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Original Article

Prostaglandin transporter, SLCO2A1, mediates the invasion and apoptosis of lung cancer cells via PI3K/AKT/mTOR pathway

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Abstract: Treatment of lung cancer involves regulation of various key factors in many signaling pathways. The prostaglandin transporter, solute carrier organic anion transporter family member 2A1 (SLCO2A1), is a promising regulatory factor of cancer cells. By analyzing the invasion and apoptosis status of lung cancer cells, and detecting the expression changes of key factors in PI3K/AKT/mTOR pathway after overexpression and knockdown of SLCO2A1 *in vitro*, this study intended to investigate the function of SLCO2A1 in mediating lung cancer cells. Results showed overexpression of SLCO2A1 could induce the invasion of lung cancer cells, and its knockdown inhibited the invasion and induced the apoptosis of cells. mTOR, AKT and S6 in PI3K/AKT/mTOR pathway were not affected by SLCO2A1. But the expression levels of p-mTOR, p-AKT and p-S6 were up-regulated or down-regulated with the overexpression or knockdown of SLCO2A1. Thus SLCO2A1 was inferred to mediate the invasion and apoptosis of lung cancer cells via PI3K/AKT/mTOR pathway. These results implied SLCO2A1 could be a regulatory factor of the invasion and apoptosis of lung cancer cells and serve as a promising target for lung cancer therapy.

Keywords: Lung cancer, SLCO2A1, PI3K/AKT/mTOR, cell invasion, cell apoptosis

Introduction

Lung cancer is a significant cause of cancer death worldwide. Different therapies have been proposed for lung cancer treatment, such as radiation therapy [1] and anti-angiogenesis therapy [2]. In recent years, molecular targeted therapy has arisen to be one of the optional therapeutic methods [3]. The identification of various lung cancer-related factors leads to the improvement of clinical outcomes. Though great achievements have been made in the diagnostic and therapeutic strategies, lung cancer is still a major threat to human health. The lung cancer cell regulatory factor characterized in this study will add more evidence for molecular targeted therapy and facilitate lung cancer treatment.

Prostaglandins (PGs) are small lipid molecules which can participate in various processes in mechanisms, such as antigen-presenting [4], inflammatory processes [5], reproduction [6] and neuronal plasticity [7]. The efficient transportation of secreted PGs is regulated by specific transporters, like solute carrier organic anion transporter family member 2A1 (SLCO2A1, alias PGT). *SLCO2A1* is a single copy gene in human genome found in a bacterial artificial chromosome (BAC) contig spanning chromosome 3q21-q22 [8]. It has been studied in bovine [9], ovine [10], mouse [11], human [12] and other organisms. This gene possesses several mRNA variants with distinct functions, which might generate from alternative splicing or alternative promoters [13, 14]. Its mRNA expresses in various tissues broadly, mainly localizing in the cyclooxygenase-positive cells [15]. Many studies have shown the relation of SLCO2A1 with diseases and have identified SLCO2A1 as a cancer-related molecule. SLCO2A1 can be one of the molecular markers for differential diagnosis between malignant follicular thyroid cancer (FTC) and benign follicular thyroid adenoma (FTA) [16]. It may also participate in the network regulating tumorigenesis [17]. However, the regulatory mechanism of SLCO2A1 in lung cancer cells remains unclear.

