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Original Article

The role of ATP8A1 in non-small cell lung cancer

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Abstract: The study objective was to investigate the expression of ATP8A1 in non-small cell lung cancer (NSCLC) and to discover the role of ATP8A1 in the carcinogenesis of NSCLC. We collected 25 cases of tumor tissues and the adjacent normal tissues from surgeries of NSCLC patients in our hospital, among which 15 cases were found with lymph node metastasis while the other 10 were not. Immunohistochemical staining was performed to compare the expression level of TAP8A1 protein in NSCLC tissues with or without lymph node metastasis and the adjacent normal tissues. Transwell and scratch assay were used to test the invasion/migration capacity of different types of NSCLC. PCR and Western Blots were performed to detect the expression of ATP8A1 and epithelial-mesenchymal transition (EMT) markers in different cells. The percentage of ATP8A1 positive cells was (39.2±8.6)% in NSCLC tissues without lymph node metastasis, which was significantly lower than that in NSCLC tissues with lymph node metastasis ((74.7±11.0)%, P<0.05) as wells as remarkably higher than that in adjacent normal tissues with no ATP8A1 expression (P<0.05). When compared with normal H1299 cells, the invasion ability of ATP8A1 knock-down cells (si-H1299) was down-regulated by (31.2±5.7)%, the migration ability was down-regulated by (23.4±7.1)%, and the gene expression level of MMP and Vimentin was significantly reduced (P<0.05) while the expression of E-cadherin was remarkably increased (P<0.05). ATP8A1 was overexpressed in NSCLC tissues which promoted the expression of MMP-9 and Vimentin as well as suppressed the expression of E-cadherin thus resulting in the elevated invasion/migration ability of NSCLC cells.

Keywords: NSCLC, ATP8A1, immunohistochemistry, invasion/migration

Introduction

Lung cancer is a malignant tumor that grows in the bronchial epithelium, and is the malignancy with the highest mortality rate so far. It can be further divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) according to the biological characteristics, of which more than 80% lung cancer patients were diagnosed as NSCLC [1]. In China, more than 350, 000 NSCLC patients die each year, and the 5-year survival rate of patients with NSCLC is as low as 10%-20% [2]. Like other malignant tumors, the distal metastasis of tumor cells is one of the major causes of poor prognosis, recurrence and death in patients with NSCLC [3]. ATP8A1, also known as ATPase II, is an important member of the P4-ATP enzyme family, and its main function is to participate in the intracellular transport of cations and ammonia phospholipids as well as some cellular metabolic activities [4]. A number of studies [5, 6] have indicated that ATPase is involved in the regulation of cancer cells apoptosis, and that by inhibition of ATPase activity or expression could lead to apoptosis of cancer cells. In addition, Kato U *et al*. [7] discovered that the phospholipid transporter complex formed by the binding of ATP8A1 with CDC50A proteins could promote cell migration by mediating the translocation of phosphatidyl ethanolamine in mammalian cell membranes, which promoted the formation of cell membrane folds. Therefore, ATP8A1 is not only involved in the regulation of cancer cells apoptosis, but may also be related to the invasion/migration ability of tumor cells. However, the expression of ATP8A1 in NSCLC tumor tissues and its effect on invasion/migration ability of NSCLC cells were rarely reported at home and

Table 1. Primer design for PCR analysis

Gene	Primer sequence (5'-3')	Length (bp)
ATP8A1	F: GGAAGAGACCGCATCCTACC	375
	R: AGAGCCAAGGGACTACGGAT	
MMP-9	F: TTTAGCAAACGTAGGGGCGG	447
	R: CTTCACGTCGAACCTGCGG	
E-cadherin	F: GCTCTGAGGAGTGGTGCATT	518
	R: GCAATTTCTCGGCCCCTTTC	
Vimentin	F: CCCTCCTTGGCTCAAACGAC	386
	R: AGAGAGGGCTGCTCTTCAGT	
β-actin	F: AAGTACTCCGTGTGGATCGG	615
	R: TCAAGTTGGGGGACAAAAAG	

abroad. In this study, we investigated the impact of ATP8A1 expression on the carcinogenesis of NSCLC by comparing its expression levels in NSCLC tissues with or without lymph node metastasis and adjacent normal tissues as well as the following cell experiments.

