The expression and correlation between chemokine CCL7 and ABCE1 in non-small cell lung cancer

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Abstract. Lung cancer is a malignant disease, and has the highest incidence and mortality worldwide. Lung cancer is also a popular subject in the field of cancer research. The molecular mechanisms of lung cancer development, invasion and metastasis need to be determined to prolong survival times and improve the quality of life. Recent studies have demonstrated that ATP-binding cassette sub-family E member 1 (ABCE1) is one of the factors that contributes to the development and metastasis of lung cancer, but the specific mechanism of this phenomenon remains unclear. A polymerase chain reaction microarray was used in the present study to screen for chemokine (C-C motif) ligand 7 (CCL7) expression in cell lines that highly expressed ABCE1, and the results showed that CCL7 was highly expressed in H1299 cells (P<0.01). The expression of CCL7 and ABCE1 in lung cancer tissues obtained from 30 patients with non-small cell lung cancer (NSCLC) was higher than that in adjacent normal lung tissues (P<0.01), and a positive correlation between the expression levels of the two genes in NSCLC was observed. These findings indicate that ABCE1 is involved in the development and progression of lung cancer through the CCL7 signaling pathway.

Introduction

ATP-binding cassette sub-family E member 1 (ABCE1) is a member of the ATP-binding cassette superfamily (1). ABCE1 acts as an RNase L inhibitor or host protein (HP) 68 and has been reported to participate in HIV-1 capsid assembly (2). After the ABCE1 gene was silenced in the human small cell lung cancer cell line NCI-H446 using RNAi technology, in vitro cell biology experiments, including cell adhesion, wound healing, migration, and invasion experiments, were performed. These assays demonstrated that the migration and invasiveness of small cell lung cancer cells were significantly inhibited (3). ABCE1 was confirmed to be one of the key factors that promotes the development and metastasis of lung cancer following the inoculation of nude mice with the lung adenocarcinoma (AC) cell line LTEP-a-2, which has upregulated ABCE1 expression (4).

Chemokine (C-C motif) ligand 7, which was also known as monocyte chemotactic factor-3 for a long period of time, can induce the majority of immune inflammatory cells, especially monocytes (5). CCL7 plays an important role in various pathologies, including cancers, auto-immune diseases and chronic inflammation (6). Monocytes have strong chemotactic ability towards tumor-associated macrophages (TAMs), and several chemokines, including CCL7, interact with cancer-associated fibroblasts (CAFs), which can influence the tumor microenvironment and promote tumor angiogenesis and infiltration by TAMs (7).

The relationship between ABCE1 and chemokine CCL7 in lung cancer has never been reported. This study attempted to determine the relationship between ABCE1 and chemokine CCL7 in lung cancer using a PCR microarray and immunohistochemistry to provide a new basis for the roles of ABCE1 and CCL7 in the pathogenesis of lung cancer.

Materials and methods

Cell culture and lentiviral packaging vector transfection. The lung cancer cell line H1299 was purchased from the Shanghai Chinese Academy of Science. The H1299 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum under the following conditions: 37°C, 5% CO₂, and an aseptic environment. The culture medium was changed every 1 or 2 days. After they reached confluence, the cells were digested with 0.25% trypsin for subculture, cryopreservation and lentivirus transfection.

The lentiviral packaging vector that overexpressed ABCE1 was purchased from JiKai Gene Chemical Technology
(Shanghai, China). The elements sequence incorporated into the GV358 vector was Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin. The restriction enzyme site was located in AgeI. Recombinant clones were screened by puromycin, and the constructs expressed green fluorescent protein (GFP) reporter genes. The experimental cells were divided into an overexpression group and an empty vector group and were seeded in six-well plates. After the cells reached 30% confluence, culture medium with enhanced transfection reagent and polybrene (5 µg/ml) were added to the wells for the transfection experiments; the amount of virus added was calculated based on the pre-experimental values of the multiplicity of infection (MOI). A fluorescence microscope was used to observe the transfection efficiency, which was 80% or greater.

RT2 Profiler™ PCR Array. Total RNA in the cells was extracted using a TaKaRa RNA extraction kit (TaKaRa Bio Inc., Dalian, China) and was stored at -80°C. An RT2 First Strand Kit (Qiagen GmbH, Hilden, Germany) was used to synthesize cDNA, and a comparative study was performed using an RT2 Profiler™ PCR Array Human Tumor Metastasis (PAHS-0282Z) chip and RT SYBR Green Master Mix. qPCR was performed using an ABI7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A number of housekeeping genes served as internal controls for sample normalization, and the 2-ΔΔCq values were compared (8).

