Original Paper

C/EBP α Suppresses Lung Adenocarcinoma Cell Invasion and Migration by Inhibiting β -Catenin

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Key Words

C/EBP α • Lung adenocarcinoma • Migration • Invasion • β -catenin

Abstract

Background/Aims: The transcription factor CCAAT/enhancer-binding protein α (C/EBP α) is a basic leucine zipper transcription factor that plays essential roles in tumor progression. Although decreased or absent C/EBP α expression in many cancers suggests a possible role for C/EBP α as a tumor suppressor, the functions of C/EBP α in lung adenocarcinoma remain unclear. **Methods:** Here, C/EBP α expression levels in 26 lung adenocarcinoma and para-carcinoma tissue samples were detected by qRT-PCR and immunohistochemistry. Cell transwell assays, wound healing assay and three-dimensional spheroid invasion assay were performed to assess the effects of C/EBP α on migration and invasion in lung adenocarcinoma cells *in vitro*. Western blotting was applied to analyze the potential mechanisms. **Results:** C/EBP α was found to be decreased in lung adenocarcinoma tissues compared to para-carcinoma tissues. Overexpression of C/EBP α significantly inhibited the migration and invasion of lung adenocarcinoma cells. In addition, C/EBP α overexpression suppressed the epithelial–mesenchymal transition (EMT) that was characterized by a gain of epithelial and loss of mesenchymal markers. Further study showed that C/EBP α suppressed the transcription of β -catenin and downregulated the levels of its downstream targets. **Conclusion:** Our data suggest that C/EBP α inhibits lung adenocarcinoma cell invasion and migration by suppressing β -catenin-mediated EMT *in vitro*. Thus, C/EBP α may be helpful as a potential target for treatment of lung adenocarcinoma.

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Introduction

Lung cancer is one of the leading types of cancer-related mortality worldwide, responsible for nearly 1.4 million deaths each year [1, 2]. Non-small-cell lung cancer (NSCLC) is

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the main histological category of lung cancer, accounting for 75–80% of all cases, and lung adenocarcinoma is one of the most common subtypes [3]. More than 50% of NSCLC patients reveal local invasion or distant metastasis when diagnosed [4, 5]. Thus, early diagnosis of NSCLC is very difficult for the majority of patients diagnosed at an advanced stage [6]. Novel insights into the regulatory mechanisms underlying NSCLC pathogenesis are critically needed.

CCAAT/enhancer-binding protein- α (C/EBP α) is a transcription factor that plays important roles in the regulation of cell proliferation and differentiation [7–11]. As a tumor suppressor, C/EBP α was found to be downregulated in several types of cancer and regulated cancer cell behaviors such as proliferation, differentiation and metabolism [12, 13]. In the lung, C/EBP α is highly expressed in alveolar type II cells, Clara cells, and alveolar macrophages [14, 15]. Low expression levels of C/EBP α were found in 50% of stage II and IIIA lung adenocarcinomas, and induction of C/EBP α led to significant growth reduction attributable to proliferation arrest, morphological changes characteristic of differentiation and apoptosis [16–18]. A recent study also showed that C/EBP α was a tumor suppressor in lung cancer and that targeted B lymphoma Mo-MLV insertion region 1 homolog (BMI1) inhibition impaired tumor growth in lung adenocarcinomas with low C/EBP α expression [19]. C/EBP family members also regulate cell migration [20]. With regard to lung adenocarcinoma cell migration and invasion, the precise function of C/EBP α and its underlying mechanism remain unclear.

In the present study, we identified significant downregulation of C/EBP α in lung adenocarcinoma tissues. Overexpression of C/EBP α strikingly suppressed the invasion and migration of lung adenocarcinoma cells. Importantly, these functional effects of C/EBP α were exerted by suppressing β -catenin-mediated the epithelial–mesenchymal transition (EMT). Thus, our findings provide a novel mechanistic role for C/EBP α in lung adenocarcinoma metastasis and a rationale for clinically exploring the use of this molecule in curbing lung adenocarcinoma aggressiveness.

