Original Article

LINC00174 Suppresses Non-Small Cell Lung Cancer Progression by Up-Regulating LATS2 via Sponging miR-31-5p

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Abstract

Objective: Dysregulation of long non-coding RNAs (lncRNAs) is associated with the progression of non-small cell lung cancer (NSCLC). This study aimed to investigate the role of long intergenic non-protein coding RNA 174 (LINC00174) in NSCLC.

Materials and Methods: In this experimental study, LINC00174 expression in NSCLC tissues and cell lines was investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Besides, cell counting kit-8 (CCK-8), 5-bromo-2'-deoxyuridine (BrdU). Transwell and Flow Cytometry assays were applied to detect the regulatory function of LINC00174 on the growth, migration and apoptosis of NSCLC cells. Bioinformatics analysis, dual luciferase reporter gene assay and RNA immunoprecipitation (RIP) assay predicted and verified the targeting relationship between LINC00174 and miR-31-5p, and between miR-31-5p and the 3´-untranslated region (3´UTR) of large tumor suppressor kinase 2 (LATS2), respectively. Western blotting was performed to detect the regulatory function of LINC00174 and miR-31-5p on LATS2 protein expression.

Results: Compared with that in normal lung tissues, LINC00174 expression in NSCLC tissues and cell lines was reduced. LINC00174 expression was negatively associated with the TNM stage of the patients. Functional experiments showed that LINC00174 overexpression inhibited NSCLC cell multiplication and migration, and induced apoptosis. Furthermore, LINC00174 targeted miR-31-5p and repressed its expression. Additionally, LINC00174 upregulated LATS2 expression through competitively binding to miR-31-5p.

Conclusion: LINC00174, as a competitive endogenous RNA, elevates LATS2 expression by adsorbing miR-31-5p, thereby inhibiting the viability and migration of NSCLC cells, and promoting apoptosis.

Keywords: Human, LATS2 Protein, Long Noncoding, Non-Small-Cell Lung Cancer, RNA

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases (1). Despite important progresses have been made in the treatment of NSCLC in recent years, the prognosis of patients with NSCLC is still not satisfactory, with a 5-year overall survival rate of <20% (2). Thus, clarifying the molecular mechanism of NSCLC progression is of great importance to further improve the prognosis of the patients.

Long non-coding RNA (lncRNA) is pivotal in the progression of tumors by regulating cancer cell viability, metastasis and resistance to treatment (3). For example, lncRNA NEAT1 is abnormally upregulated in colorectal cancer, and activates the Wnt/β-catenin signaling pathway via binding with DEAD-box helicase 5, thereby promoting colorectal cancer metastasis (4). The dysregulation of lncRNAs is also a common biological event in NSCLC (5). For instance, lncRNAs such as BRAF-activated non-protein coding RNA, growth arrest specific 6-antisense RNA 1 and maternally expressed 4 have been shown to inhibit the progression of NSCLC, while metastasis associated lung adenocarcinoma transcript 1 (MALAT1), colon cancer associated transcript 2 and HOX transcript antisense RNA function as oncogenes in NSCLC (6). Long intergenic non-protein coding RNA 174 (LINC00174) is abnormally expressed in glioma, hepatocellular carcinoma and colorectal cancer (7). However, the expression, function and mechanism of LINC00174 in NSCLC are not-well clarified.

In recent years, the interaction between lncRNA and microRNA (miRNA/miR) has attracted great attention in the field of cancer research. LncRNA can sponge miRNA to inhibit the expression and activity of miRNA, leading to upregulation of downstream target genes (8). This study is aimed to probe the molecular mechanism of LINC00174 modulating the progression of NSCLC. Here we report that LINC00174 expression is down-regulated in NSCLC, while miR-31-5p expression is increased. Functionally and mechanistically, LINC00174 represses the malignant phenotype of NSCLC cells, and the

suppressive effect of LINC00174 on the multiplication and migration of NSCLC cells depends on the miR-31-5p/large tumor suppressor kinase 2 (LATS2) axis.

Materials and Methods

Tissue samples

In this experimental study, all the patients involved in the present study were >18 years old (57.3 ± 8.4 years old). A total of 38 pairs of NSCLC tissues and adjacent tissues were collected from patients (23 males and 15 females) with NSCLC who attended Yantai Yuhuangding Hospital from 2018 May to 2019 March**.** Among the 38 cases of NSCLC, 21 cases were adenocarcinoma, and 17 cases were squamous carcinoma. None of the patients received anti-cancer therapies before the surgery. All specimens obtained during surgery were immediately stored in liquid nitrogen for subsequent experiments. The present study, with informed consent, was approved by the Ethics Committee of Yantai Yuhuangding Hospital (YHD20180146).

Cell culture

The Shanghai Cell Bank of Chinese Academy of Sciences provided the human NSCLC cell lines (95-D, H1299 and A549) and the normal bronchial epithelial cell line (HBE), which were used in the present study. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Thermo Fisher Scientific, Inc., USA) with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc., USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Thermo Fisher Scientific, Inc., USA) at 37˚C in 5% CO_2.

