LINC01342 silencing upregulates microRNA-508-5p to inhibit progression of lung cancer by reducing cysteine-rich secretory protein 3

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INTRODUCTION

Lung cancer (LC) is the most common malignancy in most countries and is still a leading cause of cancer-related death throughout the world. The high mortality of LC has resulted from the lack of LC screening and the late diagnosis caused by lacked physical symptoms in the early stages of LC [1, 2]. Traditionally LC is classified into two major histological types: non-small cell lung cancer (NSCLC) (75–85%) and small cell lung cancer (SCLC) (15–25%) [3]. The pathogenesis of LC is multifactorial, including genetic factors, environmental factors, and smoking, while LC occurrence is associated with the regulation of oncogenes and tumor suppressor genes [4]. It is reported that about 70% of LC patients were diagnosed at an advanced stage, with a 16% 5-year survival rate, and unfortunately, only 15% of LC patients were diagnosed at an early stage [5]. Thus, there is an urgency to explore the potential biomarkers for LC diagnosis and prognosis.

Long non-coding RNAs (lncRNAs) are transcripts with >200 nt in length whereas lack protein-coding ability. LncRNAs modulate various biological processes, such as cell viability, apoptosis, tumorigenesis, and immune response [6]. The involvement of several lncRNAs has been identified in LC. For instance, LINC01342 was upregulated and miR-508-5p was downregulated in LC tissues and cells. High LINC01342 expression indicated a poor prognosis of LC patients. The LINC01342/miR-508-5p silencing or miR-508-5p elevation inhibited proliferation, migration, and invasion of LC cells and promoted LC cell apoptosis, and also suppressed the in vivo tumor growth. LINC01342 bound to miR-508-5p and miR-508-5p targeted CRISP3. LINC01342 plays a prognostic role in LC and LINC01342 silencing upregulates miR-508-5p to inhibit the progression of LC by reducing CRISP3.
We performed this study to reveal the role of the LINC01342/miR-508-5p/CRISP3 axis in LC, and we speculated that LINC01342 may bind to miR-508-5p, which targets CRISP3 to affect the biological functions of LC cells, thus affecting LC development.

RESULTS

LINC01342 is upregulated in LC tissues and cells, and the upregulation indicates a poor prognosis of LC patients

At present, there are few reports about the clinical status, biological activity, or molecular mechanism of LINC01342 in LC. It was only found that LINC01342 was upregulated in OC [9]. In order to explore the expression of LINC01342 in LC tissues, we first compared the expression of LINC01342 in 106 pairs of LC tissues and adjacent normal tissues. It was discovered that the expression of LINC01342 in LC tissues was higher than that in adjacent normal tissues (Fig. 1A). Meanwhile, RT-qPCR showed that LINC01342 expression was increased in LC cell lines versus 16HBE cells, especially in A549 and SK-MES-1 cells (Fig. 1B).

To better understand the clinical significance of LINC01342 in LC, the 106 LC patients were divided into high expression group (n = 54) and low-expression group (n = 52) considering the expression of LINC01342 in all LC samples. Chi-square test showed that expression of LINC01342 was correlated with tumor-node-metastasis (TNM) stage (P = 0.034) and lymph node metastasis (LNM) (P = 0.025) (Table 1). To further study the effect of LINC01342 on the prognosis of LC patients, Kaplan–Meier analysis was performed to detect the survival of patients, and it showed that the overall survival of LC patients with high LINC01342 expression group was evidently shortened (Fig. 1C).

These findings revealed that LINC01342 was upregulated in LC tissues and cells, and this upregulation indicated a poor prognosis of LC patients.

LINC01342 silencing inhibits malignant behaviors of LC cells in vivo and in vitro

In order to further explore the effect of LINC01342 on the biological characteristics of LC cells, we selected A549 cells and SK-MES-1 cells with high LINC01342 expression. Cells were transfected with si-NC and si-LINC01342, and RT-qPCR revealed that LINC01342 was successfully downregulated (Fig. 2A). CCK-8 results showed that the downregulation of LINC01342 inhibited the proliferation of A549 and SK-MES-1 cells (Fig. 2B). The colony formation ability of cells was detected using colony formation assay, and we found that LINC01342 silencing decreased the colony formation ability of cells (Fig. 2C).

