Study on mechanism of down-regulating ikca1 molecule affecting the increment of oral squamous cell carcinoma

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Abstract: This research aimed to explore the mechanism of mediating the down-regulation of the calcium-activated potassium channel (IKCa1) gene expression in human oral squamous cell carcinoma Tca-8113 cells, thereby affecting cell proliferation and apoptosis. The expression level of IKCa1 in Tca-8113 cell line (oral squamous cell carcinoma) and HOEC cell line (human normal oral epithelial cell) was detected by RT-PCR. Then, after IKCa1 was knocked down in Tca-8113 cell line and HOEC cell line by RNA interference, and then cell proliferation levels were detected by cell counting kit 8 (CCK-8) method. Cell cycle distribution was detected by flow cytometry. Apoptosis was detected by membrane linked protein V-FITC/propidium iodide (PI) double-staining apoptosis detection kit. The protein expression level of IKCa1 was detected by Western Blot method. According to RT-PCR results, IKCa1 was significantly more expressed in Tca-8113 cell line than in HOEC cell line (P< 0.01). In addition, the mRNA expression levels in the normal oral epithelium and oral squamous cell carcinoma showed the same trend. After knocking down IKCa1 in Tca-8113 cell line, the IKCa1siRNA group significantly inhibited cell proliferation compared with the siNC control group. The results of flow cytometry showed that the proportion of apoptotic Tca-8113 cells transfected with IKCa1siRNA was significantly increased. The ratio of early apoptosis and late apoptosis of Tca-8113 cells increased (P< 0.05). To investigate the effect of IKCa1 on apoptosis, we tested the expression levels of apoptosis-related proteins. The results showed that the mRNA level of IKCa1siRNA group was significantly decreased by 44.41% compared with the control group (p< 0.01). Meanwhile, the mRNA level of Bax was significantly increased by 36.0% (p< 0.05). Our results showed that knocking down IKCa1 in Tca-8113 cells could induce cell cycle arrest and apoptosis to produce an anti-proliferation effect, thus inhibiting the expression of IKCa1 has an anti-cancer effect in oral squamous cell carcinoma.

Key words: Oral squamous cell carcinoma; Tca-8113 cell; IKCa1; Cell proliferation; Cell apoptosis.

Introduction

Cancer is one of the five leading causes of death in all societies. In the United States, cancer is the second leading cause of death after cardiovascular disease. Despite numerous advances in the treatment of cancer, oral cancer is one of the top ten causes of death due to its late detection, such as asymptomatic early-stage clinical similarities with other lesions and variation in clinical manifestations, it is one of the top ten causes of death. Although this type of cancer is rarer than other types of cancer, it is more important because the oral cavity is a strategic cavity (1-3).

The oral mucosa is pink, smooth and shiny. If everyone checks the entire mouth and mucus once a month, this is the best and most important type of test to prevent oral cancer. Very important structures, such as arteries, veins, nerves pass through the mouth and neck, the brain is very related to the mouth. If the disease develops in the upper jaw, it may also affect the brain and orbital sinuses, or if the patient is late for medical treatment, it may increase the risk of death. The disease has symptoms that everyone should pay attention to during examination, such as white, red or white and red plaques, obvious sores, hard edges, pain, burning or numbness, and obvious fungi or flower clusters (2-4).

There are many reasons for this type of cancer, and both internal and external factors may cause this type of disease. Smoking, tobacco derivatives (chewable and black smoke), alcoholic beverages consumed with tobacco, carcinogens (such as food preservatives, pheno, air pollutants), harmful radiation (such as UV and X-rays), and infections (such as Syphilis, HPV and fungal viruses). External factors affect oral cancer. Iron deficiency anemia, malnutrition (such as vitamin A deficiency), and all systemic diseases that weaken the body's immune system are some internal causes of oral cancer (1-3).

These internal and external factors activate tumor genes and turn off tumor suppressor genes, and provide conditions for cancer. New research is underway to identify tumor genes, tumor suppressor genes and oncogenic pathways that can be used. Control methods, such as gene interaction, molecular therapy, or the injection of cancer intelligence (2-5).