Cell transfection

pcDNA3.1 vectors containing the LINC00174 sequence (pcDNA3.1-LINC00174), empty vector, small interfering RNA (siRNA) targeting LATS2 (si-LATS2), scramble siRNA negative control (si-NC), miR-31-5p mimic (5′-CUUUUUGCGGUCUGGGCUUGC-3′), miR-31-5p inhibitor (5′-CGUUCGGGUCUGGCGUUUUC-3′) and the control miRNA (5′-UCACAACCUCCUAGAAAGAGUAGA-3′) were purchased from Shanghai GenePharma Co., Ltd. A549 and H1299 cells were respectively transfected with Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific, Inc., USA) as instructions.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from tissues or cells using TRIzol® reagent (Vazyme, Biotech Co., Ltd., Nanjing, China). cDNA was prepared by reverse transcription with a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd., Shiga, Japan). Next, using cDNA as a template, RT-qPCR was performed with SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd., Shiga, Japan), with housekeeping genes *U6* and *GAPDH* as the endogenous control. The relative expression of the genes was quantified with $2^{-\Delta\Delta Ct}$ method. The sequences of the primers are shown in Table 1.

Table 1: Sequences used for reverse transcription-quantitative polymerase chain reaction

Name	Primer sequences (5′-3′)
LINC00174	F: GGCCCAACACTTCCCTCAAA
	R: CAGGGAGAAACGACCTGGAG
miR-31-5p	F: GGAGAGGCAAGATGCTGGCA
	R: GTGCAGGGTCCGAGGT
LATS2	F: ACCCCAAAGTTCGGACCTTAT
	R: CATTTGCCGGTTCACTTCTGC
U6	F: GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT
GAPDH	F: AAATCCCATCACCATCTTCCAG
	R: TGATGACCCTTTTGGCTCCC

Cell counting kit-8 assay

Transfected A549 and H1299 cells in logarithmic phase were trypsinized with 0.25% trypsin (Thermo Fisher Scientific, Wilmington, DE, USA), and the cell density was accordingly adjusted to 2×10^4 cells/ml with medium. Next, the cells were seeded in 96-well plates (100 μl of cell suspension/well). The following day, 10 μl of CCK-8 solution (Biosharp Life Sciences, Biosharp, China) was added into each well. After incubation for 1 hour, the absorbance of each well at 450 nm was recorded with a microplate reader (Thermo Fisher Scientific, Wilmington, DE, USA). With the same method, the absorbance of the cells was measured every 24 hours for 3 days.

5-bromo-2′-deoxyuridine proliferation assay

A total of 1×10^5 cells/ml cells were inoculated in a 35-mm-diameter petri dish containing a glass cover slip, cultured for 1 day and synchronized with medium containing 0.4% FBS for 3 days so that the majority of cells were in G_0 phase. Subsequently, 1.0 mg/ml 5-bromo-2'-deoxyuridine **(**BrdU) reagent (BD Pharmingen, BD Biosciences, USA) was added, and the cells were cultured in complete medium at 37˚C for 2 hours. Next, the culture solution was removed; besides, the cover slips were washed in phosphate-buffered saline (PBS) three times; and the cells were accordingly fixed with methanol for 10 minutes. The dried slides were subsequently blocked with 5% normal rabbit serum (Beyotime, China), and nucleic acids were denatured with formamide (Sigma-Aldrich, China). Subsequently, the cover slips were rinsed with PBS and then incubated with the primary antibody anti-BrdU (Beyotime, China, 1: 500) at room temperature for 1 hour, while the control group was incubated with PBS. Next, the nuclei of the cells were stained with DAPI staining solution (Beyotime, China) for 2 hours at room

temperature, and finally, the number of BrdU-positive cells in 10 visual fields were observed and counted under a fluorescence microscope (Olympus, Japan), and the average number of the BrdU positive cells was calculated.

Transwell assay

Cell migration was detected with a Transwell system (Corning Inc., Corning, NY, USA). The transfected NSCLC cells were trypsinized with 0.25% trypsin (Gibco, Thermo Fisher Scientific, Inc., USA), and 1×10^5 cells/ml cell suspension was subsequently prepared with serum-free medium. Next, 200 µl of cell suspension was loaded into the upper chamber of the Transwell system, while the lower chamber was added with 600 µl of medium containing 20% FBS. Subsequently, the cells were cultured for 24 hours, and then the cells on the upper surface of the membrane were removed by a cotton swab. Notably, the cells remaining on the below surface of the membrane were then subjected to formaldehyde fixation for 15 minutes and crystal violet staining for 30 minutes. Next, five visual fields were randomly selected under a microscope (Olympus, Japan) for cell counting, and the average was accordingly recorded to represent the migration ability of NSCLC cells.

