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Inhibition of MiR-10b Restrains the Migration and Epithelial-Mesenchymal Transition of Lung Cells by Targeting LATS2 via TAZ Pathway

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: MiR-10b can promote the growth of lung cancer cells. LATS2 is reported to regulate lung cancer cell proliferation. We aimed to study the relationship between miR-10b and LATS2 in lung cancer.





Material/Methods: MiR-10b and LATS2 in lung cancer tissues and cells were measured via real-time polymerase chain reaction (RT-PCR) and western blotting. Luciferase reporter assay and mimic transfection were performed to study relation between miR-10b and LATS2. MiR-10b inhibitor was transfected to downregulate miR-10b expression and LATS2 was further downregulated. Then, the proliferation, apoptosis, migration, and invasion capacity of lung cancer cells were measured, respectively. Lung cancer cells stably transfected with LATS2 and TAZ plasmids were constructed as usual, and the effect of LATS2 overexpression on epithelial-mesenchymal transition (EMT) was determined.

Results: MiR-10b was upregulated and LATS2 was significantly downregulated in lung cancer. Inhibition of miR-10b restrained the growth of lung cancer cells and accelerated the apoptosis of lung cancer cells. LATS2 is directly bound by miR-10b and silence of LATS2 reversed its inhibitory and promotive effects. Overexpression of LATS2 inhibited the EMT of lung cancer cells by inhibiting the TAZ pathway.

Conclusions: MiR-10b was upregulated in lung cancer. Inhibition of miR-10b could restrain the development of lung cancer by increasing LATS2 expression via TAZ.

MeSH Keywords: **Enoxaparin • Lung Neoplasms • Receptors, Thyrotropin**

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Background

According to data reported by the International Agency for Research on Cancer (IARC) in 2018, lung cancer is the most familiar cancer in the world (accounting for 11.6% of all cases) [1]. In recent years, many countries have reported a significant increase in the mortality and incidence of lung cancer. In the past 20 years, despite some great progresses have been made in the diagnosis and treatment, lung cancer still presented with a 10% and 15% overall long-term survival rate [2]. One of the biggest causes of treating failure for lung cancer is metastasis. About 30% of lung cancer patients have distant metastasis at the first diagnosis, and about 50–60% of patients have metastasis during treatment. Ultimately, 80–90% of lung cancer patients die from metastasis [3]. To clarify the molecular mechanism of lung cancer invasion and metastasis, and on this basis to find and develop molecular targeted drugs, is the most important measure to improve the survival time of lung cancer patients and the prognosis and quality of life of patients.

MicroRNAs (miRNAs) are highly conserved single-stranded non-coding small RNAs consisting of 20–25 nucleotides. It can regulate target genes via influencing messenger RNA (mRNA) [4]. It is estimated that the human genome contains more than 1800 miRNAs and regulates about 30% of gene expression [5]. Single miRNAs can affect the expression of multiple genes, and a single gene can be regulated via multiple miRNAs. Abnormal miRNA expression can not only lead to tumors, but also influence the process of tumor progression. MiRNAs are reported to play an important role in tumor development. In tumors, the expression of multiple miRNAs can be abnormal, and a miRNA can also regulate multiple tumor signaling pathways through multiple target genes. Among them, miR-10b is widely studied. Ma et al. reported miR-10b was up-regulated in breast cancer tissues, and more significantly in metastatic breast cancer tissues [6]. Blomston et al. reported the expression of miR-10b was increased in pancreatic cancer and was closely related to the development of pancreatic cancer [7]. Moreover, inhibition of miR-10b in lung cancer cells inhibited the tumor development [8]. LATS2 is a tumor suppressor and human LATS2 gene is situated at chromosome 13q11–12. It is important in lung cancer [9]. LATS2 is tumor suppressor gene and participate in regulating cell cycle [10].

Summarily, this study elucidated the expression of miR-10b in lung cancer tissues and cell lines, and then explored the pivotal function of miR-10b on the apoptosis and metastasis of lung cancer, and last but not the least, further investigate the molecular mechanism.

Material and Methods

Lung cancer tissues

Lung cancer tissues and adjacent normal liver tissues used for qRT-PCR and western blot were collected from 45 lung cancer patients (23 males and 22 females) who undergoing lung resection for lung cancer between April 2014 and May 2015 at Qilu Hospital (Jinan, China). The necessary ethics approval was obtained prior to collection and experimentation.

Cell culture

Human H460, A549, H1299, H569, H358, and normal pulmonary epithelium BEAS-2B cell lines were purchased from Cell Repository, Chinese Academy of Sciences (Shanghai, China). Cells were cultured and passaged at the ratio of 1: 4 in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS, 100 mg/L streptomycin and 1×10^{-5} UI penicillin at 37°C in 5% CO₂ incubator.