Materials and Methods

Tissue samples and cell cultures

Matched pairs of lung adenocarcinoma and para-carcinoma tissue used in the study were obtained from 26 patients at Qingpu Branch Zhongshan Hospital (Shanghai, China). The tissues were stored in liquid nitrogen. Informed consent was obtained from each patient who underwent lung cancer radical resection prior to surgery and this study was conducted in accordance with a protocol approved by the Ethics Committee of Fudan University (Shanghai, China). Human lung adenocarcinoma cell lines were purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μ g/mL of streptomycin. Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂.

RNA isolation and qRT-PCR

Total RNA was prepared from tissues or cells using TRIzol reagent (TaKaRa, Dalian, China). cDNA was synthesized using a PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR Premix Ex Taq (TaKaRa) to determine the expression level of gene transcripts for C/EBP α , E-cadherin, N-cadherin, vimentin, β -catenin, cyclin D1, c-myc and β -actin. The sequences of real-time PCR primers were as follows:

C/EBP α forward 5'-ATCTGCGAGCACGAGACGTCCAT-3'; reverse 5'-CAGCTGCTTGGCTTCATCCTCCT-3';
E-cadherin forward 5'-GGACAGCCTATTTTCCCTCGAC-3'; reverse 5'-ACCCAGTCTCTCTTGTGTCTTCT-3';

N-cadherin forward 5'-ATCCCTCCAATCAACTTGCCAGA-3'; reverse 5'-CCCATTCCAAACCTGGTGTAAGA-3';

vimentin forward 5'- CAGGACTCGGTGGACTTCTCGCT-3'; reverse 5'- TTCTCCCGGAGGCGCAT-GATGTC-3';

β-catenin forward 5'- TGGGAACAGGGATTTCTCAGTC-3'; reverse 5'- TGGTCCTCGTCATT-TAGCAGTTT-3';

cyclin D1 forward 5'- TACCCCGATGCCAACCTCCTCAA-3'; reverse 5'- TTAGAGGCCACGAACAT-GCAAGT-3';

c-myc forward 5'- GACTCGGTGCAGCCGTATTTCTA-3'; reverse 5'- GAAGCCGCTCCACATACAGTCTCT-3';

β-actin forward 5'- ATCCAGGCTGTGCTGTCCCGTA-3'; reverse 5'- GAAGTCCAGGGCAACATAGCACA-3';

The relative fold change in gene expression normalized to the housekeeping gene β-actin was calculated by the $2^{-\Delta\Delta Ct}$ method.

Lentiviral infection

C/EBPα sequences were amplified and cloned into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA). The empty backbone vector was used as a control. The constructs were co-transfected with packing plasmids into 293T packing cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Virus particles were harvested 48 h after transfection. A549 and H1299 cells were infected with recombinant lentivirus-transducing units in the presence of 5 mg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA).

Transwell invasion and migration assays

Cell migration and invasion were measured using 24-well Transwell plate cell culture inserts (Corning, Cambridge, MA, USA) with or without Matrigel coating (BD Biosciences, San Jose, CA, USA). In brief, 1×10^5 infected A549 or H1299 cells in serum-free RPMI-1640 medium were placed in the upper chamber of each insert, and medium containing 10% fetal bovine serum was added to the lower chamber. After 24 h incubation, the cells remaining on the upper surface of the membranes were removed, and cells that had migrated to or invaded the lower surface were fixed, stained and counted under an inverted microscope ($\times 200$) to calculate their relative numbers. All assays were performed in triplicate.

Wound healing assay

Infected A549 and H1299 cells were seeded into six-well plates and cultured until totally confluent. Cells were then starved overnight in serum-free RPMI-1640 medium. Artificial wounds were created by a sterile 200 μ l pipette tip, and the cells were washed with serum-free medium to remove floating cells and debris. Images were taken at 0 and 24 h on a microscope (Olympus Corp., Tokyo, Japan). Migration ability was assessed by measuring changes in the size of the wounded areas.

Three-dimensional spheroid invasion assay

Cells (2×10^3) were mixed with 20% Matrigel and seeded in 24-well plates coated with 100% Matrigel (BD Biosciences), and medium was changed every other day. Five days after plating, 10 μ g/mL mitomycin C (Sigma-Aldrich) was added to the medium to block proliferation for another five days. Tumor cell outgrowth was visualized by microscopy.