Western blot analysis

Western blot assay was conducted to measure LATS2 protein expression. Cells in different groups were respectively lysed with 1 ml of RIPA lysis buffer (Biosharp Life Sciences, China) on ice for 20 minutes, and the mixture was centrifuged (1000 g, 4˚C) for 10 minutes and the supernatant was accordingly collected, with the the protein concentration of the samples detected with a BCA Protein Assay kit (Beyotimes, China). Subsequently, the protein sample was mixed with loading buffer (Biosharp Life Sciences, China) and then denatured in boiling water. Next, the samples (10 µg/lane) were dissolved by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4% stacking gel and a 12% separation gel). After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA), which were blocked with 5% skimmed milk for 30 minutes at room temperature and firstly incubated with rabbit anti-LATS2 antibody (1:1,000; ab110780, Abcam, Cambridge, UK) or anti-GAPDH antibody (1:3000, Beyotime, China) at 4˚C overnight, and secondly incubated with a horseradish peroxidase-conjugated secondary antibody (1:2,000; ab205718; Abcam, Cambridge, UK) at room temperature for 1 hour. Finally, the protein bands were developed with enhanced chemiluminescent (ECL) reagent (EMD Millipore, Billerica, MA, USA). Signal quantification was achieved with Quantity One software version 4.6.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with GAPDH as the loading control.

Luciferase reporter assay

The binding sites of miR-31-5p on LINC00174 and the 3′-untransalted region (UTR) of LATS2 were predicted by bioinformatics analysis with StarBase database, and the sequences containing the binding sites were amplified by PCR. The amplified products were inserted into the pGL3-promoter plasmid vector to construct the LINC00174 and LATS2 wild-type (WT) reporter plasmids, while mutant (MUT) plasmids were constructed by site-directed mutagenesis. The above recombinant reporter plasmids were co-transfected into 293T cells with miR-31-5p (or miR-NC). 48 hours later, the cells were collected, and a dual luciferase reporter gene assay system (Promega Corporation, Madison, WI, USA) was conducted to detect the value of luciferase activity.

RNA immunoprecipitation

Cells lysates from different groups were respectively incubated with RIP buffer containing magnetic beads conjugated with anti-human argonaute 2 (Ago2) antibody (EMD Millipore, Billerica, MA, USA), with normal mouse IgG (EMD Millipore, Billerica, MA, USA) as normal controls (NCs). The samples were subsequently incubated with Proteinase K, and then immunoprecipitated RNAs were isolated. The RNA concentration was measured by a spectrophotometer, and the RNA quality was assessed by a bioanalyzer. Purified RNAs were accordingly extracted and qPCR was performed to detect the enrichment of LINC00174.

Bioinformatics analysis

StarBase database was used to predict the binding sites among lncRNA, miRNA and the 3′UTR of mRNA, and Gene Expression Profiling Interactive Analysis (GEPIA) database was used to investigate the expression characteristics of genes in NSCLC tissues.

Flow cytometry

Annexin V-FITC/propidium iodide (PI) double staining was used to detect cell apoptosis. Cells were collected 48 hours after transfection, and the density of cell suspension was adjusted to 1×10^6 cells/mL. The cells were fixed in pre-cooled 70% ethanol and incubated overnight at 4˚C. Subsequently, 100 µL of cell suspension were centrifuged and resuspended in 200 µL of binding buffer. The resuspended cells were immediately stained with 10 µL of Annexin V-FITC staining solution and 5 µL of PI staining solution at ambient temperature for 15 minutes in the dark. Cell apoptosis was then detected with a flow cytometer (Attune NxT, Thermo Fisher, USA) at an excitation wavelength of 488 nm.

Immunohistochemistry

Immunohistochemistry (IHC) was performed to determine LATS2 protein expression in the NSCLC

samples. The NSCLC tissue samples were fixed in 10% formaldehyde and then embedded in paraffin. Subsequently, tissues sections were prepared, and the sections were dewaxed, rehydrated, and next antigen retrieval was conducted. Then the tissues were immersed in 2% H_2O_2 for 10 minutes to inactivate peroxidase, and immersed in 5% bovine serum albumin for 30 minutes to block the non-specific antigens. Then anti-LATS2 antibody (1: 200; ab110780; Abcam, Cambridge, UK) was used to incubate the tissues at 4˚C overnight in a wet box. Next, the tissues were washed with PBS and then incubated with a biotin-linked antiserum for 1 hour at room temperature in a wet box. Next, the tissues were washed by PBS again and stained with 3,3-diaminobenzidine hydrochloride. Eventually, the tissue staining was observed and scored under a microscope by two independent pathologists.

Statistical analysis

All the experiments were performed in triplicate. The data were presented as the "mean ± standard deviation", and GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) was adopted for statistical analysis. Whether the data are normally distributed or not was examined by the Kolmogorov-Smirnov test. Notably, for normally distributed data, an unpaired or paired Student's t test was executed to compare the data between two groups. Besides, comparisons among ≥3 groups were made with one-way ANOVA. If the data exhibited significant differences, Tukey's post-hoc test was then performed to compare the data between groups. For data that were not normally distributed, comparisons between two groups were made by paired-sample Wilcoxon signed-rank test. Additionally, Pearson's correlation coefficient was utilized to examine the correlation between the genes' expressions in the NSCLC samples. Statistically, $P<0.05$ is meaningful.

