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MiR-550a-3p promotes non-small cell lung cancer cell proliferation and metastasis through down-regulating TIMP2

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been identified to play a crucial regulatory role in the development and progression of malignant tumors, including lung cancer. However, the function of miR-550a-3p on the progression of non-small cell lung cancer (NSCLC) remains poorly understood.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction was employed to estimate the expression level of miR-550a-3p in NSCLC tissue and cell samples. Cell proliferation was measured by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and colony formation assays. Transwell assay was recruited to demonstrate the abilities of cell invasion and migration. Luciferase analysis and Western-blot assay were performed to elucidate the underlying mechanism of miR-550a-3p in NSCLC.

RESULTS: The level of miR-550a-3p expressed in NSCLC tissues was significantly higher than that in para-tumor control tissues. Over-expression of miR-550a-3p significantly promoted proliferation, invasion, and migration of A549 cells while knockdown of miR-550a-3p inhibited growth and metastasis of H460 cells. TIMP2 was verified as a direct target of miR-550a-3p in NSCLC. Restoration of TIMP2 rescued the influence of miR-550a-3p over-expression.

CONCLUSIONS: We demonstrated that miR-550a-3p regulated the progression of NSCLC cells through TIMP2. Thus, miR-550a-3p axis could serve as a potential therapeutic target for NSCLC treatment.

Key Words:
miR-550a-3p, NSCLC, Proliferation, Metastasis, TIMP2.

Introduction

Lung cancer, of which non-small-cell lung cancer (NSCLC) occupied 80%, still remains the leading cause of cancer-related mortality[1]. The current management of NSCLC patients includes radiotherapy, chemotherapy, and combined therapies[2]. However, the median overall survival (OS) of advanced NSCLC remains only 20-28 months[3]. Though great advancements have been made in understanding the biology of NSCLC, the underlying mechanism of NSCLC tumorigenesis and metastasis still remains unclear[4]. It is of pivotal importance to develop novel diagnostic, prognostic, and therapeutic strategies for NSCLC, especially for late-stage patients.

MicroRNAs (miRNAs), as long as 18-25 nucleotides, belong to the family of non-coding RNAs, inhibit the translation of specific target gene via directly binding to the 3′-untranslated region (3′-UTR) of mRNAs[5,6]. About 60% of human protein coding genes could regulate by different miRNAs according to several studies[7]. In carcinogenesis, miRNAs function extensively in many processions, such as tumorigenesis and metastasis[8,9]. Mounting evidence has shown that miRNAs played critical regulatory roles in various types of tumors, including NSCLC. For example, Joshi et al [10] elucidated that miRNA-148a could reduce tumorigenesis and increase apoptosis induced by TRAIL in NSCLC. Yu et al[11] has found miRNA-193a-3p and -5p suppressed the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway and then inhibited the metastasis of NSCLC. In addition, He et al[12] has reported that over-expressing miR-452 inhibits metastasis of NSCLC by repressing BMI1[12]. What's more, Wei et al [13] demonstrated miR-106b-5p regulated the expression of BTG3 to promote proliferation and reduce apoptosis in NSCLC. Thus, miRNAs are potential biomarkers for the prediction, prognosis, prevention, and treatment of NSCLC.

Previous studies[14] have found miR-550a-3p functions as an oncogene in different types of

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tumors. In colorectal cancer, miR550a/RNF43/Wnt-signaling axis was a target of Brg-1 to regulate cell metastasis. In hepatocellular carcinoma, miRNA-550a directly targeted CPEB4 and acted as a specific gene to predict metastatic trend[15]. However, the role of miR-550a-3p in tumorigenesis and progression of NSCLC remains poorly understood.

We aimed to explore the role of miR-550a-3p in NSCLC by measuring the miR-550a-3p expression levels in NSCLC tissues and cell lines. In addition, *in vitro* studies were performed to analyze the proliferation, and metastasis of NSCLC-derived A549 and H460 cells. Furthermore, we explored the underlying mechanism of miR-550a-3p in NSCLC, which could provide a potential target for NSCLC diagnosis and treatment.

Patients and Methods

Patients

NSCLC and adjacent para-tumor tissues were obtained from patients with NSCLC by surgical dissection. All the tissues were conserved in liquid nitro before further usage. All written informed consents were obtained from these patients in the Second Hospital of Jilin University, according to procedures approved by the hospital's Ethics Review Board.