Western blotting

Cell proteins were isolated using RIPA buffer supplemented with protease and phosphatase inhibitors (Applygen Technologies Inc., Beijing, China), and quantified by a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equivalent amounts of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were then blocked in 5% nonfat dried milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies obtained from Cell Signaling Technology (Danvers, MA, USA) against C/EBPα (#8178), E-cadherin (#14472), N-cadherin (#13116), vimentin (#5741), β-catenin (#8480), c-myc (#5605), and cyclin D1 (#2978). Antiserum against β-actin was obtained from Santa Cruz Biotechnology (sc-47778; Santa Cruz, CA, USA). All antisera were used at a working dilution of 1:1000. Membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibody and the bands were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

Immunohistochemistry (IHC) analysis

IHC was performed as previously described [21]. Briefly, tissues were fixed in phosphate-buffered neutral formalin, embedded in paraffin and cut into 5 μ m-thick sections. Tissue sections were deparaffinized, rehydrated, and microwave-heated in sodium citrate buffer for antigen retrieval. The sections were then incubated overnight at 4°C with a 1:200 dilution of primary antibody against C/EBPα (#8178, Cell Signaling Technology). After washing in PBS, detection of the primary antibody was performed using goat anti-rabbit HRP-conjugated secondary antibody for 1 h at room temperature. Immunolabeling was visualized with 3, 3'-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin.

Luciferase reporter assay

Luciferase reporter assay was performed as previously described [22]. Briefly, cells were seeded on 24-well culture plates and grown overnight. Cells were transiently transfected with TOPflash or FOPflash (Upstate, Lake Placid, NY, USA) along with pRL-TK (Promega, Madison, WI, USA) using GeneJuice™ transfection reagent (Novagen, Madison, WI, USA). After 48 h incubation, cells were washed twice with PBS, lysed with Passive Lysis Buffer and Dual-Luciferase reporter assays (Promega) were performed as per the manufacturer's instructions. Firefly (TOPflash or FOPflash) and *Renilla* (pRL-TK) luciferase activities were measured in the lysates using a Centro LB 960 Microplate Luminometer (Berthold Technologies, Oak Ridge, TN, USA). Firefly luciferase values were normalized to *Renilla* luciferase values to control for transfection efficiency.

Statistical analysis

All statistical analyses were carried out using SPSS 17.0 (Chicago, IL, USA). Student's *t*-test was used to compare data from two groups represented as means $\pm$ SD. Differences were considered significant at values of $P < 0.05$.

Results

The expression of C/EBPα is downregulated in lung adenocarcinoma cells and tissues

To explore the expression and significance of C/EBPα in lung adenocarcinoma, we measured C/EBPα expression in 26 lung adenocarcinoma and para-carcinoma tissue samples by qRT-PCR and IHC. qRT-PCR analysis showed that C/EBPα expression was significantly lower in lung adenocarcinoma tissues than in para-carcinoma tissues (Fig. 1A). In addition, C/EBPα protein was expressed at a much lower level in lung adenocarcinoma tissues by IHC (Fig. 1B). These experimental results showed that C/EBPα expression was decreased in cancerous tissue and thus may be an important factor that could inhibit lung adenocarcinoma.

Fig. 1. Expression of C/EBPα in lung adenocarcinoma and normal lung tissues. (A) qRT-PCR analysis of C/EBPα mRNA levels in 26 pairs of lung adenocarcinoma and para-carcinoma tissues. Normal, para-carcinoma tissue. Cancer, lung adenocarcinoma. (B) Representative staining with antibody against C/EBPα in lung adenocarcinoma and para-carcinoma tissues detected by IHC. * $P < 0.05$.

Fig. 2. Effect of C/EBPα on lung adenocarcinoma cell invasion and migration. A549 and H1299 cells were infected with C/EBPα-overexpressing lentivirus (indicated as C/EBPα) or empty vector (indicated as Con), and the stably transduced cells were analyzed for motility. (A, B) Migration and (C, D) invasion ability of A549 and H1299 cells after C/EBPα overexpression determined by Transwell assays. (E, F) Migration ability of A549 and H1299 cells after C/EBPα overexpression determined by wound healing assays. (G) Representative micrographs of indicated cultured cells in 3-dimensional spheroid invasion assays. * $P < 0.05$.

