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Original Research Article

SLC6A14 promotes cell migration and inhibits autophagy in gastric cancer by regulating JAK2/STAT3 signaling

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Abstract

Purpose: *To investigate the role of solute carrier family 6-member 14 (SLC6A14) in gastric cancer (GC) tumorigenesis.*
Methods: *The GC cell proliferation was evaluated by CCK8 and colony formation assays. Flow cytometry and wound healing assays were used to investigate cell apoptosis and migration, respectively. Immunofluorescence was used to investigate autophagy.*
Results: *The SLC6A14 was elevated in GC cells ($p < 0.001$). Overexpression of SLC6A14 increased GC cell viability and migration ($p < 0.001$), increased colony formation, and suppressed cell apoptosis ($p < 0.05$). However, knockdown of SLC6A14 inhibited GC tumorigenesis by decreasing cell viability and migration, reducing colony formation, and promoting cell apoptosis ($p < 0.001$). Overexpression of SLC6A14 decreased the LC3-II/LC3-I ratio. Silencing of SLC6A14 increased the LC3-II/LC3-I ratio and increased the fluorescence intensity of LC3. Overexpression of SLC6A14 increased phosphorylation of JAK2 and STAT3 ($p < 0.001$).*
Conclusion: *Knockdown of SLC6A14 suppresses GC cell migration and proliferation and promotes autophagy through inactivation of JAK2/STAT3 signaling.*

Keywords: *SLC6A14, Gastric cancer, Proliferation, Migration, Autophagy, JAK2/STAT3*

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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers [1]. Although the incidence and mortality of GC has reduced globally, GC still ranks as the second and third most common cancer in males and females, respectively [1]. Despite advancements in diagnostic methods and surgical procedures, overall survival of patients with GC, especially advanced GC, remains unsatisfactory [2]. Therefore, an in-depth understanding of the mechanisms associated with GC initiation and progression is needed urgently.

Because amino acid and glucose uptake is required for cancer cell growth [3], amino acid transporters are upregulated in various cancers and promote the proliferation, survival, growth, and long-term maintenance of cancer cells [4]. Solute carrier family 6 member 14 (SLC6A14) transports amino acids (except aspartate and

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glutamate) and has been shown to be involved in cellular metabolism, proliferation, angiogenesis, metastasis, apoptosis, ferroptosis, autophagy, and chemosensitivity of tumors [5]. Loss of SLC6A14 interfered with tumor cell growth and proliferation [6], however the role of SLC6A14 in GC has not yet been reported.

Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling is involved in cancer growth, apoptosis, angiogenesis, and inflammation [7,8]. In GC, JAK2/STAT3 signaling was shown to be constitutively activated and to contribute to tumor initiation and development [9]. Inhibition of JAK2/STAT3 blocked proliferation of GC, reduced inflammation, and promoted apoptosis [10]. Moreover, SLC6A14 enhanced colorectal cancer cell proliferation and metastasis through activation of JAK2/STAT3 [11]. Therefore, SLC6A14 may also regulate GC through JAK2/STAT3 signaling. The effects of SLC6A14 on GC cell proliferation, migration, apoptosis, and autophagy were investigated.

EXPERIMENTAL

Cell culture and treatment

Human GC cells (AGS, SNU-1, SGC-7901, and HGC-27) and normal gastric epithelial cells (GES-1) were purchased from American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). siRNA targeting SLC6A14 (siSLC6A14) and the negative control siRNA (siNC) were synthesized by Invitrogen. Full length SLC6A14 was cloned into the pcDNA 3.1 vector (Genepharma, Suzhou, China). AGS and SNU-1 cells at ~85 % confluence were seeded into 96-well plates and transfected with the pcDNA vector or siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Cells treated with Lipofectamine 2000 only were regarded as the control group.

Quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from cells using TRIzol (Invitrogen). RNA was reverse transcribed into cDNA and SLC6A14 mRNA expression was measured using the PreTaq II kit (Takara, Dalian, Liaoning, China) and the following primers: forward 5'-GTCGACCCTGCCTTCAAAGAACTG GTA-3' and reverse 5'-AAGCTTGCACTCTCCC CTGTTCCTA-3'. SLC6A14 mRNA expression was normalized to GAPDH (forward: 5'-TCAACGACCACTTTGTC AAGCAGAGT-3' and reverse: 5'-GCTGGTG GTCCAGGGGTCTTACT-3') using the $2^{-\triangle\triangle Ct}$ method.

