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USP3 promotes proliferation of non-small cell lung cancer through regulating RBM4

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Abstract. – OBJECTIVE: Previous studies have shown that ubiquitin specific protease 3 (USP3) is an oncogene. However, the role of USP3 in non-small cell lung cancer (NSCLC) has not been reported. This study aims to explore the expression characteristics of USP3 in NSCLC, and its regulation on the proliferative capacity of NSCLC cells.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression levels of USP3 and RNA Binding Motif 4 (RBM4) in 42 pairs of tumor tissues and adjacent tissue specimens collected from NSCLC patients. Meanwhile, the correlation between the messenger ribonucleic acid (mRNA) expressions of USP3 and RBM4, and the clinical indicators and prognosis of NSCLC patients were analyzed. At the same time, mRNA expression of USP3 in NSCLC cell lines was further verified by the qRT-PCR method. In addition, USP3 knockdown and overexpression models were constructed using lentivirus in NSCLC cell lines H1299 and SPCA1. Cell counting kit-8 (CCK-8), cell colony formation, and 5-Ethynyl-2'-deoxyuridine (EDU) assays were performed to evaluate the influence of USP3 on proliferative capacity in NSCLC cells. Finally, Dual-Luciferase reporter assay and rescue experiments were conducted to further explore its underlying molecular mechanism.

RESULTS: In this experiment, qRT-PCR results revealed that the expression level of USP3 in tumor tissues of NSCLC patients was remarkably higher than that in adjacent tissues, and the difference was statistically significant. Compared with NSCLC patients with low expression of USP3, those with high USP3 expression suffered much more advanced pathology stage and lower overall survival rate. Proliferation ability of NSCLC cells overexpressing USP3 was remarkably enhanced, while the opposite result was observed in the USP3 knockdown group. Subsequently, RBM4 expression in NSCLC tissue samples was found to be significantly reduced and negatively correlated with USP3 level. In addition, the result of Dual-Luciferase reporter assay demonstrated that USP3 can be targeted by RBM4. Rescue experiments revealed that RBM4 was responsible for NSCLC progression regulated by USP3.

CONCLUSIONS: The above studies indicated that USP3 expression was remarkably up-regulated in NSCLC tissues, which was closely related to the pathological staging and poor prognosis of NSCLC patients. Therefore, USP3 might accelerate the proliferation of NSCLC cells *via* regulating RBM4.

Key Words:
USP3, RBM4, Lung cancer, Proliferation.

Introduction

Lung cancer has become the most common malignant tumor in adults, with the highest incidence and mortality worldwide in recent years[1-3]. In China, the incidence of lung cancer ranks first and the onset age tends to be younger year by year, which seriously threatens people's life and health, and also causes a heavy social and economic burden[4,5]. With the improvement of examination methods and screening procedures, the detective rate of lung cancer in the early stage increases year by year. Surgical resection is preferred for treating lung cancer in early stage. However, postoperative recurrence and uncontrolled proliferation of lung cancer still cannot be ruled out[6-8]. Therefore, explorations on molecular mechanism of NSCLC is of great significance for developing therapeutic tumor targets[9,10].

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Ubiquitin enzyme (deubiquitinase, DUB) is a member of the protease family, which is able to target protein ubiquitin[11]. Ubiquitin C terminal hydrolase (UCHs), ubiquitin specific protease (USPs), ovarian cancer protease (OTUs), JAMM modular protease (JAMMs), machado-joseph disease (MJD), and nearly 100 DUBs have been detected. Its target proteins are widely involved in cell cycle, DNA repair, chromatin remodeling, and many cancer-related signaling pathways[12,13]. Ubiquitin specific protease 3 (USP3), a member of the USP family, is located on human chromosome 15q22.3 and consists of 520 amino acids, including one highly conserved cysteine residue and two highly conserved histidine residues participating in cell processes by mediating the deubiquitination of target proteins[14,15]. In addition, USP3 can inhibit the type I interferon pathway in the antiviral immune response by deubiquitinating the rig-i receptor[16]. Given that many DUBs play important roles in cancer-associated signaling pathways, we speculate that USP3 has great research prospects and may become a new target for cancer therapy[17,18].

