Objective: This study aimed to explore the regulatory mechanism of acid sensing ion channel 1a (ASIC1a) gene silencing-mediated PI3K/AKT signaling pathway on the progression of renal cancer cells. Methods: Human renal cancer 786-O cell line was separately transfected within five groups including blank group, negative control (NC) group, siRNA-ASIC1a group, LY294002 group and siRNA-ASIC1a + LY294002 group. mRNA and protein expressions were detected by qRT-PCR and Western blot. MTT assay, flow cytometry, transwell and scratch assays were performed to detect cell proliferation, apoptosis, invasion and migration, respectively. Results: Blank group and NC group showed no significant differences in related indexes (all P>0.05). Compared with blank group, ASIC1a expressions in siRNA-ASIC1a group and siRNA-ASIC1a + LY294002 group were significantly decreased (P<0.05), while it is not significantly different in LY294002 group (P>0.05). Compared with blank group, siRNA-ASIC1a group, LY294002 group and siRNA-ASIC1a + LY294002 group showed decreased mRNA and protein expressions of MMP-9 and Bcl-2 (all P<0.05) and phosphorylation of PI3K and AKT (all P<0.05), increased mRNA and protein expressions of Bax and caspase-3 (all P<0.05), a decreased cell proliferation, an increased cell proportion in G1 phase, a decreased cell proportion in S phase, an increased apoptosis rate, and decreased cell migration and invasion (all P<0.05), with significant differences. siRNA-ASIC1a + LY294002 group showed obvious differences in the changes of indexes (all P<0.05). Conclusion: ASIC1a gene silencing inhibited the activation of PI3K/AKT signaling pathway, in which way the proliferation, invasion and migration of renal cancer cells can be inhibited and their apoptosis promoted.

Keywords: ASIC1a gene, PI3K/AKT signaling pathway, renal cancer cells, proliferation, apoptosis, invasion

Introduction

Renal cell carcinoma ranks as the top ten lethal tumors worldwide, which incidence is only second to bladder cancer among urinary system tumors in China [1]. It has been confirmed that metastasis would occur in 30% of patients after onset [2]. Renal cell carcinoma is significantly tolerant to systematic treatment as well as chemo radiotherapy, resulting in poor treatment effect [3, 4]. The proliferation and migration of cancer cells drive the progression of renal cell carcinoma [5, 6]. With the development of molecular biologic studies of tumors in recent years, the finding of new target for treatment of renal cell carcinoma has become a focus of clinical research.

Acid sensing ion channels (ASICs) are extracellular H⁺-activated ion channels in humans, and are suspected to be involved in developing tumors, rheumatoid arthritis and cerebral injuries [7, 8]. ASIC1a is a subunit of ASICs. Recent studies have further found the importance of ASIC1a in promoting the invasion and migration of tumor [9, 10]. Weng et al. found a higher expression of ASIC1a in gliomas [11]. Carattino et al. proposed that the cell migration and cell cycle were significantly inhibited after ASIC1a silencing in liver cancer [12]. PI3K/AKT signaling pathway is an important signal transduction in human cells, which is essential to the activation of effector molecules as well as cell proliferation, apoptosis and angiogenesis [13]. Li et al. presented that inhibiting the expression of...
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Table 1. qRT-PCR primer sequence

<table>
<thead>
<tr>
<th>Term</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIC1a</td>
<td>5'-ACCTCAAC-GAGTCCCTTTA-3' R: 5'-ATTCCCCCTCCACCT-3'</td>
</tr>
<tr>
<td>PI3K</td>
<td>5'-CATCACTCTCTGCTCTAT-3' R: 5'-CAGTTGTGCAACTTCT-3'</td>
</tr>
<tr>
<td>AKT</td>
<td>5'-ACGATGAGAATGGTTGCT-3' R: 5'-TCTGCTACGGTGAAGTTGTT-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-TGACGGCTCTCCACACATG-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5'-TGAGGCTGCTTTAACTCTGGT-3'</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5'-TTGGAGATTGATGCGTGAT-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>5'-AGCTCCGAGGACAGTTTGC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GGGCTGCTTTAACTCTGGT-3'</td>
</tr>
</tbody>
</table>

Note: ASIC1a: acid sensing ion channel 1a.

