

ORIGINAL ARTICLE

LINC00632 inhibits the malignant development of non-small cell lung cancer by downregulating miR-1203

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Summary

Purpose: To uncover the role of LINC00632 in influencing metastasis of non-small cell lung cancer (NSCLC) and the potential molecular mechanism.

Methods: LINC00632 levels in paired NSCLC and paracancer tissues were detected. The correlation between LINC00632 level and pathological features of NSCLC was analyzed. In vitro proliferative and migratory abilities in NSCLC regulated by LINC00632 were assessed. In addition, nude mice bearing NSCLC were prepared to explore the in vivo effect of LINC00632 on tumor growth. The targeting relationship between LINC00632 and miR-1203 was confirmed by dual-luciferase reporter assay. The involvement of miR-1203 in regulating NSCLC cell phenotypes was finally explored.

Results: LINC00632 was lowly expressed in NSCLC tissues. Low level of LINC00632 indicated high rates of lymph node

metastasis and distant metastasis, as well as poor prognosis in NSCLC. Overexpression of LINC00632 suppressed in vitro proliferative and migratory abilities in NSCLC. Moreover, overexpression of LINC00632 inhibited tumor growth in nude mice bearing NSCLC. MiR-1203 was the downstream target of LINC00632, which was upregulated in NSCLC tissues. The inhibitory effects of LINC00632 on cell growth and metastasis in NSCLC were abolished by overexpression of miR-1203.

Conclusions: LINC00632 is downregulated in NSCLC samples, which is closely linked to metastasis and prognosis in NSCLC patients. It inhibits the malignant development of NSCLC by negatively regulating miR-1203 level.

Key words: LINC00632, MiR-120, non-small cell lung cancer, migration

Introduction

Lung cancer is highly prevalent malignancy in the world [1]. The incidence of lung cancer ranks first in men, which is also on the rise in women. So far, lung cancer has become one of the most major reasons for cancer death [1,2]. In China, the mortality of lung cancer has astonishingly increased to 465% in the past decade, which is the number one and two killer in urban and rural areas, respectively [3,4]. Based on the pathological subtypes, non-small cell lung cancer (NSCLC) accounts for 75-80% of total lung cancer cases [5,6]. The prognosis of NSCLC is poor, although great advances have

been made in clinical and experimental oncology. The 5-year survival of NSCLC is only 11% [6-8]. The high mortality of NSCLC can be attributed to strong metastatic ability and deficiency of effective strategies [9,10]. Therefore, it is urgent to develop diagnostic and prognostic hallmarks of NSCLC [11,12].

Whole-genome analysis showed that more than 90% of genes are transcribed into non-coding RNAs (ncRNAs) [13,14]. Typically, ncRNAs are classified into short ncRNAs and long ncRNAs (lncRNAs) based on the length. The latter is longer

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Received: 28/02/2020; Accepted: 02/04/2020

than 200 nucleotides (nt), and subtyped into anti-sense lncRNA, intronic transcript, large intergenic ncRNA, promoter-associated lncRNA and UTR-associated lncRNA [15,16]. LncRNAs were initially considered as transcription noises. Later, growing evidence has shown the regulatory effects of them on various life activities [17,18]. Moreover, lncRNAs are extensively involved in regulating tumor cell behaviors [14,19]. With the in-depth development of researches, more and more functions of lncRNAs in gene regulations have been revealed [19]. Through bioinformatics big data analysis, it was speculated that LINC00632 is differentially expressed in NSCLC. Therefore, we aimed to explore the role of LINC00632 in influencing the malignant development of NSCLC and its possible mechanism.

Methods

NSCLC samples

NSCLC and paracancer tissues were collected from 46 NSCLC patients undergoing surgery. None of them had preoperative chemotherapy or radiotherapy. Clinical data and follow-up data of included NSCLC patients were recorded. This study got approval by the Ethics Committee of Gansu Provincial Hospital and it was conducted after written informed consent was provided by each subject.

