Increased cell apoptosis in human lung adenocarcinoma and *in vivo* tumor growth inhibition by *RBM10*, a tumor suppressor gene

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Abstract. Tumor suppressor genes are frequently deleted or mutated in lung cancer. The RNA-binding motif protein 10 (RBM10) gene has the ability to suppress tumor activity, but the role of RBM10 during the development of lung cancer has yet to be elucidated. The current study investigated the expression levels of RBM10 in non-tumor and tumor tissues obtained from patients with adenocarcinoma using reverse transcription-polymerase chain reaction and western blot analysis, and identified a reduction in RBM10 expression in lung tumor tissue. To investigate the *in vitro* and *in vivo* function of RBM10, A549 human non-small cell lung cancer cells were transfected with the pcDNA-RBM10 vector. Flow cytometry was used to analyze the levels of apoptosis in the transfected cells. Western blot analysis was used to evaluate the expression of B-cell lymphoma 2 (Bcl-2), cleaved caspase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP) proteins in A549 cells and tissues from the A549 xenograft Bagg Albino coat (BALB/c) nude mice model. RBM10 mRNA levels were significantly decreased in adenocarcinoma cells, but not in the non-tumor tissues. The A549 cells and tumor tissues exhibited significant growth inhibition following transfection with the pcDNA-RBM10 vector, which was determined using a cell proliferation assay. Flow cytometry analysis of cells stained with Annexin V/propidium iodide indicated that the over-expression of RBM10 induced apoptosis in A549 cells. The present study demonstrated that the expression levels of Bcl-2 protein were decreased and the expression levels of cleaved caspase-3, caspase-9 and PARP proteins were significantly increased in the A549 cells and cells from *ex vivo* tumor tissues that were injected with RBM10 vector-containing *Salmonella enterica* subspecies enterica serovar typhimurium. Notably, the current study identified that the accumulated and stable overexpression of RBM10 in the xenograft BALB/c nude mice model significantly inhibited the tumor growth rate. These results may provide novel insights into the use of RBM10 for lung cancer diagnosis and therapy.

Introduction

Although there has been a decrease in the mortality rate due to improvements in clinical medicine, lung cancer has caused >1,000,000 global mortalities annually since 2008 (1). In clinical practice, lung cancer is divided into small cell lung carcinoma and non-small cell lung carcinoma and the latter includes squamous cell carcinoma, adenocarcinoma and large-cell carcinoma, according to the tumor histological type (2). Over the previous decade, the importance of acquired genetic or epigenetic changes has been recognized in the development of lung malignancy, in addition to other factors, including smoking (3). A previous study reported that a lung cancer tumor suppressor region is typically deleted in premalignant chromosomal aberrations that are associated with the development of lung cancer (4). In the early stages of lung cancer, chromosomal deletions frequently occur in tumor suppressor genes (5,6).

RNA-binding motif (RBM) genes are ubiquitous genes that encode RNA-binding proteins, which are a group of regulatory proteins that interact with RNA (7). RBM proteins are associated with a number of cellular activities, including alternative splicing and RNA degradation (8-10). The mutation of RNA-binding motif genes has been associated with cancer development due to their ability to function in the regulation of proteins at a post-transcriptional level (11-14). Previous studies have established that RBM proteins may promote cell apoptosis through a number of signaling pathways (15,16)

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and that RBMs are widely dysregulated in numerous types of cancer (17). The RNA-binding motif 10 (RBM10) gene possesses >50% conservation with the RNA binding motif 5 (RBM5) gene, which may have a role in the proliferation of cancer cells (18). RBM10 has also been implicated to affect the proliferation of cancer cells (19). In another previous study, a variant of RBM10 was identified to be significantly associated with the expression of wild type p53, which is a gene that serves an important role in the caspase apoptotic signaling pathway (19). However, to the best of our knowledge, the underlying mechanism of RBM10 in lung cancer has yet to be elucidated.

