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A gene mutation in RNA-binding protein 10 is associated with lung adenocarcinoma progression and poor prognosis

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Abstract. RBM10 regulates the expression of various genes, which are often mutated in male lung adenocarcinoma. The present study confirmed the association of the *RBM10* mutation at exon 10 with the clinicopathological data and prognosis of lung adenocarcinoma. The effect of mutant *RBM10* on regulating lung cancer cell growth and invasion was investigated *in vitro*. Tissue specimens from 50 patients with lung adenocarcinoma were subjected to Sanger sequencing for *RBM10* exon 10 mutations. Lung adenocarcinoma cells were transfected with pcDNA3.1 carrying wild type RBM10 cDNA or exon mutation cDNA for cell viability, apoptosis and invasion assays. *RBM10* exon 10 mutations were identified in 11 out of 50 patients, with a high frequency in male patients [c.763 C>T, p.Arg241Cys for 33.3% (10/30)] and were significantly associated with the American Joint Committee on Cancer stage (P=0.005), lymph node metastasis (P=0.019) and shorter 5-year survival rate compared with the wild type *RBM10* (36.4% vs. 46.5%; P=0.019). Multivariate analysis revealed that *RBM10* exon 10 mutation was an independent prognostic factor (HR=3.787; P=0.033). *RBM10* exon 10 mutation at c.763 C>T significantly promoted tumor cell proliferation and invasion capacity, whereas wild type RBM10 inhibited tumor cell invasion *in vitro*. In conclusion, *RBM 10* mutation at exon 10 (c.763 C>T) occurs frequently and is an independent prognostic predictor in lung adenocarcinoma.

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Introduction

Lung cancer accounts for 23% of all cancer-related deaths in the world in 2012 in spite of decades-long advancement in early detection, prevention, and drug development (1,2). Molecularly, lung cancer development is caused by either loss of tumor suppressor genes and/or activation of oncogenes (1,3). During lung cancer progression, tumor cells gain the ability to migrate and invade into the surrounding tissues and distant organs, like most of other human cancers (4). To date, most of lung cancer are diagnosed at advanced stages, which limits the surgical option and most of such patients are also resistant to routine chemoradiotherapy (4). Thus, identification of novel molecular targets and/or biomarkers for early detection or to predict prognosis of lung cancer could reduce disease burdens in patients.

RNA-binding protein 10 (RBM10) contains an RNA recognition motif and has been identified as a component of spliceosome complex and functions to regulate pre-mRNA splicing in the alternatively splicing pathway (5) and mRNA stabilization (6). RBM10 alteration is more frequently associated with alternative exon skipping (7), while expression of more than 90% of human genes, including tumorigenesis-related genes, is regulated by the alternative gene splicing (8). Moreover, alteration of mRNA alternative splicing, especially exon skipping (7) or exon-inclusion of the affected proteins (7,9), results in abnormal protein expression and functions in cells. RBM10 knockdown provoked alterations in 10-20% of the pre-mRNA splicing events (10), while other studies showed that RBM10 is a tumor suppressor gene (11,12) Thus, RBM10 may play an important role in suppression of human tumorigenesis. Previous studies showed that *RBM10* mutations did occur in lung and pancreatic cancers (13,14) and the Cancer Genome Atlas (TCGA) data showed that *RBM10* mutations occurred in 8% of lung adenocarcinoma and is more common in male patients. *RBM10* mutations were also reported to be more prevalent (76%) in the transversion-high group (C>T) of lung adenocarcinoma in a male cohort (14) and in 12 out of 183 of lung adenocarcinoma cases (15). Lung adenocarcinoma makes up to approximately 40% of all lung cancer cases (1). Thus, further investigation of RBM10 and its functions in lung

adenocarcinoma could provide a better understanding of the role of *RBM10* mutation in lung adenocarcinoma cell growth and invasion.

In the present study, we first assessed the clinical significance of *RBM10* exon 10 mutations in lung adenocarcinoma for association with clinicopathological features, such as tobacco smoking, age, gender, tumor histological grade, AJCC stage, lymph node metastasis, and survival. We then investigated the effect of *RBM10* exon 10 mutations on regulating lung adenocarcinoma cell proliferation, invasion, and apoptosis as well as the underlying molecular events. Our study expected to provide novel insightful information for RBM10 as a novel biomarker or therapeutic target for lung adenocarcinoma.

Materials and methods

Patients and follow-up. In the present study, we retrospectively collected paraffin-embedded tissue samples from 50 lung adenocarcinoma patients who received surgical tumor resection from the Fourth Hospital of Jinan (Shandong, China) and Shanxian Central Hospital (Shandong, China) between April 2011 and January 2015. All patients were histologically diagnosed with lung adenocarcinoma and the AJCC stage was determined according to the Seventh Edition of the AJCC Staging System for Lung Cancer (16). Patients were followed up every three months during the first year after surgery and then six months thereafter and the most recent follow-up was October 30, 2017. The median follow-up period was 44.5 months (ranged between 18.0 and 60.0 months). A laboratory protocol of this study was approved by the Fourth Hospital of Jinan and Shanxian Central Hospital Review Board according to the Declaration of Helsinki. Written informed consent was obtained from all patients to allow utilizing their tissue specimens in the present study.

Genomic DNA extraction. Paraffin-embedded tissue blocks from 50 lung adenocarcinoma and paired adjacent non-cancerous lung tissues were retrieved from the Department of Pathology of both hospitals and sectioned into 4 μm-thick tissue sections for H&E staining and confirmation of the diagnosis. After that, 10 μm tissue sections were prepared and subjected to DNA extraction using the TIANquick FFPE DNA Kit and TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Analysis of RBM10 mutations. The *RBM10* exon 10 was amplified using polymerase chain reaction (PCR) with Hot Start Taq MasterMix (Tiangen Biotech Co., Ltd.) and the specific primers (5'-GGGGTGTCCTCTAACATTGG-3' and 5'-ATGGTCTTG CCGTCGATAGT-3'). The size of the expected amplicon is 486 bp. In brief, genomic DNA of 50-100 ng was amplified in a 50 μl reaction mixture containing 25 μl of Hot Start Taq MasterMix and 5 μl of 10 μM primer mix. The PCR conditions were set to 95˚C for 2 min and followed by 40 cycles of 94˚C for 20 sec, 54˚C for 20 sec, and 72˚C for 20 sec with a final extension at 72˚C for 3 min. PCR products of 6 μl of each were analyzed in 1.5% agarose gel with 50 bp ladder DNA markers. The resulting PCR products were purified and sequenced in both directions using Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

Cell lines and culture. Human lung adenocarcinoma A549 and H1299 cell lines were obtained from Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 Medium (GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37˚C.