Oral squamous cell carcinoma is a kind of squamous cell carcinoma occurring in oral mucosa, characterized by a high degree of malignancy, the easy occurrence of local invasion and cervical lymph node metastasis, and low survival rate. Oral squamous cell carcinoma is one of the most common oral malignancies, accounting for 2 ~ 4% of all malignancies, which is the main factor lea-
Tca-8113 cell line of oral squamous cell carcinoma was purchased from Chongqing Medical University and cultured with RPMI-1640 medium containing 10% FBS (HyClone, USA) and 1% penicillin-streptomycin (Invitrogen, USA). Human normal oral epithelial HOEC cell lines were purchased from Shenzhen Haodihuatu Biotechnology Co., Ltd., and cultured with DMEM hyperglycemic medium (HyClone, USA) containing 10% FBS and 1% penicillin-streptomycin double antibody. The cells were incubated in a 37 °C incubator containing 5% CO₂. When the degree of cell fusion reached about 90%, passage culture was conducted in the ratio of 1 to 2.

RNA extraction, Reverse Transcription and PCR experiments

Total RNA in cells was extracted by TRIZOL (Invitrogen) according to the instructions. Reverse transcription and cDNA synthesis were performed according to the instructions of AidTM reverse transcription kit, and then the RT-PCR experiment was performed. Taking GAPDH as the internal reference gene, the sequence of the target gene and internal reference gene were as follows: GAPDH (forward, 5'-ATGCGTGGGCCTGAGTACGT-3', reverse, 5'-GGTCTATGAGTCCTTCAA-3'); IKCa1(forward, 5'-TGGTCTGGAGATTATGAGG-3', reverse, 5'-TCGTAAGACCTGATCTGGG-3'); Bax(forward, 5'-ATGTTGTTGAGAGCCTCAAA-3', reverse, 5'-TAACTATCTTTCGGAACCG-3'); reverse, 5'-CGTGGTCCACTGACATCA-3'). The amplification system was as follows: initial denaturation for 4 minutes at 94 °C, denaturation for 30 seconds at 94 °C, denaturation for 45 seconds at 57 °C, denaturation for 1 minute at 72 °C, and delayed termination for 10 minutes at 72 °C. In the results, 2⁻ΔΔCT value relative quantitative method was used to compare the mRNA expression differences of target genes in each group.

Cell cycle detection

Cells in each group at the logarithmic growth stage were digested with trypsin to prepare cell suspension, and cell count was performed. 1×10⁶ cells were placed in a 1.5-ml centrifuge tube, and the supernatant was discarded after centrifugation at 1500 r/min for 3 min. The cell suspension was prepared by resuspending cells with 500 μl phosphate buffer (PBS) and precooled anhydrous ethanol was added, which was shaken at the same time, and the final concentration of ethanol of fixed cells was (75%) when added to 2 ml, and then incubated overnight at 4 °C. On the second day, the fixed cells were centrifuged at 1500 r/min for 5 min, and the supernatant was thrown out. After adding 1 μl PBS, the suspended cells were centrifuged again and the supernatant was discarded. Then, 300 μl PI dye solution was added, mixed well, and stained at room temperature for 15 min. The stained cells were filtered and tested by flow cytometry.

Cell proliferation experiment

Cell proliferation rate was determined by cell counting kit 8 (CCK-8) method. Tca-8113 cells were placed in a 96-well culture plate with a density of 5000 cells per well and cultured overnight. An equal amount of serum-free medium containing 100 ul/ml CCK-8 (Dojindo, Japan) was added to each well and incubated at
37℃ for 3 h. The absorbance at 450 nm was measured by using the absorbance of the cells by means of ELX800 absorbance microplate reader (Biotek Instruments, Winooski, VT).