PI3K/AKT signaling pathway by inhibitors is conductive to promoting the cell apoptosis and the progression of renal cell carcinoma [14]. Recent studies have further confirmed that ASIC1a gene and PI3K/AKT signaling pathway intervene in the disease progression. It has been found that ASIC1a can affect the attack of ischemic brain injury by affecting PI3K/AKT signaling pathway [15]. However, it is not clear whether and how the ASIC1a gene plays a role in the invasion and migration of renal cancer cells. This study aimed to explore the effect of ASIC1a gene silencing on PI3K/AKT signaling pathway by observing the biological characteristics of renal cancer cells, in order to provide a new therapeutic target for the treatment of renal cell carcinoma.

Materials and methods

Cell culture

Human renal cancer 786-0 cell line (Boster Biological Technology Co. Ltd., Wuhan, China) was selected for cell culture. The 786-0 cells were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum (Shanghai Jingke Chemical Technology Co. Ltd., China) at 37 °C, 5% CO₂, and the medium was changed every 2-3 days. The cells in good growth condition and the logarithmic phase were transfected.

Cell grouping and transfection

The 786-0 cells were collected by routine digestion. The cells of 5 groups were seeded in 96-well culture plate: blank group (no treatment), NC group (transfection of siRNA-ASIC1a negative control), siRNA-ASIC1a group (transfection of siRNA-ASIC1a), LY294002 group (addition of inhibitor LY2940021) and siRNA-ASIC1a + LY294002 group (transfection of siASIC1a and addition of inhibitor LY294002). The antibiotic-free medium with 10% fetal bovine serum replaced the original medium the day before transfection. The cells with a density of 80-90% were transfected, according to the instruction of Lipofectamine 2000. The 20 ng/mL inhibitor LY294002 (Shanghai PrimeGene Biotechnology Co. Ltd., China) was used in LY-294002 group and siRNA-ASIC1a + LY294002 group at 30 min before the transfection of siRNA-ASIC1a. After 4-6 h of transfection, the culture medium was replaced by DMEM medium (Gibco, USA) containing 10% fetal bovine serum. siRNA-ASIC1a and siRNA-NC were designed by the BLOCK-iT™ RNAi Designer website, and the sequences were 5'-TCTTCTGCTCACCACGGG-3' and 5'-TACTCCGAACGTCCACGTTT-3', respectively. Then the cells were collected for subsequent experiments after 48 h transfection.

qRT-PCR

Ultra-pure RNA extraction kit (GenStar Bio-Solutions Co. Ltd., Beijing, China) was applied for extracting total RNA. The primers in Table 1 were synthesized by TaKaRa Biomedical Technology (Japan) Co. Ltd. The reverse transcription was performed in accordance with instructions of TaqMan MicroRNA Assays Reverse Transcription Primer (Thermo Scientific, USA) followed by reaction at 42 °C for 30-50 min, and deactivation at 85 °C for 5 s. Fluorescence quantitative PCR was followed with instructions of SYBR® Premix Ex Taq™ II kit (Xingzhi Biotechnology Co. Ltd., China) on the ABI PRISM® 7300 system (Shanghai Kunke Instrument Equipment Co. Ltd., China). The 50 μL reaction mix included 25 μL SYBR® Premix Ex Taq™ II (2×), 2 μL PCR upstream primers, 2 μL PCR downstream primers, 1 μL ROX Reference Dye (50×), 4 μL DNA templates, and 16 μL ddH₂O. Reaction procedure: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s and
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annealing at 60°C for 30 s for 32 cycles, extension at 72°C for 1 min. The results were calculated by relative quantitative method. \(2^{-\Delta\Delta Ct}\) was used to express the relative expression of each target gene. Mean value was obtained from three repeated experiments.

