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Long noncoding RNA TP73-AS1 promotes non-small cell lung cancer progression by competitively sponging miR-449a/EZH2

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ABSTRACT

Long noncoding RNAs (lncRNAs) are a type of noncoding RNA transcript that are characterized by lack of protein-coding capacity. The vital role of lncRNAs in non-small cell lung cancer (NSCLC) is attracting increasingly more attention. In the present study, we investigate the role of lncRNA antisense RNA of the TP73 gene (TP73-AS1) in NSCLC carcinogenesis. The results demonstrate that TP73-AS1 is markedly upregulated in NSCLC tissues, and functional experiments revealed that TP73-AS1 is significantly increased in NSCLC tissue and cell lines, indicating a possible oncogenic role. In loss-of-function assays, the knockdown of TP73-AS1 inhibited NSCLC cell proliferation, tumor growth and cycle progression in vivo and in vitro. Bioinformatic tools predicted that miR-449a both targeted the 3′-UTR of TP73-AS1 and EZH2, which was confirmed using luciferase reporter assay and AGO2-dependent RNA immunoprecipitate (RIP). TP73-AS1 and miR-449a were in the same RNA-induced silencing complex (RISC). In summary, the results indicate an explicit oncogenic role of TP73-AS1 in the NSCLC tumorigenesis, suggesting a TP73-AS1-miR-449a-EZH2 axis and providing new insight for NSCLC tumorigenesis.

1. Introduction

Non-small cell lung cancer (NSCLC) is one of the most common malignant lung cancers and has high mortality and poor prognosis [1,2]. The 5-year overall survival rate of NSCLC patients is still lower than 15%; however, the recurrence rate is inversely high [3]. Although the clinical therapeutic methods for NSCLC patients are gradually being developed, treatment effects do not yet classify as prospective. A potential molecular mechanism of NSCLC has gained increasingly more attention and is worth devoting further exploration.

Long noncoding RNAs (lncRNAs), acting as a type of noncoding RNA transcript, are characterized by high transcription abundance and lack of protein-coding capacity [4,5]. The aberrant expressions of lncRNAs in cancers have an increasingly important function in human diseases, including cancer and metabolic disease [6]. LncRNAs modulate the tumor cell progression, such as epigenetic regulation, genomic imprinting, and alternative splicing. For instance, lncRNA colon cancer-associated transcript 2 (CCAT2) is upregulated in epithelial ovarian cancer tissues and cell lines, and CCAT2 knockdown inhibits proliferation, promotes apoptosis and induces cell cycle arrest at the G0/G1 phase [7]. However, the underlying mechanism is still unclear. Thus, further studies of lncRNAs in cancer may be of great value in explaining the development of NSCLC.

The emerging role of lncRNAs in cancer, especially NSCLC, is attracting increasingly more attention as a potential cancer therapy. LncRNA antisense RNA to TP73 gene (TP73-AS1) is an identified oncogenic RNA in human breast cancer [8]. In the present study, we demonstrate that TP73-AS1 is markedly upregulated in NSCLC tissues indicating its clinicopathologic importance in NSCLC patients. The pathway of TP73-AS1/miR-449a-/EZH2 promotes NSCLC tumorigenesis by epigenetic regulation.

2. Materials and methods

2.1. Clinical NSCLC tissues specimens

From 2016 to 2017, a total of 45 lung cancer patients, who qualified as NSCLC by pathological diagnosis, were recruited at the First Affiliated Hospital of Dalian Medical University. The lung tumor tissue was obtained from patients by surgical resection and subsequently rapidly frozen at -80 ℃. Written consent was collected from all patients, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University.

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2.2. Cell culture

The 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 provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL, NY, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Small interfering RNAs transfection

Small interfering RNAs (siRNAs) respectively targeting TP73-AS1 and miR-449a and scrambled oligonucleotides (negative control) were designed and synthesized by Gene Pharma Company (Shanghai, China). NSCLC cells (3×10^5 per well) were seeded on 6 well plates and then transfected with siRNA or negative control (100 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The interfering efficiency was determined by qRT-PCR.

