Long non-coding RNA LINC01748 exerts carcinogenic effects in non-small cell lung cancer cell lines by regulating the microRNA-520a-5p/HMGA1 axis

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Received February 23, 2021; Accepted June 22, 2021

DOI: 10.3892/ijmm.2021.5077

Abstract. The important functions of long non-coding RNAs in the malignancy of non-small cell lung cancer (NSCLC) has been increasingly highlighted. However, whether LINC01748 functions in a crucial regulatory role still requires further research. The aim of the present study was to investigate the biological roles of LINC01748 in NSCLC. Furthermore, different experiments were utilized to investigate the mechanism of action of LINC01748 in 2 NSCLC cell lines. Reverse transcription-quantitative PCR was used to measure mRNA expression levels. Cell Counting Kit-8 assay, flow cytometry analysis and Transwell and Matrigel assays were also used to analyze, cell viability, apoptosis, and migration and invasion, respectively. A tumor xenograft model was used for in vivo experiments. RNA immunoprecipitation experiments, luciferase reporter assays and rescue experiments were used to investigate the mechanisms involved. Data from The Cancer Genome Atlas dataset and patients recruited into the present study showed that LINC01748 was overexpressed in NSCLC. Patients with high LINC01748 mRNA expression level had shorter overall survival rate compared with that in patients with low LINC01748 mRNA expression level. Then, knockdown of LINC01748 mRNA expression level reduced cell proliferation, migration and invasion, but increased cell apoptosis in vitro. Knockdown of LINC01748 also reduced tumor growth in vivo. Mechanistically, LINC01748 could act as a competing endogenous (ce)RNA to sponge microRNA(miR)-520a-5p, to increase the expression level of the target gene, high mobility group AT-hook 1 (HMGA1) in the NSCLC cell lines. Furthermore, rescue experiments illustrated that the functions exerted by LINC01748 knockdown were negated by miR-520a-5p inhibition or HMGA1 overexpression. In summary, LINC01748 acted as a ceRNA by sponging miR-520a-5p, leading to HMGA1 overexpression, thus increasing the aggressiveness of the NSCLC cells. Accordingly, targeting the LINC01748/miR-520a-5p/HMGA1 pathway may benefit NSCLC therapy.

Introduction

Lung cancer is the most common type of cancer and is the leading cause of cancer-related mortality around the world (1). Annually, ~2.1 million patients are diagnosed with lung cancer worldwide and 1.8 million patients die of this malignancy (2). Non-small cell lung cancer (NSCLC) is the predominant pathological type of lung cancer, comprising over 80% of lung cancer cases (3) and NSCLC contributes to the vast majority of cases for both morbidity and mortality (4). At present, the primary therapeutic techniques for NSCLC are surgical resection, chemoradiotherapy, targeted therapy and immunotherapy (5). Due to the lack of typical symptoms and hidden symptoms of NSCLC, patients have advanced NSCLC at the time of clinical diagnosis and thus miss the optimal treatment period (6). Commendable advancements in diagnostic and treatment methods have been made; however, the efficacy of treatment for NSCLC remains unfavorable, highlighting the importance of developing more efficacious therapies (7). According to the data from 2019, the 5-year survival rate of NSCLC globally was low, <18%, which is largely attributed to a high relapse rate and metastasis (8). Therefore, a comprehensive understanding of NSCLC pathogenesis may aid in the identification of potential diagnostic biomarkers and treatment strategies.

Long non-coding (lnc)RNAs are a family of gene transcripts, >200 nucleotides in length, which lack long open reading frames (9). lncRNAs were once considered useless; however, recent studies have confirmed that lncRNAs participate in a number of crucial and fundamental cellular biological processes (10,11). lncRNAs control gene expression at different transcriptional levels via distinctly different mechanisms (12). Aberrant expression of lncRNAs has been proved in nearly
all types of human cancer, such as bladder (13), cervical (14), gastric (15) and pancreatic cancers (16). Several lncRNAs have been found to be increased or decreased in NSCLC and have been identified as important indicators of cancer progression, such as WTA-AS (17), B4GALT1-AS1 (18) and ccAT1 (19). These lncRNAs have tumor-promoting or tumor-inhibiting effects in NSCLC and have been associated with tumor biological behavior (20).

MicroRNAs (miRNAs/miR), which are single-stranded RNA molecules, 17-21 nucleotides in length, are another type of nc RNA (21). miRNAs can decrease gene expression by base pairing with the 3'-untranslated region (UTR) of their downstream genes, ultimately degrading mRNAs or suppressing translation (22). Accumulating evidence has confirmed the importance of miRNAs in carcinogenesis and the progression of NSCLC (23-25). IncRNAs with miRNA response elements can function together as competing endogenous (ce) RNAs by sponging miRNAs, thereby sequestering miRNAs from their target mRNAs (26). Consequently, investigation of tumor-associated IncRNAs and miRNAs in NSCLC may increase the understanding into the mechanisms that contribute to NSCLC progression and aid in the development of diagnostic and therapeutic targets.