Western blot

After 48 h transfection, the cells were lysed by the protein lysate (Sigma-Aldrich Co. Ltd., USA) and the protein concentrations were measured by BCA kit (Thermo Fisher Scientific, USA). The equal amount of sample was loaded for electrophoretic separation. The proteins were transferred onto the nitrocellulose membrane, which was then blocked by 5% skimmed milk. The membrane was incubated with rabbit anti-human ASIC1a (1 μg/mL, ab94753, Abcam, UK), PI3K (1:1,000, ab151549, Abcam, UK), p-PI3K (1:1,000, ab182651, Abcam, UK), AKT (1:500, ab8805, Abcam, UK), p-AKT (1:500, ab8933, Abcam, UK), MMP-9 (1:1,000, ab73734, Abcam, UK), Bcl-2 (1:1,000, ab32124, Abcam, UK), Bax (1:2,000, ab32503, Abcam, UK), caspase-3 (1:500, ab13847, Abcam, UK) and rabbit anti-human GAPDH (1:2000, ab8226, Abcam, UK) at 4°C overnight. The membrane was washed with PBS for three times, 5 min each time, and then the membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,000, ab6785, Abcam, UK), at room temperature for 1 h. After washing three times, the membrane was immersed in the enhanced ECL reaction solutions (Pierce, USA). The relative expression level of the protein = the gray value of (the target band/the internal reference band) (Image J 2.0).

MTT assay

After 48 h transfection, cells were collected for counting, \(3 \times 10^3\)-\(6 \times 10^3\) cells at a volume of 100 μL were seeded into a 96-well plate, each group was repeated 6 wells. The following experiments were performed at 24 h, 48 h, and 72 h. The cells in each well reacted with 20 μL MTT solution at a concentration of 5 mg/mL (Gibco, USA) and incubated at 37°C for 4 h, the culture supernatant was replaced by 150 μL DMSO. Optical density (OD) value was obtained at 570 nm of the microplate reader (NYW-96M, Beijing Nuoyawei Instrument Co. Ltd., China).

Flow cytometry

Detection of cell cycle: After 48 h transfection, cells were collected and washed with cold PBS for three times. Then the cells were centrifuged at 3,000 r/min for 20 min and resuspended in PBS at a density of 1×10^5/mL. The cells were fixed with 1 mL pre-cooled 75% ethanol at 4°C for 1 h. The fixed cells were centrifuged at 1,500 r/min for 5 min followed by discarding the supernatant. The cells were obtained after twice PBS washing. The cells were incubated with 100 μL Rnase A (Thermo Fisher Scientific, USA) at 37°C in water bath for 30 min in the dark, and stained with 400 μL PI (Sigma, USA) at 4°C for 30 min in the dark. Flow cytometry (Beckman Coulter Inc., USA) was applied to detect the cell cycle by sensing red fluorescence at an excitation wavelength of 488 nm.

Detection of cell apoptosis: After 48 h cell transfection, the cells were digested with trypsin digestion without EDTA (Thermo Fisher Scientific, USA), then the cells were centrifuged at 3,000 r/min for 30 min to discard the supernatant. The cells were washed with cold PBS and centrifuged at 3,000 r/min for 30 min. Annexin-V-FITC/PI dye in HEPES buffer solution at the ratio of 1:2:50 was mixed according to the instruction of Annexin-V-FITC apoptosis detection kit (Sigma, USA). \(1 \times 10^6\) cells were resuspended in 100 μL dye liquor and mixed by shaking. Then the cells were incubated at room temperature for 15 min. 1 mL HEPES buffer solution was mixed by shaking, FITC and PI fluorescence were detected at 525 nm and 620 nm which were excited at 488 nm for cell apoptosis detection.