The cell apoptosis was determined using flow cytometry and the data suggested that LINC01342 reduction promoted the apoptosis of A549 and SK-MES-1 cells (Fig. 2D). Meanwhile, Transwell assay results showed that LINC01342 knockdown reduced the migration and invasion of A549 and SK-MES-1 cells (Fig. 2E, F).

In order to determine the effect of LINC01342 on the growth of LC cells in vivo, the xenotransplantation model was established by injecting cells stably expressing si-LINC01342 or si-NC into nude mice. The results showed that the tumor volume and weight were both suppressed after LINC01342 down-regulation (Fig. 2G).

These results indicated that silencing LINC01342 inhibited the malignant behaviors of LC cells in vivo and in vitro.

LINC01342 binds to miR-508-5p

A common mechanism of IncRNA activity is to act as bait or sponge for miRNA. In order to study the specific molecular mechanism of the biological effect of LINC01342, we predicted using the RNA22

| Parameter               | Case        | Low expression (n = 52) | High expression (n = 54) | P value |
|-------------------------|-------------|------------------------|-------------------------|---------|
| Age (years)             |             |                        |                         | 0.567   |
| < 60                    | 54          | 28                     | 26                      |         |
| ≥ 60                    | 52          | 24                     | 28                      |         |
| Gender                  |             |                        |                         | 0.686   |
| Male                    | 68          | 32                     | 36                      |         |
| Female                  | 38          | 20                     | 18                      |         |
| Smoking history         |             |                        |                         | 0.846   |
| Yes                     | 57          | 27                     | 30                      |         |
| No                      | 49          | 25                     | 24                      |         |
| Tumor size              |             |                        |                         | 0.340   |
| ≤ 3 cm                  | 54          | 29                     | 25                      |         |
| > 3 cm                  | 52          | 23                     | 29                      |         |
| Histology type          |             |                        |                         | 0.234   |
| Adenocarcinoma          | 64          | 28                     | 36                      |         |
| Squamous                | 42          | 24                     | 18                      |         |
| Lymph nodes metastasis  |             |                        |                         | 0.034   |
| No                      | 74          | 31                     | 43                      |         |
| Yes                     | 32          | 21                     | 11                      |         |
| TNM stage               |             |                        |                         | 0.025   |
| I–II                    | 69          | 28                     | 41                      |         |
| III–IV                  | 37          | 24                     | 13                      |         |

The enumeration data were analyzed using the chi-square test. TNM tumor-node-metastasis.
site that there existed binding sites between LINC01342 and miR-508-5p (Fig. 3A).

To determine whether LINC01342 directly regulates miR-508-5p, we cloned the WT or MUT binding sequence of LINC01342 into luciferase reporter vector, and co-transfected it with miR-508-5p mimic or mimic NC into A549 and SK-MES-1 cells. The results showed that miR-508-5p mimics specifically inhibited WT-driven luciferase activity in A549 and SK-MES-1 cells, while did not affect LINC01342-MUT (Fig. 3B). In addition, the RIP assay revealed that LINC01342 and miR-508-5p were more abundant in Ago2 immunoprecipitation (Fig. 3C). The findings suggest that LINC01342 can specifically bind to miR-508-5p.

Next, we compared the expression of miR-508-5p between LC tissues and adjacent normal tissues, and the results showed that the expression of miR-508-5p was reduced in LC tissues (Fig. 3D, E). Pearson test showed that there was a negative correlation between expression of LINC01342 and miR-508-5p in LC tissues (Fig. 3F). To examine the regulation between LINC01342 and miR-508-5p, we used RT-qPCR to detect the expression of miR-508-5p after silencing LINC01342, and we found that miR-508-5p was upregulated in A549 cells and SK-MES-1 cells by LINC01342 reduction (Fig. 3G).

Elevated miR-508-5p restricts malignant behaviors of LC cells

In order to understand the biological significance of miR-508-5p in LC, LC cells were transfected with miR-508-5p mimic to alter the expression of miR-508-5p, and RT-qPCR showed that the transfection was successful (Fig. 4A).

After transfection, the biological functions of LC cells were determined, and it was found through CCK-8 assay, colony formation assay, flow cytometry, and Transwell assay that the miR-508-5p elevation inhibited the proliferation, colony formation ability, migration and invasion of LC cells, and also promoted LC cell apoptosis (Fig. 4B–F).

The above data implied that miR-508-5p upregulation suppressed the malignant phenotypes of LC cells.

