Original Article

Effects of miR-132-3p on progress and epithelial mesenchymal transition of non-small cell lung cancer via regulating KLF7

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Background: MicroRNAs (miRNAs) often appear as oncogenes or tumor suppressor genes. The aim of this research was to examine miR-132-3p and Kruppel-like factor 7 (KLF7) effects in the development of non-small cell lung cancer (NSCLC).

Methods: We used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine miR-132-3p expression in tissue specimens and 6 cells (A549, H1650, H292, H1299, H1944, BEAS-2b). Luciferase report forecasted the targeting relationship between miR-132-3p and KLF7. The expression of KLF7 and interstitial protein was determined by western blot. Proliferation test and Transwell assay were adopted for examining cell development. The Cell Counting Kit-8 (CCK-8) colorimetric method was used to observe the effects of miR-132-3p and KLF7 on the proliferation, metastasis, and invasion of NSCLC tumor cells. In order to determine whether the metastasis of NSCLC tumor cells was epithelial-mesenchymal transition (EMT)-mediated, supplementary experiments with E-cadherin and vimentin were performed.

Results: An increased expression of miR-132-3p was detected in NSCLC. Its mimic promoted the proliferation of tumor cells. As an immediate site of miR-132-3p, KLF7 was reversely adjusted via miR-132-3p and restrained the development of tumor cells in NSCLC, the effects of which were attenuated via KLF7 over-expression. Besides, the presence of EMT-related diversions was confirmed in NSCLC.

Conclusions: By targeting KLF7, miR-132-3p was capable of promoting the proceeding of NSCLC tumor cells. We discovered miR-132-3p/KLF7 route may exhibit curative target for NSCLC.

Keywords: miR-132-3p; non-small cell lung cancer (NSCLC); KLF7; proliferation; invasion; migration

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Introduction

Non-small cell lung cancer (NSCLC) possesses higher incidence rate (1). The occurrence of NSCLC is a multi-step process, involving cumulative genetic and epigenetic changes (2,3). Nevertheless, pathogenesis mechanisms relating to NSCLC have not yet been fully elucidated.

MicroRNAs (miRNAs) are endogenous non-coding RNA with an average of 22 nucleotides, and exert post-transcriptive influence among animals and plants (4). The pro- and anti-cancer genes regulated by miRNAs extensively take part in metastasis and progression of NSCLC (5). Research has found that 37 miRNAs are abnormally expressed in lung cancer, and 24 of them are related to NSCLC (6,7). Nevertheless, the role and mechanism of miR-132-3p in NSCLC remain unclear.

This research aimed to elucidate the roles of miR-132-3p and KLF7 in the progression of NSCLC, in order to yield

practical direction for NSCLC treatment. In this study, the authors first found that miR-132-3p can affect NSCLC proliferation by targeting KLF7 expression, invasion, migration and EMT process.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/jtd-21-353).

Methods

Clinical specimens

A total of 30 pairs of NSCLC and peripheral normal tissue specimens were obtained from Shanghai Tongren Hospital. Age: 31–65. Sex: (male, 19 cases; female, 11 cases). The samples were stored at –80 ℃ for standby. All procedures involving human participants in this study are consistent with the Helsinki Declaration (as revised in 2013). The study has got approval from Ethics Committee of Shanghai Tongren Hospital (No. 2018-067-01) and obtained the informed consent forms of all patients.

Cell culture

Normal human pulmonary epithelial cell lines (BEAS-2B, GNHu27) and five kinds of NSCLC cell lines (A549, TCHu150) (H1944, SCSP-596) (H292, TCHu122) (H1299, TCHU160) (H1650, TCHu152) were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute 1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS) was applied to cultivate cellular systems.

Cell transfection

The miR-132-3p inhibitor was infected with A549 and H1944 severally. Separately, miR-132-3p was infected with H1650 and H1299, individually, to determine luciferase activity. In another experiment, A549 was infected with LV-antimiR-132-3p and LV-antitimiR-132-3p + shKLF7. Also, H1650 was infected with LV-miR-132-3p, LVmiR-132-3p + LV-KLF7, and LV-NC. The role of miR-132-3p and KLF7 on the expression of the abovementioned cells was measured.

Quantitative RT-PCR analysis

Trizol reagents were used to harvest total RNAs from target tissues and cells. According to manufacturer directions, we adopted deoxyribonuclease I (DNase I) to cleave DNA in the experiment. The specific primers were as follows: miR-132-3p (5'-TCC ACC AAG AAG CTG AGC GAG-3'; 5'-GTC CAG CCC ATG ATG GTT CTG TC-3'), KLF7 (5'-AGA CAT GCC TTG AAT TGG AAC G-3'; 5'-GTC CAG CCC ATG ATG GTT CT-3'). The primers for miR-132-3p and KLF7 were obtained from Novland Biopharmaceutical Technology Co., Ltd (Shanghai, China).

