

MicroRNA-15a induces cell apoptosis and inhibits metastasis by targeting BCL2L2 in non-small cell lung cancer

Tian Yang · Asmitananda Thakur · Tianjun Chen ·
Li Yang · Gao Lei · Yiqian Liang · Shuo Zhang · Hui Ren ·
Mingwei Chen

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Abstract MicroRNAs (miRNAs) play a critical role in cancer development and progression. Aberrant expression of miR-15a has recently been reported in several cancers, but its role in non-small cell lung cancer (NSCLC) still remains obscure. We investigated the effects of miR-15a on proliferation, apoptosis, and metastasis in A549 cells. Eighteen paired NSCLC and adjacent non-tumor lung tissues were surgically removed and immediately snap frozen until total RNA was extracted and confirmed by two independent pathologists. The targets of miR-15a were predicted by bioinformatics tools. RNA isolation and quantitative real-time PCR (qRT-PCR), Western blot analysis, cell proliferation assay, cell cycle analysis, cell apoptosis assay, and migration and invasion assays were done. The wild type (WT) or mutant type (MT) 3'-untranslated region (UTR) vectors were co-transfected with miR-15a or negative control into A549 cells, and after 24 h of transfection, luciferase activity was measured using the Dual-Glo luciferase assay kit. Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). *P* values of less than 0.05 were considered statistically significant. miR-15a was significantly downregulated in NSCLC than in adjacent non-cancerous tissues. miR-15a overexpression remarkably inhibited cell viability, invasion, and migration and promoted the apoptosis of NSCLC cells.

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T. Yang · A. Thakur · T. Chen · L. Yang · G. Lei · Y. Liang ·
S. Zhang · H. Ren · M. Chen (✉)
Department of Respiratory and Critical Care Medicine, The First
Affiliated Hospital, School of Medicine, Xi'an Jiaotong University,
277 Yanta West Road, Xi'an 710061, People's Republic of China
e-mail: chenmingwei64@hotmail.com

M. Chen
e-mail: chenmw36@163.com

Additionally, inhibition of miR-15a expression had the opposite effects on tumor progression, while cell cycle remained unaltered. Furthermore, we identified that BCL2L2 was a target of miR-15a and negatively regulated by miR-15a at the translational level. miR-15a acts as a tumor suppressor in NSCLC by directly targeting BCL2L2 and may serve as a potential diagnostic biomarker and therapeutic target for NSCLC.

Keywords Non-small cell lung cancer · miR-15a ·
Apoptosis · Metastasis · BCL2L2

Introduction

Lung cancer is the most common cause of death among all cancers [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85 % of all lung cancers. Despite major advances in combination chemotherapy and surgical techniques, the prognosis of NSCLC is still dismal and the 5-year survival rate with all stages and subtypes combined remains as low as 11 % [2]. Therefore, the understanding of the potential mechanisms of the tumorigenesis of NSCLC is urgently needed. Recently, accumulating evidence has shown that microRNAs (miRNAs) play a pivotal role in NSCLC pathogenesis, which provides new insights into the targeted therapy of this disease.

miRNAs are endogenous non-coding small RNAs about 20–24 nucleotides in length that function in transcriptional and posttranscriptional regulation of gene expression by pairing with complementary nucleotide sequences in the 3'-untranslated region (UTR) of a target messenger RNA (mRNA) in various biological processes including cell

proliferation, invasion, and apoptosis. Recent studies have demonstrated that miRNAs can function either as oncogenes or tumor suppressors and are aberrantly expressed in many types of human cancers. Upregulated miRNAs (miR-301b [3], miR-146a [4], and miR-361-5p [5]) function as oncogenes by negatively regulating tumor suppressor genes, whereas low-level miRNAs (miR-125b [6], miR-148b, and miR-152 [7]) could function as tumor suppressor genes and inhibit cancer by regulating oncogenes [8]. Among them, downregulation of miR-15a has been frequently reported and could be involved in the carcinogenesis and progression of many human cancers. However, the role that miR-15a plays in the carcinogenesis of NSCLC is still unclear. Overexpression of BCL2L2 was previously reported in various human cancer cell lines and primary tumors, but limited information is available about its roles or modes of action. There are reports supporting the notion that expression of this molecule is also deregulated in lung adenocarcinoma and may contribute to the pathogenesis of this disease. In the present study, for the first time, we investigated the effects of miR-15a on proliferation, apoptosis, and metastasis in A549 cells and identified miR-15a as a tumor suppressor to induce apoptosis and suppress metastasis of NSCLC cells through targeting BCL2L2.

