

Original Article

Chloride intracellular channel 4 protein promotes gastric cancer cell proliferation, invasion and migration

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Abstract: Objective: To detect the chloride intracellular channel (CLIC) 4 expression in gastric cancer (GC), and to investigate the role of the CLIC4 protein in the proliferation and migration of GC cells. Methods: Immunohistochemistry was used to detect the expression of CLIC4 in GC and normal gastric mucosa tissues. The siRNA transfection method was applied to down-regulate the CLIC4 expression in GC SGC7901 cells, and Western blot was used to detect the CLIC4 protein levels in the interference and control groups. The Cell Counting Kit 8 (CCK8) test was conducted to assess the effect of CLIC4 on SGC7901 cell proliferation, and the cell migration change was evaluated by the wound healing assay. Results: CLIC4 was majorly expressed in malignant cells, and was significantly more highly expressed in GC tissue compared to normal ($P < 0.05$). CLIC4 siRNA could significantly weaken the proliferation and migration abilities of SGC7901 cells ($P < 0.05$). Conclusion: CLIC4, which is up-regulated in GC, can enhance the proliferation and migration of SGC7901 cells, and might serve as a new biomarker for GC diagnosis and a novel target for anti-GC therapy.

Keywords: Chloride intracellular channel 4, gastric cancer, SGC7901 cell, proliferation, migration

Introduction

Gastric cancer (GC) is one of the most common and lethal digestive malignancies worldwide, with incidence and mortality rates ranking 4th and 3rd among all cancers, respectively, and the sufferers tend to become younger in recent years [1, 2]. The symptoms of early GC are usually not obvious, so most of GC patients are diagnosed at advanced stages, and have lost the chance of curative surgery [3]. Early diagnosis and treatment, and effective prevention of tumor invasion and metastasis are the key to improve GC patients' survival [4, 5]. Active mechanistic researches into GC cell proliferation and migration are of great importance in guiding clinical practice.

Recently, increasing evidence has supported that chloride intracellular channel (CLIC) is closely related to tumor initiation and progression [6, 7]. CLIC4 is a member of the CLIC family, and is widely distributed in cytoplasm and in various cell organelles, especially mitochondria

[8-10]. To the best of our knowledge, CLIC4 has not been reported in GC. To elucidate the translational role of CLIC4 in GC progression, herein we investigated its expression in GC tissues using immunohistochemistry (IHC), and studied its effect on GC cell biological behaviors by transfecting GC SGC7901 cells with CLIC4 small interfering RNA (siRNA).

Methods and materials

Reagents

The CLIC4 siRNA (5'-GCTGAAGGAGGAGGACAAGA-3') and the Dulbecco modified Eagle medium (DMEM) were purchased from Invitrogen, America; the fetal bovine serum (FBS) from Beijing TransGen Biotech, China; the rabbit-anti-CLIC4 and the rabbit-anti- β -tubulin antibodies from Santa Cruz Biotech, America; the horseradish peroxidase (HRP)-bound secondary antibody from Shanghai Kangcheng Biotech, China; the chemiluminescent HRP substrate from Millipore, America; the streptavidin-

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peroxidase (SP) immunohistochemical and diaminobenzidine (DAB) chromogenic kits from Beijing ZSGB Biotech, China; and the Cell Counting Kit 8 (CCK8) from Beyotime Biotech, China.

IHC

Written informed consent was obtained from each patient involved, and the procedures followed were in line with the Declaration of Helsinki [11] and the Good Clinical Practice [12]. The surgical GC specimens and the paired normal gastric mucosa tissues ($n = 8$, thickness <4 mm) were immersed in 4% poly-formaldehyde for 1 to 2 d, followed by tissue pruning with all layers preserved. Then the specimens were dehydrated, paraffin-embedded, cut into 5 μm slices, baked, and dewaxed. Antigen retrieval was conducted using citrate and microwave heat. Then the sections were rinsed with phosphate buffer saline (PBS) for 3 times (3 min each time), incubated in 3% H_2O_2 at 37°C for 15 min, rinsed again by PBS, added with secondary antibody working solution, incubated at 37°C for 30 min, and washed again by PBS. The HRP was then added, followed by incubation at 37°C for 15 min and PBS wash. DAB was supplied as the chromogenic agent with chromogenic time controlled by microscopic observation, and the specimens were flushed with tap water for 5 min, double-stained by hematoxylin for 8 s, washed by running water again, and baked and dehydrated. Then the slices were mounted and observed microscopically. The Image-Pro Plus software was used to analyze the optical density (OD) value of the IHC results. For each section, 5 fields were selected, and the yellow-colored areas were deemed as areas of interest for OD measurement. The integrated OD (IOD) was then obtained.