Luciferase analysis

Firstly, the WT 3'-NCR sequence as well as MT 3'-NCR sequence were cloned into psiCHECK-2 luciferase reporter vector Shanghai Beinuo Biotechnology Co., Ltd. (Shanghai, China). Then, the cell line 293T was infected with luciferase reporter vector (Shanghai Bitai Biotechnology Co., Ltd., Shanghai, China). The activity of luciferase was detected by dual luciferase reporter system (Guangzhou Huijun Biotechnology Co., Ltd, Guangdong, China).

Western blot

Cells were obtained 2 days after transfection. After quantifying through bicinchoninic (BCA), the total protein was set in lanes with 15 μg each. After blocking with fat-free milk, primary antibodies KLF7 (13125-1-AP, Proteintech), E-cadherin (20874-1-AP, Proteintech), vimentin (ab92547, Abcam), as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (60004-1-Ig, Proteintech) were incubated at 4 ℃ for 1 night. Then, the secondary antibodies were incubated together with the membranes.

Cell Counting Kit-8 (CCK-8) assay

Cells were seeded in a 96-well plate (1×10^4). Later, CCK-8 was added to the wells. The optical density (OD) value was measured under 450 nm wavelength using an Epoch reader (Shanghai Woyuan Technology Co., Ltd., Shanghai, China).

Transwell assay

A 100 μL quantity of cellular suspension and 200 μL of serum-free culture fluid were mixed into the Transwell upper chamber. Then, the complete culture medium with 400 μL 0.05% FBS were incubated together in the Transwell lower chamber for 2 days with 5% CO_2. Cells that migrated under membrane surfaces were fixed and

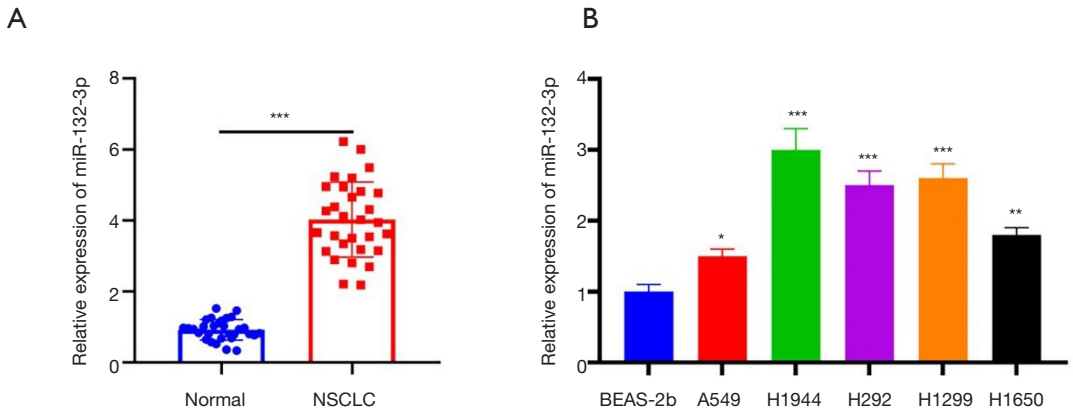

Figure 1 miR-132-3p up-regulation in NSCLC. (A,B) qRT-PCR was employed to determine miR-132-3p expression in 30 NSCLC patient tissues and 5 cell lines. *, P<0.05; **, P<0.01; ***, P<0.001. NSCLC, non-small cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

dried and dyed with crystal violet. The average field was chosen under microscope.

Edu experiment

The cells were mixed with 5-Ethynyl-2'deoxyuridine (Edu) solution, fixed with 4% paraformaldehyde, 0.5% Triton X-100 (Shanghai Tongwei Biology Co., Ltd) was used for penetration enhancement, and they were then stained with Click-iT reagent (Thermo Fisher, Waltham, MA, USA). Pictures were taken under microscope (Shanghai Yuguang Instrument Co., Ltd.).

Statistical analysis

Data was described as $\overline{x} \pm s$ and was analyzed by *t*-test between different groups. All experimental organisms were repeated 3 times. A P value <0.05 indicated a significant difference.

Results

More miR-132-3p was expressed in NSCLC

Firstly, the expression of miR-132-3p in NSCLC was determined. The outcomes revealed its expression was elevated in 5 cell lines (*Figure 1A,B*).

High miR-132-3p expression boosted NSCLC proliferation

It was revealed that miR-132-3p expression in A549, H1944, and H1299 was raised via miR-132-3p mimic transfection (*Figure 2A*). Correspondingly, the CCK-8 outcomes displayed the same trend (*Figure 2B,C,D*). The miR-132-3p mimic accelerated the growth of positive lung cancer cells (*Figure 2E*).

miR-132-3p low-expression controlled NSCLC proliferation

Low-expression of miR-132-3p was exhibited in its inhibitor transfection (detailed in *Figure 3A*). More surprisingly, the survival rate of A549, H1650, and H1299 cells lessened dramatically (*Figure 3B,C,D*). The EdU staining implied that the positive lung cancer cells were substantially lessened when exposed to miR-132-3p inhibitor (*Figure 3E*).