RNA Binding Motif 4 (RBM4), an RNA Binding Motif, is lowly expressed in tumors and affects gene splicing by targeting specific cis-acting elements[19,20]. It is reported that RBM4 can affect the nuclear export of RNA and transmembrane. In terms of physiological function, RBM4 is able to affects the differentiation of muscle cells and pancreatic cells[19,20]. However, biological functions of RBM4 in tumors are rarely reported. Therefore, in this study, we analyzed the expressions of USP3 and RBM4 in 42 pairs of NSCLC tissues and adjacent tissues, and explored the effects of USP3 and RBM4 on the biological functions of NSCLC cells. Previous studies have indicated that USP3 and RBM4 can play a role in the growth and proliferation of NSCLC cells, thus affecting the development of tumors. This study was designed to investigate the role of USP3 in the regulation of clinical parameters and prognosis in NSCLC through targeted regulation of RBM4.

Patients and Methods

Patients and NSCLC Samples

Tumor tissue specimens and paracancerous ones were collected from 42 NSCLC patients. All specimens were obtained from surgical excision, biopsy or bronchoscopy biopsy specimens from the Department of Oncology, Thoracic Surgery, and Respiratory Medicine. In addition, paracancerous tissues were harvested more than 5 cm away from cancerous tissues. None of the enrolled NSCLC patients received anti-tumor treatment such as radiotherapy or chemotherapy before surgery. The investigation was approved by the Ethics Committee of the hospital, and all patients had signed informed consent. All patients were followed up after discharge, including telephone and outpatient follow-up. The follow-up information included general conditions, clinical symptoms, and imaging examination.

Cell Lines and Reagents

Five human NSCLC cells (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell (BEAS-2B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in DMEM containing 10% FBS in a 37°C incubator with 5% CO_2.

Transfection

The control group (NC or Anti-NC) and the lentivirus (USP3 or Anti-USP3) containing the USP3 overexpression or knockdown sequences were purchased from Shanghai GenePharma Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%. Then, transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 6 h, 24 h, 48 h, and 72 h, respectively, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After cells were grown to the density over 90%, a horizontal line perpendicular to the ground was scratched with 100 μL tip, with the back line of

the plate as the reference line. After the scratches were completed, the prepared phosphate-buffered saline (PBS) solution was used for washing. Necrotic cells were removed, the medium containing 10% FBS was applied, and the cells were again placed in the original incubator for cell culture. At 6 h and 24 h, cells were captured under a microscope for observation.

5-Ethynyl-2′-Deoxyuridine (EdU) Proliferation Assay

EdU proliferation test (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, cells were incubated with 50 μM EdU for 2 h, and stained with AdoLo and 4', 6-diamidino-2-phenylindole (DAPI). The number of EdU-positive cells was detected by fluorescence microscopy. EdU-positive ratio was calculated based on the ratio of EdU-positive cell number to the total DAPI-labeled cells (blue cells).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissue samples using the TRIzol (Invitrogen, Grand Island, NY, USA) method. The reverse transcription reaction was described with reference to the AMV reverse transcription kit, and 2 μg of total RNA was added to the 20 μL system for complementary deoxyribonucleic acids (cDNAs) synthesis. Real-time PCR was subsequently performed using 2×SYBR Green PCR Master Mix. Appropriate amount of cDNA was taken as a template, with 0.4 mol/L primer, 15 μL system for amplification, and β-actin as an internal reference. The PCR reaction was carried out on a quantitative PCR reactor. The data obtained after three independent experiments were analyzed using the formula $RQ=2^{-\Delta\Delta Ct}$. Data analysis was performed using ABI Step One software and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers were as follows: USP3: Forward: 5'-CAAGCTGGGACTGGTACAGAA-3'; Reverse-5'-GCAGTGGTGCTTCCATTTACTT-3'; RBM4: Forward: 5'-GAAGCTGTTCATCGGAAACCT-3'; Reverse: 5'-GGTGGGACTGATGTTGCCC-3'; GAPDH: Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Western Blot