C/EBPα inhibits lung adenocarcinoma cell migration and invasion ability

To explore the biological function of C/EBPα in lung adenocarcinoma cells, Transwell assays and wound-healing migration assays were performed. An equal number of A549 and H1299 cells infected with a C/EBPα-overexpressing lentivirus or control lentivirus were plated. Overexpression of C/EBPα suppressed the migration and invasion of A549 and H1299 cells and resulted in a reduction of the number of invasive or migratory cells compared to the control group, as examined in Transwell migration and Matrigel invasion assays, respectively (Fig. 2A–D). Similar to its effect on cell motility in the wound-healing assay (Fig. 2E–F), C/EBPα overexpression reduced the distances migrated by A549 and H1299 cells 24 h after transfection. Furthermore, we found that the invasive capacity of C/EBPα-transfected cells was significantly impaired in 3-dimensional spheroid invasion assay (Fig. 2G). Taken together, these results strongly suggest that C/EBPα significantly decreases the migration and invasion ability of lung adenocarcinoma cells *in vitro*.

Fig. 3. C/EBPα attenuates EMT of lung adenocarcinoma cells. The expression of EMT markers in C/EBPα-overexpressing A549 and H1299 cells was measured by western blotting (A) or qRT-PCR (B). * $P < 0.05$.

Fig. 4. C/EBPα inhibits the transcription of β-catenin. (A) Transcriptional activity of the β-catenin/TCF/LEF complex in C/EBPα-overexpressing cells was measured using a luciferase assay. (B, C) The expression of downstream gene targets of β-catenin in C/EBPα-overexpressing cells was measured by western blotting (B) or qRT-PCR (C). (D) qRT-PCR analysis of β-catenin mRNA levels in lung adenocarcinoma and para-carcinoma tissues. Normal, para-carcinoma tissue. Cancer, lung adenocarcinoma. (E) Scatter plot showing the statistically significant inverse correlation between C/EBPα and β-catenin mRNA levels in lung adenocarcinoma tissues. * $P < 0.05$.

Overexpression of C/EBPα in lung adenocarcinoma cell lines inhibits cell migration and invasion by suppressing EMT

EMT is a key event in the invasion and metastasis of tumors [23]. Therefore, we examined whether overexpression of C/EBPα in A549 and H1299 cells affected EMT. The results indicated that overexpression of C/EBPα increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal markers N-cadherin and vimentin (Fig. 3A). Similar results were observed in qRT-PCR analysis showing that upregulation of C/EBPα elevated mRNA expression of E-cadherin and diminished expression of N-cadherin and vimentin mRNA in A549 and H1299 cells (Fig. 3B). These findings suggest that C/EBPα overexpression may prevent the migration and invasion of lung adenocarcinoma cells by inhibiting EMT.

C/EBPα suppresses the transcription of β-catenin

To further examine how C/EBPα is involved in cell migration and invasion signaling pathways, luciferase assays were performed using TOP Flash reporter plasmids. The TOP Flash reporter contains TCF-binding sites upstream of the luciferase gene and is a reporter

of β -catenin/TCF/LEF complex transcription activity. The luciferase activity of the TOP Flash reporter was significantly decreased by the upregulation of C/EBP α (Fig. 4A). This result indicates that inhibition of cell invasion and migration by C/EBP α is mediated by the β -catenin/TCF/LEF signaling pathway. Hence, we used western blotting to measure the protein expression level of β -catenin in the C/EBP α -upregulated cells. β -catenin protein expression was significantly decreased in C/EBP α -upregulated A549 and H1299 cells, with a simultaneous decrease in the levels of c-myc and cyclin D1, which are the downstream targets of β -catenin (Fig. 4B). In contrast with the control group, the mRNA level of β -catenin was also decreased in C/EBP α -upregulated A549 and H1299 cells (Fig. 4C).

To further confirm the correlation of C/EBP α and β -catenin *in vivo*, we used qRT-PCR to measure the expression levels of β -catenin mRNA in the same 26 pairs of human lung adenocarcinoma tissues and adjacent non-cancerous tissues that we had initially measured C/EBP α mRNA expression levels in, as described above. The β -catenin mRNA expression level was significantly greater in the cancerous tissue than in the non-cancerous tissue (Fig. 4D). The association of C/EBP α and β -catenin mRNA expression was then analyzed by Pearson's correlation test, which showed a significant negative correlation between the mRNA expression levels of these two transcripts (Fig. 4E). Together, these results strongly suggest that C/EBP α inhibits the transcription of β -catenin.

