Interference of human Na/K-ATPaseB1 subunit on proliferation and migration of gastric adenocarcinoma cell line SGC-7901

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Background and Objective: The Na/K-ATPaseB1 (ATP1B1) subunit gene is highly expressed in well-differentiated tumor cells, while it is hypoexpressed in poorly differentiated tumor cells. The expression of ATP1B1 is closely related to cell tight junction and polarity of epithelial cells. This study was to investigate the effect of specific interference of human Na/K-ATP1B1 on cell proliferation and migration of gastric adenocarcinoma cell line SGC-7901. Methods: Four shRNA plasmids specifically targeting different sites in ATP1B1 mRNA, sh150, sh295, sh562, sh765, were constructed and transiently transfected into SGC-7901 cells. Stable positive clones, shATP1B1-7901 cells, were sorted out by G418. The expression of ATP1B1 mRNA was detected by semi-quantitative RT-PCR and real-time PCR. Cell proliferation was measured by MTT; cell cycle distribution was assessed by flow cytometry, and clone forming was analyzed by the colony formation assay. Cellular migration was observed using the Transwell experiment. Results: At 24 h after transfection, the inhibition ratios of sh150, sh295, sh562, sh765 on ATP1B1 mRNA were (60.87 ± 4.38)%, (44.93 ± 2.24)%, (52.17 ± 2.60)% and (52.17 ± 2.60)% respectively in SGC-7901 cells, which were significantly higher than that of shNC control (3.00 ± 0.15)% (p < 0.05). Among the four ATP1B1 shRNAs, sh150 exerted the strongest effect (p < 0.05) and was used in the following study. Assayed by RT-PCR and real-time PCR, the expression of ATP1B1 mRNA was inhibited by (85.72 ± 5.22)% and (85.72 ± 5.22)% respectively in shATP1B1-7901 cells, in comparison with (3.3 ± 0.22)% and (4.17 ± 0.33)% in the shNC-7901 cells (p < 0.05). The proliferation ratio was higher in shATP1B1-7901 cells than in shNC-7901 and SGC-7901 cells three days after transfection (p < 0.05). The percentage of cells at S and G2/M phases in shATP1B1-7901 cells were significantly increased compared with those in shNC-7901 and SGC-7901 cells (p < 0.05). The clone formation rate of shATP1B1-7901 cells (68.50 ± 2.65)% was higher than that of shNC-7901 cells (50.00 ± 2.53)% and SGC-7901 cells (52.50 ± 2.11)% (p < 0.05). Moreover, the migration ratio of shATP1B1-7901 cells (2.80 ± 0.02)% was significantly enhanced compared to shNC-7901 (1.15 ± 0.05)% and shNC-7901 cells (1.25 ± 0.02)% (p < 0.05). Conclusion: Silencing of ATP1β1 gene can enhance the proliferation and migration capability of SGC-7901 cells.

Na+-K+ ATPase (Na+-K+ pump) is an important system for cellular energy conversion, and it is also the target of free radicals mediating cell membrane injury.1 The enzyme is constituted of subunit A and B. ATP1B1 gene is responsible for coding the B1 subunit of Na+-K+ ATPase. There are only a few reports on the role of the B1 subunit in tumor cells. The expression of Na+-K+ ATPase is decreased at different degrees in many kinds of tumor cells, which is relatively higher in highly differentiated tumor cells, while relatively lower in poorly differentiated tumor cells. In addition, the expression of ATP1B1 is closely related with the presence of tight intercellular junction and the polarity of epithelial cells. In this study, we constructed short hairpin RNAs (shRNAs) specially targeting at ATP1B1 mRNA and transfected them into gastric adenocarcinoma cell line SGC-7901 cells, thus to investigate the influence of ATP1B1 gene on proliferation and migration of tumor cells.

Materials and Methods

Reagents. The following reagents were used: newborn calf serum (Wuhan Sanli Biotechnology Co., Ltd, China), RPMI1640 culture medium and typsin (Gibco, USA), penicillin and streptomycin (North China Pharmaceutical Group Corporation, China), G418, MTT (Sigma, USA), Lipofectamine™ 2000 (Invitrogen, USA), cell total RNA rapid isolation kit (Huashun, Shanghai), RT-PCR Kit ReverTra Ace combined type (Boracker, Chengdu, China), RT-PCR, real-time PCR primers and real-time PCR kit (Takara, Dalian, China), DNA Marker II (Tiangen, Beijing), Transwell mini-chamber (8.0 μm, Milipore, USA).
Cell culture. Human gastric adenocarcinoma cell line SGC-7901 was stored in the State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and cultured in RPMI1640 medium containing 10% calf serum, 100 u/mL streptomycin and 100 u/mL penicillin at 37°C, 5% CO₂.