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from NSCLC tissue and cells using Trizol reagent (Invitrogen) according to its instructions. To synthesize complementary first strand DNA (cDNA), Reverse EasyScript One Step gDNA Removal and cDNA Synthesis SuperMix (TAKALA, Dalian, China) were performed. Then, the qRT-PCR was conducted using FastStart Universal SYBR Green Master (ROX) (Roche) reagent on a StepOne Plus system (Applied Biosystems, USA). The primer sequences were presented as following: TP73-AS1, forward, 5′-TCATTTCTGCCCCTACT CCT-3′, reverse, 5′-CAGTCACATGTCTGCGCTAAT-3′; miR-449a, forward, 5′−CCACCTCTACGCATCATTCA-3′, reverse, 5′−CCAAGCTCGT CTGGTTCTC-3′; GAPDH, forward, 5′−CCCTTCATTGACCTCAACT ACA-3′, reverse 5′-ATGACAAGCTTCCCGTTCTC-3′. Each expression level was 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 ability assay

The proliferation ability of NSCLC cells was measured using the Cell Counting Kit-8 (CCK-8) assay and colony formation assay. Briefly, cell viability was measured with the Cell Counting Kit-8 (Dojindo Laboratory, Japan) every 24 h according to the manufacturer's instructions. For colony formation assay, NSCLC cells (500 cells/well) were seeded in 6-well plates. After 2 weeks, the colonies were fixed with 4% paraformaldehyde for 5 min and then stained with 1% crystal violet for 10 min. Colonies were counted under a microscope.

2.6. Flow cytometry

Flow cytometry was conducted for apoptosis and cycle analysis. Annexin V/FITC Apoptosis Detection Kits (BD Biosciences, San Jose, CA, USA) was performed for apoptosis according to the manufacturer's instructions. The transfected NSCLC cells were stained with Annexin V-FITC and PI in binding buffer and assessed by flow cytometry (BD Biosciences). For cell cycle distribution, NSCLC cells were analyzed using flow cytometry (BD Biosciences) and propidium iodide (PI) staining (Beyotime, Shanghai, China).

2.7. Nucleus/cytoplasm fractionation

The cellular localization of TP73-AS1 was determined. Briefly, the nuclear and cytosolic fractions were separated using NUCLEI EZ PREP NUCLEI ISOLATION KIT (Sigma, USA) according to the manufacturer's instructions. The supernatant was carefully removed, and the precipitate was washed with PBS twice and resuspended in Nuclei EZ storage buffer.

2.8. Western blotting

NSCLC cells were homogenized using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. The suspension was centrifuged and then used for western blotting analysis. The proteins were separated by 10% SDS-PAGE and blotted onto a nitrocellulose PVDF membrane (Millipore, Billerica, USA). The membranes were incubated with primary antibodies (4 °C overnight, anti-EZH2, dilution 1:1,000, β-actin, 1:1000, Sigma Aldrich). Blots were developed with enhanced chemiluminescence (Millipore).

2.9. Luciferase reporter assays

Luciferase reporter assays were conducted as described previously. Wild-type and mutant TP73-AS1 containing complementary binding sites targeting mmiR-449a were generated and fused to the luciferase reporter vector psi-CHECK-2 (Promega, Madison, WI, USA). A549 cells were transfected with luciferase plasmids and miR-449a or controls. Forty-eight hours after transfection, the luciferase activity assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luciferase activity was normalized to Renilla.

2.10. RNA immunoprecipitation (RIP)

The interaction between TP73-AS1 and miR-449a was measured by RIP assay using Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA) according to instructions. For anti-AGO2 RIP, NSCLC cells were transfected with miR-449a mimics or miR-NC. Forty-eight later, RIP was performed using an AGO2 antibody (Abcam). RIP-qPCR was performed and normalized to the input levels.

2.11. In vivo tumor xenograft assay

Athymic BALB/c mice (5-week-old) were purchased and maintained under specific pathogen-free conditions according to protocols. The study was approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Dalian Medical University. A549 cells (1×10^7/ml, 0.1 ml) were transfected with sh-TP73-AS1 or sh-NC vector and subcutaneously injected into mice. Every 3 days, the size of the tumor was recorded. All mice were sacrificed after 3 weeks of injection, and the weight of tumor was measured.

2.12. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was calculated using the SPSS 19.0 software and graphed using GraphPad Prism 6.0 software. For comparison within different groups, Student's *t*-test or one-way ANOVA were performed. The final data were generated from three independent experiments. The statistical significance (P value) is set as less than 0.05.

3. Results

3.1. Elevated TP73-AS1 expression in NSCLC tissue and cells predicts a poor prognosis

In the collected tissue specimen from surgical resection, RT-PCR analysis was performed to determine TP73-AS1 expression. The clinicopathologic data are presented in Table 1. Data reveal that TP73-AS1 expression levels are significantly up-regulated in the NSCLC tissue samples compared with the adjacent non-tumor tissue (Fig. 1A).