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miR-34b inhibits the migration/invasion and promotes apoptosis of non-small-cell lung cancer cells by YAF2

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Abstract. – OBJECTIVE: Lung cancer is the leading cause of cancer death in the world and microRNAs (miRNA) have been found to be involved in the initiation and development of cancer by acting as potential oncogenes or tumor suppressor genes.

PATIENTS AND METHODS: In this study, we investigated the expression of miR-34b in non-small cell lung cancer (NSCLC) patients and discussed the molecular mechanism of miR-34b in the invasion and migration of A549 cells in vitro.

RESULTS: Our results showed that miR-34b was significantly down-regulated in primary cancer tissues when compared with the normal lung tissues. The over-expression of miR-34b inhibited migration and invasion, and promoted apoptosis of A549 lung cancer cells. Furthermore, Luciferase reporter assay validated YY1-associated factor 2 (YAF2) as a direct target of miR-34b and YAF2 expression was significantly increased in clinical NSCLC tissue samples. In addition, the over-expression of miR-34b inhibited YAF2, p-Jak2, p-STAT3 and MMP2 protein expression and promoted caspase 3 protein expression in cancer cells.

CONCLUSIONS: Our results suggest that miR-34b may inhibit migration and invasion of NSCLC cells by targeting YAF2. Thus, our findings provide new insight into the molecular mechanisms of lung cancer metastasis and miR-34b may serve as a potential target in the treatment of human lung cancer.

Key Words:
MiR-34b, Non-small cell lung cell, YAF2, JAK2/STAT3, MMP2.

Introduction

Lung cancer is a commonly diagnosed and highly aggressive tumor worldwide. Non-small cell lung cancer (NSCLC), which comprises approximately 80% of all lung cancers, is the main cause of the majority of lung cancers[1]. NSCLC can be subdivided into four major histological subtypes and NSCLC patients are frequently diagnosed at an advanced stage, suffering by metastatically or locally advanced diseases[2]. Lung cancer carcinogenesis is a multistep process through the accumulation of genetic and epigenetic alterations and is less sensitive to anticancer drugs and radiation therapy[3]. Although great efforts and progressions have been made in the study of lung cancer in recent decades, the molecular mechanisms of pathogenesis remain elusive. Thus, understanding the precise molecular biology and further elucidation of possible targets for preventing the initiation and progression of lung cancer is imperative and essential for the development of effective therapy.

MicroRNAs (miRNAs) are endogenously small non-coding RNA molecules (19-22 bases in length) that bind to imperfect sequence homology sites of mRNA's 3' untranslated regions (3'UTR), which causes either degradation or inhibition of protein translation[4]. Under normal physiological conditions, the miRNAs gene is expressed in various human tissues and specific over-expressed miRNAs may be involved in maintaining tissue homeostasis[5]. It was reported that miRNAs can modulate more than 30% of the human genes; meanwhile, about 60% of the mRNAs can interact with several different miRNAs[6]. Accumulating evidence suggests that miRNAs aberrantly expressed in many human cancers may play significant roles in human oncogenesis and metastasis[7]. The miR-34 family, which consists of miR-34a, miR-34b and miR-34c, has been demonstrated to be critical tumor suppressors in the p53 pathway

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and have important roles in reducing cell growth, inducing apoptosis and affecting cancer initiation and progression[8]. For example, miR-34a has been demonstrated to inhibit proliferation and migration of breast cancer cells through down-regulation of Bcl-2 and SIRT1[9].

Recently, the miR-34 family has also been demonstrated to be associated with the development and progression of NSCLC. For example, the plasma miR-34a and miR-34c expression were negatively correlated with lymph node metastasis in NSCLC patients[10]. Although the miR-34b gene has a high homology and shares similar set of target genes, its important roles in carcinogenesis still need to be investigated. Indeed, a recent study showed that miR-34b could inhibit the migration and invasion of metastatic prostate cancer cells by regulating the transforming growth factor-β (TGF-β) pathway[11]. In addition, the low expression of miR-34b was found in NSCLC patients, suggesting that miR-34b may play a critical role in the development of NSCLC[12]. However, the potential molecular mechanisms of miR-34b in lung cancer cells have not been documented until now.