Cell Lines and Culture

All five NSCLC-derived cell lines (A549, SPCA1, PC-9, H1299, and H460) and one normal human bronchial epithelial cell-line BEAS-2B were obtained from the American Type Culture Collection (Manassas, VA, USA). All the above cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C in humid air under 5% CO_2 conditions.

Cell Transfection

The miR-550a-3p mimics, inhibitors, negative control (NC), and inhibitors negative control (INC) were bought from Genepharma Co. (Shanghai, China) and transfected into cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The pcDNAs for TIMP2 over-expression were obtained from Hanheng (Shanghai, China) and transduced with 5 mg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). The efficiency of transfection was confirmed using quantitative Real-time polymerase chain reaction (qRT-PCR).

RNA Isolation and qRT-PCR

The total RNA of tissue samples and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression level of miR-550a-3p was detected using a SYBR Green PCR master mix Kit (TaKaRa, Dalian, China). U6 was applied as an internal control and the relative expression level was measured by $2^{-\Delta\Delta CT}$ method. Each experiment was repeated at least three times. The primers for miR-550a-3p and the U6 were synthetized by RiboBio (Guangzhou, China). Primer sequences used in this study were as follows: miR-550a-3p, F: 5'-GGGGTGTAAACATCCTCGACTG-3', R: 5'-ATTGCGTGTCGTGGAGTCG-3'; U6, F: 5'-CTCGCTTCGGCAGCACA -3', R: 5'- AACGCTTCACGAATTTGCGT -3'.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution) Assay

Cells transfected with miR-550a-3p mimics, inhibitors, negative control (NC), or inhibitors negative control (INC), respectively, were seeded in 96-well plates. Next, a total of 10 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide solution (MTT) (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were added into the medium and cells were incubated for 4 h. The medium containing MTT solution was discarded and 100 μL of dimethyl sulfoxide were added. A 550 Microplate Reader (Bio-Rad, Hercules, CA, USA) was used to determine the absorbance at 470 nm. We repeated the experiment three times.

Colony Formation Assay

A total of 300 established cells were seeded on six-well plates and cultured for 15 days. We counted the number of colonies with a diameter greater than 50 μm. The experiments were repeated three times.

Transwell Assay for Invasion and Migration

The 8-μm transwell inserts were purchased from Millipore (Billerica, MA, USA). For migration assay, A549 or H460 cells (3×10^4 per well) were added to the upper chamber in serum-free Dulbecco's Modified Eagle Medium (DMEM), and 0.5 mL of DMEM containing 10% FBS was added into the lower chamber of a 24-well

plate. After 48 h incubation, cells that failed to pass through the membrane were removed. The membrane containing cells on its lower surface was fixed with pre-cooling methanol and stained with 0.5% crystal violet. Then, the cells stained were calculated after pictures were taken using a microscope (Olympus, Tokyo, Japan) in five random visions. The assay was repeated three times independently. For invasion assay, the top chamber was coated with Matrigel (BD, Franklin Lakes, NJ, USA) and the other steps were the same as that of the migration assay.

Dual-Luciferase Assay

Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA) was recruited to detect the luciferase activity. The vector of wild type group was constructed by inserting a luciferase reporter vector with the sequence of the TIMP2 3′-UTR containing the wild type miR-550a-3p binding site. A luciferase vector harboring a mutant miR-550a-3p-binding site was also constructed and used as a control group. A549 cells were treated with the luciferase reporter vectors and miR-550a-3p mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), followed by the detection of luciferase activity.

Protein Isolation and Western-Blot

The total protein from experimental cells was extracted using radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL, USA). A total of proteins (20 μg) after that the concentration was measured using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA), were separated on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk in tris buffered saline-tween (TBST) for 1 h, the membranes were immersed with primary antibody (anti-TIMP2, or anti-β-actin) (1:2000 dilution, Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibody (1:500) at 37°C for 1 h. Then, bands were detected using the electrochemiluminescence (ECL) Plus Detection kit (Pierce, Rockford, IL, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ was considered to have statistically significance.

Results

MiR-550a-3p Over-Expressed in NSCLC Tissue Samples and Cell Lines

To validate the relationship between miR-550a-3p and NSCLC, we detected the expression level of miR-550a-3p in 82 NSCLC tissue and paired adjacent para-tumor samples as well as in five NSCLC-derived cell lines. As clearly shown in Figure 1A, the level of miR-550a-3p in NSCLC tissues was significantly higher than that in the para-tumor group. Also, miR-550a-3p expressed significantly higher in NSCLC cell lines than in normal human bronchial epithelial cell BEAS-2B cells (Figure 1B). These results indicated miR-550a-3p may play as an oncogene in the progress of NSCLC. To further investigate the function of miR-550a-3p in NSCLC cells, we chose A549 cells to over-express the miR-550a-3p level while H460 cells to knockdown the miR-550a-3p expression (Figure 1C, 1D).

