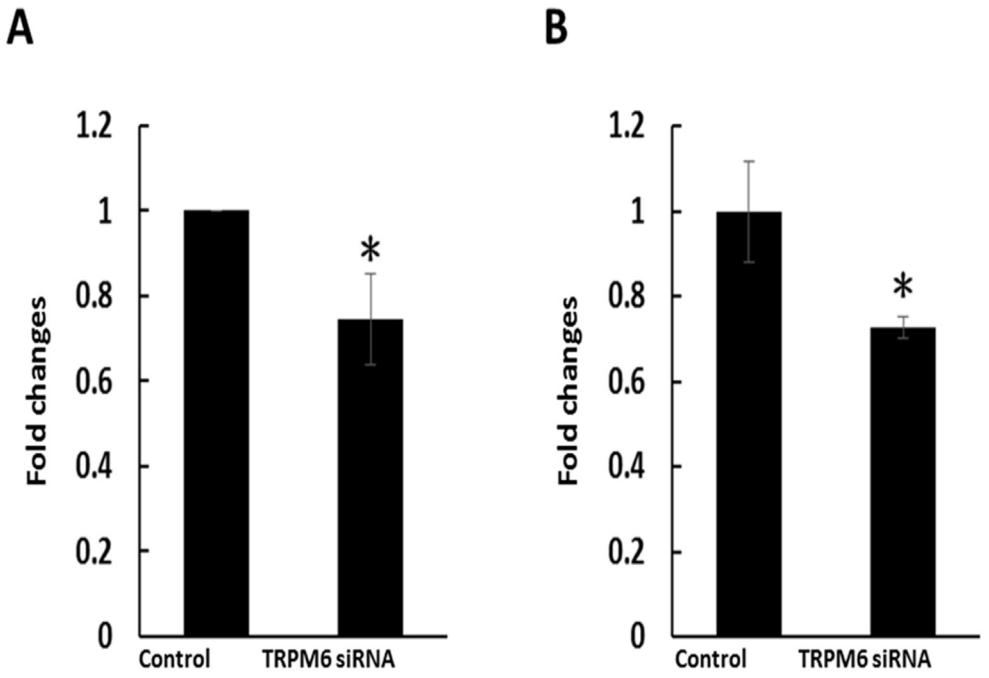

Fig. 3 The proliferation rates of the human oral cancer cell lines Ca9-22 (A), and OECM-1 (B) with *TRPM6* siRNA transfection respectively for 48 h were significantly lower than those of the oral cancer cell lines without *TRPM6* siRNA transfection (Control) (*$P < 0.05$).