Multiple lncRNAs have been associated with tumorgenesis of NSCLC, such as GASS (27), LINC02678 (28) and CCDC144NL-AS1 (29); however, whether LINC01748 plays a crucial regulatory role requires further investigation, as to the best of our knowledge the expression level and function of LINC01748 in other types of cancer has not been analyzed. Therefore, the aim of the present study was to investigate the expression profile, clinical status and biological functions of LINC01748 in NSCLC. Furthermore, different experiments were utilized to understand the mechanisms behind the carcinogenic effects of LINC01748 in NSCLC cell lines.

Materials and methods

Patient samples. Paired NSCLC and adjacent normal tissues were collected from 57 patients (33 males, 24 females; age range, 32-75 years; mean age, 56.7±7.4 years) from the Weifang Yidu Central Hospital (Weifang, China) between February 2014 and December 2015. The clinicopathological parameters of these patients, including tumor size, TNM stage and lymph node metastasis, were also collected. Adjacent normal tissues were collected 2 cm away from the tumor tissues. All patients with NSCLC were diagnosed using histopathology. The tissues were immediately washed with PBS and the tissues were stored in liquid nitrogen until RNA extraction. The inclusion criteria were as follows: i) Diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research. The exclusion criteria were as follows: i) diagnosed with NSCLC; ii) was not treated with anticancer therapies prior to surgery; and iii) agreed to take part in the research.

Cell culture. The BEAS-2B (CRL-9609™) human normal bronchial epithelial cell line was cultured in bronchial epithelial cell growth medium (Lonza Group, Ltd.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The H522 (CRL-5810™) and NCI-H460 (H460; CVCL_01459) (both from American Type Culture Collection) NSCLC cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.).

Another two NSCLC cell lines, SK-MES-1 and A549, were purchased from the National Collection of Authenticated Cell Cultures and maintained in MEM and F-12K medium, respectively and both supplemented with 10% FBS (all from Gibco; Thermo Fisher Scientific, Inc.). Penicillin-streptomycin (1%) was also used in the culture medium for all the cells. The H460 cell line is a large cell lung cancer cell line, while the H522 and A549 cell lines are lung adenocarcinoma (LUAD) cell lines. The SK-MES-1 cell line is a lung squamous cell carcinoma (LUSC) cell line. All the cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Transfection assay. Small interfering (si) RNAs targeting LINC01748 (si-LINC01748#1 and si-LINC01748#2) were purchased from Shanghai GenePharma Co., Ltd., with scrambled negative control (NC) siRNA (si-NC) as the control. The si-LINC01748#1 sequence was 5'-GTCATTTTAGCTATTTAATTTT-3'; the si-LINC01748#2 sequence was 5'-ATGATTTTACATCGTTTATACA-3'; and the si-NC sequence was 5'-CACGATAAGACATGTATTTT-3'. Guangzhou RiboBio Co., Ltd., designed and synthesized the following miRNA oligonucleotides: MiR-520a-5p mimic, NC mimic, miR-520a-5p inhibitor and NC inhibitor. The miR-520a-5p mimic sequence was 5'-UACUUAUGAGGGACGCUC-3' and the NC mimic sequence was 5'-UUGUACUACACAAAGUCUG-3'. The miR-520a-5p inhibitor sequence was 5'-AGAAAGUACUCUUCCUGGAG-3' and the NC inhibitor sequence was 5'-ACUACUGAGAGACAGUAGA-3'.

High mobility group AT-hook 1 (HMGA1)-overexpression vector pcDNA3.1-HMGA1 and empty pcDNA3.1 control was obtained from GenScript. The cells were transfected with siRNAs (100 pmol), miRNA mimic (100 pmol), miRNA inhibitor (100 pmol) or vector (4 µg) and Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ for 6 h. A Cell Counting Kit-8 (CCK-8) assay was performed after 24 h, while at 48 h post-transfection, reverse transcription-quantitative PCR (RT-qPCR), flow cytometry, migration and invasion assays, and western blot analysis were performed.

RT-qPCR. Total RNA was extracted from tissues or cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into first-strand cDNA with a PrimeScript Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). RT was performed at 37°C for 15 min, then 85°C for 5 sec. The mRNA expression levels of LINC01748 and HMGA1 were detected using a TB Green® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) and normalized to the expression level of GAPDH. The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 3 sec, 60°C for 30 sec and 72°C for 30 sec.

RNAiso Plus small RNA (Takara Biotechnology Co., Ltd.) was used to extract small RNA from the tissues and cells. The total RNA was reverse transcribed using the miR-X

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miRNA First-Strand Synthesis kit (Takara Biotechnology Co., Ltd.). Next, the expression level of miR-520a-5p was detected using a miR-X miRNA RT-qPCR TB Green® kit (Takara Biotechnology Co., Ltd.). RT was performed at 37°C for 60 min, then 85°C for 5 sec. The following thermocycling conditions were used: Initial denaturation at 95°C for 10 sec; 95°C for 5 sec and 60°C for 20 sec for 40 cycles; then 95°C for 60 sec, 55°C for 30 sec and 95°C for 30 sec. The small nuclear RNA, U6 was used as the reference control for miR-520a-5p. The 2−ΔΔCq method (30) was used for gene expression calculation.

