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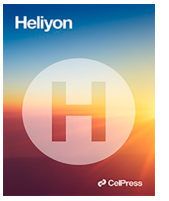

Long intergenic non-protein-coding RNA 1547 acts as a competing endogenous RNA and exerts cancer-promoting activity in non-small cell lung cancer by targeting the microRNA-195-5p/homeobox C8 axis

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ABSTRACT

Long intergenic non-protein coding RNA 1547 (LINC01547) presents a notable relationship with prognosis in patients with ovarian cancer. Herein, we examined the expression of LINC01547 in non-small cell lung cancer (NSCLC) to ascertain its clinical significance. We also explored the detailed functions of LINC01547 in regulating the aggressive phenotype of NSCLC and the molecular mechanism of action underlying its carcinogenic activities events in NSCLC. Furthermore, we applied the data acquired from the tissue specimens and the Cancer Genome Atlas (TCGA) database to analyze the level of LINC01547 in NSCLC and conducted functional assays to address the regulatory effect of LINC01547. Further, we examined the mechanistic interaction among LINC01547, microRNA-195-5p (miR-195-5p), and homeobox C8 (HOXC8) using bioinformatics prediction and luciferase reporter assay. LINC01547 was noticeably overexpressed, as affirmed by data from TCGA and our own cohort; moreover, poor prognosis was associated with increased LINC01547 levels in patients with NSCLC. LINC01547 regulates cell proliferation, colony-forming, migration, and invasion, and its absence produced tumor-repressing effects in NSCLC. Mechanistically, as a competitive endogenous RNA, LINC01547 decoyed miR-195-5p and consequently resulted in the overexpression of HOXC8 in NSCLC cells. Using rescue experiments, we found that the regulatory activities of LINC01547 deficient in repressing the malignant properties of NSCLC cells could be counteracted by hindering miR-195-5p or overexpressing HOXC8. Conclusively, LINC01547 serves as a crucial component to worsen the oncogenicity of NSCLC cells by controlling the miR-195-5p/HOXC8 axis. Thus, the newly identified competing endogenous RNA pathway may potentially be an attractive therapeutic for NSCLC management.

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1. Introduction

Lung cancer is the most common malignant tumor associated with the respiratory system, with higher morbidity and mortality rates than any other type of human cancer [1]. Currently, surgical excision is the optimal treatment method for patients with early-stage non-small cell lung cancer (NSCLC). However, over 70% of cases are identified at middle or advanced stages when they present symptoms, and by then, most have already been deprived of the best curative approach [2]. Despite revolutionary improvements in the diagnosis and management technology, merely 15% of patients with NSCLC can survive up to 5 years [3]. The possibility of relapse is on the rise because of the high aggressiveness, metastasis, and emergence of treatment-resistant phenotype [4]. Undoubtedly, unraveling the molecular events underlying NSCLC pathogenesis and developing promising therapeutic targets may aid in the improvement of clinical efficiency.

Non-coding RNAs (ncRNAs) lack protein-coding capacity and account for over 90% of transcripts [5]. Long non-coding RNAs (lncRNAs) are over 200 nucleotides in length and are defined as a novel group of ncRNA [6]. Previously, lncRNAs were referred to as "noise" in transcription events. However, several researchers recently proved that they are closely associated with numerous biological behaviors, including cell cycle, cell proliferation, apoptosis, metastasis, differentiation, autophagy, and development [7–9]. For example, a lncRNA named metastasis associated lung adenocarcinoma transcript 1 promotes osteogenic differentiation by targeting the microRNA-217/AKT serine/threonine kinase 3 axis [10]. Additionally, lncRNA RP11-295G20.2 is involved in the regulation of hepatocellular carcinoma cell growth and autophagy [11]. Accumulating studies have validated the dysregulation of lncRNA in many disorders, including human cancers [12–14]. The abnormal expression of lncRNAs may result in changes in the expression of tumor-related genes, thereby exerting tumor-promoting or anti-tumor activities [15]. Extensive work has disclosed that lncRNAs are implicated in various malignant processes associated with the pathogenesis of NSCLC [16–18].

MicroRNAs (miRNAs) are short endogenous single-stranded ncRNAs [19]. They exert their regulatory activities by directly interacting with the 3′-untranslated regions downstream of the target genes, resulting in the rapid degradation of mRNAs and/or restriction of translational processes [20]. Competing endogenous RNAs (ceRNAs) constitute a new mechanism for endogenous interactions between RNAs. MiRNAs are known to cause gene silencing by binding to mRNA, and ceRNAs can regulate gene expression by competitively binding to miRNAs. ceRNAs can disable miRNA through the binding of their miRNA response elements with miRNA, which reveals the existence of an RNA-miRNA regulatory pathway which is of great biological significance [21]. LncRNAs can control the expression of miRNAs by serving as ceRNAs and consequently affecting the onset and progression of NSCLC [22,23].

Long intergenic non-protein coding RNA 1547 (LINC01547) has been identified to be closely related to the overall survival of patients with ovarian cancer [24]. However, little is known about the possible involvement of LINC01547 in the modulation of the aggressive phenotype of NSCLC. Furthermore, the downstream effector implicated in the activity of LINC01547 are also unknown. We hypothesized that LINC01547 promoted the malignancy of NSCLC cells by targeting the miR-195-5p/*HOXC8* axis. The LINC01547/miR-195-5p/*HOXC8* pathway may have great potential as a therapeutic target for NSCLC treatment.

2. Material and methods

2.1. Sample collection

A total of 42 NSCLC tissues and adjacent normal lung tissues were obtained from patients who were diagnosed with NSCLC and received surgical resection in the Affiliated Cancer Hospital and Institute of Guangzhou Medical University. The study design was approved by the Ethics Committee of our hospital. All patients provided written informed consent. The exclusion criterion was that they had not undergone local or systemic anticancer therapies before surgery. The clinical tissues were stored in liquid nitrogen after collection.

2.2. Cell culture

NSCLC cell lines, H460 and H522, were cultured in the F–12K medium (Gibco; Thermo Fisher Scientific, MA, USA). NSCLC cell lines, A549 and SK-MES-1, were grown in 10% MEM and RPMI-1640 (Gibco; Thermo Fisher Scientific, MA, USA), respectively. Meanwhile, 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, MA, USA) was added to the abovementioned culture medium. Bronchial epithelial cell growth medium (Lonza/Clonetics Corporation, Walkersville, MD, USA) supplemented with 10% FBS was used for the culture of human non-tumorigenic bronchial epithelial cells, BEAS-2B. All cell lines were procured from ATCC (Manassas, VA, USA).

Table 1
The sequences of siRNAs.

siRNA	Sequence (5′-3′)
si-LINC01547#1	TGGCCTTTTTAAAATTCTATATT
si-LINC01547#2	GGCCTTTTTAAAATTCTATATTT
si-LINC01547#3	GCCTTTTTAAAATTCTATATTTG
si-NC	CACGATAAGACAATGTATTT

2.3. Cell transfection

miR-195-5p mimic, mimic control, miR-195-5p inhibitor and inhibitor control were purchased from RIBOBIO (Guangzhou, China). GenePharma (Shanghai, China) was the provider of specific LINC01547 small interfering (si)RNAs (si-LINC01547) and negative control siRNA (si-NC). The siRNA sequences are presented in Table 1. *HOXC8* was overexpressed in NSCLC cells by transfecting with pcDNA3.1-HOXC8 (pc-HOXC8) plasmid (Sangon; Shanghai, China). All transfection experiments were carried out using Lipofectamine® 3000 (Invitrogen, CA, USA).