Materials and methods

Tissue samples and cell lines

Eighteen paired NSCLC and adjacent non-tumor lung tissues were collected from patients who underwent primary surgical resection of NSCLC at the First Affiliated Hospital of the Medical College of Xi'an Jiaotong University. Surgically removed samples were immediately snap frozen in liquid nitrogen and stored at -80°C until total RNA was extracted and confirmed by two independent pathologists. This study was approved by the local medical ethics committee, and informed consent was obtained from all patients. A549 cells were purchased from Shanghai Institute of Cell Biology, China, and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (both from HyClone, USA) and 1 % penicillin/streptomycin (Invitrogen) at 37°C in 5 % CO_2 .

Plasmid constructions and transfection

To construct vectors of miR-15a overexpression, we synthesized chemically the pre-miR-15a and cloned pre-miR-15a into pcDNATM 6.2-GW/EmGFP-miR vector between the EcoRI and HindIII sites, and we got the predicted fragment of targeted gene (BCL2L2) through bioinformatics

analysis (<http://www.targetscan.org/>) according to base pair complementary binding of miR-15a. The seed sequence (wild type, WT) and mutagenesis (mutant type, MT) of BCL2L2 3'-UTR were chemically synthesized and cloned into pmirGLO Dual-Luciferase miRNA vector (XhoI/SacI). All sequences were synthesized by Shanghai GenePharma.

All transfection experiments were performed using LipofectamineTM 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Transfected cells were collected 24 h after transfection, and all transfections were carried out in triplicates.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from tissue samples and cell lines using TRIzol (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq II (TAKARA, Dalian, China) on the FTC-3000TM System (Funglyn Biotech Inc., Toronto, Canada). The special primer was used to synthesize miR-15a cDNA. The relative expression of genes was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method from an independent experiment, and β -actin and U6 were used to normalize mRNA and miRNA, respectively. Primers for qRT-PCR are shown in Table 1.

Western blot analysis

After a 24-h transfection, cells were lysed with RIPA lysis buffer and the protein concentration was determined. The proteins (20 μg) were electrophoresed by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 2 h at room temperature with 5 % non-fat milk. The membranes were incubated with primary antibodies

Table 1 Sequences of primers for qRT-PCR

Name	Sequence (5'-3')
miR-15a-RT	GTCGTATCCAGTGCCTGTCGTGGAGTCGGC AATTGCACTGGATACGACCACAAAC
miR-15a-F	ATCCAGTGCCTGTCGTG
miR-15a-R	TGCTTAGCAGCACATAATG
BCL2L2-F	CACAAGTGCAGGAGTGGATGG
BCL2L2-R	CCCGTATAGAGCTGTGAACTCC
U6-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCAC TGGATACGACAAAATATGG
U6-F	TGCGGGTGTCTCGCTTCGGCAGC
U6-R	CCAGTGCAGGGTCCGAGGT
β -Actin-F	AGTGTGACGTGGACATCCGCAAAG
β -Actin-R	ATCCACATCTGCTGGAAGGTGGAC

(anti-Bcl-2L2, #16026-1-AP, Proteintech; anti-BAX, #50599-2-Ig, Proteintech; anti-caspase-3, #10380-1-AP, Proteintech; anti-caspase-9, #10380-1-AP, Proteintech; β -actin, # sc-47778, Santa Cruz) overnight at 4 °C, followed by secondary anti-rabbit or anti-mouse (Pierce). Protein expression was assessed by chemiluminescence.

Cell proliferation assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 cells were seeded in 96-well plates at 3000 cells/well. After 24, 48, and 72 h of transfection, 20 μ l of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. Then, the supernatant was removed; the remaining crystals were dissolved in 150 μ l DMSO. Optical density was measured at 490 nm using a microplate reader.

Cell cycle analysis

Twenty-four hours after transfection, A549 cells were harvested, fixed with 70 % ethanol at 4 °C overnight. After centrifugation, cells were re-suspended and incubated with 0.1 mg/ml RNase A and 0.05 mg/ml propidium iodide (PI) for 30 min at 4 °C. The cell cycle was then analyzed by flow cytometry.

Cell apoptosis assay

Cell apoptosis was analyzed by Annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's protocol. After a 24-h transfection, cells were collected, centrifuged for 5 min at 1500 rpm, and re-suspended in 1 \times binding buffer. Then, 5 μ l Annexin V-FITC and 5 μ l PI were added to incubate the cells at room temperature for 15 min. The cells were then analyzed using a flow cytometer.