Western blotting. After the H1299 cells were transfected with the lentiviral vector, total protein was extracted. The BCA method was used to determine the protein concentration, and 30 µg of total protein was loaded onto a 10% SDS-PAGE gel for protein electrophoresis and then transferred onto a PVDF film, which was incubated with an ABCE1 rabbit anti-human monoclonal antibody (1:2,000 dilution; Abcam, Cambridge, MA, USA), a CCL7 rabbit anti-human polyclonal antibody (1:1,000 dilution; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and a GAPDH mouse anti-human polyclonal antibody (1:1,000 dilution; Abcam) overnight at 4°C. After the membranes were incubated with the indicated secondary antibodies (goat anti-rabbit monoclonal antibody and goat anti-mouse monoclonal antibody, 1:1,000 dilution) for 2 h at room temperature, the bands were visualized with enhanced chemiluminescence (ECL kit; Thermo Fisher Scientific, Inc.) with dark room exposure and development. The gray values of the protein bands, which represented the relative expression levels of the proteins, were determined.

Patient selection and tissue specimens. Cancer tissues and adjacent normal tissues (NTs) (located more than 2 cm from the edge of the tumor) surgically resected from 30 patients with non-small cell lung cancer (NSCLC) in the Department of Thoracic Surgery, Fourth Affiliated Hospital of China Medical University (Shenyang, China), from 2014 to 2016 were embedded in paraffin. These patients, including 13 males and 17 females with an average age of 62.4 years (range, 52 to 78 years), did not receive preoperative radiotherapy; the group included 12 cases of squamous cell carcinoma (SCC) and 18 cases of AC.

Immunohistochemical analysis. The tissue specimens were sliced, baked for 2 h, soaked in xylene for deparaffinization, subjected to benzene removal using 100% ethanol, and then subjected to gradient ethanol hydration before they were rinsed with 0.01 mol/l phosphate-buffered saline (PBS). The slices were then incubated in 0.01 M citric acid buffer (pH=6.0) for 20 min at 97°C for antigen retrieval. After endogenous peroxidase was blocked and the slides were incubated with non-immune animal serum at room temperature, each section was incubated with 50 µl of the appropriate primary antibody (ABCE1 1:500; CCL7 1:250) in a humidified chamber overnight at 4°C. After the sections were rinsed with PBS, a biotinylated secondary antibody was added to them, and the slides were incubated for 10 min at room temperature. Streptavidin peroxidase solution was then added to the slides, which were incubated for another 10 min. Approximately 100 µl of diaminobenzidine (DAB) liquid was added to each of the tissue sections, all of which were observed under a microscope for 10 min before the reaction was terminated. Hematoxylin solution was then added to the slides as a counterstain for 5 min to visualize the nuclei.

Cells with a brownish-yellow membrane and cytoplasm were considered positively stained. Ten continuous high-power fields (x400) in each slice were observed under a light microscope and given scores of 0, 1, 2 or 3 points according to the color intensity; the average score was then recorded. Fields with a positive cell rate of <5%, 5-25%, 26-50%, 51-75%, or >75% were given scores of 0, 1, 2 or 3 points, respectively. Both scores were multiplied, and the final score was categorized as follows: A score of 0-2 points was considered negative (-), a score of 3-4 points was considered weakly positive (+), a score of 5-8 points was considered moderately positive (++), and a score of 9-12 points was considered strongly positive (+++). In addition, (+) and (+++) were considered high expression, and (-) and (+) were considered low expression.

Quantitative PCR (qPCR). PCR was performed in a 96-well plate. Each well contained 20 µl of the reaction system, which included 10 µl of SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China) and was stored at -80˚C. A number of housekeeping genes served as internal controls for sample normalization, and the 2-ΔΔCq values were compared (8).

Statistical analysis. The results of the qPCR Array: The GAPDH gene was used as the internal control gene; the relative expression of the genes was calculated as ΔCq=CqTarget gene-CqInternal control, and the difference between groups was calculated as ΔΔCq=ΔCqExperimental group-ΔCqControl group. The relationship between the experimental and control groups was expressed as 2-ΔΔCq (8).

