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SLC5A8 regulates the biological behaviors of cervical cancer cells through mediating the Wnt signaling pathway

X.-M. ZHANG[1], Q.-H. MENG[2], F.-F. KONG[1], K. WANG[1], L.-J. DU[3]

[1]Department of Obstetrics, Jinan Second Maternal and Child Health Hospital, Jinan, China
[2]Department of Neonatology, Jinan People's Hospital, Jinan, China
[3]Department of Oncology, Shaanxi Ankang Central Hospital, Ankang, China

Xiaomin Zhang and *Qinghua Meng* contributed equally to this work

Abstract. – OBJECTIVE: This study aims to investigate the role of solute transport family 5 member 8 (SLC5A8) in the progress of cervical cancer (CC) to provide a theoretical basis for the treatment of CC.

PATIENTS AND METHODS: Tissues were obtained from 58 patients diagnosed with CC in our hospital. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) analysis was used to detect the expression level of SLC5A8 in CC tissues and cell lines. SLC5A8 level was up-regulated by transfection of SLC5A8 overexpression plasmid. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and flow analysis were designed to measure the cell proliferation, cell cycle, and apoptosis of CC cells.

RESULTS: The mRNA expression of SLC5A8 was down-regulated in CC tissues and cell lines. Transfection of SLC5A8 overexpression plasmid successfully over-expressed SLC5A8. In addition, an inhibited activation of Wnt signaling pathway was detected in CC cells after over-expression of SLC5A8. Besides, decreased proliferation activity and increased apoptosis were also observed in CC cells overexpressing SLC5A8 plasmid. Moreover, the impaired proliferation activity and increased apoptosis proportion of CC cells induced by SLC5A8 over-expression could be counteracted by the Wnt signaling pathway activator LiCl.

CONCLUSIONS: SLC5A8 alleviates the progression of CC by regulating the Wnt signaling pathway.

Key Words:
Cervical cancer (CC), Solute transport family 5 member 8 gene (SLC5A8), Wnt signaling pathway.

Introduction

Cervical cancer (CC), a malignant tumor of gynecology with high incidence and high mortality, is the most common cause of death in gynecologic malignancies[1]. In developing countries, a great number of CC patients are diagnosed in advanced stage[2]. Although great progress on CC treatment has been made in recent years, the treatment effects in advanced invasive CC are still limited[3,4]. Therefore, exploring the molecules and signaling pathways closely related to CC is extremely important. Solute transport family 5 member 8 (SLC5A8), located on human chromosome 12q13-23, belongs to the Na^+ and Cl^--dependent sodium-coupled solute transporte[5]. Although the abnormal expression of SLC5A8 has been found in many tumors[6-9], its role in CC has not been reported yet and needs further clarification. In this study, we collected CC tissues in our hospital and detected the expression of SLC5A8 by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot. The relationship between SLC5A8 and proliferation and apoptosis of CC cells was further analyzed, aiming to reveal the molecular mechanism of CC progress and to provide a new theoretical basis for its diagnosis and treatment.

Patients and Methods

Samples

The 58 CC specimens collected in this study were from CC patients who were treated at Jinan Second Maternal and Child Health Hospital from July 2016 to December 2018. The patients were diagnosed as CC by cervical biopsy and postoperative routine pathology. None of them received chemotherapy, radiotherapy or other special treatment before surgery. In addition, the investigation

Corresponding Author: Lianjiang Du, MM; e-mail: 5116816@qq.com

has been approved by the Jinan Second Maternal and Child Health Hospital Ethics Committee, and all patients enrolled have signed informed consent.

Cell Culture and Transfection

CC cell lines (SiHa, HeLa, C-33A, CaSki), and normal cervical epithelial cells (HUCEC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in the medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1000 U/mL penicillin and 100 μg/mL streptomycin, at 37°C constant temperature, 5% CO_2. When the cell growth reached 80-90%, trypsin was added for cell passage. After centrifugation at 1000 rpm for 5 min, the cells were resuspended in fresh medium and inoculated in a dish at 1:3. Cell transfection was performed using Lipofectamine™ 2000 (Lipo2000; Invitrogen, Carlsbad, CA, USA). Briefly, the plasmid was first diluted in 250 μL Opti-MEM® medium. At the same time, Lipo2000 was diluted in 250 μL Opti-MEM® medium and incubated for 5 min. Finally, the two solutions were mixed for a total of 500 μL, and added to the dish after 15 min incubation. SiHa cells transfected with negative vector were named as Vector group, and those transfected with SLC5A8 overexpression plasmid were named as SLC5A8 group. SiHa cells with co-treatment with the SLC5A8 plasmid and LiCl were named as SLC5A8+LiCl group.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cells were trypsinized and 4000 cells were seeded in a 96-well plate with 3 wells repeated, and cultured at 37°C in a 5% CO_2 incubator. The 96-well plate was taken at 24 h, 48 h, 72 h, and 96 h, respectively, and 20 μL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well according to the manufacturer's instructions, followed by further incubation for 4 h. Then, 150 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 10 min at room temperature. The wavelength of the microplate reader was set to 490 nm, and the cell activity was represented by the absorbance value.