Cell lines and reagents

NSCLC cell lines A549, H1299, PC-9, H358 and a bronchial epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC) (Manas-

sas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in an incubator at 37°C and 5% CO₂. Cell passage was conducted when cells were grown to 80-90% confluence.

Transfection

Cells were cultured to 30-50% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Forty-eight h later, cells were collected for future use.

Transwell migration assay

200 µL of suspension (5.0×10^5 /mL) was applied in the upper side of Transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 460 µL of medium containing 10% FBS in the bottom. After 48 h of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40×).

Wound healing assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, DMEM with 1% FBS was replaced. Twenty-four h later, wound closure was captured for calculating the percentage of wound healing (magnification 40×).

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

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment,

Table 1. Association of LINC00632 expression with clinicopathologic characteristics of non-small cell lung cancer

Parameters	Number of cases	LINC00632 expression		p value
		High	Low	
Age (years)	0.979			0.979
<60	18	11	7	
≥60	28	17	11	
Gender	0.331			0.331
Male	22	15	7	
Female	24	13	11	
T stage	0.067			0.067
T1-T2	28	20	8	
T3-T4	18	8	10	
Lymph node metastasis	0.001			0.001
No	30	24	6	
Yes	16	4	12	
Distant metastasis	0.009			0.009
No	33	24	9	
Yes	13	4	9	

and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta C_t}$ and normalized to that of β -actin or U6. LINC00632: forward: 5'-CACGCCTGTTATCCC-3', reverse: 5'-CAACCTCCGCCTCTT-3'; β -actin: forward: 5'-CGCTCTTCCAGCCTTCCTT-3', reverse: 5'-CGTTGTTGGCATACAGGTCCT-3'; miR-1203: forward: 5'-TGCAGATTATGCGGATCAAACC-3', reverse: 5'-TGCAATTCACATTTGTTGTGCTGTAG-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Dual-luciferase reporter assay

A549 and H1299 cells were pre-seeded in 24-well plates. They were co-transfected with NC mimic/miR-1203 mimic and LINC00632-WT/LINC00632-MUT, respectively. After 48-h cell culture, they were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

In vivo xenograft model

Experimental procedures of establishing *in vivo* xenograft models in nude mice were approved by the Animal Ethics and Use Committee of Gansu Provincial Hospital. A total of 15 male nude mice were randomly assigned into three groups (n=5) each. They were subcutaneously administered with A549 cells transfected with NC+NC mimic, pcDNA-LINC00632+NC mimic or pcDNA-LINC00632+miR-1203 mimic, respectively. Tumor size was weekly recorded. Six weeks later, mice were euthanized for collecting tumor tissues. Tumor volume = (width²×length)/2.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean±standard deviation. Differences between groups were analyzed by the t-test. Chi-square (χ^2) test was conducted for analyzing the relationship between LINC00632 level and clinical data of NSCLC patients. The survival curves were plotted using the Kaplan-Meier method, and log-rank