**Cell transfection**

IKCa1siRNA was transfected with liposome 2000 reagent (Invitrogen, USA). siRNA oligonucleotides were synthesized by Wuhan Cell Biotechnology Co., Ltd. The siRNA sequence was as follows: IKCa1 (sense strand) 5’-CAGCGCTGGTCTAC AAAATTCGAGCATGTGTTGGAGATCCCGTTTTCGTTAATGCTGTTAGCTGATTCCGC-3’, antisense strand 5’-GAAAGCGTGATCACCAGATTCATTTGCTTACATTAATTCTAAATCATTTCTATTTGTTTGTAGAATCCAGCGCC-3’), negative control (sense strand 5’-CAACCGGTATGACATTGACTTACATTCAAGACGTGTAAGTCAAATGTCATCA GGCT TTT TTG-3’, antisense strand 5’-AGCTCAAAAAAGCCTGATGACATTGACTTAACATTCAAGACGTGTAAGTCAAATGTCATCA GGCT TTT TTG-3’), control group. Protein levels, mRNA levels, cell proliferation, and apoptosis were detected 48 h after transfection.

**Apoptosis detection**

Apoptosis was detected using membrane protein V-FITC/propidium iodide (PI) double staining assay kit (Kgi, China). Cells were collected and stained based on manufacturer's instructions. Apoptosis data were collected and analyzed through flow cytometry.

**Western blot test**

Tca-8113 cells from the three transfection groups were collected and centrifuged at 14000×g for 10 min to prepare lysates. Total protein concentrations were quantified using BCA (Kgi, China). Equivalent amounts of protein were loaded and separated using SDS-PAGE and transferred to the nitrocellulose membrane. Sealed with 10% skim milk, and incubated in rabbit anti-human polyclonal antibody IKCa1 (Abcam, UK, ab215990) (1:500) overnight at 4 ℃, washed with PBS for 3 times the next day, and incubated with horseradish peroxidase combined with secondary antibody (Zhongsanhain Jinqiao, China, zb-2306) (1:2000) at 37 ℃ for 1 h. The expression of IKCa1 was quantitatively analyzed by the Odyssey infrared fluorescence scanning system (LICTOR) and Odyssey v1.2 software. The relative expression of the IKCa1 protein was determined by the optical density ratio of IKCa1 to β-actin.

**Statistical analysis**

SPSS13.0 was used for statistical analysis, t-test was applied to compare the samples of the two groups, and Anova was used to compare the samples of multiple groups. The statistical data were expressed as mean ± standard deviation, and P< 0.05 was considered as a statistically significant difference.

**Results**

Expression of IKCa1 in Oral squamous cell carcinoma

To investigate whether the high expression of IKCa1 in oral squamous cell carcinoma affects cell growth, we conducted the following experiment. The expression of IKCa1 in the Tca-8113 cell line (oral squamous cell carcinoma) and HOEC cell line (human normal oral epithelial cell) was detected by RT-PCR. According to the results, compared with the HOEC cell line, IKCa1 was highly expressed in the Tca-8113 cell line, with a significant difference (P< 0.001). The mRNA expression level of IKCa1 was detected in tissues. RT-PCR in 15 pairs of normal oral squamous epithelial tissues and oral squamous cell carcinoma tissues showed that the mRNA level of IKCa1 was also highly expressed in oral squamous cell carcinoma tissues, with significant differences (P< 0.001). This suggests that IKCa1 is significantly up-regulated in oral squamous cell carcinoma. See Figure 1.

**Effects of IKCa1 on cell proliferation**

In order to investigate whether the up-regulated IKCa1 in Tca-8113 cell line may affect the growth of oral squamous cell carcinoma, the following experiment was conducted. The proliferation of Tca-8113 and HOEC cells was detected by knocking down IKCa1, respectively. First, the knockdown effect of IKCa1siRNA was verified. IKCa1siRNA was transfected into the Tca-8113 cell line with transfection reagent lipo2000, and the expression of IKCa1 was detected by RT-PCR 48 hours after transfection. The results showed that IKCa1siRNA significantly reduced the mRNA expression level by 72.7% (Figure 2a, p< 0.05). Meanwhile, the expression level of IKCa1 protein was also significantly reduced by 40.7% (Figure 2b, p< 0.05). There was no significant difference in IKCa1 mRNA and protein expression between the blank control group and the negative control group (Figures 2a and 2b, p> 0.05). It proves that IKCa1siRNA can play an effective role and significantly reduce the expression of IKCa1 in cells.