Cell Cycle Analysis

Cells were digested with 0.25% trypsin and washed with phosphate-buffered saline (PBS) three times. Then, cells were fixed with 75% ethanol for 24 h at 4°C and washed with PBS containing 1% bovine serum albumin (BSA). Thereafter, cells were stained with propidium iodide (PI) and analyzed under a flow cytometer. The Cell Quest software was used to analyze the distribution proportions of cells in each phase of the cell cycle progression.

Cell Apoptosis Analysis

The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was adopted to detect the apoptosis rate of the cells. Cells were incubated in 300 μL of binding buffer first, and then 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added dropwise to the cell suspension. After shaking the well, cells were incubated in the dark for 20 min. Finally, the percentage of apoptotic cells was determined using flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA).

RNA Extraction and RT-qPCR Analysis

Tissue or cells were added with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), and lysed for 5 min at room temperature. The proposed RNA was diluted with diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) and the RNA concentration was determined by Nanodrop. The appropriate volume of RNA was used for PCR reverse transcription to synthesize complementary deoxyribose nucleic acid (cDNA). The prepared system was then added to a 96-well PCR plate according to the manufacturer's instructions, and at least 3 replicate wells were required for each sample. RT-qPCR reactions were performed on an Applied Bio Step One Plus system (Applied Biosystems, Foster City, CA, USA) to detect the expression levels of related genes, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The reaction conditions were: pre-denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s. The average of 3 replicate wells was taken as the CT value of each group. The quantitative (RQ) method was used for analysis, and the fold change with respect to the control group was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: SLC5A8, F: 5'-GGCACAAACCGGCCTTGACG-3', R: 5'-CGCTAGGTTGATCCGCAAGC-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Cells were lysed in 200 μL of radioimmunoprecipitation assay (RIPA) buffer containing 1% phenylmethylsulfonyl fluoride (PMSF; Beyotime,

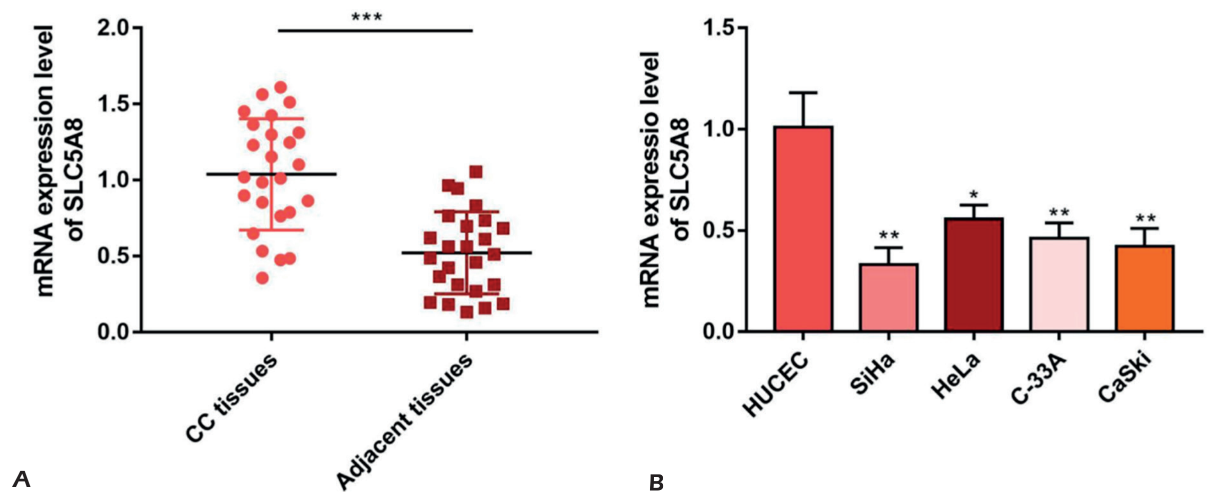

Figure 1. Expression level of SLC5A8 in CC tissues and cell lines. SLC5A8 expression was down-regulated in CC tissues and CC cell lines (SiHa, HeLa, C-33A, CaSki) compared to adjacent tumor tissues (**A**) and normal cervical epithelial cells (**B**) detected by RT-qPCR, respectively. ***$p<0.001$ *vs.* CC tissues; *$p<0.05$, **$p<0.01$ *vs.* HUCEC cells.