Migration and invasion assays

The transfected cells were trypsinized and suspended with DMEM containing 1 % fetal bovine serum (FBS). The suspended cells were seeded in the upper chamber of the Transwell insert (with Matrigel for invasion and without for migration), and DMEM containing 10 % FBS (600 μ l) was added to the lower chamber of the Transwell insert. The cells were cultured for 24 h at 37 °C. The cells in the upper chamber were removed with cotton swabs; then, the chamber membranes were fixed in 10 % methanol for 20 min and air dried. The cells were then stained with crystal violet for 30 min at 37 °C, followed by washing with PBS. The visual fields were randomly counted under high magnification.

Luciferase report assay

The WT or MT 3'-UTR vectors were co-transfected with miR-15a or negative control into A549 cells using Lipofectamine™ 2000 reagent. After 24 h of transfection, luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega) according to the manufacturer's protocol. The normalized firefly luciferase activity was calculated as the quotient of firefly/*Renilla* luciferase activity.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as means \pm SD. Student's *t* test was performed to compare the means between two samples. *P* values of less than 0.05 were considered statistically significant.

Results

miR-15a is downregulated in human NSCLC tissues

To analyze the expression of miR-15a in human NSCLC patients, we measured the expression of miR-15a in 18 pairs of human NSCLC tissues and adjacent normal tissues by qRT-PCR. As shown in Fig. 1, the expression of miR-15a was significantly decreased in 15 NSCLC tissues compared to their matched normal tissues ($P < 0.05$), suggesting that miR-15a may act as a tumor suppressor involved in lung carcinogenesis.

Overexpression of miR-15a inhibits growth of NSCLC cells

To determine the function of miR-15a in NSCLC, the A549 cells were transiently transfected with an miR-15a overexpression

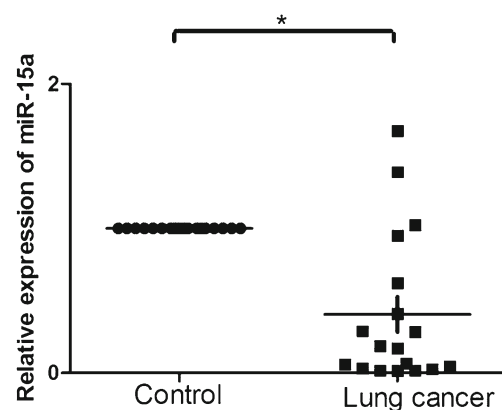


Fig. 1 Expression level of miR-15a in NSCLC tissues. qRT-PCR was used to determine the expression of miR-15a in 18 NSCLC tissues and matched adjacent non-tumor normal tissues. * $P < 0.05$

plasmid or the antisense oligonucleotide of miR-15a (anti-miR-15a) and efficiency of the transfection was examined by qRT-PCR at 24 h after transfection. The expression of miR-15a in cells with miR-15a was increased (Fig. 2a), but its expression in cells with anti-miR-15a was decreased (Fig. 2b). Next, MTT assay showed that overexpression of miR-15a could markedly inhibit cell proliferation (Fig. 2c), and A549 cells transfected with miR-15a formed significantly fewer colonies than the cells transfected with NC (Fig. 2e). Consistent with this result, we also confirmed the effect of miR-15a on proliferation and colony formation of A549 cells through transfection with anti-miR-15a (Fig. 2d, f). Inhibition of cell growth in cancer cells is usually induced by cell cycle arrest [9, 10]. To further determine whether the suppressive role of miR-15a is related to the cell cycle regulation, flow cytometric analysis was used to measure the changes in the cell cycle distribution after transfection with miR-15a. However, as shown in Fig. 2g, h, there were no significant differences among A549 cells transfected with miR-15a and anti-miR-15a compared to the control group. These results suggested that overexpression of miR-15a could inhibit cell growth without cell cycle regulation in A549 cells.

miR-15a enhances cell apoptosis in NSCLC cells

To evaluate the effects of miR-15a on NSCLC survival, the cell apoptosis assay was performed using the Annexin V and

PI double staining. As shown in Fig. 3a, miR-15a significantly promoted cell apoptosis in A549 cells compared with the negative control. Meanwhile, A549 cells transfected with anti-miR-15a resulted in a significant reduction of cell apoptosis (Fig. 3b), suggesting that miR-15a overexpression induced apoptosis in A549 cells.

miR-15a inhibits cell migration and invasion of NSCLC cells

To investigate the role of miR-15a in NSCLC metastasis, migration and invasion assays were performed. The migration ability of A549 cells was significantly inhibited in the groups transfected with miR-15a compared with the negative control (Fig. 4a, b). Meanwhile, the invasion assay showed that cell invasion was remarkably inhibited in A549 cells transfected with miR-15a (Fig. 4e, f). By contrast, the anti-miR-15a significantly increased the migration and invasion ability (Fig. 4c, d, g, h), suggesting that miR-15a function can inhibit migration and invasion in A549 cells.