In the present work, the expression level of miR-34b in tumor tissues of NSCLC patients was determined. We further investigated the mechanisms of miR-34b on migration and invasion of NSCLC cells *in vitro* and detected that this effects may be mediated by the potential target YY1-associated factor 2 (YAF2). These findings will provide new insights into the molecular mechanisms of metastasis and provide a therapeutic strategy for the treatment of lung cancer.

Patients and Methods

Clinical Tissues and Cell Culture

A total of 45 lung tissues and normal lung tissues were collected from the same NSCLC patients enrolled from June 2015 to March 2017 by the Tianjin Medical University General Hospital. The present study has obtained the patients' informed consent and has been approved by the Ethics Committee of Tianjin Medical University General Hospital.

Human NSCLC cell line A549 was purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

RNA Isolation and Expression Analysis by qRT-PCR

For determination of miRNA and mRNA levels, total RNA was extracted from tumor tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At last, the concentration of RNA was measured by using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA synthesis was then performed according to the manufacturer's recommendation. Briefly, total RNA was reverse-transcribed into cDNA using a HiFiScript cDNA Synthesis Kit (CWBIO, Beijing, China) with miRNA-specific RT primers (Ribobio, Guangzhou, China) according to the manufacturer's guidelines. The sequences of the primers were as follows: miR-34b, forward, 5'-TCTATTTGCCATCGTCTA-3', reverse, 5'-CAGGCAGCTCATTTGGAC-3'; U6, forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'; After the RT reaction, the expressions were detected using LightCycler 480 SYBR-Green I Master and the LightCycler 480 Real Time-Polymerase Chain Reaction system (both from Roche Applied Science, Indianapolis, IN, USA). The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Dual-Luciferase Reporter Assay

Target Scan (http://www.targetscan.org/) was used to predict the targeted relationship between miR-34b and YAF2. Wild- or mutant-type 3'-UTR of YAF2 were synthesized and subcloned into the pcDNA3.1/HisC vector (Invitrogen, Carlsbad, CA, USA). Then, the psiCHECK2 Firefly Luciferase reporter plasmids as well as miR-34b mimics or miR-34b negative control were used to transfect the 293T cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 24 h after transfection, Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). Normalized Luciferase activity was reported as Luciferase activity/Renilla Luciferase activity.

Cells Transfection

Cells were seeded at a density of 6 x 10^5 cells in 60-mm dishes, and grown to 80% confluency for transfection. A549 cells were transfected with miR-34b mimic or miR-34b negative control (RiboBio, Guangzhou, China). by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. Briefly, microRNA mimic in 250 μL of Opti-MEM® (Gibco, Grand Island, NY, USA) medium was added to 10 μL of Lipofectamine in

250 μL of Opti-MEM® medium and incubated for 20 min at room temperature. Cells were maintained in 3 mL of Opti-MEM® medium containing the complexes for 12 h at 37°C; then, the medium was removed and replaced with serum-supplemented medium and cultured for another 36 h. After transfection for 48 h, cells were harvested and the transfection efficiency was determined by qRT-PCR.

Cell Proliferation Assay

A549 cells transfected with miRNA for 48 h were seeded into 96-well plates and incubated at 37°C for 12 h, 24 h and 48 h, respectively. Then, 10 μL of CCK-8 solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C. The plated was incubated for 15 min at room temperature and the absorbance was measured at 490 nm using a microplate assay reader (BioTek Instruments,Winooski, VT, USA).

