Expression of CLIC1 as a potential biomarker for oral squamous cell carcinoma: a preliminary study

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Purpose: CLIC1, a member of the highly conserved class ion-channel protein family, is frequently upregulated in multiple human malignancies and has been demonstrated to play a critical role in cell proliferation, apoptosis, and invasion. However, limited is known about its expression, biological functions, and action mechanism in oral malignancies. We aimed to evaluate whether CLIC1 could be a biomarker for oral squamous cell carcinoma (OSCC).

Methods: Immunohistochemistry was used to analyze the expression of CLIC1 in tissue. CLIC1 protein and mRNA were measured through Western immunoblotting and quantitative real-time PCR. CLIC1 protein expression in plasma was detected via ELISA. A total of 72 OSCC specimens were recruited in this study for evaluation of correlations of CLIC1 with clinicopathological features and survival.

Results: CLIC1 was significantly overexpressed in tissue and plasma of OSCC patients. It was found that upregulated CLIC1 was distinctly correlated with histological grade, TNM stage, and tumor size. Meanwhile, Kaplan–Meier survival analysis showed that OSCC patients with high CLIC1 expression had remarkably poorer overall survival rate than those with low CLIC1 expression. Multivariate Cox regression analysis revealed that CLIC1 was the independent prognostic factor for overall survival rate of OSCC patients. In addition, Pearson correlation analysis showed that CLIC1 was associated with multiple tumor-associated genes.

Conclusion: These results indicated that CLIC1 acts as a molecular target in OSCC and may present a novel diagnostic marker and therapeutic target for OSCC.

Keywords: chloride intracellular channel 1, oral squamous cell carcinoma, expression, prognosis, biomarker

Introduction

It has been reported that oral squamous cell carcinoma (OSCC) is the commonest oral malignancy, with ~300,000 new cases worldwide annually.¹ Although tremendous progress has been achieved in radical surgery combined with postoperative radiotherapy/chemoradiotherapy, patient 5-year survival rate has not improved essentially in recent decades. Therefore, early detection, early diagnosis, and early treatment are very important to increase the 5-year survival rate of patients with OSCC. Novel and specific biomarkers are required to solve this problem.

Molecular changes are regarded as potential biomarkers for cancers. In OSCC, some genes have been reported as potential biomarkers, such as *KPNA2*, *HOXA13*, *HOXD13*, *EZH2*, and *LEF1*.²⁻⁵ They had certain value in the diagnosis, treatment, and prognosis of OSCC, but it was not enough to obtain sensitivity and specificity for OSCC only by them. CLIC1 is a member of the highly conserved category of chloride-channel proteins that exists in both integral membrane and soluble forms.⁶ It is abundant in the apical domains of epithelia.⁷ Recently, CLIC1 has been reported to be responsible for regulating the

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Table 1 Function of CLIC1 in different cancers

Cancer	Sample	Function	Pathway	Reference(s)
Gastric cancer	Tissue	Promotes invasion and migration and inhibits apoptosis	MAPK–Akt	14
Gallbladder cancer	Tissue	Increases drug sensitivity	–	17, 12
Glioblastoma	Tissue	Regulator of glioblastoma growth	–	18
Pancreatic cancer	Tissue	Putative oncogene	–	9
Sarcoma	Tissue	Receptor oncotargets	ERK	23
Colon cancer	Cells	Metastasis	–	11
Breast cancer	Cells	Migrates or invades	–	15
Ovarian cancer	Serum	Serological tumorigenesis biomarkers	–	13, 19, 22
Colorectal cancer	Cells	Migration and invasion	RVD	24
Endometrioid endometrial cancer	Tissue	Chemoresistance	–	21
Laryngeal cancer	Cells	Promotion of ROS production	ROS	10
Nasopharyngeal carcinoma	Tissue/plasma	Plasma tumor marker	–	8
Gallbladder cancer	Cells	Metastasis	–	20
Hepatocellular carcinoma	Cells	New targets for drug development	–	16

Abbreviations: RVD, regulatory volume decrease; ROS, reactive oxygen species.

migration, invasion, apoptosis, and drug sensitivity of cancer cells and is involved in several signaling pathways, such as MAPK–ERK, PI3K–Akt, and MAPK–p38 (Table 1).^{8–24} However, whether CLIC1 plays an important role in tumorigenesis and progression of OSCC remains elusive.