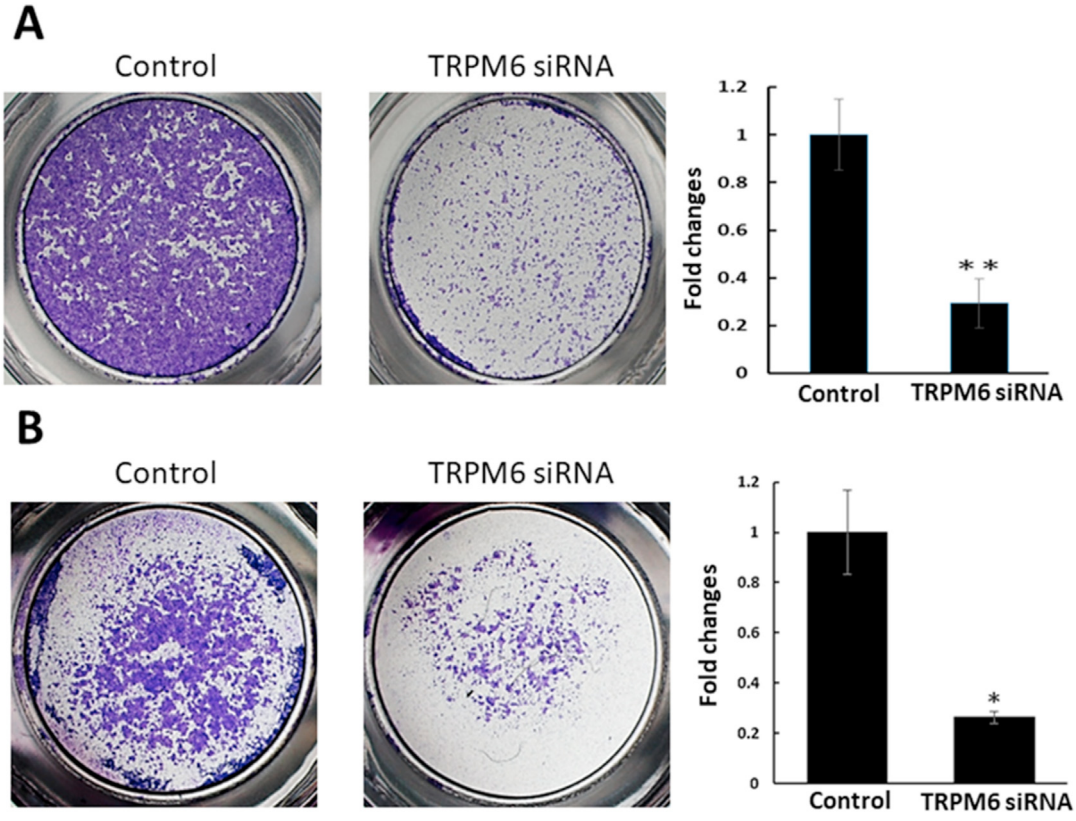

Fig. 4 Degree of migration of the human oral cancer cell lines Ca9-22 (A), and OECM-1 (B) with *TRPM6* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell line without *TRPM6* siRNA transfection (Control) (*$P < 0.05$; **$P < 0.01$). A representative result of three independent experiments is shown.

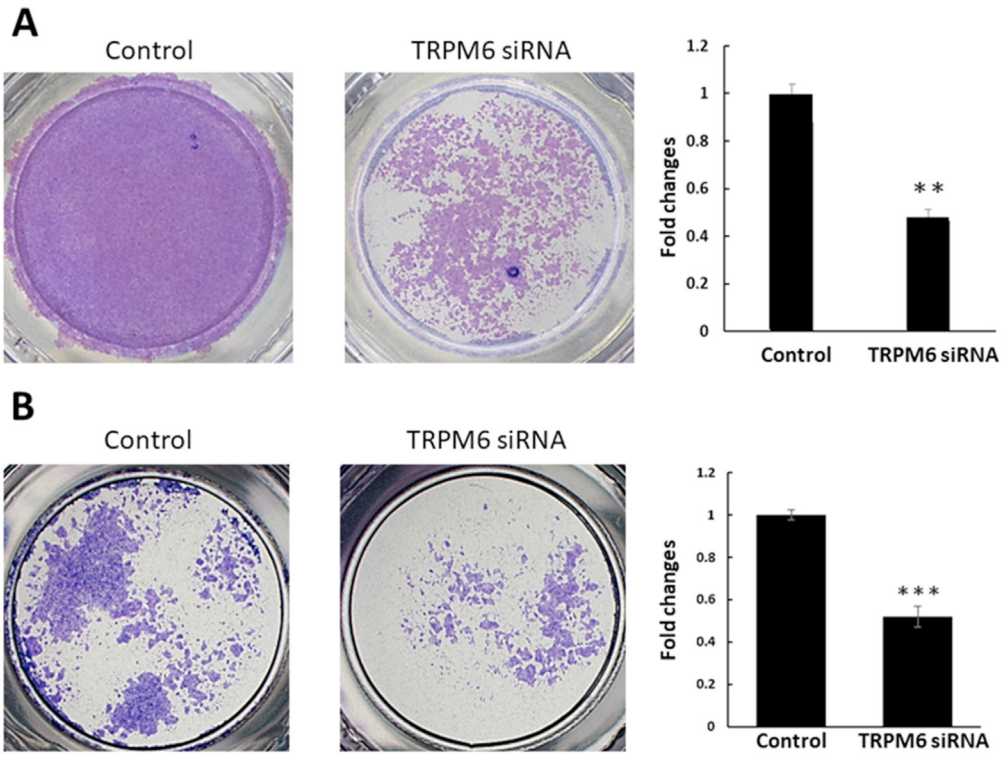

Fig. 5 Degree of invasion of the human oral cancer cell lines Ca9-22 (A), and OECM-1 (B) with *TRPM6* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without *TRPM6* siRNA transfection (control) (**$P < 0.01$; ***$P < 0.001$). A representative result of three independent experiments is shown.