Methods. Construction of shRNA plasmids. Construction of four interference plasmids. Four shRNA plasmids specifically targeting different sites in ATP1B1 mRNA, sh150, sh295, sh562, sh765, and negative control plasmid (shNC) were synthesized by Shanghai GenePharma Co. Ltd. The oligonucleotide sequences with small hairpin structures were cloned into empty vector pGPU6/GFP/Neo to construct recombinant plasmids. The size of ukaryotic expression vector pGPU6/GFP/Neo is 5117 bp (Fig.1), containing the human U6 promoter and two enzyme cutting sites, BamH I and BbsI. The vector also comprises the neomycin-resistant and green fluorescent protein (GFP) genes. After successful transfection of the recombinant plasmids into eukaryocytes, expressed GFP facilitated direct observation of the transfection rate under the inverted fluorescence microscope. The target sites and sequences of ATP1B1 are listed in Table 1.

Screening of shRNA target sites. SGC-7901 cells during logarithmic phase of growth were harvested and seeded into six-well plate. When the culture reached 80–90% confluence, transfection was performed using Lipofectamine™ 2000 according to the manufacture's instruction. In brief, SGC-7901 cells were transfected with four ATP1B1 shRNAs (sh150, sh295, shATP562, sh765) and the negative control plasmid shNC, respectively. In the blank control group, the transfection complex was replaced by RPMI1640 culture medium. The fluorescence ratio in each group was expressed GFP facilitated direct observation of the transfection rate under the inverted fluorescence microscope. The target sites and sequences of ATP1B1 are listed in Table 1.

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The expression of ATP1B1 mRNA was detected by RT-PCR using β-actin as the internal reference. The primers for ATP1B1 and β-actin were as follows: ATP1B1, (upstream) 5'-GCCAGGATACCAAGATTTCCCTCAG-3', (downstream) 5'-CCTTATCTTTACATCTGCCTTGCC-3', with the product size of 422bp; β-actin, (upstream) 5'-CACCACACCTTCTACAATGAGC-3', (downstream) 5'-GT-GATCTCTCCTCTGCATCCTGT-3', yielding a product of 659 bp.

PCR products were subjected to 1.5% agarose gel electrophoresis. The bands were observed by Bio-Rad Gel Imaging System and analyzed by Quantity One 4.5.0 Software. Relative expression of ATP1B1 = transfection efficiency × (ATP1B1 band density/β-actin band density). Inhibition of ATP1B1 mRNA = (relative expression of ATP1B1 in control group – relative expression of ATP1B1 in experiment group)/relative expression of ATP1B1 in control group × 100%. The experiment was repeated for three times and the average values were calculated.

Construction of stable shATP1B1-7901 cell line. As sh150 exerted the strongest inhibition effect on the mRNA level of ATP1B1, it was used to construct the stable shATP1B1-7901 cell line. The cells were divided into blank control group (no transfection), negative control group (transfected with shNC) and sh150 transfection group.

Positive clone cells were screened using G418 at a final concentration of 1000 mg/L 48 h after transfection. The culture medium was changed every 2–3 days. The final concentration of G418 in the culture medium was changed to 500 mg/L 10–12 days later, and cells were cultured till clones were visible to naked eyes. Clones emitting green florescence were marked under the fluorescence microscope, and then subjected to extensive culture with the concentration of G418 at 500 mg/L. The screened cells were stable transfected cells and named shATP1B1-7901. The stable transfected negative control cells were named shNC-7901. The mRNA expression of ATP1B1 was detected by RT-PCR, which was further confirmed by real-time PCR. The primer design and part of the methods were performed as described by Xiong et al.² Real-time PCR primer sequences for ATP1B1 and GAPDH were as follows: ATP1B1, (upstream) 5'-GGCA GTTGGTTTAAGATCCTCCT-3'; (downstream) 5'-AA TCTGTGTTAATCCTTGCCG-3'; GAPDH, (upstream) 5'-GAAGGTGATTAAAGATCCT-3', (downstream) 5'-GAA GA TGGTGATGGGAGA TTTCT-3'.