Transfection of siRNAs

Human miR-10b and scrambled control siRNAs were obtained from Santa Cruz Biotechnology. Human lung cancer cell lines NCI-H69 were plated into multiple-well plates with 10% FBS and DMEM in a 5% CO₂ incubator at 37°C and transfected with 80 nM miR-10b or nontarget (control) siRNAs for 72 hours by applying 2 μ L/mL Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

Establishment of LATS2 or TAZ overexpressed lung cancer cell line

A pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA, USA) was used to establish stable transfected cells overexpressing LATS2 or TAZ. To construct pcDNA3-LATS2 or pcDNA3-TAZ, the full-length human LATS2 or TAZ gene pB4 was digested with EcoRI and then inserted into an EcoRI-cleaved pcDNA3 vector. Cells were transfected with pcDNA3 or pcDNA3-LATS2 or pcDNA3-TAZ using lipofection technique on the basis of manufacturer's Lipofection protocol (Gibco BRL, Life Technologies, Rockville, MD, USA).

Cell proliferation assay

Cells were placed to 96-well plates with 2×10^3 cells per well. The Cell Counting Kit-8 (CCK-8) assay was applied to measure relative cell growth.

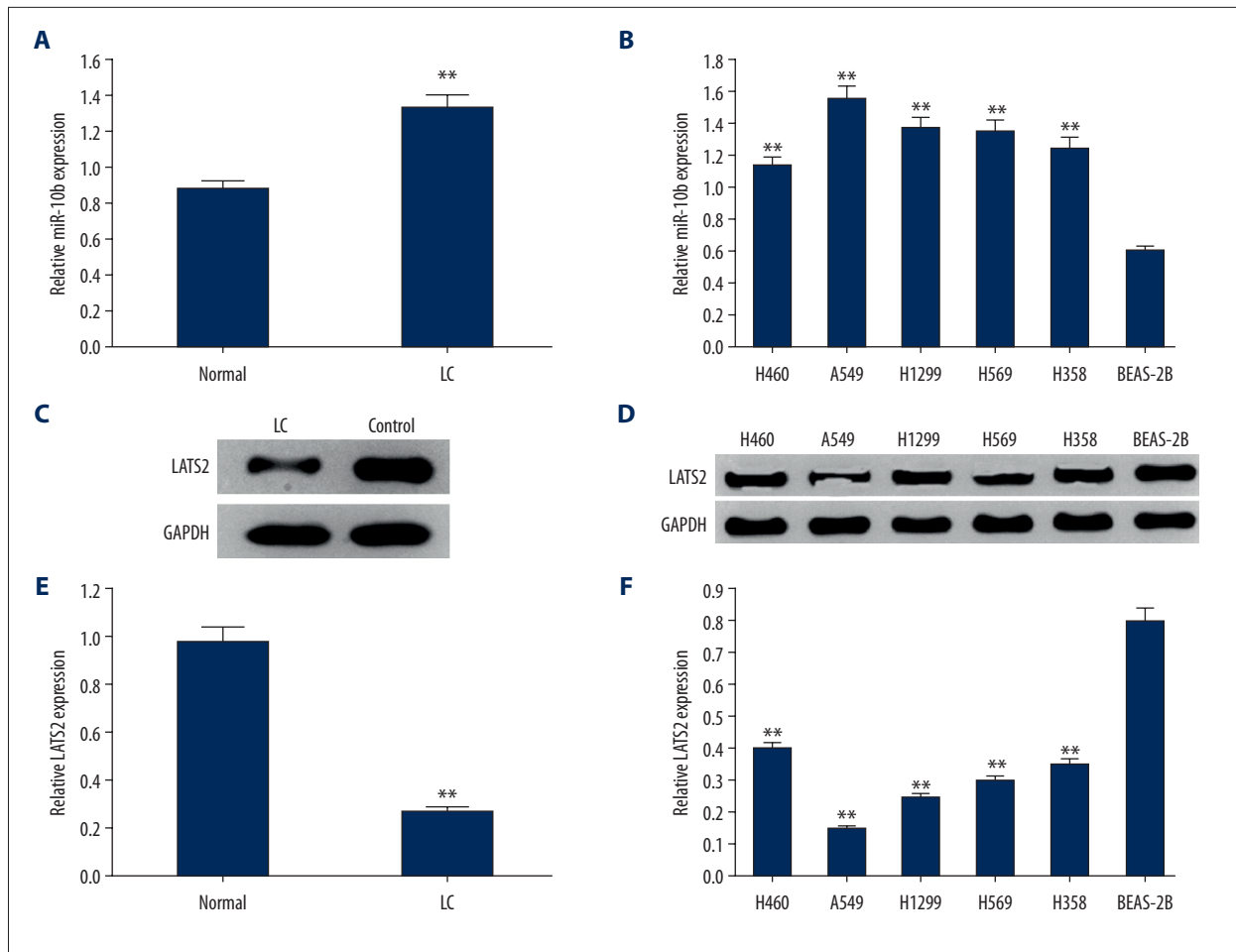


Figure 1. MiR-10b is upregulated and LATS2 is downregulated in lung cancer. (A) The relative mRNA levels of miR-10b in lung cancer tissues and the control. (B) The relative mRNA levels of miR-10b in lung cancer cell lines and the normal pulmonary epithelium BEAS-2B. The protein and mRNA level of LATS2 in lung cancer tissues and the normal tissues (C, D) or in lung cancer cell lines and the normal pulmonary epithelium BEAS-2B (E, F).