The transfected cells were lysed using PROPREP™ lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 g for 15 min at 4°C. Total protein concentration was calculated by the HCCA Protein Assay Kit (Pierce, Rockford, IL, USA). Rabbit anti-human monoclonal antibodies against USP3 and RBM4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from Genway Biotech (San Diego, CA, USA). The internal reference control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Protein samples were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA), and blocked with 5% skim milk powder for 1 h at room temperature. Primary antibodies were added for incubation overnight at 4°C shaker. On the next day, the membrane was rinsed 3 times with Tris-Buffered Saline and Tween-20 (TBST) and incubated with the second antibody for 1 h at room temperature. After that, the protein samples on the membrane were finally developed and analyzed with enhanced chemiluminescence (ECL) kit.

Dual-Luciferase Reporter Assay

A reporter plasmid was constructed by inserting target sequences. Cells were co-transfected with luciferase plasmids and transfection vectors. By measuring the intensity of the fluorescence, the relative luciferase activity was calculated.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA) statistical software. Differential expressions of USP3 in NSCLC tissues and adjacent ones were analyzed by analysis of variance (ANOVA) followed by Post Hoc Test (Least Significant Difference). USP3 level in above tissue specimens and its relationship with clinical pathological parameters were analyzed by Chi-Square test. The association between USP3 expression and survival rate and prognosis of NSCLC patients was analyzed using the Kaplan-Meier method. Cox regression model was applied for assessing potential factors that may affect the prognosis of NSCLC. Data were shown as mean ± standard deviation, and $p<0.05$ was considered statistically significant,

Results

USP3 Was Highly Expressed in NSCLC Tissues and Cell Lines

QRT-PCR was performed to examine the expression of USP3 in 42 pairs of NSCLC tumor tissues and adjacent tissue specimens, as well as in NSCLC cell lines. The results indicated that the expression level of USP3 was remarkably upregulated in NSCLC tissues compared with paracancerous ones, and the difference was statistically significant (Figure 1A). Similarly, USP3 was also found remarkably highly expressed in NSCLC cell lines (Figure 1B), among which, H1299 cells showed the lowest expression, while SPCA1 cells showed the highest. Hence, these two cell lines were selected for subsequent cell experiments.