In the present study, the expression levels of RBM10 in tissues from patients with lung cancer were investigated and it reduced expression of the RBM10 gene in lung cancer cells was detected. The pcDNA3.1 (pcDNA)-RBM10 vector was transfected into the A549 human lung cancer line and used to investigate the expression levels of pro-apoptotic proteins, including cleaved caspase-3, caspase-9, poly(ADP-ribose) polymerase (PARP) proteins and the anti-apoptotic protein B-cell lymphoma (Bcl)-2. The *in vivo* anticancer effect of RBM10 was evaluated using a xenograft Bagg albino coat (BALB/c) nude mouse model treated with *Salmonella enterica* subspecies *enterica* serovar Typhimurium containing an RBM10 or control DNA vector. The present study may provide novel insights for the use of RBM10 in lung cancer diagnosis and treatment.

Patients and methods

Patients, cells and tissues. The present study was approved by the Institutional Review Board of the Guangxi University of Chinese Medicine (Nanning, China). The lung cancer tissues were obtained from 25 patients diagnosed with primary lung adenocarcinoma by surgical resection. The paired normal lung tissues from the disease-free margins were also obtained from the same patients as controls. This study was concordant with the 1964 Helsinki declaration as well as its later amendments or comparable ethical standards. Formal written consent was obtained from each patient involved in the current study. Patient tissue samples were stored in liquid nitrogen until RNA or protein was extracted. The A549 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an incubator containing 5% CO_2.

Transfection. The cells were transfected with pcDNA3.1 or pcDNA3.1-RBM10 plasmids using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, 2 μg DNA (pcDNA3.1 vector control or pcDNA3.1-RBM10) or 5 μl Lipofectamine® 2000 was diluted in 200 μl Opti-MEM (Thermo Fisher Scientific, Inc.) and incubated at room temperature for 5 min. the diluted DNA and Lipofectamine® were combined and incubated for another 20 min at room temperature prior to their addition to 6-well plates seeded with $4x10^5$ cells/well. Cells were maintained in 37°C in an incubator containing 5% CO_2 for 48 h prior to additional experiments.

Proliferation assay. An MTT assay was used to assess the proliferation of cells according to the manufacturer's protocol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Briefly, the cells that were transfected with plasmid DNA were subsequently seeded in 96 well plates ($5x10^4$ cells/well). A total of 20 μl of 5 mg/ml MTT in PBS was added to each well and incubated at 37°C for 4 h. The cells were treated with 150 μl DMSO (Merck Millipore, Darmstadt, Germany) and agitated at room temperature for 5 min. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the absorbance of cells in each well at a wavelength of 570 nm.

Reverse transcription-polymerase chain reaction (RT-PCR). TRIzol (Thermo Fisher Scientific, Inc., USA) was used to extract 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., USA). The primer sequences were as follows: RBM10 sense, 5'-GCACGACTA TAGGCATGACAT-3'; antisense, 5'-AGTCAAACTTGTCTG CTCCA-3'; GAPDH sense, 5'-GAAGGTGAAGGTCGGAGT C3'; 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 Bio-Sciences, Pittsburg, PA, USA).

Protein extraction and western blot analysis. The extraction of proteins from lung adenocarcinoma tissue samples and A549 cells was performed as reported previously (20,21). The protein concentrations were determined using the BCA (Thermo Fisher Scientific, Inc., USA) method according to the manufacturer's protocol. A total of 20 μg of each extracted proteins were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Merck Millipore). The membrane was blocked with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology, Dallas, TX, USA) in TBS buffer with 0.01% Tween-20 (TBST) for 1 h at room temperature, followed by incubation with primary antibodies. The following primary antibodies were used at 1:500 dilution in 5% BSA and incubated at 4°C overnight: Rabbit anti-human RBM10 (Abcam, Cambridge, MA, USA; cat. no. ab26046); Bcl-2; PARP (Cell Signaling Technology, Inc.; Danvers, MA, USA; cat. no. 9532); cleaved-PARP (Cell Signaling Technology, Inc.; cat. no. 5625); caspase-3 (Cell Signaling Technology, Inc.; cat. no. 9662); cleaved caspase-3 (Cell Signaling Technology, Inc.; cat. no. 9661); β-actin antibodies (Abcam; cat. no. ab8227). Membranes were then incubated with the secondary antibody Goat anti-rabbit immunoglobulin G-horseradish peroxidase (dilution, 1:100,000 in TBST; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. The protein bands were analyzed with SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.). The intensity of the protein bands was analyzed with Quantity One software (v1709600, Bio-Rad Laboratories, Inc.).