MiR-508-5p directly targets CRISP3

CRISP3 belongs to a cysteine-rich secretory protein family and has been revealed to be upregulated in prostate cancer [18]. We set out to examine whether CRISP3 is also a target of miR-508-5p in LC. First, targetscan analysis revealed that there are potential binding sites that may be targeted by miR-508-5p in the CRISP3 gene (Fig. 5A). Dual-luciferase reporter gene assay showed that miR-508-5p mimic specifically inhibited the
expression of luciferase gene containing WT, but did not inhibit the CRISP3-MUT-binding site (Fig. 5B). Next, by examining the expression of CRISP3 in clinical samples and cell lines, we found that CRISP3 mRNA and protein expression was increased in LC tissues and cell lines (Fig. 5C–F). Correlation analysis showed that CRISP3 expression was negatively correlated with miR-508-5p expression, but was positively correlated with LINC01342 expression (Fig. 5G). In A549 and SK-MES-1 cells, upregulation of miR-508-5p and CRISP3 reduction suppressed malignancy of LC cells (Fig. 6A). The viability, colony formation ability, migration, and invasion of the transfected cells were determined, and it was found that the downregulation of CRISP3 repressed viability, colony formation ability, migration, and invasion of LC cells, and facilitated the LC cell apoptosis (Fig. 6B–F). These results suggest that downregulated CRISP3 suppresses the malignancy of LC cells.

**DISCUSSION**

LC is the main cause of cancer-related mortality, contributing to ~25.3% of all cancer deaths, with a poor prognosis [19]. This study was performed to investigate the role of the LINC01342/miR-508-
5p/CRISP3 axis in LC, and our results indicated that LINC01342 promoted the malignant behaviors of LC cells through regulating miR-508-5p/CRISP3, thus contributing to LC progression.

To begin with, we detected the expression of LINC01342 in tissues and cells and found that it was upregulated in LC tissues and cells, respectively, compared with adjacent normal tissues and normal bronchial epithelial cells 16HBE. Due to the limited literature reporting LINC01342, we can only find in a recent study that LINC01342 expression was increased in the OC tissue samples [9]. Considering this aberrant upregulation of LINC01342, we analyzed its role in the prognosis of LC patients. The patients were divided into the high and low LINC01342 expression groups based on the median value of LINC01342 expression to detect the association between LINC01342 expression and clinicopathological characteristics of LC patients. It was found in our findings that LC patients with high LINC01342 expression had a later TNM stage and severer LNM in contrast to those with low LINC01342 expression. Moreover, we also found that LINC01342 upregulation indicated a poorer survival rate in LC patients. Thus, we concluded that LINC01342 functioned as an oncogene in LC. Moreover, we knocked LINC01342 down in LC cells to observe its role in cancer cell growth. Through the CCK-8 assay, colony formation assay, flow cytometry, scratch test, and Transwell assay, we found that the knockdown of LINC01342 inhibited proliferation, migration, and invasion, and inhibited apoptosis of LC cells. Similarly, Zhang et al. have revealed that downregulation of LINC01342 repressed the proliferative and metastatic abilities of OC cells [9]. The identification of LINC01342 in human cancer cell biological functions demands further explorations.

As predicted through a bioinformatic website, there existed a binding region between LINC01342 and miR-508-5p. Furthermore, this binding relationship between LINC01342 and miR-508-5p was confirmed using dual-luciferase reporter gene assay and RIP assay. MiR-508-5p expression was also detected in our research and we found that it was downregulated in LC tissues and cell lines. Consistently, Liu et al. have reported that the expression of miR-508-5p was decreased in glioma tissues and cell lines [20], and it has been demonstrated as well that miR-508-5p was downregulated in hepatocellular carcinoma tissues versus the non-tumorous tissues [21]. These data revealed the downregulation of miR-508-5p in human cancers. Therefore, we regulated miR-508-5p expression in LC cells to assess its role during LC cell growth. It was found through a series of essays in our study that the elevation of miR-508-5p inhibited the malignant episodes of LC cells in vivo and in vitro. Similar to our findings, a study has proposed that miR-508-5p upregulation markedly suppressed the proliferation, migration, and invasion of melanoma cells [13], and miR-508-5p has been found to reduce glioma cell growth as well [22]. Furthermore, we found through the bioinformatic analysis and confirmed using dual-luciferase reporter gene assay that CRISP3 was a target gene of miR-508-5p. Herein, we detected the expression of CRISP3 and assessed its role in LC cells. It was found in our results that CRISP3 was upregulated in NSCLC patients after chemotherapy [17]. Furthermore, a publication has elucidated that CRISP3 facilitated cell motility and invasion in prostate cancer [16]. The low expression of CRISP3 has been reported to predict a favorable prognosis in patients with mammary carcinoma, and also weakened the migration and invasion of mammary carcinoma cells [23].