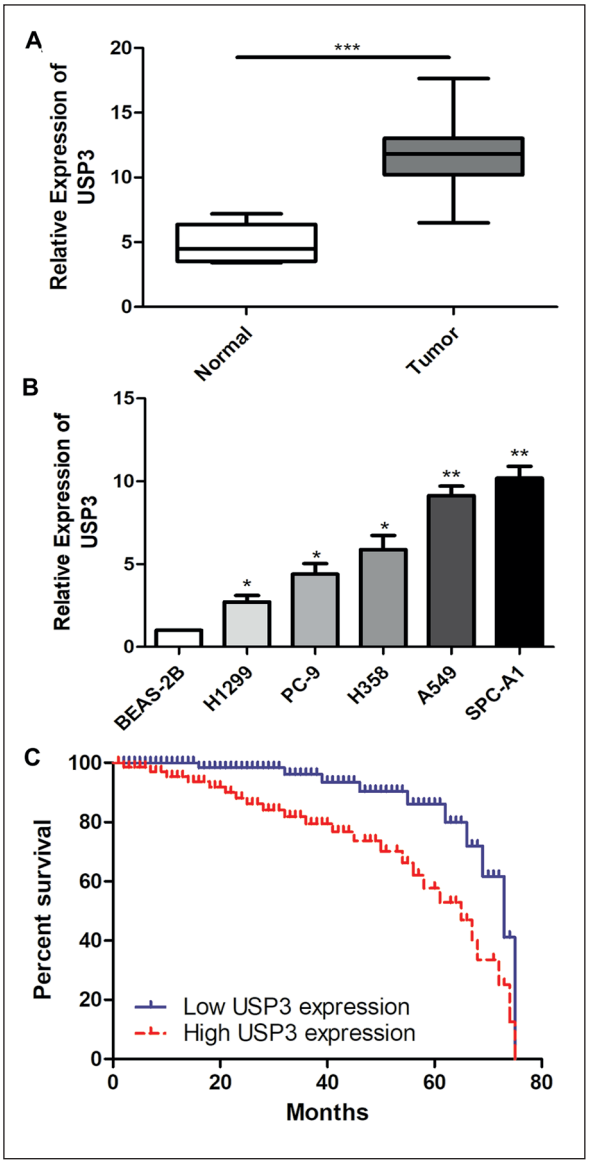

Figure 1. USP3 is highly expressed in NSCLC tissues and cell lines. **A,** QRT-PCR was used to detect the difference in expression of USP3 in NSCLC tumor tissues and adjacent tissues; **B,** QRT-PCR was used to detect the expression level of USP3 in NSCLC cell lines; **C,** Kaplan-Meier survival curve of NSCLC patients based on USP3 expression. The prognosis of patients with high expression of USP3 was significantly worse than that of low expression group. Data are mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

USP3 Expression Was Correlated with Pathological Staging and Overall Survival in NSCLC Patients

According to the detected mRNA level of USP3, the 42 pairs of NSCLC tissue and paracancerous tissue samples were divided into high and low expression groups. In addition, Chi-square test was performed to figure out the correlation between USP3 expression and age, gender, pathological stage, lymph node or distant metastasis of NSCLC patients. As shown in Table I, high expression of USP3 was unrelated with age, gender, lymph node or distant metastasis in NSCLC patients. USP3 level was significantly correlated to pathological stage. Subsequently, the Kaplan-Meier survival curves revealed that high USP3 expression was remarkably associated with poor prognosis of NSCLC patients, suggesting that USP3 might be a new biological indicator for predicting the prognosis of NSCLC ($p<0.05$; Figure 1C).

USP3 Promotes Cell Proliferation in NSCLC Cell Lines

To explore the effect of USP3 on proliferation ability of NSCLC cells, USP3 overexpression and knockdown models were first successfully constructed (Figure 2A). Subsequently, the results of CCK-8, colony formation, and EdU assay demonstrated that the proliferation of NSCLC cells overexpressing USP3 remarkably increased compared with control group, while the opposite results were obtained in USP3 knockdown group (Figure 2B-2D).

RBM4 is a Direct Target of USP3

It was found that overexpression of USP3 remarkably decreased protein and mRNA expressions of RBM4, while USP3 knockdown significantly upregulated it (Figure 3A). In addition, after the RBM4 overexpression vector was transfected into H1299 cell line, the protein and mR-

Table I. Association of USP3 and RBM4 expression with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	No. of cases	USP3 expression		*p*-value	RBM4 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.204			0.474
< 60	16	10	6		5	11	
≥ 60	26	11	15		11	15	
Gender				0.355			0.525
Male	21	12	9		7	14	
Female	21	9	12		9	12	
T stage				0.011			0.011
T1-T2	26	17	9		6	20	
T3-T4	16	4	12		10	6	
Lymph node metastasis				0.095			0.974
No	29	17	12		11	18	
Yes	13	4	9		5	8	
Distance metastasis				0.057			0.057
No	26	16	10		7	19	
Yes	16	5	11		9	7	

