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Original Article

LINC00472 suppresses non-small cell lung cancer progression via regulating miR-23a-3p/CCL22 axis

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Abstract

Long non-coding RNA (lncRNA) LINC00472 has a close connection with the development of tumors. The aim was to explore the role of LINC00472 on NSCLC cell biological function in vivo and its potential mechanisms. The mRNA levels of LncRNA 00472 and microRNA-23a-3p, were determined by RT-qPCR. Cell Counting Kit-8, cell scratches and western blot assays were used to analyze the proliferation, migration and level of apoptosis-associated proteins. Luciferase reporter assay validates the binding between LINC00472/CCL22 and miR-23a-3p. LINC00472 and CCL22 were lowly expressed in NSCLC tissues and cells, while miR-23a-3p expression was upregulated. LINC00472 overexpression significantly depressed NSCLC cell cellular behavior, whereas promoting cell death. MiR-23a-3p could reverse these above-mentioned biological behavior changes caused by LINC00472 overexpression. Additionally, LINC00472 increased CCL22 expression through sponging miR-23a-3p. Knocking down CCL22 antagonized the inhibitory effect of LINC00472 on NSCLC cell survival. LINC00472 may reduce the cellular growth, and accelerate death of NSCLC through increasing CCL22 expression by targeting miR-23a-3p.

Keywords: NSCLC, LINC00472, miR-23a-3p, CCL22.

1. Introduction

Non-small cell lung cancer (NSCLC) is a kind of main lung cancer type [1]. Because of the lack of dependable molecular markers for early detection, NSCLC patients often present with locally advanced and metastatic disease at the time of their initial diagnosis [2, 3]. In the meantime, patients with advanced NSCLC have only a 15% 5-year survival rate, and the recurrence rate after surgery is very high [4]. Therefore, in-depth study of the mechanisms of NSCLC progression and identifying new early diagnostic and therapeutic targets are essential to clinical NSCLC therapy.

As interest in the in-depth research of the cellular biological function of lncRNA has grown, studies have confirmed that lncRNA plays an important role in modulating gene expression, epigenetic changes, cell cycle, histone modification and chromatin remodeling in tumor progression [5-8]. For instance, lncRNA LINC-PINT is highly expressed in clinical samples of NSCLC patients, and overexpression of LINC-PINT exhibits antitumor activity in NSCLC cells [9]. Moreover, LINC00346 upregulation inhibits NSCLC cell invasion and metastasis through regulating JAK-STAT3 pathway [10]. In spite of this, the specific role and molecular mechanism of the majority of lncRNAs in NSCLC remains to be fully understood. It is reported that colorectal cancer expresses low levels of LINC00472, a gene located on chromosome 6q13 [11], osteosarcoma [12] and pancreatic cancer [13], and inhibited tumor cell growth. Seo et al. recently reported that LINC00472 is expressed at low levels in NSCLC and would be able to be as a biomarker for NSCLC prognosis [14]. Yet, the functions and potential regulatory mechanisms of LINC00472 in NSCLC have not been fully elaborated.

A microRNA is a small, non-coding RNA molecule that plays a role in gene expression by inhibiting or degrading messenger RNA (mRNA) [15]. As a result, miRNAs have been found to play an important role in tumor proliferation, differentiation, apoptosis, drug resistance, as well as other functions [16]. Furthermore, these highly sensitive and specific miRNAs can serve as biomarkers for targeted therapeutics, especially in patients with advanced NSCLC [17]. MiR-23a-3p is regarded as an important oncogenic molecule, which has been shown to be abnormally upregulated in pancreatic cancer [18], as well as

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prostate cancer [19], etc, and ectopic expression of miR-23a-3p stimulates tumor cell malignant phenotypes. It was previously demonstrated that miR-23a-3p targeting PTEN facilitated biological behaviors of NSCLC cells in vitro [20]. However, there are no more studies about miR-23a-3p in NSCLC progression, especially the upstream and downstream regulatory mechanisms.