To determine the role of CLIC1 in OSCC, we examined CLIC1 mRNA and protein expression in OSCC tissues and plasma. Moreover, we analyzed the association between CLIC1 expression and clinicopathological characteristic in OSCC. In addition, we elucidated the prognostic role of CLIC1 in OSCC patients. Finally, we found that CLIC1 was associated with multiple tumor-associated genes.

Methods

Sample preparation and clinicopathological data

An immunohistochemistry analysis was conducted on OSCC specimens collected from 72 patients who had undergone radical surgical resection (without prior radiotherapy or chemotherapy) from January 2012 to February 2013 at the Research Center of Pathology, Chongqing Medical University. A total of 26 pairs freshly frozen OSCC and corresponding noncancerous tissue samples were used for quantitative real-time (qRT) PCR and Western blot; 76 plasma samples included 54 OSCC plasma samples collected from OSCC patients and 22 healthy controls. All tissue and blood samples were collected at the Stomatological Hospital of Chongqing Medical University, China from 2017 to 2018 and used for qRT-PCR, Western blotting, and ELISA analysis.

This study was approved by the Ethics Committee of the Stomatological Hospital of Chongqing Medical University, and all patients provided informed written consent before enrollment. Collection and processing of tissue and blood samples were conducted in accordance with the Declaration of Helsinki.

All tissue samples and blood samples were collected from patients and stored using the standard procedure.²⁵ These samples had undergone only one freeze–thaw cycle before the study was carried out. Telephone interviews were conducted routinely for at least 6 months during follow-up. Time from diagnosis to death or last follow-up visit was regarded as survival time and was checked at the last telephone interview in February of 2018, regardless of whether the patient was alive or not.

Immunohistochemistry

Standard immunoperoxidase staining was used for immunohistochemical staining and CLIC1 protein expression in normal and malignant samples evaluated. Each section was semiquantitatively scored based on the extent and intensity of immunoreactivity: 0, 10% immunoreactive cells; 1, ≤25% immunoreactive cells; 2, 25%–75% immunoreactive cells; and 3, >75% immunoreactive cells. Additionally, staining intensity was graded on a 0–3 scale: 0, negative; 1, weak; 2, intermediate; and 3, strong. The final score was designated as the sum of extent and intensity and samples categorized as negative (0), weak (1–2), moderate (3), and strong staining (4–6). For statistical calculations, moderate and strong staining

Table 2 Quantitative real-time PCR primers used in this study

Gene	Primer sequence	
<i>CLIC1</i>	Forward: AATCAAACCCAGCACTCAATG	Reverse: CAGCACTGGTTTCATCCACTT
<i>GAPDH</i>	Forward: CAACGACCCCTTCATTGACC	Reverse: CGCCAGTAGACTCCACGACAT
<i>PA28B</i>	Forward: CTTTCCAGGAGGCTGAGGAA	Reverse: GGGAAGTCAAGTCAGCCACA
<i>MMP2</i>	Forward: GCGCCGTCGCCCATCATCAA	Reverse: AGCTCTCCTTGGGGCAGCCA
<i>MMP9</i>	Forward: GGGACGCAGACATCGTCATC	Reverse: TCGTCATCGTCGAAATGGGC
<i>ANXA7</i>	Forward: GCTATCCCCCAACAGGCTAC	Reverse: CACGTTCTGAGTTCCTGCT
<i>GSN</i>	Forward: CCGCTGTCGCCACCAT	Reverse: GAATTCTCCACACGCCAGA
<i>MMP12</i>	Forward: CCAACGCTTGCCAAATCCT	Reverse: CCACGGTAGTGACAGCATCAA
<i>MMP13</i>	Forward: ACCCTGGAGCACTCATGTTTCTA	Reverse: TGGCATCAAGGGATAAGGAAGGGT
<i>SERPINB5</i>	Forward: CACGAGTTGTGCTCCTCGC	Reverse: CATACAGAACGTGGCCTCCA

scores were considered positive and other final scores considered negative.

Western immunoblotting

Total protein extracts from tissue were separated by 10% SDS-PAGE (20 mg per lane), then transferred onto a polyvinylidene fluoride membrane. Membranes were blocked and then probed with primary antibodies against CLIC1 (1:800 dilution; Abcam, Cambridge, UK) and GAPDH (1:500 dilution; Sino Biological, Beijing, China) at 4°C overnight. The secondary antibody (1:500 dilution; SAB, China) was placed under infrared light at room temperature for 1 hour and visualized using an enhanced chemiluminescence-detection reagent.