Ectopic Expression of miR-550a-3p Affected Cell Proliferation in NSCLC

We employed MTT and colony formation assays to detect the effect of miR-550a-3p on cell proliferation. Over-expression of miR-550a-3p significantly promoted cell growth of A549 cells; however, down-regulation of miR-550a-3p markedly inhibit the proliferation of H460 cells (Figure 2A, 2B). In addition, A549 cells transfected with miR-550a-3p mimics formed more colonies than the NC group while H460 cells treated with miR-550a-3p inhibitors formed less colonies than the INC group (Figure 2C, 2D). These data suggested miR-550a-3p could promote cell proliferation of NSCLC.

Abnormal miR-550a-3p Level Influenced Cell Invasion and Migration

Metastasis still remains the main lethal factor of NSCLC, so we further probed the function of miR-550a-3p on cell metastasis. By using transwell invasion assay, we found that miR-550a-3p mimics enhanced the invasion ability of A549 cells but miR-550a-3p inhibitors decreased the invasion of H460 cells compared with relative

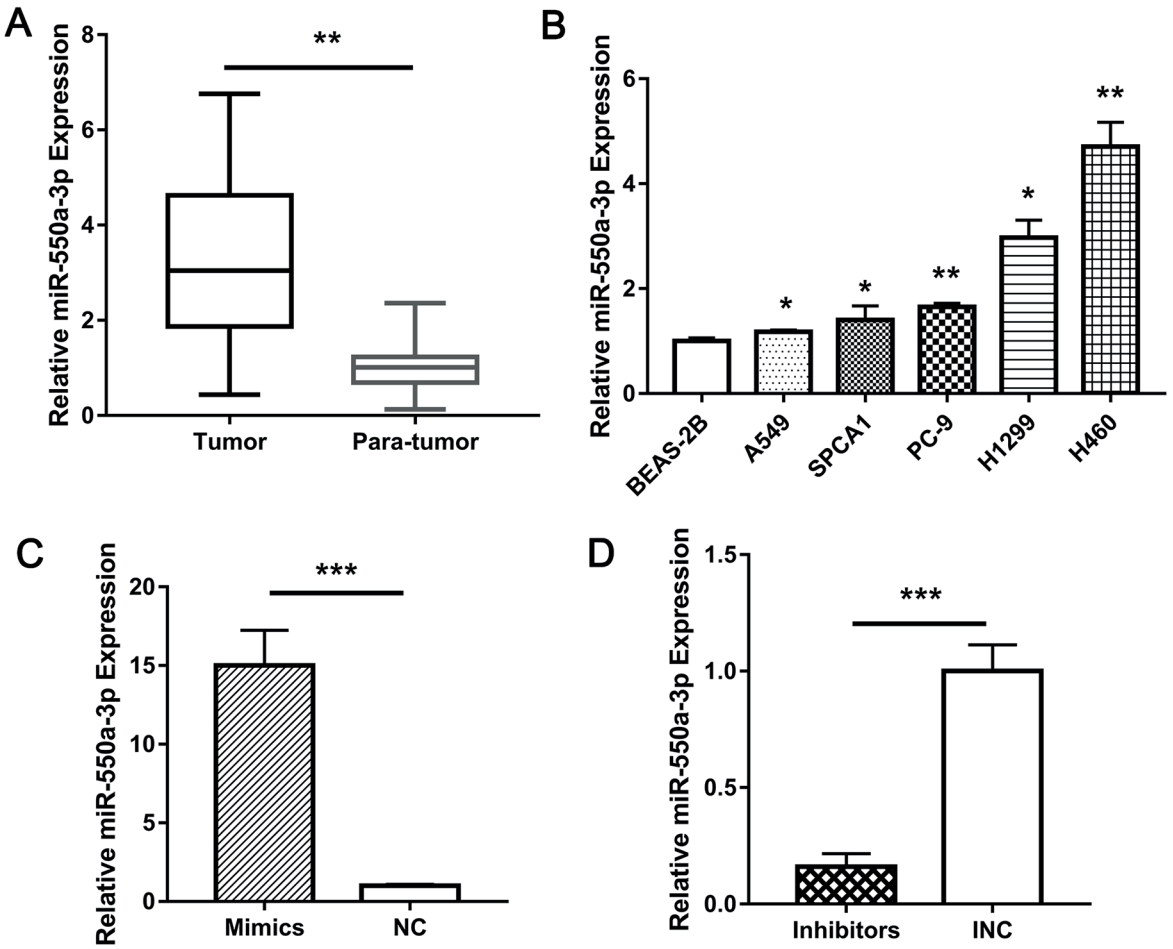

Figure 1. miR-550a-3p was down-regulated in NSCLC tissues and cell lines. ***A,*** Analysis of the expression level of miR-550a-3p in 21 NSCLC samples and 21 normal tissues. ***B,*** Analysis of miR-550a-3p expression level in NSCLC cell lines (A549, SPCA1, PC-9, H1299, H460) and lung/brunch normal epithelial cell line BEAS-2B cells. ***C,*** Expression of miR-550a-3p in miR-550a-3p mimics treated A549 cells. ***D,*** expression of miR-550a-3p in miR-550a-3p inhibitors treated H460 cells. *p<0.05, **p<0.01, ***p<0.001.