Monocytes and dendritic cells express chemokine (C-C motif) ligand 22 (CCL22), a member of the chemokine family. Blocking of CCL22 could accelerate tumor deterioration by affecting the M1/M2 macrophage balance [21, 22]. It has been reported that CCL22 overexpression represses the malignancy of NSCLC cells by inhibiting represses cell epithelial-mesenchymal transition [23]. It remains a mystery how CCL22 regulates NSCLC progression.

This research concentrated on the expression pattern and regulatory relationship among LINC00472, miR-23a-3p and CCL22 in NSCLC vivo and vitro. We report a new axis of LINC00472/miR-23a-3p/CCL22 is involved in the occurrence and development of NSCLC. The findings may provide new ideas for diagnosing and treating NSCLC.

2. Materials and Methods

2.1. Clinical samples collection

40 NSCLC patients chosen in our hospital from April 2020 to August 2021 were involved in this study, and NSCLC tumor tissues and corresponding adjacent normal tissues within 2 cm of the tumor tissue margin) were collected from these NSCLC patients. The inclusion criteria were any patients who had not received any form of anti-tumour therapy preoperatively. Exclusion criteria included patients with NSCLC who had received radiotherapy, or chemotherapy, as well as patients who exhibited an unclear clinical stage of NSCLC. Collected tumor tissues were reconfirmed by at least 3 specialists in pathology at our hospital. Tissue samples were frozen at -80°C for storage..

2.2. Cell culture

NSCLC cells (H1581, H1975) and human lung epithelial cells (BEAS-2B). H1581 cells (cat. no. YB-H0624) were obtained by Ybscience company (Shanghai, China). H1975 (cat. no. BNCC340345) and BEAS-2B (cat. no. BNCC359274) cells were purchased from Beina Biotechnology Institute (Beijing, China). These above-mentioned cells were grown in DEME medium (Lonza, Switzerland) supplemented with 10%FBS in a 5% CO_2 incubator at 37°C.

2.3. Cell transfection

The pcDNA plasmid-mediated LINC0047 overexpression vector (OE-LINC00472; 5'-AACCAAACCTACCCACAACG-3'), siRNA-CCL22, and their non-target controls (OE-NC or si-NC) were purchased Biological Engineering Co. Ltd (Shanghai, China). MiR-233a-3p mimic and its scrambled controls (miR-NC) were obtained from Genechem (China). Afterwards, these vectors were transfected into cells by Lipofectamine 3000 (Invitrogen, USA). The transfection efficiencies were evaluated using RT-qPCR at 48 h after transfection.

2.4. RT-qPCR assay

Total RNAs were extracted from NSCLC tissue samples and cells (H1581 and H1975) by TRIzol reagent (Life Technologies, USA) as per the kit instructions. RNAs were then reverse-transcribed into cDNA. Using cDNA as a template for PCR reaction, the SYBR Green PCR kit (Toyobo, Japan) was used for PCR reaction on Stepone real-time PCR systems (ABI company, CT, USA). As an internal reference, GAPDH was used for lncRNA/mRNA, while U6 was used for miRNA analyses. By using the$2^{-\Delta\Delta Ct}$ method, the relative expression level of each target gene was calculated. PCR primer sequences (Table 1).

2.5. Nucleocytoplasmic separation assay

In accordance with manufacturer's instructions, PARIS™ kit (Ambion, AM1921, USA) was used for RNA separation in H1581 and H1975 cells. The relative mRNA level of LINC00472 in cytoplasm and nucleus was determined by RT-qPCR. The nuclear and cytoplasmic controls were respectively GAPDH and U6.

2.6. Cell proliferation assay

H1581 and H1975 cells were kept into 96-well plates at 4×10^3 cells/well. Each well was incubated for another 2 hours with CCK-8 reagent (Sigma, USA) after cells were cultured for 24, 48, 72, and 96 h at 37°C and 5% CO_2. The cell proliferation viability was detected by enzyme-labeling instrument (Thermo Fisher Scientific, USA) at 450 nm.

2.7. Wound-healing assay

DMEM complete medium was used to seed and grow H1581 and H1975 cells at a density of 3 104 cells/well until they reached 80% confluence. A scratch created with a 200-L sterile pipette tip was then applied to the cells and cultured in serum-free DMEM medium for 24 hours.