Cell Apoptosis Assay

Apoptosis was evaluated microscopically and by a flow cytometry-based assay using the annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining kit (BD Biosciences, Franklin Lakes, NJ, USA). Apoptotic cells were indicated as annexin-V-positive. Cells were trypsinized, washed twice with PBS and centrifuged at 1000 rpm for 5 min at room temperature. Then, the cells were resuspended in 1x binding buffer, 5 μL of PI and FITC were added per 1 x 10^5 cells, cells were vortexed and incubated for 15 min at room temperature in the dark. Subsequently, cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound Healing Assay

The cells transfected with miRNA were trypsinized and seeded in 24-well plates, cultured overnight in complete medium. The monolayers of cells were scratched using a 200-μl pipette tip. Cells were then washed with culture medium to remove cellular debris and allowed to culture again up to 24 h in serum-free medium. Images were captured under an Eclipse Ti-U (Nikon, Kanagawa, Japan) inverted microscope following wounding (0 and 24 h). The relative surface traveled by the leading edge was assessed using Image-Pro Plus version 6.0 software.

Invasion Assays

The cells (2.5×10^4 cells) in the serum-free medium were planted to the upper transwell chamber (Corning Incorporated, Corning, NY, USA; 24-well insert, pore size: 8 mm) of the insert pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The complete medium (500 μl) containing 10% FBS was placed to the lower chambers as a chemoattractant. After 40 h of incubation, the cells that did not migrate or invade through the pores were carefully wiped off by wet cotton swab. The inserts were then stained with 20% methanol and 0.2% crystal violet, imaged with an Eclipse Ti-U inverted microscope, and invaded cell number per field was counted by Image-Pro Plus version 6.0.

Western Blot Analysis

Total proteins from cells were homogenized using RIPA lysis buffer. The protein concentrations were determined by the BCA protein reagent (Pierce, Waltham, MA, USA). Then, these proteins were separated through SDS/PAGE before being transferred to the polyvinylidene difluoride (PVDF) membrane. The primary antibodies were as follows: rabbit polyclonal anti-YAF2 antibody (1:500, ab15251, Abcam, Cambridge, MA, USA), mouse monoclonal β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), horseradish peroxidase (HRP) -conjugated anti-rabbit secondary antibody (1: 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-mouse secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was washed by Tris-Buffered Saline and Tween 20 (TBST) three times with 10 min each time. The anti-β-actin antibody was used as the internal reference. Finally, the active bands were visualized with an enhanced chemiluminescence (ECL) system.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Data are shown as means ± standard deviation (SD), and One-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test were used to examine the differences among the three groups. The comparison between two different groups was performed by the *t*-test and differences were considered statistically significant if $p<0.05$.

Results

Expression Level of MiR-34b in Clinical Specimens

The expression level of miR-34b in tumor tissues and non-tumor tissues of NSCLC patients

was determined by qRT-PCR. The results showed that the relative expression of miR-34b was significantly lower than that in non-tumor tissues (p<0.01) (Figure 1), suggesting that the low expression of miR-34b might be associated with poor overall survival in NSCLC patients.

The Effects of MiR-34b on the Proliferation and Apoptosis of A549 Cells

Considering the correlation between miR-34b expression and lung cancer progression, we further investigated the effect of miR-34b on the proliferation and apoptosis of lung cancer cell. A549 cells were transfected with miR-34b mimic or miR-34b mimic negative control and qRT-PCR results showed that miR-34b was significantly up-regulated in cells transfected with mimic compared to that of the control group and mimic negative group (Figure 2A).

A549 cells transfected with miR-34b mimic revealed pronounced growth inhibition compared to that transfected with a mimic negative control group (Figure 2B). Consistently, the results of flow cytometry showed that the percentage of apoptotic cells in the miR-34b mimic group was much higher than that in the control group and mimic negative group (Figure 2C and D). Collectively, these results suggested that the up-regulation of miR-34b inhibited growth and promoted apoptosis in A549 cells.

The Effects of MiR-34b on the Migration and Invasion of A549 Cells

To explore whether miR-34b suppresses metastasis of lung cancer cells, wound healing/scratch assays and transwell migration and invasion assays were performed. A549 cells that were transfected with miR-34b mimic were significantly reduced in the wound healing and transwell assays compared with that of the control (Figure 3A and B). These results demonstrated that the over-expression of miR-34b reduced the abilities of migration and invasion in A549 cells, confirming the biological importance of miR-34b in the inhibition of lung cancer progression.