negative control group (Figure 3A). Similarly, up-regulation of miR-550a-3p significantly increased the migrated numbers of A549 cells; however, down-regulation of miR-550a-3p decreased the numbers of H460 cells (Figure 3B). These results showed that miR-144-5p could influence the invasion and migration abilities of NSCLC cells.

TIMP2 was a Direct Target of miR-550a-3p

The online database TargetScan (http://www.targetscan.org/) was used to search for potential miR-550a-3p targets. TIMP2 was found to be one of the predicted miR-550a-3p targets (Figure 4A). In order to verify the assumption, the luciferase assay was conducted. The results suggested that transfection of miR-550a-3p mimics reduced luciferase activity in A549 cells transfected with TIMP2-3′-UTR-wild type, but not TIMP2-3′-UTR-mutant plasmid vector (Figure 4B). Furthermore, miR-550a-3p mimics transfection decreased the TIMP2 protein levels in A549 cells and miR-550a-3p inhibitors treatment increased the TIMP2 protein expression in H460 cells compared with negative control group, relatively (Figure 4C, 4D, 4E). These data indicated TMP2 as a direct target of miR-550a-3p in NSCLC.

Up-Regulation of TIMP2 Offset the Function of miR-550a-3p in NSCLC Cells

To confirm the role of TIMP2 in the effects of miR-550a-3p in NSCLC, we restored TIMP2