Plasmid construction and cell transfection. The wild-type full-length *RBM10* cDNA was amplified using PCR according to the coding sequence of human RBM10 from GenBank, whereas *RBM10* cDNA carrying exon 10 mutation at c.763 C>T was amplified using PCR with specific primer sets. These cDNA fragments were then cloned into pcDNA3.1 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.). After amplification and DNA sequencing confirmation, these plasmids were named pcDNA3.1-RBM10w and pcDNA3.1-RBM10m, respectively and used for cell transfection.

For cell transfection, A549 and H1299 cells were seeded into 6-well plates at a density of $1x10^6$ cells/well and cultured in RPMI1640 with 10% FBS overnight to reach approximately 70% confluency and then transfected with Lipofectamine2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. We prepared three groups of transfections, i.e., the negative control (transfected with pcDNA3.1 only), wild type (transfected with pcDNA3.1-RBM10w) and the mutation group (transfected with pcDNA3.1-RBM10m). After that, cell growth medium was refreshed with complete growth medium 6 h after transfection and the cells were further incubated for 48 h.

Quantitative reverse transcriptase-polymerase chain reaction. TRIzol (Thermo Fisher Scientific, Inc.) was used to isolate RNA following the manufacturer's protocol. For reverse transcription, the components in a total volume of 10 μl were used as follows: 3 μg total RNA; 10 mM deoxyribonucleotide triphosphate; 0.5 μg oligo deoxythymine; 20 U RNasin®; 200 U Maloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.). The primer sequences were as follows: RBM10 sense, 5'-GCACGACTA TAGGCATGACAT-3'; antisense, 5'-AGTCAAACTTGTCTG CTCCA-3'; GAPDH sense, 5'-GAAGGTGAAGGTCGGAGT C-3'; antisense, 5'-GAAGATGGTGATGGGATTTC-3'. PCR was performed with 25-30 cycles as follows: 95˚C for 30 sec; 55˚C for 30 sec; 72˚C for 1 min. Densitometry analysis was performed with ImageMaster VDS-CL Image Master 1.0.3.7 software (GE Healthcare, Chicago, IL, USA).

Cell viability CCK-8 assay. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). In brief, gene transfected A549 and H1299 cells were detached from cell culture dishes using trypsin and re-seeded into 96-well plates at a density of $1x10^4$ per well and cultured for 24 h, 48 h, and 72 h, respectively. At the end of each experiment, 10 μl of CCK-8 reagent was added into each well and cells were further incubated for 90 min. The absorbance values were then measured at 450 nm using a plate reader (Eppendorf, Hamburg, Germany) and the inhibitory rate was then calculated.

Flow cytometric apoptosis assay. The flow cytometer assay was performed to assess the changed cell apoptosis after gene transfection. Specifically, A549 and H1299 growing in the exponential phase were transfected with pcDNA3.1 plasmids carrying wild type RBM10 cDNA or RBM10 exon 10 mutation at c.763 C>T, or vector-only for 24 h. The cells were then detached using trypsin and re-seeded into 6-well plates at a density of $1x10^5$ per well and cultured in a serum-free medium for 24 h. After that, cells were harvested in ice-cold phosphate buffered saline (PBS) and apoptotic cells were detected using the FITC Annexin-FITC/PI Apoptosis Detection kit (cat. no: 4A; Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The stained cells were then measured with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The experiments were in triplicate and repeated at least three times.

Transwell tumor cell invasion assay. Tumor cell invasion ability was assessed by using the Transwell chamber assay (EMD Millipore, Billerica, MA, USA). Briefly, A549 and H1299 cells were transfected with pcDNA3.1-RBM10 wild type or mutated cDNA or vector-only. After 24 h of incubation, the cells were suspended in serum-free RPMI 1640 and plated on the upper chamber of the polycarbonate Transwell filters that were pre-coated with Matrigel (BD Biosciences), while the lower chamber was filled with RPMI 1640 supplemented with 10% FBS. After 24 h incubation, the non-invaded cells in the upper chamber were removed using cotton swabs, whereas the cells invaded into the low surface of the filters were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells were photographed in five random fields under a Nikon TS100-F inverted microscope (Nikon, Tokyo, Japan) and then counted.

Western blot analysis. A549 and H1299 cells were transfected with pcDNA3.1-RBM10 wild type or mutated cDNA or vector-only for 48 h. Total cellular protein was then extracted using the radioimmunoprecipitation assay lysis buffer and protein concentrations were measured using bicinchoninic acid (BCA) kit (CWBiotech, Beijing, China). Equal amounts of protein samples (20 μg each) were heated at 100˚C for 10 min and then separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidenedifluoride (PVDF) membrane (EMD Millipore). The membranes were incubated in 5% bovine serum albumin (BSA) for 2 h at the room temperature and then incubated with primary antibodies at a dilution of 1:1,000 against Numb (1:1,000; cat. no: 18701-1-AP), Notch-1 (1:1,000; cat. no: 10062-2-AP), Fas (1:1,000; cat. no: 13098-1-AP), E-cadherin (1:1,000; cat. no: 20874-1-AP), and CyclinD1 (1:1,000; cat. no: 60186-1-Ig; all from ProteinTech Group, Inc., Chicago, IL, USA) at 4˚C overnight. On the next day, the membranes were washed with Tris-based saline-Tween 20 solution (TBST) three times and then incubated with a secondary antibody at a dilution of 1:1,000 (1:1,000; cat. no: AP124P; EMD Millipore) for 1 h at the room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL)-Plus western blotting detection reagents (EMD Millipore).