Results

LINC00174 expression is reduced in human NSCLC tissues

First, RT-qPCR was employed to probe *LINC00174* expression in paired NSCLC tissues and paracancerous tissues. As against that in normal tissues, *LINC00174* expression in cancer tissues was markedly downregulated (Fig.1A). *LINC00174* expression in NSCLC cell lines was also dramatically lower than that in a normal bronchial epithelial cell line HBE (Fig.1B). *LINC00174* expression in lung squamous cell carcinoma and lung adenocarcinoma was respectively analyzed by searching GEPIA database, and the results showed that *LINC00174* expression in both lung squamous cell carcinoma and lung adenocarcinoma was downregulated relative to that in normal tissues (Fig.1C). Additionally, *LINC00174* expression was negatively correlated to the TNM stage of NSCLC patients (Fig.1D), suggesting that the low expression of *LINC00174* could probably be relevant to the progression of NSCLC.

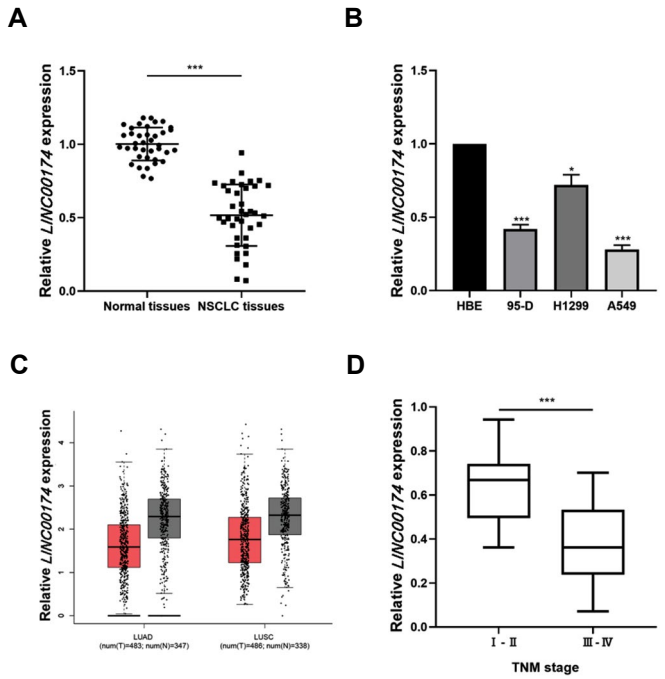

Fig.1: *LINC00174* is expressed at low level in NSCLC. **A.** RT-qPCR was used to detect the expression of *LINC00174* in NSCLC tissues (n=38) and adjacent normal tissues (n=38), and the results indicated that *LINC00174* was down-regulated in NSCLC. **B.** RT-qPCR was employed to detect the expression of *LINC00174* in normal bronchial epithelial cells and NSCLC cell lines, and the results indicated that *LINC00174* was down-regulated in NSCLC cell lines. **C.** Gene Expression Profiling Interactive Analysis (GEPIA) database was applied to analyze the expression of *LINC00174* in normal tissues and NSCLC (lung adenocarcinoma and lung squamous cell carcinoma) tissues, and the results indicated that *LINC00174* was down-regulated in NSCLC. **D.** The correlation between the expression levels of *LINC00174* and the patient's TNM stage (I-II, n=21; III-IV, n=17) was analyzed with Student's t test. *; P<0.05, ***; P<0.001, NSCLC; Non-small cell lung cancer, RT-qPCR; Reverse transcription-quantitative polymerase chain reaction, and TNM; Tumor node metastasis.

Effect of *LINC00174* on the growth and migration of NSCLC cells

We then explored the possible biological functions of *LINC00174* in tumor progression. A549 and H1299 cells were transfected with pcDNA3.1-*LINC00174* and si-*LINC00174*, respectively (Fig.2A, B). CCK-8 and BrdU assays highlighted that, as against that of the control group, the growth of A549 cells transfected with pcDNA3.1-*LINC00174* was greatly inhibited, while the growth of H1299 cells transfected with si-*LINC00174* was demonstrably enhanced (Fig.2C, D). In addition, the results of the Transwell assay revealed that *LINC00174* overexpression inhibited the migration of A549 cells relative to the control, while knocking down *LINC00174* promoted the migration of H1299 cells (Fig.2E). Additionally, *LINC00174* overexpression induced the apoptosis of NSCLC cells, while its knockdown

inhibited the apoptosis of NSCLC cells (Fig.2F). These data uncovered that *LINC00174* repressed the malignant biological behaviors of NSCLC cells.