The following primers were used: LINC01748 forward, 5'-TGGTACGGCACTCGTCTC-3' and reverse, 5'-CTGGTACAACGGATTTGTCG-3'; HMG2A forward, 5'-CTGCTACACGGCAACA-3' and reverse, 5'-CACCTCACCTGTTGTGGA-3'; GAPDH forward, 5'-GGGAAATGGTGAAGGT-3' and reverse, 5'-CACTCACTGTTGTGGA-3'; miR-520a-5p forward, 5'-TCGGCAGGUCACAGAGGA-3' and reverse, 5'-AACGCTTCAGAATTTTGCGT-3'.

**CCK-8 assay.** Cellular proliferative ability was determined using a CCK-8 assay. In short, the transfected cells were seeded, at a concentration of 2x10^3 cells/well into 96-well plates. After culturing for different periods of time (0, 24, 48 and 72 h), the cells were detached, counted and prepared as a single-cell suspension in FBS-supplemented culture medium. A microplate reader was used to measure the absorbance at 450 nm. Finally, the collective absorbance values were used to plot a cell growth curve.

**Flow cytometry analysis.** To determine the apoptotic rate, the NSCLC cells, transfected with different factors, were treated with EDTA-free trypsin, washed twice with ice cooled PBS and centrifuged at room temperature at 1,000 x g for 5 min. The collected cells were then resuspended in 195 µl Annexin V-FITC binding buffer from an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology). Next, the cell suspension was supplemented with 5 µl Annexin V-FITC and 10 µl PI, followed by a 20-min incubation at 20°C without light. Finally, the apoptotic cells were evaluated using FACScalibur flow cytometer (BD Biosciences). CellQuest software v.2.9 (BD Biosciences) was used for data analysis.

**Migration and invasion assays.** Transwell chambers (8.0 µm; Corning, Inc.) were used for both assays. For the migration assay, the cells were detached, counted and prepared as a single-cell suspension in FBS-free basolateral medium (Gibco; Thermo Fisher Scientific, Inc.). Next, 200 µl cell suspension, containing 5x10^4 cells, was seeded into the upper chambers. The basolateral chambers were loaded with 600 µl 20% FBS-supplemented culture medium. The Transwell chambers were incubated for 24 h at 37°C, then fixated with 4% paraformaldehyde at room temperature for 30 mins. The migrated cells that had passed through the pores and attached to the underside of the membrane were stained with 0.5% crystal violet at room temperature for 30 min. The cells which had not migrated or invaded were gently cleaned with a cotton bud. Finally, images of the migrated cells were captured using an inverted light microscope (x200 magnification; Olympus Corp.). The number of migrated cells in five random fields of view was counted using an inverted light microscope. For the invasion assay, Matrigel (Corning, Inc.) was used to coat the upper chambers and polymerized by incubating at 37°C for 2 h, and experimental procedures were repeated in the same fashion as the migration test.

**Tumor xenograft model.** Lentiviruses were produced using a second-generation lentiviral system. LINC01748 short hairpin RNA (shRNA; sh-LINC01748) and non-targeting scramble shRNA (sh-NC) were purchased from Shanghai GenePharma Co., Ltd., and inserted into the pLKO.1 vector (Addgene, Inc.). The sh-LINC01748 sequence was 5'-CCCGGTCATTCTAAGCTATTATTCTTCCAGGAAATTAAATGATTCTTTG-3' and the sh-NC sequence was 5'-CCCGGCAAGGAGACAATTTTATATTCTTCCAGGAAATTAAATGATTCTTTG-3'. The following vectors, alongside pSPAX2 and pMD2.G (both from Addgene Inc.), were transfected into 293T cells (The National Collection of Authenticated Cell Cultures) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). In total, 30 µg plasmids were used for transfection, and the ratio of each plasmid (pSPAX2: pMD2.G: pLKO.1) was 1:1:2. After incubation for 5 h at 37°C with 5% CO₂, the culture medium was replaced with fresh DMEM medium containing 10% FBS, 1% glutamax, 1% non-essential amino acids and 1% sodium pyruvate solution (all from Gibco; Thermo Fisher Scientific, Inc.). Subsequent to incubation for 48 h at 37°C with 5% CO₂, the lentiviruses harboring either sh-LINC01748 or sh-NC were collected by ultracentrifugation at 4°C for 2 h (1,000 x g), mixed with polybrene (5 µg/ml; Sigma-Aldrich; Merck KGaA) and F-12K medium, were transfected into the A549 cells with a multiplicity of infection 5. The A549 cells were incubated with 2 µg/ml puromycin to select stably transfected cells.

The animal experiment was approved by the Ethics Committee of Animal Experiments at Weifang Yidu Central Hospital (Shandong, China). Male BALB/c nude mice (mean weight ± SD, 20.4 ± 1.1 g), aged 4–6 weeks, were purchased from Beijing HFK Bioscience (Beijing, China). A total of 6 mice were used, and housed under specific pathogen-free conditions at 25°C and 55% humidity, with a 10:14 light/dark cycle and ad libitum access to food and water. The flanks of the mice were subcutaneously inoculated with 2x10^6 A549 cells expressing sh-LINC01748 or sh-NC, which were resuspended in 100 µl PBS. The size of the tumors was detected every 5 days with a calliper. On day 30 after cell inoculation, the mice were euthanized by cervical dislocation, and the tumor xenografts were resected, weighed and imaged. The tumor volume was calculated using the following formula: Volume=0.5 x length x width\(^2\).