Materials and methods

Source of clinical specimens

25 cases of NSCLC tissues and the paired adjacent normal tissues (about 5 cm from tumor tissues) were obtained from radical surgeries of NSCLC patients in our hospital. All clinical specimens were fixed, dehydrated, paraffin embedded and made into 5 μm-thick pathological sections. All 25 cases were diagnosed as NSCLC with 6 cases being adenocarcinoma and 19 cases being squamous-cell carcinoma, among which 15 cases were diagnosed with lymph node metastasis while the other 10 without. There were 14 male patients and 11 female patients at the age of 34-65 years old, and all patients provided written informed consent for study participation. The study protocols were approved by the ethics committees of Shandong Provincial Hospital affiliated to Shandong University.

Reagents and cell lines

NSCLC cell line H1299 was purchased from ATCC (USA). The ATP8A1 knock-down si-H1299 cell line was constructed for the experiments in this study. DMEM culture medium, trypsin and FBS were purchased from Gibco (Gaithersburg, MD). The BCA kit, total-RNA extraction kit, total-protein extraction kit and immunohistochemistry kit were purchased from Beyotime Biotechnology (Shanghai, China). ATP8A1 rabbit polyclonal antibody, MMP-9 rabbit monoclonal antibody, E-cadherin mouse monoclonal antibody, Vimentin mouse monoclonal antibody and the goat anti-rabbit, goat anti-mouse secondary antibodies were purchased from Abcam (Cambridge, MA).

PCR test for gene expression

Total RNA was extracted by total-RNA extraction kit and reverse-transcribed into cDNA, which was then subjected for RT-PCR analysis. Primers used in this study were designed according to the gene sequences provided by NCBI (https://www.ncbi.nlm.nih.gov/), as listed in **Table 1**. The PCR products were collected and 10 μl from each group was loaded for gel electrophoresis; the results were photographed by a Bio-Rad gel imaging analyzer and quantified by Quantity One software. The final results were shown as the relative content of the target gene compared with β-actin.

Western blot analysis of protein expression

Cells were digested by trypsin treatment, centrifuged to remove the culture medium, and washed by ice old PBS. Total protein extraction kit was used to extract the proteins, and the concentration was detected by BCA kit. 75 μg of proteins in each group was loaded for SDS-PAGE, and transferred to a wet nitrocellulose membrane. The membrane was blocked with 5% no-fat milk in TBST (150 mM NaCl, 20 mM Tris, 0.05% Tween-20) for 2 h at room temperature and incubated with indicated antibodies at 4°C overnight. After incubation with secondary antibodies, the band density was measured with a computer-assisted image-analysis system and analyzed by Image-J software (normalized against β-actin levels).

Immunohistochemistry analysis for protein expression

Clinical pathological sections were immunohistochemically stained by using a purchased commercial kit, and the negative control was stained with PBS. The PAT8A1 positive cells were decided by yellow-to-brown staining of cytoplasm. For each section, positive cells from 5 different visual fields under 400× objective len were counted, and the mean value of the 5 visual fields was designated as the number of positive cells in a section.

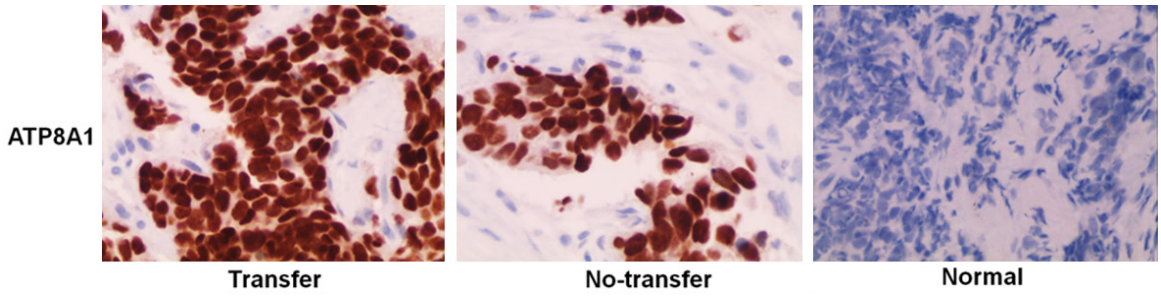

Figure 1. Immunohistochemistry analysis of the expression of ATP8A1 in different tissues.

