

Overexpression of CUGBP1 is associated with the progression of non-small cell lung cancer

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Abstract The multifunctional RNA-binding protein CUGBP1 regulates multiple aspects of nuclear and cytoplasmic messenger RNA (mRNA) processing, including splicing, stabilization, and translation of mRNAs. Previous studies have shown that CUGBP1 is overexpressed in non-small-cell lung cancer (NSCLC) tissues, but the pathological functions of CUGBP1 in tumorigenesis and development are unknown. Here, we provide the first evidence demonstrating the clinicopathological significance of CUGBP1 in NSCLC. Using immunohistochemistry, the levels of CUGBP1 expression in NSCLC tissues and adjacent non-cancerous tissues were examined and determined to be associated with differentiation. Short hairpin RNA-induced downregulation of CUGBP1 promoted apoptosis and decreased proliferation in the A549 NSCLC cell line. Moreover, Western blot analysis indicated that the depletion of CUGBP1 increased the protein levels of cyclin D1, BAD, BAX, *Jun D*, and E-cadherin, while the cyclin B1 level decreased. Knockdown of CUGBP1 decreased β -catenin and vimentin levels and increased E-cadherin expression, suggesting that CUGBP1 may contribute significantly to epithelial to mesenchymal transition (EMT) progression. These results demonstrate the importance of CUGBP1 in the biological and pathological functions of NSCLC and indicate its potential as a therapeutic target for NSCLC.

Keywords Non-small-cell lung cancer · CUG-binding protein 1 · Apoptosis · Proliferation · Prognosis

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Introduction

Lung cancer is the leading cause of cancer mortality worldwide [1–3]. It can be classified into two main groups: small-cell lung cancer (SCLC), accounting for 15 % of all lung cancers, and non-small-cell lung cancer (NSCLC), accounting for 85 % [4]. Although the rates of early screening and diagnosis have improved in recent years, the prognosis of most NSCLC patients remains poor, with a 5-year survival rate of less than 15 % [5]. Effective treatment measures that significantly prolong survival and improve the quality of life in NSCLC patients are lacking. Therefore, it is imperative to discover new therapeutic methods and drug targets for the treatment of NSCLC.

CUGBP1, a member of the CUGBP and embryonic lethal abnormal vision-like factor (CEFE) family of human RNA-binding proteins, was first identified as a protein that binds to the CUG RNA repeats that are expanded in the 3'-untranslated region (UTR) of the gene encoding myotonic dystrophy protein kinase (DMPK), which is associated with DM1 (myotonic dystrophy type 1) [6–8]. It has been shown that CUGBP1 regulates many posttranscriptional processes, including alternative splicing, translation, deadenylation, and messenger RNA (mRNA) decay, which is conducted by binding to GU-rich elements (GREs) found in the 3'-UTR regions of short-lived transcripts [9–11]. Considering that CUGBP1 is involved in embryonic and cardiac development, skeletal muscle and adipose tissue differentiation, and germ cell formation, it may also play a significant role in tumorigenesis and the deterioration of certain tumors [12, 13]. Some studies have indicated that CUGBP1 plays an important role in modulating proteins during differentiation, cellular stress, or tumorigenesis and participates in the process of apoptosis in esophageal and liver cancer [12, 14]. Recently, a study revealed that CUGBP1 was overexpressed in cancer tissues, which may independently predict a poor prognosis in NSCLC [15].

However, the role of CUGBP1 in the pathogenesis of NSCLC remains unclear. To investigate the functional role of CUGBP1 in NSCLC, CUGBP1 expression was knocked down, and the resulting effects on proliferation, apoptosis, cell cycle distribution, and invasion of NSCLC cells were examined.