Discussion

Although a decrease or the complete absence of C/EBP α has been associated with poor prognosis and metastasis in a multitude of human tumors [18, 24], the functions of C/EBP α in lung adenocarcinoma remain unclear. In this study, we revealed the critical role of C/EBP α in lung adenocarcinoma and its underlying mechanisms. We observed that elevated C/EBP α expression was negatively correlated with lung adenocarcinoma cell migration and invasion. We also found that C/EBP α transcriptionally suppresses the β -catenin gene, thereby constituting an important signaling pathway that directly affects EMT and invasion of lung adenocarcinoma cells. Collectively, our results strongly suggest that downregulated C/EBP α expression causes high β -catenin expression and critically contributes to lung adenocarcinoma pathogenesis and aggressive behavior.

C/EBP α has been found to exhibit tumor suppressor activity in many types of cancer [25, 26]. Bennett et al. reported that C/EBP α is epigenetically downregulated in head and neck squamous cell carcinoma [18]. Shi et al. demonstrated that overexpression of C/EBP α significantly suppressed the proliferation of primary hepatocarcinogenesis cells *in vitro* and reduced the growth of subcutaneous hepatocarcinogenesis xenografts in nude mice [27]. Importantly, loss of C/EBP α in mouse respiratory epithelium led to respiratory failure at birth due to an arrest in the type II alveolar cell differentiation program [15]. Lung-specific C/EBP α knockout mice developed pulmonary adenocarcinoma, suggesting that the loss of C/EBP α could be an important event in the multistep process of lung tumorigenesis [19]. In this study, we found that C/EBP α expression was significantly decreased in lung adenocarcinoma tissues compared to para-carcinoma tissues. These results are consistent with findings by Halmos et al., who found that C/EBP α expression was more frequently downregulated in adenocarcinoma compared to normal lung [16]. We also found that overexpression of C/EBP α significantly inhibited lung adenocarcinoma cell migration and invasion ability, suggesting that C/EBP α may be an important factor affecting lung adenocarcinoma metastasis.

EMT is associated with functional changes relevant to metastasis, e.g. increased migratory and invasive abilities. During EMT, epithelial cells lose their epithelial polarity and cell-cell contacts and gain mesenchymal phenotypes with increased migratory and invasive capabilities. Accumulating evidence has shown that promoting EMT leads to tumor cell migration and invasion [28-30]. Previously, Hongbo et al. showed that enhanced C/EBP α expression successfully inhibited hepatocellular carcinoma metastasis by inhibiting EGFR/ β -catenin signaling pathway-mediated EMT *in vitro* and *in vivo* [25], suggesting that C/

EBPα affected tumor cell invasive activity associated with EMT. In our study, we demonstrated that C/EBPα-overexpression enhanced E-cadherin expression and decreased N-cadherin and vimentin expression in lung adenocarcinoma cells, suggesting that C/EBPα reduced cell migration and invasion by suppressing EMT in lung adenocarcinoma.

A variety of signaling pathways are involved in the activation of EMT programs, including Wnt/β-catenin signaling [31]. However, the mechanistic relationship between EMT and the Wnt/β-catenin pathway is not entirely clear. Chromatin immunoprecipitation with high-throughput sequencing indicated that EMT induces a switch from the β-catenin/E-cadherin/Sox15 complex to the β-catenin/Twist1/TCF4 complex, which then binds to cancer stem cell-related gene promoters [32]. In our study, we found that C/EBPα suppressed β-catenin/TCF/LEF complex transcriptional activity and downregulated levels of β-catenin and its downstream targets. Pearson's correlation test showed a significant negative correlation between mRNA expression levels of C/EBPα and β-catenin in human lung adenocarcinoma tissues. Considering that translocation of β-catenin into the nucleus leads to the loss of E-cadherin and subsequent induction of EMT [33], we speculated that C/EBPα-inhibited EMT may be associated with the β-catenin signaling pathway.

In conclusion, we have demonstrated that C/EBPα inhibited lung adenocarcinoma cell migration and invasion by suppressing EMT mediated by the β-catenin signaling pathway. Our findings provide novel mechanistic insights into the role of C/EBPα in lung adenocarcinoma pathogenesis, and suggest a potential therapeutic approach for lung adenocarcinoma therapy.

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Disclosure Statement

All authors declare no conflicts of interest.

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