BCL2L2 is a potential target of miR-15a

We predicted hundreds of potential miR-15a target genes using bioinformatics method (<http://www.targetscan.org/>), which showed that the 3'-UTR of BCL2L2 contains a seed sequence matched with mature miR-15a. To evaluate the direct effect of miR-15a on BCL2L2 expression, we constructed reporter plasmids containing wild-type or mutant

Fig. 2 miR-15a induces apoptosis of A549 cells. **a, b** Apoptosis was analyzed using Annexin V and PI kit after treatment with miR-15a and anti-miR-15a. The data represent three independent experiments and a representative picture is shown. * $P < 0.05$

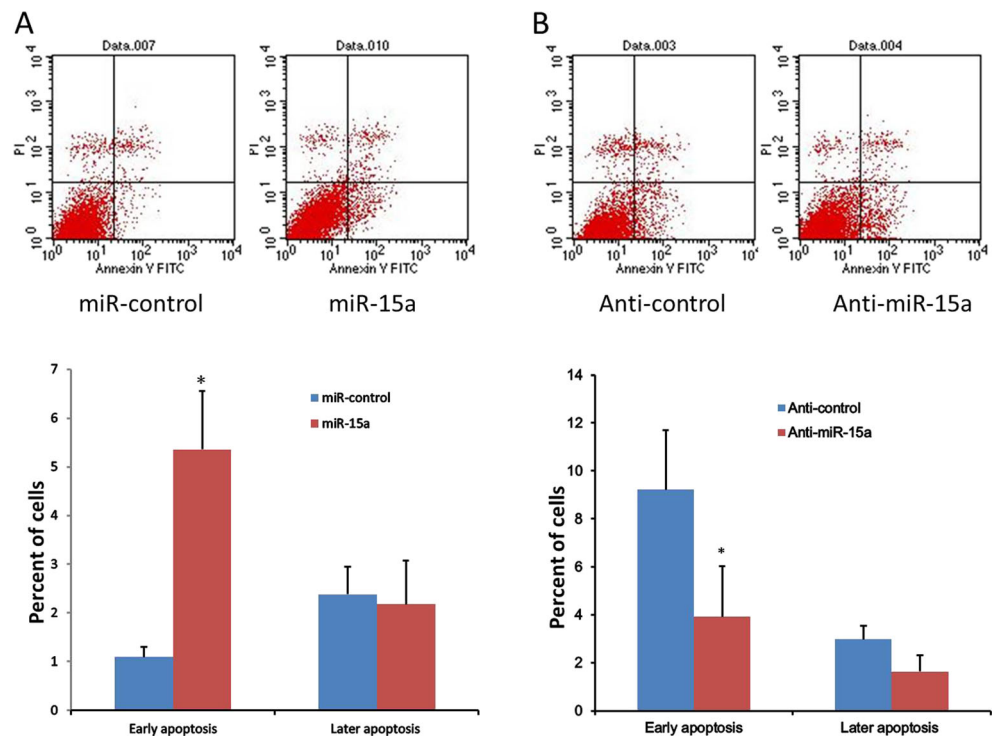


Fig. 3 miR-15a inhibits cell growth in A549 cells. **a, b** The expression of miR-15a in A549 cells by transfection of miR-15a and anti-miR-15a using qRT-PCR compared with the negative control. **c, d** The effects of miR-15a and anti-miR-15a on A549 cell proliferation were determined by MTT assay. **e, f** Representative results of colony formation of A549 cells after transfection of miR-15a and anti-miR-15a. **g, h** Cell cycle distribution was analyzed after treatment with miR-15a. The data represent three independent experiments and representative pictures are shown. * $P < 0.05$

