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Original Research Article

KLK4 affects the biological behavior of oral squamous cell carcinoma through Wnt/β-catenin and PI3K/Akt signaling pathways

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Abstract

Purpose: *To investigate the effect of kallikrein 4 (KLK4) on oral squamous cell carcinoma (OSCC), and its mechanism of action.*
Method: *Human tongue SCC cell line TCA-8113 was cultured, and the interference fragment and irrelevant sequence fragment of KLK4 gene were designed and synthesized. These fragments were constructed into pgcsi-h1 plasmid. Then, the TCA-8113 cells were cultured for 24 h, followed by transfection, after which TCA-8113-NC and TCA-8113-siKLK4 cells were obtained. The mRNA expression of KLK4 was determined using quantitative real-time-polymerase chain reaction (PCR). Proliferative potential was assessed using MTT method, while apoptosis was determined by flow cytometry. Furthermore, cell migration was evaluated by scratch test, and Transwell cell invasion experiment was used to determine cell invasion. Immunoblot assay was applied to determine the expressions of E- and N-cadherins, vimentin, uPA, Wnt1, β-Catenin, p-pi3k and p-Akt.*
Result: *Compared with TCA-8113-nc, the expression of KLK4 in TCA-8113-siKLK4 cells was significantly down-regulated, while, relative to TCA-8113-NC, TCA-8113-siKLK4 cells had significantly reduced cell proliferation, invasion and migration, and enhanced apoptosis ($p < 0.05$). Moreover, there was significant up-regulation of E- and N-cadherins, vimentin, uPA, Wnt1, β-Catenin, p-pi3k and p-Akt in TCA-8113-siKLK4 group, relative to TCA-8113-NC ($p < 0.05$).*
Conclusion: *KLK4 enhances the multiplication, metastasis and invasive potential of OSCC cells, but suppresses apoptotic changes via activation of Wnt/β-catenin/PI3K/Akt signal route. The potential of Kallikrein-4 as biomarker for OSCC cells should be determined in suitable animal models.*

Keywords: *KLK4, Wnt /β-Catenin, PI3K/Akt signal route, OSCC*

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INTRODUCTION

It has been reported that OSCC is a very debilitating illness with high mortality, and it is responsible for more than 20 % of all head and neck malignancies [1]. With societal development and rapid changes in lifestyles, the incidence of oral squamous cell carcinoma has gradually increased due to poor living habits, environmental pollution and other factors. Studies have found that invasiveness and metastatic changes are crucial events

responsible for mortality of persons with carcinoma [2].

Kallikrein-4 (KLK4) which belongs to kallikrein gene group, is a serine protein kinase gene highly distributed in several tissues and organs of the body. Some researchers have found that KLK4 is vital for tumor growth and metastasis, and due to its involvement in multiplication, apoptosis and metastasis of cancerous cells, it is a biomarker for various cancers [3]. Research has revealed that KLK4 has vital relationship with the pathogenesis of oral SCC, although not much is known about the underlying mechanism [4].

This research was done to study the effect of KLK4 on biological behavior of oral SCC cells, as well as the underlying mechanism.

EXPERIMENTAL

Cells

Human tongue SCC TCA-8113 cells (Shanghai Yaji Biotechnology Co. LTD.) were used in this study.

Equipment and reagents

The instruments used, and their suppliers (in parenthesis) were: high-speed refrigerated centrifuge (Shanghai Luxiang Yi Centrifuge Instrument Co. Ltd., model: TGL-16M); biological microscope (Shenzhen Sinico Optical Instrument Co. Ltd., model: XK-DZ002); phosphate buffer (Shanghai Yuanye Biotechnology Co. Ltd); real-time quantitative PCR (Shanghai Hongshi Medical Technology Co. Ltd., model: SLAN-96P); RPMI-1640 culture medium (Shanghai Junrui Biotechnology Co. Ltd.); fetal bovine serum (Shanghai Jianglin Biotechnology Co. Ltd.); flow cytometer (Beijing Amag Trading Co. Ltd., model: BD FACSCanto Ⅱ); constant-temperature incubator (Shanghai Senxin Experimental Instrument Co. Ltd., model: DRP-9082); rabbit anti-mouse Caspase-3 monoclonal antibody (Shanghai Yisheng Biotechnology Co. Ltd.); Bax rabbit anti-mouse polyclonal antibody (Wuhan Emijie Technology Co. Ltd.); Bcl-2 rabbit anti-mouse polyclonal antibody (Shanghai Hufeng Chemical Co. Ltd.); p-akt rabbit anti-polyclonal antibody (Beijing Baolaibo Technology Co. Ltd.), and P-akt rabbit anti-polyclonal antibody (Shanghai Xinfan Biotechnology Co. Ltd.).