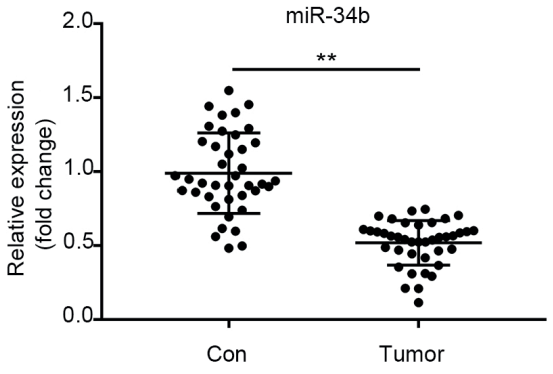

Figure 1. MiR-34b was downregulated in NSCLC. The expression of miR-34b was decreased in tumor tissues compared to that in the non-tumor tissues. Data are presented as means ± SD. **p<0.01.

MiR-34b Regulated YAF2 Expression Directly

To detect the molecular mechanism by which miR-34b suppresses the growth and metastasis of lung cancer cells, we predicted the putative target genes of miR- miR-34b in human cells using the standard online software (TargetScan, http://www.targetscan.org). Among the predicted candidates, YAF2 was of interest in this study. To test whether miR-34b directly targets YAF2, the wild-type or mutant 3'UTR sequence of YAF2 was cloned into a Luciferase reporter vector. Luciferase assay revealed that miR-34b significantly reduced Luciferase activities by directly bounding to YAF2 3 'UTR. However, mutation of the putative miR-34b binding sites in the YAF2 3'UTR abrogated Luciferase response to miR-34b (Figure 4A). Of note, the expression level of YAF2 was significantly higher in tumor tissues of NSCLC patients compared to that non-tumor tissues (Figure 4B). These results illustrated that miR-34b may have a direct impact on the expression of YAF2.

The Mechanisms of MiR-34b on the Growth and Migration of A549 Cells

YAF2 has been demonstrated to play critical roles in cell survival and apoptosis[13,14]. To further confirm that YAF2 acts as a target of miR-34b, we examine the mRNA or protein levels of YAF2 and caspase 3 in A549 cells transfected with miR-34b mimic or miR-34b mimic negative control. As shown in Figure 5A-C, the level of YAF2 was dramatically reduced and caspase 3 expression level was significantly increased in the miR-34b mimic group compared to that in the control group and mimic negative group.

To further explore the mechanism of growth and migration inhibition induced by miR-34b, we examined the effect of miR-34b on the expression of those effectors that are associated with cancer development. As shown in Figure