2.8. Luciferase reporter assay

In order to generate WT pGL3-LINC00471/CCL22 vectors (WT-LINC00472/CCL22), wild-type (WT) sequences of LINC00472 and CCL22 3′UTR were inserted into pGL3 vector (Promega, USA). Furthermore, mutant (Mut) LINC00472 and CCL22 3′UTRs containing miR-23a-3p binding sites were cloned into pGL3 vectors (Mut-LINC00472/CCL22). A Dual-Luciferase Reporter Assay System (Promega, USA) was used to detect luciferase activity 48 hours after transfection.

2.9. Western blotting

Total proteins were extracted from each group of H1581 and H1975 cells using RIPA lysate buffer (Sigma, USA)

Table 1. Primer sequences.

Primer name	Sequences
LINC00472	3’-GATGGCAGCTGTCTCTCTCC-5’
LINC00472	5’-GGGCCTCTCTGACCGTATCT-3’
miR-23a-3p	3’-ATCACATTGCCAGGGATTTCC-5’
miR-23a-3p	5’-CTCGCTTCGGCAGCACA-3’
CCL22	3’-CAGGACTACATCCGTCACCC-5’
CCL22	5’-TGAGTAAAGGTGGCGTCGTT-3’
GAPDH	3’-GGGCCAAAAGGGTCATCATC-5’
GAPDH	5’-ATGACCTTGCCCACAGCCTT-3’
U6	3’-CTCGCTTCGGCAGCACA-5;
U6	5’-AACGCTTCACGAATTTGCGT-3’

containing 1% phenylmethanesulfonyl fluoride (PMSF; Sigma, USA). Absorbance measurement at 562 nm was performed using a BCA quantitative kit (Beyotime, China) to determine protein concentration. Transferring proteins (30 μg/lane) onto PVDF membranes was carried out using 12% SDS-PAGE. 5% skimmed milk was applied to block these membranes for 1 h at room temperature, followed by three washings with TBST. Next, membranes were incubated primary antibodies against Bax (1:1000, ab182733,), Bcl-2 (1:2000, ab32124), CCL22 (1:2000, ab1416) and GAPDH (1:2000, ab8245) overnight at 4°C, and further incubated with corresponding horseradish peroxide-labeled secondary antibody (1:2000, ab6877) for 1 h at room temperature. Enhanced chemiluminescence was used to measure the protein using an enhanced chemiluminescence kit (ECL; Beyotime, Shanghai, China), and Image J software (Version 1.8.0, NIH, Bethesda, MD, USA) was used for analyzing the gray value of protein bands.

2.10. Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, USA) and Prism 9.0 software (GraphPad Software Inc., La Jolla, USA) were performed to analyze the data and graphs in this study. We tested all data for normality, and those with normal distributions were expressed as mean ± standard deviation (SD). Comparisons between two groups were made using the t-test, and comparisons among multiple groups were made using the one-way ANOVA of variance followed by Tukey post hoc test. Every in vitro experiment was repeated three times. Statistical significance was defined as a $P < 0.05$.

3. Results

3.1. LINC00472 was lowly expressed in NSCLC

First of all, LINC00472 mRNA level in NSCLC was analyzed by RT-qPCR. It was found that LINC00472 mRNA level in NSCLC significantly decreased (Figure 1A). Similarly, the expression of LINC00472 in NSCLC cells (H1581 and H1975) was lower, compared with normal BEAS-2B cells (Figure 1B). Kaplan–Meier plot ($P=0.019$, Figure 1C) showed that the high expression of LINC00472 was significantly associated with better overall survival of LUAD patients. Additionally, Nuclear-cytoplasmic RNA isolation assay demonstrated that LINC00472 mainly localized in the cytoplasm and not in nucleus (Figure 1D). We divided the patients into two groups (H-LINC00472, L-LINC00472) according to the median value of LINC00472 expression [0.49, 0.02~1.35]. There was a greater degree of involvement and more distal metastases of lymph nodes in patients with low LINC00472 expression compared with patients with high LINC00472 expression. And the LINC00472 expression level was not related to the age and gender of the patients (Table 2). Overall, low LINC00472 expression was observed in NSCLC tissues and cells, and exceptionally low LINC00472 expression may be closely associated with the NSCLC malignant progression.