KLF7 was a downstream target of miR-132-3p

Our findings supported that KLF7 is a downstream target of miR-132-3p (*Figure 4A*). The activity of mimic miR-132-3p was substantially lessened in H1650 via WT-KLF7 3'UTR transfection (*Figure 4B*). Curiously, in H1650, MUT-KLF7 3'UTR transfection did not cause any changes (*Figure 4C*). The activity of mimic miR-132-3p in H1299 was same as in H1650 (*Figure 4D*). Similarly, In H1299, MUT-KLF7 3'UTR transfection did not lead to any changes (*Figure 4E*).

miR-132-3p negatively regulated KLF7

As shown in *Figure 5A,B*, low expression of KLF7 was exhibited via shKLF7 transfection. The expression of

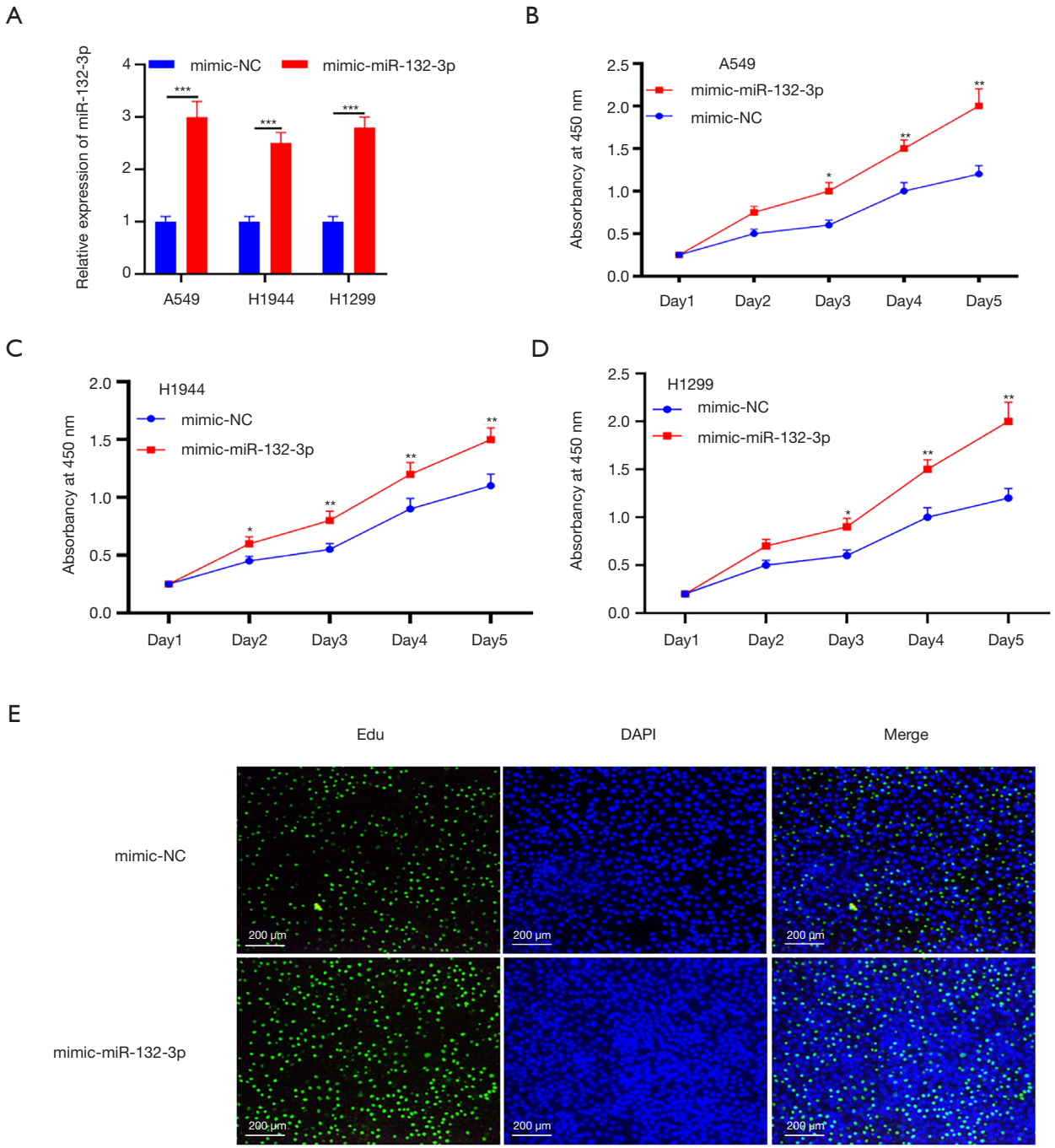

Figure 2 High expression of miR-132-3p boosted NSCLC proliferation. (A) miR-132-3p expression was checked via qRT-PCR; (B,C,D) survival rate of 3 cells infected with highly-expressed miR-132-3p; (E) EdU staining experiment. *, P<0.05; **, P<0.01; ***, P<0.001. NSCLC, non-small cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; EdU, 5-Ethynyl-2'deoxyuridine.