Cell culture

GC SGC7901 cell lines were from our own laboratory. The adherent SGC7901 cells were inoculated in the DMEM medium with 100 mL/L FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and were cultured at 37°C with 5% CO_2 and 90% relative humidity. The cells underwent trypsinization and passage every 2-3 d, and those at the logarithmic growth phase were used in the following experiments.

CLIC4 siRNA transfection

The SGC7901 cells were seeded into the 12-well plate, with each well added with 900 μL

DMEM medium. For the experimental group, 100 μL OPTI, 10 μL CLIC4 siRNA, and 2 μL Lipofectamine RNAiMAX were mixed well, kept at room temperature (RT) for 20 min, and then added into the corresponding wells. For the control group, 100 μL OPTI and 2 μL Lipofectamine RNAiMAX were mixed and placed at RT for 20 min, before added to the wells. Then the plate was incubated at 37°C with 5% CO_2 for 1 d.

Wound healing assay

The cells from the experimental and control groups were inoculated into the 12-well plate, and cultured to monolayer fusion state. The 200 μL sterile pipette tip was applied to vertically scratch the bottoms of the wells, followed by PBS wash to get rid of the disassociated cells. The culture medium with 10 mL/L FBS was then added, and the wells were observed using an inverted microscope after 1 and 2 d, respectively, with the relative distance of the cells migration to the scratch area measured. The assay was repeated for 5 times.

CCK8 test

After transfected with CLIC4 siRNA and control siRNA for 1 d, respectively, the cells in the experimental and control groups were trypsinized, and 100 μL of the cell suspension was transferred to the 96-well plate, followed by incubation overnight to make cells attached to the wall. On the following day, 10 μL CCK8 solution was added into each well, followed by incubation for 1-4 h. The absorbance value of each well was measured at a wavelength of 450 nm using a microplate spectrophotometer. The test was repeated for 3 times.

Western blot

The cells underwent lysis on ice, and were then centrifuged using a pre-cold centrifuge at 12,000 rpm ($r = 8.5$ cm) for 20 min. The protein supernatant was obtained, and then isolated using the 4-20% SDS-PAGE electrophoresis. The wet transfer system (Bio-Rad) was applied to transfer the protein to the polyvinylidene fluoride (PVDF) membrane, followed by block for 2 h. The primary antibody (dilution, 1:200) was added before incubation overnight. On the second day, the PVDF membrane was washed by PBST for 4 times (15 min/time). The HRP-bound secondary antibody (dilution, 1:5,000)