Then, IKCa1 was knocked down in Tca-8113 cell line and HOEC cell line, respectively, and the proliferation of the cells was detected by CCK-8 method. According to the experimental results, IKCa1siRNA group can significantly inhibit cell proliferation compared with siNC control group after IKCa1 knockdown in Tca-8113 cell line (Figure 2c). Moreover, compared
Figure 2. Knocking down IKCa1 Inhibits the Proliferation of Tca-8113 Cells; (A, B) Effects of IKCa1 - specific siRNA on IKCa1mRNA and protein expression levels. "nc" represents cells transfected with pgensil-hk+liposomes. "sr1" represents the transfection of Tca-8113 cells with pgensil-IKCa1sirna+liposome. "b" (blank) represents cells transfected with ROMI-1640+liposomes. Compared with the blank group (b), *p < 0.05. (C, D) After knocking down IKCa1 in Tca-8113 cell line and HOEC cell line, the proliferation of the cells was detected by CCK-8 method, ***p < 0.001.

Effects of IKCa1 on cell cycle and apoptosis
In order to investigate whether the inhibition of cell proliferation after knockdown of IKCa1 was caused by affecting the process of cell cycle, flow cytometry was applied to analyze the cell cycle distribution. The results showed that compared with the control group, the Tca-8113 cells in G0/G1 phase increased from 71.6 ±3.4% to 91.7 ±4.6% (P< 0.001), and the cells in S phase decreased from 23.8 ±1.1% to 7.1 ±0.8% (P < 0.01). Therefore, after knocking down IKCa1, the cells were blocked in G0/G1 phase.

In order to further study whether IKCa1 can affect apoptosis, flow cytometry was carried out. The results showed that the proportion of apoptotic cells in Tca-8113 cells transfected with IKCa1siRNA was significantly increased (3.9 ±0.52% VS 6.9 ±1.12%) (Figure 3). The proportion of early apoptosis and late apoptosis of Tca-8113 cells increased significantly (P< 0.05) (The percentage of apoptosis was determined via the sum of early and late apoptosis).

Effect of IKCa1 on the expression of apoptotic protein
To explore the mechanism of IKCa1 affecting apoptosis, we detected the expression of apoptosis-related proteins. The mRNA expression of anti-apoptosis protein Bcl-2 and apoptosis-promoting protein Bax was detected by RT-PCR after IKCa1siRNA was transfected into Tca-8113 cells. The results showed that the mRNA level of Bcl-2 in IKCa1siRNA was significantly decreased by 44.41% compared with the control group (Figure 4a, p< 0.01). At the same time, the mRNA level of Bax was significantly increased by 36.0% (Figure 4b, p< 0.05). Therefore, IKCa1 may affect tumor growth through the apoptosis-related pathway.

Discussion
Oral squamous cell carcinoma of the mouth is the most common type of squamous cell carcinoma of the head and neck, which is one of the top ten most common cancers worldwide. Despite routine treatment regimens, patients with oral squamous cell carcinoma have poor prognosis and short survival. The incidence of oral squamous cell carcinoma is increasing year by year, but the molecular mechanism of tumor proliferation is still unclear. Therefore, it is necessary to identify new molecular markers and study effective therapeutic drugs to inhibit the growth of oral squamous cell cells (18, 19).

A large number of studies have confirmed that ion channels are one of the structural units that regulate the
physiological functions of cells, such as growth, differentiation, and proliferation. During cell proliferation, Ca²⁺, K⁺, aquaporin and Ca²⁺ channels are activated (20, 21), and these and other ion channels are considered to be important factors in the proliferation of normal cells and cancer cells (22). The K⁺ channel helps to set up membrane potential and ACTS as a driving force for Ca²⁺ influx, which is also crucial for the regulation of cell growth and plays a role in carcinogenesis (23-25). Ca²⁺ dependent potassium channels (KCa) also regulate cell cycle progression, attracting increasing attention as a potential therapeutic target. Previous studies have found that IKCa1 is highly expressed in many malignant tumors (6-9). IKCa1 is one of the K⁺ channels regulating the proliferation and migration of cancer cells (26-28), mainly controlling the late G1 phase, G1/S transition period and G2/M phase. We investigated the role of IKCa1 in the development of oral squamous cell carcinoma at the molecular level using RNA interference techniques. In this study, we demonstrated that decreased expression of KCa1 had a strong anticancer effect in human oral squamous cell lines. In addition, the inhibitory effect of KCa1 expression on cell proliferation was associated with cell cycle arrest and apoptosis induction in oral squamous cell carcinoma.