In vitro transwell invasion assay of human OSCC cell cultures with TRPM6 gene knock-down

The degree of invasion of the oral cancer cell line Ca9-22 (Fig. 5A) and OECM-1 (Fig. 5B) with *TRPM6* siRNA transfection for 48 h was significantly decreased respectively as compared with the oral cancer cell lines without *TRPM6* siRNA transfection. ($P < 0.01$, Ca9-22; $P < 0.001$, OECM-1).

Intracellular calcium measurement

The degree of intracellular calcium activity in human oral cancer cell line Ca9-22 (Fig. 6A) and OECM-1 (Fig. 6B) with *TRPM6* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without *TRPM6* siRNA transfection ($P < 0.05$, Ca9-22; $P < 0.05$, OECM-1).

Discussion

Reviewing English literature, there is no study concerning the association of TRPM6 in human oral cancer.[11] Recently, only one study of TRPM2 protein overexpression in human lingual squamous cell carcinoma was reported by Zhao et al.[19] In the current study, significant upregulation of TRPM6 protein expression in human OSCC tissue specimens in comparison with human normal oral mucosa were confirmed by immunohistochemical and Western blot analyses. Compatible with the *in vivo* data, overexpression of TRPM6 protein was also demonstrated in human oral cancer cell lines as compared with a human normal oral mucosa primary culture. Therefore, our results are compiled to the finding of Zhao et al.,[19] suggesting both TRPM2 and TRPM6 proteins have been overexpressed in human OSCC.

Additionally, a significantly higher TRPM6 protein expression was noted in OSCC patients with higher pathologic stage and with lymph-node metastasis in comparison with those without. Moreover, significant decreases in the proliferation rate, degree of migration and invasion of the oral cancer cells with *TRPM6* siRNA transfection were noted as compared with the oral cancer cells without *TRPM6* siRNA transfection. Taken together, the experimental evidences in the current study indicated that TRPM6 have been associated with in human OSCC, and could have potential implication to the development of human OSCC. Thus, the current study, to the best of our knowledge, is the first experiment of TRPM6 on human OSCC. Finally, the present study has also been the first report to confirm the intracellular calcium concentration being significantly decreased when compared TRPM6 knock-down with the oral cancer cell lines without knock-down.

A significant overexpression of TRPM6 protein was observed in human oral precancer cell line DOK and oral cancer cell lines as compared with the primary culture of normal oral mucosa in the current study. Upregulation of TRPM6 protein was also noted in human oral cancer cell lines as compared with DOK. A significantly higher TRPM6 protein expression was noted in human OPMDs with ED in comparison with human normal oral mucosa, which indicated that TRPM6 protein could be associated with malignant progression of human OPMDs. Hence, taking the data