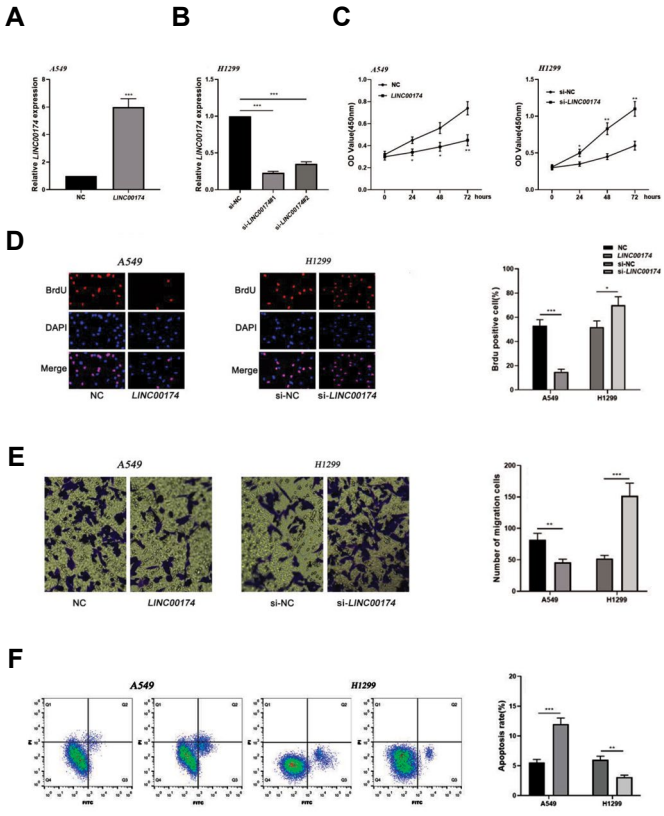

Fig.2: *LINC00174* inhibits NSCLC cell proliferation and migration. **A, B.** RT-qPCR confirmed that the *LINC00174* overexpression and knockdown cell models were successfully constructed. **C.** CCK-8 assay and **D.** BrdU assay were used to detect the proliferation of NSCLC cells, the results of which indicated that *LINC00174* negatively regulated the proliferation of NSCLC cells. **E.** Transwell assay was used to detect the migration of NSCLC cells, the results of which indicated that *LINC00174* negatively regulated the migration of NSCLC cells. **F.** Flow cytometry was used to detect the apoptosis of NSCLC cells, the results of which indicated that *LINC00174* positively regulated the apoptosis of NSCLC cells. *; P<0.05, **; P<0.01, ***; P<0.001, NSCLC; Non-small cell lung cancer, and RT-qPCR; Reverse transcription-quantitative polymerase chain reaction.

miR-31-5p is a target of *LINC00174*

Bioinformatics analysis with StarBase suggested that there was a potential binding site between *LINC00174* and miR-31-5p (Fig.3A). Dual luciferase reporter gene assay was performed to verify the above prediction, and the findings indicated that miR-31-5p greatly decreased *LINC00174*-WT luciferase activity but had no effect on that of *LINC00174*-MUT (Fig.3B). RIP assay was conducted to verify the interaction between *LINC00174* and miR-31-5p. The results indicated that compared with the non-specific IgG group, *LINC00174* and miR-31-5p were specifically enriched by anti-Ago2 antibodies, indicating that *LINC00174* could directly bind with miR-31-5p (Fig.3C). Furthermore, the effects of *LINC00174* overexpression and knockdown on miR-31-5p expression were examined, and it was found that *LINC00174* overexpression remarkably inhibited miR-31-5p expression as against that of the control, while knocking down *LINC00174* led to a higher expression of miR-31-5p (Fig.3D). The present study also analyzed miR-31-5p expression in NSCLC tissues using bioinformatics tools (StarBase database) and RT-qPCR. The results demonstrated that miR-31-5p expression in cancer tissues was higher than that in paracancerous tissues (Fig.3E, F). In addition, RT-qPCR revealed that *LINC00174* negatively regulated miR-31-5p expression (Fig.3G). The above results indicated that miR-31-5p was the downstream target of *LINC00174*, and miR-31-5p was negatively regulated by it.