3.2. LINC00472 suppressed NSCLC cell malignant behavior

To begin with, pcDNA-LINC00472 vector (OE-LINC00472) was transfected into H1581 and H1975 to increase LINC00472 expression level (Figure 2A). In the functional analysis, overexpression of LINC00472 repressed the proliferation activity of H1581 and H1975 cells (Figure 2B). Moreover, overexpression of LINC00472 suppressed the migration ability of H1581 and H1975 cells (Figure 2C). In addition, H1581 and H1975 cells overexpressing LINC00472 showed increased Bax protein

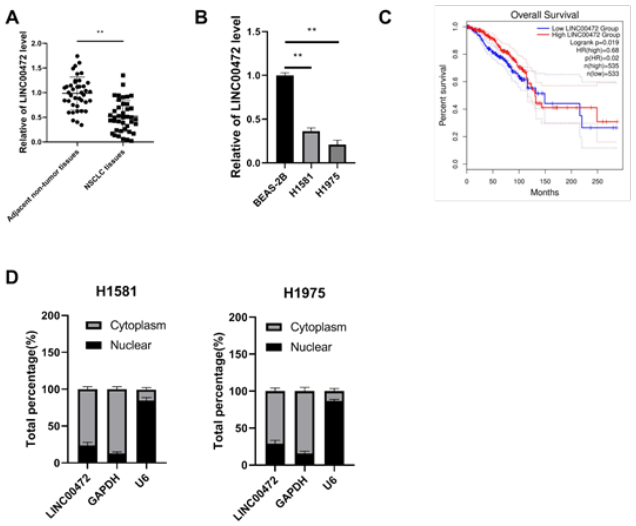

Fig. 1. LINC00472 was lowly expressed in NSCLC. (A-B) LINC00472 expression detected by RT-qPCR$^{**}P < 0.01$ vs. BEAS-2B. (C) Association between LINC00472 expression and overall survival of LUAD patients assessed in the GEPIA database. (D) LINC00472 expression detected by RT-qPCR.

Table 2. The clinical pathological features of 40 NSCLC patients according to LINC00472 expression.

Characteristics		H-LINC00472 N=(20)	L-LINC00472 N=(20)	X^2/t	P value
Age (years)		49.39 ± 3.24	50.44 ± 4.52	0.8444	0.4038
Gender	Male	9	12	0.9023	0.3422
	Female	11	8		
T stage	T_1~T_2	7	11	1.616	0.2036
	T_3~T_4	13	9		
N stage	N_1	1	7	12.53	**0.0019**
	N_2	3	8		
	N_3	16	5		
M stage	M_0	3	10	5.584	**0.0181**
	M_1	17	10		

Note: T, tumor size and/or primary location; N: lymph node involvement; M, presence or absence of distal metastases.

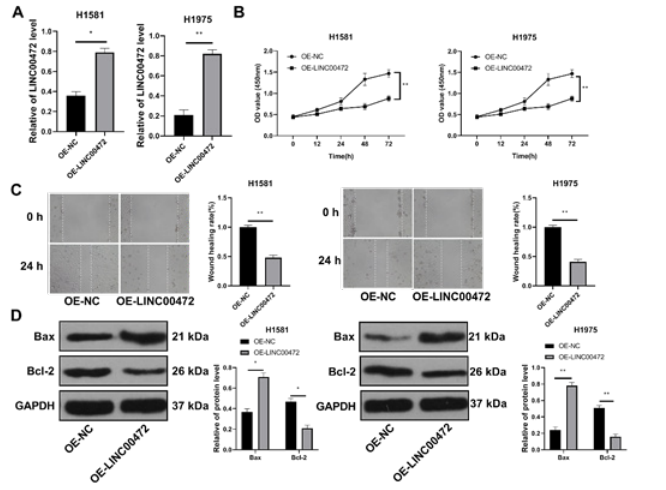

Fig. 2. LINC00472 overexpression suppressed cell proliferation, and migration and induced apoptosis in NSCLC cells. (A) LINC00472 expression assessed by RT-qPCR. (B) cell proliferation viability evaluated by CCK-8 assay. (C) cell migration ability evaluated by wound-healing assay. (D) Bax/Bcl-2 expression evaluated by western blotting. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. OE-NC.

