Original Article

Effect of CtBP2 silencing on proliferation, autophagy and EMT of non-small cell lung cancer

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Abstract: Lung cancer cell proliferation and EMT are important factors leading to metastasis and recurrence. CtBP2 is a cellular phosphoprotein and an important transcription factor, but the role of CtBP2 in non-small cell lung cancer (NSCLC) remains unclear. Lung cancer A549 cells and normal bronchial epithelial cell line 16HBE cells were cultured in vitro. CtBP2 mRNA and protein expression were analyzed by Real time PCR and Western blot. A549 cells were divided into a control group, a NC group and a CtBP2 siRNA group followed by analysis of cell proliferation by MTT assay, cell migration by cell scratch assay, expression of autophagy genes ATG5 and Beclin1 by Western blot and expression of EMT-related transcription factors Snail and Slug by real time PCR. Compared with normal 16HBE cells, CtBP2 expression was significantly elevated in A549 cells (P<0.05). CtBP2 siRNA was transfected into A549 cells, and it significantly down-regulated CtBP2 expression, inhibited cell proliferation and migration, promoted the expression of ATG5 and Beclin1, and inhibited the expression of Snail and Slug (P<0.05). In addition, CtBP2 siRNA promoted E-cadherin expression, decreased N-cadherin expression, and inhibited NF-κB expression. Targeting CtBP2 can inhibit the NF-κB signaling pathway, regulate autophagy, inhibit tumor cell proliferation and EMT, and then inhibit the occurrence and development of lung cancer.

Keywords: Lung cancer, CtBP2, NF-κB, autophagy, proliferation, EMT

Introduction

Lung cancer has a high incidence in the world with one of the highest mortality rates. Up to one million people die of lung cancer every year. It is a globally recognized high-grade malignant tumor with characteristics of high incidence and mortality rate [1, 2]. According to histological classification, lung cancer is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Non-small cell lung cancer accounts for more than 80% of lung cancer, including squamous cell carcinoma, adenocarcinoma and large cell carcinoma [3]. At present, the treatment for lung cancer is mainly surgery and chemotherapy combined with radiotherapy. Despite the rapid development of diagnosis and treatment technology, treatment approaches for non-small cell lung cancer have been developed. As lung cancer patients are often already at advanced stages at the time of diagnosis, the prognosis is unsatisfactory with a 5-year overall survival rate of only 10-15% [4, 5]. Lung cancer is characterized by abnormal expression of cell proliferation-related genes and abnormal cell cycle progression [6]. Finding and identifying genes and their mechanisms involved in cell growth regulation is critical to the development of effective treatment and diagnostic strategies for lung cancer in clinic.

In the physiological and pathological state of the body, autophagy is one of the self-degrading pathways of cells, which is evolutionarily conserved and well characterized. The formation of autophagosomes is the formation of vesicles in which the components to be degraded are encapsulated by lipid bilayer membranes. The lysosome then fuses with the vesicles and degrades the vesicles and their internal matter [7, 8]. Autophagy can maintain cell homeostasis under normal physiological conditions, can fight tumors, and has stress resistance; which is an important mechanism of autonomic stabilization [9]. In the process of malignant tumor development, autophagy has the dual role of tumor suppression and tumor...
promotion, so the role of autophagy in tumorigenesis has gradually become a research hotspot [10]. Lung cancer cell proliferation and epithelial-mesenchymal transition (EMT) are important factors leading to metastasis and recurrence of lung cancer [11, 12]. The C-terminal binding protein (CtBP) is a cellular phosphoprotein that is recognized in the interaction with the C-terminus of the adenovirus E1A protein [13]. This family includes two genes encoded by CtBP1 and CtBP2 in mammals [14]. CtBP2 participates in several cellular processes such as cell proliferation and apoptosis and is involved in tumor pathogenesis [15, 16]. However, the role of CtBP2 in non-small cell lung cancer has not been fully elucidated. The aim of the present study was to investigate CtBP2's role in NSCLC.