Shanghai, China) on ice and shaken for 30 min. The optical density (OD) value of the sample was measured using a microplate reader, and the sample concentration was calculated with reference to the protein standard solution. The appropriate volume of the protein sample was taken according to the concentration, and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After the electrophoresis was completed, the protein was transferred on 0.45 mm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Thereafter, the PVDF membrane was blocked with a 5% skim milk powder blocking solution for 1 h and washed 3 times with Tris Buffered Saline-Tween-20 (TBST) for 10 min each time. The primary antibody was incubated overnight at 4°C and the secondary antibody was incubated for 2 h at room temperature. Finally, an appropriate amount of enhanced chemiluminescence (ECL) working solution was applied to the membrane in the darkroom, and the membrane was placed in a chemiluminescent gel imager for imaging.

Statistical Analysis

The data were analyzed by Graph Pad 7.03 statistical software (La Jolla, CA, USA). Differences between the two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by Post Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Expression Level of SLC5A8 in CC Tissues

We first extracted the RNAs from tissue and cell samples. RT-qPCR results showed that the expression of SLC5A8 was significantly lower in CC tissues than in adjacent tissues (Figure 1A). In addition, compared to HUCEC cells, the expression level of SLC5A8 in CC cell line (SiHa, HeLa, C-33A, CaSki) was also reduced (Figure 1B).

Over-Expression of SLC5A8 Inhibited the Activation of Wnt Signaling Pathway

As a tumor-suppressor gene, SLC5A8 has been reported in many tumors. To further investigate the molecular regulation of SLC5A8 in the malignant progression of CC, SiHa cells were transfected with SLC5A8 overexpression plasmid or negative vector, respectively. As shown in Figure 2A, 2B, the expression level of SLC5A8 in SiHa cell was significantly higher after transfection with SLC5A8 overexpression plasmid than that in Vector group, suggesting that the transfection was successful and could be carried out in the following experiments. The Wnt signaling pathway plays a very important role in cancer cell apoptosis, proliferation, and invasion[10]. Herein, we examined the protein expression level of the Wnt signaling pathway-related molecules after overexpression of SLC5A8. The results in Figure 2C, 2D showed that the protein expressions of β-catenin and C-Myc in SLC5A8 group were significantly lower than those in Vector group,