**Cellular fractionation assay.** A Cytoplasmic and Nuclear RNA Purification kit (Norgen Biotek Corp.) was used to separate the cytoplasmic and nuclear fractions of the NSCLC cells, which was followed by determination of the relative LINC01748 mRNA expression level in both fractions using RT-qPCR.
Bioinformatics analysis. TCGA database (https://portal.gdc.cancer.gov/) was used to examine mRNA expression level of LINC01748, miR-374a-5p, miR-374b-5p, miR-520a-5p, miR-525-5p and HMGA1 in LUAD and LUSC. The prediction of LINC01748 target miRNAs was performed using The Encyclopedia of RNA Interactomes (ENCORI) and StarBase online tool (starBase 3.0; http://starbase.sysu.edu.cn/). ENCORI and TargetScan (http://www.targetscan.org/), bioinformatic tools were used to analyze miRNAs and identify the downstream targets of miR-520a-5p.

Luciferase reporter assay. The HMGA1 3' UTR and LINC01748 fragments with the wild-type (WT) miR-520a-5p-binding site were prepared by Shanghai GenePharma Co., Ltd., and inserted into the psiCHECK2 luciferase reporter vector (Promega Corporation), yielding the HMGA1-WT and LINC01748-WT reporter vectors. Similarly, mutant (Mut) reporter plasmids containing HMGA1-Mut and LINC01748-Mut were constructed by Shanghai GenePharma Co., Ltd. For the reporter assay, Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to co-transfect the generated WT or Mut reporter vectors and miR-520a-5p/NC mimic into the NSCLC cells. Luciferase activity was detected using a Dual-Luciferase Reporter Assay system (Promega Corporation) 2 days later. Renilla luciferase activity was normalized to the firefly luciferase activity.

RNA immunoprecipitation (RIP) assay. RIP experiments were conducted utilizing an EZ-Magna RIP RNA binding protein immunoprecipitation kit (EMD Millipore). The NSCLC cells were treated with RIP cell lysis buffer and the cell lysates were collected. The supernatant was obtained after centrifugation at 4°C for 1,000 x g for 15 min. The supernatant (10%) was used as the ‘input’ and the rest was incubated in RIP buffer containing magnetic beads conjugated with Argonaut 2 (Ago2) or normal IgG control antibodies (EMD Millipore). The magnetic beads were processed with protease K to detach the proteins, then incubated overnight at 4°C. After the extraction of the immunoprecipitated RNA, the enrichment of LINC01748, miR-520a-5p and HMGA2 were analyzed using RT-qPCR.

Western blot analysis. Total protein was isolated from cells or tumor xenografts utilizing ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a bichinchoninic acid assay kit (Beyotime Institute of Biotechnology). Following 10% SDS-PAGE, the separated proteins (30 μg/lane) were transferred onto PVDF membranes, which were then blocked for 2 h at room temperature with 5% skimmed milk diluted with TBS containing 0.1% Tween-20. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies targeting HMGA1 (cat. no. ab129153; 1:1,000 dilution) or GAPDH (cat. no. ab128951; 1:1,000 dilution) (both from Abcam). After incubation for 12 h, the membranes were further incubated at room temperature with a HRP-labelled secondary antibody (cat. no. ab205718; 1:5,000 dilution; Abcam) for 1 h. Finally, the immunoblots were visualized using an ECL Western Blotting Substrate kit (Abcam).

Statistical analysis. All experiments were repeated three times and all the results are presented as the mean ± SD. Significant differences between two groups were analyzed using a paired or unpaired Student's t-test. Comparisons between multiple groups were analyzed using one-way analysis of variance followed by a Tukey's post hoc test. Pearson's correlation analysis was used to detect the correlation between gene expression levels. According to the median expression value of LINC01748, all patients with NSCLC were divided into low or high LINC01748 expression groups. The association between LINC01748/miR-520a-5p/HMGA1 expression level and the clinicopathological parameters in patients with NSCLC was assessed using a χ² test. Kaplan-Meier analysis and the log-rank test were utilized for analysis of overall survival time. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC01748 is upregulated in NSCLC and is associated with poor prognosis. LINC01748 mRNA expression status in patients with LUAD and LUSC was first analyzed using data from TCGA database. As compared with that in normal tissues from healthy individuals, LINC01748 was increased in LUAD and LUSC tissues (Fig. 1A). NSCLC and adjacent normal tissues were collected from patients with NSCLC recruited into the present study and LINC01748 mRNA expression level was detected using RT-qPCR. The LINC01748 mRNA expression level was increased in the NSCLC tissues compared with that in the adjacent normal tissues (Fig. 1B). An increase in LINC01748 mRNA expression level was associated with a larger tumor size, TNM stage and lymph node metastasis (Table I). In addition, LINC01748 mRNA expression level in the NSCLC cell lines was increased compared with that in the normal BEAS-2B cell line (Fig. 1C). Furthermore, patients with NSCLC and high LINC01748 mRNA expression levels showed shorter overall survival time compared with that in patients with low LINC01748 mRNA expression level (Fig. 1D). These findings suggested that LINC01748 may contribute to cancer progression in NSCLC.