Real-time quantitative PCR

Total RNA was isolated from tissue using Trizol reagent (Takara, Kusatsu, Japan). The primers used in this study were shown in Table 2. RNA was reverse-transcribed into cDNA with a ReverTra Ace qRT-PCR kit (Takara, Japan). qRT-PCR was performed on a StepOnePlus RT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The reaction comprised an initial denaturation step of 95°C for 30 minutes, followed by 39 cycles of 95°C for 5 seconds, 60°C for 34 seconds, and a final extension step of 95°C for 10 minutes. The mRNA-expression level of the target gene in each sample was normalized to the GAPDH control.

ELISA

Blood samples in heparin tubes were centrifuged (2,000 g) at 4°C for 10 minutes to obtain plasma and frozen at -80°C for further analysis. CLIC1 protein levels in plasma were measured with human ELISA kits according to the manufacturer's

instructions. The results were processed with an automated analyzer (PerkinElmer Inc., Waltham, MA, USA).

Statistical analysis

Data were analyzed using SPSS 20.0. All data are expressed as means $\pm$ SD. Independent Student's *t*-tests and χ^2 tests were used to assess the effects of CLIC1 on tissue and plasma. The Kaplan–Meier test was used for univariate survival analysis. The Cox proportional-hazard model was used for multivariate analysis and for determining 95% CIs. Pearson correlation coefficients were used to assess correlations among tumor-associated genes. Results were regarded as significant at $P < 0.05$.

Results

CLIC1 significantly upregulated in OSCC tissue

To evaluate CLIC1-expression status in human OSCC tissue, 26 pairs of OSCC and noncancerous oral tissue were collected to examine CLIC1 mRNA expression by qRT-PCR. Results showed that *CLIC1* mRNA expression was upregulated in OSCC tissue (2.31 ± 1.27) compared with noncancerous oral tissue (0.34 ± 0.32 , $P = 0.003$; Figure 1C). CLIC1 protein expression was higher in OSCC tissue (1.16 ± 0.44) than paired noncancerous oral tissue (0.41 ± 0.93) via Western blot, and this was consistent with qRT-PCR results. Furthermore, CLIC1 protein expression in different histological grades showed significant differences ($P < 0.001$; Figure 1D and E).

CLIC1 expression associated with clinicopathological features in OSCC patients

To elucidate the biological functions of CLIC1 in OSCC development, the expression of this protein in OSCC was

Figure 1 CLIC1 expression was significantly increased in oral squamous cell carcinoma (OSCC) tissue.

Notes: (A) CLIC1 expression in normal, well differentiated, moderately differentiated, and poorly differentiated OSCC tissue via IHC. (B) Average staining scores of CLIC1 expression in normal, well differentiated, moderately differentiated, and poorly differentiated OSCC tissue. (C) Quantitative real-time PCR showed CLIC1-expression levels in 26 tissue pairs ($P=0.002$). (D) Western blotting of CLIC1 protein expression. (E) CLIC1 protein expression in normal tissue, well differentiated OSCC tissue, moderately differentiated OSCC tissue, and poorly differentiated OSCC tissue via Western blot. P -values calculated by Student's t -test. Data presented as mean $\pm$ SD. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

compared with normal control tissue via IHC. Our results showed that CLIC1 is localized mainly in the nucleus and can also be observed in membranes and cytoplasm of cancer cells. Its protein expression differed in tumors of different differentiation (Figure 1A). Approximately 70.83% (51 of 72) of the OSCC patients displayed positive CLIC1-immunoreactive cells, while only 10.00% (two of 20) of the patients had positive immunoreactive cells in the normal control group ($P<0.001$; Figure 1B). In addition, there were significant CLIC1 proteins of histological grade. Then, we evaluated correlations between CLIC1 expression and histopathological parameters in the OSCC patients. Table 3 showed the CLIC1 overexpression remarkably related to

histological grade ($P=0.049$), TNM stage ($P=0.025$), and tumor size ($P=0.021$), but not with age, sex, or lymph-node metastasis. These results indicated a potential role of CLIC1 expression in promoting incursionary OSCC phenotypes.