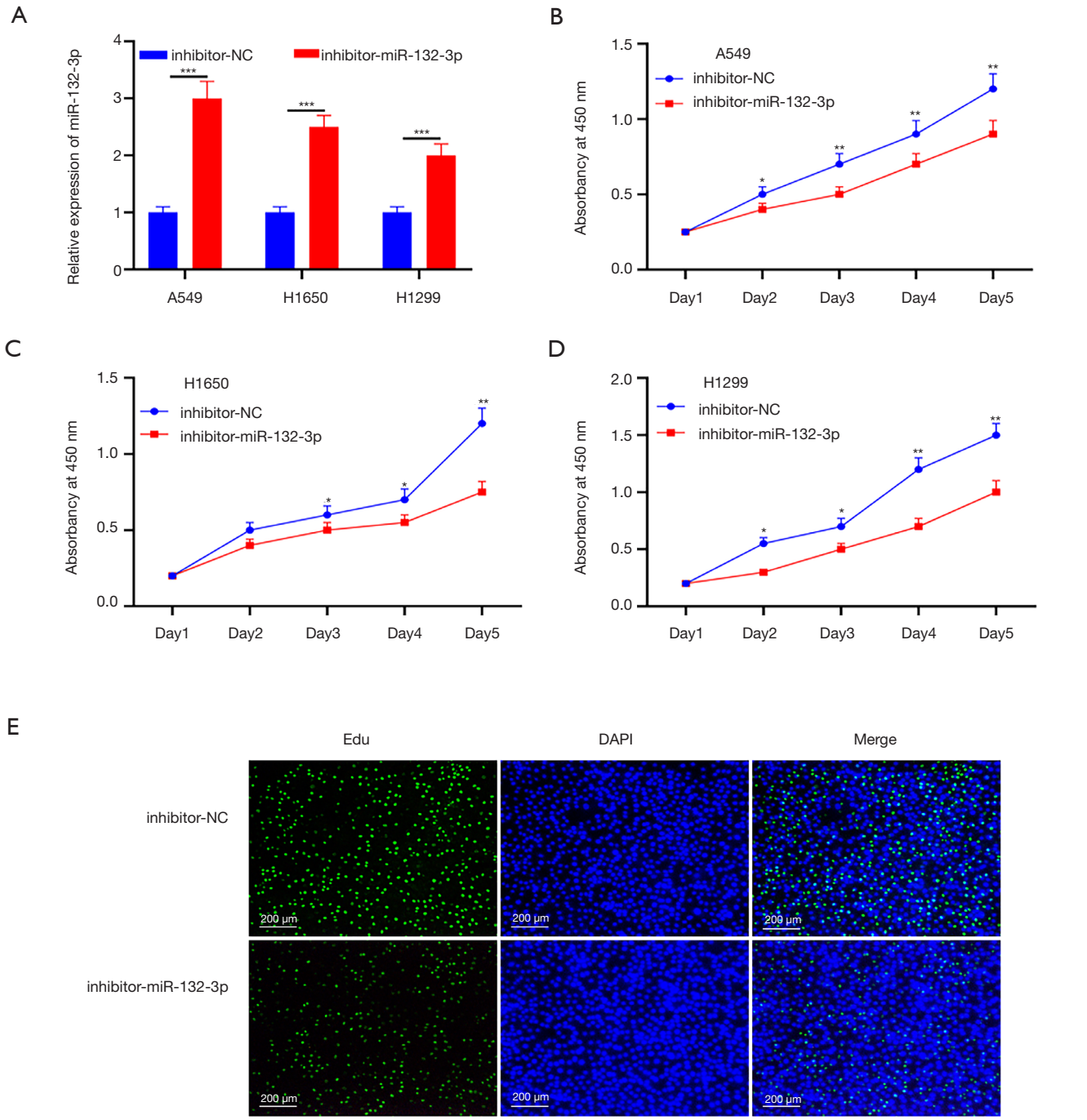

Figure 3 miR-132-3p low-expression restrained NSCLC proliferation. (A) qRT-PCR was applied to detect miR-132-3p expression via transfection with its inhibitor. (B,C,D) Survival rate of 3 cells infected with miR-132-3p restrainer. (E) EdU staining experiment. *, P<0.05; **, P<0.01; ***, P<0.001. NSCLC, non-small cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; EdU, 5-Ethynyl-2'deoxyuridine.