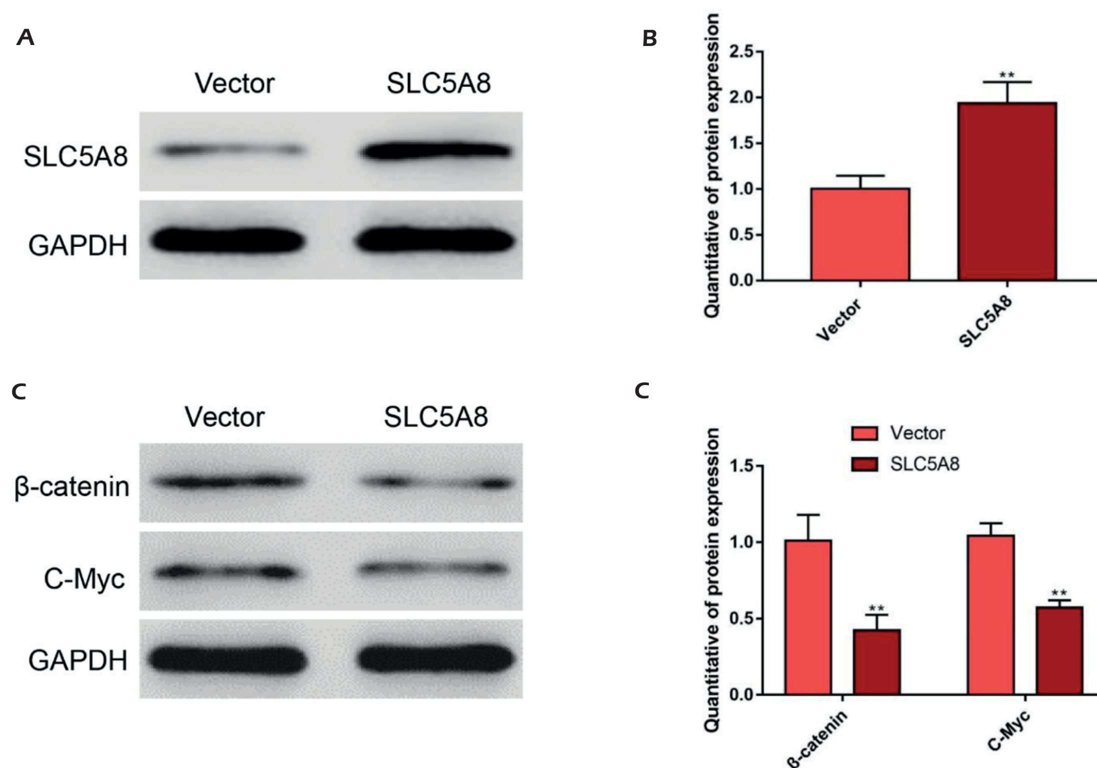

Figure 2. Over-expression of SLC5A8 inhibited the activation of the Wnt signaling pathway. **A,** SLC5A8 was successfully up-regulated after transfection of SLC5A8 overexpression plasmid by Western blot. **B,** Quantitative of SLC5A8 protein expression level. **C,** Expressions of β-catenin and C-Myc were inhibited in SiHa cells overexpressing SLC5A8. **D,** Quantitative of β-catenin and C-Myc protein expression level. $^{**}p<0.01$ *vs.* Vector group.

indicating that high expression of SLC5A8 inhibited the activation of the Wnt signaling pathway in SiHa cells.

Over-Expression of SLC5A8 Impaired the Proliferation Activity of SiHa Cells

To explore the effect of SLC5A8 on SiHa proliferation activity, we performed an MTT assay. The results showed that the proliferation activity was significantly inhibited in SiHa cells overexpressing SLC5A8. However, LiCl partially reversed the decreased proliferation activity induced by SLC5A8 overexpression (Figure 3A). In addition, we also explored the effect of SLC5A8 on the cell cycle of SiHa cells. As shown in Figure 3B, the proportion of G1 phase cells in SiHa cells overexpressing SLC5A8 was significantly higher, whereas the proportions of cells in the S and G2 phase were lower than those in Vector group. Notably, the above trends were alleviated after the additional treatment of the Wnt signaling pathway activator LiCl.

Over-Expression of SLC5A8 Promoted the Apoptosis of SiHa Cells

Considering that over-expression of SLC5A8 inhibited the proliferation activity of SiHa cells, we further examined the effect of SLC5A8 on SiHa cell apoptosis. We found that SLC5A8 was closely linked to the apoptosis of SiHa cells, which was verified in Figure 4A, 4B. The proportion of apoptosis cells in SLC5A8 group was higher than that in Vector group and SLC5A8+LiCl group. Further, we detected the anti-apoptosis gene Bcl-2 and pro-apoptosis gene Bax in different groups, respectively. As shown in the Figure 4C-4E, Bcl-2 was significantly down-regulated while Bax was significantly up-regulated in SiHa cells overexpressing SLC5A8. However, after treatment with LiCl, the expression modulation was significantly antagonized.

Discussion

In recent years, the incidence of CC greatly increases, as well as its recurrence rate, which seriously threatens women's health[1]. With the wide application of cervical exfoliative cytology screening and HPV testing, CC and its precan-