Cell culture

The TCA-8113 cells were cultured in RPMI-1640 medium. When the cell density reached about 85 %, 0.25 % trypsin was added, and the culturing was stopped when the cells became rounded. The medium was changed daily during the culturing process.

Cell transfection

Interference fragment and irrelevant fragment of KLK4 gene were designed and synthesized. The fragments were constructed into PGCSI-H1 plasmid, and were transfected into TCA-8113 cells after 24-h culture. The cells were cultured, with medium replacement with 2 mL of non-serum basal medium, followed by culturing for 2 h. After incubation at room temperature for 24 h, complete medium (200 g/mL) was added to the culture plate for antibiotic screening. The selected cell groups were sub-cultured to obtain stable target cell lines. Then, TCA-8113-nc and TCA-8113-siKLK4 cells were cultured at 37 ℃ in a 5 % CO_2 incubator at constant temperature. When the cell density was more than 90 %, the cells were harvested and used for subsequent investigations.

Quantitative real time- polymerase chain reaction (qRT-PCR)

The KLK4 mRNA expression in each group was assayed with qRT-PCR, following extraction of total RNA and reverse-transcription of the RNA to cDNA. Thereafter, qPCR was carried out in line with standard procedures, with GAPDH as internal reference gene. Relative concentrations of mRNA were calculated using the $2^{\Delta\Delta Ct}$ procedure.

MTT assay

Cell multiplication was determined using MTT method: TCA-8113-NC and TCA-8113-siKLK4 cells inoculated in 96-well culture plates were cultured for 24, 48 and 72 h. Each group of cells was set up in 5 wells. Proliferation potential was measured using MTT assay. Results from multiple measurements were averaged.

Flow cytometry

Cell apoptosis in each group was measured flow cytometrically. In Scratch test, TCA-8113-nc and TCA-8113-siKLK4 cells were cultured in serum-free medium after addition of mitomycin C (1 μg/mL), for 1 h. The cells in each group were scratched with a 200-μL pipette tip, after which the cells were examined under a light microscope and photographed. The distances migrated in each group were compared between the two groups at 0, 24 and 48 h.

Transwell assay

Invasiveness potential was determined with Transwell method. The TCA-8113-NC and TCA-8113-siKLK4 cells were cultured and prepared into cell suspensions, and 800 μL 20 % FBS was put into Transwell lower compartment, while the upper chamber contained 200 μL cell suspension. The invaded cells were subjected to 20-min fixation in 4 % formaldehyde solution, followed by 5-min staining with 0.5 % crystal violet. Unreacted dyestuff was washed off with distilled water, and the invaded cells were examined microscopically.

Western blot assay

Western blot assay method was used to determine the protein expression levels of E-cadherin, N-cadherin, Vimentin, urokinase-type plasminogen activator (uPA), Wnt1, β-catenin, p-PI3K and p-Akt, in each group.

Statistical analysis

The SPSS 22.0 software was used for statistical analyses of data. Measurement data are expressed as mean ± standard deviation (SD). Two-group comparison of measurement data was done with one-way ANOVA and LSD *t*-test. Values of $p < 0.05$ were indicative of statistical significance of differences.

RESULTS

Expression level of KLK4

Table 1 shows that, compared with TCA-8113-NC, the expression level of KLK4 in TCA-8113-siKLK4 group was significantly decreased ($p < 0.01$).

Table 1: Relative expression level of KLK4 in each group

Group	KLK4
Tca-8113-NC	0.97±0.11
Tca-8113-iKLK4	0.26±0.04
T	13.564
P-value	<0.01

Effect of treatment on cell proliferation

The proliferation of TCA-8113-siKLK4 group was significantly decreased at 48 h and 72 h, relative to those of TCA-8113-NC. In contrast, cell proliferation potential at 24 h was comparable in both groups ($p > 0.05$). These data are presented in Table 2.