Materials and methods

Patients

Eighty NSCLC patients were selected from the Department of Thoracic Surgery in the Affiliated Hospital of Qingdao University, treated by curative surgical resection and never received chemotherapy or radiotherapy before. Their tumor tissues and pericancerous tissues were collected for immunohistochemical staining by the Department of Pathology. The clinical characteristics analyzed included gender, age, histology, TNM stage, N stage, and differentiation; data were recorded according to the World Health Organization (WHO) and the Union for International Cancer Control (UICC). Research approval was obtained from the Ethics Committee of the Affiliated Hospital of Qingdao University, and written informed consent was obtained from all participants.

Immunohistochemistry

The specimens were formalin-fixed and paraffin-embedded. After dewaxing and hydration, the slides were washed with phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked with 3 % hydrogen peroxide. After heating, 1 % hydrogen peroxidase was used to block endogenous peroxidase activity. The specimens were then incubated with anti-CUGBP1 (Santa Cruz Biotechnology, Shanghai, China, diluted 1:200 in PBS) for 90 min at room temperature. After washing with PBS, the samples were incubated with secondary antibodies for 60 min at room temperature. Finally, DAB staining was performed, followed by counterstaining of all sections with hematoxylin.

Cell culture and CUGBP1 depletion

The human NSCLC cell lines A549 (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were grown in the designated complete medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10 % fetal bovine serum (FBS, Summit Biotechnology, Ft. Collins, CO). The cells were cultured as a monolayer in a humidified atmosphere containing 5 % CO₂ at 37 °C in 100 % humidity. For lentivirus packaging, A549 cells were transfected with CUGBP1 short hairpin RNA (shRNA) (a

GU-repeat sequence UGUUUUUUGU) [10] or control shRNA. After 36 h of transfection, the media containing the packaged lentivirus was gathered and passed through 0.45- μ m filters. A549 cells were subcultured in six-well tissue culture plates at 5×10^4 cells per well, and the prepared lentiviral particles were added after 48 h of culture at a multiplicity of infection (MOI) of 50.

Real-time qPCR analysis

Five days following infection, A549 cells were harvested for total RNA extraction, and cDNA was prepared from each sample using 10 ng of total RNA for reverse transcription. The primers were as follows: CUGBP1 (forward 5'-GTCA GTGGTGGACCTGACCT-3', reverse 5'-AGGGGTCTAC ATGGCAACTG-3'). Actin was used as a control housekeeping gene (forward 5'-TGA CTTCAACAGCGACACCCA-3', reverse 5'-CAC CCTGTTGCTGTAGCCAAA-3'). All primers were synthesized and purified by reverse-phase high-performance liquid chromatography (HPLC) at the central laboratory of our hospital. The PCR conditions were as follows: 1 cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 20 s. The expression of CUGBP1 was measured by normalization of the cycle threshold (Ct) with the control housekeeping gene, calculated by the formula $2^{-\Delta\Delta Ct}$. The entire process was conducted on the BioRad Connect Real-Time PCR platform.

Western blot analysis

Whole cell lysates were prepared in buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4 % sodium dodecyl sulfate, 10 % glycerol). After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes for immunoblotting. After blocking with 5 % non-fat milk for 1 h, the nitrocellulose membranes were incubated with specific antibodies: the primary antibodies include anti-CUGBP1 (Santa Cruz, sc-20003, dilution 1:1000) and anti-GAPDH (Santa Cruz Biotechnology, sc-32233, dilution 1:3000); the secondary antibodies are goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005, dilution 1:5000). A chemiluminescence reagent (NEN, Boston, MA) was employed to detect the signal, which was visualized by autoradiography. Densitometry analysis was performed with ImageJ software (NIH, Bethesda, MD).

Cell proliferation assay

The CCK-8 assay was performed to assess the level of cell proliferation. Cell lines with CUGBP1 shRNA or control shRNA were seeded into 96-well plates and incubated for 24, 48, and 72 h. Two hours prior to incubation completion, 10 μ l of CCK-8 was added to each well. The absorbance of

each well was then evaluated using a microplate reader at 450 nm. All of the experimental procedures were repeated at least three times independently.

