Lentiviral vector-mediated RBM5 overexpression downregulates EGFR expression in human non-small cell lung cancer cells

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Abstract

Background: RNA binding motif 5 (RBM5) is a tumor suppressor gene that modulates apoptosis through the regulation of alternative splicing of apoptosis-related genes. Our previous studies suggested that RBM5 expression was negatively correlated with the expression of epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) tissues. This study was aimed at determining whether RBM5 is able to regulate EGFR expression.

Methods: Both in vitro and in vivo studies were carried out to determine the effect of RBM5 on the expression of EGFR. Lentiviral vector-mediated RBM5 overexpression was employed in lung adenocarcinoma cell line A549. A549 xenograft mice were treated with recombinant RBM5 plasmid carried by attenuated Salmonella typhi Ty21a. Real-time quantitative polymerase chain reaction and Western blot were carried out to detect RBM5 and EGFR expression.

Results: Both in vivo and in vitro studies indicated that the expression of EGFR mRNA and protein was decreased significantly in the RBM5 overexpression group compared to control groups as shown by real-time PCR and Western blot analysis. We identified that RBM5 overexpression inhibited EGFR expression both in A549 cells and in A549 xenograft mouse models.

Conclusions: Our study demonstrated that EGFR expression is regulated by RBM5 in lung adenocarcinomas cells either in a direct or indirect way, which might be meaningful with regards to target therapy in lung cancer.

Keywords: RNA binding motif 5, Epidermal growth factor receptor, Non-small cell lung cancer, Lentiviral vector, A549, Xenograft mice model

Background

Lung cancer is one of the most common malignant tumors and remains the leading cause of cancer death both in males and females globally [1]. Among all lung cancer subtypes, non-small cell lung cancer (NSCLC) accounts for approximately 87% of all lung cancer cases, and has a poor prognosis; the overall five-year survival rate is 18.2% [2]. Molecularly, NSCLC development is believed to be initiated by the activation of oncogenes or inactivation of tumor suppressor genes [3].

Epidermal growth factor receptor (EGFR) (also known as HER-1 or Erb1) is a cell-surface receptor belonging to the ErbB tyrosine kinase receptor family, which also includes HER-2/neu (ErbB2), HER-3 (ErbB3), and HER-4 (ErbB4). EGFR activation is associated with cell apoptosis, proliferation, angiogenesis, invasion, and metastasis, which plays an important role in carcinogenesis and tumor progression in human epithelial cancers, including NSCLC [4]. These actions are accomplished through activation of the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways [5]. EGFR and PI3K initiate malignant neoplastic transformation via a combinatorial genetic network composed of other pathways, including the Tor, Myc, G1 Cyclins-Cdks, and Rb-E2F pathways [6], and drive cells through the restriction point of late G1 into S phase [7]. A series of anticancer agents directly targeting EGFR were developed and proved to be effective [8-13], but the clinical benefits of EGFR-TKIs are limited by primary or
acquired resistance [14]. Therefore, EGFR inhibition by an upper regulator seems to be more attractive. Yet the EGFR upstream regulatory mechanisms are still not well understood. Further insights into important molecular regulators of EGFR are needed for the development of novel therapeutics.

RNA-binding motif protein 5 (RBMS5) (also known as LUCA-15 or H37) maps to the human chromosomal locus 3p21.3, which is strongly associated with lung cancer [15]. It is reported to be downregulated in 73% of primary NSCLC specimens [16] and is also found in other human cancers. However, the precise mechanism by which RBMS5 mediated tumor suppression still remains to be clarified. Present studies are mostly focused on the regulation of apoptosis by the alternative splicing of correlated genes, such as Bax, Bcl-2, cleaved caspase-3, caspase-9, and P53 [17-21]. Only a few researchers noticed another mechanism of negative regulation of cell proliferation, inducing cell cycle arrest in G1 by down-regulating cyclin A and phosphorylated RB expression [17], which might also be involved in the malignant neoplastic transformation initiated by the EGFR and PI3K signaling pathway. These observations draw our interest in regard to the relationship between RBMS5 and EGFR. We conducted a series of investigations to clarify the relationship between RBMS5 and an important regulator of cell proliferation, EGFR. We detected RBMS5 and EGFR expression in 120 paired resected NSCLC tumor tissues and adjacent normal tissues in a previous study, which suggested that the RBMS5 expression was negatively correlated with the expression of EGFR in NSCLC tissues [22]. Afterwards, we inhibited EGFR expression in the lung adenocarcinoma cell line NCI-H1975 using small interfering RNA, and found that RBMS5 expression was not directly regulated by EGFR in non-smoker-related lung adenocarcinomas [23]. Herein, we hypothesized that inhibition of EGFR in lung adenocarcinomas might be achieved via RBMS5 overexpression. The objective of this study was to assess whether forced RBMS5 expression in lung adenocarcinoma cell line A549 cells and A549 xenografts could suppress the expression of EGFR, which would suggest that one of the mechanisms of potential tumor suppressor activity of RBMS5 in NSCLC is initiated via the inactivation or inhibition of EGFR.