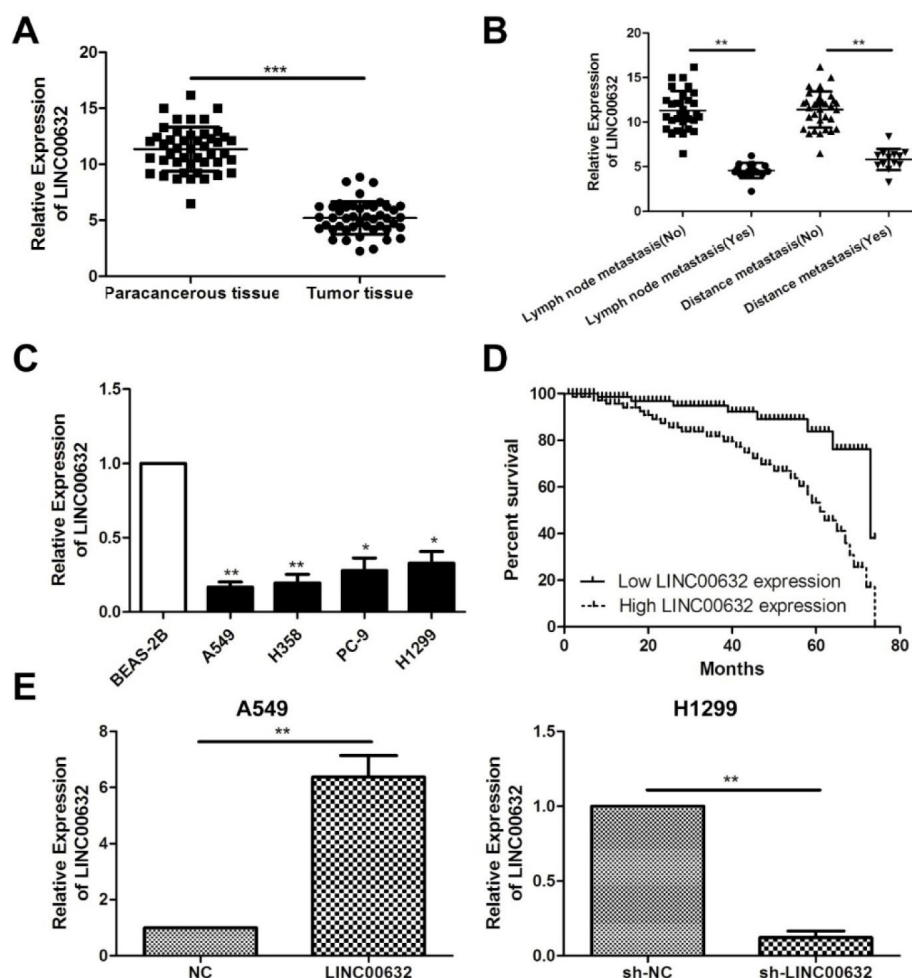


Figure 1. Downregulated LINC00632 in NSCLC samples. **A:** LINC00632 level in NSCLC tissues and paracancer tissues. **B:** LINC00632 level in NSCLC patients either with lymph node metastasis, distant metastasis or not. **C:** LINC00632 level in NSCLC cell lines. **D:** Overall survival in NSCLC patients with high or low level of LINC00632. **E:** Transfection efficacy of pcDNA-LINC00632 and sh-LINC00632 in A549 and H1299 cells, respectively. Data are expressed as mean±SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

test was performed to assess differences between groups. $P < 0.05$ was considered as statistically significant.

Results

Downregulated LINC00632 in NSCLC samples

Compared with paracancer tissues, LINC00632 was downregulated in NSCLC tissues (Figure 1A). We collected clinical and follow-up data of included NSCLC patients which showed that LINC00632 level was correlated to the rates of nodal metastasis and distant metastasis in NSCLC patients (Table 1). Lower LINC00632 level was seen in

NSCLC patients with nodal or distant metastasis than in those without metastases (Figure 1B). Similarly, LINC00632 was lowly expressed in NSCLC cell lines as well (Figure 1C). Moreover, survival analysis demonstrated a poor prognosis in NSCLC patients expressing low level of LINC00632 (Figure 1D). It seems that LINC00632 may be a novel hallmark predicting the malignant development of NSCLC.