Flow cytometry for apoptosis and cell cycle assessment

Cell apoptosis was measured using an annexin-V apoptosis analysis kit according to the manufacturer's protocol (C1052, Beyotime Institute of Biotechnology, Jiangsu, China). Forty-eight hours after transfection with Lv-shCUGBP1 or Lv-shCon, the resulting A549 cells were centrifuged at $3000\times g$ for 5 min. Next, at room temperature, the transfected cells were washed with PBS, resuspended in 100 μ l of buffer, and incubated with annexin-V for 15 min in the dark. After adding 400 μ l of buffer to the cell suspension, fluorescence-activated cell sorting analysis was performed using a flow cytometer and CellQuest software. Similarly, after transfection of CUGBP1 shRNA or control shRNA, the A549 cells were collected by trypsinization, washed with PBS, and fixed with 70 % cold ethanol overnight. The ethanol-suspended cells were then collected, washed, and stained with propidium iodide (PI) for 30 min in the dark at room temperature. Finally, the cell cycle assessment was performed by determining the DNA content using a Cell Cycle and Apoptosis Analysis Kit (C1052, Beyotime Institute of Biotechnology, Jiangsu, China), according to the manufacturer's protocol and analyzed by Cell Lab Quanta Beckman Coulter.

DAPI (4',6-diamidino-2-phenylindole) staining

The A549 cells transfected with CUGBP1 shRNA and control shRNA were seeded in six-well plates at a density of 2×10^5 /well. The culture media was then changed to that including 10 % CCS (charcoal stripped FCS). Next, the cells were immobilized onto poly-(L-lysine)-coated slides with 4 % paraformaldehyde for 15 min, followed by permeabilization with 0.1 % Triton X-100. After washing the slides with PBS, the transfected cells were cultured with 50 ml of DAPI staining solution for 5 min. All of the above procedures were

Table 1 Expression of CUGBP1 in NSCLC and normal tissues

	Case	CUGBP1 expression		χ^2	P value
		Positive (%)	Negative (%)		
Tumor group	80	55 (68.8)	25 (31.2)	7.7356	0.0054
Control group	80	28 (35.0)	52 (65.0)		

conducted at room temperature. A Nikon Eclipse Ti fluorescent microscope (Nikon TI-SR, JAPAN) was employed to observe the transfected cells at emission wavelengths of 340–380 nm.

Statistical analysis

The experimental results were evaluated by Student's *t* test and expressed as the means \pm SD. All statistical analyses were performed with SPSS 17.0 software. The level of statistical significance was set at $P<0.05$.

Results

CUGBP1 is highly expressed in NSCLC tissues

To explore the important role of CUGBP1 in NSCLC, immunohistochemistry was employed with the CUGBP1 antibody on 80 NSCLC tissues along with 80 normal tissues. As shown in Fig. 1, CUGBP1 expression was localized in the nucleus. Importantly, the level of CUGBP1 expression in the NSCLC tissue samples was significantly higher ($P<0.05$) than that in the control group samples (Table 1). The relationships between the expression of CUGBP1 and clinicopathological variables were then analyzed. The expression of CUGBP1 was associated with differentiation, and maybe also with the TNM stage; no statistically significant differences were detected between CUGBP1 expression and the other variables (Table 2).

Fig. 1 IHC staining for CUGBP1 in NSCLC patients: CUGBP1 expression is located in the cytoplasm. **a** Positive expression of CUGBP1; **b** negative expression of CUGBP1