**Lentiviral vectors construction and lentivirus infection**

Lentiviral vectors containing green fluorescence protein (GFP) were employed in order to achieve high efficiency of introduction and subsequent stable expression of RBMS5 in A549 cells. Recombined pGC-LV-GV287-GFP vector with the RBMS5 (NM_005778) gene (LV-RBMS5) and pGC-LV-GV287-GFP with a scrambled control sequence (LV-GV287) were constructed by Genechem Company (Genechem, Shanghai, China). A549 cells were then infected with the above lentiviral vectors. A total of $5 \times 10^5$ A549 cells were seeded in a six-well cell plate and further incubated for 12 hours to reach 30% confluent, and then infected with LV-RBMS5 (RBMS5 overexpression group), LV-GV287 (negative control group), and no infection (non-transfected control group) by replacing the infection medium containing recombinant vectors at a multiplicity of infection (MOI) of 20 plaque-forming units (p.f.u.) per cell. Plates were then incubated for 24 hours prior to having their media changed to fresh, virus-free media. Three days later, the GFP density contained by lentivirus was detected to evaluate the efficiency of infection, and cells were harvested for Western blot and real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

**Establishment of A549 xenografts**

The use of animals in this study was in accordance with animal care guidelines, and the protocol was approved by Jilin University Animal Care Committee. A549 xenografts were established and the RBMS5 gene was delivered into xenografts by attenuated *Salmonella* according to a previous study [19]. Briefly, BALB/c athymic nude female mice (nu/nu); between four and five-weeks-old) were purchased from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). A549 cells (1 × 10^5) were suspended in 100 μl PBS and injected subcutaneously into the right flank region of nude mice. 5 × 10^5 *Salmonella enterica* serovar typhimurium cells (competence) (obtained from the China-Japan Union Hospital of Jilin University, Jilin, China) were purchased and 1 μg GV287-RBMS5 or 1 μg GV287 plasmids and cooled for 15 minutes on ice. The plasmids were electro-transfected into the competence under the conditions as follows: capacitance = 25 μF, voltage = 1.25 kV (12.5 kV/cm). Then the recombinant attenuated salmonellae were quickly transferred into Luria-Bertani (LB) agar medium for proliferation at 37°C and stored at ~80°C.

The tumor-bearing mice were randomly divided into three groups (six mice per group) at day 21 after cell injection. The mice were treated at day 28 and 35, respectively, through a tail vein as follows: (a) control group (50 μl PBS); (b) negative control group (attenuated
Salmonella-carrying GV287) (10^8 colony-forming units (CFU) per 50 μl PBS); (c) RBM5 overexpression group (attenuated Salmonella-carrying GV287-RBM5) (10^8 CFU per 50 μl PBS). The mice were sacrificed on day 42 and the tumors were removed. One part of the tumor was fixed in Trizol™ reagent (Invitrogen, Carlsbad, United States) according to the manufacturer’s instructions. Primers were made by Genechem (Genechem, Shanghai, China). Selected primer sequences included RBM5 forward 5′-CCATCCAGAGAGGATATTC-3′, RBM5 reverse 5′-CGGCTTACACCTGTGTTCTCT-3′, EGFR forward 5′-ATGAGATGGAAGACCGG-3′, EGFR reverse 5′-CGGCAGGATGGAGAT-3′, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-TGACTTCTAACAGCGACACCCA-3′, and GAPDH reverse 5′-CACCCTGTGCTGACCCAAA-3′.