LINC01748 knockdown decreases NSCLC cell proliferation, migration and invasion but augmented NSCLC cell apoptosis. The RT-qPCR data, shown in Fig. 1C, revealed that among the 4 NSCLC cell lines, LINC01748 mRNA expression level was higher in the H460 and A549 cell lines compared with that in the other 2 NSCLC cell lines. Therefore, the H460 and A549 cell lines were used as models for subsequent functional assays. A total of 2 siRNAs targeting LINC01748 were used to avert off-target effects and transfection of the two different siRNAs decreased LINC01748 expression in NSCLC cell lines (Fig. 2A). The proliferative ability of the NSCLC cells significantly decreased following LINC01748 knockdown (Fig. 2B). In addition, the apoptosis of the NSCLC cells was increased following LINC01748 knockdown (Fig. 2C). Furthermore, the migration (Fig. 2D) and invasion (Fig. 2E) of the NSCLC cell lines was hindered following transfection with si-LINC01748. Taken together, LINC01748 could have carcinogenic effects and be integral to the malignancy of the NSCLC cells.

LINC01748 serves as a miR-520a-5p sponge. To understand the mechanisms involved for the function of LINC01748, the subcellular location of LINC01748 in the NSCLC cells was
determined. Using a cellular fractionation assay, most of the LINc01748 was found in the cytoplasm of the NSclc cells (Fig. 3A), suggesting that LINc01748 could act as a ceRNA or miRNA sponge. Bioinformatics prediction was performed using ENCORI, which identified 11 miRNAs complementary to LINc01748 (Fig. 3B). Using data from TCGA database, miR-374a-5p, miR-374b-5p, miR-520a-5p and miR-525-5p (Fig. 3C-F) were found to be downregulated in LUAD and

Table I. Association between LINc01748 mRNA expression level and the clinicopathological factors in patients with non-small cell lung cancer.

| Clinicopathological factors          | High (n=29) | Low (n=28) | P-value<sup>a</sup> |
|--------------------------------------|------------|------------|---------------------|
| Sex                                  |            |            | 0.596               |
| Male                                 | 18         | 15         |                     |
| Female                               | 11         | 13         |                     |
| Age, years                           |            |            | 0.792               |
| <60                                  | 14         | 12         |                     |
| ≥60                                  | 15         | 16         |                     |
| Tumor size, cm                       |            |            | 0.033               |
| <3                                   | 12         | 20         |                     |
| ≥3                                   | 17         | 8          |                     |
| TNM stage                            |            |            | 0.031               |
| I+II                                 | 13         | 21         |                     |
| III+IV                               | 16         | 7          |                     |
| Lymph node metastasis                |            |            | 0.035               |
| Negative                             | 10         | 18         |                     |
| Positive                             | 19         | 10         |                     |

<sup>a</sup>χ² test.

Figure 1. LINc01748 expression in NSCLC. (A) Using The Cancer Genome Atlas database, LINc01748 expression in LUAD and LUSC was analyzed. Relative LINc01748 mRNA expression level in (B) NSCLC and adjacent normal tissues and (C) NSCLC cell lines was detected using reverse transcription-quantitative PCR. (D) Kaplan-Meier analysis was used to investigate the association between LINc01748 mRNA expression level and overall survival rate in patients with NSCLC. ***P<0.001 and **P<0.01. NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; FPKM, fragments per kilobase of transcript per million mapped reads.
LUSC; therefore, they were selected for subsequent investigation. After LINC01748 knockdown, miR-520a-5p was clearly increased, but the levels of the other 3 miRNAs were unchanged (Fig. 3G). Thus, miR-520a-5p could be the downstream target of LINC01748.

Luciferase reporter and RIP assays were then performed to confirm the direct binding between miR-520a-5p and LINC01748 (Fig. 3H). The luciferase activity of LINC01748-WT was reduced following miR-520a-5p overexpression, while LINC01748-Mut activity was unaltered following miR-520a-5p mimic co-transfection (Fig. 3I). In the RIP assay, both miR-520a-5p and LINC01748 were enriched in the Ago2-containing beads but not in the IgG-containing beads (Fig. 3J). Subsequently, miR-520a-5p mRNA expression levels were lower in NSCLC tissues compared with that in adjacent normal tissues (Fig. 3K). The decreased miR-520a-5p...
mRNA expression level was associated with TNM stage and lymph node metastasis (Table II). In addition, the mRNA expression levels of miR‑520a‑5p and LINC01748 showed an inverse correlation in the NSCLC tissues from 57 patients (Fig. 3L). Data from TCGA was also used to analyze the association between miR‑520a‑5p mRNA expression level and patient survival rate in patients with NSCLC. However, miR‑520a‑5p mRNA expression level was not associated with overall survival rate in patients with LUAD and LUSC (Fig. 3M). Overall, LINC01748 could act as a miR‑520a‑5p sponge in NSCLC.