Table 2: Cell proliferation capacity in each group

Group	Time (h)	Proliferation capacity
Tca-8113-NC	24	0.34±0.03
	48	0.47±0.04
	72	0.82±0.08
Tca-8113-iKLK4	24	0.31±0.02
	48	0.38±0.04[ab]
	72	0.63±0.07[abc]

[a]$P > 0.05$, compared with TCA-8113-NC group at 24 h;
[b]$p < 0.05$, compared with TCA-8113-NC group at 48 h;
[c]$p < 0.05$, compared with TCA-8113-NC group at 72 h

Apoptosis

As shown in Table 3, there was significantly higher degree of apoptosis in TCA-8113-siKLK4 group than in TCA-8113-NC group ($p > 0.05$).

Table 3: Cell apoptosis in each group

Group	Apoptosis (%)
Tca-8113-NC	4.85±0.14
Tca-8113-siKLK4	17.49±3.74
t	7.552
P-value	<0.001

Cell migration

Cell migration potential at 24 and 48 h were significantly lower in TCA-8113-siKLK4 group than in TCA-8113-NC group ($p < 0.05$). These results are shown in Figure 1.

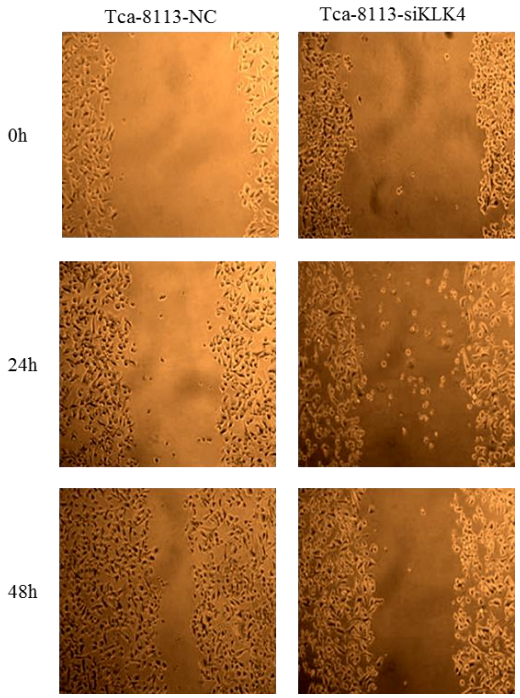

Figure 1: Comparison of cell migration capacity between the 2 groups

Invasion capacity of cells

Figure 2 shows significant reduction in the invasion ability of TCA-8113-siKLK4 cells, relative to TCA-8113-NC cells ($p < 0.05$).

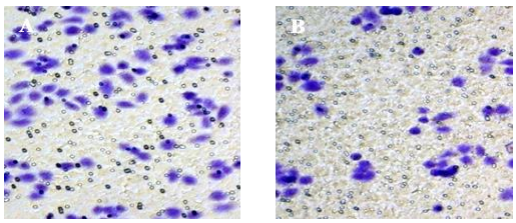

Figure 2: Invasion capacity of cells in each group. A: TCA-8113-NC group; B: TCA-8113-siKLK4 group)

Relative concentrations of E- and N-cadherins, vimentin and uPA

Results from Western blotting showed that, compared with TCA-8113-NC group, E-cadherin level in TCA-8113-siKLK4 group was markedly up-regulated, while those of N-cadherin, Vimentin and uPA were significantly decreased. These results are presented in Figure 3.

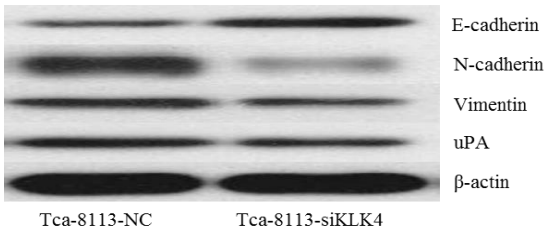

Figure 3: Comparison of relative concentrations of E- and N-cadherins, Vimentin and uPA amongst the groups

Relative concentrations of Wnt1, β-catenin, P-PI3K and P-Akt

Results from immunoblot assay (Figure 4) showed that relative protein levels of Wnt1, β-catenin, P-PI3K and P-Akt in TCA-8113-siKLK4 group were significantly lower than the corresponding expression levels in TCA-8113-NC cells.