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Small RNAs were extracted by applying RNAiso Kit for Small RNA, and reverse-transcribed into complementary DNA using a Mir-X™ miRNA First-Strand Synthesis Kit (TaKaRa; Dalian, China). For miR-195-5p quantification, PCR amplification was carried out by using Mir-X miRNA qRT-PCR TB Green® Kit (TaKaRa; Dalian, China). Expression of miR-195-5p was normalized with U6 snRNA. Total RNA was extracted by using TRIzol® reagent (Invitrogen, CA, USA). To determine the expression levels of *LINC01547* and *HOXC8*, PrimeScript™ RT Reagent Kit and TB Green® Premix Ex Taq™ II (TaKaRa; Dalian, China) were employed to carry out reverse transcription and PCR amplification, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference control for LINC01547 and *HOXC8*. All data were analyzed using the $2^{-\Delta\Delta Cq}$ method [25]. The sequences of all primers are presented in Table 2.

2.5. Transwell migration and invasion assays

The assays were performed as described [26]. Transwell inserts (8 μm pore size) precoated with Matrigel (BD Biosciences) were used for assessing cell invasion. After trypsinization, the transfected cells were resuspended in an FBS-free basal medium. A 200 μL cell suspension containing 4×10^4 cells was placed in the upper chambers whereas 600 μL of 10% FBS-supplemented culture medium in the lower chambers served as the chemoattractant. After a day, the invaded cells were fixed in 100% methanol and stained with crystal violet. Subsequently, the cells were visualized under a light microscope (Olympus; Tokyo, Japan). Transwell migration assays were executed using Transwell inserts without Matrigel and a similar procedure was followed.

2.6. Cell counting Kit-8 (CCK-8) assay

The assay was performed as previously reported [27]. A cell suspension was prepared with cell density adjusted to 3×10^4 cells/mL. A 100 μL cell suspension was seeded into 96-well plates, followed by incubation at 37 °C. Cells were harvested daily until day 3, and cell proliferation was monitored by probing with 10 μL CCK-8 solution (Beyotime; Shanghai, China). After a 2h cultivation, the absorbance at a wavelength of 450 nm was read on a microplate reader.

2.7. Colony formation assay

The colony formation assay was conducted according to a previous study [28]. To perform this assay, transfected cells were resuspended in a fresh culture medium and then seeded into 6-well plates. Each well was covered with 2 mL cell suspension containing 500 cells. Cells were grown under normal conditions. On day 14 of cell culture, the cells were stained with 0.1% crystal violet. Finally, the newly formed colonies were counted on a light microscope.

Table 2
Primer sequences used for qRT-PCR.

Gene	Sequence (5′-3′)
LINC01547	Forward: AGGCCTCACAGAGGGAAACAC
	Reverse: GGGGTTTGTAATCGCTGTTGTC
HOXC8	Forward: TGAGCTCCTACTTCGTCAACCC
	Reverse: TGGTAGCCTGAGTTGGAGATGC
GAPDH	Forward: ACCTGACCTGCCGTCTAGAAAA
	Reverse: TTGAAGTCAGAGGAGACCACCTG
miR-195-5p	Forward: TCGGCAGGUAGCAGCACAG
	Reverse: CACTCAACTGGTGTCGTGGA
miR-339-3p	Forward: TCGGCAGGCGGCUCUGUCGU
	Reverse: CACTCAACTGGTGTCGTGGA
miR-449a	Forward: TCGGCAGGUGGCAGUGUAUUG
	Reverse: CACTCAACTGGTGTCGTGGA
miR-449b-5p	Forward: TCGGCAGGAGGCAGUGUAUUG
	Reverse: CACTCAACTGGTGTCGTGGA
miR-589-5p	Forward: TCGGCAGGUGAGAACCACGUC
	Reverse: CACTCAACTGGTGTCGTGGA
U6	Forward: GCTTCGGCAGCACATATACTAAAAT
	Reverse: CGCTTCACGAATTTGCGTGTCAT

2.8. Xenograft tumor growth model

The experimental procedures involving nude mice were approved by the Institutional Animal Care and Use Committee of Affiliated Cancer Hospital and the Institute of Guangzhou Medical University. The short-hairpin RNAs targeting LINC01547 (sh-LINC01547) and short-hairpin RNAs control (sh-NC; both from GenePharma) were inserted into pLKO.1 vector, and transfected into 293T cells using Lipofectamine® 3000. After 2 days, the lentiviruses were harvested and transfected into A549 cells. Eventually, the selection of stably transfected A549 cells was carried out using puromycin.

Subsequently, A549 cells that stably overexpressed sh-LINC01547 or sh-NC were subcutaneously injected into the BALB/c nude mice (HFK Bio-Technology; Beijing, China). The size of tumor xenografts was recorded weekly and used for the calculation of tumor volume. All mice were euthanized at 5 weeks post-injection and the tumor xenograft tissues were collected.

2.9. Nuclear/cytoplasmic fractionation experiment

The nuclear and cytoplasmic fractions were separated from NSCLC cells using a Cytoplasmic and Nuclear RNA Purification kit (Norgen Biotek Corp). Then, the nuclear and cytoplasmic RNAs were extracted and subjected to qRT-PCR for the determination of LINC01547 localization.

2.10. Bioinformatics prediction

The binding interaction between LINC01547 and miR-195-5p was forecasted by using the miRDB (http://mirdb.org/) and StarBase (http://starbase.sysu.edu.cn/) databases. miRDB and TargetScan (http://www.targetscan.org) were used to search for the downstream targets of miR-195-5p.

2.11. RNA immunoprecipitation (RIP)

RIP was carried out as previously reported [29]. This assay was carried out using EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, Massachusetts, USA) in line with the supplier's instructions. RIP cell lysis buffer was used to prepare the NSCLC whole cell lysates. Thereafter, cell lysates were incubated with magnetic beads conjugated with anti-argonaute2 (Ago2) or IgG control (Millipore, Massachusetts, USA) at 4 °C overnight. After proteinase K treatment, immunoprecipitated RNAs were extracted, and tested using qRT-PCR.

2.12. Luciferase reporter assay

Luciferase reporter assay was performed as previously reported [30]. The wild-type (WT) sequences of LINC01547 and *HOXC8* were constructed using GenePharma and inserted into the psiCHECK™-2 vector, thus, generating the WT-LINC01547 and WT-HOXC8 recombinant luciferase reporter vectors. In the same way, the mutant (MUT) vectors, namely MUT-LINC01547 and MUT-HOXC8, were also constructed. For the reporter assay, miR-195-5p mimic or mimic control alongside WT or MUT vectors were transfected into NSCLC cells. Forty-eight hours after cultivation, the luciferase activity was assessed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, Wisconsin, USA).

2.13. Western blotting

Western blotting was performed as previously described [31]. Cultured cells were treated with radioimmunoprecipitation assay (RIPA) buffer (KeyGen; Nanjing, China) containing a protease inhibitor cocktail (Beyotime, Shanghai, China) for total protein isolation. A BCA Kit (KeyGen, Wageningen, the Netherlands) was applied for the quantification of protein in each sample. Proteins (20 μg) were electrophoresed using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), after which they were transferred to polyacrylamide difluoride membranes. After blocking with 5% skimmed milk for 2 h, the membranes were incubated overnight at 4 °C with primary antibodies targeting HOXC8 (ab79690; Abcam, Cambridge, MA, USA) or GAPDH (ab128915; Abcam, Cambridge, MA, USA). Thereafter, the membranes were incubated with a secondary antibody (ab205718; Abcam, Cambridge, MA, USA) the next day, and protein signals were detected by employing the New Super ECL Kit (KeyGen BioTech, Wageningen, the Netherlands).