miR‑520a‑5p targets HMGA1 in NSCLC cells. To understand the roles of miR‑520a‑5p in NSCLC, miR‑520a‑5p mimic was transfected into the H460 and A549 cell lines to upregulate miR‑520a‑5p (Fig. 4A), followed by a series of functional experiments. Compared with that in the NC mimic‑transfected NSCLC cells, cell proliferation (Fig. 4B), migration and invasion (Fig. 4C) was decreased, while cell apoptosis (Fig. 4D) was increased in cells transfected with miR‑520a‑5p mimics. Using bioinformatics tools, HMGA1 (Fig. 4E) was identified as a potential candidate target gene of miR‑520a‑5p and was selected for experimental confirmation as a result of its crucial regulatory effects on NSCLC progression (31‑34).

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To investigate whether miR‑520a‑5p could affect HMGA1 mRNA and protein expression level, NSCLC cells were transfected with miR‑520a‑5p mimic or NC mimic and HMGA1 mRNA and protein expression level was analyzed in the cells. Transfection with the miR‑520a‑5p mimic resulted in a significant decrease in HMGA1 mRNA and protein expression level (Fig. 4F and G, respectively) in the NSCLC cells. Furthermore, luciferase activity in the NSCLC cells expressing HMGA1‑WT with miR‑520a‑5p mimics was decreased, but this effect was counteracted when the binding sequences were mutated (Fig. 4H). Utilizing TCGA data, it was found that HMGA1 mRNA expression level was significantly upregulated in LUAD and LUSC tissues (Fig. 4I). Altogether, miR‑520a‑5p could directly target HMGA1 in NSCLC and could have antioncogenic activity.

LINC01748 regulates the miR‑520a‑5p/HMGA1 axis in NSCLC. After identifying that miR‑520a‑5p could target HMGA1 in NSCLC, the associations between LINC01748, miR‑520a‑5p and HMGA1 were further investigated in NSCLC cell lines. HMGA1 mRNA and protein expression levels (Fig. 5A and B, respectively) was significantly reduced in the NSCLC cells following LINC01748 knockdown. RIP assays confirmed that LINC01748, miR‑520a‑5p and HMGA1 were enriched in the Ago2‑containing beads compared with that in the IgG control (Fig. 5C). In addition, HMGA1 mRNA expression level was increased in NSCLC tissues (Fig. 5D) and was associated with tumor size, TNM stage or lymph node metastasis (Table III). An increase in HMGA1 mRNA expression level was positively correlated with LINC01748 mRNA expression level (Fig. 5E). In addition, there was a negative correlation between HMGA1 and miR‑520a‑5p mRNA expression levels in NSCLC tissues (Fig. 5F). Notably, rescue experiments illustrated that the increased miR‑520a‑5p mRNA (Fig. 5G) and decreased HMGA1 mRNA and protein (Fig. 5H and I) expression levels in LINC01748 knockdown NSCLC cells were restored following transfection with the miR‑520a‑5p inhibitor. Overall, these data indicated that LINC01748 could positively control HMGA1 expression in the NSCLC cells by acting as a decoy for miR‑520a‑5p.

Table II. Association between miR‑520a‑5p mRNA expression level and clinicopathological factors in patients with non‑small cell lung cancer.

| Clinicopathological factors | miR‑520a‑5p mRNA expression level | P‑value<sup>a</sup> |
|----------------------------|----------------------------------|---------------------|
|                            | High (n=29)                      | Low (n=28)          |                        |
| Sex                        |                                  |                     | 0.790                 |
| Male                       | 16                               | 17                  |
| Female                     | 13                               | 11                  |
| Age, years                 |                                  |                     | 0.113                 |
| <60                        | 11                               | 15                  |
| ≥60                        | 18                               | 13                  |
| Tumor size, cm             |                                  |                     | 0.596                 |
| <3                         | 15                               | 17                  |
| ≥3                         | 14                               | 11                  |
| TNM stage                  |                                  |                     | 0.016                 |
| I+II                       | 22                               | 12                  |
| III+IV                     | 7                                | 16                  |
| Lymph node metastasis      |                                  |                     | 0.017                 |
| Negative                   | 19                               | 9                   |
| Positive                   | 10                               | 19                  |