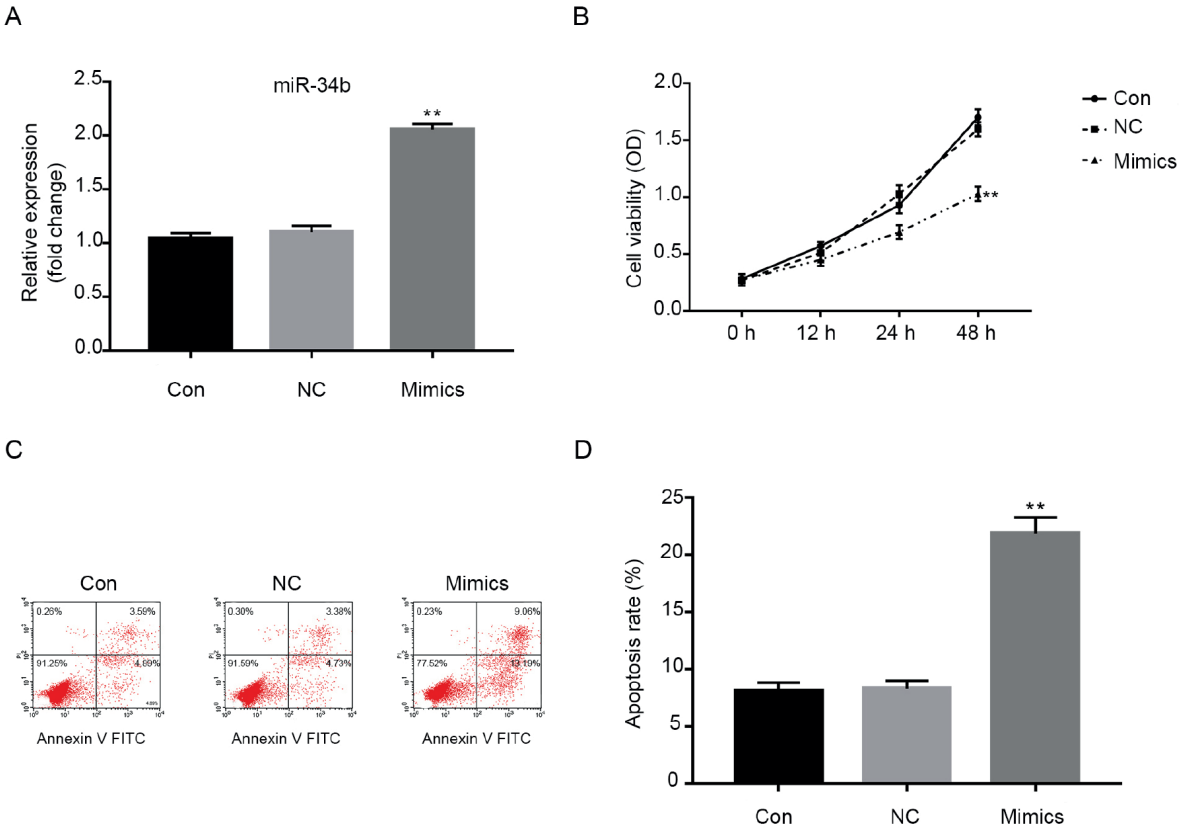

Figure 2. MiR-34b inhibited cell proliferation and promoted apoptosis ability. ***A,*** The expression of miR-34b in cells transfected with miR-34b mimics were significantly higher than that in the control group and NC group. ***B,*** Cells transfected with miR-34b mimics significantly suppressed the proliferation of NSCLC in comparison with the control and NC group. ***C-D,*** The apoptosis rate of cells transfected with miR-34b mimics was crucially increased compared with the control and NC group. Data are presented as means ± SD. ***p*<0.01.

6A, the over-expression of miR-34b significantly decreased p-Jak2, p-STAT3 and MMP2 protein levels in A549 cells. Consistently, the mRNA expression of MMP2 was also dramatically reduced in the miR-34b mimic group compared to that in the control group and mimic negative group (Figure 6B).

Discussion

NSCLC is the most common cause of lung cancer with relatively higher mortality around the world[1]. Great efforts have been made in investigating the pathological mechanisms and potential therapeutic methods of NSCLC. Accumulating evidence has demonstrated that miRNAs play essential roles in the classification and pathogenesis of NSCLC[15]. In this work, the mechanisms of miR-34b in inhibiting growth and metastasis of NSCLC cells were studied *in vitro*.

MiRNAs are a class of small single-stranded noncoding RNA molecules that function in mRNA silencing and the post-transcriptional regulation of gene expression[4]. It has been reported to be associated with the progression of many human cancers[7]. Recent studies have reported that the dysregulated expression of several miRNAs, such as miR-5702[16], miR-106b[17], miR-192[18] and miR-224[19] are involved in the development of lung cancer. The miR-34 family (miR-34a, miR-34b and miR-34c) consists of 3 miRNAs and its low expression has been shown to be correlated with the poor prognosis in NSCLC patients[10]. MiR-34b is an important member that shares a primary transcript on chromosome 11q23 with miR-34c[8]. Previous studies showed that the promoter region of miR-34b is the direct target p53; thus, it may participate in the induction of many cellular processes such as apoptosis, cell cycle arrest and senescence[20]. Indeed, miR-34b has been demonstrated to be a

