Long non-coding RNA LINC00339 facilitates the tumorigenesis of non-small cell lung cancer by sponging miR-145 through targeting FOXM1

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ABSTRACT

Non-small cell lung cancer (NSCLC) is one of leading causes of cancer-related death worldwide. Long noncoding RNAs (lncRNAs) has been identified to modulate the tumorigenesis of NSCLC. However, the precise molecular mechanism of lncRNAs in the course is still unclear. Results showed that LINC00339 was significantly up-regulated in NSCLC tissue and cells, which indicated the poor prognosis of NSCLC patients. Loss-of-function experiments showed that LINC00339 silencing inhibited the proliferation and invasion, accelerated the apoptosis, and suppressed the tumor growth of NSCLC cells in vitro and in vivo. Luciferase reporter assay and RNA immunoprecipitation (RIP) revealed that LINC00339 promoted the NSCLC progression via FOXM1 via targeting miR-145. In conclusion, our results identify the important role of the LINC00339/miR-145/FOXM1 axis in the NSCLC tumorigenesis, providing neoteric mechanism for the NSCLC tumorigenesis.

1. Introduction

Non-small cell lung cancer (NSCLC) is the major subclass of lung cancer, acting as one of the leading causes of cancer-related death worldwide [1,2]. Thousands numerous new prevalence-incidence cases of NSCLC patients were reported every year [3]. The major challenge for the NSCLC clinical therapy is the delay of suspected patients who diagnosed at advanced or metastatic stages [4]. The poor prognosis of NSCLC individuals is largely due to the inability to diagnose at early stage [5]. Therefore, it is urgent to uncover the potential critical molecular mechanism of NSCLC pathogenesis and progression.

Long noncoding RNAs (lncRNAs) are transcripts with longer than 200 nucleotides, while without protein-coding potential [6–8]. The protein-coding sequences occupy only approximate 2% of the human genome. With the help of next-generation sequencing (NGS) and whole-genome sequencing technology, huge quantity of non-coding RNAs have been discovered [9,10]. Increasing number of lncRNAs have been identified to be involved in the tumorigenesis, including thyroid cancer [11], NSCLC [12,13]. For example, lncRNA NEAT1 was significantly upregulated in NSCLC tissues and closely associated with advanced TNM stages, lymph node metastasis, distant metastasis, and poor prognosis, through miR-181a-HMGB2 and acting as a competitive sponge of miR-181a [14].

LINC00339 is a new identified oncogenic lncRNA in human cancers. For example, LINC00339 was upregulated in glioma tissue as well as in glioma cell lines, and the high-expression of LINC00339 in glioma tissues was positively correlated with glioma vasculogenic mimicry formation [15]. However, the role of LINC00339 in NSCLC is still unclear. In this study, we found that lncRNA LINC00339 was significantly high-expressed in NSCLC tissue and cells. Moreover, we hypothesized that lncRNA LINC00339 might promote the genesis of NSCLC cells by facilitating FOXM1 via miR-145.

2. Materials and methods

2.1. Clinical tissue specimens

A total of 45 lung cancer patients who underwent resection at the First Affiliated Hospital of Dalian Medical University from July 2016 to October 2017 were recruited into the study. There is no any patients had received preoperative chemotherapy before this study. All tissue samples were excised in surgery and snap-frozen in liquid nitrogen. The NSCLC was identified by two experienced pathologists. This study was approved by the Ethic Committee of The First Affiliated Hospital of Dalian Medical University. All written informed consents had been collected from every patient before surgery.

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2.2. Cell lines

Human NSCLC cell lines (SK-MES-1, Calu-3, A549, H460) were provided by the American Type Culture Collection (ATCC, USA), and normal human bronchial epithelial cells (NHBE) and HEK-293 T cells were p provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human NSCLC cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA). Normal human bronchial epithelial cells and HEK-293 T cells were cultured in RPMI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured in a 5% CO_2 humidified atmosphere at 37 °C.

2.3. Oligonucleotides transfection

Oligonucleotides targeting LINC00339 (si-LINC00339) and miR-145 (miR-145 inhibitor) and their negative controls (NC) were synthesized by GenePharma Bio Company (Shanghai, China). Oligonucleotide sequences (20 nM) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection of 48 h, cells were used for further assays. The sequences were shown as following: si-LINC00339-1#, sense, 5′-GGG AAGAUGUCUCAGCAGACG-3′; si-LINC00339-2#, sense, 5′-GGAUGUC AGGAAGCAGUCACG-3′; si-LINC00339-3#, sense, 5′-GGAUGAAGCUC GUAGCAGACG-3′.