Flow cytometry. Flow cytometry was used to analyze the number of apoptotic cells following transfection with the
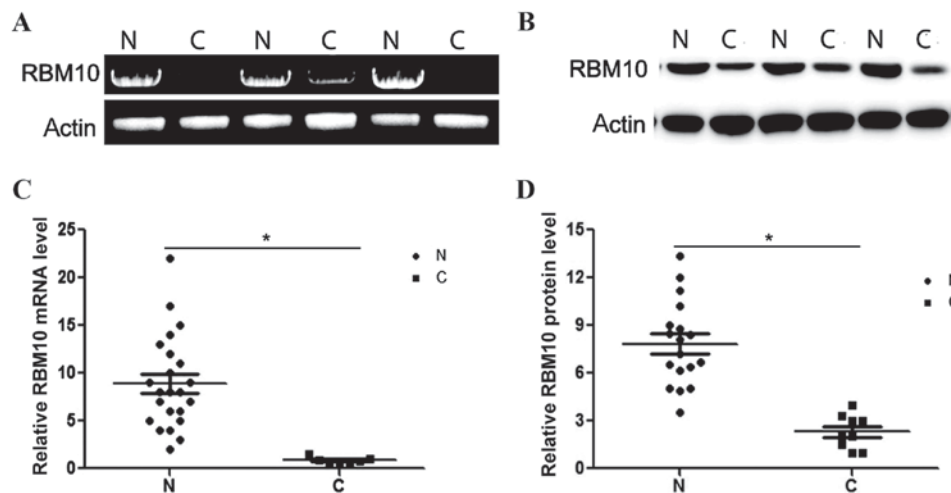
Figure 1. Expression levels of RBM10 in control tissues and tissues from patients with lung cancer. (A) reverse transcription-polymerase chain reaction analysis of the expression levels of RBM10 mRNA in 3 control and 3 lung cancer tissues, with b-actin mRNA as control. (B) Western blot analysis of RBM10 protein expression levels in 3 tumors and 3 control tissues, with b-actin as control. Relative expression levels of RBM10 (C) mRNA and (D) protein in control and cancer tissues. N, normal control tissues; C, cancerous tissues; RBM10, RNA-binding motif protein 10. Data are presented as means ± standard deviation from three independent experiments, *P<0.05.

described plasmid DNA vectors. Briefly, 1×10^6 A549 cells were harvested and resuspended in PBS. The total of 5 μl Annexin V (1 μg/ml) (Beckman Coulter Inc., Brea, CA, USA) was added to cells and incubated at room temperature for 15 min. Subsequently, propidium iodide (1 μg/ml) was added to the cells for 5 min at room temperature. All staining incubation steps were performed in dark.

In vivo tumor growth. A total of 16 BALB/c nude mice (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were used. All mice used were female, aged 4-6 weeks and weight >20 g prior to initiation of experiments. Mice were randomly assigned to experimental groups. All mice were kept up to 4 per cage and maintained in the vivarium room of Guangxi University of Chinese Medicine (Nanning, China) with free access to water and food. Mice were injected with 3×10^5 A549 cells subcutaneously. Tumor diameters were measured with digital calipers starting from the 8th day following injection. The tumor bearing cells were treated with pcDNA as the control group or pcDNA-RBM10. The plasmids were carried by an attenuated *Salmonella enterica* subsp. *enterica,* serovar Typhimurium strain using electroporation as reported previously (22,23). Briefly, *S. enterica* subsp. *enterica,* serovar Typhimurium cells (1×10^8 colony-forming units per 50 μl) transfected with pcDNA or pcDNA-RBM10 plasmids were injected into mice via the tail vein on day 7, 28 and 35 following subcutaneous injection. The diameter of the tumors was measured every 3 days for 42 days with digital calipers. The weight of tumors was assessed following the sacrifice of the mice by gradual CO_2 asphyxiation. A secondary physical method, cervical dislocation, was used to assure death. All the animal experiments were performed under the regulation of the Animal Care Committee in Guangxi University of Chinese Medicine.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The specific test used for each experiment to determine significance is indicated in the fig. legends. Data are representative of results obtained in at least 3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of RBM10 are significantly reduced in lung cancer tissue. The expression levels of RBM10 mRNA and protein in lung cancer tissues was analyzed using RT-PCR and western blot analysis in 5 pairs of lung tumor tissues and non-tumor tissues. Fig. 1A and B present the expression levels of RBM10 mRNA and protein in the analyzed control and tumor tissues. The relative expression levels of RBM10 mRNA and protein in tumor and control tissues were determined, indicating that the levels of RBM10 mRNA and protein were reduced in the tumor tissues (P<0.05; Fig. 1C and D). The relative expression levels of RBM10 mRNA in the tumor tissues demonstrated a 4.3-fold decrease compared with the RBM10 mRNA levels in the normal tissues (P<0.05; Fig. 1C). The results of the western blot analysis indicated that the level of RBM10 protein was decreased by 3.2-fold in the tumor tissues compared with the control tissues (P<0.05; Fig. 1D).