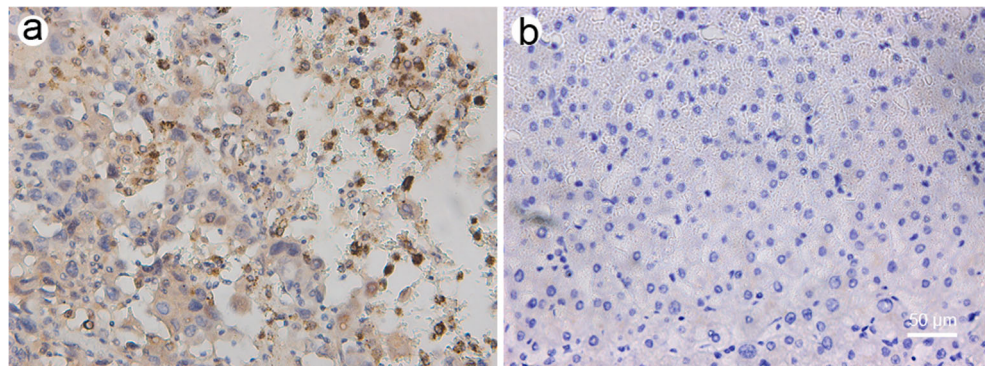
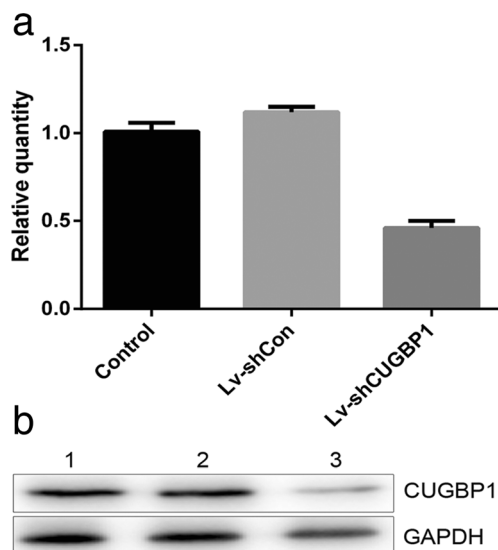
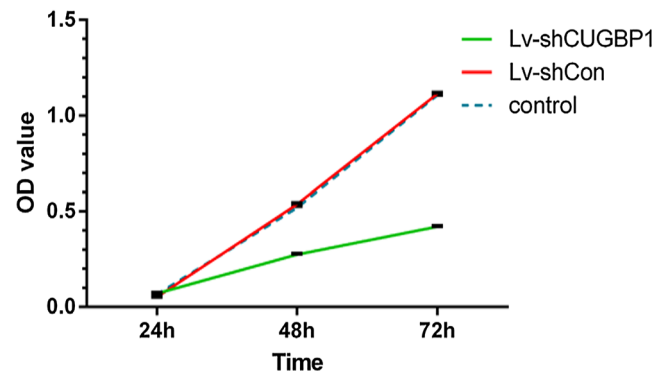


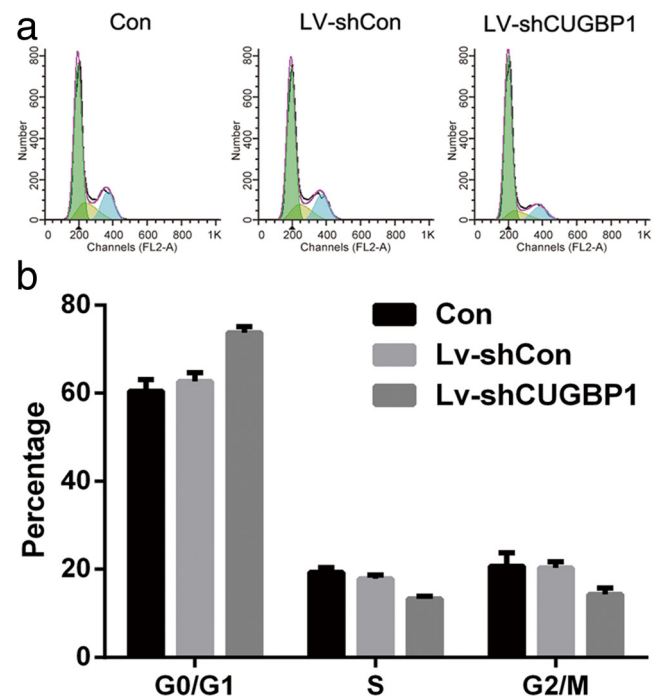
Table 2 Relationship between CUGBP1 expression and clinicopathological factors