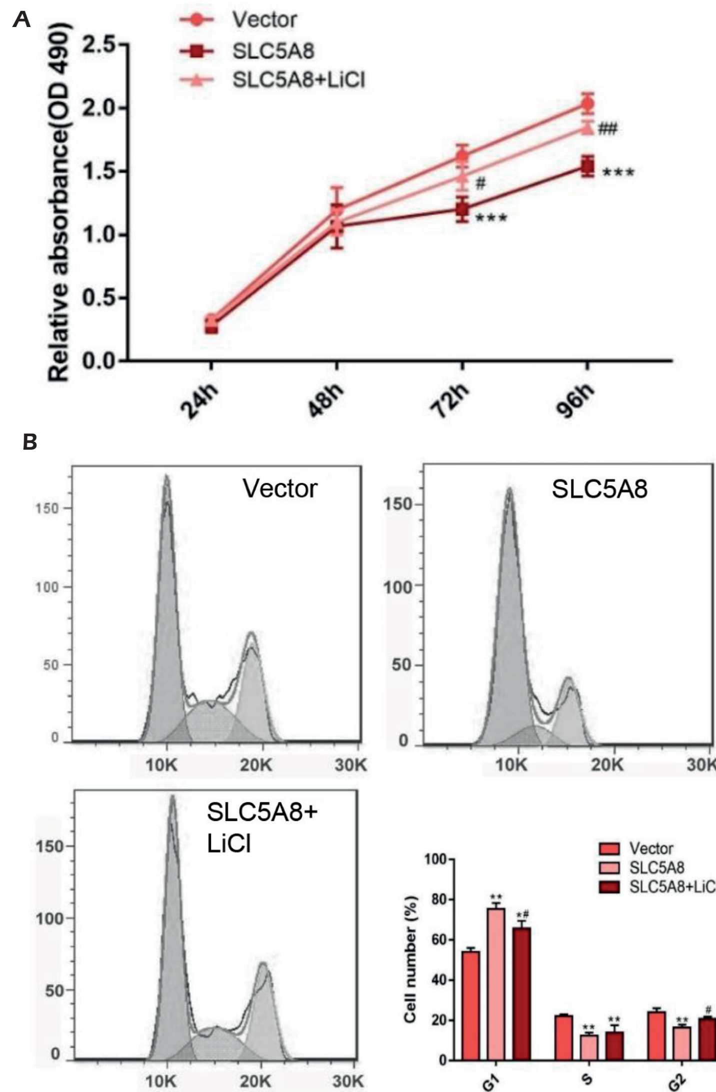

Figure 3. Effects of SLC5A8 on the proliferation activity and cell cycle of SiHa cells. **A,** Proliferation activity of SiHa cells was suppressed by over-expression of SLC5A8, whereas co-treatment with LiCl could restore it. **B,** Over-expression of SLC5A8 resulted in a higher cell number in G1 phase and lower cell number in S and G2 phase, and this trend could be resisted by LiCl. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* Vector group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* SLC5A8 group.

cerous lesions could be detected, diagnosed, and treated at an early stage[11]. A large number of studies[12,13] have found that HPV is widely involved in the occurrence and progression of CC. However, HPV infection will not inevitably result in the occurrence of CC[14,15]. Therefore, some scholars speculated that certain genes could independently induce the occurrence and progression of CC[16]. At present, increasing evidence indicates that there are many abnormally expressed genes in CC attribute to the tumorigenesis, such as the activation of oncogenes Ras[17,18], the inactivation of tumor suppressor genes PTEN[19,20], etc.

Solute transport (SLC) has many species, including approximately 55 families and 362 members as the largest transporter in human cells[21]. As a tumor-suppressor gene, SLC5A8 has a tissue-specificity[22]. In addition, the expression of SLC5A8 is down-regulated in many tumor tissues such as breast cancer[6], colon cancer[7], gastric cancer[8]. These findings strongly suggested that SLC5A8 is tightly involved in tumorigenesis.

Based on this, we first examined the expression level of SLC5A8 in CC tissues and corresponding adjacent tissues, and we found that the expression level of SLC5A8 in the later was significantly