Cell viability and apoptosis assays

The AGS and SNU-1 cells were transfected with pcDNA vector or siRNA for 24 h, seeded into 96-well plates, and then incubated for another 24 h. Cells were then treated with 10 µL of CCK8 reagent (Beyotime, Beijing, China) for 2 h. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). For flow cytometry, transfected AGS and SNU-1 cells were harvested and resuspended in the binding buffer from the Annexin FITC, PI Staining Kit (Thermo Fisher Scientific). The cells were stained with 5 µL of PI and 5 µL of Annexin V in the dark (Thermo Fisher Scientific) and then analyzed using a FACS flow cytometer (Life Technologies, Darmstadt, Germany) to determine apoptotic ratios.

Cell proliferation and migration assays

The AGS and SNU-1 cells were seeded into 6-well plates and transfected with a pcDNA vector or an siRNA. The cells were cultured in RPMI-1640 medium for 10 d, fixed in methanol, stained with crystal violet, and observed using a microscope (Olympus, Tokyo, Japan). Image J v.1.46 software was used to count the colonies. For the cell migration analysis, AGS and SNU-1 cells were seeded into 6-well plates and transfected with a pcDNA vector or an siRNA. A pipette tip was used to generate a scratch in the middle of each well. After 24 h, the cells were observed by microscopy (Olympus) and Image J software was used to measure wound widths.

Immunofluorescence

The AGS and SNU-1 cells were transfected with siRNA and a GFP-LC3-expressing plasmid (pEGFP-LC3; Cell Biolabs, San Diego, CA, USA) for 24 h. Cells were fixed with 4 % paraformaldehyde and then stained with DAPI (Sigma-Aldrich, Milwaukee, WI, USA). Immunofluorescence was evaluated using a confocal microscope (Olympus).

Western blot

Cells were lysed in RIPA buffer (Beyotime) and protein concentrations of cell lysates were determined using a BCA kit (Thermo Fisher Scientific). Protein samples were separated by 10 % SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in 5 % bovine serum albumin and

probed with specific antibodies: anti-SLC6A14 and anti-β-actin (1:2000, Abcam, Carlsbad, CA, USA), anti-PARP and anti-cleaved PARP (1:2500, Abcam), anti-Bax and anti-Bcl-2 (1:3000, Abcam), anti-LC3-I and anti-LC3-II (1:3500, Abcam), anti-p-JAK2 and anti-JAK2 (1:4000, Abcam), and anti-p-STAT3 and anti-STAT3 (1:4500, Abcam). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Abcam). Immunoreactivities were visualized by enhanced chemiluminescence (Sigma-Aldrich).

Statistical analysis

Experiments were performed in triplicate and data are expressed as mean ± SEM. Data were analyzed by the student's t test or one-way analysis of variance in SPSS software. *P*-values < 0.05 were considered statistically significant.

RESULTS

SLC6A14 level in GC

Data from GEPIA (http://gepia.cancer-pku.cn/) showed that SLC6A14 is upregulated in GC tissues (n = 408) compared to normal tissues (n = 211; Figure 1 A). Ualcan analysis (http://ualcan.path.uab.edu/index.html) also confirmed up-regulation of SLC6A14 in tumor tissues (n = 415; Figure 1 B). The SLC6A14 expression was higher in human GC cells (AGS, SNU-1, SGC-7901, and HGC-27) than in GES-1 cells (Figures 1 C and D) indicating an association between SLC6A14 and GC.

Effect of SLC6A14 on GC cell survival

To investigate the effect of SLC6A14 on GC, AGS and SNU-1 cells were transfected with a pcDNA vector or an siRNA. Transfection with pcSLC6A14 increased SLC6A14 protein expression and transfection with siSLC6A14 decreased SLC6A14 expression (Figure 2A). Overexpression of SLC6A14 increased AGS and SNU-1 cell viability and knockdown of SLC6A14 decreased AGS and SNU-1 cell viability (Figure 2B). The SLC6A14 overexpression suppressed AGS and SNU-1 cell apoptosis and SLC6A14 knockdown promoted AGS and SNU-1 cell apoptosis (Figure 2C). SLC6A14 overexpression enhanced BCL-2 protein expression and reduced cleaved PARP and Bax protein expression in AGS and SNU-1 cells (Figure 3). However, silencing of SLC6A14 downregulated BCL-2 protein expression and upregulated cleaved PARP and Bax protein expression (Figure 3).