Flow cytometry

Cells in each group were fully digested by trypsin, centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded and washed with phosphate-buffered saline (PBS). Cell density was adjusted to be 1×10^6 per mL. Staining was performed according to Annexin V-FITC-PI kit instructions, and flow cytometry was used for detection.

Transwell assay

We added 200 μ L 2×10^5 /mL single cell suspension was to the upper chamber. Then, 500 μ L medium containing chemokines was added to the lower chamber. After culture at 37°C with 5% CO₂ for 48 hours, the matrix glue and cells in the upper room were carefully removed with cotton swabs. Crystal violet was used for staining, and a microscope was used to observe. Ten 100-fold fields were taken, counted, and averaged.

Scratch assay

We seeded 1.0×10^5 cells into each well of 12-well plate. We transfected cells and incubated the dishes at 37°C until cells reached 100% confluence. An artificial gap was scratched using a 100 μ L Pipette nozzle. Wells were observed and photographed under an inverted phase contrast microscope. The software program HMIAS-2000 was used to calculate the cell migration distance (μ m).

Western blotting

The protein samples were extracted from cells and protein samples were fractionated by SDS-PAGE (7.5–10% polyacrylamide gels). The primary antibodies against CARMA (Stressgen Bioreagents, Ann Arbor, MI, USA; rabbit polyclonal), TTP53 (Cell Signaling; rabbit polyclonal) and CTNNB1 (Cell Signaling; rat specific, rabbit polyclonal) were used, with GAPDH (anti-GAPDH antibody from Research Diagnostics, Concord, MA, USA) as an internal control.

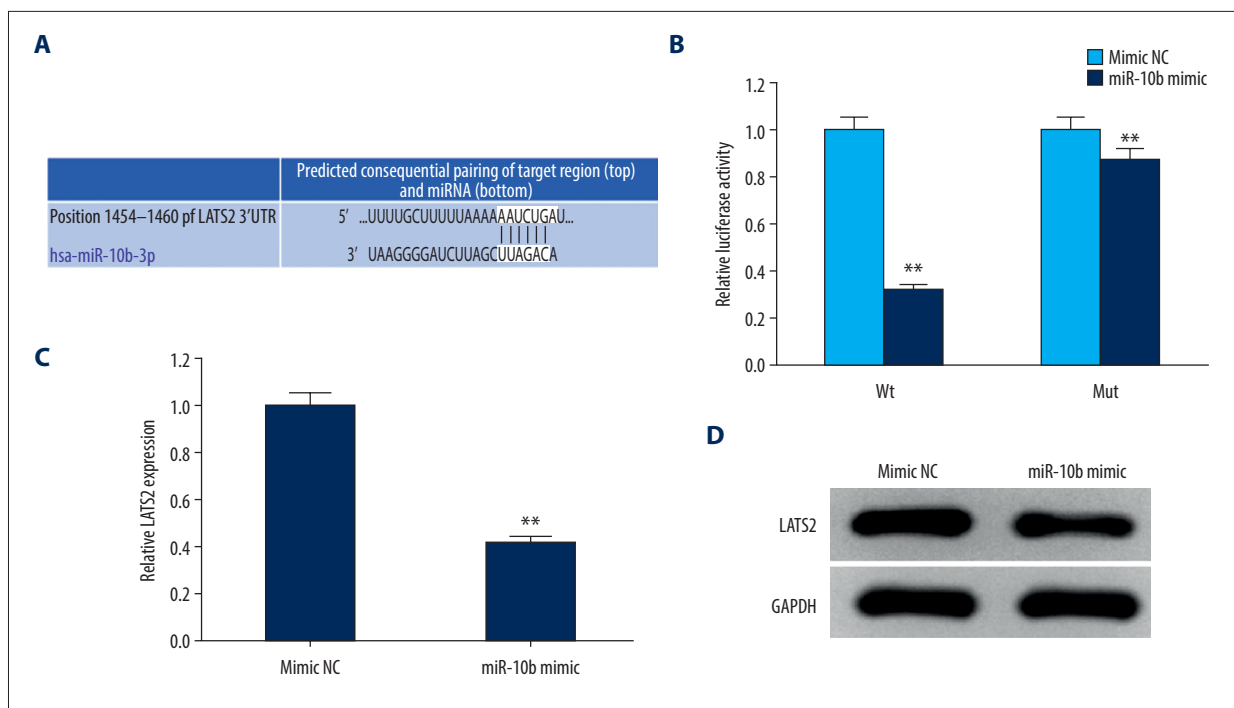


Figure 2. LATS2 is a target gene of miR-10b. (A) The site between 3'UTR of LATS2 and miR-10b. (B) Fluorescence reporting vector experiment verified LATS2 is a target gene of miR-10b. Overexpression of miR-10b decreased the relative mRNA level (C) and protein expression (D) of LATS2.

Real-time polymerase chain reaction (RT-PCR)

Total RNA of cell lines or tumor tissues was extracted by total RNA Isolation System (Promega, Madison, WI, USA). cDNA was reverse transcribed from 1 µg total RNA per sample using anchored oligo-dT primers (Reverse-iT First Strand Synthesis; ABgene). RT-qPCR was performed by using the LightCycler and the FastStart DNA Master SYBR Green 1 kit (Roche Applied Sciences). Primers of forward and reverse were tabulated in supplementary profile and chemically synthesized by Sangon Technology Company.