In this study, according to RT-PCR results, IKCa1 was significantly more highly expressed in Tca-8113 cell lines than in normal oral epithelial HOEC cell lines (P<0.001) (Figure 1a), and the expression level of IKCa1 in oral squamous cell carcinoma was higher than that in normal oral epithelial tissues according to RT-PCR results. After knocking down IKCa1 in the Tca-8113 cell line, IKCa1siRNA significantly inhibited cell proliferation compared with siNC in the control group (Figure 2c). Moreover, compared with the HOEC cell line, the Tca-8113 cell line showed more significant changes in proliferation after knocking down IKCa1 (Figures 2c, 2d). Therefore, inhibiting IKCa1 expression can specifically inhibit the proliferation of Tca-8113 cells. It can be seen that IKCa1 can be used as a marker of malignant tumor in the oral squamous cell system, providing a new molecular marker for accurate detection and treatment in clinical practice. Compared with the control group, Tca-8113 cells with IKCa1 knockdown increased from 71.6±3.4% to 91.7±4.6% (P<0.01) in G0/G1 phase, and decreased from 23.8±1.1% to 7.1±0.8% (P<0.001) in S phase. Flow cytometry showed that the proportion of apoptotic tca-8113 cells transfected with IKCa1siRNA was significantly increased (3.9±0.52% VS 6.9±1.12%). The ratio of early apoptosis and late apoptosis of Tca-8113 cells increased (P<0.05). (Figure 3) Therefore, it can be seen that the effect of IKCa1 on cell proliferation may be caused by apoptosis. To investigate the effect of IKCa1 on apoptosis, we tested the expression levels of apoptosis-related proteins. The results showed that the mRNA level of IKCa1siRNA was significantly decreased by 44.41% compared with the control group (Figure 4a, p<0.01). Meanwhile, Bax mRNA level was significantly increased by 36.0% (Figure 4b, p<0.05). It is well known that Bcl-2 family members play a decisive role in regulating apoptosis and determining cell fate by regulating the balance between proapoptotic (Bax, BAK, and BH3) and anti-apoptotic (Bcl-2 and its homology) proteins. When the ratio of Bax to Bcl-2 increases, the mitochondrial permeability conversion hole opens, and then releases apoptotic mitochondrial protein to activate caspase and induce apoptosis. However, previous studies have not linked IKCa1’s anticancer effect to this apoptotic pathway. Thus, to our knowledge, this is the first study to investigate the involvement of the Bcl2 family in IKCa1-mediated apoptosis of human oral squamous cell carcinoma cells. However, further confirmation that the activation of the Bcl2 family regulated apoptotic pathway is KCa1 inhibiting effect and the specific mechanism of action needs to be further confirmed by more experiments.

The most recent studies have focused on the malignant cell behavior of KCa1 in cancer (29-34). This study also demonstrated its effect on cell proliferation in oral squamous cell carcinoma. Therefore, we can effectively inhibit the expression of KCa1 through genetic and pharmacological means according to its characteristics, thus significantly reducing the proliferation of tumor cells and changing the susceptibility of tumors to establish cancer therapies, thus increasing the efficacy of existing therapies. In this paper, the effects of IKCa1 on the proliferation of oral squamous cell carcinoma cell lines and their mechanisms were studied to further observe the effects of RNA interference on cell cycle and apoptosis, to provide more evidences for clinical diagnosis and accurate treatment of oral squamous cell carcinoma cell lines and provide more possibilities for the development of new therapeutic drugs (35-51).

In conclusion, our results show that IKCa1 is significantly up-regulated in oral squamous cell carcinoma. Knocking down IKCa1 in Tca-8113 cells can induce cell cycle arrest and apoptosis to produce an anti-proliferation effect, so inhibiting IKCa1 expression has a strong anticancer effect in oral squamous cell carcinoma. This study can provide more evidence for the research and development of clinical therapeutic drugs and the research of drug resistance mechanisms.

References