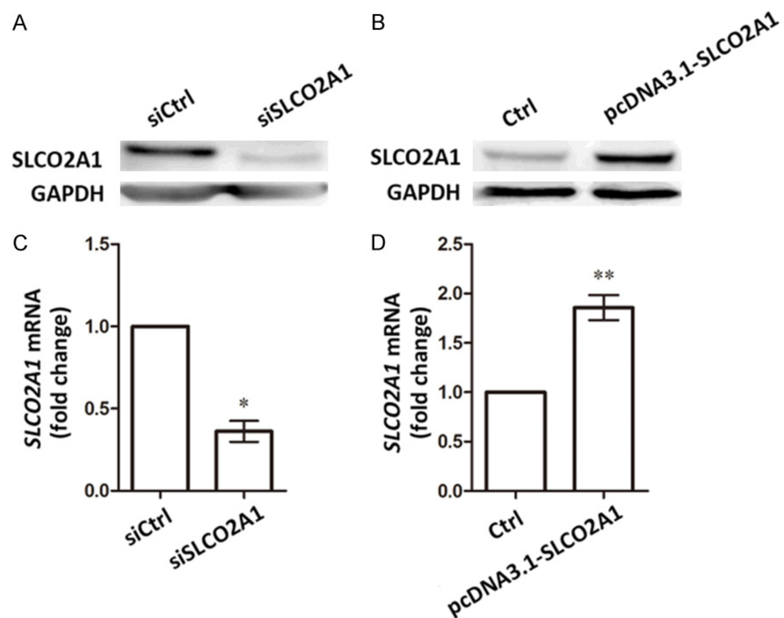

Figure 1. Expression levels of SLCO2A1 in transfected cells. A and B. SLCO2A1 protein levels after knockdown (siSLCO2A1) or overexpression (pcDNA3.1-SLCO2A1). C and D. *SLCO2A1* mRNA levels after knockdown (siSLCO2A1) or overexpression (pcDNA3.1-SLCO2A1). **indicates the difference was extremely significant ($P < 0.01$) and *indicates the difference was significant ($P < 0.05$).

In this study, we focused on the functions of SLCO2A1 in mediating the invasion and apoptosis of lung cancer cells and tried to reveal the mechanisms in these processes. The expression vector and the specific siRNA of SLCO2A1 were used to respectively overexpress or knockdown SLCO2A1. Then changes in the cell invasion and apoptosis were tested. The expression changes of key factors in PI3K/AKT/mTOR pathway were further analyzed to reveal the regulatory mechanisms of SLCO2A1. This study aimed to examine the potential possibility of SLCO2A1 being a therapeutic target for human lung cancer and to illustrate its mechanism.

Materials and methods

Cell culture

Human non-small cell lung cancer (NSCLC) cell line H460 (purchased from Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured at 37°C with 5% CO_2. Each 10 cm plastic dish contained 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone), penicillin (100 U/mL) and 100 mg/mL Streptomycin (KeyGen, Nanjing, China).

Plasmids and siRNA transfection

The SLCO2A1 expression vector (pcDNA3.1-SLCO2A1) was constructed by sub-cloning the coding sequence of wild-type *SLCO2A1* into pcDNA3.1 (+). The sequence was confirmed by sequencing. The empty vector pcDNA3.1 (+) was used in the control group. The *SLCO2A1*-specific siRNA siSLCO2A1 and the control siRNA (no silencing) were synthesized by GenePharma Co (Shanghai, China). Cell transfections were conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manuals. Stable SLCO2A1 transfectants were generated under G418 (Gibco, Paisley, UK) selection as described before [18].

Matrigel invasion assay

The invasion assay of H460 cells *in vitro* was conducted on the transwell system with 8 μm pore size membranes coated by BD Matrigel™ Matrix (BD Biosciences, NY, USA). The transfected cells were starved for 24 hours and harvested. In each well, cell suspension with 5×10^4 cells was added to the upper chamber containing serum-free media. The lower chamber was filled with DMEM with 10% FBS. After 12 hours of incubation, the invaded cells were fixed with 70% ethanol. Then the cells were stained with 0.1% crystal violet and sealed on slides. Eight visual fields (100×) per chamber were randomly chosen and photographed. The migrated cells of test groups and the control group were counted and compared.