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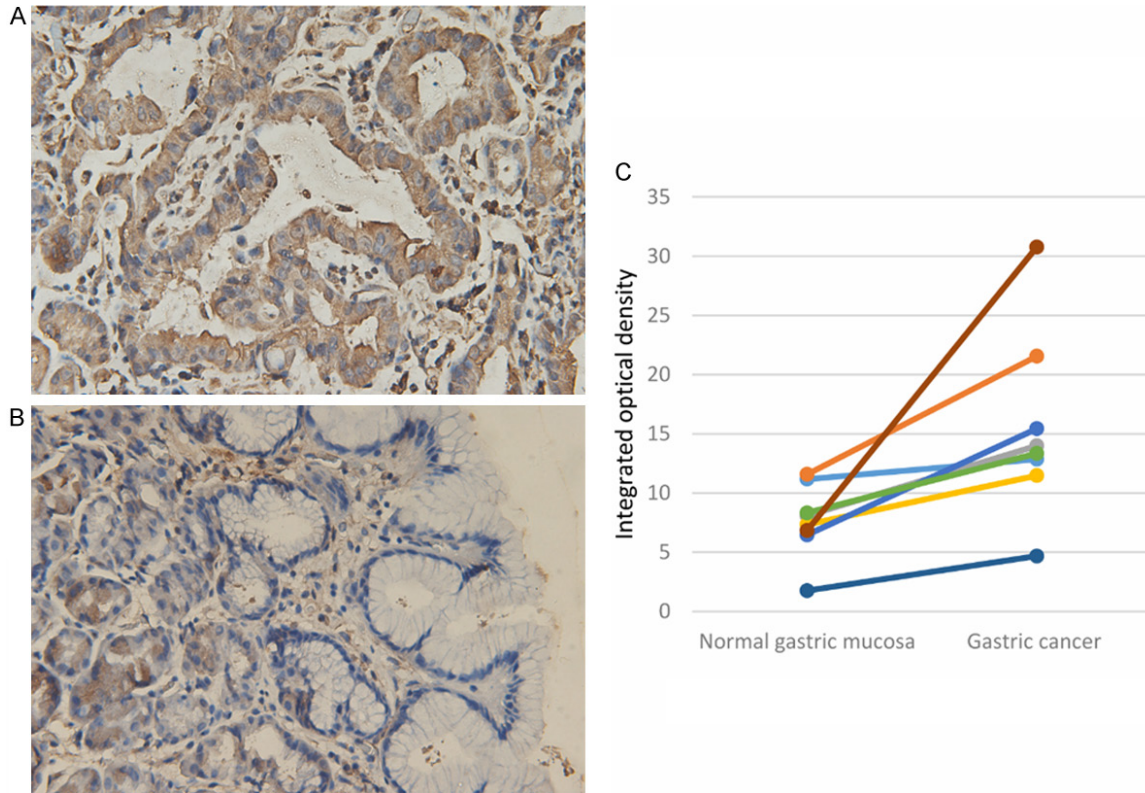


Figure 1. Expression of the chloride intracellular channel 4 (CLIC4) protein using immunohistochemistry. The gastric cancer cells (A) were brown- or yellow-stained, while the normal gastric mucosa epithelia (B) were lightly dyed (high power field, $\times 400$). CLIC4 was majored expressed in malignant cells rather than the tumor stroma. The integrated optical density value of the gastric cancer tissue was significantly higher than that of the normal mucosa (C, $n = 8$).

was then added. The immune complex was added with chemiluminescent HRP substrate for development and photo-taking. The molecular weight of the target band was analyzed using the gel image analysis system, and the relative content of the protein was assessed with the net OD value.

Statistical analysis

The data were presented as mean \pm standard deviation (SD)/standard error (SE), and the difference between the 2 groups was compared using the independent sample t test. The Sigmaplot 12 software was applied for data analysis, and a difference was considered significant with a 2-sided P value <0.05 and very significant with $P < 0.01$.

Results

CLIC4 was more highly expressed in the GC tissue

The GC cells were brown- or yellow-stained using IHC, while the normal gastric mucosa epi-

thelia were lightly dyed. CLIC4 was majored expressed in the malignant cells rather than in the tumor stroma. The IOD value of the GC tissue was significantly higher than that of the normal mucosa (15.52 ± 7.72 vs. 7.70 ± 3.06 , $t = 2.664$, $P = 0.019$) (Figure 1).

CLIC4 siRNA markedly decrease the CLIC4 expression in GC cells

The total protein was extracted in the experimental and control groups, respectively, and the CLIC4 protein expression was tested using Western blot. The CLIC4 protein level was significantly lower in the siRNA interference group than the control group (135.06 ± 7.02 vs. 73.24 ± 8.00 , $t = 5.808$, $P = 0.001$) (Figure 2).