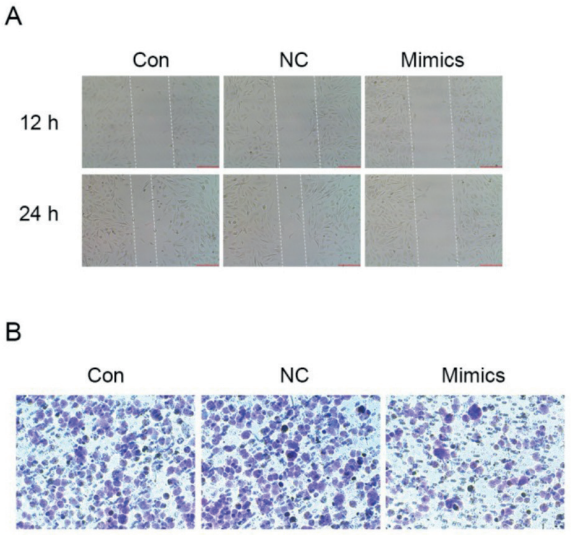

Figure 3. Impact of miR-34b on cell invasion and migration. ***A,*** Cell migration was significantly inhibited in miR-34b mimics compared with control and NC group (x100). ***B,*** The invasion rate was significantly resisted by miR-34b mimics. Data are presented as means ± SD. $^{**}p<0.01$.

tumor suppressor in a number of malignancies including colorectal, pancreatic and soft tissue sarcomas[21]. In lung cancer, the over-expression of miR-34b could increase the radiosensitivity of the p53 wild-type A549 cell line, but not the p53-negative H1299 cell line[22]. Consistent with the results of these findings, our results showed that miR-34b expression was down-regulated in the tumor tissue of NSCLC patients. Further *in vitro* experiments indicated that the overexpression of miR-34b could inhibit the migration and invasion of A549 NSCLC cells. This effect of miR-34b might be relevant to the inhibition of cell growth and promotion of cell apoptosis. However, whether p53 may be involved in the regulatory effect needs to be investigated in future studies.

Many genes and signaling pathways have been demonstrated to be the targets of miR-34b in the regulation of cancer progression and development. For example, the TGF-β signaling pathway is involved in the inhibitory effects of miR-34b on the migration and invasion of metastatic prostate cancer cells[11]. Moreover, topoisomerase 2 alpha (TOP2A), maternal embryonic leucine zipper kinase (MELK), centromere protein F (CENPF) and SRY-box 1 (SOX1) are recently identified as the regulated genes of miR-34b and may contribute to the proliferation and metastasis of small cell lung cancer cells[12]. YAF-2 was first isolated and characterized as a protein that can interact with transcription factor Yin Yang 1 (YY1) during muscle differentiation and development[23]. YAF2 may play a crucial role in regulating the mechanism of p53-mediated apoptosis by interacting with the key protein programmed cell death 5 (PDCD5)[24]. It should be noted that YY1 was also the direct target of miR-34 family and the overexpression of miR-34 family suppressed tumorigenesis of gastric cancer cells through down-regulation of YY1[25]. These findings led us to speculate that YAF-2 might also have a close relationship with the miR-34 family. Indeed, our results of the Luciferase activity assay and Western blot demonstrated that miR-34b could target the 3'UTR of YAF-2 and regulated its expression in A549 cells. The increased expression of YAF-2 in tumor tissue of NSCLC patients further emphasized our