Clinical characteristics	Case	CUGBP1 expression		P value
		Positive	Negative	
Total	80	55	25	
Gender				0.4605
Male	47	29	18	
Female	33	23	10	
Age				0.4238
<60	41	26	15	
≥60	39	28	11	
Histology				0.3883
Squamous	24	17	7	
Adenocarcinoma	56	34	22	
TNM stage				0.0056
I+II	25	13	12	
III	55	45	10	
N stage				0.3031
N ₀	38	23	15	
N ₁ +N ₂	42	30	12	
Differentiation				0.0173
Well/moderate	37	17	20	
Poor	43	31	12	

**Fig. 2** The expression levels of CUGBP1 mRNA and protein in A549 cells. **a** Expression analysis of CUGBP1 mRNA in A549 cells by real-time qPCR after infection with lentivirus. Data represent the means±SD of four independent experiments. **b** Protein levels of CUGBP1 in A549 cells after infection with lentivirus were determined with antibodies against CUGBP1, using GAPDH as an internal control. The data represent one of four separate experiments. 1, Control; 2, Lv-shCon; 3, Lv-shCUGBP1**Fig. 3** The detection of A549 cellular proliferation after knockdown of CUGBP1. The CCK-8 assay indicated decreased proliferation of A549 cells after CUGBP1 knockdown compared with that of the two control groups

CUGBP1 expression is downregulated by transfection with CUGBP1 shRNA

Highly specific and apparently non-toxic interfering RNA has been widely used to knock down the expression of target genes [16]. To examine the functions of CUGBP1 in NSCLC, A549 cells were collected following transfection with CUGBP1 shRNA or control shRNA. As demonstrated by real-time qPCR analysis, the transcription level of CUGBP1 was significantly decreased in A549 cells transfected with Lv-

**Fig. 4** Knockdown of CUGBP1 blocked cell cycle progression in A549 cells. **a** Cells were grown to 95 % confluence after transduction, and the cell cycle distribution was explored by flow cytometry analysis. **b** The percentage of A549 cells in the G0/G1, S, and G2/M phases. Data represent the means±SD of three independent experiments

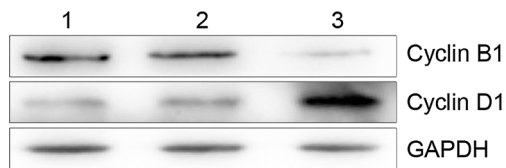


Fig. 5 Effect of CUGBP1 depletion on cell cycle regulatory factors. The expression of cyclin B1 and cyclin D1 was determined by Western blot analysis. GAPDH was used as an internal control. The data represent one of three separate experiments. 1, Control; 2, Lv-shCon; 3, Lv-shCUGBP1

shCUGBP1 compared with cells transfected with Lv-shCon or control cells (Fig. 2a). Western blot analysis further confirmed that the level of CUGBP1 expression was downregulated significantly by transfection with CUGBP1 shRNA (Fig. 2b). These results indicated that the knockdown of CUGBP1 in A549 cells significantly decreased CUGBP1 expression at the mRNA and protein levels.

Suppression of CUGBP1 inhibits A549 cell proliferation

Oncogenes are typically overactivated to promote the proliferation of tumor cells and impact their invasion and migration. CUGBP1 can control the eukaryotic cell cycle by efficiently binding to the 5'-UTR region of the cyclin-dependent kinase inhibitor [12]. The proliferation of A549 cells was detected after transfection with CUGBP1 shRNA or control shRNA using the CCK-8 assay. The results demonstrate that the OD values of A549 cells transfected with Lv-shCUGBP1 (48 h, 0.274 ± 0.005 ; 72 h, 0.421 ± 0.004) were significantly lower than those of A549 cells transfected with Lv-shCon (48 h, 0.537 ± 0.007 ; 72 h, 1.114 ± 0.006) or control cells (48 h, 0.519 ± 0.010 ; 72 h, 1.108 ± 0.002) (Fig. 3, $P < 0.001$), which suggests that the depletion of CUGBP1 can inhibit A549 cell proliferation.