2.14. Statistical analysis

Data are expressed as mean ± standard deviation (SD), and the normal distribution of data was checked before data analysis. The comparison between two groups was performed applying Student's *t*-test, whereas the comparison among multiple groups was performed using ANOVA (one-way) and Tukey's posthoc test. Survival curves were plotted and compared using the Kaplan-Meier method and log-rank test, respectively. $P < 0.05$ indicates a statistically significant difference.

3. Results

In this study, we attempted to examine the expression pattern and specific functions of LINC01547 in NSCLC. Furthermore, we explored the mechanisms by which LINC01547 exerts its tumor-promoting actions in NSCLC. Our work uncovered a novel ceRNA pathway involving LINC01547, miR-195-5p and *HOXC8* in NSCLC.

3.1. LINC01547 is overexpressed in NSCLC tumor tissues and correlates with poor prognosis in NSCLC patients

We analyzed LINC01547 expression levels in lung cancer using the data from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). LINC01547 was strikingly upregulated in Lung Adenocarcinoma (LUAD) and Lung Squamous Cell Carcinoma (LUSC) (Fig. 1A). LINC01547 expression in the 42 pairs of NSCLC and adjacent normal lung tissues collected from patients was determined by using qRT-PCR and was found to be significantly higher in NSCLC tissues relative to normal tissues (Fig. 1B). Thus, consistent with the TCGA results, our results showed that LINC01547 expression level was higher in NSCLC. To evaluate the correlation between LINC01547 level and survival of patients with NSCLC, 42 NSCLC patients were divided into low expression and high groups expression according to the median expression value of LINC01547 level. Kaplan-Meier survival analysis showed that patients with high LINC01547 expression levels had lower overall survival than patients with lower LINC01547 levels (Fig. 1C). Furthermore, to determine whether LINC01547 played a role in NSCLC progression, qRT-PCR was performed in the four NSCLC cell lines. We found that all four NSCLC cell lines overexpressed LINC01547 compared with the BESA-2B cell line. (Fig. 1D). Notably, as H460 and A549 cells showed relatively higher levels of LINC01547 among the four NSCLC cell lines, they were used in the subsequent experiments.

3.2. LINC01547 promotes NSCLC cell proliferation, migration and invasion

We knocked down LINC01547 expression by transfecting a specific small interfering RNA; to prevent off-target effects, three siRNAs targeting LINC01547 were applied to entirely abrogate LINC01547 expression. The transfection efficiencies in H460 and A549 cells were confirmed by qRT-PCR (Fig. 2A). Results showed that si-LINC01547#1 and si-LINC01547#2 were applied in the subsequent assay. CCK-8 assay and colony formation assays were performed to investigate the effect of LINC01547 on cell proliferation. The results showed that LINC01547 knockdown by si-RNA significantly reduced the cell proliferation rate. (Fig. 2B and C). Furthermore, we next examined the effect of LINC01547 on NSCLC migration ability by performing transwell migration assays. The results showed that LINC01547 knockdown suppressed the migration ability of H460 and A549 cells. (Fig. 2D). Similarly, the invasion abilities of the two

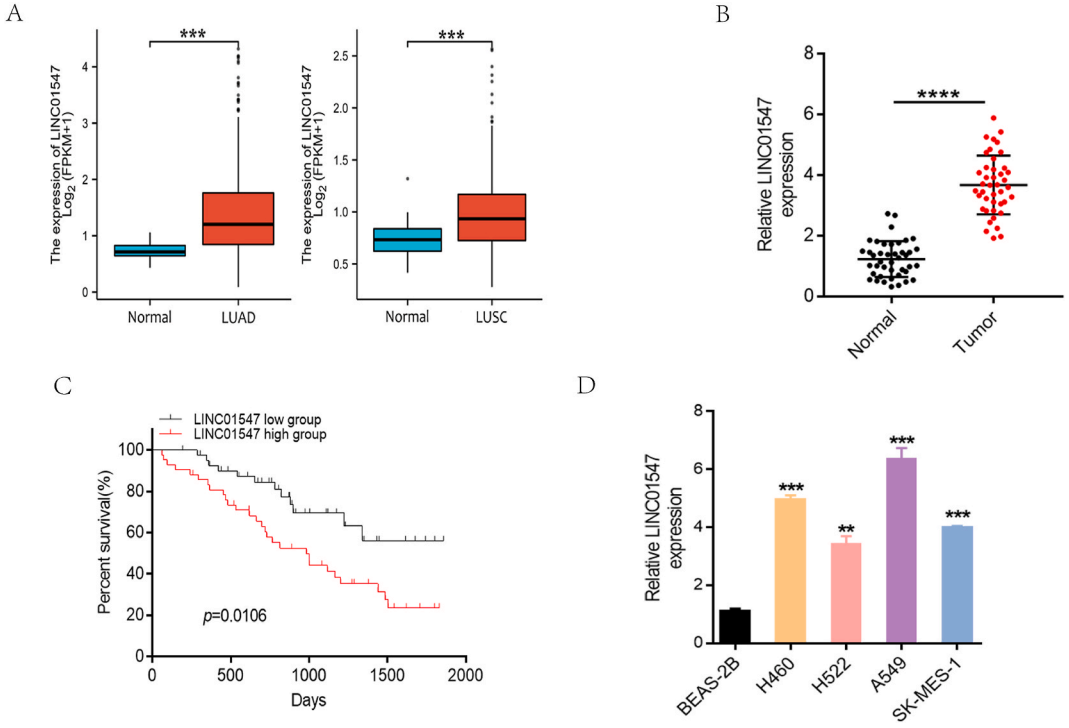

Fig. 1. Overexpressed LINC01547 in NSCLC. (A) Expression of LINC01547 in LUAD and LUSC from TCGA database. (B) The measurement of LINC01547 in NSCLC tissues was conducted utilizing qRT-PCR. (C) The relationship between LINC01547 level and overall survival in NSCLC patients. (D) LINC01547 expression in a panel of NSCLC cell lines.
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