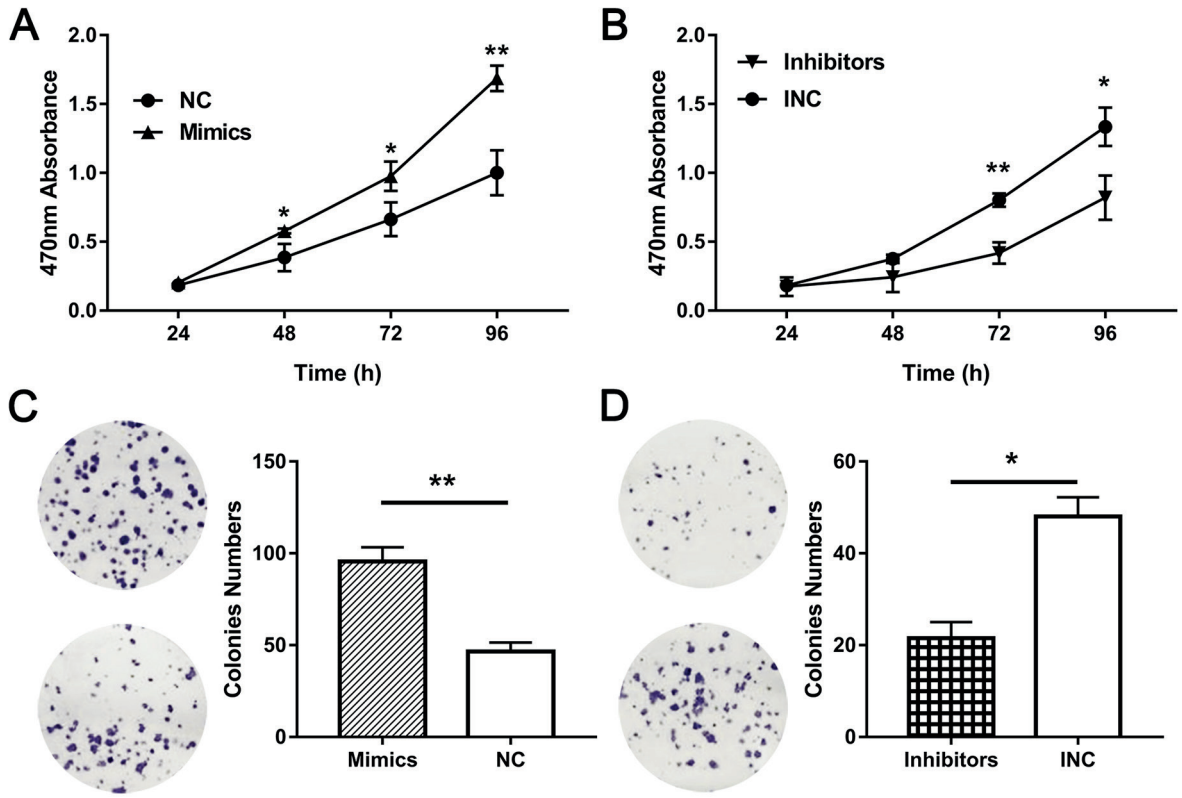

Figure 2. miR-550a-3p effected the proliferation of NSCLC cells. ***A-B,*** MTT assay was performed to determine proliferation of A549 (A) or H460 (B) cells treating with miR-550a-3p mimics or inhibitors compared to each negative control. ***C-D,*** Colony formation assay was performed to determine the growth of A549 (C) or H460 (D) cells transfected with mimics or inhibitors, respectively. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

expression of miR-550a-3p mimics treated A549 cells. The expression of TIMP2 protein was significantly restored (Figure 5A, 5B). Further studies showed that TIMP2 restoration rescued the effect of miR-550a-3p on the proliferation (Figure 5C) and metastasis (Figure 5D, 5E) of A549 cells. These results demonstrated miR-550a-3p function as an oncogene *via* TIMP2 in NSCLC cells.

Discussion

In recent years, miR-550a-3p has been identified to participate in several pathological progresses of many diseases. Such as, miR-550a-3p could function as a potential blood biomarker for Alzheimer's disease[16]. Also, circulating miR-550a-3p was found having significant changes in patients with idiopathic and postmenopausal osteoporosis and fragility fractures[17]. In patients with diabetes, miRNA-550a interferes with vitamin D metabolism in peripheral B cells[18]. What is more, circulating miR-550a-3p could act as a biomarker for severe acute pancreatitis associated with acute lung injury[19]. Also, miR-550a-3p was related to different types of tumors, including breast cancer, lung adenocarcinoma, colorectal cancer and hepatocellular carcinoma[14,15,20]. However, the specific functions of miR-550a-3p played in NSCLC have not been explained. In our study, we first found the expression of miR-550a-3p in NSCLC was significantly higher than that in adjacent para-tumor tissue samples, indicating a novel biomarker for NSCLC diagnosis.

Furthermore, we conducted gain- and loss- of function experiments to study how miR-550a-3p effects the NSCLC progressing. We found that up-regulation of miR-550a-3p level could significantly promote the proliferation of NSCLC cells but knockdown of miR-550a-3p significantly inhibit H460 cell growth. This indicated a novel target for the block of tumor growth. As cancer metastasis still remains the main death-related factor of NSCLC, we next demonstrated that