Suppression of CUGBP1 blocks the cell cycle in A549 cells

It is known that cell proliferation is regulated by the cell cycle, which includes a series of phases. To explore the role of CUGBP1 in regulating the cell cycle, flow cytometry was employed to determine whether the depletion of CUGBP1 affects the cell cycle in A549 cells. As the results show, the population of Lv-shCUGBP1-transfected A549 cells in the G0/G1 phase increased significantly (73.79 ± 1.31 %,

$P < 0.05$), and the populations of cells in both the S (13.33 ± 0.56 %, $P < 0.05$) and G2/M (14.28 ± 1.48 %, $P < 0.05$) phases were decreased compared with those of the control cells (G0/G1, 60.45 ± 2.67 %; S, 19.31 ± 1.06 %; G2/M, 20.67 ± 3.12 %). However, there were no statistically significant differences between cells transfected with Lv-shCon (G0/G1, 62.62 ± 2.00 %, S, 17.83 ± 0.86 %, and G2/M, 20.29 ± 1.44 %) and control cells (Fig. 4). To further confirm that the knockdown of CUGBP1 impacts the cell cycle in A549 cells, two cell-cycle-related proteins, cyclin B1 and cyclin D1, were examined in cells transfected with Lv-shCUGBP1 or Lv-shCon and control cells. It was found that the depletion of CUGBP1 induced the downregulation of cyclin B1 and an upregulation of cyclin D1 (Fig. 5). All of the above results indicate that the suppression of CUGBP1 blocks the cell cycle.

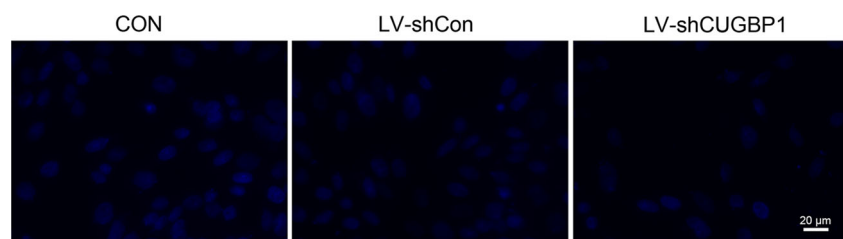
Depletion of CUGBP1 induces apoptosis in A549 cells

The clinicopathological function of CUGBP1 is crucial for detecting the mechanism of oncogenesis in NSCLC. In oral cancer cells, it has been found that CUGBP1 is related to mRNAs that encode proapoptotic factors and modulate the process of apoptosis [17]. To explore the effect of CUGBP1 on apoptosis, DAPI staining was performed in the A549 cell lines. The results showed that apoptosis was increased in cells transfected with Lv-shCUGBP1 compared with cells transfected with Lv-shCon or control cells (Fig. 6). Next, the expression levels of apoptosis-related proteins (such as BAD, BAX, and Jun D) were investigated by Western blot analysis. As shown in Fig. 7, the knockdown of CUGBP1 increased the level of expression of all three proteins, indicating that CUGBP1 is associated with apoptosis. We then utilized the annexin-V kit to explore the characteristics of apoptosis induced by CUGBP1 in A549 cells. It was found that, compared with control cells, there was significant variance in both early apoptosis (15.5 to 13.0 %, $P < 0.05$) and late apoptosis (17.7 to 10.9 %, $P < 0.05$) in A549 cells transfected with Lv-shCUGBP1 (Fig. 8).