RT-qPCR was carried out using a Thermal Cycler Dice Real Time System (TaKaRa, Osaka, Japan) using Prime Script™ RT Master Mix (TaKaRa, Osaka, Japan). A two-step cycling condition was used for EGFR, RBM5, and GAPDH as follows: 95°C for 30 seconds followed by 40 cycles of 95°C for five seconds, and then 60°C for 30 seconds. A dissociation curve was generated for all three genes using the following conditions: 95°C for 15 seconds, 55°C for 30 seconds, and then 95°C for 15 seconds. The expression levels of the RBM5 and EGFR genes were normalized to the internal control GAPDH, respectively, to obtain the relative threshold cycle (ΔCt), and the relative expression between control A549 cells and infected cells was calculated using the comparative Ct (ΔΔCt) method (ΔΔCt = ΔCt of control cells – ΔCt of infected cells) or 2-ΔΔCt.

Statistical analysis
All experiments were performed at least in triplicate. All data were presented as means ± standard deviation (SD). Statistical significance was determined by analysis of t-test using SPSS version 17.0 (SPSS Inc., Chicago, United States). A P value of less than 0.05 was considered statistically significant.

Results
Infection efficiency of lentivirus vectors
In order to achieve high efficiency of introduction and subsequent stable expression of RBM5 in A549 cells, we tried to import this gene by infecting A549 cells with RBM5 lentiviral vectors containing GFP. The recombinant lentivirus vector LV-RBM5 was successfully constructed and infected A549 cells. The stably infected A549 cells expressed GFP after infection by the lentiviral vectors at different MOIs. GFP expression was detected three days after infection using fluorescence microscopy. The efficiency of the infection (averaged proportion of GFP-expressing cells on the total cell count) was approximately 80% at an MOI of 20 (Figure 1). Consequently, an MOI of 20 was chosen for the next steps of this study.

Overexpression of RBM5 in A549 cells infected with lentiviral vectors
RT-qPCR analysis demonstrated that the relative expression level of RBM5 mRNA was markedly increased in the RBM5 overexpression group (13.32 ± 2.16), compared with that in the negative control group (1.00 ± 0.09) and the non-transfected control group (0.65 ± 0.07; P <0.01; Figure 2A). There was no statistical difference
Figure 1 Infection efficiency of LV-RBM5 in A549 cells by GFP detection (100×). Lentiviral vector-mediated RBM5 expression was visualized by fluorescence microscopy three days after infection. Comparing the assessment in a bright field with the assessment in fluorescent field revealed an infection efficiency of over 80%. A, bright field; B, fluorescent field; CON, control group with no transfection; NC(OE), negative control group transfected with GFP lentiviral vectors LV-GV287; OE, RBM5 overexpression group transfected with GFP lentiviral vectors LV-RBM5.

Figure 2 Overexpression of RBM5 in A549 cells by lentivirus-mediated gene expression system. A549 cells were infected with lentiviral vector LV-RBM5. Three days after infection, the relative RBM5 mRNA and protein expression were determined by RT-qPCR and Western blot analysis respectively. β-actin was used as an internal control. A, RT-qPCR analysis for RBM5 mRNA in different groups. B, Western blot analysis for RBM5 protein in different groups. C, Quantification of RBM5 protein levels relative to β-actin. CON, non-transfected control group; NC(OE), negative control group; OE, RBM5 overexpression group. Data shown are means ± SD of three separate experiments. **P <0.01 indicates significant difference as compared to the control.
of RBM5 mRNA expression between the negative control and the non-transfected control (P >0.05). Furthermore, the effectiveness of the lentiviral infection of LV-RBM5 was also confirmed by Western blot analysis. The protein expression of RBM5 in A549 cells was significantly higher in the RBM5 overexpression group than that in the control groups (Figure 2B).