Scratch assay

The cells were seeded into a 96-well plate and cultured in serum-free DMEM medium after cell attachment. When the cell fusion reached 90-100%, approximately 4-5 scratches at the same width were made slowly and perpendicularly by a 10 μL pipette at the bottom of each well. The cell migration distance in the scratched area was measured with an inverted microscope (XDS-800D, Shanghai Caikon Optical Instrument Co. Ltd., China) at 0 h and 24 h after scratching and photographed in randomly selected visual fields. Three replicates and three repeats were set in each group.
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Transwell assay

The transwell chambers (Jrdun Biotechnogy (Shanghai) Co. Ltd., China) were put into a 96-well plate. The bottom membrane of the transwell chamber was covered with matrigel dilution (1:8) (Shanghai Qcbio Science & Technologies Co. Ltd., China) and dried at room temperature. The cells were resuspended and diluted to 1×10⁵/mL with RPMI 1640 medium. 200 μL cell suspension was added into the upper chamber and 600 μL RPMI 1640 medium containing 20% fetal bovine serum was added into the lower chamber. After 24 h routine culture, the cells were fixed with 4% paraformaldehyde (Beijing Leagene Biotech. Co. Ltd., China) for 15 min, stained with 0.5% crystal violet solution (Beijing Solarbio Science & Technology Co. Ltd., China) for 15 min, and then washed with PBS for three times. Transmembrane cells were counted at five visual fields (200×) randomly.

Statistical analysis

SPSS 21.0 statistical software was applied for data analysis. The measurement data were expressed as mean ± standard deviation (X ± sd). One-way analysis of variance was used for multiple group comparison, and Student-Neuman-Keuls method was used for pairwise comparison. P<0.05 indicated a statistically significant difference.

Results

Expression of ASIC1a, PI3K, AKT, MMP-9, Bcl-2, Bax and caspase-3 mRNA

Compared with the blank group, the expressions of ASIC1a, PI3K, AKT, MMP-9 and Bcl-2 mRNA were significantly decreased in siRNA-ASIC1a group and siRNA-ASIC1a + LY294002 group (all P<0.05), the expressions of Bax and caspase-3 mRNA were increased significantly (all P<0.05), but ASIC1a mRNA expression was not significantly different in LY294002 group (P>0.05). The expressions of PI3K, AKT, MMP-9 and Bcl-2 mRNA were significantly decreased (all P<0.05), and the expressions of Bax and caspase-3 mRNA were increased significantly (all P<0.05). The related index between blank group and NC group were indifferent (all P>0.05). Compared with siRNA-ASIC1a group, only ASIC1a mRNA expression was significantly increased in LY294002 group (P<0.05). Compared with siRNA-ASIC1a group, siRNA-ASIC1a + LY294002 group showed significantly decreased expressions of PI3K, AKT, MMP-9 and Bcl-2 mRNA (all P<0.05), and the expressions of Bax and caspase-3 mRNA were significantly increased (all P<0.05). See Figure 1.

Expression of ASIC1a, PI3K, p-PI3K, AKT, p-AKT, MMP-9, Bcl-2, Bax and caspase-3 proteins

Compared with the blank group, the expressions of ASIC1a, MMP-9, Bcl-2, PI3K and AKT phosphorylation proteins were significantly downregulated (all P<0.05), and Bax and caspase-3 protein expressions were upregulated (all P<0.05) in siRNA-ASIC1a group and siRNA-ASIC1a + LY294002 group. ASIC1a protein expression was not statistically different between blank group and LY294002 group (P>0.05), but the expressions of MMP-9, Bcl-2, PI3K and AKT phosphorylation protein were sig-
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Compared with the blank group, the cell proliferation in siRNA-ASIC1a group, LY294002 group, and siRNA-ASIC1a + LY294002 group was rather decreased (all P<0.05). There was no significant difference between the blank group and the NC group (P>0.05). Compared with the siRNA-ASIC1a group, the cell proliferation was not significantly different in LY294002 group (P>0.05), but significantly decreased in siRNA-ASIC1a + LY294002 group (P<0.05). See Figure 3.