SLC6A14 promoted GC cell migration of GC

Transfection with pcSLC6A14 increased the number of AGS and SNU-1 colonies and transfection with siSLC6A14 decreased the number of AGS and SNU-1 colonies (Figure 4A and Figure 4B). The SLC6A14 overexpression promoted AGS and SNU-1 cell migration and SLC6A14 knockdown repressed AGS and SNU-1 cell migration (Figure 4C and Figure 4D) demonstrating the anti-invasive effect of SLC6A14 silencing on GC.

SLC6A14 suppressed GC cell autophagy

The LC3-I protein expression was elevated and LC3-II protein expression downregulated in AGS and SNU-1 cells overexpressing SLC6A14 (Figure 5 A). Silencing of SLC6A14 upregulated the LC3-II/LC3-I ratio, which promotes autophagy (Figure 5 A). Moreover, immunofluorescence analysis showed that knockdown of SLC6A14 increased the fluorescence intensity of LC3-II in AGS and SNU-1 cells (Figure 5 B) indicating the pro-autophagic effect of SLC6A14 silencing on GC.

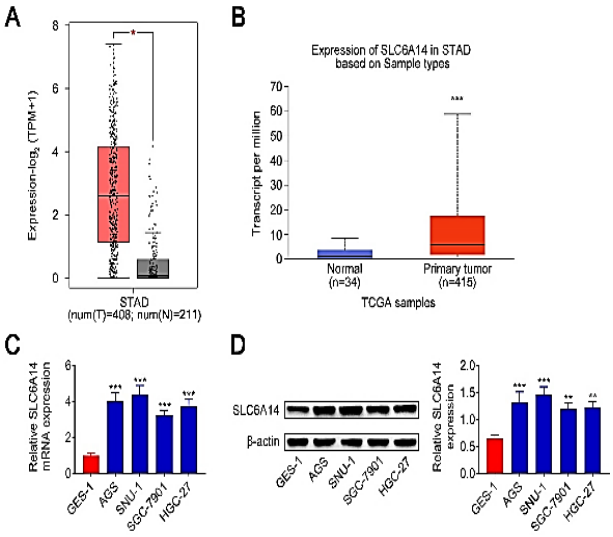

Figure 1: SLC6A14 elevation in GC tissues and cells compared to normal tissues. (A) SLC6A14 in GC tissues (n = 408) compared to normal tissues (n = 211) based on GEPIA (http://gepia.cancer-pku.cn/) data. (B) SLC6A14 in GC tissues (n = 415) compared to the normal tissues (n = 34) based on Ualcan analysis (http://ualcan.path.uab.edu/index.html). (C) SLC6A14 mRNA expression in human GC cells (AGS, SNU-1, SGC-7901, and HGC-27) compared to normal gastric epithelial cells (GES-1). (D) SLC6A14 protein expression in human GC cells compared to GES-1 cells. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

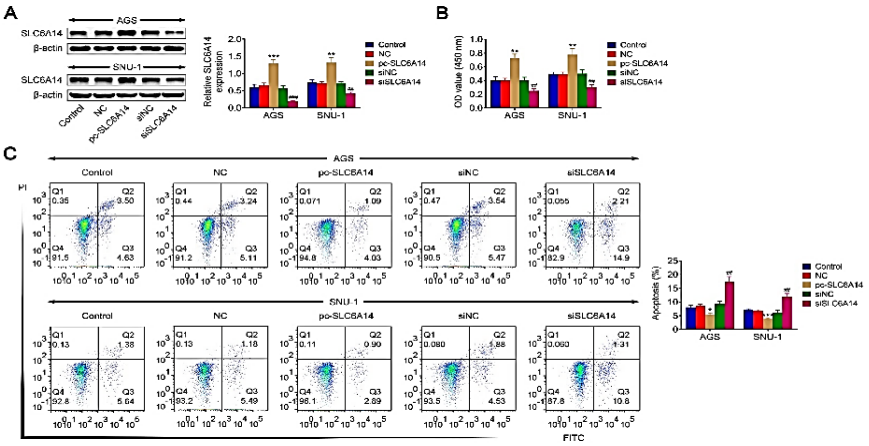

Figure 2: Effect of SLC6A14 on GC cell survival. (A) Transfection with pcSLC6A14 on SLC6A14 protein expression in AGS and SNU-1 cells and transfection with siSLC6A14 on SLC6A14 protein expression, (B) Overexpression and knockdown of SLC6A14 on AGS and SNU-1 cell viability, (C) Overexpression and knockdown of SLC6A14 on AGS and SNU-1 cell apoptosis. *, **, *** vs. Negative Control (NC): $p < 0.05$, $p < 0.01$, $p < 0.001$. #, ##, ### vs. siNC: $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively