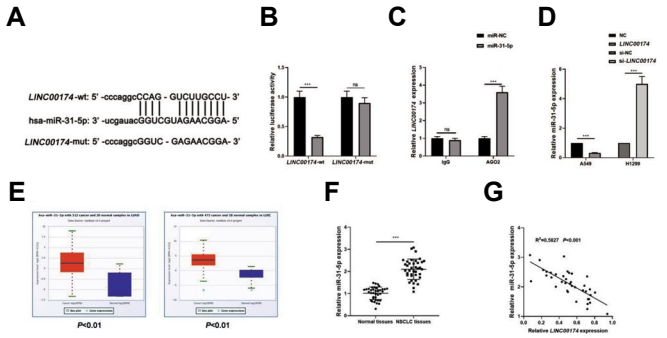

Fig.3: miR-31-5p is the target of *LINC00174*. **A.** StarBase (http://starbase.sysu.edu.cn/index.php) predicted the binding site between *LINC00174* and miR-31-5p. **B.** Dual luciferase reporter gene assay validated the binding association between *LINC00174* and miR-31-5p. **C.** RIP assay was conducted to verify the interaction between *LINC00174* and miR-31-5p. **D.** RT-qPCR was applied to detect the effect of overexpression or knockdown of *LINC00174* on miR-31-5p expression, and the results indicated that *LINC00174* negatively regulated the expression of miR-31-5p in NSCLC cells. **E.** StarBase database and **F.** RT-qPCR were employed to analyze the expression of *miR-31-5p* in NSCLC tissues (n=38) and normal lung tissues (n=38), and the results showed that *miR-31-5p* was up-regulated in NSCLC tissues. **G.** *LINC00174* was negatively correlated with the expression of miR-31-5p in NSCLC samples (n=38). ***; P<0.001, ns; No statistical significance, NSCLC; Non-small cell lung cancer, RT-qPCR; Reverse transcription-quantitative polymerase chain reaction, and miR; microRNA.

LINC00174 functions by inhibiting miR-31-5p

To clarify whether *LINC00174* inhibits the malignancy of NSCLC cells by suppressing miR-31-5p expression, miR-31-5p mimics were transfected into A549 cells with *LINC00174* overexpression, and miR-31-5p inhibitors were subsequently transfected into H1299 cells with *LINC00174* knockdown (Fig.4A, B). The findings revealed that the transfection of miR-31-5p mimics partially reversed the effects of *LINC00174* overexpression on the multiplication, migration and apoptosis of A549 cells, while miR-31-5p inhibitors partially counteracted the effect of knocking down *LINC00174* on the malignant biological behaviors of H1299 cells (Fig.4C-F).

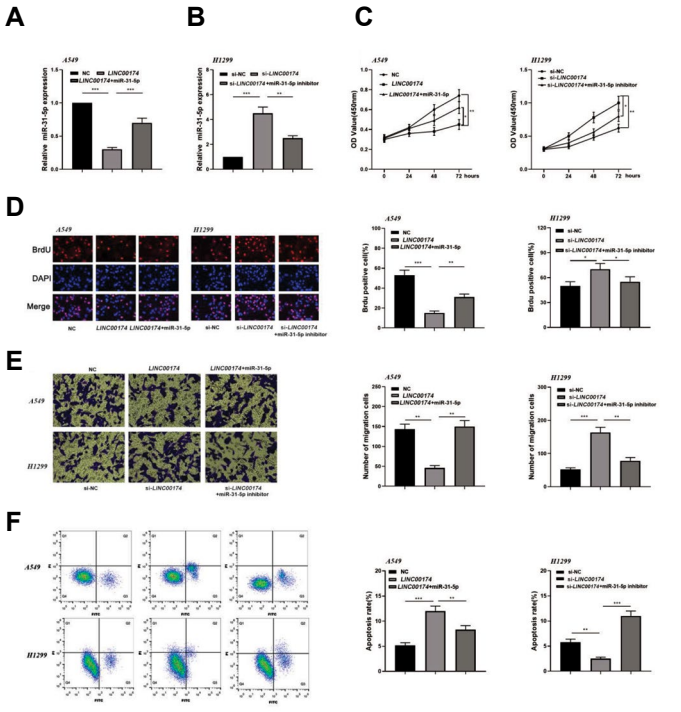

Fig.4: miR-31-5p partially reverses the inhibitory effect of *LINC00174* on NSCLC. NSCLC cells with *LINC00174* overexpression or knockdown were transfected with **A.** miR-31-5p mimics or **B.** miR-31-5p inhibitor, respectively, and the miR-31-5p expression levels in NSCLC cells were detected by RT-qPCR. **C.** CCK-8 and **D.** BrdU assay were used to detect the proliferation of NSCLC cells after transfection, the results of which showed that miR-31-5p counteracted the biological function of *LINC00174*. **E.** Transwell assay was used to detect the migration of NSCLC cells after transfection, the results of which showed that miR-31-5p counteracted the biological function of *LINC00174*. **F.** Flow cytometry was used to detect the apoptosis of NSCLC cells after transfection, the results of which showed that miR-31-5p counteracted the biological function of *LINC00174.* *; P<0.05, **; P<0.01, ***; P<0.001, NSCLC; Non-small cell lung cancer, RT-qPCR; Reverse transcription-quantitative polymerase chain reaction, and miR; MicroRNA.

LINC00174 upregulates LATS2 by inhibiting miR-31-5p

StarBase database predicted that LATS2 mRNA is a hidden target of miR-31-5p (Fig.5A). Luciferase reporter gene assay suggested that miR-31-5p greatly decreased LATS2-WT luciferase but that of LATS2-MUT was not significantly affected (Fig.5B). GEPIA database was used to analyze LATS2 expression in NSCLC. It was found that, relative to that in normal tissues, LATS2 expression in NSCLC was markedly downregulated (Fig.5C). Furthermore, miR-31-5p restrained LATS2 expression at the mRNA and protein level in NSCLC cells, while miR-31-5p inhibitors had opposite effects (Fig.5D). These data confirmed that LATS2 mRNA was a target of miR-31-5p, and that miR-31-5p inhibited its expression and translation via binding to the 3′UTR of LATS2 mRNA.