LINC00632 inhibited the migratory ability in NSCLC

We constructed LINC00632 overexpression and knockdown models in A549 and H1299

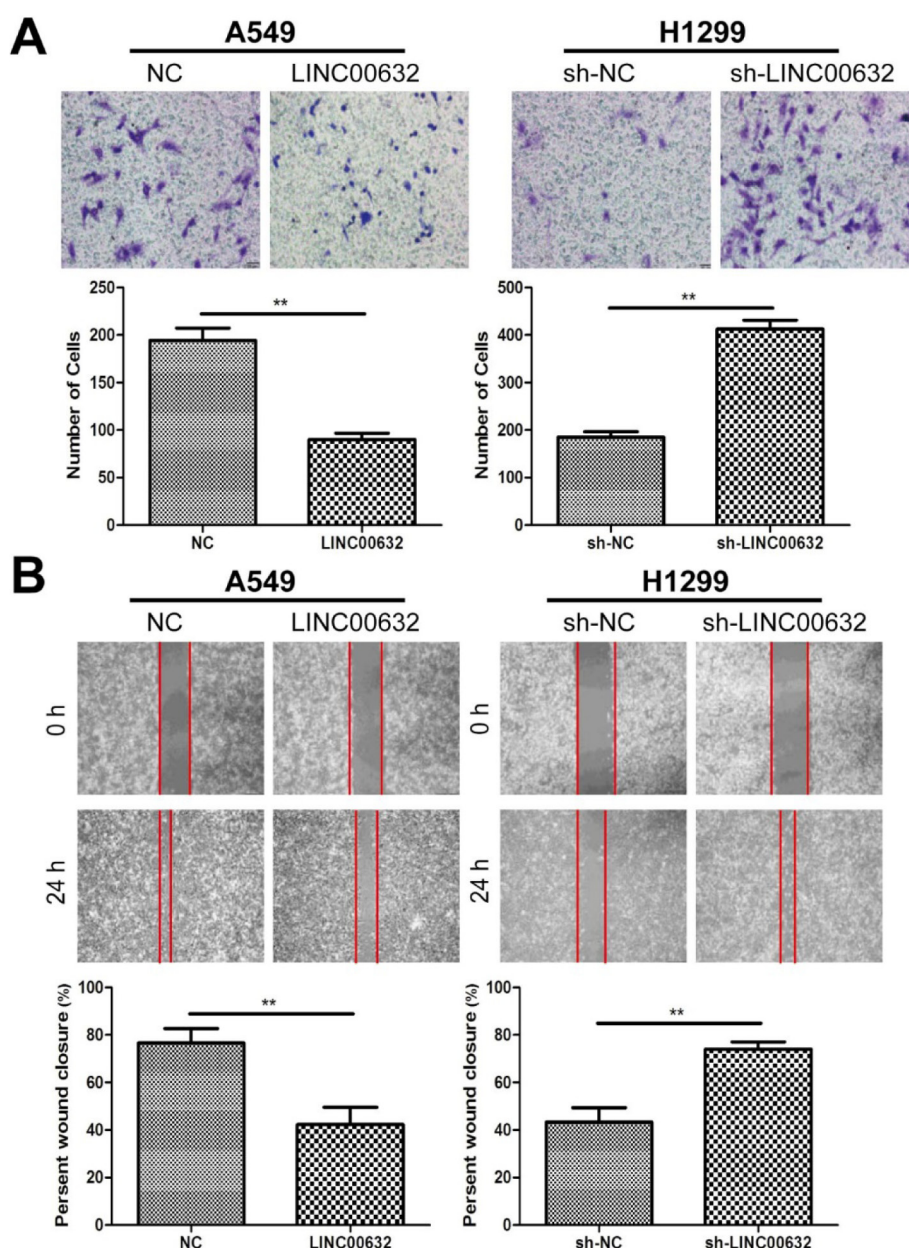


Figure 2. LINC00632 inhibited the migratory ability in NSCLC. **A:** Migration in A549 and H1299 cells influenced by LINC00632 (magnification 40×). **B:** Wound healing assay in A549 and H1299 cells influenced by LINC00632 (magnification 40×). Data are expressed as mean±SD. ** $p < 0.01$.

cells, respectively (Figure 1E). Overexpression of LINC00632 markedly decreased the migratory cell number and wound closure percentage in A549 cells (Figure 2A and 2B, left). Conversely, knockdown of LINC00632 in H1299 cells obtained the opposite results (Figure 2A and 2B, right).