CONCLUSION

In conclusion, we found that the knockdown of LINC01342 upregulates miR-508-5p to inhibit the progression of LC through
the inhibition of CRISP3. This study may provide novel biomarkers for LC prognosis and treatment. However, the detailed mechanism was not ulteriorly explored in this study due to limited conditions, and we would perform further studies in the future.

MATERIALS AND METHODS

Ethics statement

Written informed consent was acquired from all patients before this study. The protocol of this study was confirmed by the Ethics Committee of The First Hospital of China Medical University and based on the ethical principles for medical research involving human subjects of the Helsinki Declaration. Animal experiments were strictly in accordance with the Guide to the Management and Use of Laboratory Animals issued by the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of The First Hospital of China Medical University.

Study subjects

One hundred and six pairs of primary LC tissues and matched adjacent normal tissues were harvested from patients (70 males and 36 females, ages 26–75 years) accepted surgical resection in The First Hospital of China Medical University, and all of the cancer tissues were confirmed by pathological diagnosis.

Cell culture

LC cell lines (A549, PC-9, SK-MES-1, H1299, and H1975) and normal bronchial epithelial cell line 16HBE (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) containing 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 units/mL penicillin (Haoo, Heilongjiang, China) and 100 μg/mL streptomycin (Ruiyang Technology Co., Ltd., Beijing, China).

Cell transfection and grouping

A549 and SK-MES-1 cells were seeded onto six-well plates and transfected with oligonucleotides or plasmids using Lipofectamine 2000 (Invitrogen, CA, USA). The cells were classified into: the small interfering RNA (si)-negative control (NC) group (transfection of NC si-RNA), si-LINC01342 group (transfection of si-RNA-LINC01342), mimic NC group (transfection of miR-508-5p mimic NC), miR-508-5p mimic (transfection of miR-508-5p mimic), short hairpin RNA (sh)-NC group (transfection of sh-RNA NC), sh-CRISP3 group (transfection of sh-CRISP3 sh-RNA), overexpressed (oe)-NC group (transfection of unrelayed LINC01342 plasmid), oe-LINC01342 group (transfection of plasmid overexpressing LINC01342), oe-LINC01342 + miR-508-5p mimic group (transfection of plasmid overexpressing LINC01342 and miR-508-5p mimic) and oe-LINC01342 + sh-CRISP3 group (transfection of plasmid overexpressing LINC01342 and CRISP3 sh-RNA). MiR-508-5p mimic and its NC were acquired from RiboBio Co., Ltd. (Guangdong, China).
(Shanghai, China); si-LINC01342, oe-NC, oe-LINC01342, and si-NC were purchased from GenePharma Co., Ltd. (Shanghai, China); sh-CRISP3 and sh-NC were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China).

**Cell counting kit-8 (CCK-8) assay**
According to manufacturers' information, the cell proliferation was determined using CCK-8 kits (Dojindo, Shanghai, China). Cells were plated onto 96-well plates and incubated for 0–4 days. A microplate reader was used to detect the absorbance of cells at 450 nm, and then the proliferation rate was calculated [24].

**Colony formation assay**
Cells that had been transfected for 24 h were seeded onto a six-well plate at 1000 cells/well for 14 days culture. Then, cells were fixed and stained with 0.5% crystal violet dye solution. The colonies (a colony contains over 50 cells) were counted under a microscope [25].

**Flow cytometry**
The apoptosis of cells was determined using the fluorescein isothiocyanate apoptosis detection kits (BioLegend, CA, USA) referring to a publication [26].

**Transwell assay**
The migration and invasion of cells were determined using Transwell chambers (BD Biosciences, NJ, USA) as previously described [27]. Matrigel was used in the invasion assay but not the migration assay.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**
Total RNA was extracted using Trizol kits (Invitrogen, CA, USA) and reversely transcribed into cDNA. The SYBR Green PCR Kit (Takara) was used for the quantification analysis. U6 and glyceraldehyde phosphate dehydrogenase (GAPDH) were used as internal references. Data were analyzed using 2−ΔΔCt method and primer sequences (RiboBio) were shown in Table 2.