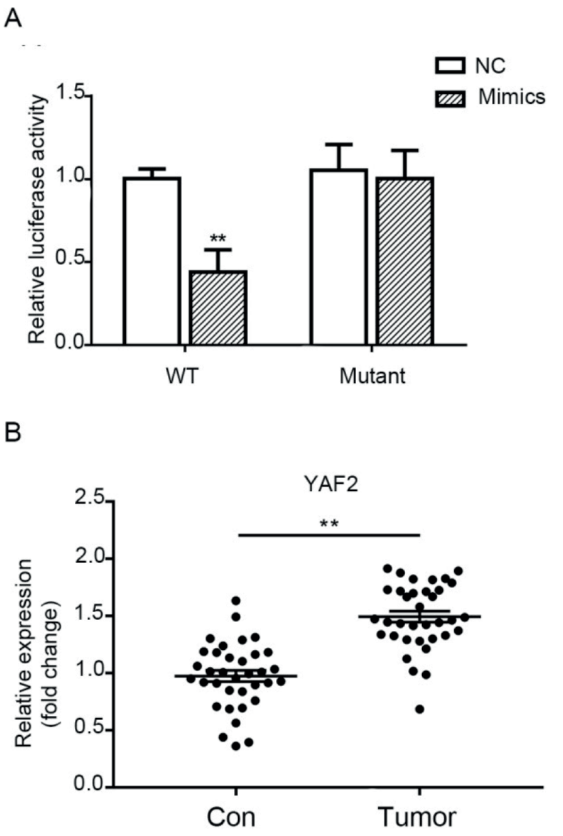

Figure 4. MiR-34b regulating mechanism on YAF2. ***A,*** MiR-34b mimics significantly reduced the relative Luciferase activity in the WT group; there is no remarkably difference in the mutant group. ***B,*** The expression of YAF2 was significantly increased in tumor tissues. YAF2 may act as an oncogene in NSCLC. Data are presented as means ± SD. $^{**}p<0.01$.

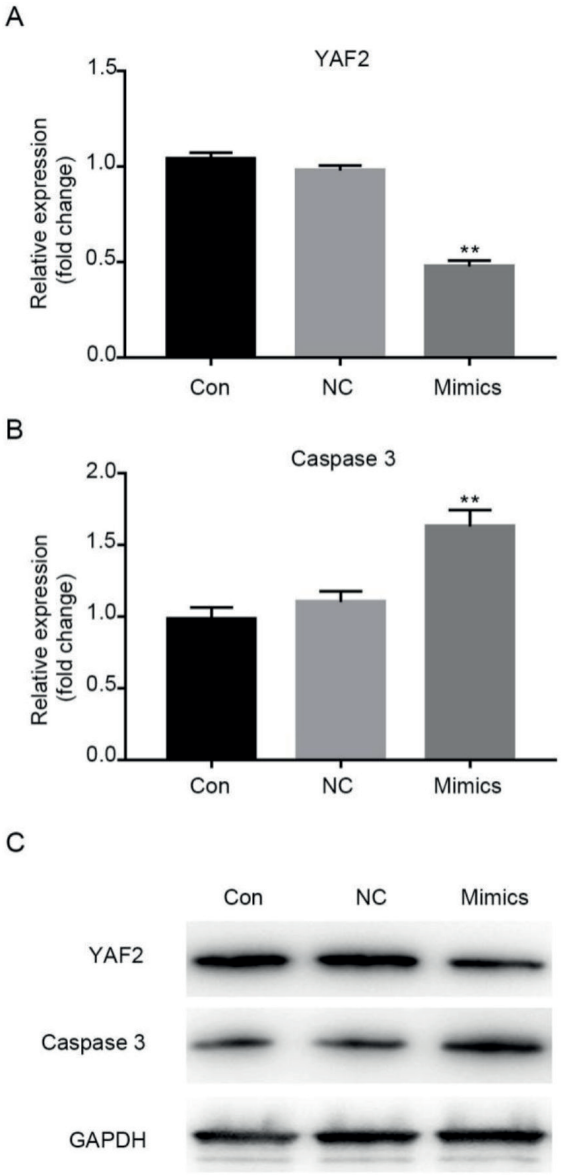

Figure 5. MiR-34b regulated the expression of YAF2 and Caspase 3. ***A,*** The expression levels of YAF2 in cells transfected with miR-34b mimic was significantly downregulated. ***B,*** The expression of Caspase 3 in cells transfected with miR-34b mimic was significantly increased compared with the Con and NC group. ***C,*** MiR-34b may regulate YAF2 stability by directly targeting its 3'UTR in A549 cells. The expression of YAF2 was negatively correlated with Caspase 3. Data are presented as means ± SD. $^{**}p<0.01$.

in vitro cell data, which indicated that YAF2 was a direct and functional target of miR-34b and its expression was negatively correlated with the development of NSCLC.