A549 cells transfected with pcDNA-RBM10 exhibit reduced cell proliferation and increased levels of apoptosis. To further investigate the role of RBM10 in the proliferation of tumor cells, pcDNA or pcDNA-RBM10 were transfected into A549 cells for 48 h prior to evaluating the expression levels of RBM10. Fig. 2A presents the expression levels of RBM10 mRNA and protein in cells transfected with the control vector pcDNA or pcDNA-RBM10 and the increased expression levels of RBM10 mRNA and protein indicated the transfection and expression of the pcDNA vector. The expression levels of RBM10 mRNA and protein in cells effective transfected with pcDNA-RBM10 was significantly increased compared with
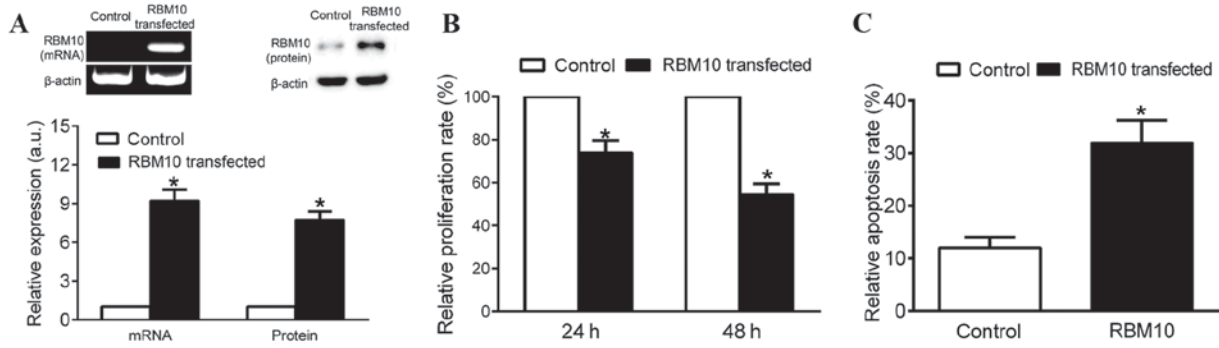
Figure 2. *In vitro* anti-tumor analysis of RBM10 mRNA levels in A549 cells. (A) The assessment of RBM10 mRNA and protein expression levels in A549 cells transfected with pcDNA3.1 (control) and pcDNA3.1-RBM10 and the relative expression levels of RBM10 mRNA and protein in cells transfected with pcDNA3.1 and pcDNA3.1-RBM10. The analysis was performed using reverse transcription-polymerase chain reaction and western blot analysis. (B) Cell proliferation and (C) levels of apoptosis in cells transfected with pcDNA3.1 and pcDNA3.1-RBM10. The cells transfected with pcDNA3.1 are used as a control. Data are presented as means ± standard deviation from three independent experiments. *P<0.05. RBM10, RNA-binding motif protein 10; a.u., arbitrary units.
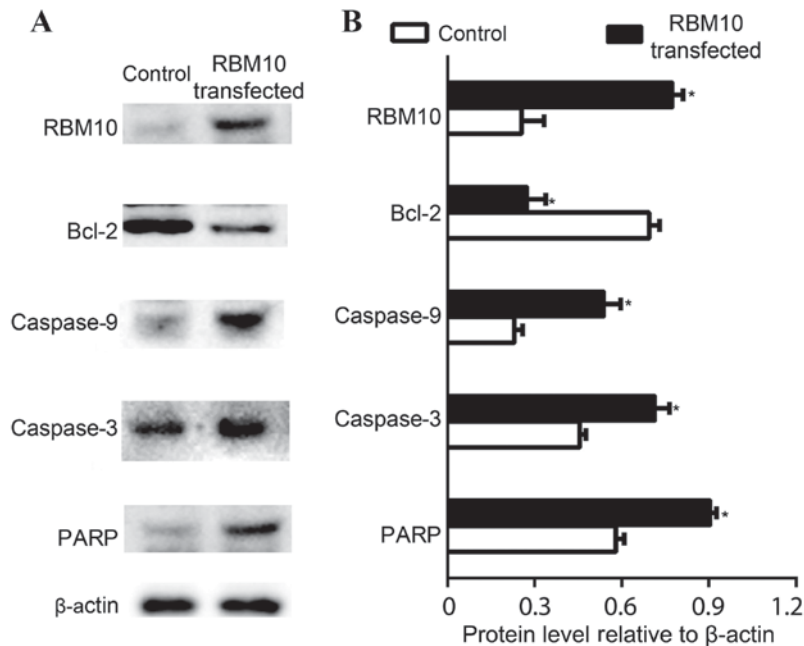