Statistical analysis

SPSS 22.0 software was used to perform *t*-test and variance analysis. Significance was defined as $P < 0.05$. Data was presented as mean \pm SEM. using *t*-tests for 2-group comparisons.

Results

miR-10b was upregulated and LATS2 downregulated in lung cancer

There were 45 lung cancer tissues and normal liver tissues selected, and the expression of miR-10b in these tissues were determined by qRT-PCR. Results are shown in Figure 1A. As shown, the relative mRNA levels of miR-10b in lung cancer

tissues were all significantly increased compared with the normal ($P < 0.01$). To further verify the abnormal expression of miR-10b in L lung cancer, we used qRT-PCR to measure the mRNA levels of miR-10b in lung cancer cell lines H460, A549, H1299, H569, H358, and normal pulmonary epithelium BEAS-2B. Results showed (Figure 1B) the relative mRNA levels of miR-10b in lung cancer cell lines were all upregulated compared with normal liver cell lines BEAS-2B ($P < 0.01$). The relative mRNA and protein level of LATS2 in lung cancer tissues and cell lines were determined via RT-PCR and western blotting, respectively. Results of RT-PCR showed relative mRNA levels of LATS2 in lung cancer tissues were reduced compared to the normal tissues (Figure 1C). The protein level of LATS2 in lung cancer tissues was also decreased compared with that of normal group (Figure 1D). Results of RT-PCR and western blotting showed LATS2 expression in H460, A549, H1299, H569, and H358 cells were all reduced compared with that in BEAS-2B cell lines (Figure 1E, 1F).

miR-10b directly targets to LATS2

Bioinformatics website was used to predict the candidate target gene of miR-10b, and we found that LATS2 has a specific binding site of miR-10b. To determine that miR-10b realize its function by targeting the 3'-UTR of LATS2 (Figure 2A), EGFP fluorescence reporting vector experiment was performed in A549 cells. Results showed the relative luciferase activity of

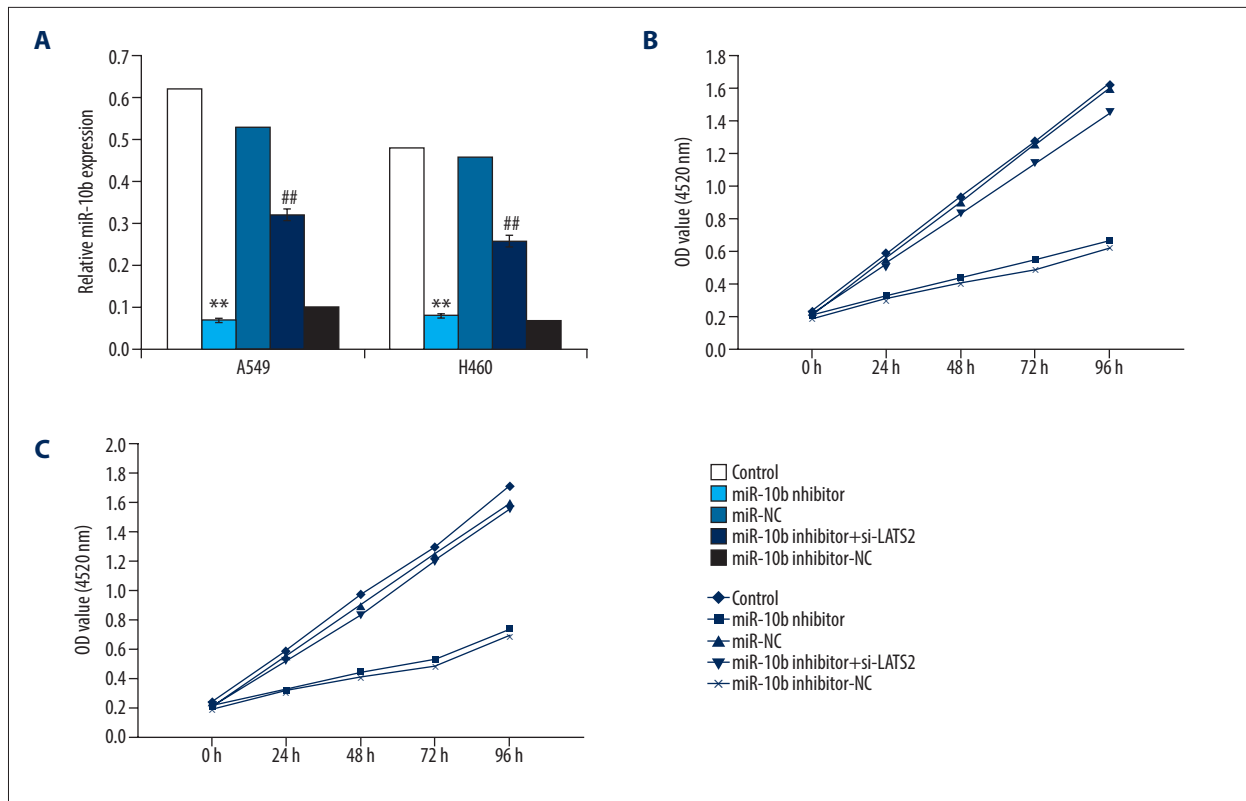


Figure 3. Inhibition of miR-10b restrained lung cancer cell proliferation and inhibition of LATS2 reversed the restrain role. (A) The relative mRNA levels of LATS2 in different groups. Inhibition of miR-10b restrained A549 cell (B) and H460 (C) cell proliferation and inhibition of LATS2 reversed the restrain role.