Statistical analysis. Association of *RBM10* mutations with clinicopathological characteristics was assessed using the Chi-square test. Association of *RBM10* mutations with five-year survival rate was assessed using Kaplan-Meier method and the log-rank test and the multivariate analysis was performed using Cox regression test. Moreover, all *in vitro* experiments were in triplicates and repeated at least three times and the data were expressed as a mean ± standard deviation. Student's t-test was used to analyze the mean difference between two groups, while multi group comparisons of the means were analyzed by using one-way analysis of variance (ANOVA) test followed by the post-hoc Student-Newman-Keuls test. A $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using the Statistical Package of Social Sciences (v19.0; SPSS, Inc., Chicago, IL, USA).

Table I. Association of RBM10 mutations with clinicopathological characteristics of lung adenocarcinoma patients.

		RBM10 exon10 status		
Variables	No.	Mutant	Wild-type	P-value
Age (years)				0.728
≤60	27	5	22	
>60	23	5	18	
Gender				0.033
Female	20	1	19	
Male	30	10	20	
Smoking status				0.780
No-smoking	30	7	23	
Smoking	20	4	16	
Differentiation				
Poor-differentiated	13	5	8	0.195
Moderate	25	5	20	
Well-differentiated	12	1	11	
Tumor size				0.793
T1	23	5	18	
T2	21	4	17	
T3	6	2	4	
Lymphatic metastasis				0.012
N0	30	3	27	
Yes	20	8	12	
AJCC stage				0.005
I	23	2	21	
II	11	1	10	
III	16	8	8	

Results

Patients' characteristics. In this study, we analyzed 50 lung adenocarcinoma patients, including 30 males and 20 females with a median age of 59.0 years old (ranging from 42 to 77 years at the time of diagnosis). Among these patients, there were 20 smokers and 30 non-smokers. Tumor stage T1,

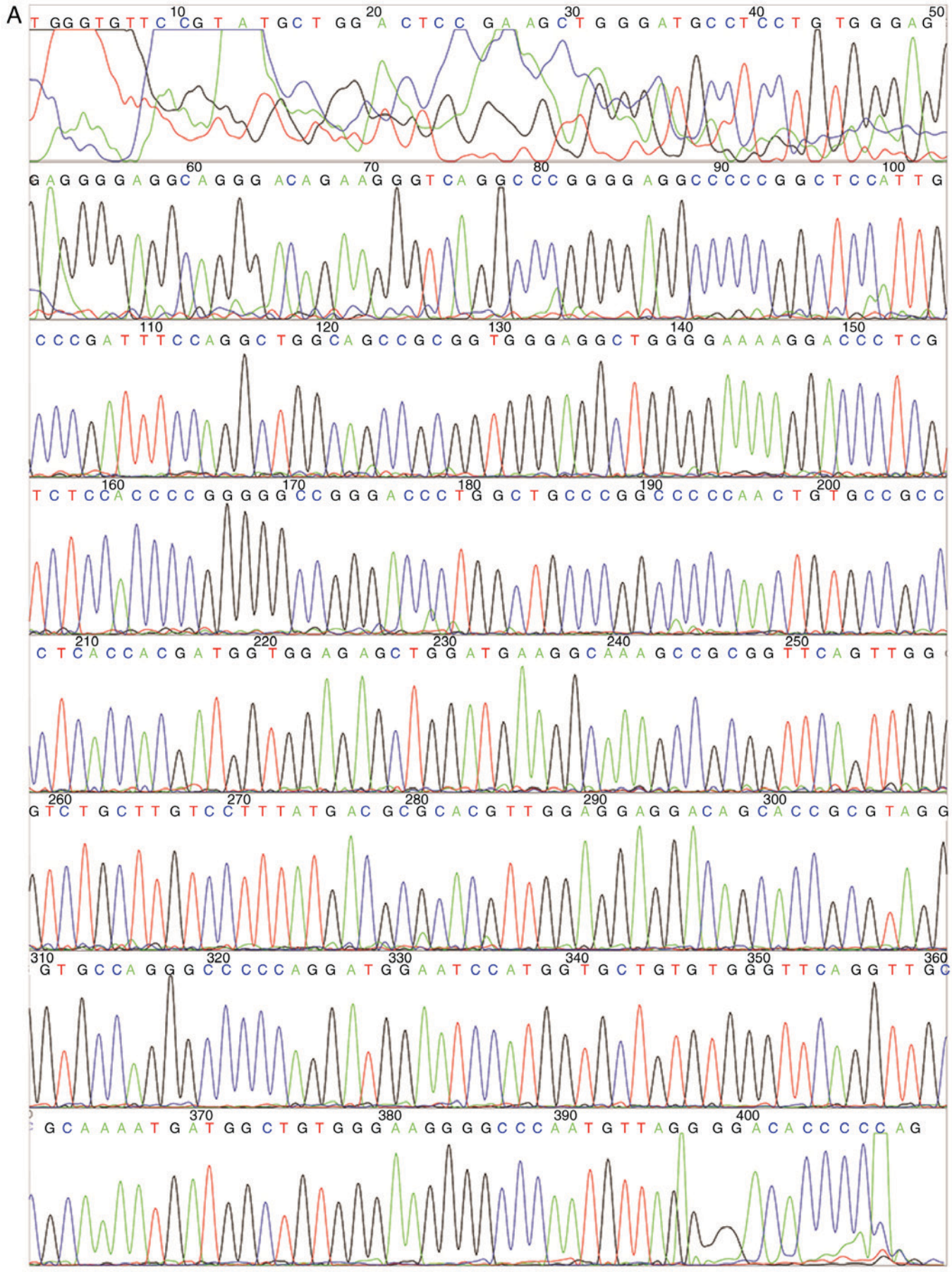

Figure 1. *RBM10* exon 10 DNA sequence histogram. (A) DNA sequencing of RBM10 exon 10 in adjacent non-cancerous lung tissue from the same patient.