LINC00632 bound to miR-1203 in NSCLC

Binding sequences in the 3'UTR of LINC00632 and miR-1203 were predicted by bioinformatics method. Overexpression of miR-1203 remarkably decreased luciferase activity in wild-type LINC00632 vector, verifying the binding between miR-1203 and LINC00632 (Figure 3A). MiR-1203 was downregulated in A549 cells overexpressing LINC00632 and it was upregulated in H1299 cells transfected with sh-LINC00632 (Figure 3B). In

NSCLC tissues, miR-1203 was upregulated and its level was negatively correlated to LINC00632 level (Figure 3C, 3D).

MiR-1203 reversed the regulatory effect of LINC00632 on in vitro migratory ability in NSCLC

To explore the biological function of miR-1203 in NSCLC, miR-1203 mimic and inhibitor were constructed. Higher level of LINC00632 was observed in H1299 cells with co-silence of LINC00632 and miR-1203 than those with solely knockdown of LINC00632 (Figure 4A). Co-overexpression of LINC00632 and miR-1203 could downregulate LINC00632 in A549 cells compared with those overexpressing LINC00632 (Figure 4B). Interestingly, the inhibitory effect of LINC00632 on the migratory ability in NSCLC was abolished by miR-1203 (Figure 4C).

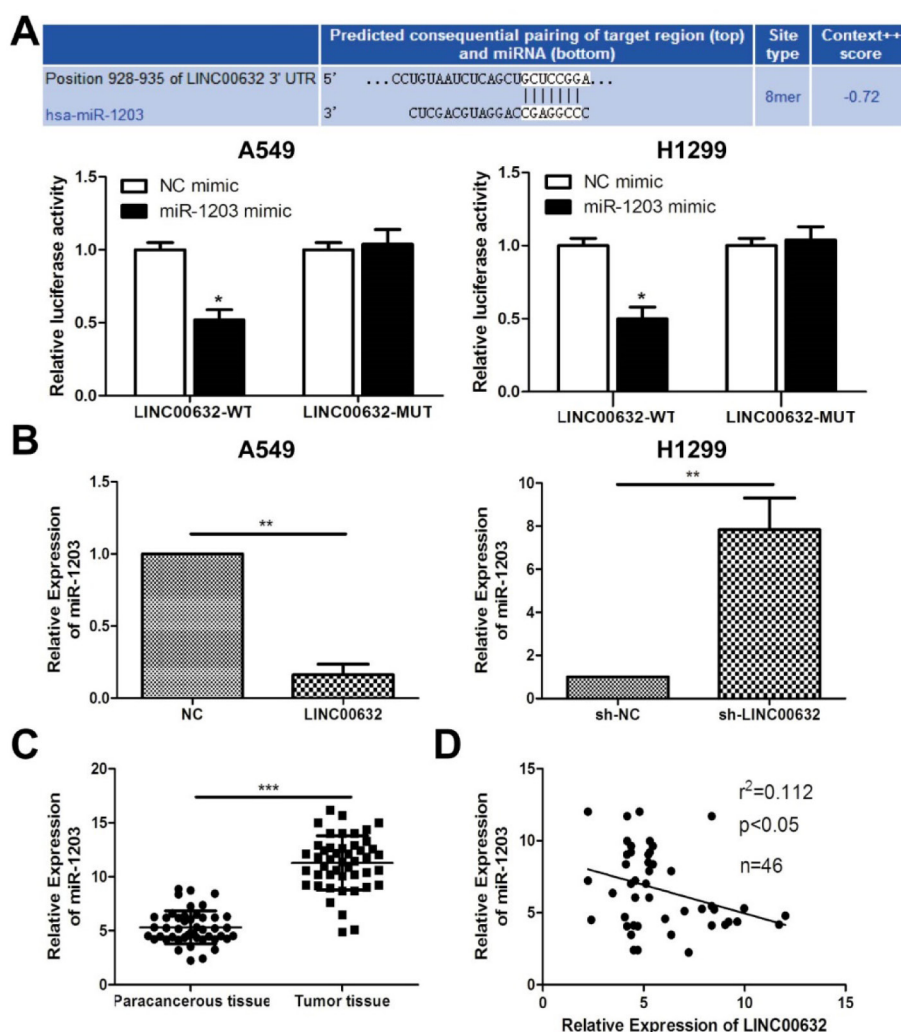


Figure 3. LINC00632 bound to miR-1203 in NSCLC. **A:** Binding sequences in the 3'UTR of miR-1203 and LINC00632 (upper lane). Luciferase activity in A549 and H1299 cells co-transfected with NC mimic/miR-1203 mimic and LINC00632-WT/LINC00632-MUT (bottom). **B:** MiR-1203 level in A549 and H1299 cells influenced by LINC00632. **C:** MiR-1203 level in NSCLC tissues and paracancer tissues. **D:** A negative correlation between expression levels of LINC00632 and miR-1203 in NSCLC tissues. Data are expressed as mean \pm SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