<sup>a</sup>χ<sup>2</sup> test. miR, microRNA.
Figure 3. LINC01748 sponges miR-520a-5p. (A) Cellular location of LINC01748 in NSCLC cells analyzed using cellular fractionation. (B) In total, 11 predicted target miRNAs of LINC01748 were identified using The Encyclopedia of RNA Interactomes. Using TCGA database, the mRNA expression levels of (C) miR-374a-5p, (D) miR-374b-5p, (E) miR-520a-5p and (F) miR-525-5p in LUAD and LUSC were analyzed. (G) Following knockdown of LINC01748, the mRNA expression level of miR-374a-5p, miR-374b-5p, miR-520a-5p and miR-525-5p was determined using reverse transcription-quantitative PCR. *P<0.01 compared with si-Nc. (H) The binding site in LINC01748 was complementary to miR-520a-5p. (I) The measurement of luciferase activity was analyzed in the NSCLC cells following cotransfection with LINC01748-WT or LINC01748-Mut and miR-520a-5p mimics or NC mimic. **P<0.01 compared with NC mimic. (J) RIP assays demonstrated that LINC01748 and miR-520a-5p could be precipitated by the anti-Ago2 antibody. ***P<0.01 compared with IgG. (K) Relative miR-520a-5p mRNA expression level in NSCLC tissues. (L) The correlation between miR-520a-5p and LINC01748 expression in NSCLC tissues. *P<0.05, **P<0.01 and ***P<0.001. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NC, negative control; si, short inhibiting; miR, microRNA; WT, wild-type; Mut, mutant.
Figure 4. miR-520a-5p directly targets HMGA1. (A) The transfection efficiency of miR-520a-5p mimic. After miR-520a-5p was overexpressed, (B) cell proliferation (C) migration and invasion and (D) apoptosis was analyzed in the NSCLC cell lines. (E) The binding site for miR-520a-5p within the HMGA1 3’-UTR was predicted. HMGA1 (F) mRNA and (G) protein expression level in miR-520a-5p-overexpressing NSCLC cells. (H) Luciferase activity was measured in the NSCLC cells following cotransfection with HMGA1-WT or HMGA1-Mut and miR-520a-5p mimic or NC mimic. (I) The mRNA expression level of HMGA1 in LUAD and LUSC was analyzed in The Cancer Genome Atlas datasets. The cut-off value is the average value of HMGA1 level in LUAD and LUSC. **P<0.01 and ***P<0.001. NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; WT, wild-type; Mut, mutant; miR, microRNA; NC, negative control; FPKM, fragments per kilobase of transcript per million mapped reads; HMGA1, high mobility group AT-hook 1.
LINC01748 knockdown represses NSCLC progression via the miR-520a-5p/HMGA1 axis. Lastly, rescue experiments were conducted to ascertain whether LINC01748 regulated the malignancy of NSCLC cells via the miR-520a-5p/HMGA1 axis. Initially, the efficiency of the miR-520a-5p inhibitor in the NSCLC cells was confirmed using RT-qPCR (Fig. 6A). NSCLC cells with LINC01748 knocked down were further transfected with miR-520a-5p inhibitor or NC inhibitor. The proliferation of the NSCLC cells was significantly suppressed following LINC01748 knockdown and this effect was partly rescued by miR-520a-5p downregulation (Fig. 6B). In addition, the knockdown of LINC01748 promoted NSCLC cell apoptosis and this effect was neutralized by the miR-520a-5p inhibitor (Fig. 6C). Furthermore, migration and invasion assays revealed a decrease in the migration and invasion (Fig. 6D and E) of the NSCLC cells, but transfection with miR-520a-5p inhibitor offset these repressive effects.

Meanwhile, pcDNA3.1-HMGA1 (Fig. 7A) or empty pcDNA3.1 plasmid was transfected alongside si-LINC01748 into the NSCLC cells. Proliferation (Fig. 7B) was significantly reduced, whereas cell apoptosis (Fig. 7C) was increased in the LINC01748 knockdown NSCLC cells, and these effects were abolished following transfection with pcDNA3.1-HMGA1. In addition, overexpression of HMGA1 abrogated the suppressive effect of si-LINC01748 on NSCLC cell migration (Fig. 7D) and invasion (Fig. 7E). In short, the aforementioned results suggested that the miR-520a-5p/HMGA1 axis mediated LINC01748-induced promotion of NSCLC malignancy.

Targeting LINC01748 suppresses NSCLC tumor growth in vivo. To investigate the effect of LINC01748 on tumor growth in vivo, a tumor xenograft model was established using A549 cells expressing sh-LINC01748 or sh-NC. Initially, the efficiency of sh-LINC01748 transfection was assessed using RT-qPCR and the results are shown in Fig. 8A. Compared with that in the sh-NC group, the sh-LINC01748 group showed substantially reduced tumor growth (Fig. 8B) with a notably lower tumor size (Fig. 8C) and weight (Fig. 8D). Expression analysis of the tumor xenografts revealed that LINC01748 was notably downregulated in xenografts from the sh-LINC01748 group (Fig. 8E), suggesting the successful and sustained silencing of LINC01748. Furthermore, the miR-520a-5p mRNA expression level was increased (Fig. 8F), while the HMGA1 mRNA and protein expression levels (Fig. 8G and H, respectively) were decreased in the tumor xenografts originating from sh-LINC01748-transfected A549 cells. Thus, the knockdown of LINC01748 inhibited NSCLC tumor growth in vivo.

Discussion

The regulatory activities of lncRNAs in NSCLC pathogenesis have received increasing attention and recognition (35-37). Thus, investigating the specific functions of lncRNAs would be instrumental in developing effective targets for NSCLC therapy. The important roles of lncRNAs in the malignancy of NSCLC have been increasingly highlighted (38-40); however, the majority of lncRNA expression patterns and their exact roles are not completely understood. The present study increased the understanding of the lncRNA-miRNA-mRNA network by revealing that LINC01748 could act as a ceRNA of miR-520a-5p, whereas miR-520a-5p directly targeted HMGA1 to exacerbate the oncogenesis of NSCLC.