CLIC4 siRNA significantly reduce the proliferation ability of GC cells

The CCK8 test revealed that the CLIC4 siRNA-transfected SGC7901 cells had significantly lower OD values than the control group (0.892 ± 0.014 vs. 1.057 ± 0.030 , $t = -4.608$, $P =$

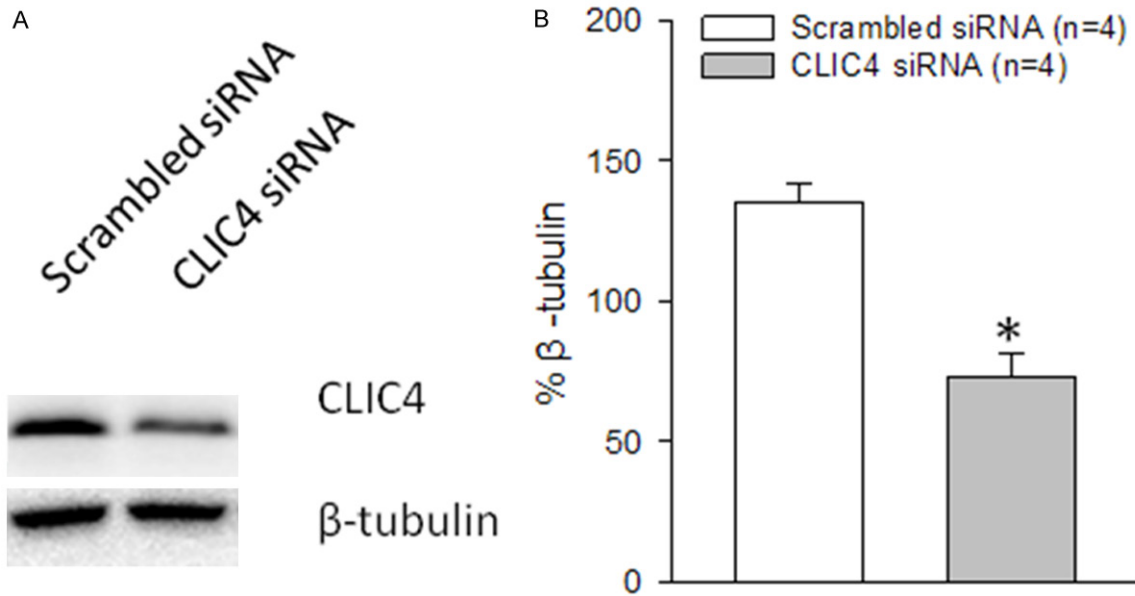


Figure 2. The CLIC4 expression in SGC7901 cells before and after CLIC4 siRNA treatment using Western blot. Both the band intensity (A) and the relative content (B) revealed that the CLIC4 protein level was significantly lowered after the CLIC4 siRNA interference. siRNA, small interfering RNA; CLIC4, chloride intracellular channel 4; *, $P < 0.05$.

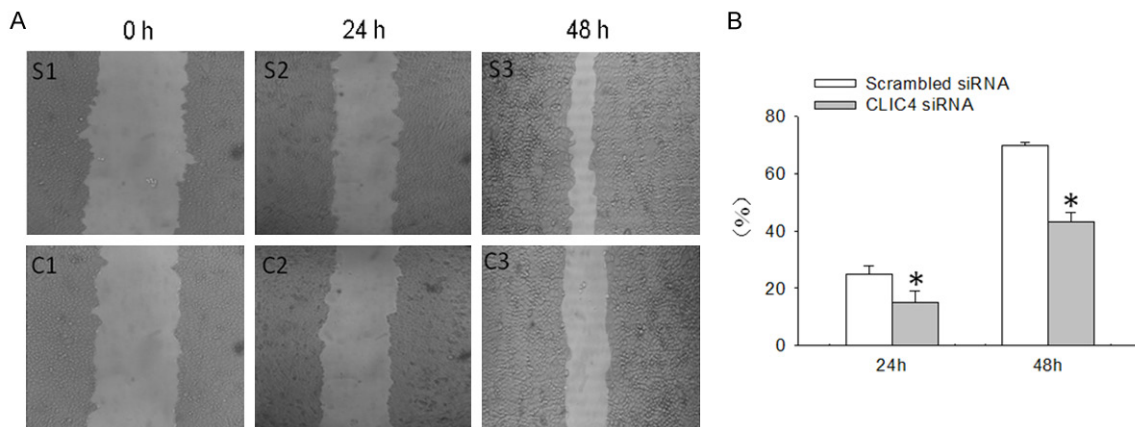


Figure 3. The effect of CLIC4 on the migration ability of the SGC7901 cells. The cell migration figure (A, $\times 100$) and the relative cell migration ratio (B, $n = 5$) showed that the wound healing ratios were significantly smaller in the CLIC4 siRNA-treated group 1 and 2 days after the scratch, compared to the control group. S, scrambled siRNA; C, CLIC4 siRNA; siRNA, small interfering RNA; CLIC4, chloride intracellular channel 4; *, $P < 0.05$.