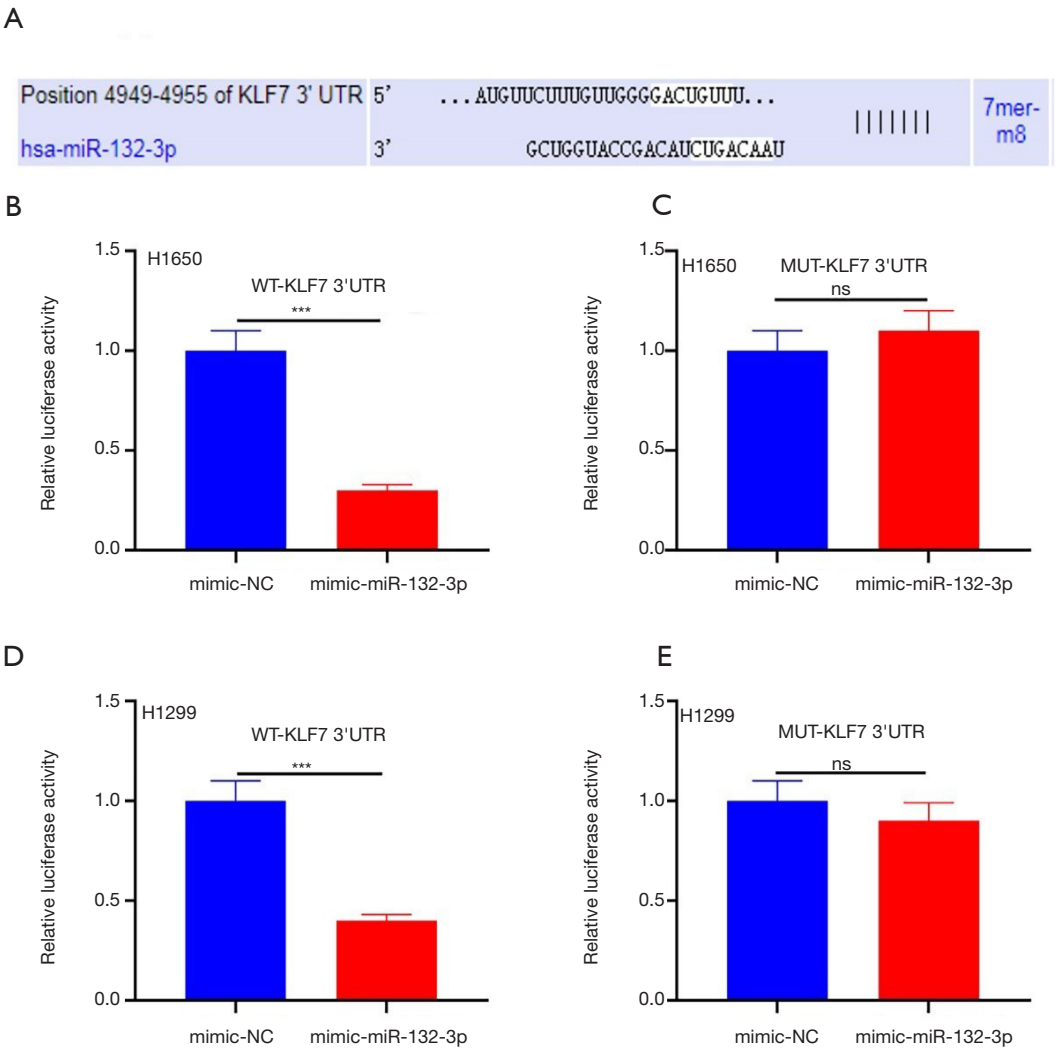

Figure 4 KLF7 was a downstream target of miR-132-3p. (A) miR-132-3p and KLF7 3'UTR had binding sites; (B,C,D,E) double luciferase activity was used to measure the transfection activity of miR-132-3p highly-expressed in 2 cells. ***, P<0.001; ns, P>0.05.

KLF7 was increased via A549 cells transfected with LV-anti-miR-132-3p (*Figure 5C,D*). Yet, LV-KLF7 transfection displayed the opposite trend (*Figure 5E,F*). Additionally, KLF7 expression in H1650 displayed the opposite trend (*Figure 5G,H*).

miR-132-3p promoted cell development via adjusting KLF7

The outcomes of CCK-8 revealed that the cell vitality in A549 appeared substantially lower in LV-anti-miR-132-3p + shKLF7 (*Figure 6A*). The cell viability of LV-miR-132-3p was higher than LV-miR-132-3p + LV-KLF7 (*Figure 6B*). Transwell assay showed that the number of cells decreased in LV-anti-miR-132-3p + shKLF7 (*Figure 6C*). Meanwhile, Transwell results indicated that the number of cells raised in LV-miR-132-3p (*Figure 6D*).