wild type (WT) in miR-10b mimic group was significantly decreased than mimic normal control (NC) group, while there had no significant difference between miR-10b mimic group and mimic NC group in Mutant (Mut) type (Figure 2B). Moreover, results of RT-PCR and western blotting showed LATS2 expression was markedly decreased after transfection of miR-10b mimic (Figure 2C, 2D) ($P < 0.01$). Those indicated LATS2 has a specific binding site of miR-10b.

Inhibition on LATS2 reverses the role of miR-10b on lung cancer cell proliferation

To study the influence of LATS2 on the role miR-10b on lung cancer, we divided lung cancer cells A549 and H460 into 5 groups: control, miR-10b inhibitor, miR-NC, miR-10b inhibitor+si-LATS2 and miR-10b inhibitor+si-NC group. The relative mRNA levels of miR-10b in the 5 groups were determined by RT-PCR. Results (Figure 3A) showed miR-10b expression in miR-10b inhibitor was reduced stably than the control and miR-NC group ($P < 0.01$). After transfection with si-LATS2, miR-10b expression was markedly increased compared with miR-10b inhibitor group ($P < 0.01$). CCK-8 assay was taken to estimate the role of miR-10b expression on lung cancer cell growth. As results showed inhibition of miR-10b restrained the proliferation of

lung cancer cells A549 and H460, while further inhibition of LATS2 abolished the inhibition role (Figure 3B, 3C).

LATS2 inhibition reverses the role of miR-10b on lung cancer cell apoptosis

The results of flow cytometry showed inhibition of miR-10b in A549 and H460 cells significantly promoted cell apoptosis compared to that of the control group, while further inhibition of LATS2 reversed the promotive action of miR-10b inhibition of lung cancer cell apoptosis (Figure 4A). Then, we used western blotting to determine the protein expression of caspase-3, Bax, and Bcl-3 in the 5 groups. Results showed inhibition of miR-10b significantly increased caspase-3 and Bax expression, while it decreased Bcl-3 expression (Figure 4B). However, the expression of caspase-3, Bax, and Bcl-3 were reversed after transfection of si-LATS2.

LATS2 inhibition reverses the role of miR-10b on lung cancer cell growth

The results of Transwell assay showed silence of miR-10b weakened the migration ability of A549 and H460 cells, while the inhibition role was abolished after inhibition on LATS2 (Figure 5A).