Materials and methods

Main instruments and reagents

Non-small cell lung cancer A549 cell line and normal bronchial epithelial cell line 16HBE cells were preserved in our laboratory and frozen in liquid nitrogen. DMEM medium, fetal bovine serum (FBS), and cyan chain double antibody were purchased from Hyclone (USA). Dimethyl sulfoxide, MTT powder was purchased from Gibco; trypsin-EDTA digest was purchased from Sigma (USA). PVDF membranes were purchased from Pall Life Sciences, EDTA from Hyclone (USA), Western blot related chemical reagents were from Shanghai Biyuntian Biotechnology Co., Ltd., ECL reagents were purchased from Shanghai Shenggong Biological Co., Ltd. The RNA extraction kit, reverse transcription kit, and lipo2000 reagent were from Invitrogen (USA). CtBP2 siRNA and negative control (NC) were synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The Leica DFC 300 FX inverted microscope was purchased from Leica (Japan).

A549 cell culture and grouping

After being thawed at 37°C, A549 cells were centrifuged at 1000 rpm for 3 min, resuspended in 1 ml of fresh medium, and transferred to a 5 ml cell culture flask. Two ml of fresh medium was added and cells were cultured in a saturated humidity incubator at 37°C, 5% CO2 for 24-48 hours. A549 cells were seeded in a culture dish at 1×10^6 cells/cm^2 and cultured in a culture medium containing 10% FBS, 90% high glucose DMEM medium (containing 100 U/ml penicillin, 100 ug/ml streptomycin) at 37°C with 5% CO2. Three to eight generation cells in logarithmic growth phase were selected for experiments. A549 cells were divided into 3 groups, control group: normal cell culture; NC group and CtBP2 siRNA group, respectively and transfected with CtBP2 negative control or CtBP2 siRNA in A549 cells.

Transfection of CtBP2 siRNA into A549 cells

CtBP2 siRNA and CtBP2 negative controls were transfected into A549 cells. The CtBP2 siRNA sequence was 5’-CTTTGGATTCAGCGTCATA-3’, 5’-GTTCTCATTTGGACATGCA-3’. The CtBP2 negative control sequence was 5’-AUAGGUGGCAUCAUCUGG-3’, 5’-AUATCUGGATCUCUGUGG-3’. After the cell density reached 70-80%, CtBP2 siRNA and negative control liposomes were separately added to 200 ul of serum-free medium, mixed well for 15 min of incubation. The mixed lipo2000 was mixed with CtBP2 siRNA and negative control dilutions, for 30 min of incubation. The serum of the cells was removed, gently rinsed with PBS, 1.6 ml of serum-free medium was added, and each system was cultured at 37°C 5% CO2, Three to eight generation cells in logarithmic growth phase were selected for experiments. A549 cells were divided into 3 groups, control group: normal cell culture; NC group and CtBP2 siRNA group, respectively and transfected with CtBP2 negative control or CtBP2 siRNA in A549 cells.

Real-time PCR detection of CtBP2, ATG5 and Beclin1 expression

Total RNA was isolated using Trizol reagent and reversely transcribed into cDNA according to the kit instructions. The primer sequences were shown in Table 1. Real-time PCR reaction conditions: 55°C 1 min, 92°C 30 s, 58°C 45 s, 72°C 35 s, for a total of 35 cycles. GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards were calculated. Based on the standard CT value, a stan-
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Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>AGTACCAGCTGTGGTGGG</td>
<td>TAAAGACCAGGATGCTCGTT</td>
</tr>
<tr>
<td>CtBP2</td>
<td>CTCGTACACCTGCACCTAAAG</td>
<td>GCACGTACAGGATTGCTTT</td>
</tr>
<tr>
<td>ATG5</td>
<td>TGAGAGTGATCGTGCGGAT</td>
<td>CTGTACCTGCACTAAGCTGC</td>
</tr>
<tr>
<td>Beclin1</td>
<td>CGGATGGTACGAGTA</td>
<td>GAGTCTGAAGATCGTGTG</td>
</tr>
</tbody>
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standard curve was drawn and then the semi-quantitative analysis was carried out using the $2^{-\Delta\Delta Ct}$ method.