Numerous lncRNAs are dysregulated in NSCLC and have been associated with the regulation of malignant tumor...
characteristics (17,41,42). For example, MALAT1 (43), dGcR5 (44) and NcK1-AS1 (45) were found to be increased in NScLc tissues and were associated with the oncogenicity of NScLc. By contrast, LINc01089 (46), LINc01089 (47) and dHRS4-AS1 (48) are found to be decreased in NScLc and could play anti-carcinogenic roles. However, the detailed roles of LINc01748 in NScLc remain unclear; thus, there is an urgent requirement for further investigation. In the present study, there was an increase in the mRNA expression level of LINc01748 in NScLc tissues in data from TCGA database and in patients with NScLc recruited into the current study. Compared with that in patients with low LINc01748mRNA expression level, patients with high LINc01748 mRNA expression level displayed shorter overall survival time. Then, knockdown of LINc01748 revealed that LINc01748 could maintain the malignancy of the NScLc cells. The knockdown of LINc01748 attenuated NScLc cell proliferation, migration and invasion, but promoted apoptosis in vitro. In addition, LINc01748 knockdown decreased tumor growth in vivo.

The molecular mechanisms behind the tumor-promoting activities of LINc01748 in NSCLC require further investigation. The ceRNA theory is recognized as a novel mechanism of cytoplasmic lncRNAs. In this theory, lncRNAs exert ‘sponge-like’ activity for miRNAs, consequently lowering the regulatory effects of miRNAs on downstream genes (50).

Next, the subcellular location of LINc01748 in the NSCLC cells was investigated and whether LINc01748 functioned as a ceRNA or natural miRNA sponge. The results revealed that LINc01748 was a cytoplasmic lncRNA, which could directly interact with the miR-520a-5p. Subsequent mechanistic experiments, such as luciferase reporter assays, RIP assays, molecular analysis and rescue experiments showed that LINc01748 could act as a ceRNA to sponge miR-520a-5p, thereby increasing the mRNA expression level of the miR-520a-5p target, HMGA1, in NSCLC cells. Specifically, to the best of our knowledge, a new ceRNA pathway, the LINc01748/miR-520a-5p/HMGA1 pathway, in NSCLC was identified for the first time.

miR-520a-5p has been investigated in numerous studies and was found to be expressed at low levels in chronic myelogenous leukemia (51) and breast cancer (52), and have anticarcinogenic effects (51,52). A previous study has reported low miR-520a-5p mRNA expression level in NSCLC (53). The results in the present study further corroborate the tumor-inhibiting effect of miR-520a-5p in NSCLC cells. CDK4 (53), GOT2 (54), ORMDL3 (52) and STAT3 are proven targets of miR-520a-5p.
Therefore, the putative target of miR-520a-5p was investigated and HMGA1 was selected for experimental confirmation as a result of its crucial regulatory effects on NScLc progression (31-34). Increased HMGA1 mRNA expression levels in NSCLC were found to be significantly associated with clinical stage, differentiation, TNM stage and poor prognosis (33). Furthermore, HMGA1 was verified as an independent predictive factor for the prognosis of patients with NSCCL (33). HMGA1 exerts pro-oncogenic activities in NSCCL and was associated with aggressive phenotypes (31-34).

Previously, 2 lncRNAs, VPS9d1-AS1 (55) and TRPM2-AS (56), were associated with the regulation of HMGA1 in NScLc cell lines. In the present study, to the best of our knowledge, it was confirmed for the first time that miR-520a-5p was sponged by LINc01748 and was negatively regulated by this lncRNA in NScLc cell lines. In addition, HMGA1 was found to be under control of the LINc01748/miR-520a-5p axis in 2 NSCLC cell lines at the posttranscriptional level. Furthermore, the effects of LINc01748 knockdown in 2 NSCLC cells lines were negated by decreasing the expression levels of miR-520a-5p/HMGA1. Therefore, the carcinogenic role of LINc01748 in the NSCLC cell lines was accomplished by sequestering miR-520a-5p and weakening the inhibitory effect of the latter on HMGA1.

In the present study, the oncogenic activities of the LINc01748/miR-520a-5p/HMGA1 axis was investigated in 2 NSCLC cell lines. LINc01748 was found to be over-expressed in NSCLC tissues and was associated with poor patient prognosis. This lncRNA sponged miR-520a-5p, leading...
to HMGA1 overexpression; thus, exacerbating the aggressiveness of the NSCLC cell lines. Accordingly, targeting the LINC01748/miR-520a-5p/HMGA1 pathway may be beneficial as a NSCLC therapy. The role of LINC01748 in other types
of cancer have never been examined, which highlights the novelty of the present study.

Acknowledgements

Not applicable.

Funding

This research was supported by Weifang Yidu Central Hospital.

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors’ contributions

This study was designed by YT and JC, and confirm the authenticity of the data. JC analyzed the data. All the experiments were performed by YT, FX, LX and JC. YT and JC wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of Weifang Yidu Central Hospital (Shandong, China) approved the present research. The animal experiment was approved by the Ethics Committee of Animal Experiments at Weifang Yidu Central Hospital (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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