Cell apoptosis assay

Cell apoptosis was detected with Annexin V-Cy5 Apoptosis Kit (BioVision, California, USA) and fluorescence activated cell sorting (FACS) analysis. Transfected cells were suspended in 1× Binding Buffer with annexin V-Cy5 (1:1000) and propidium iodide (PI, 1 mg/mL). After 5 min of incubation at room temperature, the cells were analyzed with Becton Dickinson FACSCalibur

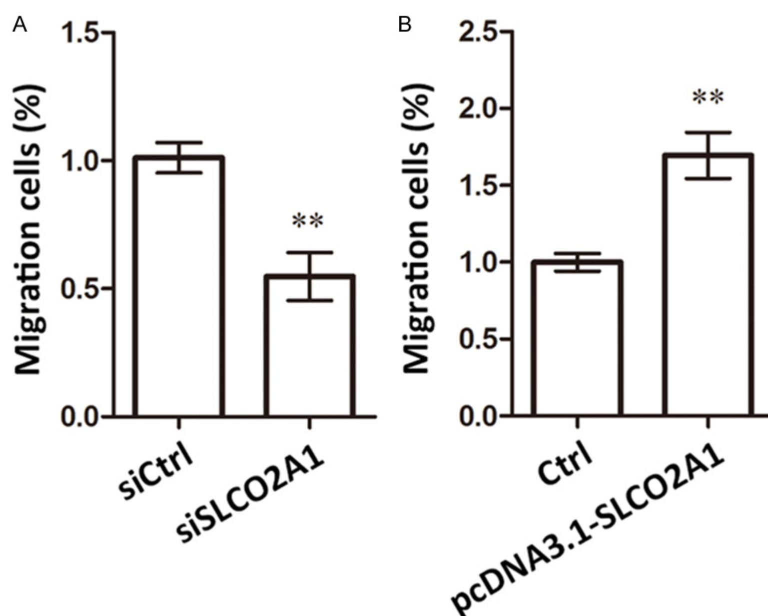

Figure 2. Percentage of migration cells after overexpression or knockdown of SLCO2A1. A. Percentage of migration cells after knockdown of SLCO2A1 (siSLCO2A1). B. Percentage of migration cells after overexpression of SLCO2A1 (pcDNA3.1-SLCO2A1).

Flow Cytometer (BD Biosciences). The total apoptotic cells included the cells in early apoptosis stages (annexin V-Cy5 positive and PI negative), and the cells in late apoptosis stages (annexin V-Cy5 positive and PI positive).

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from each group of transfected cells (2×10^5 each) using Trizol reagent (Invitrogen) following the manufacturer protocol. The first-strand cDNA was synthesized with iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA). qRT-PCR system contained Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, USA). *GAPDH* specific primers (Fw: 5'-GGTGAAGGTCGGAGTCAACGGA-3' and Rv: 5'-GAGGGATCTCGCTCCTGGAAGA-3') were used to amplify the internal reference gene. A pair of specific primers (Fw: 5'-CTGTGGAGACAATGGAATCGAG-3' and Rv: 5'-CACGATCCTGTCTTTGCTGAAG-3') was used to test the *SLCO2A1* mRNA level.

Western blot analysis

Protein was extracted from transfected cells with CelLytic™ M (Sigma, Saint Louis, USA). Protein samples were separated on 10-12% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene defluoride (PVDF) membranes. The blot was blocked in PBST (1× PBS with 0.1% triton) and incubated with primary antibodies overnight at 4°C. Then the blot was incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies. Positive bands were developed by enhanced chemiluminescence and analyzed by a densitometer.

Statistical analysis

All experimental were repeated three times and results were represented as the mean ± standard deviation (SD). Statistical analyses were performed by the SPSS Statistics 19 software (IBM, New York, USA). *P*-values were calculated using the one-way analysis of variance (ANOVA). Values with $P < 0.05$ were considered statistically significant.