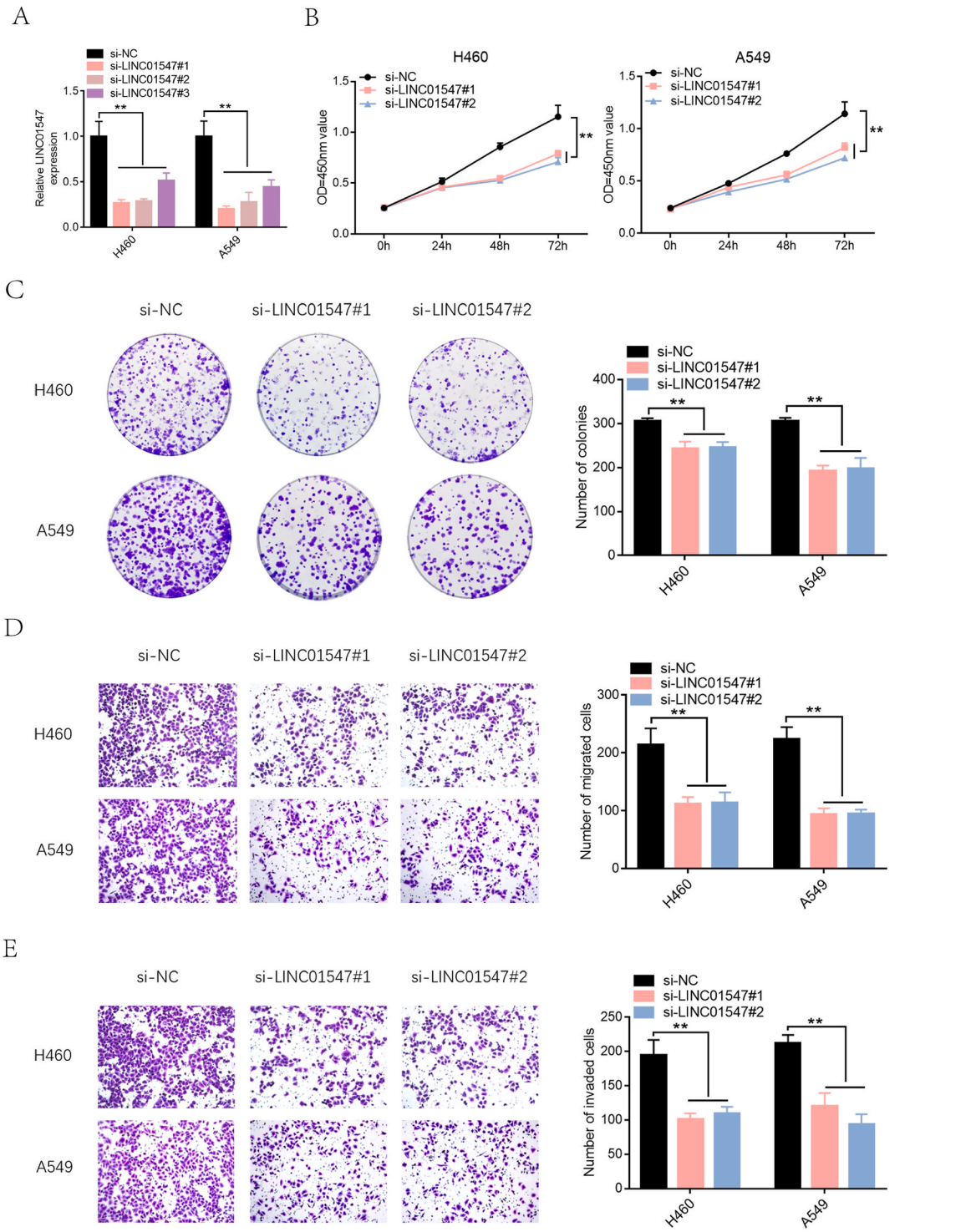

Fig. 2. LINC01547 interference restricts the aggressive behaviors of NSCLC cells. (A) The efficiency of si-LINC01547 transfection in NSCLC cells. (B, C) The proliferative and colony-forming abilities of NSCLC cells after LINC01547 ablation. (D, E) The repressing effect of si-LINC01547 on NSCLC cell motility.
**P < 0.01.

cell lines were significantly suppressed by LINC01547 knockdown (Fig. 2E). Taken together, these results indicate that LINC01547 exhibits an oncogenic role in NSCLC cell proliferation, migration and invasion.

3.3. LINC01547 acts as an miR-195-5p sponge

Next, we investigated the mechanisms underlying the cancer-promoting activities of LINC01547 in NSCLC. Cell fractionation experiment proved that LINC01547 was mostly located in the cytoplasm of NSCLC cells (Fig. 3A). Evidence strongly suggests that many RNA transcripts serve as ceRNAs by competitively binding common miRNAs. Accordingly, we explored whether LINC01547 exerted modulatory functions as a ceRNA. An investigation of the potential miRNA that may interact with LINC01547 using miRDB (http://www.mirdb.org/mirdb/index.html) and StarBase (http://starbase.sysu.edu.cn/) databases, respectively, predicted 51 and 46 miRNAs, of which five overlapping miRNAs (Fig. 3B) were selected for further verification. To verify the predicted findings, LINC01547 expression was entirely knocked down and its effect on the expression of the five candidate miRNAs was evaluated. qRT-PCR results indicated that knocking down LINC01547 led to a remarkable increase in the expression of miR-195-5p alone in H460 and A549 cells, but without any change in the expression levels of the other four candidate miRNAs (Fig. 3C). For this reason, we selected miR-195-5p for further analyses. Afterward, we predicted the direct binding site of miR-195-5p to LINC01547(Fig. 3D). To further verify the binding between LINC01547 and miR-195-5p, we performed the dual luciferase assays using the psiCHECK™-2 vector containing the full LINC01547 sequence. The luciferase reporter assays showed that, compared with the negative control, luciferase activity was reduced by the miR-195-5p mimic. To exclude non-specific binding, we also engineered the psiCHECK™-2 vector containing the LINC01547-MUT, as expected, the miR-195-5p mimic failed to suppress the luciferase activity of LINC01547-MUT (Fig. 3E).

Furthermore, miRNAs bind to Ago2, a core component of RNA-induced silencing complexes that regulate gene expression after transcription. To verify whether LINC01547 was bound to miR-195-5p in this way, anti-Ago2 RIP assays were performed in H460 and A549 cells. As shown in Fig. 3F, an abundance of LINC01547 and miR-195-5p was found in the Ago2-associated immunoprecipitants. Taken together, these results confirmed that LINC01547 acted as an miR-195-5p sponge in NSCLC.

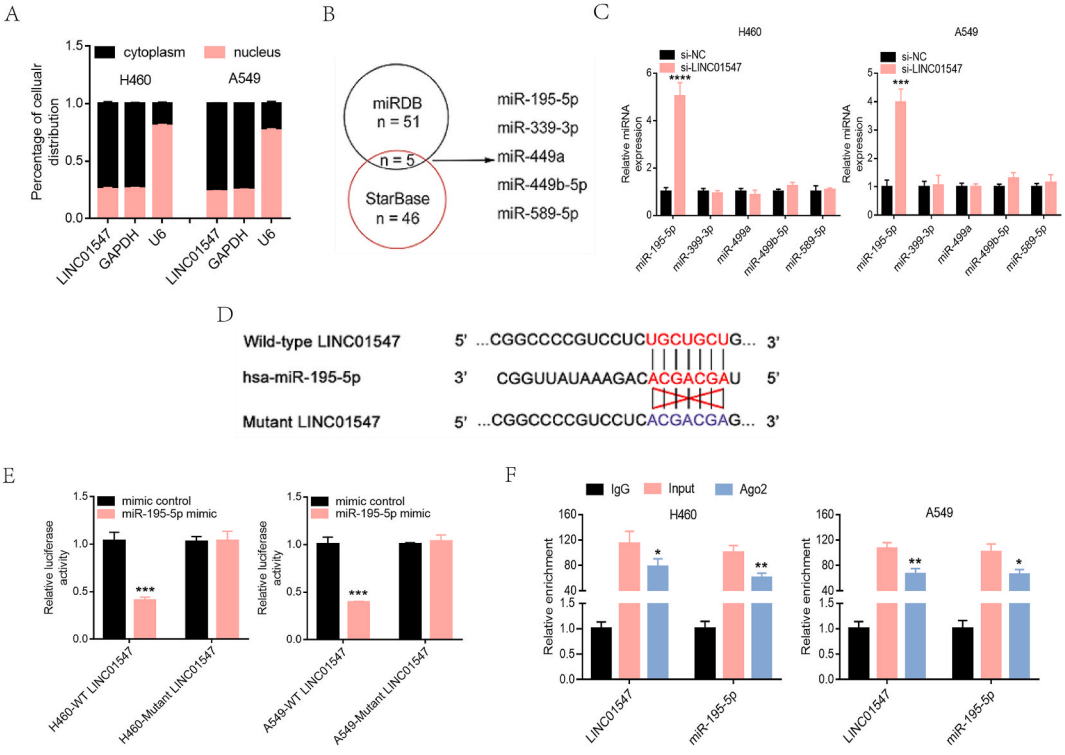

Fig. 3. LINC01547 serves as an miR-195-5p sponge. (A) Subcellular location of LINC01547 in NSCLC cells. (B) The overlap miRNAs predicted by miRDB and StarBase. (C) Expression of five candidates was measured in LINC01547-silenced NSCLC cells. (D) The binding sequences between LINC01547 and miR-195-5p. The mutant site was also presented. (E) Luciferase activity was monitored in NSCLC cells, which were cotransfected with miR-195-5p mimic or mimic control and WT-LINC01547 or MUT-LINC01547. (F) The direct interaction between LINC01547 and miR-195-5p was certified utilizing RIP.
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