Figure 3. Evaluation of apoptosis-associated genes in cells transfected with pcDNA3.1 and pcDNA3.1-RBM10. (A) mRNA expression levels of RBM10, Bcl-2, cleaved caspase-9, cleaved caspase-3, PARP in CTRL cells and cells transfected with RBM10. The transfection of RBM10 was validated by the over expression of RBM10 gene. (B) Quantitative analysis of the expression of apoptosis-associated genes, including Bcl-2, caspase-9, caspase-3 and PARP. The expression levels of β-actin were used as a control. Cleaved caspase-9 and cleaved caspase-3 are labeled as caspase-9 and caspase-3. Data are presented as means ± standard deviation from three independent experiments. *P<0.05. RBM10, RNA-binding motif protein 10; Bcl-2, B cell lymphoma 2; PARP, poly (ADP-ribose) polymerase.

the control cells (P<0.05; Fig. 2A). The proliferation of A549 cells transfected with pcDNA-RBM10 was reduced compared with cells transfected with pcDNA (P<0.05; Fig. 2B). The cells transfected with pcDNA-RBM10 demonstrated an increased level of apoptosis compared with cells transfected with pcDNA, which indicates that RBM10 may serve an important role in inducing cancer cell apoptosis (P<0.05; Fig. 2C).

RBM10 enhances cell apoptosis by modulating the expression levels of apoptotic genes. The expression levels of apoptosis associated genes, including Bcl-2, caspase-3, caspase-9 and PARP, were detected in cells transfected with pcDNA or pcDNA-RBM10. Bcl-2 is one of the key members of the Bcl-2 family of regulator proteins that are involved in the regulation

of cell apoptosis (20). The expression levels of Bcl-2 protein were decreased significantly in cells transfected with RBM10 (P<0.05; Fig. 3A and B). Cleaved caspase-3, cleaved-9 and PARP expression levels were significantly increased in cells transfected with pcDNA-RBM10 compared with cells transfected with pcDNA (P<0.05; Fig. 3A and B). These results indicate that RBM10 may promote apoptosis via inducing the expression of caspase-3, caspase-9 and PARP and decreasing Bcl-2 expression levels.