0.002), indicating a weaker proliferative potential.

CLIC4 siRNA significantly weakens the migration ability of GC cells

The scratch assay showed that the wound healing ratios (%) were significantly smaller in the CLIC4 siRNA-treated group 1 (14.748 ± 4.041 vs. 24.958 ± 2.780 , $t = -4.804$, $P = 0.003$) and 2 days (42.925 ± 3.504 vs. 67.342 ± 1.080 , $t =$

-6.352 , $P = 0.007$) after the scratch, compared to the control group, suggesting a weaker *in vitro* cell migration ability (Figure 3).

Discussion

CLIC is a newly found independent sub-family of the chloride channel, and has 7 members including p64, CLIC1-5, and parhormin. Most of the members are expressed in cell organelles. The CLIC proteins have a wide range of func-

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tions, and are involved in the modulation of cell cycling, signal transduction, cell apoptosis, and cytoskeleton reconstruction [6, 7]. CLIC4 is widely expressed in mitochondria, nucleus, cytoplasm, Golgi apparatus, endoplasmic reticulum, and microfilament, and on cell membrane, and plays vital roles in regulation of membrane potential, cell development, cell apoptosis, tumor initiation, etc. [8-10].

CLIC4 might exert tumor type-specific functions, either enhancing or inhibiting tumor progression, which might be because of the relative CLIC4 location in the cancer. Ronnov-Jessen *et al.* [13] initially proposed the important role of CLIC4 in the formation of breast cancer-associated myofibroblast, and found that the CLIC4 expression could be significantly up-regulated by transforming growth factor (TGF). Shukla *et al.* [14] revealed that CLIC4 was highly expressed in the microenvironment of many kinds of human cancers. The malignant cell-secreted TGF could increase the CLIC4 expression in tumor stromal cells, and the over-expressed CLIC4 could promote the transformation of fibroblast to myofibroblast, thus stimulating epithelial-mesenchymal transition (EMT) and enhancing the invasion and migration abilities of the tumor. However, Suh *et al.* [15] uncovered that the CLIC4 transcription level was significantly down-regulated in various tumors especially breast, ovarian, and kidney cancers. They also found that the CLIC4 expression was lost at the early stage of tumor progression, and the exogenous CLIC4 treatment could inhibit tumor growth [16].

Our study for the first time investigated the role of CLIC4 in GC. We found that the CLIC4 protein level was significantly higher in the GC tissue in relative to the paired normal gastric mucosal epithelia using IHC, and that it was majorly expressed in malignant cells rather than the tumor stroma. We then applied the CLIC4 siRNA transfection method to down-regulate the CLIC4 expression in GC SGC7901 cells. The CCK8 test showed that the *in vitro* proliferation ability of SGC7901 cells was significantly decreased after the CLIC4 expression was silenced by the siRNA, which suggests that the CLIC4 protein can enhance GC cell proliferation. The wound healing assay demonstrated that the *in vitro* migration ability of GC cells was significantly weakened after the CLIC4 expres-

sion in SGC7901 cells was interfered with, indicating that CLIC4 can effectively promote the migration and metastasis potentials of GC cells. Our results showing that CLIC4 which was mainly located in the tumor cells could be potentially supported by its abovementioned location-specific role in malignancies.

However, the specific intracellular mechanism remains obscure, which requires further investigation, and which is the major focus of our future work. Our preliminary work suggested that CLIC4 might function through the modulation of the secretion of exosomes. Its regulation of other molecular events including signaling transduction, membrane electrophysiological alteration, cell adhesion, integrin trafficking, and EMT might also play a role in its pro-GC progression activity [17]. The differential expression of CLIC4 in malignant cells and the tumor stroma might also be of great further research interest.

In conclusion, CLIC4, which is up-regulated in GC, can enhance the proliferation and migration of SGC7901 cells, and might serve as a new biomarker for GC diagnosis and a novel target for anti-GC therapy.

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Disclosure of conflict of interest

None.

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