Real-time PCR was performed using the SYBR green method and the reaction system was prepared according to the instruction of the real-time PCR kit. ATP1B1 gene expression was detected by the BIO-RAD iCycler IQ real-time RCR reactor. The reaction for each sample was performed in triplicate. The reaction conditions were as follows: 95 for 2min, followed by 40 cycles of 95°C for 10s, 55°C for 10s, and 72°C for 30 s. The inhibition ratio of ATP1B1 mRNA was calculated.

MTT assay. Stable cell lines and routinely cultured SGG-7901 cells during logarithmic phase of growth were divided into blank control group (no transfection), negative control group (transfected with shNC) and sh150 transfection group. Cells were digested with 0.25% tynsin-0.01% EDTA to prepare single cell suspension. Then 200 μl cell suspension was seeded into a 96-well plate at a density of 1500 cells/well. Starting from the next day, five wells were removed from each group every day for seven successive days, and the absorption (A) was measured at 570 nm. The experiment was repeated for three times and the average values were calculated. The cell proliferation rate = A value on Day N/A value on Day 1 × 100%.
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Detection of cell cycle distribution. Cells during logarithmic phase of growth were divided into three groups as mentioned above and prepared a single cell suspension. Then cells were fixed in 70% ethanol at -20°C and kept at 4°C. Before analysis, cells were washed with PBS and treated with 1% Triton X-100, 0.01% RNase and 0.5% propidium iodide. The DNA content was detected on the Profile II flow cytometer with an excitation wavelength at 488 nm and analyzed by Multicycle software. Proliferation index was calculated using the following formula:

$$PI = \frac{S\ phase + G_2/M\ phase}{G_0/G_1\ phase + S\ phase + G_2\ phase} \times 100\%$$

The experiment was repeated three times and the average values were calculated.

Clone formation assay. Cells during logarithmic phase growth were divided into three groups as mentioned above and digested with 0.25% trypsin-0.01% EDTA to prepare a single cell suspension. After counting and dilution, the cells were seeded into six-well plate at a density of 200 cells/well. Three parallel wells were set for each group. Cells were cultured in 2 ml RPMI1640 containing 10% calf serum at 37°C, 5% CO2. The culture medium was changed every three days. The culture was terminated 10–14 days later. The culture medium was removed and cells were washed with PBS for twice, followed by methanol fixation for 15min and Giemsa staining. Cells that migrated to the outer surface of the filter membrane were counted under a microscope. Five visual fields (× 200) were randomly selected from each filter membrane to calculate the average value. Each experiment was repeated three times. The migration rate (%) = (number of cells that migrated to the outer surface of the filter membrane/number of seeded cells) × 100%.

Statistical analysis. All data were expressed as mean ± SD. Variance analysis and paired comparison were performed using SPSS 13.0 Software package. p < 0.05 was considered statistically significant.

Results

Successful transfection of ATP1B1 shRNAs into SGC-7901 cells. At 24h after transfection, the transfection efficiency of four ATP1B1 shRNAs, sh150, sh295, sh562, sh765, and shNC negative control in SGC-7901 cells reached 70–80%. The mRNA expression of ATP1B1 was significantly decreased after transfection with ATP1B1 shRNAs compared with transfection with shNC (p < 0.05). The inhibition ratios of sh150, sh295, sh562, sh765 and shNC on ATP1B1 mRNA were (60.87 ± 4.38)%, (44.93 ± 2.24)%, (49.28 ± 2.02)%, (52.17 ± 2.60)% and (3.00 ± 0.15)%, respectively. Therefore, sh150 was chosen to be used in the following experiments.

Inhibition effect of stable transfection of shATP1B1 on ATP1B1 mRNA. Construction of stable transfected cells was successful. The photofluorogram of stable shATPB1-7901 cell line is shown in Fig.3. RT-PCR showed that the mRNA expression of ATP1B1 was significantly inhibited in shATP1B1-7901 cells [(85.72 ± 5.22)%) than in shNC-7901 cells [(3.3 ± 0.22)%) (p < 0.05) (Fig. 4). Real-time RCR showed that the inhibition rate of clone formation rate (%) = (number of clones/number of seeded cells) × 100%
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ATP1B1 mRNA was (87.53 ± 3.23)% in shATP1B1-7901 cells, which was significantly higher than that in shNC-7901 (4.17 ± 0.33)% and SGC-7901 cells (p < 0.05).