The miR-132-3p/KLF7 axis adjusted interstitial protein

As displayed in *Figure 7A,B*, the expression of in cells infected with LV-anti-miR-132-3p + shKLF7 was greatly reduced, while the expression of vimentin exhibited an opposite trend (*Figure 7A,B*). Surprisingly, LV-miR-132-3p + LV-KLF7

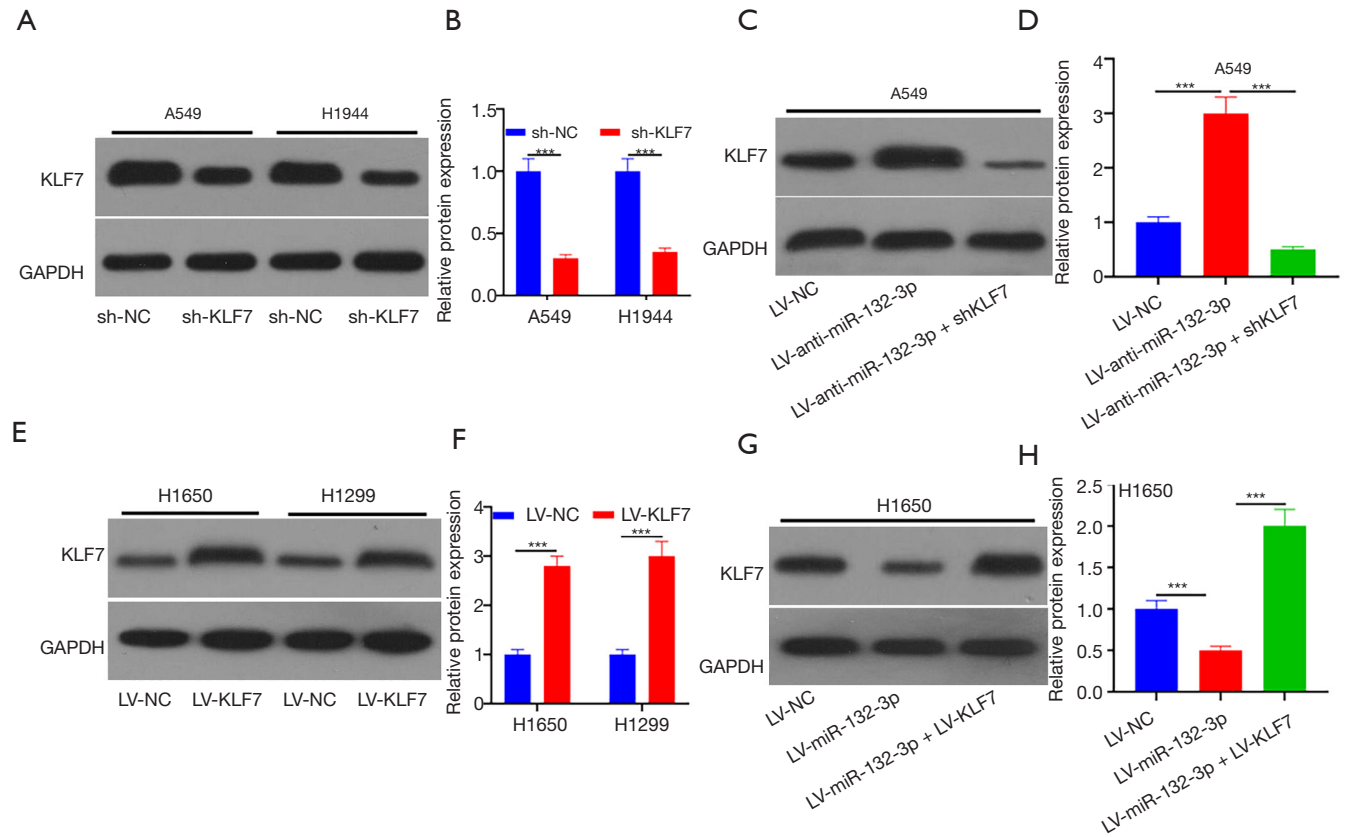

Figure 5 miR-132-3p negatively regulated KLF7. (A,B) Western blot was used to detect KLF7 expression via shKLF7 influence; (C,D) Western blot was used to detect KLF7 expression via LV-anti-miR-132-3p and shKLF7 influence; (E,F) Western blot was utilized for detecting KLF7 expression via LV-KLF7 affect; (G,H) Western blot was used to detect KLF7 expression via LV-miR-132-3p and LV-KLF7 influence. ***, P<0.001

transfection not only increased E-cadherin expression but also reduced the expression of vimentin (*Figure 7C,D*).

Discussion

The tumor that is NSCLC is complex and malignant (8). The pathogenic factors of NSCLC include environment, diet, infection, host genes, and so on. Abnormal gene expression is the key factor of NSCLC, especially the abnormal expression of oncogenes or tumor suppressor genes (9-16). Abnormal expression of miR-132-3p has been confirmed in glioma, breast cancer, and bladder cancer (17-24). However, the role of miR-132-3p in the progression of NSCLC has remained unclear.

In this study, we discovered more miR-132-3p in tissue specimens and cells. A negative relationship was detected between KLF7 and miR-132-3p. In addition, miR-132-3p mimic facilitated the development of NSCLC tumor. Nevertheless, the inhibitor of miR-132-3p exhibited a contrasting result. The above-mentioned results hinted that miR-132-3p performed an oncogenic role, while KLF7 exerted an anti-oncogenic role in NSCLC.