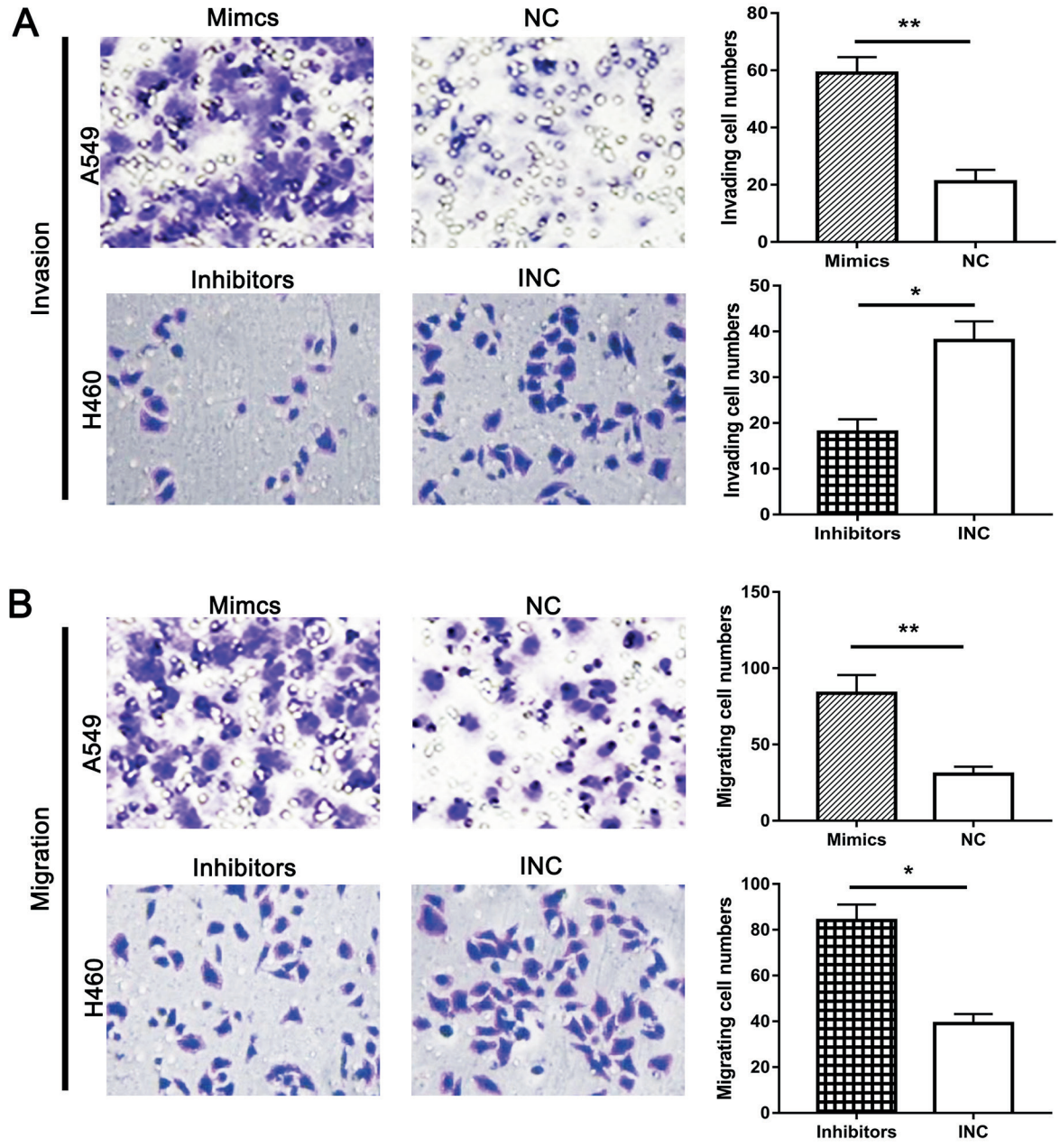

Figure 3. miR-550a-3p effected the invasion and migration of NSCLC cells. ***A,*** Transwell invasion assay was used to detect the invasion ability of miR-550a-3p mimics treated A549 cells or miR-550a-3p inhibitors treated H460 cells. ***B,*** Transwell migration assay was used to detect the migration ability of miR-550a-3p mimics treated A549 cells or miR-550a-3p inhibitors treated H460 cells. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