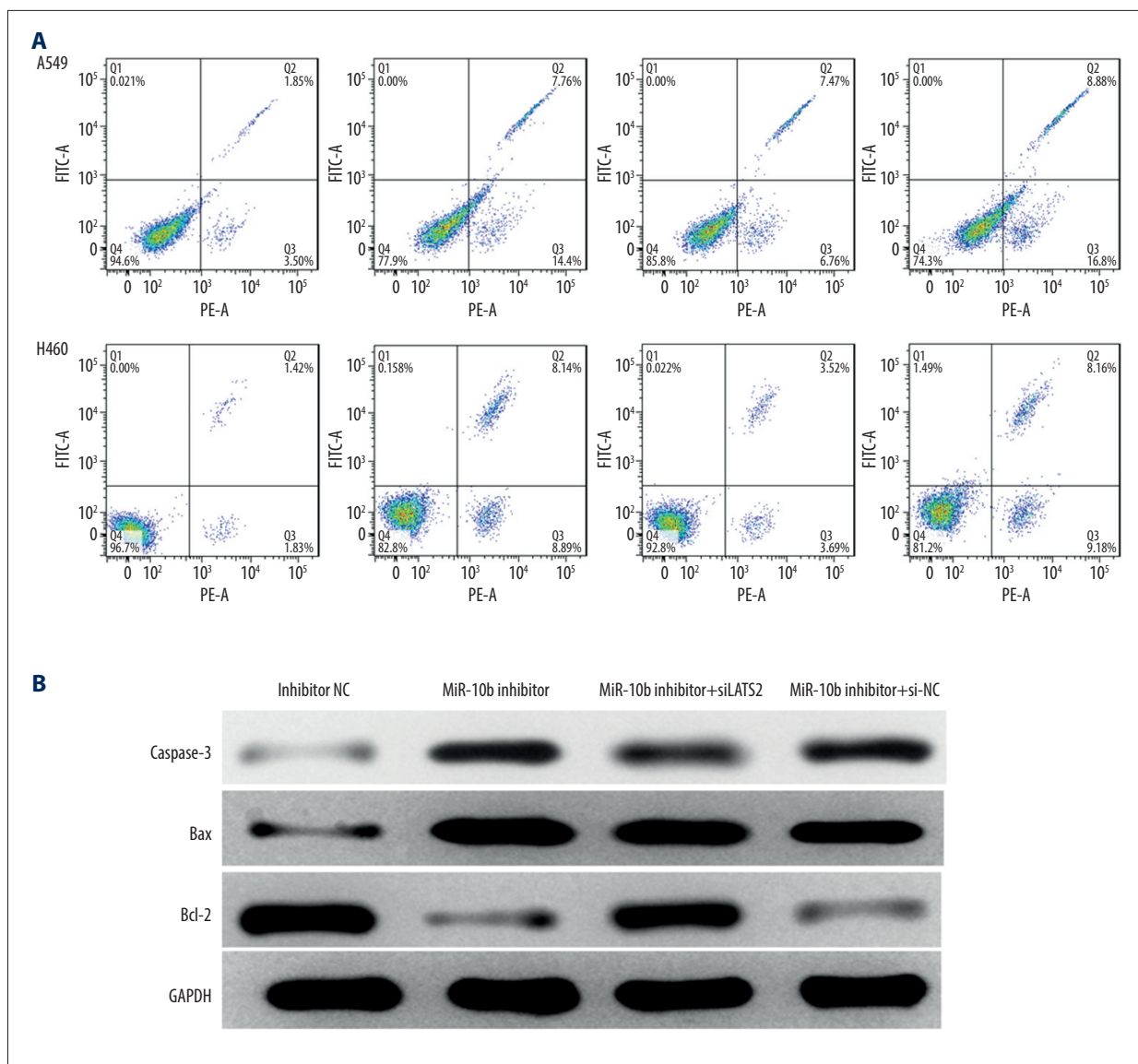


Figure 4. LATS2 reversed the role of miR-10b on lung cancer cell apoptosis. **(A)** A549 cell and H460 cell apoptosis situation in different groups. **(B)** The protein expression of caspase, Bax and Bcl-2 in different groups.

Moreover, western blotting was used to measure the protein expression of N-cadherin, E-cadherin, Vimentin, and Snail-1. As shown, the protein expression of N-cadherin, Vimentin, and Snail-1 in miR-10b inhibitor group were significantly decreased compared with the control and miR-NC group. However, the protein expression was markedly decreased in miR-10b inhibitor+si-LATS2 group compared with miR-10b inhibitor group (Figure 5B). For E-cadherin, inhibition of miR-10b increased its expression, while further inhibition of LATS2 reversed the increase. Scratch wound healing assay was performed to measure the contribution of LATS2 and miR-10b on A549 and H460 cell migration. Results showed that inhibition of miR-10b constrained the migratory property of H460 cells, yet inhibition of LATS2 reversed this effect (Figure 5C).

Overexpression of LATS2 inhibits epithelial mesenchymal transformation of lung cancer cells

To study how LATS2 affect the epithelial mesenchymal transformation of lung cancer cells, A549 cells were divided into 4 groups: LATS2 overexpression, LATS2-NC, TAZ overexpression, TAZ-NC, and LATS2+TAZ overexpression. The protein expression values of TAZ, N-cadherin, E-cadherin, Vimentin, and Snail-1 in the 4 groups were determined by western blotting. Results are shown in Figure 6. As shown, overexpression of LATS2 significantly increased the expression of TAZ, N-cadherin, Vimentin, and Snail-1, and decreased E-cadherin expression. Co-overexpression of TAZ abolished the increase role of LATS2 on TAZ, N-cadherin, Vimentin, and Snail-1.