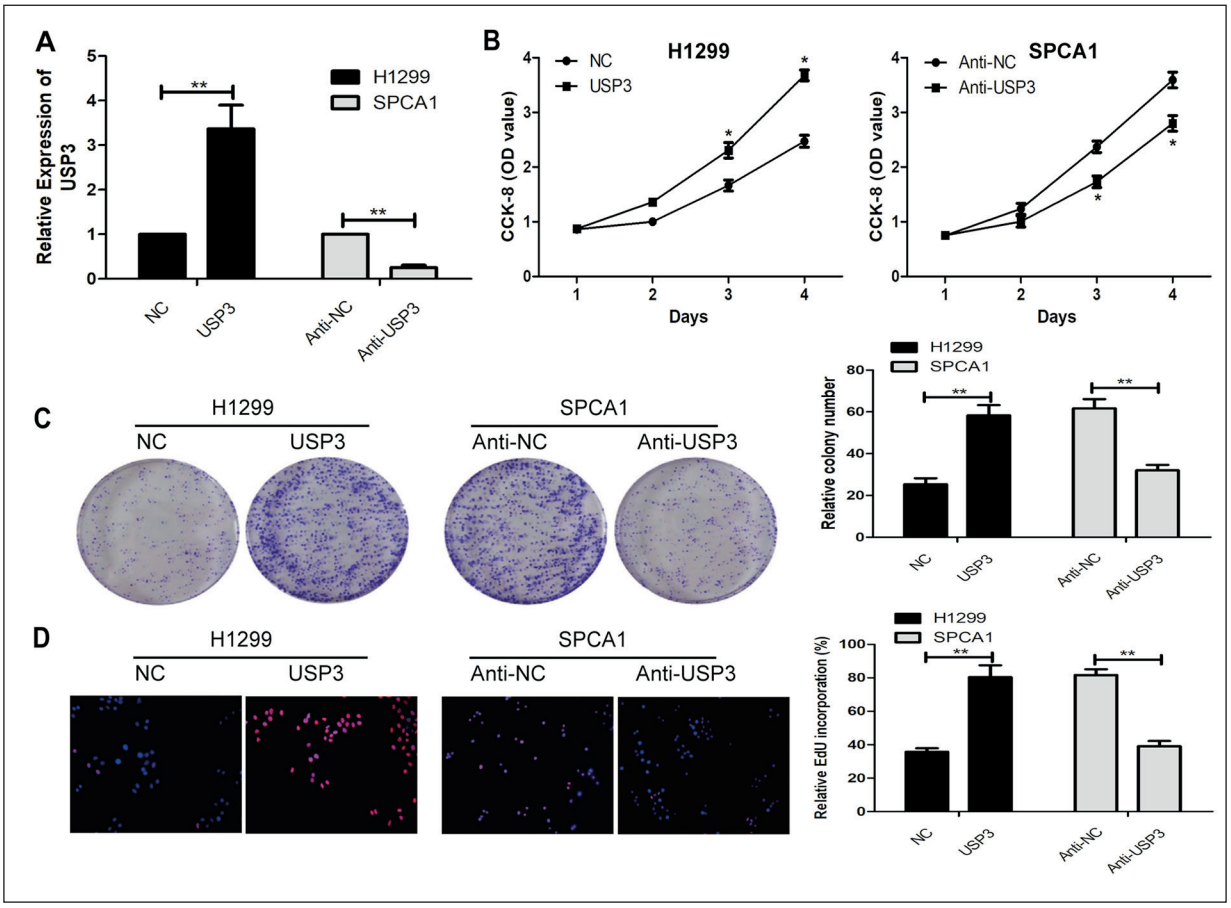

Figure 2. USP3 can promote the proliferation of NSCLC cells. **A,** QRT-PCR verified the transfection efficiency of USP3 overexpression vector in H1299 cell line and USP3 knockout vector in SPCA1 cell line; **B,** CCK-8 assay was used to detect viability in H1299 and SPCA1 cell lines; **C,** Colony formation assay was performed to detect relative colony number in H1299 and SPCA1 cell lines (magnification: 10×); **D,** EdU assay was used to detect EdU-positive ratio in H1299 and SPCA1 cell lines (magnification: 20×). Data are expressed as mean ± SD, *$p < 0.05$, **$p < 0.01$.