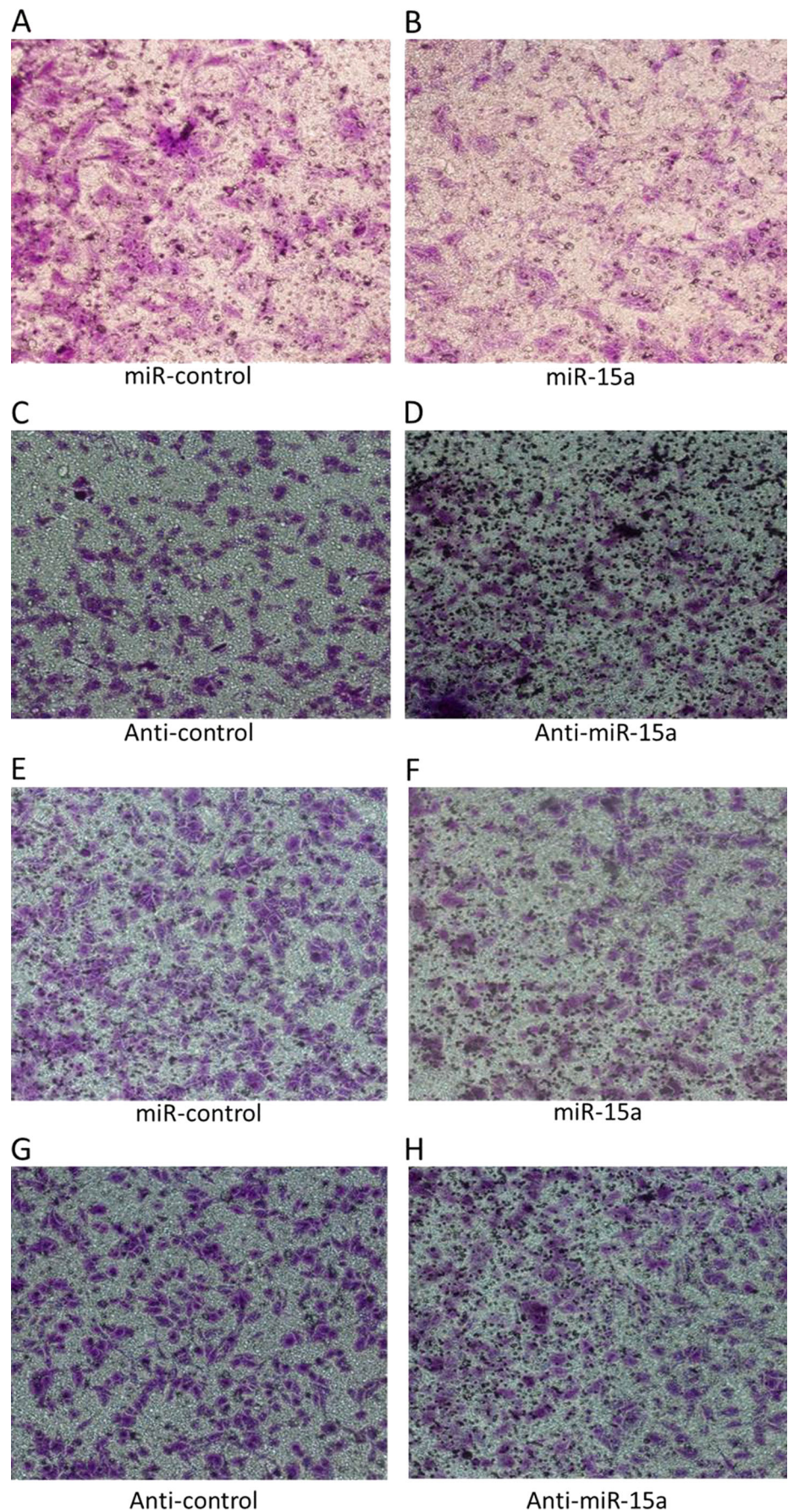
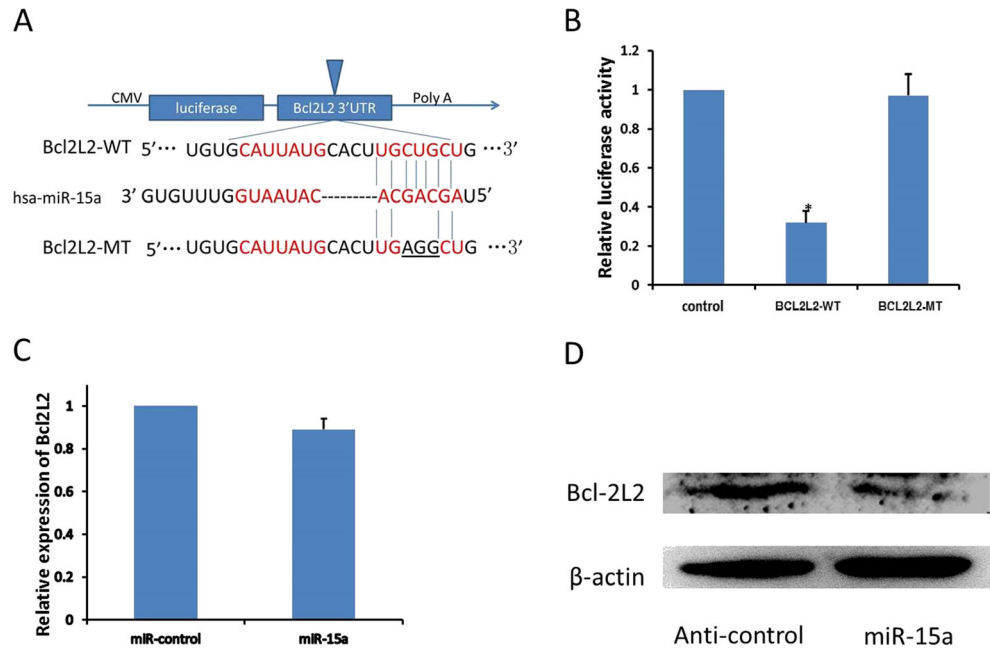


