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, RBM10 mutation at exon 10 (c.763 C>T) occurs frequently and is an independent prognostic predictor in lung adenocarcinoma.

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 Shaxian 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 Shaxian 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'-GGGGTGTTCCTTACACATTGG-3' and 5'-ATGGTCTTGCCGTGAGAT-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% CO₂ 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 1x10⁶ 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 deoxynucleotidyl; 20 U RNasin; 100 U Maloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.). The primer sequences were as follows: **RBM10** sense, 5'-GCCACGACTATAGCCATGACAT-3'; antisense, 5'-ATGCAAACTTGGTCCTGCTCCA-3'; GAPDH sense, 5'-GAAGGTGAGGTCGGACT-3'; antisense, 5'-GAAGATGTTGAGTGGATTTC-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 1x10⁵ 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 cytometry 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. 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 albumin (BSA) for 2 h at the room temperature and then incubated with secondary antibody at a dilution of 1:1,000 (1:1,000; cat. no: AP124P; EMD Millipore) followed by enhanced chemiluminescence (ECL)-Plus western blotting detection reagents (EMD Millipore).

Statistical analysis. Association of RBM10 mutations with clinicopathological characteristics was assessed using the Statistical Package of Social Sciences (v19.0; SPSS, Inc., Chicago, IL, USA). All statistical analyses were performed 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).

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

| Variables                  | No. | Mutant | Wild-type | P-value |
|----------------------------|-----|--------|-----------|---------|
| Age (years)                |     |        |           |         |
| ≤60                        | 27  | 5      | 22        | 0.728   |
| >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         |         |

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).
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.
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 (χ²=10.751, P=0.005) and lymph node metastasis (χ²=6.294, P=0.012), and male patients (χ²=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 RBM10 wild type; χ²=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)                            |     |                          |         |
| ≤60                                    | 31  | 48.0                     | 0.786   |
| >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   | 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 1. Continued. (C) Kaplan-Meier curve analysis of 5-year survival rate of lung adenocarcinoma patients stratified by RBM10 exon 10 mutation.
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 downregulated 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 studies 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 RBM10 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
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 frequency 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.

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