Cell cycle

Compared with the blank group, the cell proportion in G1 phase was significantly increased in siRNA-ASIC1a group, LY294002 group and siRNA-ASIC1a + LY294002 group, and the cell proportion in S phase was significantly decreased (all P<0.05). There was no significant difference between blank group and NC group (P>0.05). Compared with siRNA-ASIC1a group, LY294002 group has no significant difference (P>0.05). Compared with siRNA-ASIC1a group,
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the cell proportion in G1 phase was significantly increased in siRNA-ASIC1a + LY294002 group, and the cell proportion in S phase was significantly decreased (all P<0.05). See Figure 4.

Cell apoptosis

Compared with the blank group, the apoptosis rate was increased in siRNA-ASIC1a group, LY294002 group, and siRNA-ASIC1a + LY294002 group (all P<0.05). No significant difference was found between the blank group and the NC group (P>0.05), or between siRNA-ASIC1a group and LY294002 group (P>0.05). The apoptosis rate in siRNA-ASIC1a + LY294002 group was significantly increased when compared with the siRNA-ASIC1a group (P<0.05). See Figure 5.

Cell migration

Compared with the blank group, the cell migration was decreased in siRNA-ASIC1a group, LY294002 group, and siRNA-ASIC1a + LY294002 group (all P<0.05). The blank group and the NC group showed similar cell migration, so did siRNA-ASIC1a group and LY294002 group (P>0.05). Compared with the siRNA-ASIC1a group, the cell migration in siRNA-ASIC1a + LY294002 group was significantly decreased (P<0.05). See Figure 6.

Cell invasion

Compared with the blank group, the cell invasion was decreased in siRNA-ASIC1a group, LY294002 group, and siRNA-ASIC1a + LY294002 group (all P<0.05). It is not significantly different between the blank group and the NC group (P>0.05), or between siRNA-ASIC1a group and LY294002 group (P>0.05). Compared with the siRNA-ASIC1a group, the cell invasion in siRNA-ASIC1a + LY294002 group was significantly decreased (P<0.05). See Figure 7.

Discussion

Renal cancer is also known as renal adenocarcinoma and renal cell carcinoma, occurs mainly in the epithelial system of renal uriniferous tubule. It is one of the most common malignant tumors in clinic and its incidence has gradually increased in recent years [16]. Heredity, obesity, high blood pressure and unhealthy living habits like smoking are risk factors leading to the onset of renal cancer [17]. Surgical treatment and comprehensive medical treatment are the major treatment of renal cancer, but surgical resection is often difficult to apply to patients with advanced renal cancer [18]. Although some progresses have been made on current cytotoxic drugs and targeted drug research, the poor prognosis still tortures patients with advanced renal cancer [19]. Therefore, great significance is attached to understanding the specific pathogenesis of renal cancer and finding new diagnostic markers and treatment strategies. From the perspective of tumor molecular biology and targeted biotherapy, this study found a high expression of ASIC1a gene in renal cancer cells and ASIC1a gene-silencing can cope with the activation of PI3K/AKT signaling pathway, thereby inhibiting the proliferation and invasion while promoting the apoptosis of renal cancer cells.

ASIC1a is a member of the epithelial sodium channel/degradation superfamily. The ASIC1a subgene can mediate the internal flow of Ca^{2+}. The extensive biological function of ASIC1a has important pathological significance, so it has become a research hotspot in orthopedic diseases and nerve system diseases [20]. Previous studies have confirmed that the inhibition of ASIC1a expression could reduce the imbalance of articular chondrocyte matrix metabo-
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It has been confirmed that ASIC1a is greatly involved in the progression of cancer. Jin et al. believed that the overexpression of ASIC1a could accelerate the proliferation of liver cancer cells by activating LEF-TCF transcription factors and was related to the prognosis of patients with liver cancer [23]. In addition, increased ASIC1a expression in prostate cancer cells further confirmed its role in cancer progression [24]. PI3K/AKT signaling pathway has been proved to occupy a decisive position in tumor progression and prognosis [25]. A study on the pathogenesis of PI3K/AKT signaling pathway in renal cancer confirmed that PI3K and AKT phosphorylation levels were

Figure 4. Detection of cell cycle by flow cytometry. A: Cell cycle distribution; B: Cell cycle distribution cartogram. Compared with the blank group, *P<0.05; compared with the siRNA-ASIC1a group, #P<0.05. NC: negative control.