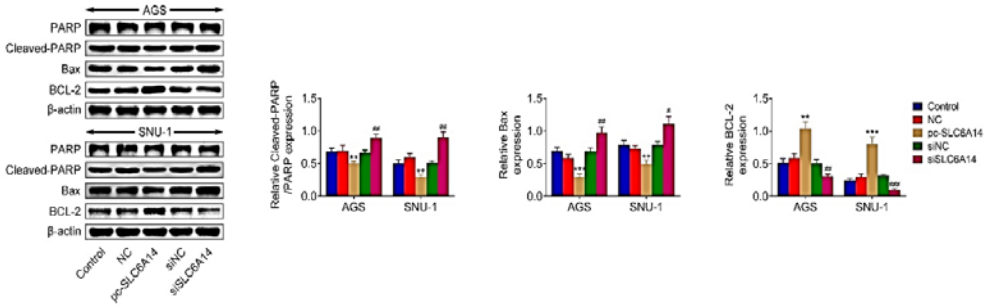

Figure 3: Effect of SLC6A14 on BCL-2 protein expression, cleaved PARP and Bax expression in AGS and SNU-1 cells. *, **, *** vs. NC: $p < 0.05$, $p < 0.01$, $p < 0.001$. #, ##, ### vs. siNC: $p < 0.05$, $p < 0.01$, $p < 0.001$

SLC6A14 activated JAK2/STAT3

Protein expression of JAK2 and STAT3 was not affected in AGS and SNU-1 cells upon transfection with the pcDNA vector or siRNA (Figure 6). However, JAK2 and STAT3 phosphorylation increased upon SLC6A14 overexpression and expression of p-JAK2 and p-STAT3 decreased uponSLC6A14 knockdown (Figure 6) indicating that knockdown of SLC6A14 suppressed JAK2/STAT3 activation in GC.

DISCUSSION

Solute carriers that transport vitamins, lipids, sugars, and amino acids have been implicated in tumor pathogenesis [12]. Overexpression of SLC6A6 predicted a poor GC prognosis and induced GC tumorigenesis [13]. In this study, it has been shown that SLC6A14 promotes oncogenesis in GC by promoting cell proliferation and migration and by suppressing apoptosis and autophagy.

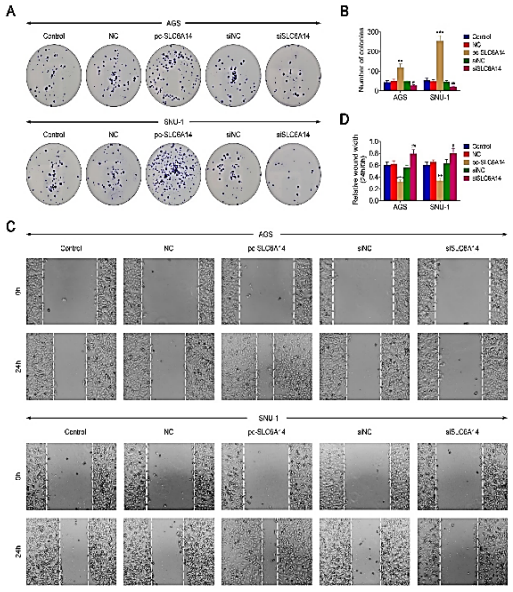

Figure 4: Effect of SLC6A14 on GC cell migration (A) Overexpression and knockdown of SLC6A14 on AGS and SNU-1 cell proliferation, (B) Overexpression and knockdown of SLC6A14 on the number of AGS and

SNU-1 colonies, (C) Overexpression and knockdown of SLC6A14 on AGS and SNU-1 cell migration, (D) Relative wound widths of AGS and SNU-1 cells transfection with pc-SLC6A14 or siSLC6A14. **, *** vs. NC: $p < 0.01$, $p < 0.001$. #, ## vs. siNC: $p < 0.05$, $p < 0.01$