Results

Transfection with plasmids or siRNA regulated target gene expression

After transfected with pcDNA3.1-SLCO2A1 or the specific siRNA, cells of the test groups and the control groups were analyzed from both the protein level and the mRNA level of SLCO2A1. Western blot results showed that the expression level of SLCO2A1 protein in the siSLCO2A1 group was more than two times lower than that in the control group (**Figure 1A**), implying the translation process of SLCO2A1 was suppressed by siSLCO2A1. Similarly, the protein level of SLCO2A1 almost doubled after the cells were transfected with pcDNA3.1-SLCO2A1 (**Figure 1B**), which indicated that transfection with pcDNA3.1-SLCO2A1 could increase the protein level of SLCO2A1. The mRNA level of *SLCO2A1* was tested by qRT-PCR and results showed as expected, that compared to the control groups, the level was about two times lower in siSLCO2A1 group than in the control group ($P < 0.05$) (**Figure 1C**), possibly owing to the *SLCO2A1* mRNA degradation caused by its specific siRNA. The expression level of *SLCO2A1* in

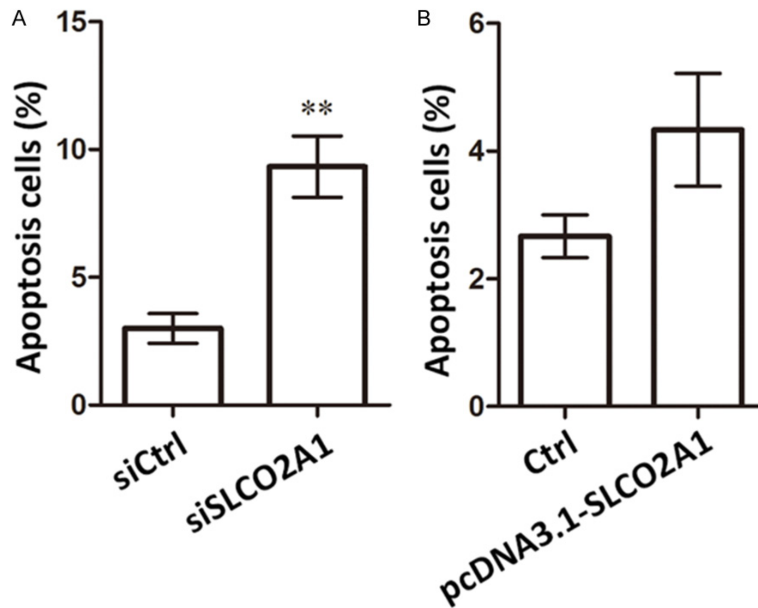

Figure 3. Percentage of apoptosis cells after knockdown or overexpression of SLCO2A1. A. Percentage of apoptosis cells after knockdown of SLCO2A1 (siSLCO2A1). B. Percentage of apoptosis cells after overexpression of SLCO2A1 (pcDNA3.1-SLCO2A1).

the pcDNA3.1-SLCO2A1 group was nearly two folds higher ($P < 0.01$) (**Figure 1D**). So both the mRNA and the protein levels of SLCO2A1 were up-regulated after the cells were transfected with pcDNA3.1-SLCO2A1; contrariwise, they were down-regulated after the cells were transfected with siSLCO2A1.

Interfering with SLCO2A1 affected lung cancer cell invasion

The cell invasion assay was conducted with Matrigel method. Results showed that knockdown of SLCO2A1 led to about 50% decrease of invaded cells compared to the control group ($P < 0.01$) (**Figure 2A**). But when SLCO2A1 was overexpressed, the percent of invaded cells increased to about 1.7 times as much as the control group ($P < 0.01$) (**Figure 2B**). These results implied the functions of SLCO2A1 in mediating lung cancer cell invasion. Since knockdown of SLCO2A1 decreased the amount of invaded cells and overexpression of SLCO2A1 promoted the cell invasion, interfering with SLCO2A1 might help to suppress the invasion of lung cancer cells.

Interfering with SLCO2A1 induced lung cancer cell apoptosis

Cell apoptosis was tested by FACS and results reflected an extremely significant difference between the SLCO2A1 knockdown cells and the control group ($P < 0.01$) (**Figure 3A**). After knockdown of SLCO2A1, the percentage of apoptosis cells was significantly increased. But the percentage of apoptosis cells did not change significantly after the overexpression of SLCO2A1 (**Figure 3B**). According to these results, interfering with SLCO2A1 was supposed to induce the apoptosis of lung cancer cells.