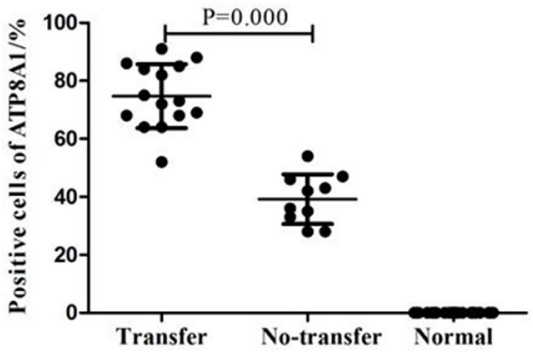

Figure 2. The expression of ATP8A1 protein in different tissues of NSCLC patients. The percentage of ATP8A1 positive cells in NSCLC without lymph node metastasis was significantly lower than that in NSCLC with lymph node metastasis (P<0.05).

Transwell analysis of cell invasion ability

40 μl of 1 mg/ml ECM gel (diluted by FBS-free DMEM medium precooled in 4°C overnight) was added into each chamber, and incubated in a 37°C incubator with 5% CO_2 for 5 hours. Excess liquid was removed and 70 μl FBS-free DMEM medium was added in each chamber before incubation in the 37°C incubator with 5% CO_2 for another 0.5 hours.

Cells in logarithmic growth phase were cultured in serum-free medium for 1 day, and cultivated to prepare cell suspensions at the concentration of 1×10^5 cells/ml. Add 200 μl of the suspension into the upper chamber and 500 μl of DMEM medium with 10% FBS into the lower chamber of the 24-well plate. After 7 days of regular culture, 4% paraformaldehyde was added to fix the cells for 15 min and 0.25% crystal violet was used to stain the cells for 25 min. Chambers was gently washed with ddH_2O for several times and dried in a sterile clean bench. Photos were taken and the invaded cells were counted.

Cell scratch assay to detect the cell migration ability

Cells were detached by trypsin treatment and resuspended to the concentration of 1.5×10^5 cells/ml. Each well of the 6-well plate was inoculated with 3 ml of the suspension and cultured for 20-24 hours. Lineation scratches were performed with a 10 μl pipette tip at the bottom of the well and the exfoliated cells was washed off with PBS. FBS-free DMEM medium was used for regular culture in the next 24 hours and the pictures of cells near the scratch were taken by a microscope.

Statistical analysis

Statistical analysis was performed using the SPSS19.0 statistical program. Counting was presented in percentage (mean ± standard deviation), and t test was performed to compare the differences between groups (P<0.05 was considered statistically significant).

Results

Expression of ATP8A1 protein in different tissues

As shown in **Figures 1** and **2**, the percentage of ATP8A1 positive cells in NSCLC without lymph node metastasis was (39.2±8.6)%, which was significantly lower than that in NSCLC with lymph node metastasis ((74.7±11.0)%, P<0.05). No expression of ATP8A1 protein was detected in adjacent normal tissues.