Depletion of CUGBP1 inhibits the process of EMT in A549 cells

In epithelial to mesenchymal transition (EMT), when epithelial cells obtain the abilities of fibroblast-like cells,

Fig. 6 The analysis of cellular apoptosis after depletion of CUGBP1. DAPI staining indicating that silencing of CUGBP1 increased apoptosis in A549 cells



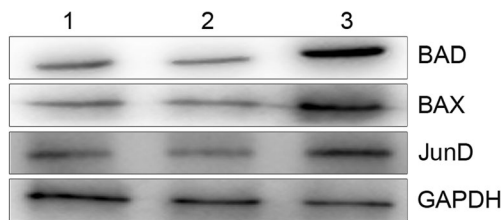


Fig. 7 The expression levels of apoptosis-related proteins in A549 cells with knockdown of CUGBP1. The detection of apoptosis-related proteins by Western blot analysis showing increased expression of BAD, BAX, and *Jun D* following CUGBP1 knockdown in A549 cells. 1, Control; 2, Lv-shCon; 3, Lv-shCUGBP1

intercellular adhesion between the cells reduces and motility increases, further promoting tumor progression [18]. Thus, we initially explored the correlation between EMT in cancer and CUGBP1. We know that E-cadherin loss, high vimentin and β -catenin expression are considered to be fundamental events in EMT. The expression of EMT-related proteins in A549 cells with or without CUGBP1 knockdown was analyzed by Western blot analysis. As the results show, the level of E-cadherin was upregulated in A549 cells with knockdown of CUGBP1 compared with the negative control groups; in contrast, the levels of β -catenin and vimentin were both downregulated (Fig. 9). These results indicate that CUGBP1 potentially contributes to the development of EMT in NSCLC.

Discussion

According to the statistics, approximately 1.2 million new cases of NSCLC are diagnosed each year, and the overall 5-year survival rate remains at approximately 10–15% [17]. An important reason for this poor prognosis is that the tumor has often spread beyond the primary site before diagnosis [4]. Therefore, it is extremely urgent to develop effective

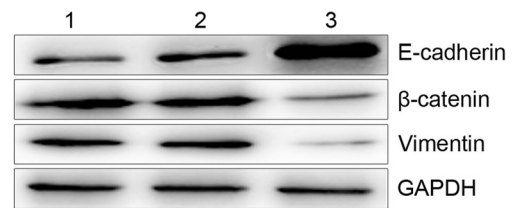


Fig. 9 The expression of EMT regulatory molecules in A549 cells after the depletion of CUGBP1. Results of Western blot analysis of EMT regulatory molecules showing increased expression of E-cadherin with decreased vimentin and β -catenin expression after CUGBP1 knockdown in A549 cells. 1, Control; 2, Lv-shCon; 3, Lv-shCUGBP1

measures, such as predictive factors, for the diagnosis and treatment of NSCLC [19]. CUGBP1, an RNA-binding protein, is defined as a posttranscriptional regulatory network with the function of regulating cell growth, motility, and apoptosis [20]. Recent studies have indicated that CUGBP1 plays a significant role in the regulation of apoptosis, proliferation, and cell cycle in tumor cells by binding to target transcripts upon T cell activation [10, 12, 13], and the high expression of CUGBP1 prevented apoptosis by inducing the expression of p21 under stress conditions in HeLa cells [21, 22]. Although one study recently indicated the overexpression of CUGBP1 in NSCLC, the role of CUGBP1 in the pathogenesis of NSCLC remained unclear. In the current study, we first examined the level of CUGBP1 expression and its association with clinical characteristics in 80 NSCLC patients. In our study, we demonstrated that the expression of CUGBP1 in the NSCLC tissues was significantly higher than that in the adjacent non-cancerous tissues, and this overexpression is associated with TNM stage and differentiation.