Overexpression of RBM5 inhibits EGFR expression in A549 cells

To explore whether RBM5 is able to directly regulate EGFR expression, we examined EGFR mRNA by RT-qPCR, and EGFR protein by Western blot analysis in A549 cells infected by different lentiviral vectors. As seen in Figure 3A, compared to negative control group and non-transfected control group, cells in the RBM5 overexpression group showed a significant decrease in EGFR expression (by 28.5% and 26.6%, respectively; P <0.001 and P <0.001, respectively). Additionally, Western blot analysis showed that the protein expression of EGFR in the RBM5 overexpression group was significantly lower than that in the control groups (Figure 3B).

Suppression of EGFR expression by RBM5 overexpression in A549 xenograft tumors

To further test our hypothesis, the mice model of A549 xenograft was established as described in the Methods section. To ensure that the recombinant attenuated salmonellae-carrying plasmids preferentially localized in the xenograft tumors, the kinetics of bacterium distribution in the xenograft tumor and different organs of the tumor-bearing mice were monitored in a previous study [18]. At the 28th and 35th day after implantation, the tumor-bearing mice were treated with attenuated Salmonella-carrying plasmid through a tail vein. The mice were sacrificed 42 days after implantation and the tumors were removed to monitor the tumor sizes and to determine RBM5 and EGFR expression by RT-qPCR and Western blot analysis. H&E staining were performed to observe histopathological performance on A549 xenografts. We observed that the mRNA and protein expression of RBM5 were significantly increased in the RBM5 overexpression group as compared to those in control groups, while EGFR expression was decreased significantly in the RBM5 overexpression group as compared to those in control groups (Figure 4). H&E staining

Figure 3 EGFR expression was suppressed by RBM5 overexpression in A549 cells. A549 cells were infected with lentiviral vector LV-RBM5. Three days after infection, the relative EGFR mRNA and protein expression were detected by RT-qPCR and Western blot analysis, respectively. β-actin was used as an internal control. A, RT-qPCR analysis for EGFR mRNA in different groups. B, Western blot analysis for EGFR protein in different groups. C, Quantification of EGFR protein levels relative to β-actin; CON, non-transfected control group; NC(OE), negative control group; OE, RBM5 overexpression group. Data shown are means ± SD of three separate experiments. **P <0.01 and ***P <0.001 indicates significant difference as compared to the control.
Figure 4 (See legend on next page.)
showed that there were a large number of cancer nests in control, and that the tumor tissue could survive in a good state (Figure 4).

**Discussion**

The RBM5 gene is a tumor suppressor gene (TSG) that is located within a 370 kb overlapping lung cancer allelic loss region on 3p21.3 [24]. There is increasing evidence suggesting that downregulation of RBM5 plays an important role in NSCLC occurrence, progression, metastasis, and drug resistance [16,18,21,22,25,26], yet the mechanisms are still not well clarified. Present studies on RBM5 anti-tumor mechanisms are mostly focused on its apoptosis induction role, such as: RBM5 overexpression enhanced TRAIL-, TNF-alpha-, Fas-, and PS3-mediated apoptosis [20,27,28], increased the expression of Stat5b, BMP5 [29], Bax [17], and proapoptotic Casp-2 L [30], and decreased the expression of Amplified In Breast Cancer 1 (AIB1), proto-oncogene Pim-1, caspase antagonist BIRC3 (cIAP-2, MIHC), and cyclin-dependent kinase 2 (CDK2) [29]. Rac1 and β-catenin were upregulated when RBM5 was knocked down [26]. Our previous study confirmed previous findings and further demonstrated that exogenous expression of RBM5 inhibited the A549 cell growth in vivo and in vitro, and re-sensitized A549/DDP cells to cisplatin by enhancement of mitochondria apoptosis [18,19,21]. Our recent study demonstrated for the first time an inverse correlation between the expression levels of RBM5, and transforming growth factor alpha (TGF-α) signaling factors, EGFR, and KRAS in NSCLC tissues [22], which suggested that the presence of a complex regulatory network between those genes was involved in tumor suppression and oncogenic expression. Although several studies found that the molecular mechanism of RBM5 tumor suppression involved cell proliferation inhibition [17,29,31], the precise mechanisms underlying such inhibition have been poorly understood. Here, we demonstrate that overexpression of RBM5 suppressed EGFR expression, both in lung adenocarcinoma cell line A549 cells and in A549 xenograft tumors. This effect occurs in NSCLC cells expressing a lower level of RBM5 [17]. Previously, we have proved that RBM5 expression was not directly regulated by EGFR [23], however, the results in the current study indicate that RBM5 might manipulate EGFR expression as an upstream gene, which may be a predominant mechanism by which RBM5 mediates tumor suppression.