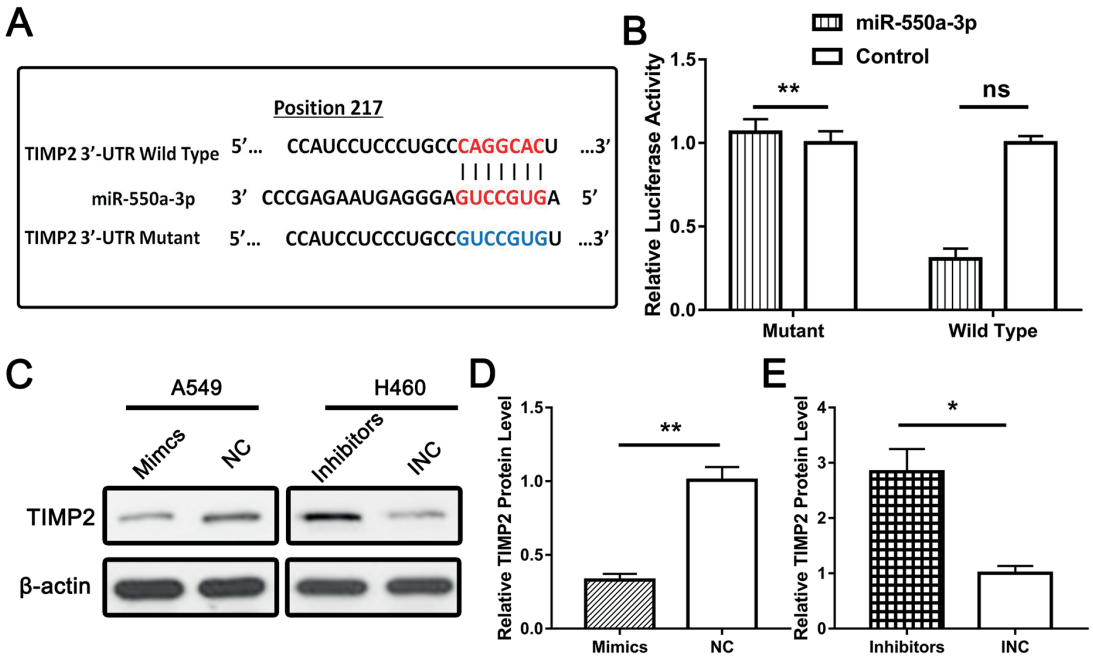

Figure 4. TIMP2 was a direct target of miR-550a-3p. ***A,*** The predicted binding sites of miR-550a-3p in the 3'-UTR of TIMP2. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C,*** Levels of TIMP2 and β-actin protein measured by Western-blot in miR-550a-3p over-expression A549 cells and miR-550a-3p knockdown H460 cells. ***D, E,*** the relative protein level of TIMP2 and β-actin. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ns: non-sense.

up-regulation of miR-550a-3p promoted cell invasion and migration, but inhibition of miR-550a-3p remarkably reduced the ability of cell metastasis. Our data indicated a potential biological point for effective strategy for the treatment of NSCLC.

To further explore the underlying oncogene role of miR-550a-3p in NSCLC, we investigated potential targets of miR-550a-3p and revealed that TIMP2 is a direct target of miR-550a-3p. TIMP2 was an important inhibitor of matrix metalloproteinases (MMPs), which has been broadly studied in human tumors and has been identified to be intensively related to the invasive ability and metastasis of many types of tumor cells including NSCLC[21]. Over-expression of TIMP-2 could protect B16F10 melanoma cells from apoptosis *via* reducing the invasion and angiogenesis while down-regulation of TIMP2 enhances cancer metastasis in hepatocellular carcinoma *via* HIF-1α/miR-210/HIF-3α regulatory feedback circuit[22,23]. Also, high levels of TIMP-2 indicated poor outcome in invasive bladder cancer[24]. Furthermore, TIMP2 could act as a target gene of different miRNAs in several cancers. For example, miR-106a targeted TIMP2 to promote gastric cancer cell invasion and metastasis[25]. TIMP2, together with ING4, acted as targets for miR-761 to promote proliferation and metastasis of NSCLC[26]. Furthermore, miR-20a and miR-106a functioned as oncogenes and enhanced the invasion of human glioma stem cells by directly repressing TIMP-2[27]. To verify whether TIMP2 was a functional target of miR-550a-3p, dual-luciferase assay was employed in A549 cells. Also, protein level of TIMP2 was measured in miR-550a-3p expression interference cells and showed a negative relationship between miR-550a-3p and TIMP2 level. Also, TIMP2 over-expression could reverse the effects of miR-550a-3p mimics, which further confirmed miR-550a-3p accelerated NSCLC cell proliferation, invasion, and migration *via* TIMP2.

However, in this study, we only aimed at TIMP2, which had limitation in the molecular mechanism of miR-550a-3p. As the mechanism of miRNA regulation is a network system, one miRNA could target different genes while one gene could be regulated by different miRNAs.