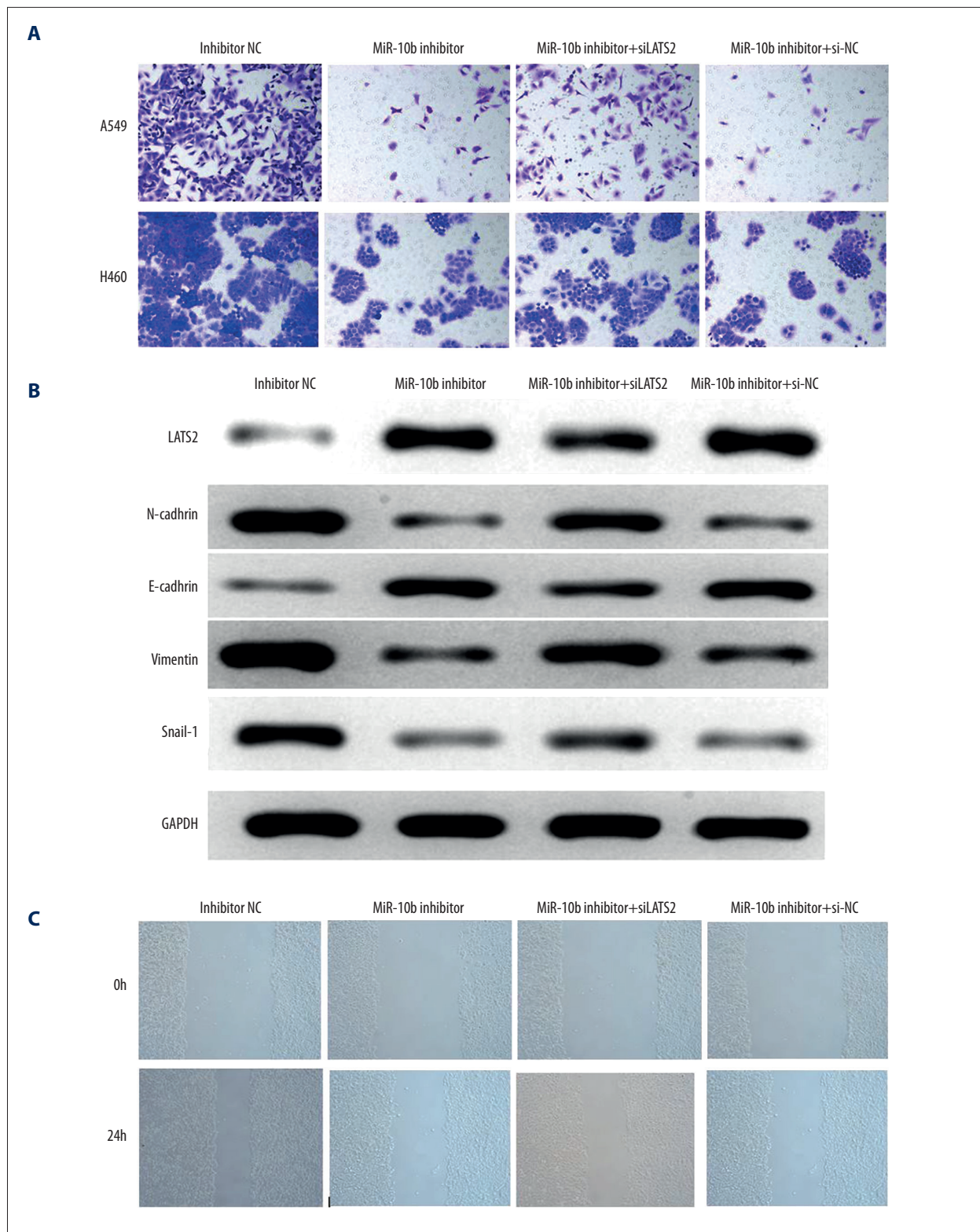


Figure 5. LATS2 reversed the role of miR-10b on lung cancer cell migration and invasion. **(A)** Inhibition of miR-10b restrained lung cancer cell migration and invasion, and inhibition of LATS2 reversed the restrain role. **(B)** The protein expression of LATS2, N-cadherin, E-cadherin, Vimentin, and Snail-1 in different groups by western blotting. **(C)** The invasion situation of H460 cells with inhibition of miR-10b and/or inhibition of LATS2.