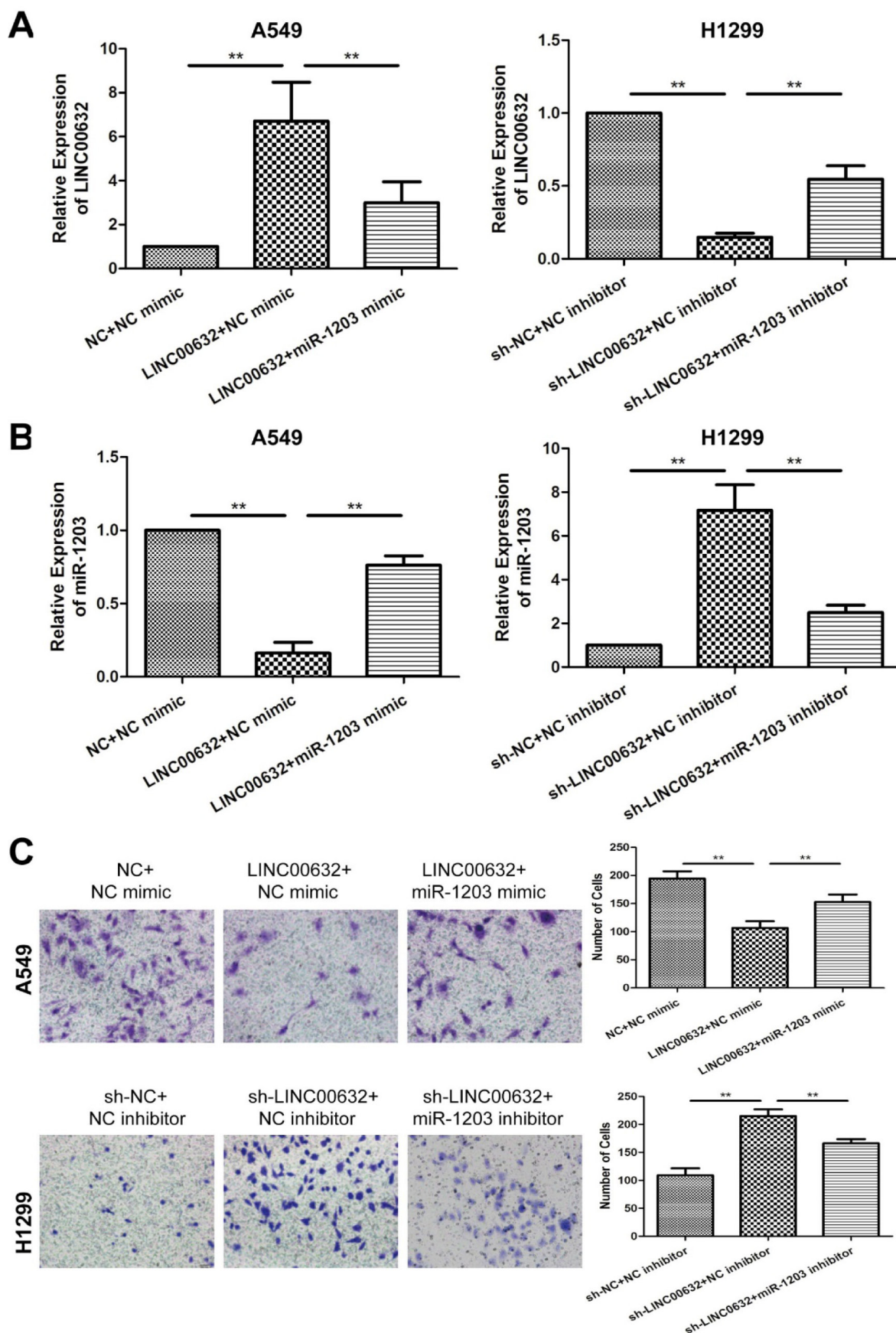


Figure 4. MiR-1203 reversed the regulatory effect of LINC00632 on *in vitro* migratory ability in NSCLC. **A:** LINC00632 level in A549 and H1299 cells influenced by both LINC00632 and miR-1203. **B:** MiR-1203 level in A549 and H1299 cells influenced by both LINC00632 and miR-1203. **C:** Migration in A549 and H1299 cells influenced by both LINC00632 and miR-1203 (magnification 40×). Data are expressed as mean±SD. ***p*<0.01.