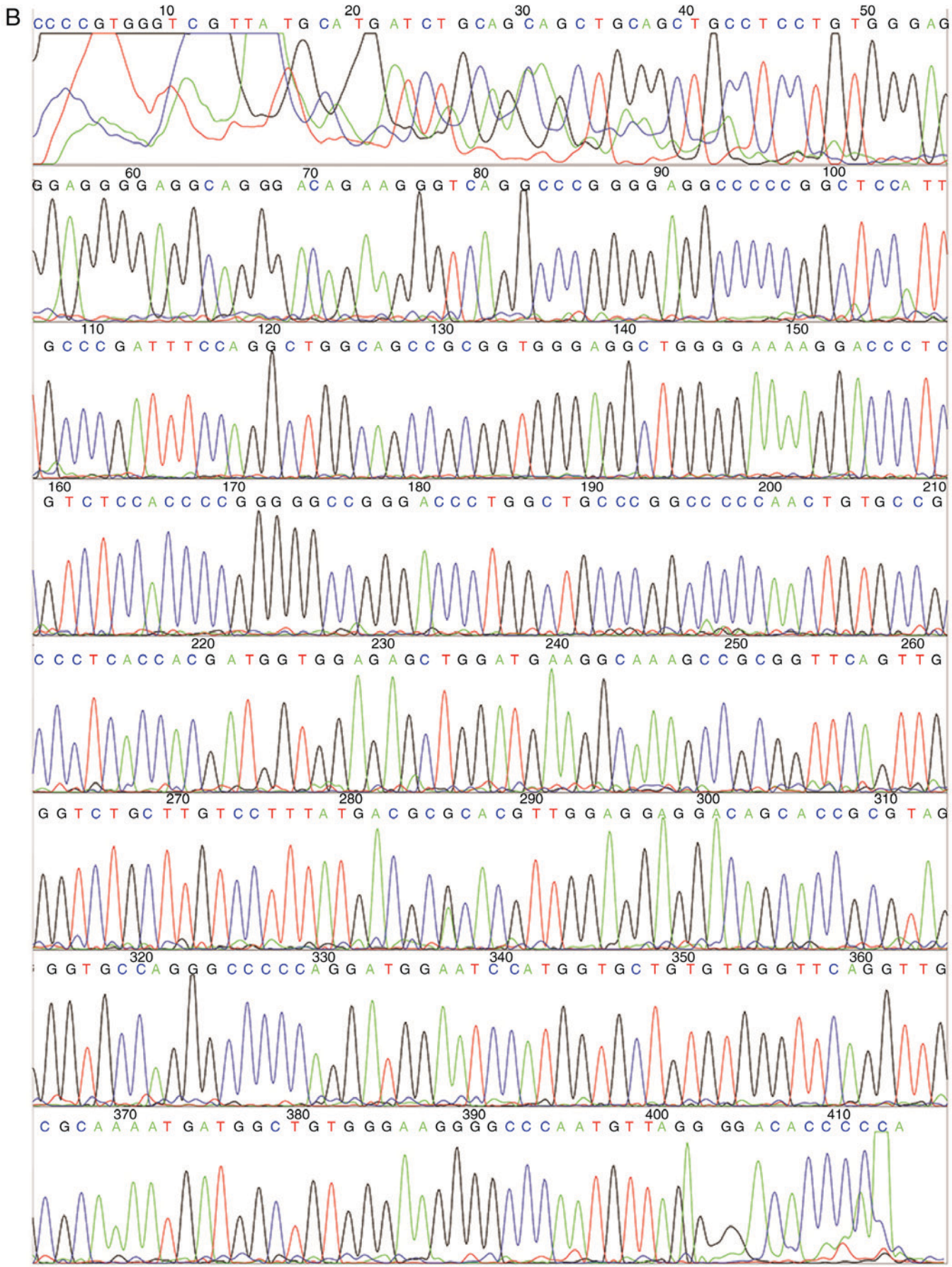

Figure 1. Continued. (B) DNA sequencing of *RBM10* exon 10 at c.763 C>T in a patient with lung adenocarcinoma

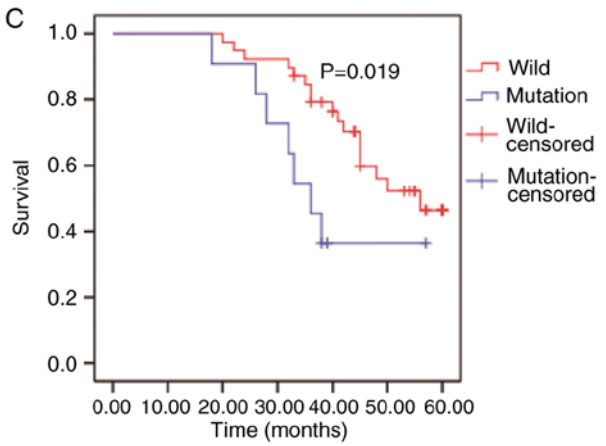

Figure 1. Continued. (C) Kaplan-Meier curve analysis of 5-year survival rate of lung adenocarcinoma patients stratified by *RBM10* exon 10 mutation.

T2 and T3 were observed in 23 (46%), 21 (42%), and 6 (12%) patients, respectively, while 30, 8, and 12 patients had N0, N1 and N2 stage tumors, respectively. The AJCC staging showed that there were 23 patients at stage I, 11 at stage II, and 16 at stage III. Tumor differentiation was 12 well, 25 moderate, and 13 poor-differentiated cases (Table I). The last follow-up was conducted on October 30, 2017 and these patients had a 5-year survival rate of 41.5%.

Association of RBM10 exon 10 (R241C) mutation with clinicopathological characteristics of lung adenocarcinoma patients. *RBM10* exon 10 mutations were detected in 11 (22%) of 50 patients, whereas there was no mutation found in all normal tissues (Fig. 1A and B). All 11 patients had *RBM10* exon 10 mutation c.763 C>T, which causes a replacement of arginine with cysteine at codon 241 (R241C) (Fig. 1B).

We then associated the RBM10 (R241C) mutation with the clinicopathological characteristics of lung adenocarcinoma patients and found that *RBM10* mutation was significantly associated with the AJCC stage (χ^2=10.751, P=0.005) and lymph node metastasis (χ^2=6.294, P=0.012), and male patients (χ^2=5.614, P=0.033). However, *RBM10* (R241C) mutation was not associated with tobacco smoking, age of patients and tumor size and differentiation (Tables I and II).