Fig. 4 BCL2L2 is a potential target of miR-15a. **a** Diagram of the BCL2L2 3'-UTR containing reporter constructs. **b** The relative luciferase activity was measured in A549 cells after co-transfection of the BCL2L2 luciferase construct with either miR-15a or control. **c** BCL2L2 mRNA level was analyzed by qRT-PCR and normalized to β -actin expression. **d** Western blot analysis of BCL2L2 protein level in A549 cells transfected with miR-15a or miR-control. Data represent mean \pm SEM from three independent experiments. * P <0.05



3'-UTR of BCL2L2, which was cloned into the luciferase gene, respectively (Fig. 5a). As shown in Fig. 5b, miR-15a significantly suppressed the luciferase activity of the BCL2L2-WT 3'-UTR (P <0.05.), without effect on the BCL2L2-MT in A549 cells. In addition, although overexpression of miR-15a had no effect on the mRNA expression of BCL2L2 (Fig. 5c), it significantly decreased the protein level of BCL2L2 (Fig. 5d), indicating that miR-15a suppressed BCL2L2 expression posttranscriptionally in A549 cells. Consistently in these cells, the downstream effectors of BCL2L2 such as caspase-9, caspase-3, and BAX were also increased by miR-15a overexpression (Fig. 6a). On the contrary, in A549 cells with anti-miR-15a, the downstream protein levels of BCL2L2 were dramatically inhibited (Fig. 6b). Taken together, these results suggest that miR-15a suppresses BCL2L2 expression posttranscriptionally and regulates cell apoptosis and metastasis in A549 cells.

Discussion

miRNAs have been implicated in the regulation of multiple biological processes, including cell proliferation, apoptosis, differentiation, and cell death. Previous studies showed that miR-15a was commonly decreased in several human cancers. However, its underlying molecular mechanism remains controversial. miR-15a was significantly underexpressed in primary multiple myeloma (MM), and the aberrant expression of miR-15a could play a role in the tumorigenesis of MM by

modulation of angiogenesis through targeting VEGF-A [11]. Shi et al. reported that miR-15a inversely correlated with AP4 protein level in colorectal cancer and further defined a double-negative feedback loop involving miR-15a and AP4 that stabilizes epithelial-mesenchymal transition (EMT) and metastatic progression [12]. Moreover, it was found that downregulation of miR-15 in cancer-associated fibroblasts (CAFs) promoted tumor growth and invasiveness through reduced posttranscriptional repression of *Fgf-2* and its receptor *Fgfr1*, which act on both stromal and tumor cells in prostate cancer [13].

In our study, there were 13 tissue samples of adenocarcinoma, 4 samples from squamous cell carcinoma, and 1 sample of large cell carcinoma. The expression of miR-15a was significantly decreased in 15 NSCLC tissues compared to their matched normal tissues (P <0.05). Two samples of adenocarcinoma and one of squamous cell carcinoma had higher expression of miR-15a. However, statistically significant differences were observed between NSCLC tissues compared to their matched normal tissue samples. We demonstrated that miR-15a expression was significantly decreased in NSCLC tissues. Bandi et al. indicated that miR-15a induced cell cycle

Fig. 5 miR-15a inhibits cell migration and invasion in A549 cells. **a-d** Cell migration ability of A549 cells transfected with miR-15a and anti-miR-15a was evaluated by Transwell analysis and compared with the negative control. **e-h** Cell invasion ability of A549 cells transfected with miR-15a and anti-miR-15a was evaluated by Matrigel invasion chamber and compared with the negative control