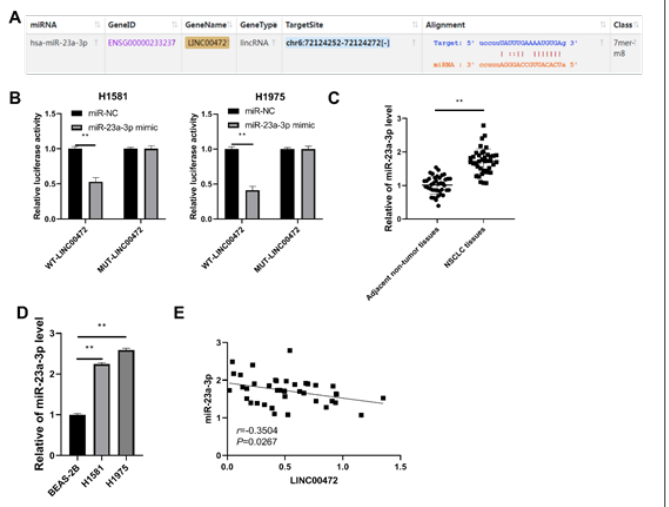

Fig. 3. MiR-23a-3p was a direct target of LINC00472. (A) Potential binding sites between LINC00472 and miR-23a-3p were predicted based on Starbase. (B) Luciferase activity. $^{**}P < 0.01$ vs. miR-NC. (C) the expression of miR-23a-3p detected by qRT-PCR. $^{**}P < 0.01$ vs. Adjacent non-tumor tissues. (E) the correlation between LINC00472 and miR-23a-3p by Pearson analysis.

expression and decreased Bcl-2 protein expression (Figure 2D). These data suggest that LINC00472 acted as a tumor suppressor and effectively inhibited NSCLC cell growth in vitro.

3.3. MiR-23a-3p directly targeted LINC00472

Subsequently, the complementary sequences between LINC00472 and miR-23a-3p were predicted by Starbase database (Figure 3A). Luciferase report assay demonstrated that miR-23a-3p mimic inhibited the luciferase activity of WT- H1581/H1975 cells, but did not influence the luciferase activity of MUT cells (Figure 3B). miR-23a-3p was highly expressed in NSCLC tissues and cells (H1581 and H1975) compared with normal tissues and cells (BEAS-2B), respectively (Figures 3C and 3D). Furthermore, Pearson's correlation analysis demonstrated a negative relationship between LINC00472 expression and miR-23a-3p expression in NSCLC tissues (Figure 3E). These above results showed that LINC00472 could directly target miR-23a-3p.

3.4. LINC00472 restrained NSCLC cell growth by downregulating miR-23a-3p expression

As depicted in Figure 4A, miR-23a-3p mimic markedly upregulated the miR-23a-3p expression level and weakened increased LINC00472 expression induced by OE-LINC00472 transfection in H1581 and H1975 cells. By transfecting miR-23a-3p mimics into H1581 and H1975 cells, LINC00472 overexpression partially reversed the inhibition of proliferation and migration (Figures 4B and 4C). In addition, miR-23a-3p mimic up-regulated Bcl-2 expression and down-regulated Bax expression (Figure 4D). Taken together, LINC00472 repressed the malignant growth of NSCLC cells by downregulating miR-23a-3p expression.

3.5. LINC00472 alleviated NSCLC cell malignant progression by targeting the miR-23a-3p/CCL22 axis

Finally, the interaction between LINC00472 and CCL22 in NSCLC cells was verified by rescued functional