MTT assay to detect cell proliferation

The logarithmic growth phase A549 cells were inoculated into the 96-well culture plates with 10% fetal bovine serum DMEM culture medium at a dose of $5 \times 10^4$ cells. After 24 hours of culture, the supernatant was removed, and the cells were placed in the wells to be tested at intervals of 24 h after addition of 20 ul of sterile MTT. Three replicate wells were set at each time point. Cell culture was continued for 4 h, followed by removal of the supernatant, addition of DMSO 150 μl/well and shaken for 10 min followed by analysis of absorbance (A) value at 570 nm by a microplate reader.

Cell scratch test

Cell migration ability was determined using an in vitro wound healing assay. In a 6-well plate, a horizontal line was evenly drawn with a marker using a ruler, about every 0.5-1 cm, across the well. The cells of each group were inoculated into these 6-well plates, and cultured in DMEM medium containing 10% fetal bovine serum after transfection for 48 hours in a single layer, and then cultured for 12 hours in serum-free medium. Using a 100 ul pipet tip to scratch the horizontal line of the cell perpendicular to the marker line. The cells were washed 3 times with PBS followed by addition of serum-free medium. Scratches in the cell growth were observed on an inverted Lycra inverted microscope. From each sample 10 field of view counts were made and the average was taken.

Western blot

The cell proteins of each group were extracted: the lysate was added, the cells were lysed on ice for 15-30 min, disrupted by sonication for 5 s×4 times, centrifuged at 4°C, 10,000×g for 15 min. The protein was quantified by BCA assay and stored at -20°C. The isolated protein was separated in 10% SDS-PAGE, transferred to PVDF membrane, blocked with 5% skim milk powder, and incubated with 1:1000, 1:1500, 1:2000, 1:1000 dilution of primary antibody CtBP2, NF-κB, and E-cadherin and N-cadherin monoclonal antibody, respectively at 4°C overnight. After PBST washing, 1:2000 goat anti-rabbit secondary antibody was incubated with the membrane in the dark for 30 min followed by washing with PBST, developed using chemiluminescence, and exposure to X-ray in order to observe the results. X-film and strip density measurements were separately scanned.
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**Statistical analysis**

All data were shown in mean ± standard deviation (SD) and analyzed by SPSS 11.5 software. Comparison of difference between two groups was performed by student t-test. The differences among multiple groups were analyzed by ANOVA and Dunnett’s test. P<0.05 was considered statistically significant.

**Results**

**Expression of CtBP2 in lung cancer A549 cells and normal bronchial epithelial cells**

CtBP2 mRNA (Figure 1A) and protein (Figure 1B) expression were significantly elevated in A549 cells compared with those in normal 16HBE cells (P<0.05).

**Effect of CtBP2 siRNA on CtBP2 expression in lung cancer A549 cells**

CtBP2 siRNA transfection was significantly down-regulated over the expression of CtBP2 mRNA (Figure 2A) and protein (Figure 2B) (P<0.05).

**Effect of CtBP2 siRNA on proliferation of lung cancer A549 cells**

Transfection of CtBP2 siRNA significantly reduced the proliferation of A549 cells compared with NC group (P<0.05) (Figure 3).

![Figure 2](image1.png)  
**Figure 2.** Effect of CtBP2 siRNA on the expression of CtBP2 in lung cancer A549 cells. A. Real time PCR was used to detect the expression of CtBP2 mRNA in lung cancer A549 cells; B. Western blot was used to detect the expression of CtBP2 protein in lung cancer A549 cells. Compared with the control group, *P<0.05.