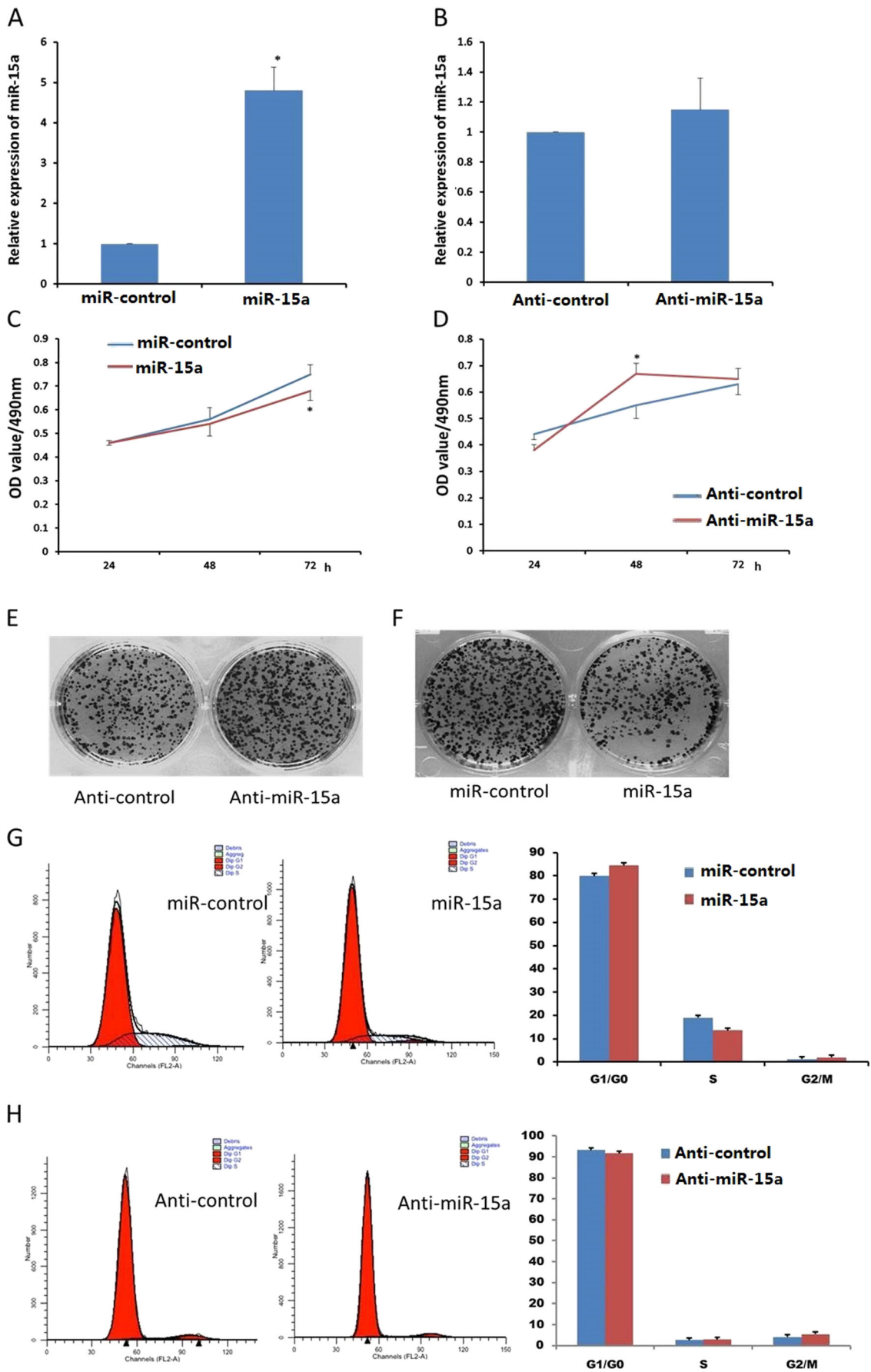
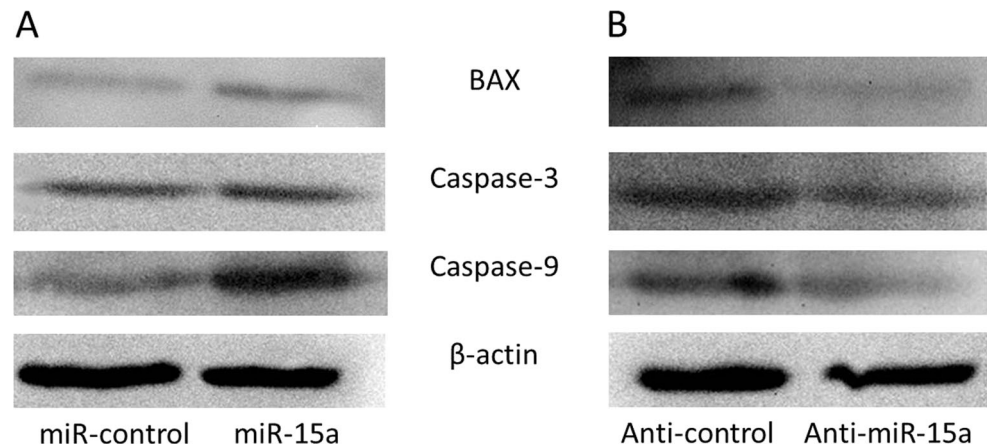