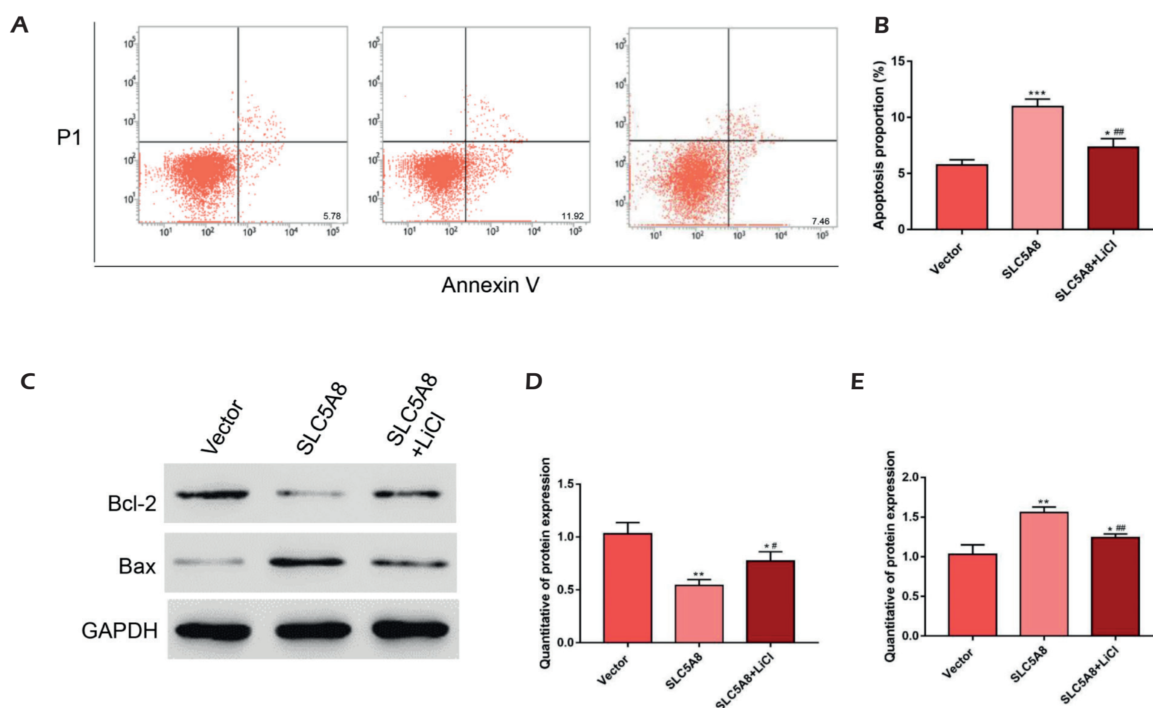

Figure 4. Effects of SLC5A8 on the apoptosis of SiHa cells. **A-B,** Apoptosis proportion of SiHa cells increased after overexpression of SLC5A8 plasmid, while LiCl could alleviate it. **C,** Bcl-2 was significantly down-regulated while Bax was significantly up-regulated after SiHa cells highly expressed SLC5A8. **D,** Quantitative of Bcl-2 expression level. **E,** Quantitative of Bax expression level. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* Vector group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* SLC5A8 group.

lower than that in the former. Besides, we detected down-regulated expression level of SLC5A8 in different CC cell lines (SiHa, HeLa, C-33A, CaSki) compared to HUCEC cells. These results strongly suggested a close relationship between SLC5A8 and CC.

The anti-tumor effect of SLC5A8 has been extensively analyzed[22]. Transfection efficacy of SLC5A8 overexpression plasmid was first verified. Subsequently, we found that the protein expression levels of C-Myc and β-catenin were down-regulated by overexpression of SLC5A8, indicating that the Wnt signaling pathway was inactivated. The Wnt signaling pathway is a conserved signaling pathway in the early progress of organisms, and the disorder of this pathway can cause various diseases such as tumors[10]. A large number of experiments have verified this conclusion. Kishore et al[23] reported that Vitamin K3 (menadione) suppresses the epithelial-mesenchymal-transition and Wnt signaling pathway in human colorectal cancer cells. Akrami et al[24] indicated that PLGF knockdown could induce apoptosis through the Wnt signaling pathway in gastric cancer stem cells. In addition, Li et al[25] illustrated that inhibition of DNMT could suppress the stemness of colorectal cancer cells by down-regulating the Wnt signaling pathway.

To deeply explore the effects of SLC5A8 and the Wnt signaling pathway on cell cycle and apoptosis of CC, SiHa cells were transfected with vector plasmid, SLC5A8 overexpression plasmid or SLC5A8 overexpression plasmid + LiCl (an activator of Wnt signaling pathway). It was found that the proliferation activity of SiHa cells was significantly reduced after overexpression of SLC5A8, and the proportion of cells in the G1 phase was also enhanced. In addition, flow cytometry results showed that the cell apoptosis rate of SiHa cells overexpressing SLC5A8 was higher than that of Vector group. Surprisingly, the above changes caused by overexpressed SLC5A8 were attenuated after LiCl treatment, suggesting that SLC5A8 acted as an inhibitor in CC progress by regulating the Wnt signaling pathway.

Cell invasion and migration are extremely important events in the progress of cancer. Thus,

our further research will be focused on exploring the influence of SLC5A8 on the invasion and migration of SiHa cells and the underlying mechanisms, so as to better clarify the regulatory role of SLC5A8 in CC progress.

Conclusions

This paper revealed that SLC5A8 acted as a suppressor in the progression of CC by regulating the Wnt signaling pathway. Thus, these results indicated that SLC5A8 might serve as a potential diagnostic and therapeutic molecular marker for CC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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