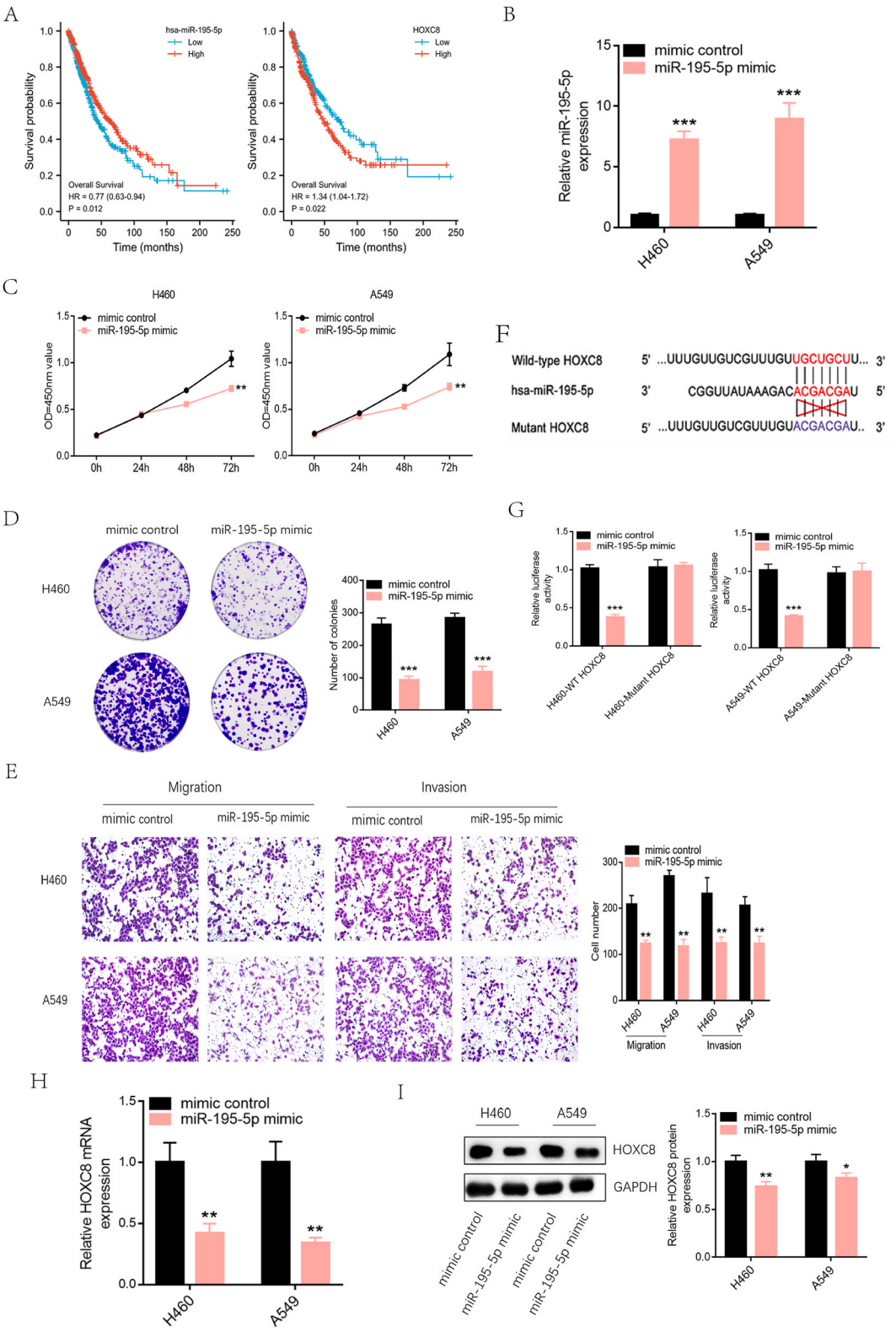

Fig. 4. miR-195-5p directly targets HOXC8. (A) The relationship between miR-195-5p、HOXC8 level and overall survival in NSCLC patients. (B) The transfection efficiency of miR-195-5p mimic in NSCLC cells. (C, D) The proliferative and colony-forming abilities of miR-195-5p-overexpressed NSCLC cells. (E) The motility of NSCLC cells in the presence of miR-195-5p upregulation. (F) The complementary WT and MUT binding site between miR-195-5p and HOXC8 3′-UTR. (G) The measurement of luciferase activity triggered by WT-HOXC8 or MUT-HOXC8 was implemented in miR-195-

5p mimic-transfected or mimic control-transfected NSCLC cells. (H, I) HOXC8 expression in NSCLC cells after miR-195-5p mimic treatment.
*P < 0.05, **P < 0.01, ***P < 0.001.

3.4. *LINC01547 decoys miR-195-5p, thereby regulating HOXC8 in NSCLC*

Data from the TCGA database showed that, patients with higher *HOXC8* levels had poorer overall survival, whereas those with higher miR-195-5p levels had greater overall survival (Fig. 4A). Similarly, we verified the effects of miR-195-5p on cells proliferation, migration and invasion. MiR-195-5p was overexpressed in miR-195-5p mimic-transfected NSCLC cells in contrast to control mimic-transfected cells (Fig. 4B). The CCK-8 proliferation assay results, revealed that cell proliferation was significantly suppressed with the miR-195-5p mimic. Similar effects were also observed in colony formation assays (Fig. 4C and D). Moreover, transwell migration and invasion assays indicated that overexpression of miR-195-5p decreased cell migration and invasion (Fig. 4E).