ATP8A1 promotes the invasion/migration ability of NSCLC cells

When compared to normal H1299 cells, the invasion capacity of ATP8A1 knock-down (si-H1299) cells was down-regulated by (31.2±

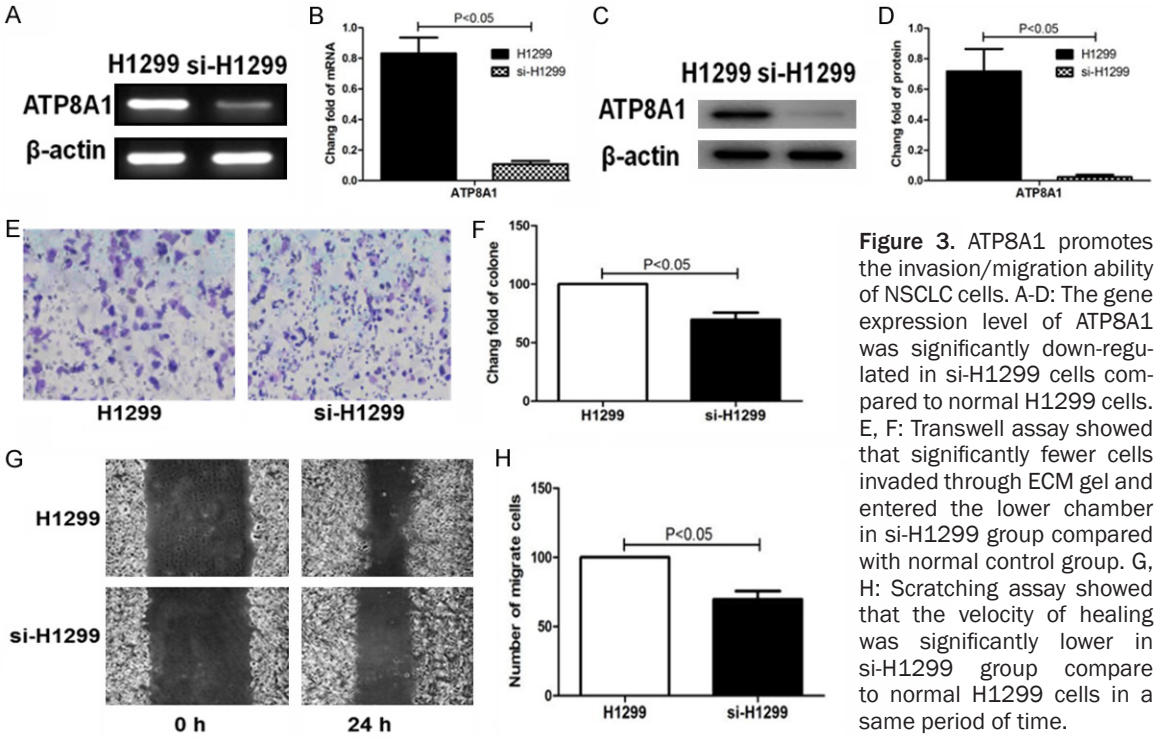

Figure 3. ATP8A1 promotes the invasion/migration ability of NSCLC cells. A-D: The gene expression level of ATP8A1 was significantly down-regulated in si-H1299 cells compared to normal H1299 cells. E, F: Transwell assay showed that significantly fewer cells invaded through ECM gel and entered the lower chamber in si-H1299 group compared with normal control group. G, H: Scratching assay showed that the velocity of healing was significantly lower in si-H1299 group compare to normal H1299 cells in a same period of time.

5.7)% while the migration capacity was down-regulated by (23.4±7.1)% (**Figure 3**).

Effect of ATP8A1 on the expression of EMT markers in NSCLC

As shown in **Figure 4A**, **4B**, the relative mRNA expression content of MMP-9, E-cadherin and Vimentin in ATP8A1 knock-down H1299 cells (si-H1299) was (0.55±0.10), (1.03±0.21) and (0.50±0.08), respectively, which was significantly distinguished from those in normal H1299 cells (the number was (0.83±0.12), (0.71±0.09) and (1.03±0.17), respectively ($P<0.05$).

As shown in **Figure 4C**, **4D**, the relative protein expression content of MMP-9, E-cadherin and Vimentin in ATP8A1 knock-down H1299 cells (si-H1299) was (0.44±0.07), (0.87±0.18) and (0.49±0.14), respectively, which was significantly distinguished from those in normal H1299 cells (the number was (0.74±0.13), (0.63±0.09) and (0.97±0.27), respectively ($P< 0.05$).