Bonnal et al. found that RBM5 was a component of complexes involved in 3′ splice site recognition, and regulates alternative splicing of apoptosis-related genes, including the Fas receptor, switching between isoforms with antagonistic functions in programmed cell death [28]. It may be the same mechanism that explains how EGFR expression was suppressed by overexpression of RBM5. That is, upregulated RBM5 recognized the 3′ splice site of the pre-mRNAs of EGFR and led to more alternatively spliced mRNAs and less matured mRNAs of EGFR. However, we could not definitively conclude what the alternatively spliced mRNAs are. The alternatively spliced mRNAs might be mRNAs of other genes, generate protein isoforms of the same gene which harbor different functions, or degenerated.

For many years, chemotherapy has been the standard first-line systemic treatment for advanced NSCLC, but the clinical outcomes were unpromising. The advent of EGFR tyrosine kinase inhibitors (TKIs) changed the treatment paradigm. Nevertheless, the clinical application is restricted by limitations [8-14], including: (1) patients should be selected on the basis of EGFR mutations rather than EGFR amplification or overexpression; and (2) primary or acquired drug resistance after a short time of usage. Previous studies found that in tumor biopsy samples, 55 to 61% of the samples were EGFR-positively and 32 to 45% had EGFR amplification, without fully overlapping each other [32-34]. Yet, the occurrence of EGFR gene mutations was only 10 to 40% [35-38]. Our present findings suggest that overexpression of RBM5 could inhibit EGFR expression by either direct or indirect ways in A549 cells. The cell line A549 was chosen for this study not only because it has the lowest RBM5 expression in seven different lung cancer cell lines [17], but also because it has wild-type EGFR-positive expression and gene amplification [39,40], which are more common in NSCLC. It might be concluded that NSCLC with EGFR-positive expression or gene amplification could be treated by exogenous RBM5, resulting in EGFR suppression. Our results could have a potential implication for lung cancer treatment, and uncover a
new promising therapeutic strategy to suppress the EGFR pathway, which is induced by the overexpression of RBM5. Taken together, our study demonstrates a prospective meaning that overexpression of RBM5 in NSCLCs would lead to tumor suppression through EGFR inhibition. RBM5 may act as a novel therapeutic target in terms of gene therapy.

Nevertheless, there were still several limitations in the present study. Firstly, as we have focused on the A549 cell line, additional experiments involving other cancer cell lines or normal and/or immortalized cell lines would help to verify this relationship between these two genes. Secondly, as the upstream regulation of EGFR is still not well understood, further studies concerning whether there are other mechanisms involving in this process are warranted in order to confirm the specific mechanisms of EGFR expression suppression. Thirdly, the relationship between RBM5 and EGFR mutation is yet further. Further investigation is required to determine whether RBM5 is able to modulate EGFR expression when EGFR mutations exist.

Conclusions
We demonstrate that EGFR expression is regulated by RBM5 in vivo and in vitro in a direct or indirect way, and that may be one of the predominant mechanisms by which RBM5-mediated tumor suppression. These findings also indicate that RBM5 is the upstream regulator of the EGFR pathway.

Abbreviations
RBM5: RNA binding motif 5; EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; RPWI: Roswell Park Memorial Institute; GFP: green fluorescence protein; TV: LV-RBM5: Recombined pGC-LV-GV287-GFP vector with the RBM5 (NM_005778) gene; TV: LV-RBM5: pGC-LV-GV287-GFP with a scrambled control sequence; M0: multiplicity of infection; p.f.u.: plaque-forming units; RT-qPCR: real-time quantitative polymerase chain reaction; CFU: colony-forming units; TBST: tris-buffered saline and Tween-20 solution; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; TSC: tumor suppressor gene; TKIs: tyrosine kinase inhibitors.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
ZZS performed all the experiments and drafted the manuscript. JZY and RWL participated in the RNA and protein extraction. HL and JZ participated in the data analysis. KW contributed to the research design, data collection, and critically revising the manuscript. All authors have read and approved the final version of the manuscript.

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