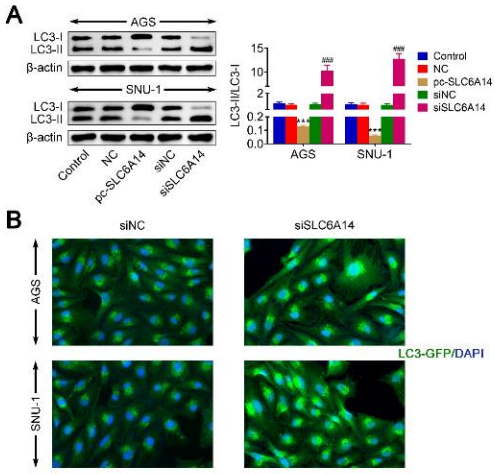

Figure 5: Effect of SLC6A14 on GC cell autophagy. (A) Overexpression of SLC6A14 on LC3-I and LC3-II protein expression and knockdown of SLC6A14 on the LC3-II/LC3-I ratio in AGS and SNU-1 cells, (B) Knockdown of SLC6A14 on the fluorescence intensity of LC3 in AGS and SNU-1 cells. *** vs. NC: $p < 0.001$. ### vs. siNC: $p < 0.001$

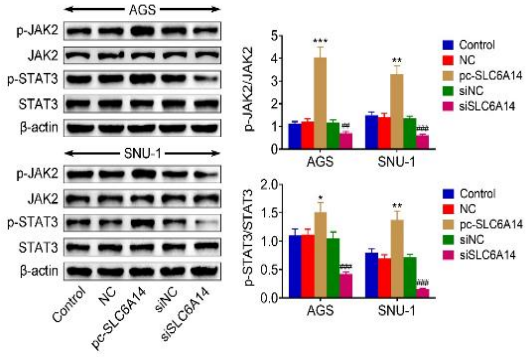

Figure 6: Overexpression and knockdown of SLC6A14 on JAK2 and STAT3 phosphorylation in AGS and SNU-1 cells. *. **, *** vs. NC: $p < 0.05$, $p < 0.01$, $p < 0.001$. ##, ### vs. siNC: $p < 0.01$, $p < 0.001$

Data from the TCGA database showed that SLC6A14 was upregulated in GC tissues, and that SLC6A14 was also elevated in GC cells. The role of SLC6A14 in GC prognosis and diagnosis should be investigated by analyzing the relationships between SLC6A14 expression and clinical parameters of GC patients. In this study, functional assays revealed that knockdown of SLC6A14 promoted GC cell apoptosis and inhibited GC cell proliferation and migration. In addition, it has been shown that the epithelial-to-mesenchymal-transition (EMT) contributes to GC invasion and metastasis [14]. Therefore, SLC6A14 may promote the EMT during GC tumorigenesis.

Autophagy regulates the degradation and recycling of defective proteins and organelles, thus autophagy regulates the intracellular homeostatic processes during GC development. Autophagy can play a dual role in GC by promoting cell survival or promoting cell death [15]. Protective autophagy involves the conversion of LC3-I to LC3-II, activates autophagosomes, and inhibits proliferation, metastasis, the EMT, and angiogenesis of GC [16]. Blocking of SLC6A14 has been shown to induce autophagy in pancreatic cancer by inactivating mTORC1 signaling [17]. Knockdown of SLC6A14 has been shown to induce the conversion of LC3-I to LC3-II and enhance the fluorescence intensity of LC3 indicating that silencing of SLC6A14 promotes autophagy in GC cells.

Activation of JAK2/STAT3 signaling was shown to contribute to cell proliferation, metastasis, the EMT, and angiogenesis in GC [10]. In addition, blocking of JAK2/STAT3 signaling promoted autophagy in GC [18]. Moreover, SLC6A14 upregulated p-JAK2 and p-STAT3 and stimulated tumor proliferation and metastasis in colorectal cancer cells [11]. It has been shown that knockdown of SLC6A14 decreased JAK2 and STAT3 phosphorylation in GC cells. Specific inhibitors of JAK2/STAT3 signaling should be used in future experiments to confirm that SLC6A14 promotes GC via activation of JAK2/STAT3 signaling.

CONCLUSION

The SLC6A14 functions as an oncogene in GC. Inhibition of SLC6A14 promotes apoptosis and autophagy and suppresses cell proliferation and migration of GC cells via inactivation of JAK2/STAT3 signaling. However, the role of SLC6A14 in gastric tumor xenografts should be investigated in future research.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We 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 the authors. Haifeng Wang and Jindao Wang designed the study, performed the experiments, supervised the data collection, and analyzed and interpreted the data. Jindao Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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