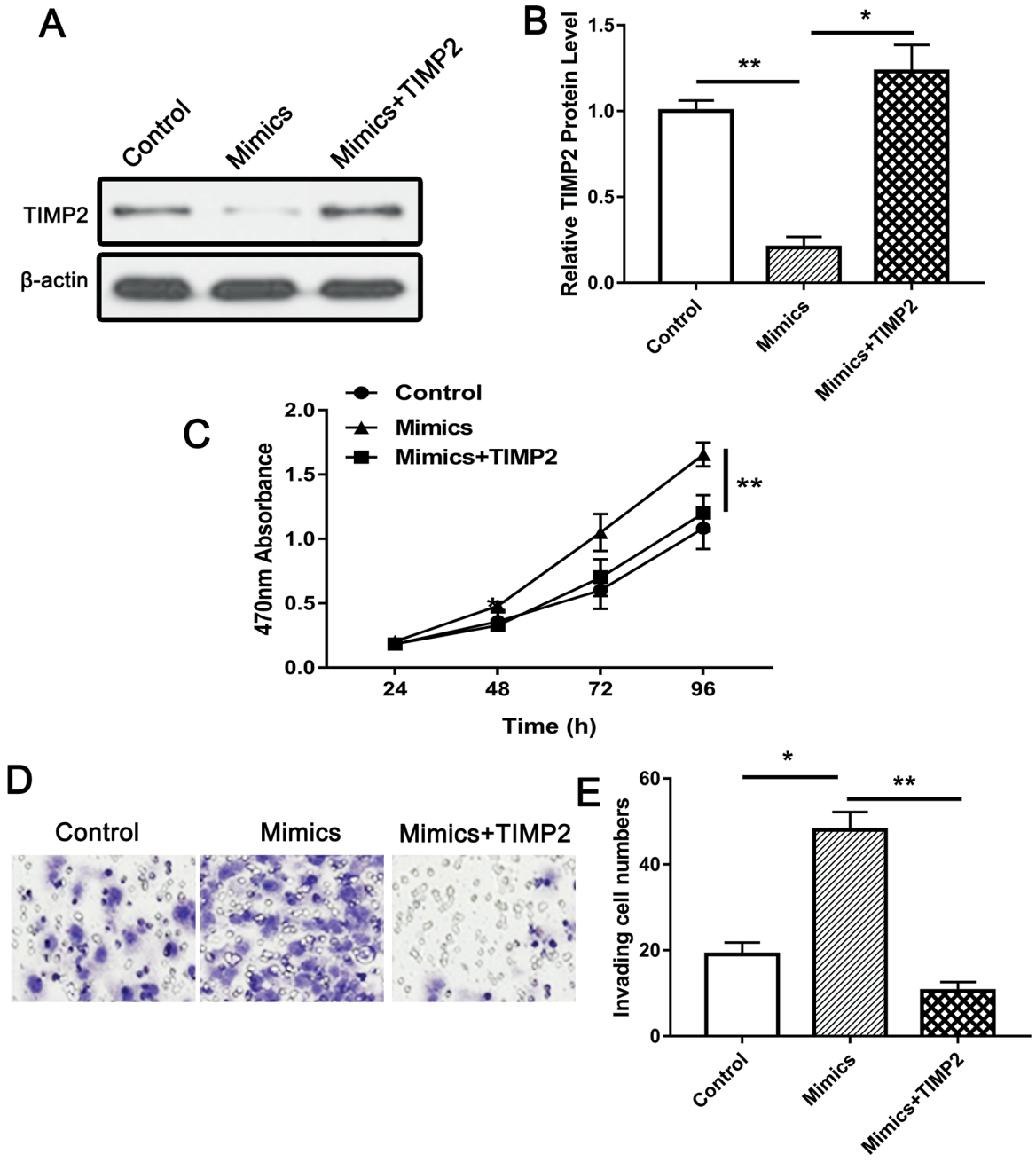

Figure 5. TIMP2 rescued the effects of miR-550a-3p mimics in A549 cells. ***A,*** Western-blot analyses of TIMP2. β-actin was used as an internal control. ***B,*** Relative protein band densities of TIMP2. ***C,*** Analysis of the cell proliferation ability by MTT assay in control, mimics, or mimics+TIMP2 treated A549 cells; ***D-E,*** Cell invasion ability was measured by transwell assay; Data are represented as the mean ± SD of three replicates. *p<0.05, **p<0.01, ***p<0.001.

Though further studies are needed, our study demonstrated the function of miR-550a-3p in NSCLC for the first time and elucidated TIMP2 as a target gene involved in the regulation mechanism of miR-550a-3p partially.

Conclusions

We demonstrated miR-550a-3p may function as an oncogene in NSCLC and promote cell proliferation and metastasis for the first time via

down-regulating TIMP3, suggesting miR-550a-3p as a potential therapeutic target for NSCLC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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