Association of RBM10 mutation (R241C) with poor prognosis of patients. We then assessed the association of *RBM10* exon 10 mutation (R241C) with 5-year survival rate of these patients, As shown in Fig. 1C, patients carrying the *RBM10* (R241C) mutation had much shorter 5-year survival rate (36.4% vs. 46.5 of RBM 10 wild type; χ^2=5.466, P=0.019; Fig. 1C and Table II). Our multivariate analysis revealed that *RBM10* exon 10 mutation and tumor differentiation and lymphatic metastasis were all independent prognostic factors (Table III).

RBM10 (R241C) mutation induction of tumor cell proliferation and modulation of Numb and Notch expression in vitro. After that, we further assessed the effect of *RBM10* (R241C) mutation on regulation of lung cancer cell proliferation and found that viability of A549 and H1299 cells after transfection with wild type RBM10 was slightly inhibited compared with that of blank plasmid (P<0.01; Fig. 2), whereas *RBM10* (R241C) mutation significantly induced A549 and H1299 cell viability (Fig. 2). Furthermore, *RBM10* (R241C) mutation also modulated expression of Numb and Notch proteins, which are the key regulatory proteins of NSCLC cell proliferation (17). As shown in Fig. 3, transfection with plasmid carrying *RBM10* (R241C) mutated

Table II. Univariate analysis of NSCLC patient prognosis (n=50).

Variables	No.	5-year survival rate (%)	P-value
Age (years)			0.786
≤60	31	48.0	
>60	29	42.7	
Gender			0.875
Female	20	48.2	
Male	30	32.7	
Smoking status			0.780
Smoking	20	36	
No-smoking	30	49.3	
Differentiation			<0.001
Low	13	13.5	
Moderate	25	48.9	
High	12	69.8	
Tumor size			0.144
T1	23	57.7	
T2	21	46.2	
T3	6	22.2	
Lymphatic metastasis			<0.001
N0	30	26.9	
N1	8	67.6	
N2	12		
AJCC stage			<0.001
I	23	72.4	
II	11	49.9	
III	16	20.8	
RBM10 exon10 gene status			0.019
Mutation	11	36.4	
Wild type	39	46.5	

Table III. Multivariate analysis of NSCLC patients' prognosis.

Variables	P-value	HR	95% confidence interval	
			Lower	Upper
RBM10 mutation	0.033	3.787	1.112	12.895
Differentiation	<0.001	0.03	0.153	0.909
Lymphatic metastasis	0.029	4.306	1.165	15.91

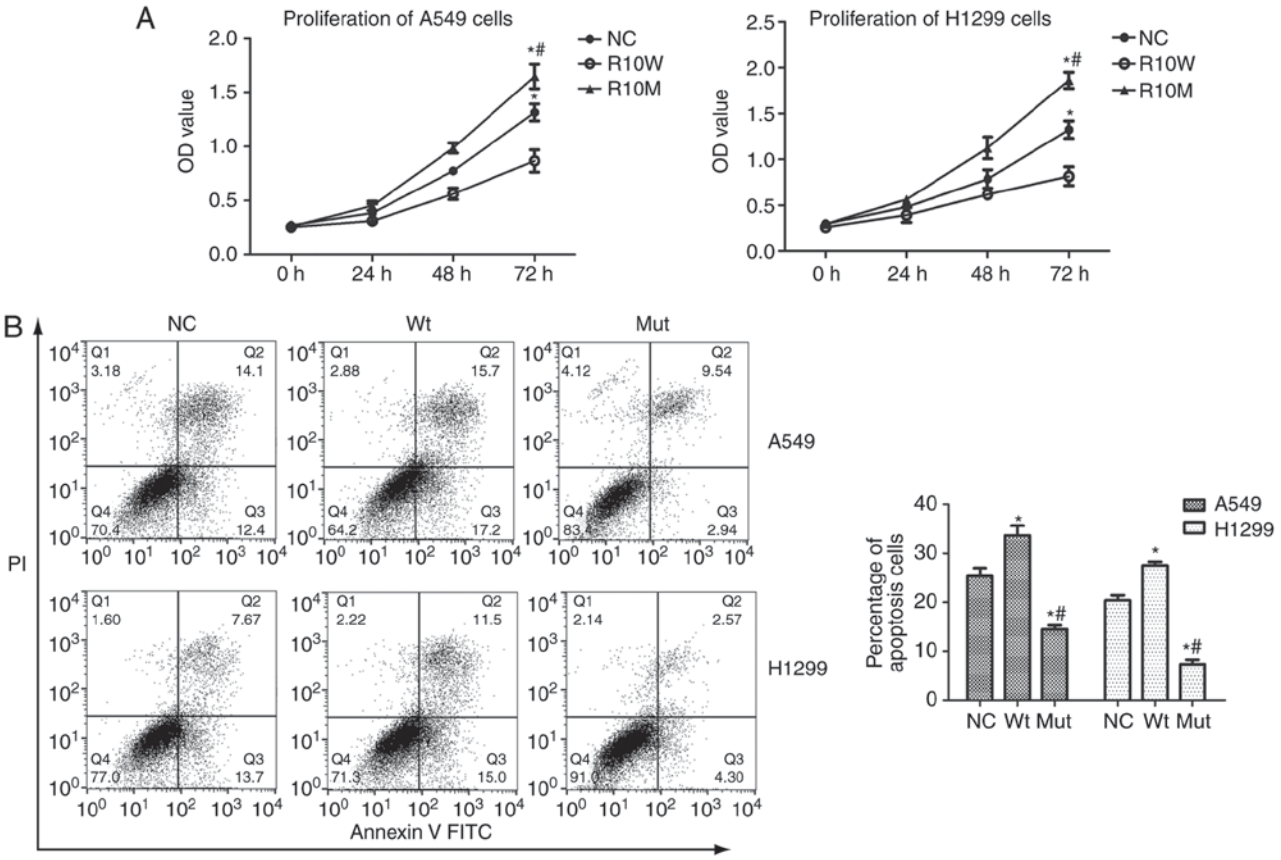

Figure 2. Effect of *RBM10* mutation on regulation of lung adenocarcinoma malignant behaviors. (A) Cell viability CCK-8 assay. Lung adenocarcinoma A549 and H1299 cells were grown and transfected with *RBM10* (R241C), wild type, or vector-only (NC) and then subjected to CCK-8 assay. The experiment was repeated three times. (B) Flow cytometric assay. The duplicated A549 and H1299 cells were subjected to flow cytometric apoptosis assay. The graph on the bottom is the quantitative data of flow cytometric assay results. The experiment was repeated three times. *P<0.05 vs. the NC group; #P<0.05 vs. the Wt group. NC, negative control; Wt, wild-type; Mut, mutant-type; OD, optical density.