![Figure 3](image2.png)  
**Figure 3.** Effect of CtBP2 siRNA on proliferation of lung cancer A549 cells. Compared with the control group, *P<0.05.
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Effect of CtBP2 siRNA on migration of lung cancer A549 cells

Compared with NC group, transfection of CtBP2 siRNA significantly decreased lung cancer A549 cells migration (P<0.05) (Figure 4).

Effect of CtBP2 siRNA on the expression of ATG5 and Beclin1

CtBP2 siRNA significantly upregulated the expression of autophagy genes ATG5 and Beclin1 compared with NC group (P<0.05) (Figure 5).

Effect of CtBP2 siRNA on the expression of EMT-related transcription factors Snail and Slug in lung cancer A549 cells

Down-regulation of CtBP2 in A549 cells caused a significant decrease of Snail and Slug expression (P<0.05) (Figure 6).

Effect of CtBP2 siRNA on the expression of EMT-related proteins E-cadherin, N-cadherin and NF-κB in lung cancer A549 cells

Transfection of CtBP2 siRNA promoted E-cadherin expression and decreased N-cadherin expression (Figure 7) as well as reduced NF-κB expression (Figure 8).

Discussion

Tumor invasion and metastasis are important causes of recurrence and poor prognosis of non-small cell lung cancer (NSCLC) and even death. NSCLC is prone to transfer and infiltration [17, 18]. CtBP2 is involved in tumorigenesis and progression by inhibiting tumor cell apoptosis by mediating EMT and inhibiting tumor suppressor [15]. However, the role of CtBP2 in non-small cell lung cancer remains unclear. The results of this study showed that CtBP2 mRNA and protein were significantly increased in lung cancer cell line A549 compared with those in normal bronchial epithelial cells, suggesting involvement of CtBP2 in lung cancer. In the present study, CtBP2 siRNA expression was significantly decreased in cells,
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and it was confirmed that down-regulation of CtBP2 expression inhibited tumor cell proliferation and migration. These results suggest that CtBP2 can inhibit the proliferation and metastasis of lung cancer.

Autophagy is an important regulatory mechanism of the body. On the one hand, autophagy can inhibit the tumor by maintaining the stability of the normal cell genome. On the other hand, in the tumor environment, the up-regulation of autophagy may also make the tumor cells adapt to a stressful environment, promote tumor proliferation and activity, thereby promoting tumor development [15]. In malignant tumors such as lung cancer, inhibition or overexpression of autophagy key genes such as ATG5 and Beclin1 regulates the dynamics of autophagy, affecting the malignant proliferation or phenotype of NSCLC cell lines [19]. The process of EMT is closely associated with tumor invasion and metastasis [20]. EMT involves multiple genes. Increased expression of Snail and Slug promotes EMT and decreases the expression of cell adhesion factor E-cadherin, which not only blocks the adhesion between cells and adjacent cells, but also leads to the invasion and metastasis of tumor cells to normal cells. Meanwhile, increased expression of N-cadherin also causes epithelial cells to lose cell polarity, lose epithelial phenotypes such as junctions with the basement membrane, and migrate and invade tumor cells [21, 22]. Autophagy and EMT occur in multiple signaling pathways. NF-κB is involved in the inflammatory response, tumor and immune response of cells, and its activation can lead to autoimmune diseases, chronic inflammation and tumorigenesis [23, 24]. This study demonstrates that targeting CtBP2 up-regulates the expression of key autophagy genes such as ATG5 and Beclin1, promotes autophagy to protect and maintain cell stability; while down-regulation of CtBP2
inhibits Snail and Slug expression, increases E-cadherin expression and decreases N-cadherin expression, which in turn inhibits the occurrence of EMT, thereby delaying the migration of tumor cells.

Conclusion

Targeting CtBP2 can inhibit the occurrence of lung cancer by regulating NF-κB signaling pathway, regulating autophagy, inhibiting tumor cell proliferation and EMT. This study demonstrates that CtBP2 can be used as a novel therapeutic target for treating lung cancer, providing a new reference for the diagnosis and treatment of lung cancer.

Disclosure of conflict of interest

None.

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