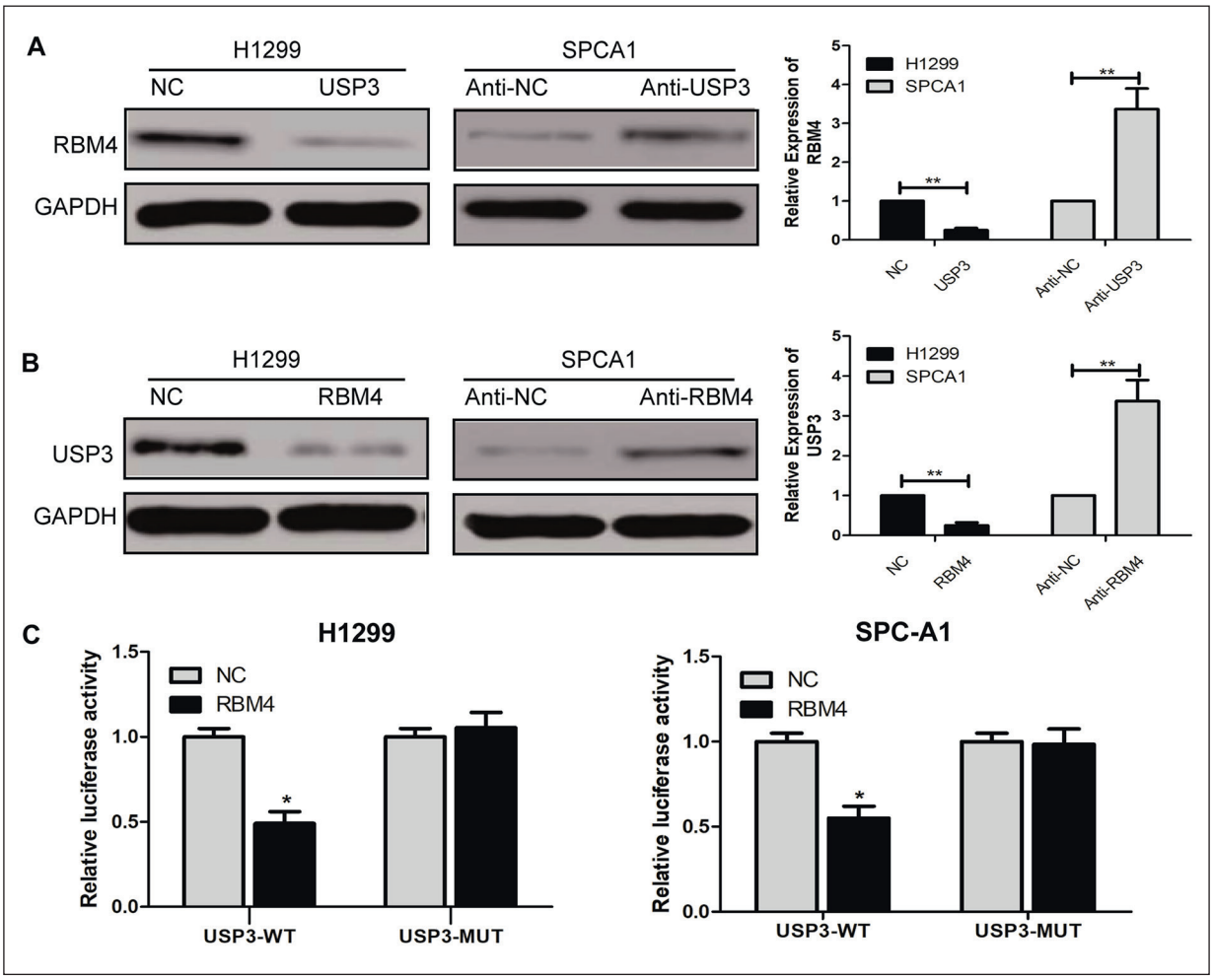

Figure 3. USP3 can direct target RBM4. **A,** Western Blotting and qRT-PCR verified the changes in RBM4 expression after transfection of USP3 overexpression vector in the H1299 cell line and the USP3 knockout vector in the SPCA1 cell line; **B,** Western Blotting and qRT-PCR verified the changes in USP3 expression after transfection of RBM4 overexpression vector in the H1299 cell line and the RBM4 knockout vector in the SPCA1 cell line; **C,** Results of the dual luciferase reporter gene assay indicated that overexpression of RBM4 significantly attenuated the luciferase activity of the wild type USP3 vector without attenuating the luciferase activity of the mutant vector, validating the direct targeting of USP3 and RBM4. Data are expressed as mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

NA expressions of USP3 remarkably decreased. Conversely, knockdown of RBM4 knockdown in SPCA1 cells upregulated protein and mRNA expressions of USP3 (Figure 3B). To further validate the targeting relationship between RBM4 and USP3, Dual-Luciferase reporter assay was performed. The data revealed that overexpression of RBM4 remarkably attenuated the luciferase activity of the wild-type USP3 vector, while the luciferase activity in mutant-type vector was unchangeable, further demonstrating that USP3 can be targeted by RBM4 through their binding sites (Figure 3C).

RBM4 Modulated USP3 Expression in Human Lung Cells

To further explore the interaction between USP3 and RBM4 in NSCLC cells, RBM4 was up-regulated in NSCLC cells overexpressing USP3, and RBM4 was down-regulated in NSCLC cells with silenced USP3 (Figure 4A). Subsequently, the effects of the above treatments on the function of NSCLC cells were further explored by CCK-8 and colony formation assays. The results demonstrated that USP3 could counteract the effect of RBM4 on the proliferation of NSCLC cells (Figure 4B and 4C).