Considering that miR-31-5p is targeted by *LINC00174*, the present study also examined the effect of the *LINC00174*/miR-31-5p axis on LATS2 expression in NSCLC cells. Western blotting showed that *LINC00174* overexpression promoted LATS2 expression, and that the effect of *LINC00174* was partially reversed by miR-31-5p (Fig.5E). Furthermore, RT-qPCR uncovered that LAST2 expression was positively correlated with that of *LINC00174* and negatively correlated with that of miR-31-5p in NSCLC samples (Fig.5F, G). LATS2 expression in cancer tissues and adjacent tissues of 38 patients with NSCLC was also detected by IHC. It showed that the expression of LAST2 protein was markedly reduced in NSCLC tissues (Fig.5H, I). These data implied that *LINC00174* could upregulate LATS2 expression in NSCLC via repressing miR-31a-5p.

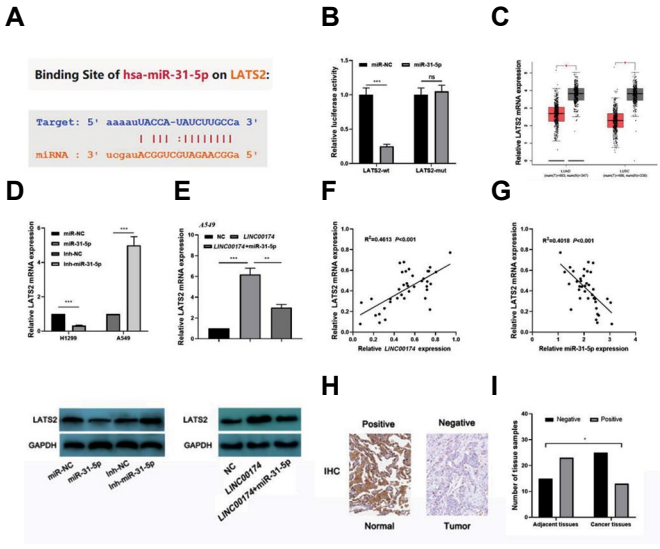

Fig.5: *LINC00174* upregulates LATS2 mRNA by decoying miR-31-5p. **A.** StarBase database was used to predict the binding site between miR-31-5p and LATS2 mRNA. **B.** Dual luciferase reporter gene assay was adopted to verify the targeted binding relationship between miR-31-5p and LATS2 mRNA. **C.** The Gene Expression Profiling Interactive Analysis (GEPIA) database was used to analyze the mRNA expression of LATS2 in normal tissues and NSCLC (lung adenocarcinoma and lung squamous cell carcinoma) tissues, which suggested that LATS2 was underexpressed in NSCLC tissues. **D, E.** The mRNA (upper) and protein (below) expression levels of LATS2 were detected by RT-qPCR and western blot. **F, G.** The correlations between *LINC00174* and LATS2 mRNA, and between miR-31-5p and LATS2 mRNA were analyzed. **H, I.** IHC was used to detect the expression of LATS2 expression in NSCLC samples, which showed that LATS2 expression was reduced in NSCLC tissues. *; P<0.05, **; P<0.01, ***; P<0.001, ns; No statistical significance, NSCLC; Non-small cell lung cancer, RT-qPCR; Reverse transcription-quantitative polymerase chain reaction, and miR; microRNA.

Discussion

LncRNAs play crucial roles in cancer biology through diverse mechanisms. In the nuclei, lncRNAs can regulate gene expression by directly interacting with DNA or chromatin-regulatory factors, transcription factors and RNA-binding proteins, functioning as enhancers, baits or scaffolds; in the cytoplasm, lncRNAs interact with mRNA and regulate the stability or translation of mRNAs; furthermore, lncRNAs serve as competitive endogenous RNAs (ceRNAs), reducing the inhibitory effect of miRNA on mRNA (5, 9). For example, in colorectal cancer, small nucleolar RNA host gene 1 is induced by Sp1 transcription factor (SP1), and, as the ceRNA of miR-154-5p, it reduces the ability of miR-154-5p to inhibit

cyclin D2 expression, thus promoting cell multiplication (10). Since lncRNAs can be detected in body fluids such as blood, urine and saliva, they also have the potential to act as biomarkers. It has been reported that lncRNA MALAT1 expression in the serum of patients with NSCLC is greatly higher than that in healthy controls (11). The levels of nuclear paraspeckle assembly transcript 1, CDKN2B-AS1 and sprouty RTk signaling antagonist 4-IT1 levels are increased in the plasma of patients with NSCLC (12). LINC00174 adsorbs miR-320 to increase oncogene S100A10 expression in hepatocellular carcinoma, thereby promoting the growth and metastasis of hepatocellular carcinoma cells while inhibiting apoptosis (13). In glioma, LINC00174 acceletrates the multiplication and metastasis of cancer cells, and inhibits apoptosis via modulating the miR-152-3p/solute carrier family 2 member 1 axis (7). The present study demonstrated that LINC00174 expression in NSCLC tissues and cell lines was evidently lower than that in normal tissues and cell lines, and that LINC00174 expression was negatively correlated with the TNM stage of patients with NSCLC. In addition, *in vitro* functional experiments confirmed that LINC00174 inhibited the viability and migration of NSCLC cells. Our data indicated that LINC00174 acted as a tumor suppressor in NSCLC.