cDNA was able to inhibit Numb protein expression, which negatively regulates NSCLC cell proliferation (17) (P<0.01; Fig. 3). In contrast, Notch expression in A549 and H1299 cells was significantly increased after transfection with the plasmid carrying *RBM10* (R241C) mutated cDNA (P<0.01; Fig. 3).

RBM10 (R241C) mutation suppression of A549 and H1299 cell apoptosis through Fas down-regulation. We performed FITC Annexin-FITC/PI Apoptosis assay to assess the effect of RBM10 (R241C) mutation on A549 and H1299 cell apoptosis. Our data showed that tumor cell apoptosis rate was reduced after transfection with *RBM10* (R241C) mutation compared with wild type *RBM10* (P<0.01; Fig. 2B). Moreover, expression of Fas protein was decreased in tumor cells after transfection with RBM10 mutated cDNA compared with that of wild type *RBM10* (P<0.01; Fig. 3).

RBM10 (R241C) mutation promotion of A549 and H1299 cell invasion through downregulation of E-cadherin protein. We also assessed the effect of *RBM10* (R241C) mutation on NSCLC cell invasion capacity. Our data showed that *RBM10* (R241C) mutation promoted A549 cell invasion capacity, whereas *RBM10* wild type inhibited tumor cell invasion (P<0.01; Fig. 4). Moreover, E-cadherin expression was down-regulated in *RBM10* (R241C) mutation group compared with *RBM10* wild type and blank plasmid group (P<0.01; Fig. 3).

Discussion

RBM10 is localized at chromosome Xp11.23-q13.3 (18), coding a protein that contains two RNA recognition motifs, two Zinc fingers, bipartite nuclear localization signals, a glycine (G)-patch, one arginine/serine-rich domain, and an OCtamerREpeat (OCRE) domain (19). RBM10 is able to regulate mRNA alternative splicing and affect protein expression (7,9). Aberrant RBM10 expression could alter exon-skipping or exon-inclusion of the affected proteins (7,9). Previous studied showed disruption of alternative splicing of mRNA in promotion of cancer progression due to gene transcripts involving in cell proliferation, apoptosis, DNA-damage response, angiogenesis, and metastasis (9,20). In our current study, we found that *RBM10* exon 10 mutations occurred in 11 of 50 lung adenocarcinomas and was associated with tumor stages, lymph node metastasis, and poor survival. Our data also showed that *RBM10* exon 10 mutation at c.763 C>T significantly promoted lung adenocarcinoma cell proliferation and invasion capacity *in vitro*. Future studies are warranted to investigate *RBM 10* protein as a predicative biomarker for lung adenocarcinoma progression and prognosis.

Indeed, recent studies have shown that RBM10 depletion or mutation was associated with human cancer development, including lung and pancreatic cancers (13,15). *RBM10*

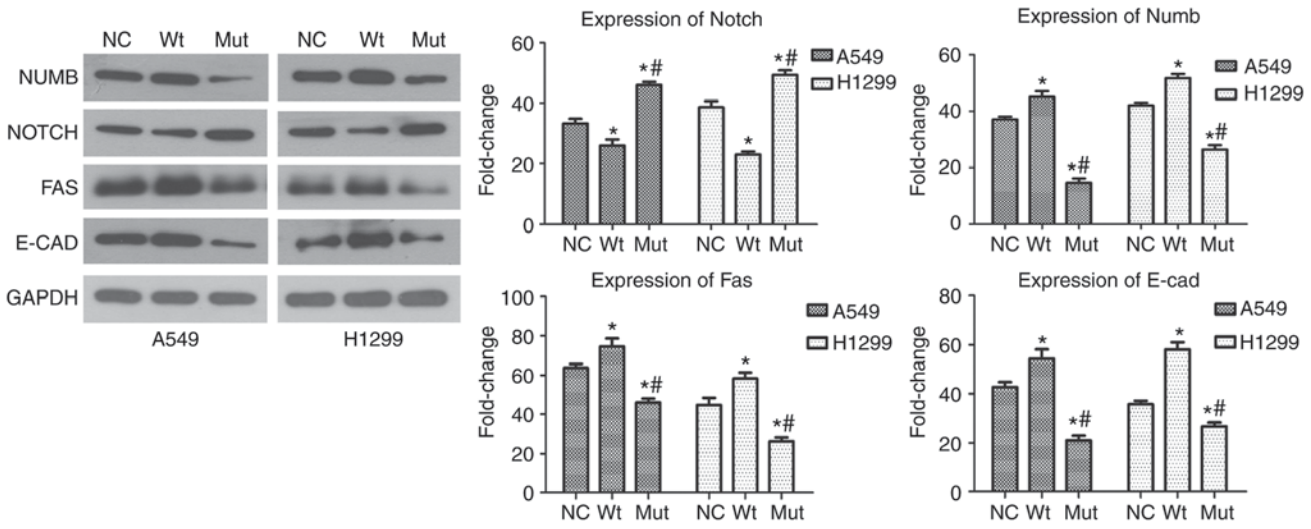

Figure 3. Effect of *RBM10* mutation on the regulation of protein expression in lung adenocarcinoma cells. Lung adenocarcinoma A549 and H1299 cells were grown and transfected with *RBM10* exon 10 c.763 C>T, wild type or vector-only and then subjected to western blot analysis. The bar graphs are quantitative data of the western blots. The experiment was repeated three times. *P<0.05 vs. the NC group; #P<0.05 vs. the Wt group.