EMT was accompanied by changes in tumor cytomorphology, adhesion between cells and between cells and stroma lost, but the invasion and migration existed, which promoted tumor metastasis in the body (25). Numerous studies have shown that the differentiated miRNA expression in tumors is highly related to the occurrence of tumor EMT (26,27). Our research investigated E-cadherin and vimentin, the markers of EMT, to explore any possible latent mechanisms. It has been shown that miR-132-3p controls

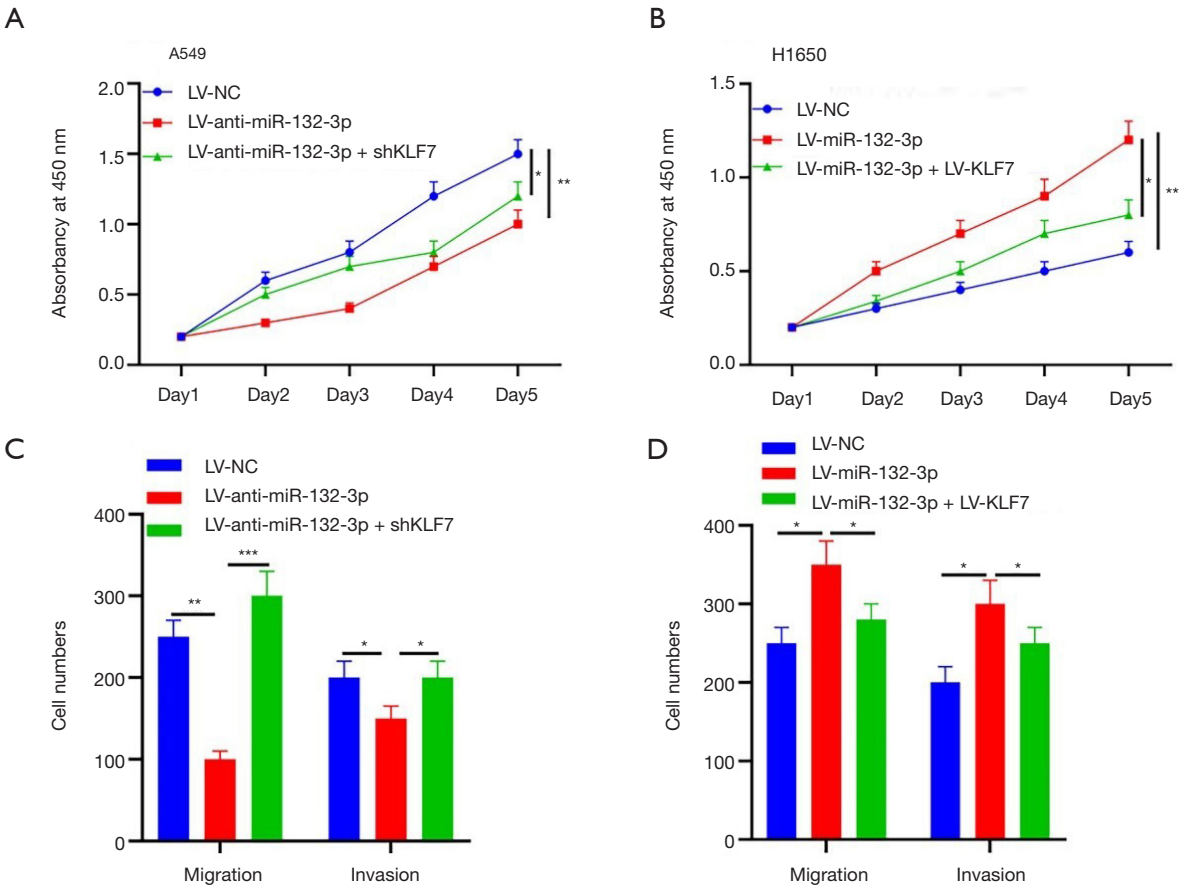

Figure 6 miR-132-3p controlled cell development via adjusting KLF7. (A,B) Survival rate of A549 and H1650 cells transfected with shKLF7/LV-KLF7 influence; (C,D) movement of A549 and H1650 cells infected with shKLF7 as well as LV-KLF7 affect. *, P<0.05; **, P<0.01; ***, P<0.001.

EMT and inactivates the PI3K/Akt pathway in NSCLC. In addition, Li *et al.* have found that regulating KLF7 can inhibit NSCLC invasion, migration, and EMT (28). In the current study, miR-132-3p/KLF7 was shown to be involved in the progression of NSCLC via adjusting relevant EMT expression.

At present, the treatment measures for NSCLC mainly include surgical excision and chemotherapy, however, its survival rate is poor, especially the chemical resistance of cancer cells seriously reducing the survival rate of NSCLC patients (29). In recent years, the search for new treatment methods has become a hot topic of research. To be sure, studies of targets such as exosomes, FOXO1 signaling pathway, miRNAs, PI3K/Akt signaling pathway, Wnt signaling pathway, LncRNAs, NF-κB signaling pathway are possible for in-depth understanding and treatment of NSCLC (30). In the future, more clinically viable drugs will need to be developed for these targets.