Fig. 6 miR-15a increases the downstream protein levels of BCL2L2. **a** Protein levels of caspase-9, caspase-3, and BAX were measured by Western blot after transfection of miR-15a and miR-control into A549 cells. **b** Western blot results of caspase-9, caspase-3, and BAX in A549 cells after transfection of miR-15a. β -Actin was used as an internal control



arrest by targeting G1 cyclins and contributed to the tumorigenesis of NSCLC [14]. However, in the present study, forced overexpression of miR-15a suppressed cell proliferation and induced apoptosis, without affecting the cell cycle; then, we supposed that miR-15a indirectly inhibited cell survival through inducing apoptosis. Furthermore, miR-15a overexpression could suppress invasion of NSCLC cells. Taken together, these data suggested that miR-15a might be a novel tumor suppressor in NSCLC.

BCL2L2, also known as BCL-W, is a prosurvival member of the Bcl-2 protein family and functions as an oncogene. Upregulation of BCL2L2 was reported in various human cancers, such as gastric cancer [15], colon cancer [16], and cervical cancer [17]. The expression of BCL2L2 was repressed to enhance cell apoptosis activity in colon cancer cells transfected with miR-195 [16]. In addition, recent studies have shown that the expression of BCL2L2 was significantly associated with infiltrative morphotypes [15]. The overexpression of BCL2L2 in gastric cancer cells increased their migratory and invasive potentials by activating the PI3K/Akt pathway [18] or matrix metalloproteinase 2 (MMP-2) [19]. Some BCL-2 family members share homology with BCL-2 and have anti-apoptotic properties, while others have pro-apoptotic properties. BCL2L2 is increased in a variety of malignancies, including lung cancer. Recent studies showed that BCL2L2 overexpression was significantly associated with differentiation status, tumor stage and poor prognosis [20], and induced apoptosis [21] in lung adenocarcinoma. Selective silencing of BCL2L2 induces spontaneous apoptosis in lung cancer cell lines and sensitizes these cells to cytotoxic agents and radiation. However, lung cancers do vary in their dependence on BCL-2 family members for apoptotic resistance and that the balance in BCL-2 proteins likely contributes to this sensitivity. miRNAs are non-coding RNAs which were much conserved during evolution and have emerged lately as potent regulators of gene expression and cell survival. These miRNAs are able to regulate expression of at least one third of

all human genes and play a critical role in a variety of normal biological processes, including cell differentiation, apoptosis, and development. BCL2L2 is a prosurvival member of the BCL2 protein family which acts as an inhibitor of apoptosis. Although overexpression of BCL2L2 was previously reported in various human cancer cell lines and primary tumors, limited information is available about its roles and modes of action. In our study, although BCL2L2 was negatively regulated directly by miR-15a in NSCLC cells as shown by dual luciferase assays and Western blot, miR-15a has no effect on the BCL2L2 mRNA levels detected by qRT-PCR, suggesting that miR-15a negatively regulates BCL2L2 expression at the posttranscriptional level. Moreover, the downstream proteins of BCL2L2 were also further identified. Therefore, these results indicated that miR-15a might induce apoptosis and suppress metastasis by targeting BCL2L2 in NSCLC.

Our study has certain limitations. As the sample size for tissue specimens is relatively small, we did not analyze for variation in expression of miR-15a between tissue samples of NSCLC. It is clinically valuable to study the differential expressions in NSCLC subtypes; we believe our results will encourage future research with a large cohort to investigate the difference among NSCLC subtypes and promote in vivo experiments to verify the candidature of BCL2L2 as a therapeutic target in NSCLC.

In conclusion, we found that miR-15a was significantly downregulated in clinical specimens of NSCLC. By targeting BCL2L2, overexpression of miR-15a can indirectly decrease the cell viability through repression of apoptosis and also inhibit metastasis in NSCLC cells. Further study will be needed to clarify the potential of miR-15a and BCL2L2 as a therapeutic target and clinical biomarker in NSCLC.

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Conflicts of interest None

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