Inhibition effect of stable transfection of shATP1B1 on proliferation of SGC7901 cells. As shown in Figure 5, the proliferation rate was significantly higher in shATP1B1-7901 cells than in shNC-7901 and SGC-7901 cells (p < 0.05).

Influence of stable transfection of shATP1B1 on cell cycle of SGC7901 cells. The ratios of cells in S and G2/M phases were significantly increased in shATP1B1-7901 cells compared with those in shNC-7901 and SGC-7901 cells (p < 0.05). There was no significant difference in cell cycle distribution between shNC-7901 and SGC-7901 cells. The proliferation index was remarkably higher in shATP1B1-7901 cells [(69.08 ± 2.43)%] than in shNC-7901 cells [(51.74 ± 2.38)%] and SGC-7901 cells [(52.88 ± 0.9)%] (p < 0.05).

Effect of stable transfection of shATP1B1 on clone formation of SGC7901 cells. The clone formation rate was significantly higher in shATP1B1-7901 cells (68.50 ± 2.65%) than in shNC-7901 cells [(50.00 ± 2.53)%] and SGC-7901 cells [(52.50 ± 2.11)%] (p < 0.05).

Effect of stable transfection of shATP1B1 on migration of SGC7901 cells. Cells that migrated to the outer surface of the filter membrane were counted under a microscope. The migration ratio of shATP1B1-7901 cells was (2.80 ± 0.02)%, which was
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Discussion

The main functions of Na+-K+-ATPase include maintaining cell permeability, cell volume, an intracellular environment of low levels of Na+ and high levels of K+, and cell membrane resting potential.3 The B subunit, a regulatory subunit of Na+-K+-ATPase, is constituted of glycoproteins. It has the typical structure and function of adhesion molecules, and is an important factor for polarized localization of Na+-K+-ATPase.4 In mammals, the B subunit has three subtypes, including B1, B2 and B3. B1 is the most common one and is located on the cell membrane.4,5 So far, the functions of each subtype are still unclear. The B subunit can stabilize and regulate maturation of the structure and function of the whole enzyme, regulate the affinity of the enzyme with positive ions, and deliver the subunits to the cell membrane.6,7

It has been reported that Na+-K+-ATPase not only regulates energy conversion, but also is a key factor in cell growth and differentiation. The expression of Na+-K+-ATPase is decreased at different rates in tumor tissues of many cancer patients, which may be mainly due to the change in the expression of its B subunit, as indicated by in-depth studies. Hypoexpression of ATP1B1 has been detected in suprarenal epithelioma, lung cancer, hepatocellular carcinoma, hormone dependent prostatic carcinoma.1,8 In addition, the expression of ATP1B1 is closely related with the presence of tight intercellular junction, the polarity of epithelial cells, and the differentiation type of tumor cells. Overexpression of ATP1B1 is associated with tight intracellular junction and polarized epithelia, whereas hypoexpression of ATP1B1 is related to loose intracellular junction and unpolarized epithelia. Increasing the expression of ATP1B1 in cells that lack tight junction and polarity can inhibit reconstruction of epithelial polarity, cell invasiveness and activity. Therefore, it is suggested that a decrease in ATP1B1 expression is related with the progress of cancer.

We designed four shRNAs targeting ATP1B1, and chose the strongest one, sh150, to construct the stable cell line shATP1B1-7901 successfully. The results revealed that transfection of ATP1B1 shRNA into SGC-7901 cells significantly promoted cell proliferation and clone formation, increased the proliferation index and migration potential, and induced cell cycle arrest at S phase compared with control cells.

Decreased expression of ATP1B1 mediated by ATP1B1 shRNA may change ATPase activity and intracellular Na+-K+ concentrations subsequently, resulting in an unstable intracellular environment. Moreover, interstitial gap junction proteins, which play an important role in cell proliferation, differentiation, and physiological processes, such as metabolism, growth and development, would be influenced after the expression of ATP1B1 is decreased. As a result, attenuated intracellular junction and blocked intercellular communication promote growth, migration and invasion capabilities of tumor cells.9

Currently, investigations on ATP1B1 gene mainly focus on over-expression of ATP1B1.2 ATP1B1 is also used for gene chip analysis to study spectrum expression.10 In the present study, we silenced the expression of ATP1B1 using shRNA, which degraded ATP1B1 mRNA.11 Silencing of ATP1B1 gene can enhance the proliferation and migration capability of SGC-7901 cells, which may provide some evidence for using ATP1B1 gene in the diagnosis and treatment of cancer.

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