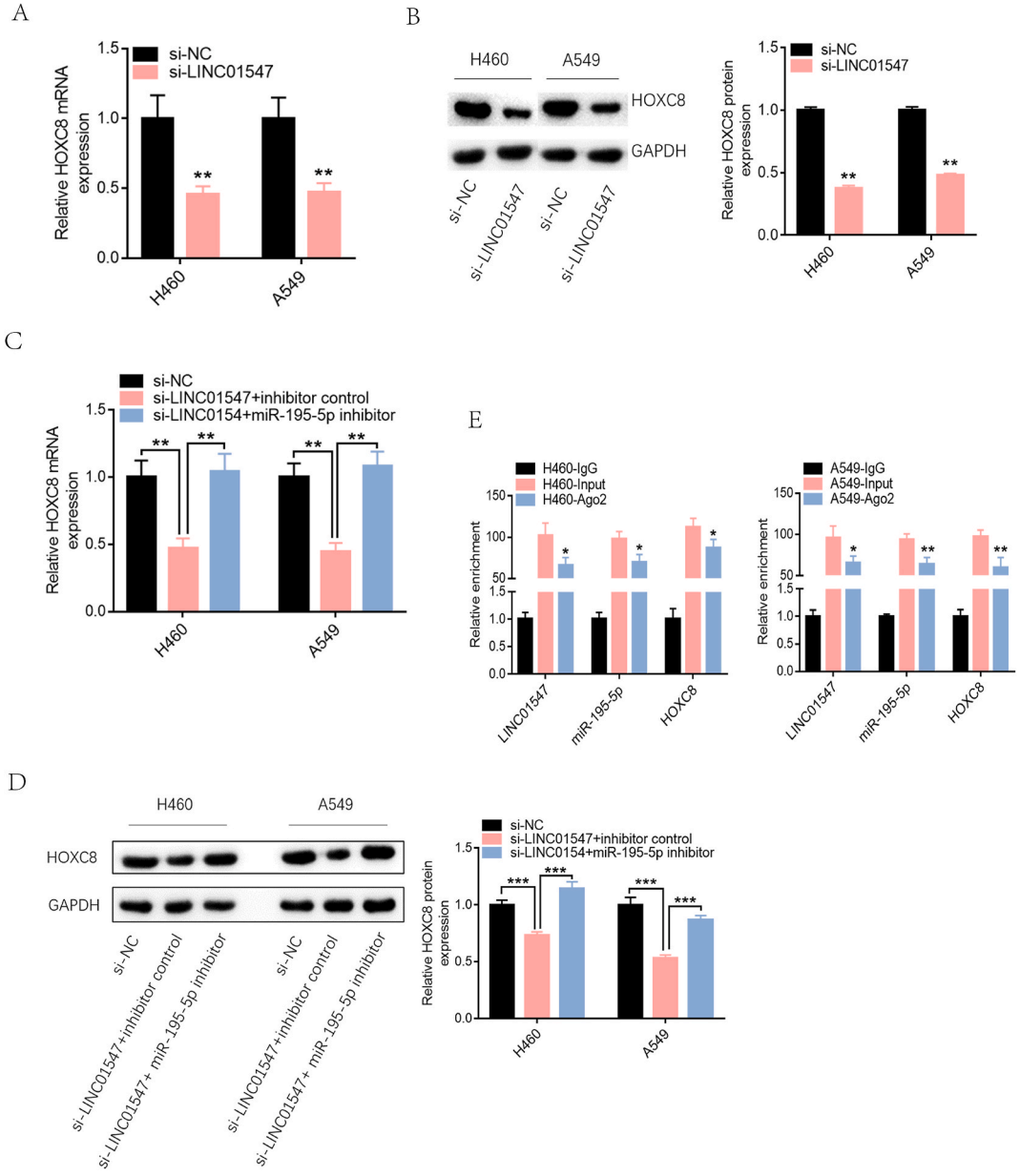

Fig. 5. LINC01547 sequesters miR-195-5p and consequently controls HOXC8 in NSCLC. (A, B) LINC01547 was ablated in NSCLC cells, and subjected to the measurement of HOXC8 expression. (C, D) LINC01547-silenced NSCLC cells were cotransfected with miR-195-5p inhibitor or inhibitor control, followed by detection of HOXC8 level. (E) RIP assay further corroborate the direct interaction among LINC01547, miR-195-5p and HOXC8 in NSCLC.
*P < 0.05, **P < 0.01, ***P < 0.001.

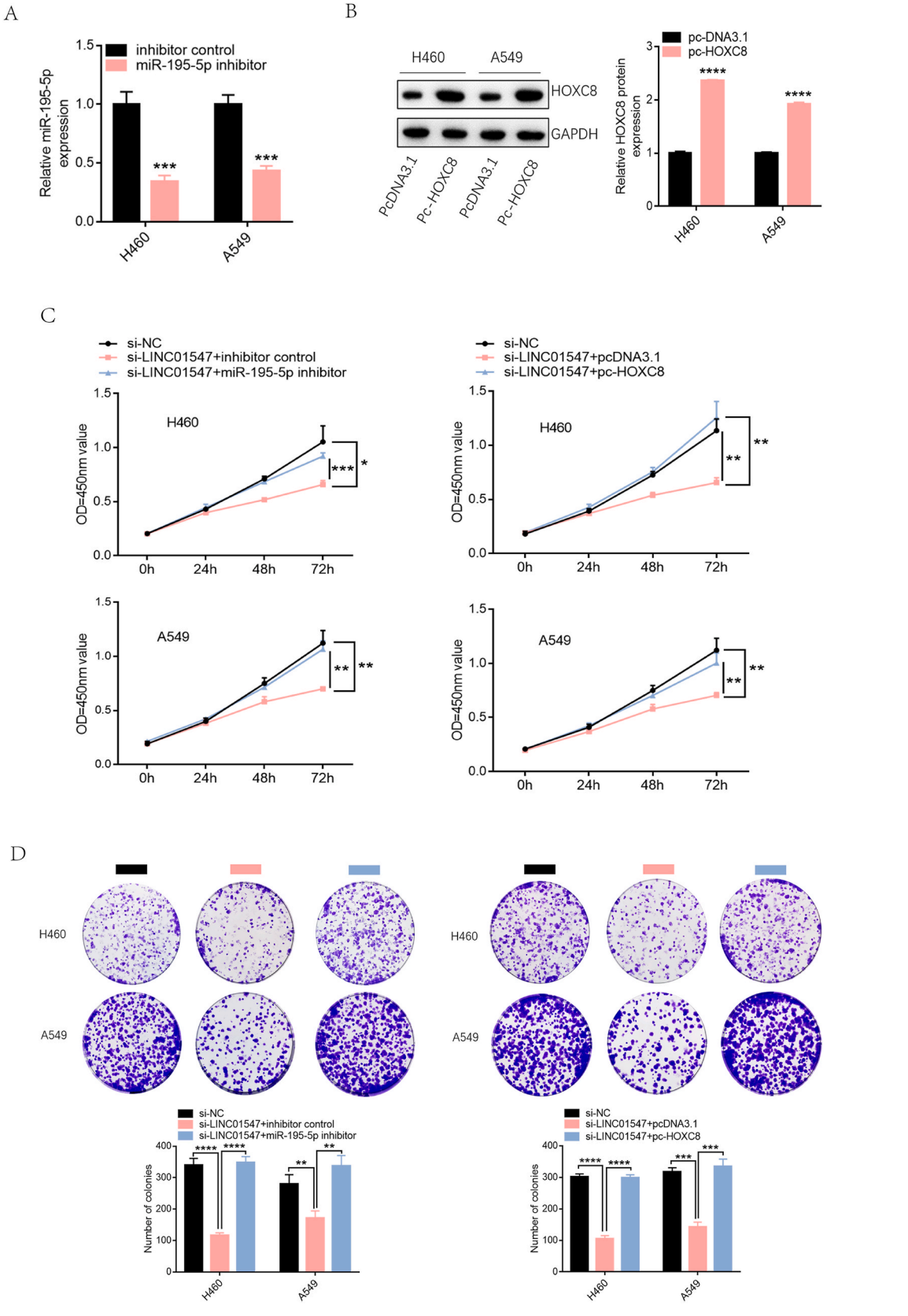

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Fig. 6. miR-195-5p/HOXC8 axis mediates the inhibitory influences of si-LINC01547 on NSCLC cell proliferation. (A, B) The efficiency of miR-195-5p inhibitor and pc-HOXC8 transfection in NSCLC cells. (C, D) LINC01547-silenced NSCLC cells underwent cotransfection of miR-195-5p inhibitor or pc-HOXC8, followed by the evaluation of cell proliferative and colony-forming abilities.
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