Next, we explored the ability of CUGBP1 to control cell proliferation and apoptosis, and the potential correlation with EMT. First, we assessed its effects on cell proliferation in A549 cells with knockdown of CUGBP1 by the CCK-8 assay. The results demonstrated that the depletion of CUGBP1 significantly decreased cell proliferation. Considering that cell

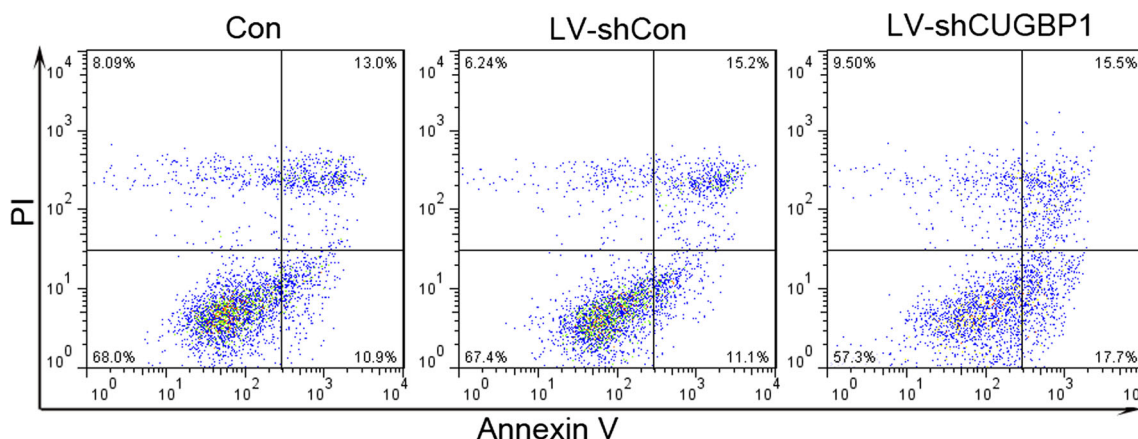


Fig. 8 The detection of apoptosis characteristics induced by knockdown of CUGBP1. Depletion of CUGBP1 induced both the early and late stage of apoptosis in A549 cells

growth and proliferation are impacted by cell cycle progression, flow cytometry was employed for cell cycle analysis. It was determined that the G0/G1 phase was upregulated while the S and G2/M phases were downregulated in the CUGBP1 shRNA group compared with the control group. Moreover, the knockdown of CUGBP1 induced a downregulation of cyclin B1 and an upregulation of cyclin D1. These results suggested that CUGBP1 may regulate the process of proliferation by controlling the cell cycle. We then investigated the effect of CUGBP1 on cell apoptosis using DAPI staining, which revealed an obvious increase in apoptosis following the knockdown of CUGBP1. The annexin-V kit was used to detect the apoptotic features of A549 cells by flow cytometry, and significant differences were observed in both early and late apoptosis in A549 cells transfected with CUGBP1 shRNA compared with the control groups. Moreover, the levels of apoptosis-related proteins, such as BAD, BAX, and *Jun D*, were examined by Western blot analysis, and the results showed that all three proteins were upregulated in cells with CUGBP1 depletion. All of the above data indicate that CUGBP1 plays an important role in mediating cellular apoptosis. Currently, tumor invasion and metastasis are associated with EMT, suggesting that epithelial cells can gain an enhanced ability to migrate after losing polarity [23]. Western blot analysis was used to assess the levels of E-cadherin, β -catenin, and vimentin expression, which can regulate EMT in tumor cells. It was found that the knockdown of CUGBP1 induced the up-regulation of E-cadherin and the down-regulation of β -catenin and vimentin. Thus, the invasive ability was decreased prominently in A549 cells transfected with CUGBP1 shRNA. These results indicate that CUGBP1 may contribute to the EMT process.

In conclusion, this study demonstrates the importance of CUGBP1 in biological and pathological functions of NSCLC and indicates CUGBP1 as a potential therapeutic target for NSCLC. However, the mechanism of CUGBP1 in the pathogenesis of NSCLC has not been elucidated, and further research will likely be continued in the future.

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Conflicts of interest None

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