Evaluation of CLIC1 expression predicts poor prognosis in patients

Among the 72 OSCC patients, only 64 patients' survival information was collected through phone-call follow-up. Histopathological subtype ($P=0.001$), TNM stage ($P=0.046$), and lymph-node metastasis ($P<0.001$) were significantly relevant to survival time by Kaplan–Meier survival analysis. Survival figures showed that the survival time of the

Table 3 Association of CLIC1 expression with the clinicopathological characteristics of oral squamous cell carcinoma

	Category	Cases	CLIC1		
			Positive cases, n (%)	χ^2	P-value
Age, years	<60	28	18 (64.29)	0.019	0.888
	≥60	44	29 (65.91)		
Sex	Male	47	30 (63.83)	0.125	0.723
	Female	25	17 (68.00)		
Location	Tongue	18	12 (66.67)	1.558	0.669
	Buccal mucosa	17	13 (76.47)		
	Gingiva	8	6 (75.00)		
	Others*	29	16 (55.17)		
Histological grade	Well differentiated	32	16 (50.00)	6.042	0.049 [#]
	Moderately differentiated	20	15 (75.00)		
	Poorly differentiated	20	16 (80.00)		
TNM stage	0–I	20	9 (45.00)	5.023	0.025 [#]
	II–IV	52	38 (69.23)		
Lymph-node metastasis	Negative	58	35 (60.34)	2.18	0.139
	Positive	14	12 (85.71)		
Tumor size (cm)	<3	30	15 (50.00)	5.3	0.021*
	≥3	42	32 (76.19)		

Notes: *Included floor of mouth, lips and palate; [#]statistically significant. P-values determined using Kruskal–Wallis and χ^2 tests.

CLIC1-positive patients was obviously lower than that of patients with CLIC1-negative expression ($P=0.025$; Table 4, Figure 2). To identify risk factors of prognosis precisely, univariate Cox regression analysis was used. These data revealed that CLIC1 expression, histological grade, and lymph-node metastasis were adversely correlated with postoperative survival time, suggesting that overexpression of CLIC1 is a risk factor for OSCC (Table 5).

Determination of CLIC1 as a plasma OSCC biomarker

As CLIC1 was overexpressed in OSCC tissues, we speculated that CLIC1 could be detected in blood samples of OSCC patients. To determine this possibility, a sensitive ELISA was conducted to detect the expression of CLIC1 protein in plasma. Plasma samples of 30 OSCC patients without any treatment, 14 who had undergone resection only, ten who

Table 4 Univariate analysis of overall survival of oral squamous cell carcinoma patients

	Category	Cases (n)	Mean survival time, months (95% CI)	P-value
Age, years	<60	22	56.818 (46.840–66.796)	0.38
	≥60	42	55.490 (48.087–62.892)	
Sex	Male	43	54.680 (47.618–61.742)	0.746
	Female	21	54.828 (44.793–64.863)	
Location	Tongue	17	62.035 (53.044–71.026)	0.231
	Buccal mucosa	15	46.558 (31.198–61.917)	
	Gingiva	7	70.167 (68.676–71.685)	
	Others*	25	53.520 (43.876–63.963)	
Histological grade	Well differentiated	30	62.182 (55.205–69.158)	0.001 [#]
	Moderately differentiated	16	63.063 (56.012–70.113)	
	Poorly differentiated	18	38.333 (24.606–52.060)	
TNM stage	0–I	19	66.819 (60.844–72.793)	0.046 [#]
	II–IV	45	51.386 (43.666–59.105)	
Lymph-node metastasis	Negative	51	61.833 (55.942–67.725)	<0.001 [#]
	Positive	13	33.359 (21.257–45.461)	
Tumor size (cm)	<3	27	58.213 (50.116–66.310)	0.833
	≥3	37	53.304 (45.157–61.451)	
CLIC1	Negative	26	63.917 (56.705–71.129)	0.025 [#]
	Positive	38	50.431 (42.098–58.765)	

Notes: *Floor of mouth, lips, and palate; [#]statistically significant. P-valued determined using log-rank test.

Figure 2 CLIC1 was correlated with overall survival rate in oral squamous cell carcinoma patients.