Interfering with SLCO2A1 affected PI3K/AKT/mTOR pathway

The expression levels of six key factors in PI3K/AKT/mTOR pathway were tested after cells were transfected with pcDNA3.1-SLCO2A1 or siSLCO2A1 (**Figure 4**). After the overexpression of SLCO2A1, the expression levels of mTOR, AKT and S6 were almost the same as those in the control group. But p-mTOR, p-AKT and p-S6 were all up-regulated, increasing to 1.5 times, two times and almost two times as the control group, respectively. In cells where SLCO2A1 was interfered, expression change of mTOR, AKT or S6 could not be detected. But the expression levels of p-mTOR, p-AKT and p-S6 were down-regulated to almost half as those in the control group. It seemed that the overexpression or knockdown of SLCO2A1 could not affect mTOR, AKT or S6 in PI3K/AKT/mTOR pathway. But SLCO2A1 might regulate the expression of p-mTOR, p-AKT and p-S6. Thus SLCO2A1 might affect the PI3K/AKT/mTOR pathway and further mediate the invasion and apoptosis of lung cancer cells.

Since the three factors, p-mTOR, p-AKT and p-S6, instead of the other three tested factors, might correspond to the expression changes of SLCO2A1, we further compared the possible changes of them when inhibiting the PI3K/AKT/mTOR pathway and inhibiting the expression of SLCO2A1. Here, BEZ235 was chosen as the specific inhibitor of PI3K/AKT/mTOR pathway [19, 20]. As expected, when the pathway was inhibited, the expression levels of p-mTOR, p-AKT and p-S6 were all down-regulated to

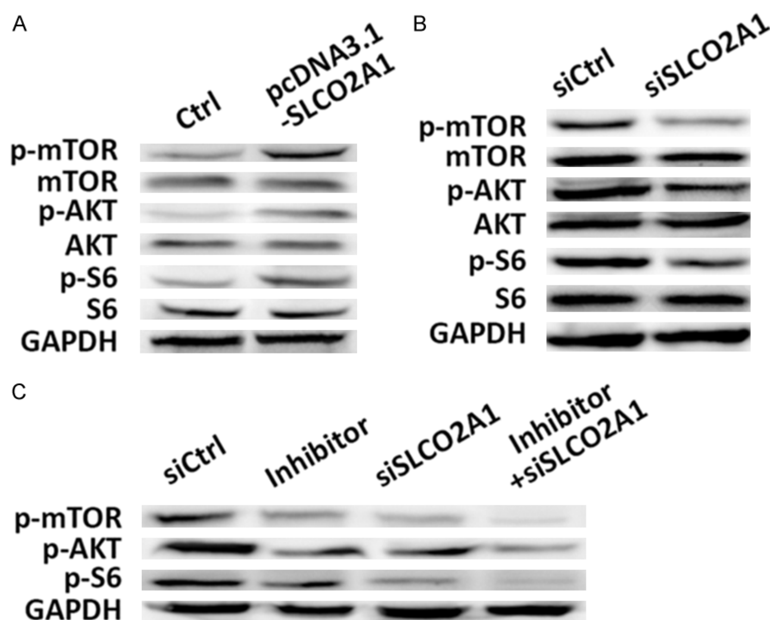

Figure 4. Expression changes of main factors in PI3K/AKT/mTOR pathway. A. Expression changes of six factors after the overexpression of SLCO2A1. B. Expression changes of six factors after the knockdown of SLCO2A1. C. Expression changes of p-mTOR, p-AKT and p-S6 after inhibiting PI3K/AKT/mTOR pathway with inhibitor or interfering with SLCO2A1.

about half of the control group. Knockdown of SLCO2A1 had the similar effects, decreasing the expression of the three factors to about 50%. When inhibiting PI3K/AKT/mTOR pathway and interfering with SLCO2A1 at the same time, the down-regulation of these three factors were more apparent. Their expression levels were reduced to 20% compared to the control group. These results indicated that interfering with SLCO2A1 might inhibit PI3K/AKT/mTOR pathway, like the inhibitor BEZ235. It was implied that SLCO2A1 could be a key factor regulating PI3K/AKT/mTOR pathway and one of the potential therapeutic targets for lung cancer.