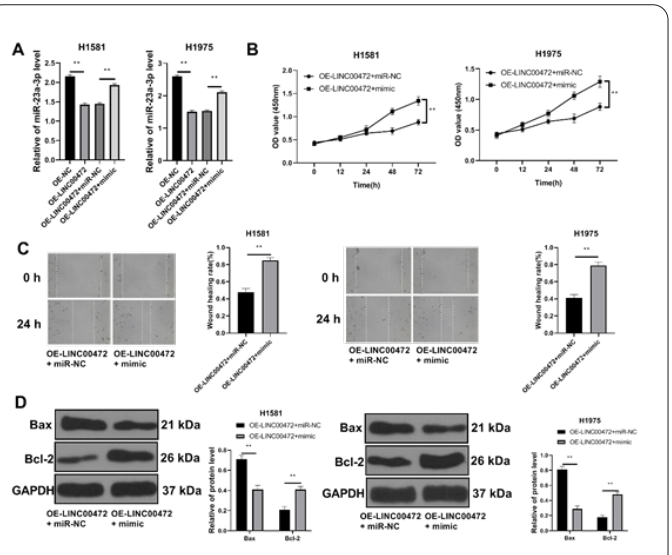

Fig. 4. LINC00472 restrained NSCLC cell growth by downregulating miR-23a-3p expression. (A) MiR-23a-3p determined by RT-qPCR. (B) cell proliferation viability evaluated by CCK-8 assay. (C) cell migration ability by wound-healing assay. (D) Bax/Bcl-2 expression evaluated by western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$.

experiments. As demonstrated in Figure 5A, the expression of CCL22 in H1581 and H1975 cells transfected with siRNA-CCL22 (si-CCL22) was significantly decreased. Meanwhile, knocking CCL22 down alleviated the inhibitory effects of LINC00472 in H1581 and H1975 cells. Results from these phenotypic experiments showed that CCL22 down-regulation restricted cell proliferation and migration, and enhanced apoptosis in H1581 and H1975 cells, where these above changes could be blocked by additional transfection of LINC0047 overexpression vector (Figures 5B-D). Collectively, LINC00472 facilitated NSCLC cell survival via modulating the miR-23a-3p/CCL22 pathway.

4. Discussion

Tumor patients have experienced a significant impro-

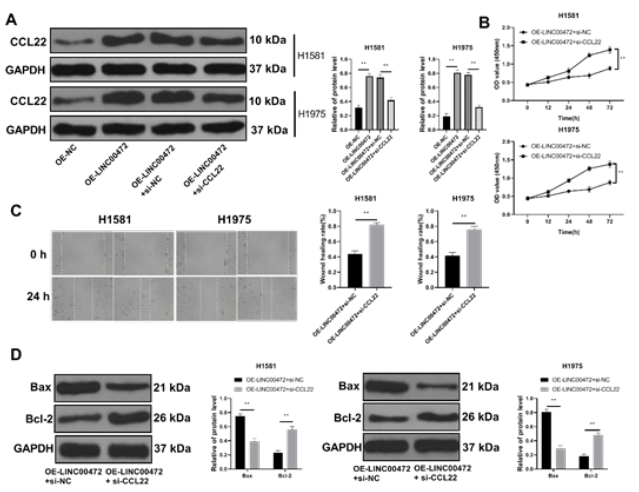

Fig. 5. LINC00472 alleviated NSCLC cell malignant progression by targeting the miR-23a-3p/CCL22 axis. H1581 and H1975 cells were transfected with *pcDNA*-LINC00472 vector (OE-LINC00472), OE-NC, OE-LINC00472+si-CCL22, OE-LINC00472+si-NC and then used in the following experiments. (A) CCL22 protein expression determined by western blotting. (B) cell proliferation viability evaluated by CCK-8 assay. (C) cell migration ability evaluated by wound-healing assay. (D) Bax/Bcl-2 expression evaluated by western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$.

vement in survival times due to targeted therapy based on tumor-specific molecular targets, improved their quality-of-life, and provided a new direction for clinical treatment of NSCLC [24]. Thus, exploring the signal transduction pathway and molecular mechanisms in the NSCLC progression is helpful in finding more potential specific therapeutic targets, which is very important for early diagnosis, prognosis prediction and formulation of effective treatment strategies. In this study, we demonstrated that LINC00472 is lowly pressed in NSCLC tissues and cells, and found its mechanism of NSCLC cells through modulating miR-23a-3p/CCL22 pathway.