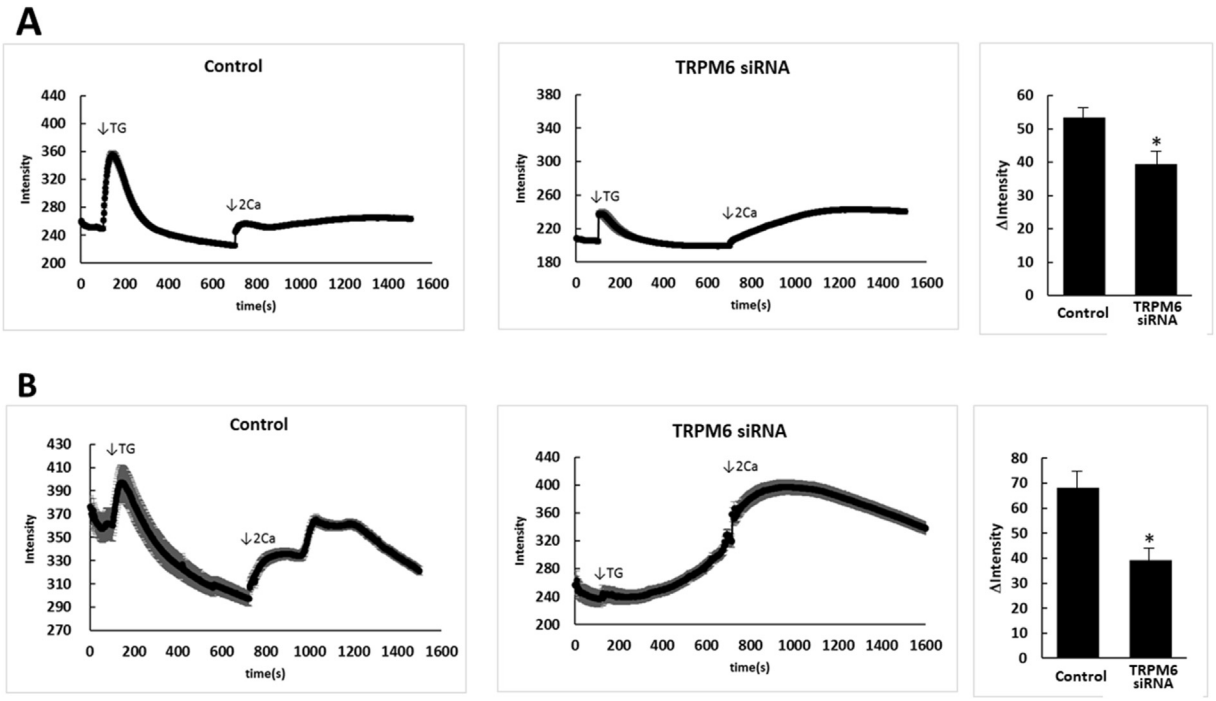

Fig. 6 Degree of intracellular Ca^{2+} activity in human oral cancer cell line Ca9-22 (A) and OECM-1 (B) with *TRPM6* siRNA transfection for 48 h was significantly decreased as compared with the oral cancer cell lines without *TRPM6* siRNA transfection (control) (*$P < 0.05$). A representative result of three independent experiments is shown.

for human OPMDs and OSCCs altogether, the present study, to the best of our knowledge, is the first experiment to demonstrate the potential association of the overexpression of TRPM6 in human OPMD.

There is a shift of membranous staining to cytoplasmic and nuclear staining of TRPM6 proteins from normal mucosa to lesions of OPMD and OSCC, which is consistent to our previous study on another calcium channel (Orai1/STIM1) during human oral squamous cell carcinogenesis;[20] and it is also compatible to the findings of significant localization of TRPM2 protein in nuclei of oral cancer cells,[19] and of prostate cancer cells when compared with non-cancerous cells.[21] Therefore, it is claimed that the subcellular localization of TRPM2 and TRPM6 proteins could implicate to the cancer cell proliferation.[21]

On the other hand, TRPM2, but not TMPM6, has been implicated as a potential target of treatment in some cancers;[22,23] additionally, autophagy-related proteins were considered being associated with prognosis and chemoresistance of gastric cancer;[24,25] however, these have not yet been confirmed in human oral cancer study. Furthermore, TRPM2, not yet for TRPM6, could be activated by ADP-ribose, which is a mitochondrial metabolite produced by oxidative stress.[26–28] Thus, further study is worthwhile to investigate whether the upregulation of TRPM6 is mediated by autophagy and mitochondrial function in human OPMD and OSCC.

In conclusion, the present study would be the first experiment to demonstrate TRPM6 overexpression during human oral squamous cell carcinogenesis. In future, we intend to investigate the potential key roles of autophagy and mitochondrial function to upregulate TRPM6 during human oral squamous cell carcinogenesis.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgement

The work was supported by a grant from the Ministry of Science and Technology, Taiwan (MOST-109-2314-B-037 -015 -MY2).

References

1. Health Promotion Administration Ministry of Health and Welfare. *Cancer registry annual report 2011 Taiwan*, vols. 3–17; 2014.
2. Wang YY, Tail YH, Wang WC, et al. Malignant transformation in 5071 southern Taiwanese patients with potentially malignant oral mucosal disorders. *BMC Oral Health* 2014;14:99.
3. Wright A, Shear M. Epithelial dysplasia immediately adjacent to oral squamous cell carcinoma. *J Oral Pathol* 1985;14: 559–64.
4. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000;1: 11–21.
5. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003;4:517–29.
6. Hajnoczky G, Davies E, Madesh M. Calcium signaling and apoptosis. *Biochem Biophys Res Commun* 2003;304:445–54.
7. Lipskaia L, Lompre AM. Alteration in temporal kinetics of Ca^{2+} signaling and control of growth and proliferation. *Biol Cell* 2004;96:55–68.