Notes: Kaplan–Meier plots of overall survival of patients with positive and negative CLIC1-expression scores. P-values calculated by log-rank test.

had undergone resection and chemotherapy, and 22 healthy controls were examined. CLIC1 protein expression was significantly higher in OSCC plasma than that of healthy controls ($P=0.023$), CLIC1 protein expression obviously lower in resection patients than those of OSCC patients ($P=0.039$), and CLIC1-expression levels in resection- + chemotherapy-treated patients aberrantly lower than in resection-only patients and healthy controls ($P=0.002$ and $P=0.000$, respectively; Figure 3A). Moreover, CLIC1-expression level in OSCC patients' plasma was also obviously higher in stage I–II than III–IV ($P=0.002$; Figure 3B). However, there was no statistical difference between moderate differentiation and good differentiation ($P=0.459$; Figure 3C).

Correlation of *CLIC1* and tumor-associated gene expression in OSCC tissue

In order for profound understanding of CLIC1, tumor-associated genes were detected. In OSCC tissue, *CLIC1* mRNA-expression level of was positively correlated with

PA28B, *MMP2*, *MMP9*, *MMP13*, and *MMP12* mRNA-expression levels, whereas *CLIC1* mRNA expression level was negatively correlated with *ANXA7*, *GSN*, and *SERPINB5* mRNA-expression levels (Figure 4). Correlations between mRNA expression of *GSN*, *PA28B*, *MMP2*, *MMP9*, and *MMP13* were significantly different from those of *CLIC1* ($P=0.043$ and $P<0.001$).

Discussion

In this research, our results suggested that CLIC1 plays an important role in the tumorigenic potential of human OSCC. CLIC1 mRNA and protein were overexpressed in OSCC tissue and CLIC1 protein closely associated with patients' overall survival. Investigation indicated that CLIC1 is an independent metastasis and prognostic factor in OSCC. Moreover, CLIC1 protein was highly expressed in OSCC plasma and associated with therapy. Furthermore, our data demonstrated that CLIC1 may be involved in the pathway of development and metastasis of OSCC. Therefore, we consider CLIC1 has potential as a novel molecular therapeutic target and a possible outcome predictor.

CLIC1 has been reported to be upregulated in human malignant tumors of the colon, brain, liver, stomach, and lung.^{14,22,24,26,27} It was evaluated to be the most penetrating receptor among commonly dysregulated proteins within three sarcomas.²³ This protein plays an important role in the metastasis of colonic cancer.²⁴ In gliomas, CLIC1 expression is inversely related to patient survival.²⁷ Moreover, CLIC1 participates in regulation of aggression in human lung adenocarcinoma.²⁶ Consistently with upregulated CLIC1 expression in a large number of tumors, our data suggested that CLIC1 mRNA and protein were highly expressed in OSCC tissue. Firstly, our study discovered that CLIC1 exists mainly in the nucleus, cytoplasm, and cell membranes of OSCC patients. Next, this research found that increased CLIC1 protein

Table 5 Multivariate analysis of overall survival of oral squamous cell carcinoma patients

	Category	HR (95% CI)	P-value
CLIC1 expression	Negative	5.354 (1.463–19.586)	0.011 ^a
	Positive		
Histological grade	Well differentiated	0.180 (0.038–0.859)	0.032 ^a
	Moderately differentiated		
	Poorly differentiated		
TNM stage	0–I	1.680 (0.413–6.836)	0.469
	II–IV		
Lymph-node metastasis	Negative	4.743 (1.607–14.001)	0.005 ^a
	Positive		
Tumor size (cm)	<3	1.197 (0.244–5.871)	0.825
	≥3		

Note: ^aStatistically significant.

Figure 3 Elevated CLIC1 protein levels in oral squamous cell carcinoma (OSCC) plasma samples via ELISA.

Notes: (A) Plasma CLIC1 protein levels of healthy controls (n=22), OSCC patients (n=30), resection operation (RO) patients (n=14), and RO + chemotherapy (CT)-treated patients (n=10). (B) Plasma CLIC1 protein levels of TNM stage (P=0.009). (C) Plasma CLIC1 protein levels of histological grade (P=0.459). *P<0.05; **P<0.01; ***P<0.001; P-values calculated by Student's t-test.

expression was relevant not only to tumor progression but also to poor patient survival, indicating its materiality in OSCC advancement. Notably, Cox multivariate analysis suggested that CLIC1-overexpression level was an independent

prognostic factor for OSCC patients. Therefore, CLIC1 is a potential molecular therapeutic target for OSCC.