Recently, increasing numbers of lncRNAs, which emerged as significant regulators in tumor progression, have been reported to play pro-oncogenic or depending on the genetic background of the tumor, these compounds play tumor-suppressive roles [25]. For example, Liu et al. found that LINC00472 suppresses the cell survival of oral squamous cell carcinoma by upregulating the expression of ELF3 via absorbing miR-455-3p [26]. Wang et al. reported that LINC00472 inhibits p-p65 and p-IκBα by binding to IKKβ and then impairs the cell growth of breast cancer in vivo and in vitro [27]. It is worth noting that LINC00472 restrains the invasion and metastasis of lung adenocarcinoma cells by binding to RNA-binding protein YBX1 [28]. No research has shown the specific functions and mechanisms of LINC00472 in NSCLC. The findings from the present study manifested that the expression of LINC00472 in NSCLC tissues and cells was significantly lower compared with paired non-tumor tissues and normal cells. Overexpression of LINC00472 impaired NSCLC cell proliferation and migration while inducing cell apoptosis, which was consistent with previous studies on LINC00472 in other cancers [26-28].

LncRNA commonly absorbs miRNAs to alleviate the inhibitory roles of miRNAs on target gene expression, thus taking part in the occurrence of tumors [29, 30]. As expected, LINC00472 could also sponge various miRNAs to regulate tumors progression, for example, LINC00472 shows anti-tumor potentials in colorectal cancer [11] or osteosarcoma [12] through miR-196a/PDCD4 or miR-300/FOXO1 pathway, respectively. Based on the ceRNA hypothesis, we further analyzed the downstream regulatory gene of LINC00472 in NSCLC and predicted that miR-23a-3p was a potential target gene of LINC00472 by Starbase database. MiR-23a-3p enhances deterioration effects in multiple tumors, including NSCLC [20, 31, 32]. miR-23a-3p strongly increases cell proliferation of NSCLC cells by inhibiting PTEN [20]. Interestingly, one previous study has revealed that LINC00472 could sponge miR-23a-3p to exert their anti-cancer effects in pancreatic cancer [13], but the interaction between LINC00472 and miR-23a-3p in NSCLC is currently unknown. In this study, miR-23a-3p was overexpressed in NSCLC. Luciferase reporter and PCR analysis confirmed that LINC00472 directly targeted miR-23a-3p and negatively modulated its expression. Meanwhile, our rescue experiments consistently validated that miR-23a-3p attenuated the anti-oncogenic actions of LINC00472 in NSCLC cells, suggesting that the anti-tumor effects of LINC00472 in NSCLC may be achieved by downregulating miR-23a-3p expression.

CCL22 is one such chemokine that decreases tumor growth in NSCLC. For example, lncRNA HOTAIR encourages NSCLC cell progression in vitro by inhibiting the expression of CCL22 [33-36]. Moreover, CCL22 activation impairs proliferation and EMT process in A 549 cells [23]. In the present study, the relationship between miR-23a-3p and CCL22 was predicted and verified by TargetScan database and luciferase experiments. The expression trend of CCL22 was consistent with that of LINC00472, it was low-expressed in NSCLC. Cellular functional analysis showed that down-regulated CCL22 attenuated the inhibitory effects of LINC00472 on NSCLC cell malignant phenotypes. Our results indicated that LINC00472 suppressed NSCLC progression by miR-23a-3p/CCL22 pathway.

Nevertheless, besides CCL22, other downstream target genes of LINC00472/miR-23a-3p axis involved in NSCLC tumorigenesis should be investigated in further research. Additionally, the relationship between clinic pathological features of NSCLC patients and LINC00472 has not been studied well, and the regulation effects of the ceRNA network were not validated by in vivo experiments, these studies will be carried out in subsequent researches.

The current research revealed that lncRNA LINC00472 inhibited NSCLC cells malignant behavior via miR-23a-3p/CCL22 pathway. These results support the potential role of LINC0047 as the effective diagnostic and therapeutic target for NSCLC.

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Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Availability of data and material

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

Authors' contributions

YS conducted the experiments and wrote the paper; LYS, LY, JX, YY, YG, LH and LX analyzed and organized the data; DH conceived, designed the study and revised the manuscript.

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