Statistical analysis software provided by Qiagen GmbH was used for statistical analysis and mapping. P<0.05 was considered to indicate a statistically significant difference.
Western blotting: Black bands on the PVDF film, which indicated positive results, were scanned by a gel imaging system for quantitative analysis based on the gray values. The protein band of GAPDH was used as the control. SPSS21.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA) was used for the analysis. Measurement data are presented as the mean ± standard deviation and were analyzed using the paired sample t-test and one-way ANOVA with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Immunohistochemistry and qPCR: SPSS 21.0 statistical analysis software (SPSS, Inc.) was employed. Measurement data are presented as mean ± standard deviation and were analyzed using the paired sample t-test. P<0.05 was considered to indicate a statistically significant difference, and correlations were evaluated using Pearson correlation analysis.

Results

**Transfection of the NSCLC cell line H1299 using a lentiviral vector with ABCE1 overexpression.** The H1299 cells were transfected with Eni.S and polybrene. As the LV-GV358-ABCE1 and LV-GV358 constructs contained GFP, the transfected cells were observed to have visible green fluorescence in the cytoplasm when viewed under a fluorescence microscope. The transfection efficiency was 80% or greater. No fluorescence was observed in the untreated control cells (Fig. 1).

**Screening of ABCE1-related genes by a PCR Array chip.** Total mRNA in the LV-GV358-ABCE1- and LV-GV358-transfected H1299 cells was extracted and then reverse transcribed to obtain cDNA fragments. Using the cDNA as the template, we used an RT² Profiler™ PCR Array Human Tumor Metastasis chip for qPCR. The experiments were performed in triplicate, and in all, nine tumor metastasis-related genes were screened by statistical analysis. The difference in CCL7 expression was the most significant (Fig. 2; Table I), and the CCL7 gene was selected as the primary research target from the group of ABCE1-related metastasis genes.

**Western blotting.** The expression of CCL7 in LV-GV358-ABCE1-transfected H1299 cells (0.73±0.019) was significantly higher than that in LV-GV358-transfected H1299 cells (0.32±0.019) and normal H1299 cells (0.35±0.021), P<0.01, and the expression of ABCE1 in LV-GV358-ABCE1-transfected H1299 cells (0.56±0.016) was significantly higher than that in LV-GV358-transfected H1299 cells (0.37±0.016) and normal H1299 cells (0.34±0.003), P<0.01 (Fig. 3).

**Immunohistochemistry.** The expression of the CCL7 and ABCE1 proteins was mainly localized in the cytoplasm, but CCL7 was also expressed in some fibroblasts and capillary endothelial cells. The expression level of CCL7 in lung cancer tissues (8.6±0.58) was higher than that in adjacent NTs (8.6±0.58), P<0.01, and the expression level of ABCE1 in lung cancer tissues (9.13±0.6) was higher than that in adjacent NTs (2.13±0.29), P<0.01 (Fig. 4). The rate of positive CCL7 expression in lung cancer tissues was 70%, and the rate of positive ABCE1 expression in lung cancer tissues was 87%.

**qPCR.** The relative expression levels (ΔCq values) of CCL7 mRNA in NSCLC tissues and adjacent tissues were 10.66±0.41.
and 13.93±0.39, respectively, P<0.01, and the ΔCq values of ABCE1 mRNA in NSCLC tissues and adjacent tissues were 8.29±0.33 and 10.31±0.27, respectively, P<0.01. The mRNA expression of CCL7 was positively correlated with that of ABCE1 in NSCLC, with a Pearson correlation coefficient of r=0.6944, P<0.01 (Fig. 5).

Discussion

Due to its malignancy and threat to human health, lung cancer is a hot topic in the field of cancer research. The incidence and mortality of lung cancer are currently increasing annually, the age at onset is decreasing, and the disease is widespread and occurs worldwide (8). In recent years, with the development of molecular biology, more cancer genes and pathogenic mechanisms have been identified; however, the survival and disease remission rates of lung cancer are still low. Lymph node and organ metastases are important factors in determining the degree of malignancy in lung cancer; thus, further investigations into the mechanism of lung cancer metastasis are essential.