RBM10 decreases tumor size in tumor-bearing mice. The *in vivo* antitumor effect of RBM10 in mice was investigated by transplanting tumor cells into mice. The mice were then injected with *S. enterica* subsp. *enterica*, serovar Typhimurium
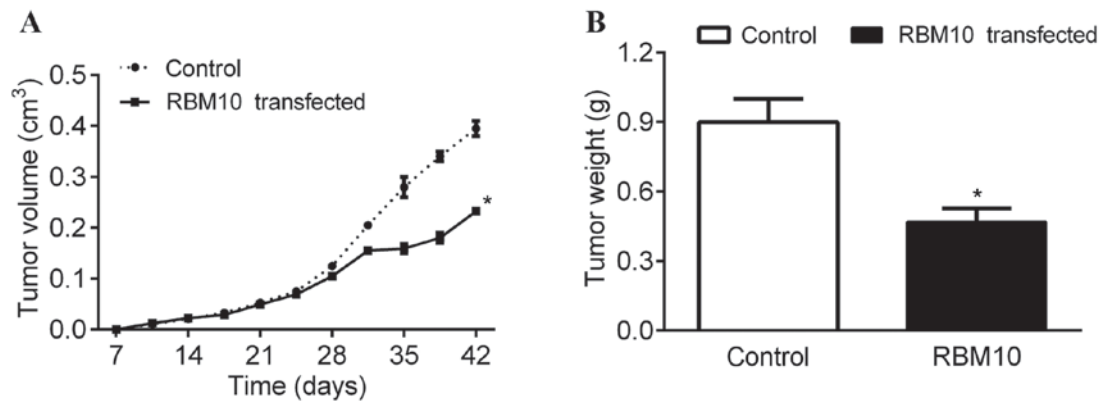
Figure 4. *In vivo* assessment of anti-tumor function of RBM10 in tumor-bearing BALB/c nude mice. The mice were transplanted with 5×10^5 A549 cells on day 0 to establish tumor formation. *Salmonella* carrying pcDNA3.1 or pcDNA3.1-RBM10 are injected every 2 days following the inoculation of the tumor. (A) Assessment of tumor size in mice injected with *Salmonella* carrying pcDNA3.1 (control) or pcDNA3.1-RBM10 every 2 days following the induction of tumor. (B) Weights of tumors in mice treated with *Salmonella* carrying pcDNA3.1 (control) or pcDNA3.1-RBM10 on day 35. Data are presented as means ± standard deviation from three independent experiment. *P<0.05. RBM10, RNA-binding motif protein 10.

carrying RBM10 at day 7, 28 and 35 following the transplantation of the tumor cells, and the tumor size was monitored. The present study identified that the two groups of mice possessed tumors of a similar size until day 24 (Fig. 4A). However, the tumors from the mice treated with *S. enterica* subsp. *enterica,* serovar Typhimurium carrying pcDNA-RBM10 were smaller in size after day 24 compared with the tumors from the control mice (P<0.05; Fig. 4A). In addition, the mice treated with *S. enterica* subsp. *enterica,* serovar Typhimurium carrying pcDNA-RBM10 presented with tumors of a lighter weight compared with the tumors from control mice that were treated with the control pcDNA vector (P<0.05; Fig. 4B). These results indicate that the *in vivo* accumulation and expression levels of RBM10 may reduce the growth rate of the tumors.

Discussion

Lung cancer is one of the predominant causes of cancer-associated mortality globally at present (21). Although previous studies have focused on the diagnosis and treatment of lung cancer, the 5-year survival rate for patients requires improvement (2,22,23). Therefore, further studies are required into the underlying molecular mechanisms of lung cancer using *in vitro* cellular and *in vivo* animal models. Previous studies (24,25) have demonstrated that RBM10 serves an important role in promoting cell apoptosis as well as being an RNA binding protein that is involved in the regulation of co-transcriptional modification of pre-mRNA (19). Certain studies have established a positive correlation between the expression of RBM10 and pro-apoptotic factors in breast tumor samples (19,24). Another previous study demonstrated that there was co-expression of RBM10 and caspase-3 in breast cancer specimens (24). Notably, RBM10 and RBM5 are paralogues and RBM10 shares ~50% identity with RBM5, which is important as RBM5 has been established to be involved in cancer suppression (19). This indicates that RBM5 and RBM10 may possess similar abilities or overlapping functions. The underlying mechanisms of RBM5 are yet to be elucidated and there are a number of previous studies (24,25) investigating the role of RBM10.