Reportedly, miRNAs targets and inhibits the translation of mRNA or reduces the stability of mRNA, thus playing a regulatory role in the development of cancer (1, 14). For example, miR-449b-3p inhibits the epithelial-mesenchymal transformation of NSCLC cells via targeting IL-6 and modulating the JAK2/STAT3 signaling pathway (15). miR-128-3p promotes the growth and migration of NSCLC cells via activating the Wnt/β-catenin and TGF-β pathways (16). miR-31-5p plays different roles in different tumors, either promoting or inhibiting cancer progression. For example, miR-31-5p facilitates the growth, migration and invasion of colorectal cancer cells by targeting NUMB endocytic adaptor protein (17). In hepatocellular carcinoma, miR-31-5p inhibits the malignant biological behaviors via modulation of SP1 transcription factors (18). Some previous studies report that miR-31-5p expression in NSCLC is elevated, and its overexpression is dramatically correlated with the unfavorable prognosis of patients with NSCLC (19-22). The present study confirmed that miR-31-5p expression in NSCLC tissues was markedly higher than that in the normal tissues, suggesting that miR-31-5p works as an oncomiR in NSCLC.

ceRNA is a vital regulatory mechanism in cancer biology. The ceRNA network links the function of protein coding mRNA with that of non-coding RNA (23). For example, HOXD-AS1, as the ceRNA of miR-147a, upregulates pRB expression, thus promoting the multiplication and cell cycle progression of NSCLC cells, and inhibiting cell apoptosis (24). MALAT1 competitively sponges miR-124 to upregulate STAT3 expression, thus accelerating the malignant progression of NSCLC (25). MiR-31-5p is also modulated by ceRNA mechanisms. For example, in bladder cancer, circular-bromodomain PHD finger transcription factor expedites the multiplication and metastasis of bladder cancer cells by regulating the the miR-31-5p/RAB27A axis (26). MiR-31-5p, which is decoyed by long intergenic non-protein coding RNA 1234, inhibits the expression of MAGE family member A3 in hepatocellular carcinoma, thus reducing the multiplication, invasion and drug resistance of hepatocellular carcinoma cells (27). The present study revealed that LINC00174 functioned as the ceRNA of miR-31-5p and negatively regulates its expression. In addition, it was found that miR-31-5p could reverse the inhibitory effect of LINC00174 on the multiplication and migration of NSCLC cells.

LATS2 has been reported to be a tumor suppressor, and it is vital in regulating the cell cycle, mitosis and genomic stability (28). LATS2 forms a positive feedback loop with p53: it promotes the tumor-suppressive effect of p53 by binding or inactivating MDM2; in turn, p53 directly regulates the transcription of LATS2 (29). In addition, LATS2 is the key regulator of the Hippo signaling pathway, which is closely relevant to the occurrence and progression of multiple types of cancers. It has been reported that low expression of LATS2 in NSCLC is associated with poor prognosis (30, 31). In NSCLC, LATS2 blocks the growth, migration and invasion of NSCLC cells: LATS2 overexpression leads to the phosphorylation of the oncoprotein YY1 associated protein 1, which is a downstream effector of Hippo signaling, resulting in increased expression of E-cadherin, and downregulation of vimentin and matrix metalloproteinase 9 (32). Previous studies report that miR-31-5p can target peroxisomal biogenesis factor 5, tensin 1, CDK1 and other genes involved in the regulation of hepatocellular carcinoma, colon adenocarcinoma and renal cell carcinoma (33-35). In addition, miR-31-5p modulates the drug sensitivity of colorectal cancer cells by targeting LATS2 (36). Here we demonstrated that LATS2 was expressed at low level in NSCLC and negatively modulated by miR-31-5p, but positively regulated by LINC00174. Our data provide a novel mechanism explaining the dysregulation of LAST2 in NSCLC.

Conclusion

On all accounts, LINC00174 is lowly expressed in NSCLC tissues and cell lines, and that LINC00174 functions as a ceRNA to inhibit the multiplication and migration of NSCLC cells via the miR-31-5p/LATS2 axis. To our best knowledge, this is the first study on the role of LINC00174 in lung cancer, and our data extend the understanding of the mechanism of NSCLC progression. In the following studies, *in vivo* experiments are necessary to further validate our demonstrations, and more patients should be enrolled to verify the potential of LINC00174 as a prognostic marker.

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in this study.

Authors' Contributions

Me.S.; Designed this study. X.C., Ma.S., W.J., L.C.; Performed the experiments. X.C., Ma.S.; Analyzed and interpreted the experimental data. X.C., W.J., Ma.S.; Wrote the draft. X.C., W.J.; Performed the revision. Me.S.; Confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

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