2.4. Quantitative real-time PCR

Total RNA was extracted from tissues or cells using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. PrimeScript RT Reagent Kit (Bio-Rad, Hercules, CA, USA) was used to synthesize complementary DNA (cDNA). The qRT-PCR was performed using SYBR Premix Ex Taq (7500Fast; ABI, Foster City, CA, USA). The primer sequences were shown as follows: LINC00339, sense, 5′-CGTGGTTCAGACCAGAC ACA-3′, antisense, 5′-TGGGCTGGTTCTAGTTGGTT-3′; miR-145, sense, 5′-ACGTGGGGTTTCAGTTT-3′, anti-sense, 5′-TTTTCATATGGTGT GGG-3′; GAPDH, sense, 5′-GACTCATGACCACAGTCCATGC-3′, anti-sense, 5′-AGAGGCAGGGATGATGTTCTG-3′. Each expression levels were calculated with the $2^{-\triangle\triangle Ct}$ method and GAPDH acted as the endogenous control to normalize the data. The PCR was performed in triplicate.

2.5. Proliferation assay

The proliferation ability of NSCLC cells were tested using colony forming efficiency assay and CCK-8 assay. Briefly, for colony forming efficiency assay, NSCLC cells (500 cells/well) were seeded in 6-well plates. After 2 weeks, the colonies were fixed with 4% paraformaldehyde and stained with crystal violet for 10 min. Colonies were examined and counted under microscope. For CCK-8, 10 μl solution was added into cells, which were incubated at 37 °C for 2 h. The absorbance at 450 nm was measured using a spectrophotometer every day. The assays were repeatedly performed in triplicate.

2.6. Transwell invasion assay

For invasion assays, matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) were performed according to the manufacturer's instructions. Briefly, NSCLC cells were placed at the upper surface of chamber. 24 h later, the invasive cells through the member were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The invaded cells number was counted in 5 randomly selected microscopic views and photographed.

2.7. Western blotting analysis

Total protein was extracted from NSCLC cells as previously described with RIPA solution containing protease inhibitor. The lysates of total protein were centrifuged to remove the cellular debris and then separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, USA). The membranes were incubated with antibody, including anti-FOXM1 (1:1000 dilution) and GAPDH (1:500 dilution). Parallel blotting of GAPDH served as the internal control. The blots were visualized via chemiluminescence using an ECL kit (Amersham Biosciences, Piscataway, NJ, USA).

2.8. Luciferase reporter assays

Sequences of plasmid containing the wild-type or mutant 3′-UTR of FOXM1 and LINC00339 were co-transfected with miR-145 mimics or control. The transfection was conducted using Lipofectamine 2000 reagent according to the manufacturer's protocol. Cells were lysed and assayed for luciferase activity after 48 h transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase activity was normalized against firefly luciferase activities.

2.9. RNA immunoprecipitation (RIP)

The RIP assay was performed for the relationship between LINC00339 and miR-145 using an EZMagna RIP RNA-binding protein immunoprecipitation kit (Millipore, USA) according to the manufacturer's instructions. RIP buffer, with magnetic beads coated with anti-human argonaute 2 (Ago2) antibodies (Millipore, USA) was incubated with cell lysis solution. IgG (Millipore, USA) was used as a negative control (input group). RNA was purified from RNA protein and detected by RT-PCR using PrimeScript 1 st Strand cDNA Synthesis Kit (Takara, China).

2.10. cytoplasm/nucleus fraction isolation assay

The cytoplasm/nucleus fraction isolation assay was performed using a PARIS Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

2.11. Xenograft animal experiments

A total of 10 male nude mice were used in this study. NSCLC cells were stably transfected with lentiviral vector for shRNA-LINC00339 (sh-LINC00339) or shRNA-control (sh-NC). Then, the cells were subcutaneously injected into nude mice at concentration of 1×10^7 cell per 1 ml. After that, the tumor volume was measured once every three days. Once the experiment completed, the mice were sacrificed and the tumors were removed to weight. All animal assays were performed in accordance with protocols approved by the Animal Experimentation Ethics Committee of the First Affiliated Hospital of Dalian Medical University.

2.12. Statistical analysis

All data were presented as mean ± standard deviation (SD). The statistical analysis was performed using SPSS (IBM, Armonk, NY, USA) and diagrams were mapped using GraphPad Prism software. Student's t-test, one-way ANOVA and χ^2 tests were performed to calculate the statistic difference. The overall survival analysis was performed using Kaplan-Meier analysis. P value less than 0.05 indicated statistical significance.