In conclusion, miR-132-3p could directly target KLF7 expression and regulate EMT transformation, thus accelerating the migration and invasion of NSCLC cells. This provided strong data support for a better understanding of miR-132-3p regulating NSCLC development. More importantly, the miR-132-3p/KLF7 axis presents a new clinical approach for the diagnosis, treatment, and prevention of NSCLC metastasis.

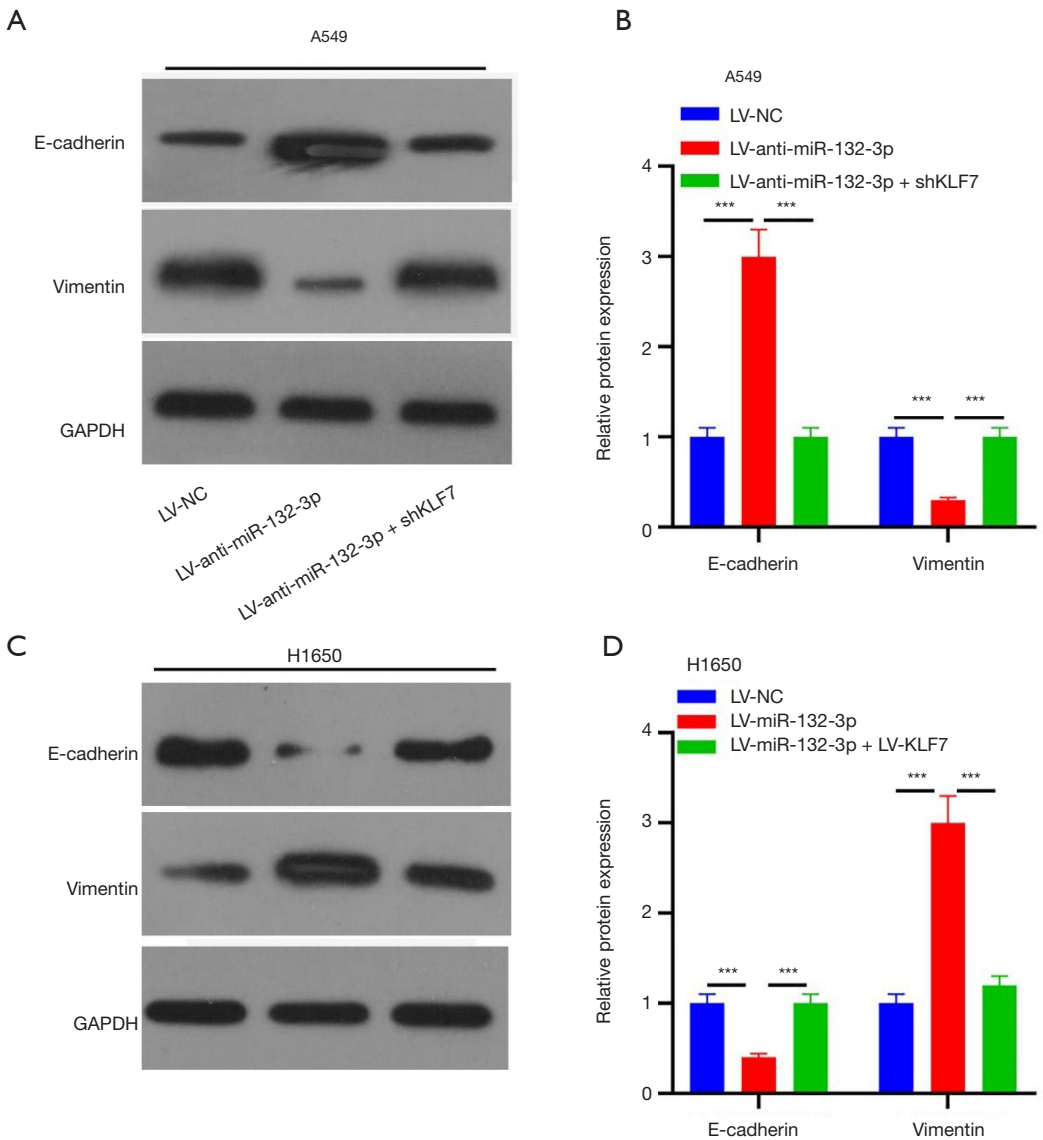

Figure 7 The miR-132-3p/KLF7 axis adjusted interstitial protein. (A,B) We applied western blot to measure E-cadherin and vimentin expression via shKLF7-related transfection in A549; (C,D) Western blot was utilized for determining E-cadherin and vimentin expression through LV-KLF7-related transfection in H1650. ***, P<0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/jtd-21-353

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jtd-21-353). The authors have no conflicts of

interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures involving human participants in this study are consistent with the Helsinki Declaration (as revised in 2013). The study has got approval from Ethics Committee of Shanghai Tongren Hospital (No. 2018-067-01) and obtained the informed consent forms of all patients.

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