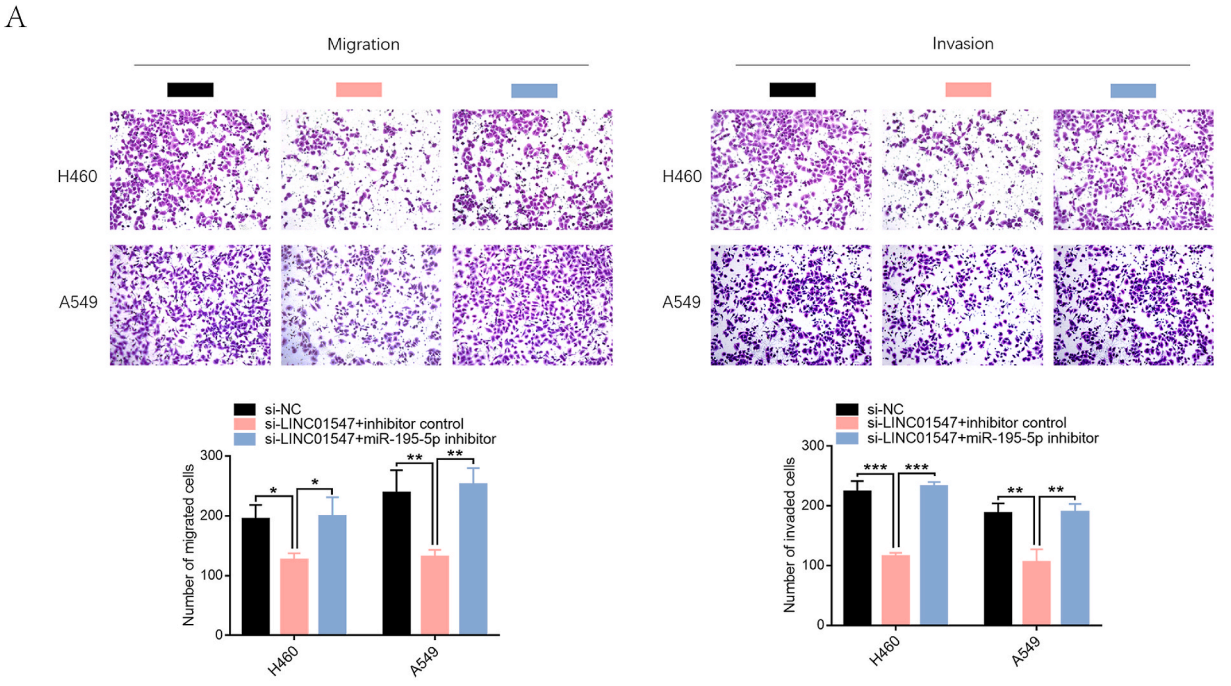

Fig. 7. miR-195-5p/HOXC8 axis mediates the inhibitory influences of si-LINC01547 on NSCLC cell motility. (A, B) NSCLC cells in the presence of LINC01547 ablation were cotransfected with miR-195-5p inhibitor or pc-HOXC8. Cell motility was detected in each group.
*P < 0.05, **P < 0.01.

Our earlier findings showed that LINC01547 was directly bound to miR-195-5p and was negatively regulated by it, suggesting that LINC01547 may function as a ceRNA. An online search of the miRNA target prediction database (miRDB and StarBase) showed, that *HOXC8* was the putative target of miR-195-5p (Fig. 4F). A luciferase reporter assay was employed to confirm the predicted relationship and test our hypothesis of LINC01547 functioning as a ceRNA. We constructed the psiCHECK™-2 vector containing either the WT or MUT *HOXC8*. These plasmids were transfected into H460 and A549 cells with miR-195-5p or negative control and the results showed that the miR-195-5p mimic reduced the luciferase activity of WT-*HOXC8*, but not that of MUT-*HOXC8* (Fig. 4G). These results indicated that *HOXC8* was a target of miR-195-5p. Additionally, *HOXC8* expression was significantly reduced when miR-195-5p was overexpressed in H460 and A549 cells, as shown in Fig. 4H. Moreover, western blotting also shown corroborated the results (Fig. 4I).

Overall, our data demonstrated that *HOXC8* was the direct target of miR-195-5p, which was sponged by LINC01547. Therefore, we subsequently investigated whether LINC01547 affected *HOXC8* expression by interacting with miR-195-5p. Interestingly, the depletion of LINC01547 resulted in a decline in the expression of *HOXC8* in NSCLC cells (Fig. 5A and B). Meanwhile, co-transfection with miR-195-5p inhibitor was able to offset the LINC01547-dependent reduction in *HOXC8* expression (Fig. 5C and D). Anti-Ago2 RIP assay demonstrated an increased enrichment of LINC01547, miR-195-5p and *HOXC8* in the Ago2 group (Fig. 5E). In short, these data suggest that LINC01574 serves as a ceRNA for miR-195-5p, and the downregulation of LINC01547 lowers *HOXC8* level by sponging miR-195-5p.

3.5. *The miR-195-5p/HOXC8 axis mediates the action of si-LINC01547 in NSCLC cells*

Rescue experiments were conducted to investigate the contributions of miR-195-5p/HOXC8 complex to LINC01547 function in NSCLC. Initially, the efficiency of miR-195-5p inhibitor and pc-HOXC8 plasmid transfection was tested (Fig. 6A and B). Next, by performing CCK-8 proliferation assays and colony formation assays, we found that when cells with LINC01547 knockdown were transfected with miR-195-5p inhibitor or pc-HOXC8, either the reduced miR-195-5p or increased *HOXC8* levels counteracted the corresponding suppression of proliferation and colony-forming ability via reduced LINC01547 expression in H460 and A549 cells (Fig. 6C and D). Furthermore, transwell migration and invasion assays indicated that loss of LINC01547 resulted in restriction in the

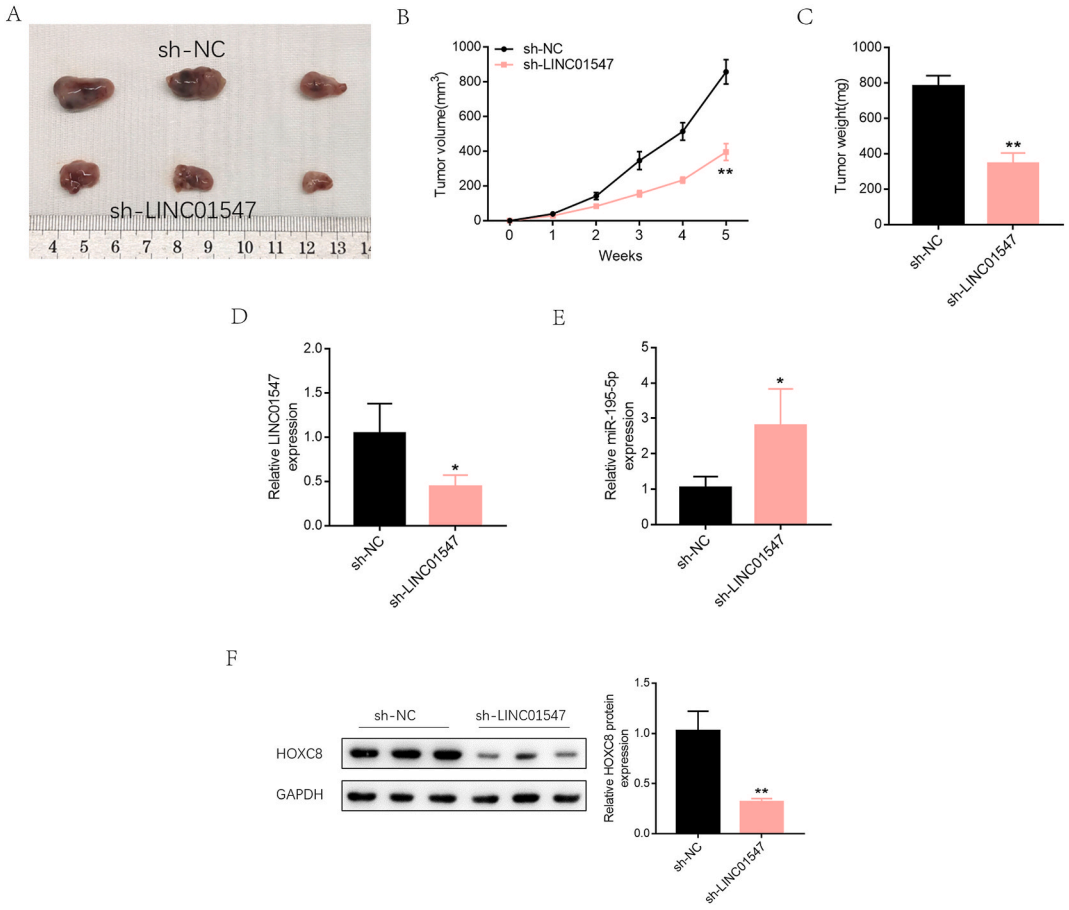

Fig. 8. LINC01547 downregulation hinders growth in vivo. (A) Typical images of tumors. (B, C) The growth curves and weight of tumors. (D–F) LINC01547, miR-195-5p and HOXC8 levels in tumor xenografts.

motility of NSCLC cells, which could be recovered by treatment with miR-195-5p inhibitor (Fig. 7A) or overexpression of *HOXC8* (Fig. 7B). Taken together, LINC01547 knockdown inhibited the malignancy of NSCLC by targeting the miR-195-5p/*HOXC8* axis.