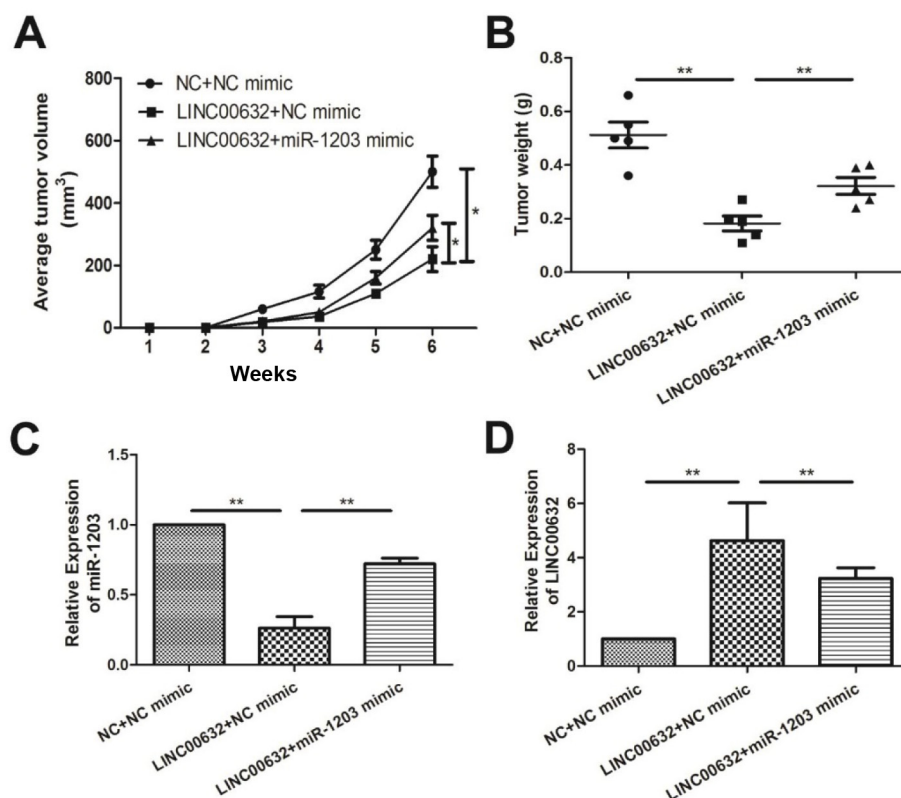


Figure 5. Knockdown of LINC00632 inhibited NSCLC progression *in vivo*. **A:** Average tumor volume in nude mice administered transfected A549 cells. **B:** Tumor weight in nude mice administered transfected A549 cells. **C:** MiR-1203 level in NSCLC tissues collected from nude mice administered transfected A549 cells. **D:** LINC00632 level in NSCLC tissues collected from nude mice administered transfected A549 cells. Data are expressed as mean±SD. * $p < 0.05$, ** $p < 0.01$.

Knockdown of LINC00632 inhibited *in vivo* progression of NSCLC

Nude mice were administered transfected A549 cells and potential influences of LINC00632 and miR-1203 on the growth of NSCLC were observed. Smaller tumor volume and lower tumor weight were found in NSCLC mice overexpressing LINC00632 than those with co-overexpression of LINC00632 and miR-1203 (Figure 5A,5B). We collected NSCLC tissues from mice of each group. The relative level of miR-1203 in tumor tissues was lower in mice overexpressing LINC00632 compared with those co-overexpressing LINC00632 and miR-1203 (Figure 5C). LINC00632 level in NSCLC tissues was higher in mice overexpressing LINC00632 than the other two groups (Figure 5D).

Discussion

As one of the fastest growing, highest morbidity and highest mortality malignancies in the world, NSCLC seriously threatens human health and life [1-3]. Although various therapeutic methods, such as surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy are applied,

the prognosis of NSCLC is poor, and the 5-year survival is around 15% [4-6]. Metastasis is the main cause of the deterioration and death of NSCLC. Nevertheless, fast, effective, simple and convenient biomarkers for early detection of NSCLC are lacking, which are urgently required to improve the survival [7,8,10-12].