As a specific inhibitor of RNase and a key enzyme in the interferon-dependent 2-5A/RNase L pathway, ABCE1 plays an important physiological role in the regulation of the stability of cell RNA (9). ABCE1 also plays an important role in the initiation, extension and termination of eukaryotic protein translation, as well as in ribosome recycling (10,11). Ren et al (12) found that ABCE1 was highly expressed in human lung AC and metastatic lymph nodes and was associated with clinical stage. Gao et al (13) observed that the expression levels of ABCE1 was correlated with histopathological type, but not with age, gender, the grade of tumor differentiation. In AC, the expression level of ABCE1 protein were higher than that in the squamous carcinoma. Recently, a series of studies revealed that ABCE1 may be a new interaction protein for

Table I. Genes differentially expressed between ABCE1 overexpression and empty vector groups.

| Gene   | Description                                      | Fold change | P-value |
|--------|--------------------------------------------------|-------------|---------|
| CCL7   | Chemokine (C-C motif) ligand 7                   | 12.49       | 0.0048  |
| TIMP3  | TIMP metalloproteinase inhibitor 3               | 5.63        | 0.0154  |
| CXCR2  | Chemokine (C-X-C motif) receptor 2              | 3.37        | 0.0338  |
| ETV4   | Ets variant 4                                   | 3.16        | 0.0401  |
| TNFSF10| TNF superfamily member 10                       | 3.11        | 0.0262  |
| SERPINE1| Serpin peptidase inhibitor, clade E, member 1 | -5.41       | 0.0124  |
| CXCL12 | Chemokine (C-X-C motif) ligand 12               | -5.02       | 0.0228  |
| MMP11  | Matrix metalloproteinase 11                     | -4.87       | 0.0250  |
| ITGA7  | Integrin, α7                                    | -4.76       | 0.0498  |

ABCE1, ATP-binding cassette sub-family E member 1.
β-actin and that the binding of ABCE1 to β-actin requires the Fe-S cluster domain (14,15). These results show that ABCE1 is highly expressed in many malignant tumor cells, indicating that this protein is closely related to the proliferation, invasion and metastasis of lung cancer.

Chemokine CCL7, which was initially identified as a cytokine in mononuclear cells, acts on a variety of target cells, including neutrophils, eosinophils, basophils, natural killer cells, T lymphocytes and other inflammatory cells, as well as dendritic cells and mononuclear cells, particularly mononuclear cells (16). Further research has shown that CCL7 has functions in many diseases. For example, Tsuneyama et al. (17) found that CCL7 and mononuclear cell infiltration were present in the portal area of the liver in more than 80% of patients.
with primary biliary cirrhosis, suggesting that elevated CCL7 expression is associated with biliary cirrhosis. The study by Edman et al. (18) found that CCL2 and CCL7 selectively enhanced the differentiation of Nurr1+ precursors into dopaminergic (DA) neurons. Gonzalez et al. (19) confirmed that CCL7 plays a dual role in renal tubulointerstitial fibrosis by altering the extracellular matrix, an effect that is detrimental at the early stage but beneficial at the later stage.

The role of chemokine CCL7 in tumor growth and metastasis is very complicated, as studies have shown that CCL7 not only promotes tumor metastasis but also inhibits the growth of some malignant tumors (20, 21). As CCL7 can play a chemotactic role in many leukocyte subsets, which identify and kill tumor cells, some researchers conducted anti-tumor experiments using mast cells transfected with CCL7. Interestingly, the tumor cells did not die, but the surrounding tumor tissue was infiltrated with TAMs, eosinophils, neutrophils, granulocytes and lymphocytes (22, 23). In addition, a large number of dendritic cells accumulated around the peripheral vasculature of the tumor. Wetzel et al. found that transfection with a virus containing CCL7 inhibited the growth of cervical cancer cells in humans (24). While this study was supported by the National Natural Science Foundation of China (grant no. 30973502).

In the present study, screening using an RT2 Profiler™ PCR Array chip showed that the change in the mRNA expression of chemokine CCL7 was significant in NSCLC cell lines that exhibited upregulation of ABCE1, showing that the expression of these two genes is strongly correlated during the processes of NSCLC invasion and metastasis. Western blotting was performed to verify the high expression of CCL7 protein in NSCLC cells that overexpressed ABCE1. The expression of CCL7 and ABCE1 in NSCLC tissues was significantly higher than that in adjacent tissues, as confirmed by immunohistochemistry and qPCR, and a positive correlation between the two genes was observed. These results indicate that CCL7 and ABCE1 are closely associated with the development and metastasis of NSCLC. ABCE1 may change the tumor microenvironment through the chemokine CCL7 pathway; this is a new direction for future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
The authors declare that they have no competing interests. 

Competing interests

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