Discussion

This study tested the effects of overexpression and knockdown of SLCO2A1 on the invasion and apoptosis of lung cancer cells *in vitro*. Knockdown of SLCO2A1 was proved to suppress cell invasion and induce cell apoptosis, and its overexpression could induce cell invasion. Expression analysis of the key factors in PI3K/AKT/mTOR pathway revealed that SLCO2A1 might mediated the invasion and apoptosis of lung cancer cells via regulating factors such as p-mTOR, p-AKT and p-S6. These results could provide confident evidence for clinical treatment of lung cancer.

Related factors have been reported to influence the invasion and apoptosis of lung cancer cells. For example, a high level expression of hepatoma-derived growth factor (HDGF) may increase the possibility of tumor relapse and aggravate non-small cell lung cancer [21]. Knockdown of monocarboxylate transporters MCT1 and MCT4 can reduce the invasion of human cancer cells [22]. Overexpression of S-Phase kinase associated protein 2 (SKP2) will lead to the invasion [23] and its down-regulation tends to inhibit the growth of lung cancer cells [24]. Here, SLCO2A1 was found to have similar effects on lung cancer cells, like the above factors. Overexpression and knockdown experiments detected its influence on the invasion and the apoptosis of lung cancer cells. These results inferred that SLCO2A1 might be a key factor to control lung cancer cells. Regulation of SLCO2A1 could serve as a potential entry point to lung cancer treatment.

The association of PI3K/AKT/mTOR pathway with human cancer has been revealed by numerous studies since 1990s. A tumor suppressor called PTEN can dephosphorylate the phospholipid products of PI3-kinase [25]. High mutation frequency of *PIK3CA* gene has been found in human cancers [26, 27] and mutations of some key factors in this pathway are related to lung adenocarcinoma [28]. This pathway is also an important genetic event for human glioblastomas (GBMs) [29]. In this study, the impacts of SLCO2A1 on PI3K/AKT/mTOR pathway were analyzed. Three of the tested factors in this pathway, p-mTOR, p-AKT and p-S6, could respond to the overexpression and knockdown of SLCO2A1. The expression levels of these factors were increased or decreased with the overexpression or knockdown of SLCO2A1. It seemed that SLCO2A1 could be a promoting factor for PI3K/AKT/mTOR pathway,

thus mediating cell invasion and apoptosis via regulating some key factors in PI3K/AKT/mTOR pathway. It was possible that SLCO2A1 could participate in some other pathways involving in lung cancer, like Wnt pathway [30] and MEK-ERK pathway [31], and constitute a complex regulatory network with various factors and pathways to mediate the invasion and apoptosis of lung cancer cells. Such research could uncover significant information for lung cancer therapy.

In summary, SLCO2A1 exhibits the ability to mediate the invasion and apoptosis of lung cancer cells, possibly via regulating PI3K/AKT/mTOR pathway. Knockdown of SLCO2A1 can inhibit lung cancer cell invasion and induce cell apoptosis. Overexpression of SLCO2A1 can promote cell invasion. SLCO2A1 can regulate the expression of p-mTOR, p-AKT and p-S6, and acts as a promoting factor of PI3K/AKT/mTOR pathway. So SLCO2A1 is one of the promising targets for the clinical treatment of lung cancer. Further research on SLCO2A1 and important signaling pathways will allow a better understanding of related mechanisms and provide more therapeutic strategies.

Disclosure of conflict of interest

None.

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