Discussion

Lung cancer is one of the most common malignant tumors with the highest morbidity and mortality, of which more than 80% of patients were diagnosed with NSCLC. Since NSCLC has no typical clinical symptoms and NSCLC cancer cells are prone to lymph node metastasis resulting in deterioration of the disease, the study of genes involved in NSCLC metastasis and development of targeted drugs against NSCLC metastasis are emerging into a hot spot of researches [8].

ATP8A1 is the first P4 type ATPase found in mammals which mainly localizes in mammalian red blood cells and is over expressed in the precursor and mature red blood cells. Previous studies [9] showed that over-expressed ATP8A1 participates in the induction of red blood cell migration through promotion of ectropion of medial phosphatidylserine in these cells. In recent years, multiple researches [10, 11] discovered that human-derived CDC50 protein is a key component in the process of P4 type ATPase mediated intracellular and extracellular transportation, which forms a phospholipid transporter complex with ATP8A1 and promotes the turnover of phosphatidylserine and phosphatidylethanolamine in the cell membranes thus elevating the migration capacity of cells. As for cancer cells, high rates of invasion and migration are the major characteris-

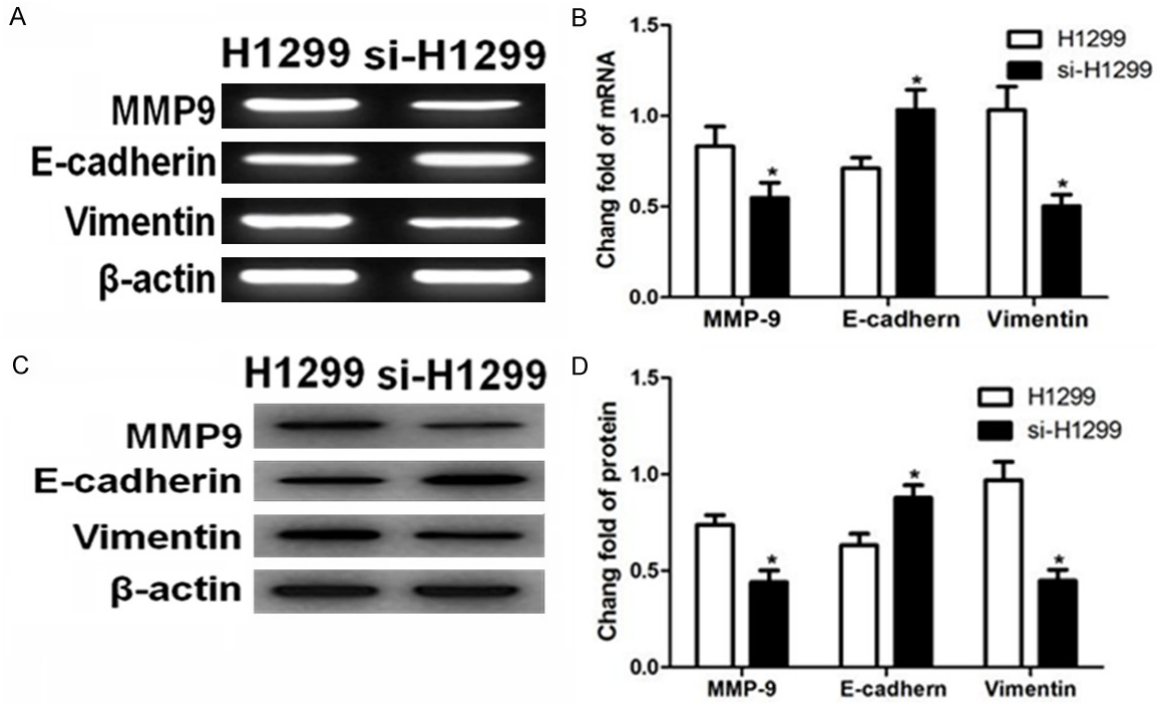

Figure 4. The effect of ATP8A1 on the expression of EMT markers in NSCLC. A, B: ATP8A1 knock-down resulted in significant up-regulation of E-cadhern mRNA expression as well as down-regulation of MMP-9 and Vimentin mRNA. C, D: ATP8A1 knock-down resulted in significant up-regulation of E-cadhern protein expression as well as down-regulation of MMP-9 and Vimentin proteins. *indicated that the differences were statistically significant between si-H1299 and the normal control cells.