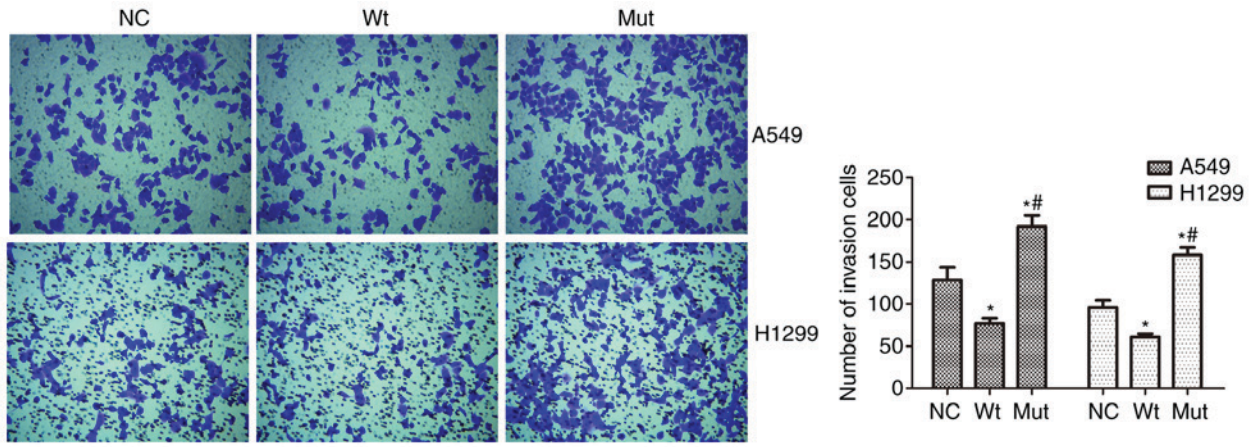

Figure 4. Effect of *RBM10* mutation on the regulation of lung adenocarcinoma cell invasion. Lung adenocarcinoma A549 and H1299 cells were grown and transfected with *RBM10* (R241C), wild type or vector-only and then subjected to a Transwell tumor cell invasion assay. The bottom graph is the quantitative data of the Transwell assay results. Magnification, x100. The experiment was repeated three times. *P<0.05 vs. the NC group; #P<0.05 vs. the Wt group.

missense somatic and frame shift mutations were identified in lung adenocarcinoma (15) with a frequency of 8% (14). An *in vitro* study demonstrated that *RBM10* exon 10 mutation led to enhancement of lung adenocarcinoma cell proliferation (19). Our current study further confirmed these findings; however, our data showed a higher frequency of *RBM10* exon 10 mutation (R241C) in lung adenocarcinoma patients (22%) and such a mutation was associated with advanced tumor stage, lymph node metastasis, and poor survival of patients. Furthermore, the TCGA data showed that *RBM10* mutations were enriched in male lung adenocarcinoma (14). In the present study, we found that 10 out of these 11 patients carrying this mutation were male, confirming the previous data (14).

RBM10 mutations contributed to lung cancer development (19), but the mechanisms by which RBM10 mutation promotes lung adenocarcinoma development remain to be elucidated. To explore the effect of mutant RBM10 on regulating lung adenocarcinoma cell growth and invasion capacity, we constructed plasmids carrying mutant RBM10 (R241C) or wild type RBM10 cDNA and transfected them into lung adenocarcinoma A549 and H1299 cells. Our data showed that RBM10 exon 10 (R241C) mutation significantly promoted tumor cell growth and invasion capacity compared with controls. Lung cancer A549 cells showed activation of the Notch pathway, which was responsible for increased tumor cell malignant behaviors (9). However, loss of Numb resulted in progression of NSCLC through the activation of Notch protein (17). We, therefore, investigated the effect of RBM10 mutation on expression of Notch and Numb proteins and found that Notch expression in A549 and H1299 cells after transfected with RBM10 mutated cDNA was upregulated compared with that of RBM10 wild type and vector-only. In contrast, Numb expression was downregulated in cells transfected with RBM10 mutation as compared with that of with wild type and blank plasmid. Based on these results, we concluded that RBM10 mutation promoted NSCLC cell proliferation through modulation of Numb and Notch expression.

Furthermore, the irrational increase in cell number is considered one of the hallmarks of carcinogenesis, which occurs due to either the increase in cell proliferation or defect in cell death (21). A previous study reported that p53, Fas, tumor necrosis factor (TNF)-α, and death receptor (DR)-5 were able to activate an alternative apoptotic pathway distinct from the well-established apoptotic pathway that is mediated by cytochrome C (22). Our current results indicated that the apoptotic rates of RBM10 (R241C) transfected A549 and H1299 cells were significantly decreased as compared to that of RBM10 wild type and vector-only. Downregulation of Fas protein expression in tumor cell surface resulted in evasion of apoptosis (22). For example, in HeLa cells, a cervical cancer cell line, RBM10 deletion resulted in apoptosis inhibition and contributed to carcinogenesis and tumor progression by alternative splicing of Fas (23). In our current study, we found that expression of Fas protein was lower in A549 and H1299 cells after transfected with RBM10 (R241C) compared to that of wild type and vector-only. Although our current study is just a proof-of-principle, Fas downregulation may be related to the RBM10 exon 10 mutation and the inhibition of lung adenocarcinoma cells apoptosis.

In addition, tumor cell migration and invasion capacity is one of the characteristics during tumor progression and metastasis (9). During tumorigenesis, cells lose normal homeostasis control and transform into premalignant or malignant phenotypes. These alterations are gained through mutations or loss of cell growth-critical genes or through epigenetic changes in genomic DNA that could lead to the silence of tumor suppressor genes or the activation of certain oncogenes (9). In our current study, we assessed the significance of RBM10 (R241C) in promoting lung adenocarcinoma invasion. We confirmed that RBM10 (R241C) significantly increased lung adenocarcinoma invasion compared to those transfected with RBM10 wild type and vector-only. At the gene level, our data showed that E-cadherin expression was reduced after RBM10 (R241C) transfection in lung adenocarcinoma cells, suggesting that RBM10 regulated E-cadherin, further confirmed that decrease in E-cadherin expression contributed to NSCLC cell migration (24).