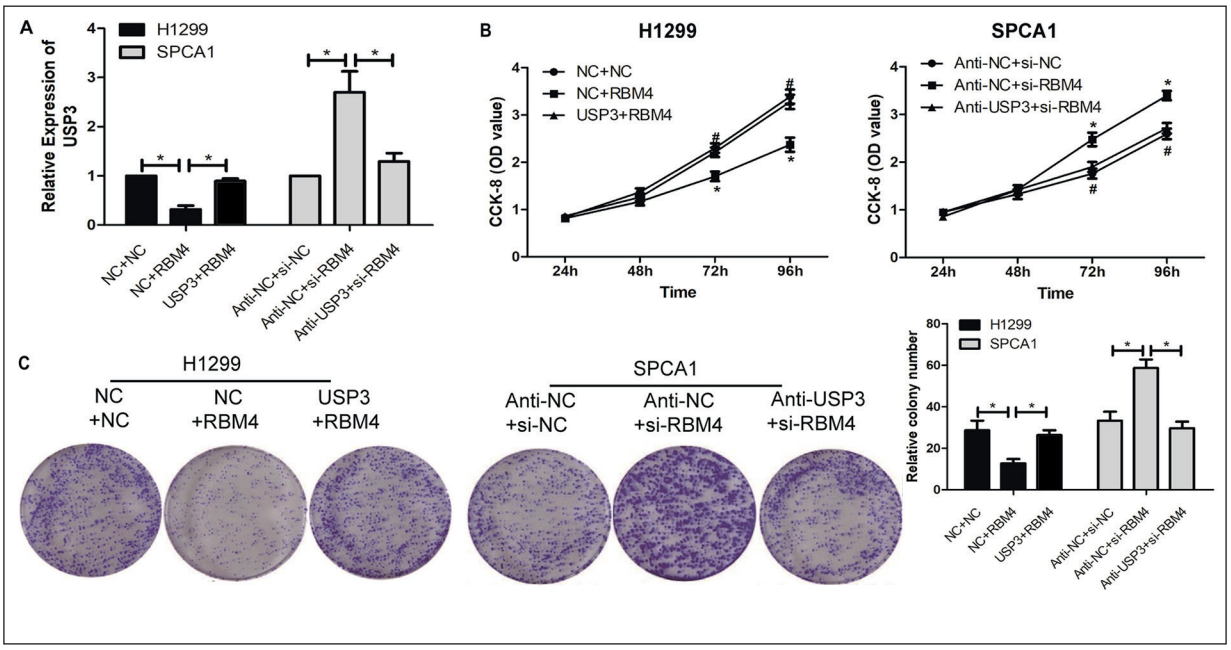

Figure 4. USP3 regulates the expression of RBM4 in NSCLC tissues and cell lines. **A,** USP3 expression levels in USP3 and RBM4 co-transfected NSCLC cell lines were detected by qRT-PCR; **B,** CCK-8 assay was used to viability in co-transfected cells; **C,** Colony formation assay was performed to detect the number of colonies in co-transfected cells (magnification: 10×). Data are expressed as mean ± SD, $*^{\#}p < 0.05$.

Discussion

Among many malignant tumors, the incidence and mortality of NSCLC have been consistently high. In time diagnosis of lung cancer can improve the cure rate and survival rate, which is of great significance to improve the prognosis of lung cancer patients[1-3]. Fiberoptic bronchoscopy is the main diagnostic method for lung cancer, while brush examination, needle aspiration, and irrigation are important supplements for biopsy. The advantage is that the identification of benign and malignant cells is mainly based on morphological characteristics without relying on immunocytochemistry. Cytological diagnosis is also faced with severe test of clinical surgical treatment: for some severe surface ulcerations and hilar lymph node lesions, it is particularly important to analyze the morphological characteristics of brush-detected cells[4-6]. Therefore, finding differentially expressed genes in lung cancer and analyzing their correlation with clinical prognosis will help to improve the diagnosis and treatment of NSCLC, and improve the clinical prognosis of patients[7-10].

Our findings uncovered that USP3 exerted a carcinogenic role, while RBM4 was a tumor-suppressor gene in NSCLC. To further explore the effects of USP3 and RBM4 on the biological functions of NSCLC, USP3 overexpression/knockdown models were constructed. Functional experiments illustrated the promotive effect of USP3 on the proliferation of NSCLC cells.

Ubiquitination of proteins plays a very important role in regulating many intracellular biological functions, such as DNA damage repair, cell cycle progression, signal transduction, etc.[11,12]. The ubiquitination modification can be divided into two types, namely, monoubiquitination and polyubiquitination[12,13]. As a member of the ubiquitin specific protease (USP) family, USP3 can interact with its target protein to remove ubiquitin molecules binding target proteins, thereby inhibiting degradation of target proteins or mediating downstream biological functions of target proteins[14-16].

RBM4 is a key molecule of RNA splicing factor. Our findings showed that RBM4 was lowly expressed in NSCLC tissues[19-22]. In this study, the binding relation between USP3 and RBM4 was first predicted by bioinformatics method and further verified by Dual-Luciferase reporter assay. Notably, rescue experiments illustrated the involvement of RBM4 in USP3-regulated phenotype changes of lung cancer cells. Therefore, a regulatory loop USP3/RBM4 has been identified that was responsible for the deterioration of NSCLC.

Conclusions

In summary, USP3 is upregulated in NSCLC, which is significantly associated with the pathological stage and poor prognosis of NSCLC patients. USP3 might promote the proliferation of NSCLC cells *via* targeting RBM4.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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