In the current study, the expression of RBM10 mRNA and protein was examined in clinical lung cancer tissues and this indicated that the expression levels of RBM10 mRNA and protein are significantly decreased in the tumor tissues (Fig. 1A and B). These results indicate that RBM10 mRNA may serve an important role in lung cancer. To further evaluate the suppressive role of RBM10 in cancer development, RBM10-containing plasmid DNA was transfected into A549 cells and this demonstrated that the proliferation and growth rate of A549 cells were significantly inhibited when RBM10 was overexpressed (Fig. 2A). Additionally, there was an inhibition of proliferation and an increase in the levels of apoptosis in cells transfected with pcDNA-RBM10 (Fig. 2B and C).

To investigate the underlying molecular mechanisms of the antitumor effect of RBM10, the expression levels of antitumor associated genes were analyzed in cells transfected with RBM10. The current study effectively overexpressed RBM10 by transfecting lung cancer cells with pcDNA-RBM10 (Fig. 3A). Subsequently, the expression levels of Bcl-2 were investigated. The abnormal expression of Bcl-2 has been associated with various forms of cancer, including lung cancer (26). The present study demonstrated that the overexpression of RBM10 in A549 cells reduced the expression levels of Bcl-2 (Fig. 3A) and this was also associated with an enhanced level of apoptosis in the cancer cells (Fig. 2C). These results are concordant with previous studies that demonstrate the reduction of Bcl-2 may facilitate the apoptosis of small-cell lung cancer cells (27). The expression levels of caspase-9, caspase-3 and PARP were analyzed in A549 cells with endogenous or overexpressed levels of RBM10 mRNA (Fig. 3). The current study demonstrated an increased expression of caspase-9, caspase-3 and PARP in cells upon overexpression of RBM10 mRNA. This is concordant with a previous study that demonstrates that the decrease in expression levels of RBM10 increased the expression of caspase-3 and caspase-9 (28). Concordant with previous studies, the results in the current study indicate that the downregulation of Bcl-2 and upregulation of cleaved caspase-3, caspase-9 and PARP are associated with an increase in the level of apoptosis in cancer cells (6,29).

Based on the aforementioned clinical examination and *in vitro* studies (24,25), this study focused on the *in vivo* anticancer effect of the overexpression of RBM10 by treating mice with attenuated *Salmonella* carrying pcDNA3.1 (control group) or pcDNA3.1-RBM10. *Salmonella* is a facultative anaerobe that is able to grow in aerobic and anaerobic conditions and, therefore, may be used to deliver numerous types of therapeutic agents (30). This technique has been used to provide tumor-targeting bacteria that are able to deliver genes that encode pro-drug-converting enzymes, angiogenic inhibitors or cytokines (31-33). Primarily, this has been investigated for use in clinical trials for gene therapy (34) and the current study demonstrates its use as a carrier for delivering the RBM10 gene into mice. The present *in vivo* study identified that mice treated with pcDNA and pcDNA-RBM10 possess tumors of a similar size until day 24 following tumor implantation. After day 24, mice treated with pcDNA-RBM10 exhibited a reduced tumor growth rate and a reduction in tumor weight (Fig. 4A and B). However, the current study did not assess the *in vivo* transfection efficiency of the vector or the optimal dosage for *in vivo* use and this remains to be investigated.

In conclusion, the current study established that tumor tissues from patients with lung cancer have decreased expression levels of RBM10 mRNA and protein. To further investigate the role of RBM10 mRNA in lung cancer, the *in vitro* anticancer effects of RBM10 mRNA were evaluated by transfecting the lung cancer cells with a vector containing the RBM10 gene. The current *in vitro* study demonstrated that cells transfected with RBM10 DNA vector have a reduction in cell proliferation and an increased level of apoptosis. The present study also established that cells transfected with RBM10 have decreased expression of Bcl-2 and increased expression of caspase-9, caspase-3 and PARP, which are genes that are established to enhance cancer cell apoptosis (35-37). Additionally, the present study identified that tumor bearing mice that were treated with *Salmonella* carrying pcDNA3.1-RBM10 presented with a decreased tumor growth rate and reduced tumor weight compared with control mice, which suggests that RBM10 may be involved in cancer suppression. The current study may provide a novel marker for the diagnosis of lung cancer and a potential chemotherapeutic treatment.

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