Increasing attention has been paid to blood research. CLIC1 has also been detected in the blood of patients, suggesting that

Figure 4 Correlations between CLIC1 and PA28B, MMP2, MMP9, ANXA7, GSN, MMP12, MMP13, and SERPINB5 mRNA in OSCC according to qRT-PCR data.

Notes: (A) Correlation between CLIC1 and PA28B. (B) Correlation between CLIC1 and MMP2. (C) Correlation between CLIC1 and MMP9. (D) Correlation between CLIC1 and ANXA7. (E) Correlation between CLIC1 and GSN. (F) Correlation between CLIC1 and MMP12. (G) Correlation between CLIC1 and MMP13. (H) Correlation between CLIC1 and SERPINB5. r-values calculated by Pearson correlation analysis. P-values calculated by Student's t-test.

Abbreviations: OSCC, oral squamous cell carcinoma; qRT-PCR, quantitative real-time PCR.

CLIC1 could be useful as a blood marker. CLIC1 proteins secrete from tumor cells and can enter the circulation.²⁸ Chang et al first reported that there exists the possibility of CLIC1 as a plasma marker for nasopharyngeal carcinoma.⁸ CLIC1 has been confirmed to be overexpressed in ovarian cancer patient serum.²² It seems to have recently become a popular view that CLIC1 may be applied as general cancer screening markers. We detected CLIC1 protein in plasma samples of OSCC patients, and found that its expression level was significantly higher than in normal controls. CLIC1 protein expression in plasma was also correlated with advanced tumor status. Furthermore, it was closely associated with treatment. These results indicated that CLIC1 expression could be regarded as a biomarker and in indices for tumor metastasis and prognosis in OSCC. However, CLIC1 needs to be evaluated with further testing of larger patient cohorts to avoid false positives or false negatives before clinical application. In addition, because CLIC1 is associated with histological grade, TNM, and tumor size, but not with lymph-node metastasis, it may require coordination with other biomarkers for diagnosis and treatment of OSCC. CLIC1 could be included in a group of select biomarkers for early detection.

Although the potential role of CLIC1 in cancer is not clear, there have been many advances. Some studies have reported that CLIC1 is linked to many tumor-associated proteins. Wei et al found that downregulation of CLIC1 increased maspin expression and reduced MMP2, MMP9, MMP12, and VEGF expression. Conversely, upregulation of CLIC1 decreased maspin expression and increased MMP2, MMP12, VEGF, and MMP13 expression.²⁹ Zhang et al provided the novel insight that CLIC1 has a vital role in hepatocarcinoma metastasis, perhaps via regulation of gelsolin and annexin A7 expression.³⁰ Other research reported that knockdown of *PA28B* increased tumor invasion and migration through upregulation of CLIC1.³¹ What is more, CLIC1 may be correlated with the regulation of integrin-family proteins, which leads to subsequent regulation of the PI3K–AKT, MAPK–p38, and MAPK–ERK pathways.¹⁴ To evaluate the biological function of CLIC1 in OSCC pathogenesis further, we compared CLIC1 with tumor-associated genes using PCR. We found that CLIC1 was relevant to a variety of tumor genes, especially *PA28B*, MMP2, and MMP13, whereas *PA28B* and matrix metalloproteinases (MMPs) played an important role in tumor metastasis and invasiveness.^{31,32} These data indicated that CLIC1 may be involved in the pathway of development and metastasis of OSCC. In vitro experiments and experiments on animals are required to elucidate the mechanism of CLIC1 acting in OSCC.

Conclusion

In summary, this study detected the expression of CLIC1 in OSCC tissue and plasma. The results pointed to CLIC1 perhaps being a novel biomarker for OSCC diagnosis, prognosis, and even early screening. The potential use of CLIC1 as an OSCC biomarker needs to be evaluated with further testing of larger patient cohorts and in-depth mechanical experiments.

Acknowledgment

This work was supported by funds provided by the Program for Innovation Team Building at the Institutions of Higher Education in Chongqing in 2016, Chongqing Municipal Key Laboratory of Oral Biomedical Engineering of Higher Education, Chongqing Research Program of Basic Research and Frontier Technology (PYZD201601), the Scientific and Technological Research Program of the Health and Family Planning Commission of Chongqing Province (2017ZDXM019 and 2016MSXM046), and Science and Technology Projects Foundation of Chongqing Yubei District Agricultural Bureau (2017-37).

Disclosure

The authors report no conflicts of interest in this work.

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