However, there are several limitations in the current study; for example, we didn't assess protein levels after transfection of the wt and mutant RBM10; we didn't investigate the effect of the RBM10 mutant in alternative splicing although we detected expression of related proteins.

Our current study confirmed that RBM10 exon 10 (R241C) mutation at p.763 C>T frequently occurred in lung adenocarcinoma, especially in male patients. RBM10 (R241C) mutation was associated with AJCC stage, lymph node metastasis, and shorter 5-year survival rate of lung adenocarcinoma patients. Our *in vitro* data demonstrated that RBM10 (R241C) mutation induced lung adenocarcinoma cell proliferation, blocked tumor cell apoptosis, and promoted tumor cell invasion. However, it remains unknown why RBM10 mutation frequently occurred in male lung adenocarcinoma patients. It may be related to the chromosomal localization of RBM10 gene at Xp11.23-q13.3 (10), since man only carries one X chromosome and any alteration could show a dominant phenotype.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XWW conceived and designed the experiments. LLY and XWW prepared the manuscript. LLY, XMW and ML performed the experiments. YMX and XFZ interpreted the results. LLY and JL analyzed the data.

Ethics approval and consent to participate

The study protocol was approved by the Fourth Hospital of Jinan and Shanxian Central Hospital Review Board according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Herbst RS, Heymach JV and Lippman SM: Lung cancer. N Engl J Med 359: 1367-1380, 2008.
3. Cooper WA, Lam DC, O'Toole SA and Minna JD: Molecular biology of lung cancer. J Thorac Dis 5 (Suppl 5): S479-S490, 2013.
4. Hong WK, Bast RC, Hait WN, Kufe DW, Pollock RE, Weichselbaum RR, Holland JE and Frei E: Chapter 78: Cancer of the Lung. People's Medical Publishing House, 2010.
5. Glisovic T, Bachorik JL, Yong J and Dreyfuss G: RNA-binding proteins and post-transcriptional gene regulation. FEBS Lett 582: 1977-1986, 2008.
6. Mueller CF, Berger A, Zimmer S, Tiyerili V and Nickenig G: The heterogenous nuclear riboprotein S1-1 regulates AT1 receptor gene expression via transcriptional and posttranscriptional mechanisms. Arch Biochem Biophys 488: 76-82, 2009.
7. Wang Y, Gogol-Döring A, Hu H, Fröhler S, Ma Y, Jens M, Maaskola J, Murakawa Y, Quedenau C, Landthaler M, *et al*: Integrative analysis revealed the molecular mechanism underlying RBM10-mediated splicing regulation. EMBO Mol Med 5: 1431-1442, 2013.
8. Pan Q, Shai O, Lee LJ, Frey BJ and Blencowe BJ: Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat Genet 40: 1413-1415, 2008.
9. David CJ and Manley JL: Alternative pre-mRNA splicing regulation in cancer: Pathways and programs unhinged. Genes Dev 24: 2343-2364, 2010.

10. Sutherland LC, Thibault P, Durand M, Lapointe E, Knee JM, Beauvais A, Kalatskaya I, Hunt SC, Loiselle JJ, Roy JG, *et al*: Splicing arrays reveal novel RBM10 targets, including SMN2 pre-mRNA. BMC Mol Biol 18: 19, 2017.
11. Ji Y, Xie S, Jiang L, Liu L, Li L, Luo L, Chen Y, Zhang J, Yu L, Zhang Y, *et al*: Increased cell apoptosis in human lung adenocarcinoma and in vivo tumor growth inhibition by RBM10, a tumor suppressor gene. Oncol Lett 14: 4663-4669, 2017.
12. Hernández J, Bechara E, Schlesinger D, Delgado J, Serrano L and Valcárcel J: Tumor suppressor properties of the splicing regulatory factor RBM10. RNA Biol 13: 466-472, 2016.
13. Witkiewicz AK, McMillan EA, Balaji U, Baek G, Lin WC, Mansour J, Mollaee M, Wagner KU, Koduru P, Yopp A, *et al*: Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. Nat Commun 6: 6744, 2015.
14. Cancer Genome Atlas Research Network: Comprehensive molecular profiling of lung adenocarcinoma. Nature 511: 543-550, 2014.
15. Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, Cho J, Suh J, Capelletti M, Sivachenko A, *et al*: Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 150: 1107-1120, 2012.
16. Ganti AK, Huang CH, Klein MA, Keefe S and Kelley MJ: Lung cancer management in 2010. Oncology (Williston Park) 25: 64-73, 2011.
17. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, *et al*: Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci USA 106: 22293-22298, 2009.
18. Johnston JJ, Teer JK, Cherukuri PF, Hansen NF, Loftus SK; NIH Intramural Sequencing Center (NISC), Chong K, Mullikin JC and Biesecker LG: Massively parallel sequencing of exons on the X chromosome identifies RBM10 as the gene that causes a syndromic form of cleft palate. Am J Hum Genet 86: 743-748, 2010.
19. Zhao J, Sun Y, Huang Y, Song F, Huang Z, Bao Y, Zuo J, Saffen D, Shao Z, Liu W and Wang Y: Functional analysis reveals that RBM10 mutations contribute to lung adenocarcinoma pathogenesis by deregulating splicing. Sci Rep 7: 40488, 2017.
20. Kaida D, Schneider-Poetsch T and Yoshida M: Splicing in oncogenesis and tumor suppression. Cancer Sci 103: 1611-1616, 2012.
21. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
22. Israels LG and Israels ED: Apoptosis. Oncologist 4: 332-339, 1999.
23. Inoue A, Yamamoto N, Kimura M, Nishio K, Yamane H and Nakajima K: RBM10 regulates alternative splicing. FEBS Lett 588: 942-947, 2014.
24. Ma K, Fan Y, Dong X, Dong D, Guo Y, Wei X, Ning J, Geng Q, Wang C, Hu Y, *et al*: MTA1 promotes epithelial to mesenchymal transition and metastasis in non-small-cell lung cancer. Oncotarget 8: 38825-38840, 2017.