3.6. LINC01547 depletion obliterates tumor growth in vivo

We conducted a xenograft tumor growth model to investigate the in vivo roles of LINC01547 in NSCLC growth. In contrast to the sh-NC group, the mice with LINC01547 stable knockdown manifested slow tumor growth (Fig. 8A and B). Additionally, the weight of the tumor xenografts was reduced with LINC01547 ablation (Fig. 8C). At the molecular level, the LINC01547-silenced tumors exhibited reduced LINC01547 expression levels and increased miR-195-5p levels relative to the sh-NC group (Fig. 8D and E). Furthermore, HOXC8 protein expression was enhanced in xenografts that were harvested from the sh-LINC01547 group (Fig. 8F). These results confirmed that the depletion of LINC01547 hindered the in vivo growth of tumors.

4. Discussion

Over 50,000 lncRNAs have been characterized in the human genome [32], and they have captured scientific attention by their importance in cancer onset and progression [33–35]. Dysregulated lncRNAs in NSCLC have received extensive coverage, which provides a novel perspective on the pathogenesis of NSCLC [36–38]. Concretely, lncRNAs function as modulators in the malignant process of NSCLC [39], making them attractive targets for managing the lethal disease. In this study, we examined the expression of LINC01547 in NSCLC to clarify its clinical significance. Furthermore, we explored the detailed functions of LINC01547 in regulating the aggressive phenotype of NSCLC. Our results showed that LINC01547 promoted cell proliferation, migration and invasion by increasing *HOXC8* expression by interacting with miR-195-5p, similar to a ceRNA mechanism.

LINC01547 was found associated with the overall survival of patients with ovarian cancer [24]. However, the expression profile and roles of LINC01547 in NSCLC are currently mostly undefined. In the current study, a noticeable overexpression of LINC01547 was affirmed by data from TCGA and our cohort. In addition, poor prognosis was ascertained in patients with NSCLC characterized by increased LINC01547 levels. Functionally, the absence of LINC01547 produced tumor-repressing effects in NSCLC, as it regulates cell growth and metastasis. Overall, the aforementioned observations indicate LINC01547 as a promising diagnostic biomarker and a therapeutic target for NSCLC.

Through the current study, we have provided a novel insight into the molecular events underlying LINC01547 function in NSCLC, which have thus far been ambiguous. The cellular location of lncRNAs provides clues regarding the molecules mediating their biological functions [40]. Nuclear lncRNAs regulate gene expression by interacting with various molecules such as DNA, RNA and protein or cis or trans regulation of gene transcription, affecting mRNA splicing, stability and translation [41,42]. For the cytoplasmic lncRNAs, the ceRNA theory is the most established mechanism that explains their activities. LncRNAs mostly transregulate gene expression at the post-transcriptional level. They can decoy miRNAs through their miRNA response elements, and consequently control gene expression at the post-transcriptional level by inhibiting mRNAs through miRNA-triggered dysregulation of mRNAs or restrictions in translation. Thus, they can regulate the translation and degradation of mRNAs, or participate in the regulation of intracellular signaling pathways [42,43].

We confirmed LINC01547 as a cytoplasmic lncRNA through the nuclear/cytoplasmic fractionation experiment, which provides basic theoretical frameworks to support lncRNA as a ceRNA or miRNA sponge. Based on bioinformatics prediction, miR-195-5p had a complementary binding site within LINC01547. A strong interaction between LINC01547 and miR-195-5p was subsequently detected by using luciferase reporter assay and RIP. A ceRNA network is composed of lncRNA, miRNA, and mRNA; thus, we subsequently searched for the downstream targets of miR-195-5p through online databases that predicted miRNA targets. Next, several mechanistic studies proved *HOXC8* as the direct target of miR-195-5p. *HOXC8* was found to be indirectly controlled by LINC01547 in an miR-195-5p-mediated mechanism. Accordingly, LINC01547 was confirmed to sequester miR-195-5p away from *HOXC8* in NSCLC cells. The interaction among LINC01547, miR-195-5p, and *HOXC8* delineates a ceRNA pathway in NSCLC.

Many studies have indicated the involvement of miR-195-5p in carcinogenesis and cancer progression. For instance, miR-195-5p is weakly expressed in prostate cancer [44], melanoma [45], gastric cancer [46], and cervical cancer [47], and has anti-carcinogenic roles. In NSCLC, a low miR-195-5p expression level presented a noticeable correlation with many aggressive pathological parameters and poor clinical outcomes [48,49]. Functionally, the involvement of miR-195-5p in effecting the malignancy of NSCLC has been extensively recognized [50–53]. Consistent with these observations, our current research revealed the decreased levels of miR-195-5p in NSCLC and its corresponding cancer-repressing activity. Notably, *HOXC8*, an important member of the homeobox gene family, is either overexpressed or underexpressed in several solid tumor tissues, such as esophageal, breast, and pancreatic cancers. It is closely related to the occurrence and development of malignant tumors, invasion and metastasis, induction of angiogenesis and other biological behaviors and prognosis [54–56]. However, it has rarely been reported in NSCLC. This study found it to function as a crucial mediator of miR-195-5p action in NSCLC. We conducted a sequence of rescue experiments, which highlighted that the regulatory activities of LINC01547, which is deficient in repressing the malignant properties of NSCLC cells, can be counteracted by hindering miR-195-5p or overexpressing *HOXC8*. We uncovered the significance of the regulatory circuity LINC01547/miR-195-5p/*HOXC8* in NSCLC progression and showed that LINC01547 exerted oncogenic behaviors through competitively binding to miR-195-5p in NSCLC cells.

Our study had one limitation. The nude mice with the xenograft tumor growth model were divided into two groups, with only three samples in each group. The sample size was insufficient. We will resolve the limitation in future studies using larger sample sizes.

5. Conclusion

Our study confirms LINC01547 as a crucial pathological component in NSCLC oncogenicity. By directly interacting with miR-195-5p, LINC01547 promoted the expression of *HOXC8*, which consequently modulated the malignant characteristics of NSCLC. Accordingly, the LINC01547/miR-195-5p/*HOXC8* pathway may have great potential as therapeutic target for NSCLC management.

Declarations

Ethics Statement

This study and experimental procedures were approved by the Experimental Animal Ethics Committee of Guangzhou Medical University (GD2019-046). All animal rearing and experiments were conducted in strict accordance with the institutional code of care and use of laboratory animals.

Author contribution statement

Zeyong Jiang, Jian Zhao: Conceived and designed the experiments.
Wenjie Wu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Siyu Zhu, Yonghui Wu: Performed the experiments; Analyzed and interpreted the data.
Lu Dai: Contributed reagents, materials, analysis tools or data.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18015.

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