Emerging sequencing technologies have shown the transcription information of over 85% of the human genome [13]. Surprisingly, studies of the mammalian genome have found that very few RNA transcripts can encode protein products [13,14]. LncRNAs are believed to be basic regulators of transcription [15,17]. The complicated network involving lncRNAs participates in almost every aspect of biological activity [14,19]. Tumor-associated lncRNAs have been well concerned [19]. These certain lncRNAs are differentially expressed in normal tissues and tumor tissues. In this paper, LINC00632 was downregulated in NSCLC samples and its level was associated with metastasis and overall survival in NSCLC patients. Subsequently, our findings revealed that LINC00632 inhibited the migratory ability in NSCLC cells and tumor growth in nude mice bearing NSCLC. We may consider that LINC00632 is a promising hallmark of NSCLC.

LncRNA-miRNA interaction influences pathological processes [17,19]. To illustrate the molecular mechanism underlying the role of LINC00632 in NSCLC, its downstream target should be searched. Bioinformatics prediction and dual-luciferase reporter assay verified that miR-1203 was the target binding LINC00632. A negative correlation was identified between expression levels of LINC00632 and miR-1203 in NSCLC samples. Notably, miR-1203 was able to abolish the regulatory effects of LINC00632 on *in vitro* migratory ability and *in vivo* tumor growth in NSCLC. To sum up, our study has proven that LINC00632 inhibited the malignant development of NSCLC via targeting and negatively regulating miR-1203.

Conclusions

LINC00632 is downregulated in NSCLC samples, which is closely linked to metastasis and prognosis in NSCLC patients. It inhibits the malignant development of NSCLC by negatively regulating miR-1203. LINC00632 may serve as a biomarker for the progression and prognosis of NSCLC, thus providing a new target for the treatment of NSCLC.

Conflict of interests

The authors declare no conflict of interests.

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