To further understand the mechanism of miR-34b-induced inhibition of lung cancer metastasis, we examined the effect of miR-34b on the expression of proteins that are associated with cancer development. Janus kinase 2 (Jak2) is a crucial member of the JAK family which plays an important role in cell proliferation, differentiation and apoptosis[26]. Signal transducer and activator of transcription 3 (STAT3) is an important transcription factor of intracellular signaling and aberrant STAT3 signaling can promote initiation and progression of human cancers by either inhibiting apoptosis or inducing cell proliferation, angiogenesis, invasion, and metastasis[27]. It should be noted that JAK-STAT3 signaling has recently been demonstrated to be the key target of miRNAs in regulating tumor cell proliferation, survival, invasion and immunosuppression[28]. Indeed, our results also demonstrated that the protein levels of Jak2 and STAT3 were significantly reduced under the over-expression of miR-34b. Moreover, the expression of matrix metalloproteinase (MMP)-2,

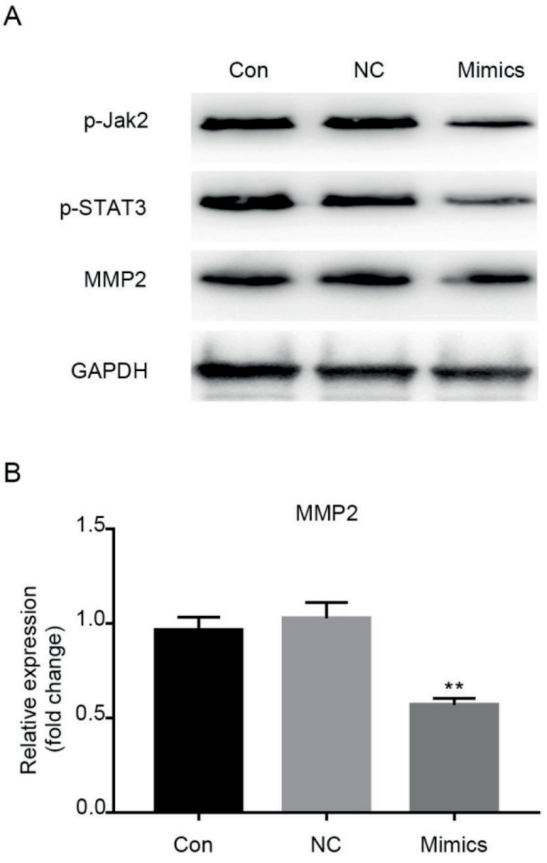

Figure 6. MiR-34b inhibited the JAK2/STAT3 signaling pathway and the expression of MMP2. ***A,*** MiR-34b significantly suppressed the expression of p-JAK2 and p-STAT3. ***B,*** The expression of MMP2 was significantly decreased in cells transfected with miR-34b mimics. Data are presented as means ± SD. $^{**}p<0.01$.

which is a member of the MMP family that have important roles in tumor invasion and metastasis, has also been inhibited by miR-34b, indicating that miR-34b may inhibit the proliferation and promote apoptosis through the extracellular matrix degradation[29]. However, further research is required to investigate whether these proteins are the direct targets of miR-34b as well as their interacted relationships during the lung cancer development.

Conclusions

We found that miR-34b may be a key tumor suppressor miRNA during the development of NSCLC and it can inhibit the migration and invasion of lung cancer cells by targeting YAF-2. Thus, miR-34b may serve as a potential therapeutic candidate in the treatment of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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