**Western blot analysis**
Total protein in tissues or cells was extracted. The proteins were conducted with gel electrophoresis, transferred onto membranes, and incubated with primary antibodies CRISP3 (1:1000, C9996, Sigma-Aldrich, MI, USA) and GAPDH (1:1000, ab8245, Abcam, CA, USA), and then were incubated with relative secondary antibody for 1 h. Subsequently, the bands were visualized using the enhanced chemiluminescent detection system (Bio-Rad, CA, USA) and the gray value was determined.
Dual-luciferase reporter gene assay

The fragments of LINC01342 wild type (WT), LINC01342 mutant type (MUT), CRISP3-WT, and CRISP3-MUT containing miR-508-5p-binding site were designed by GenePharma. According to the instructions, the pmir-GLO vector was used to insert the fragments into the luciferase reporter molecule. A549 and SK-MES-1 cells were seeded in six-well plates and transfected with miR-508-5p mimic or mimic NC. Then, a total of 100 ng pmir-GLO reporter vector containing each fragment was transfected into A549 and SK-MES-1 cells. After transfection, the cells were incubated at 37 °C and with 5% CO2. The luciferase activity was determined by a dual-luciferase reporter gene detection system (Promega, WI, USA).

RNA immunoprecipitation (RIP) assay

According to the manufacturer’s information, Magna RIP™ RNA-binding protein immunoprecipitation Kit (Millipore Inc., MA, USA) and the Aga2 antibody (Abcam) or immunoglobulin G (IgG) antibody (the NC) were used for RIP assay. The immunoprecipitated RNA was confirmed by RT-qPCR.

Table 2. Primer sequence.

| Gene        | Sequence (5′ → 3′)               |
|-------------|---------------------------------|
| LINC01342   | F: GTTTGACTTGTTCAGGCACA         |
|             | R: GTCCTCCAAAGACGAGAACAG        |
| miR-508-5p  | F: TACTCCAGAGGGCGTCATCATG       |
| CRISP3      | F: AAATCATGGAAAATAAGGGAATCTG    |
|             | R: CCAAGAAGCAGACATTGGCGATTG     |
| U6          | F: CTCGCTTGGCGCCGACACA         |
|             | R: AAGCGCTTCCAGAATTTGCGT        |
| GAPDH       | F: CATCAGATCACATTGGATCGT        |
|             | R: CCATCAGGCCCACAGTTTCC         |

F forward, R reverse, miR-508-5p microRNA-508-5p, GAPDH glyceraldehyde phosphate dehydrogenase.

Subcutaneous tumorigenesis in nude mice

BALB/c nude mice aging 4–6 weeks were subcutaneously injected with A549 or SK-MES-1 cells that had been stably transfected with si-LINC01342 or si-NC. Each group contained five nude mice. The longest and shortest diameters of xenografts were recorded with a caliper every week, and the tumor volume was calculated (volume = 0.5 × length × width²). Injected for 28 days, all mice were euthanized with the xenografts collected and weighed [24].

Statistical analysis

All data analyses were conducted using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The measurement data were expressed as mean ± standard deviation. The t-test was performed for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups, followed by Tukey’s post hoc test. Correlations among expression of LINC01342, miR-508-5p, and CRISP3 were analyzed using the Pearson test. P value < 0.05 was indicative of statistically significant difference.

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ACKNOWLEDGEMENTS

We would like to give our sincere gratitude to the reviewers for their constructive comments.

AUTHOR CONTRIBUTIONS

QS contributed to study design and manuscript editing. ZX and QS contributed to experimental studies. HW and LZ contributed to data analysis.

FUNDING

None.

COMPETING INTERESTS

The authors declare no competing interests.

CONSENT FOR PUBLICATION

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Written informed consents were acquired from all patients before this study. The protocol of this study was confirmed by the Ethics Committee of The First Hospital of China Medical University and University for the ethical principles for medical research involving human subjects of the Helsinki Declaration. Animal experiments were strictly in accordance with the Guide to the Management and Use of Laboratory Animals issued by the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of The First Hospital of China Medical University.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41420-021-00613-x.

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