8. Rizzuto R, Pinton P, Ferrari D, et al. Calcium and apoptosis: facts and hypotheses. *Oncogene* 2003;22:8619—27.
9. Bell N, Hann V, Redfern CP, Cheek TR. Store-operated Ca^{2+} entry in proliferating and retinoic acid-differentiated N- and S-type neuroblastoma cells. *Biochim Biophys Acta* 2013;1833:643—51.
10. Cui C, Merritt R, Fua L, Pan Z. Targeting calcium signaling in cancer therapy. *Acta Pharm Sin B* 2017;7:3—17.
11. Stokłosa P, Borgström A, Kappel S, Peinelt C. TRP channels in digestive tract cancers. *Int J Mol Sci* 2020;21:1877.
12. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577—80.
13. Sarbia M, Loberg C, Wolter M, et al. Expression of bcl-2 and amplification of c-myc are frequent in basaloid squamous cell carcinomas of the esophagus. *Am J Pathol* 1999;155:1027—32.
14. Kimura Y. Studies on lactate dehydrogenase isoenzymes in a cell line (Ca9-22) derived from carcinoma of the gingiva. *Kokubyo Gakkai Zasshi* 1978;45:20—35.
15. Yang CY, Meng CL. Regulation of PG synthase by EGF and PDGF in human oral, breast, stomach, and fibrosarcoma cancer cell lines. *J Dent Res* 1994;73:1407—15.
16. Chang SE, Foster S, Betts D, Marnock WE. DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. *Int J Cancer* 1992;52:896—902.
17. Wang YY, Wang WC, Sue CW, Hsue CW, Yuan SS, Chen YK. Overexpression of sprouty 1 protein in human oral squamous cell carcinogenesis. *J Dent Sci* 2021;16:21—8.
18. Pan Z, Zhao X, Brotto M. Fluorescence-based measurement of store-operated calcium entry in live cells: from cultured cancer cell to skeletal muscle fiber. *JoVE* 2012;60:3415.
19. Zhao LY, Xu WL, Xu ZQ, et al. The overexpressed functional transient receptor potential channel TRPM2 in oral squamous cell carcinoma. *Sci Rep* 2016;6:38471.
20. Wang YY, Wang WC, Sue CW, Hsue CW, Yuan SS, Chen YK. Expression of Orai1/STIM1 in human oral squamous cell carcinogenesis. *J Dent Sci* 2022;17:78—88.
21. Zheng X, Sikka SC, Huang L, et al. Novel role for the transient receptor potential channel TROM2 in prostate cancer cell proliferation. *Prostate Cancer Prostatic Dis* 2010;13:195—201.
22. Perraud AL, Fleig A, Dunn CA, et al. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature* 2001;411:595—9.
23. Sano Y, Inamura K, Miyake A, et al. Immunocyte Ca^{2+} influx system mediated by LTRPC2. *Science* 2001;293:1327—30.
24. Cao QH, Liu F, Yang ZL, et al. Prognostic value of autophagy related proteins ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer. *Am J Transl Res* 2016;8:3831—47.
25. Ge J, Chen Z, Huang J, et al. Upregulation of autophagy-related gene-5 (ATG-5) is associated with chemoresistance in human gastric cancer. *PLoS One* 2014;9:e110293.
26. Perraud AL, Schmitz C, Scharenberg AM. TRPM2 Ca^{2+}-permeable cation channels: from gene to biological function. *Cell Calcium* 2003;33:519—53.
27. Perraud AL, Shen B, Dunn CA, et al. NUDT9, a member of the Nudix hydrolase family, is an evolutionarily conserved mitochondrial ADP-ribose pyrophosphatase. *J Biol Chem* 2003;278:1794—801.
28. Perraud AL, Takanishi CL, Shen B, et al. Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. *J Biol Chem* 2005;280:6138—48.