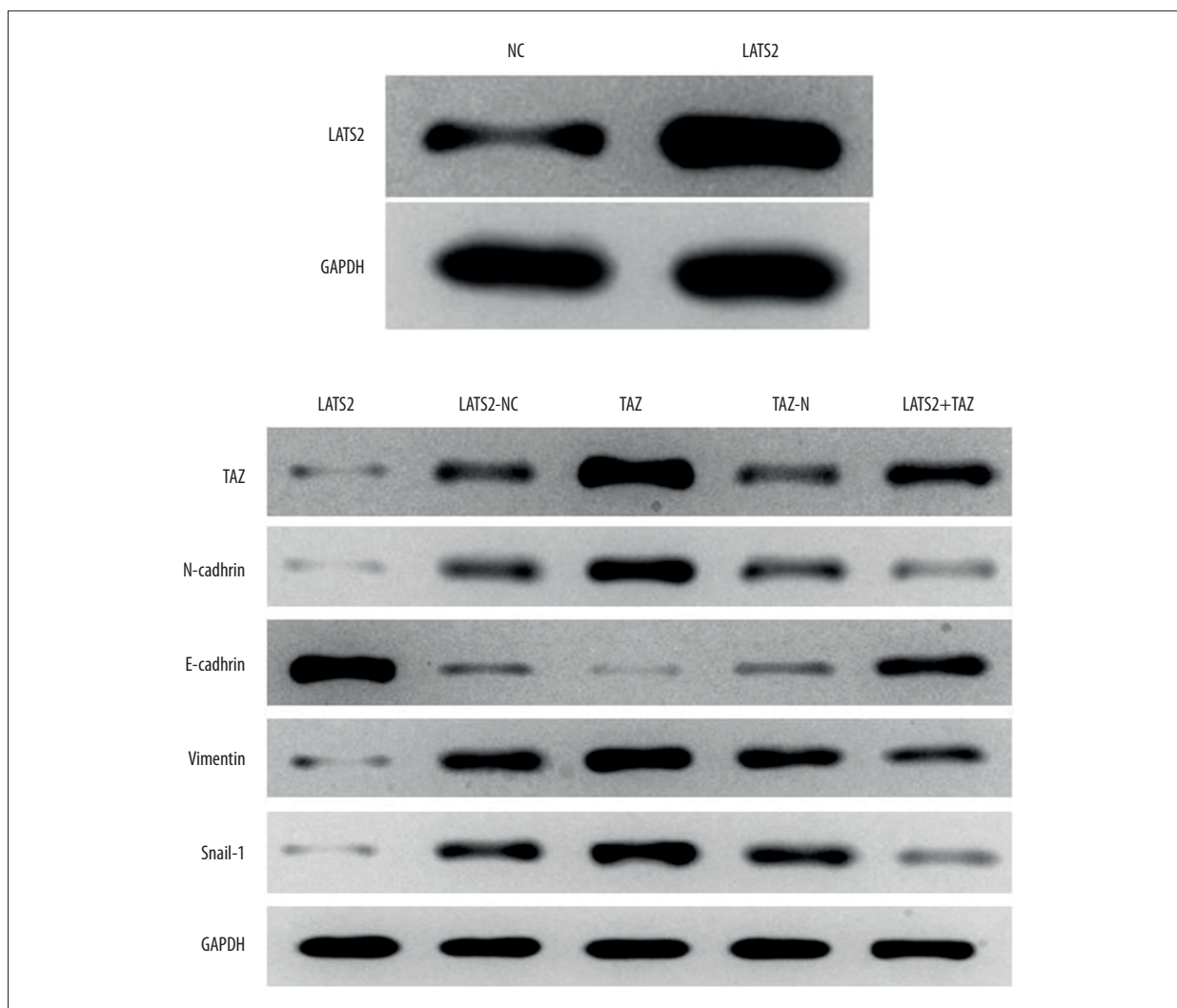


Figure 6. The protein expression of TAZ, N-cadherin, E-cadherin, Vimentin, and Snail-1 in H460 cells with overexpression of LATS2 and TAZ by western blotting.

Discussion

With the continuous improvement of medical standards, early prevention, diagnosis and treatment of lung cancer have made great progress, but the morbidity and mortality of lung cancer are still increasing year by year [11]. At present, the main clinical tumor markers of lung cancer have certain significance in the diagnosis and pathological classification of lung cancer, but their sensitivity and specificity are relatively low. MiRNAs are classified as proto-oncogenes or tumor suppressors and takes participate in many processes, such as cell apoptosis, metabolism, and differentiation, by targeting different transcripts [12].

MiR-10b is closely related to many tumors. It is highly expressed in metastatic cancer cell lines and metastatic breast tumors from patients [6]. MiR-10b was related to high-grade malignancy; this association held true for various cancer types [13].

Liu et al. found that miR-10b promoted the growth of non-small cell lung cancer (NSCLC) cells and is a potential therapeutic target for NSCLC intervention [14]. In our study, we found miR-10b was significantly increased in lung cancer tissues and cell lines. LATS2 can maintain the dynamic balance of cells. The LATS family has extensive transcriptional regulation, maintenance of genetic stability and inhibition of cell migration. Previous studies reported that LATS2 acted as a tumor suppressor gene. Studies showed YAP and TAZ are both phosphorylation targets of LATS2, and the phosphorylated YAP and TAZ cannot enter nucleus to work [15]. LATS2 was reported to regulate lung cancer cell proliferation [16]. Our study showed that LATS2 was significantly decreased in lung cancer compared to that of the control group. Moreover, we verified that miR-10b directly targeted to LATS2. To explore the role of miR-10b on lung cancer cell proliferation, apoptosis, invasion, and migration, we decreased miR-10b expression to

study the change of lung cancer cell proliferation, apoptosis, invasion, and migration ability. Moreover, inhibition of LATS2 was co-constructed with inhibition of miR-10b, and explored the reverse role of LATS2 on miR-10b. We concluded from these results that miR-10b inhibition restrained the proliferation, migration, and invasion of lung cancer cells, and promoted the apoptosis of lung cancer cells. Inhibition of LATS2 reversed the role of miR-10b on lung cancer cells.

Epithelial mesenchymal transformation (EMT) is a key factor for transforming tumor cells from epithelioid phenotype to mesenchymal phenotype, which can promote tumor invasion, metastasis, and drug resistance [17]. It has some properties that present with reduced expression of E-cadherin and conversion of cellular keratin to Vimentin. EMT is important in regulating tumor metastasis in tumor microenvironments. Epithelial tumor cells mainly showed downregulation of epithelial marker E-cadherin and upregulation of mesenchymal marker Vimentin during the transformation to mesenchymal cells with stronger invasion and migration ability. Many studies have shown that miRNAs can directly target transcription molecules of EMT. E-cadherin is a calcium-adhesion protein

responsible for cell-cell adhesion and tissue cytoskeleton. Reduced expression results in the transformation of cells into mesenchymal cells with greater motility and invasivity [18]. The reduction of E-cadherin expression is regulated by the interaction of various transcription factors. TAZ exerts its function as a transcription coactivator that contains a WW domain and modulates mesenchymal differentiation as well as multiple organ development [19]. Accumulating reports demonstrate that TAZ plays an important role in breast cancer development [20]. To further unveil the association between LATS2 and TAZ, overexpression of LATS2, overexpression of TAZ, overexpression of LATS2 and TAZ were performed, respectively. Results showed overexpression of LATS2 inhibited EMT, while overexpression of TAZ abolished the inhibition role.

Conclusions

MiR-10b was upregulated in lung cancer. Inhibition of miR-10b can suppress the proliferation, invasion and migration of lung cancer by increasing LATS2 expression and EMT via TAZ.

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