tics of malignancy as well as the major cause of death in cancer patients. Since the early last century, scientists at home and abroad have begun to study the mechanism of NSCLC cell invasion and migration, and now after over a hundred years of hard work, the theory of NSCLC metastasis regulation with multiple factors, multiple mechanisms and multiple genes is gradually been improved [12]. At present, the genes involved in metastasis or signaling pathway and the target genes are the hot spots in the study of NSCLC invasion/migration mechanism, and are also important markers for clinical pathological staging and prognosis. Since ATP8A1 is closely related to the migration ability of mammalian cells [13, 14], we hypothesized that its expression could participate in the regulation of NSCLC cell metastasis and could be a potential drug target.

In this study, we compared the expression of ATP8A1 protein in NSCLC tumor tissues and adjacent normal tissues, and the results showed that the ATP8A1 protein expression in the 15 cases of lymph node metastasis NSCLC tissues was significantly higher than that in the 10 cases of NSCLC without metastasis ($P<0.05$), and that no expression of ATP8A1 protein was detected in normal adjacent tissues. These results suggested that the expression of ATP8A1 could be related with the carcinogenesis of NSCLC, and that its overexpression may promote the tumorigenesis of NSCLC by elevating the invasion and migration ability of cancer cells. Further cell experiments revealed that the NSCLC cell line with siRNA interfered ATP8A1 gene expression (si-H1299) was associated with reduced ability of invasion and migration. Collectively, the above results suggested that overexpression of ATP8A1 could promote the invasion and migration ability of H1299 and accelerate the progression of NSCLC.

Our studies also discovered that the expression of MMP-9 and vimentin was significantly down-regulated ($P<0.05$) while the expression of E-cadherin was significantly up-regulated ($P<0.05$) in ATP8A1 knock-down cell line (si-H1299). *MMP-9*, *vimentin* and *E-cadherin* are

three important genes that have been proved to play significant roles in the EMT process [15], a process during which epithelial cells were transferred into mesenchymal cells and empowered with invasion and migration capacities. The EMT process is also associated with decreased stem cell characteristics, increased apoptosis and senescence, as well as elevated immunosuppression, and plays a central role in development, tissue healing, organ fibrosis and carcinogenesis. The increased EMT in cancer cells contributes to the inhibition of the expression of intercellular junction proteins, which results in the decreased mutual connection between cells and promotes the cancer cells to invade and migrate into adjacent healthy tissues [16].

MMP-9 is an important member of the matrix metalloproteinases (MMPs) family, and has been proved to be the most significant proteolytic enzyme during EMT. Akter H et al. [17] discovered that overexpressed MMP-9 protein promotes the invasion and migration ability of gastric cancer cells through ERK pathway. Vimentin protein, an important intermediate filament protein, is considered to be an interstitial marker during EMT, which is mainly expressed in mesenchymal cells and not found in normal cells. The abnormal overexpression of vimentin is a significant marker for EMT [18], and is closely related to lymph node metastasis, clinical stage and prognosis of lung cancers [19]. The role of E-cadherin in cancer cell EMT is the opposite with MMPs and vimentin, which is the promotion of interactions between cells and cell membrane materials. As an epithelial marker of EMT, E-cadherin inhibits the migration of tumor cells while its down-regulation is related to elevated invasion and migration capacity of tumor cells [20, 21].

In summary, ATP8A1 is overexpressed in NSCLC tumor tissues and promotes the invasion/migration ability of NSCLC cells through up-regulating the expression of MMP-9 and vimentin as well as down-regulating of E-cadherin.

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Disclosure of conflict of interest

None.

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