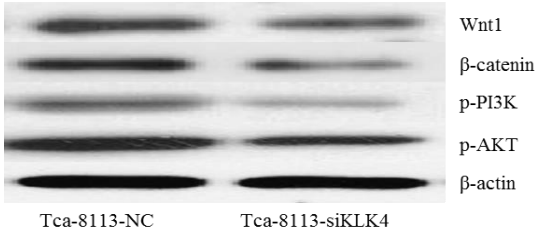

Figure 4: Levels of Wnt1, β-catenin, P-PI3K and P-Akt in each group

DISCUSSION

Oral squamous cell carcinoma (SCC) is one of the most common malignant tumors of the head and neck. Since the symptoms of oral SCC are not usually obvious at the early stage of the disease, most patients are diagnosed in the clinic at the late stage. Moreover, due to changes in lifestyle as a result of socio-economic advancements, the incidence of oral SCC has gradually increased, even in the younger population. Due to the anatomical location of oral cavity, the treatment of oral SCC often brings great bodily injury and dysfunction to patients, leading to serious impact on their quality of life and mental health [5]. Kallikrein-4 (KLK4) belongs to the serine protease group of chymo-proteases. Studies have found that KLK4 is expressed in a variety of normal and cancerous tissues, and it degrades extracellular matrix proteins, a process which is an important aspect of enzyme-linked activation in carcinogenesis and cancer invasion [6].

Some scholars have found that KLK4 is associated with malignancies of the oral cavity, neck and face, and it influences the proliferation, apoptosis, metastasis, invasion, differentiation and drug resistance of malignant tumors from different sources through different mechanisms [7]. The purpose of this study was to determine the influence of KLK4 on the biological behavior of oral squamous cell carcinoma cells, and mechanism involved.

The damage done to the body by malignant tumor cells is reflected in uncontrolled cell proliferation and failure of apoptotic mechanisms. Abnormal cell proliferation is one of the important biological behaviors in tumor deterioration, while cell apoptosis is one of the important factors that regulate tumor cell proliferation [8]. In this study, MTT assay was used to measure cell proliferation, and flow cytometry was used to measure apoptosis. It was found that after KLK4 gene interference, cell proliferation was significantly reduced, while apoptosis was significantly enhanced, suggesting that KLK4 promoted the proliferation of oral SCC cells and inhibited cell apoptosis. These results are consistent with those reported in an earlier study [9].

Invasive and metastatic changes are typical behaviors of cancerous cells, and they are also important factors that predispose carcinoma subjects to death. Invasive and metastatic changes in tumors are intricate processes involved in cancer progression. They are two different stages of the same process: invasion is

the premise for metastasis, while metastasis is the final result of invasion [10]. In this study, the invasion and migration of cells were significantly reduced after KLK4 gene interference, suggesting that KLK4 significantly promoted the invasion and migration of oral SCCs.

During epithelial-mesenchymal transformation, epithelial cells, under the influence of some factors, gradually decrease their cell polarity and intercellular adhesion capacity, and become mesenchymal cells with morphology and characteristics associated with malignant tumors. It has been reported that epithelial-mesenchymal transformation enhances tumor invasion and metastasis, and also generates cancer cells with stem cell characteristics which are important in tumor metastasis [11]. At present, it is believed that the major markers of epithelial-mesenchymal transformation comprise decreases in E-cadherin protein level, and increases in protein concentrations N-cadherin and Vimentin. Moreover, uPA is an important protease associated with epithelial-mesenchymal transformation, and it is widely present in malignant tumor cells. It has been reported that the expression of uPA in metastatic tumor cells is significantly higher than that in non-metastatic tumor cells [12]. This investigation revealed that E-cadherin level was significantly increased after KLK4 gene interference, while the expression levels of N-cadherin, Vimentin and uPA were significantly decreased. These results suggest that KLK4 enhanced epithelial-mesenchymal transformation in oral SCC.

An important regulatory pathway currently considered to be involved in cell proliferation is the Wnt/β-catenin signal route, with β-catenin as a key factor which is closely associated with the occurrence, differentiation, invasion and metastasis of malignant tumors [13]. Studies have shown that KLK4 functions not only through Wnt/β-catenin signal route, but also through PI3K/AKT) signaling pathway [14]. Indeed, the PI3K/AKT route contributes to the epithelial cell interstitial transformation process [15]. In this study, the expression levels of Wnt1, β-catenin, p-PI3K and p-Akt were significantly decreased after KLK4 gene interference. This study suggests that KLK4 may exert a carcinogenic effect through the Wnt/β-catenin and PI3K/AKT signaling pathways.

CONCLUSION

The results demonstrate that KLK4 enhances the multiplication, metastasis and invasiveness of OSCCs, and inhibits their apoptosis through stimulation of Wnt/β-catenin/PI3K/AKT signal routes. The potential of Kallikrein-4 as a biomarker for OSCC cells should be determined in suitable animal models.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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