Figure 5. Detection of cell apoptosis by flow cytometry. A: Cell apoptosis flow chart; B: Cell apoptosis rate histogram. Compared with the blank group, *P<0.05; compared with the siRNA-ASIC1a group, #P<0.05. NC: negative control.
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directly proportional in renal cancer cells, but inversely proportional to the PTEN expression, a tumor suppressor gene. And the inactivation of PTEN could help to activate PI3K/AKT signaling pathway and promote cancer progression [26]. More and more studies agreed that ASIC1a and PI3K/AKT signaling pathways could work together in the pathogenesis of some diseases. For example, Liu et al. found that ASIC1a could affect the pathogenesis of many nerve system diseases by affecting the activation of PI3K/AKT signaling pathway [27]. Zhang et al. confirmed that ASIC1a could affect the drug resistance of liver cancer cells by affecting the activation of PI3K/AKT signaling pathway [28]. However, it is unclear whether the ASIC1a and

Figure 6. Detection of cell migration by scratch assay. A: Cell migration images; B: Migration distance histogram. Compared with the blank group, *P<0.05; compared with the siRNA-ASIC1a group, #P<0.05. NC: negative control.

Figure 7. Detection of cell invasion by Transwell (200×). A: Cell invasion images; B: Cell invasion rate. Compared with the blank group, *P<0.05; compared with the siRNA-ASIC1a group, #P<0.05. NC: negative control.
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PI3K/AKT signaling pathways were related to the pathogenesis of renal cancer.

This study found that ASIC1a gene silencing could affect the biological characteristics of renal cancer cells by inhibiting PI3K/AKT signaling pathway. Matrix metalloproteinases, as an important enzyme for degrading extracellular matrix, is essential to tumor progression [29]. Study has confirmed that the activation of PI3K/AKT signaling pathway can promote the expression of MMP-9 and tumor invasion [30]. In this study, the decrease of MMP-9 expression in siRNA-ASIC1a group and siRNA-ASIC1a + LY294002 group further confirmed that the role of gene silencing of ASIC1a and the inhibition of PI3K/AKT signaling pathway in reducing cell motility and tumor invasion. The ratio of Bax/Bcl-2 is regarded as a close indicator of tumor apoptosis, and it can also determine the expression of caspase-3, a pro-apoptotic factor [31]. In this study, ASIC1a gene silencing and PI3K/AKT signaling pathway inhibition could promote the decrease of Bcl-2 expression and increase the expressions of Bax and caspase-3. The change of Bcl-2, Bax and caspase-3 expression was significant in the siRNA-ASIC1a + LY294002 group. These findings confirmed the protective role of ASIC1a gene silencing and PI3K/AKT signaling pathway inactivating renal cancer progression. The PI3K and AKT expressions were decreased in the siRNA-ASIC1a group, suggesting that ASIC1a gene silencing affected renal cancer cells by inhibiting PI3K/AKT signaling pathway. To further confirm this finding, the cell apoptosis, migration and invasion were detected in this study. Compared with other groups, the apoptosis in siRNA-ASIC1a group and LY294002 group was significantly increased while the cell migration and invasion were decreased. Changes in siRNA-ASIC1a + LY294002 group were more significant compared with those in siRNA-ASIC1a group and LY294002 group. However, only the relationship between gene and pathway and their impact on the biological characteristics of tumor cells were investigated in this study. Whether the experimental process is affected by other factors still needs more experimental confirmation.

In summary, ASIC1a gene silencing can significantly inhibit PI3K/AKT signaling pathway, thereby inhibiting the proliferation, invasion and migration of renal cancer cells while promoting their apoptosis. ASIC1a can be a target of great potential and importance to treat renal cancer.

Disclosure of conflict of interest

None.

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