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Circular RNA hsa_circRNA_103809 promotes lung cancer progression via facilitating ZNF121-dependent MYC expression by sequestering miR-4302

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ABSTRACT

Lung cancer characterized with malignant cell growth is the leading cause of cancer-related deaths. In recent years, several circular RNAs (circRNAs) have been reported to participate in lung cancer progression. However, the correlation between circular RNA (circRNA) and lung cancer still remains to be further investigated. In this study, we screened out a highly expressed circular RNA hsa_circRNA_103809 in lung cancer tissues. We showed hsa_circRNA_103809 could serve as a prognostic biomarker for patients with lung cancer. Furthermore, we found that hsa_circRNA_103809 knockdown significantly suppressed lung cancer cell proliferation and invasion *in vitro* and delayed tumor growth *in vivo*. In mechanism, we identified hsa_circRNA_103809 as a sponge of miR-4302 targeting ZNF121. By sequestering miR-4302, hsa_circRNA_103809 promoted the expression of ZNF121 which consequently enhanced MYC protein level in lung cancer cells. Through rescue assays, we demonstrated hsa_circRNA_103809 contributed to lung cancer cell proliferation and invasion via facilitating ZNF121-dependent MYC expression by sponging miR-4302. In conclusion, our findings illustrated a novel hsa_circRNA_103809/miR-4302/ZNF121/MYC regulatory signaling pathway in lung cancer progression.

1. Introduction

Lung cancer characterized with malignant cell growth is the leading cause of cancer-related deaths worldwide [1]. Lung cancer could be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). As the environmental pollution, the incidence and mortality of lung cancer are quickly increasing [2]. The five-year survival rate of patients with lung cancer remains quite dismal [3]. Therefore, understanding the mechanism of carcinogenesis and identifying effective therapeutic targets for lung cancer are urgently required.

Circular RNAs (circRNAs), characterized with a covalently closed continuous loop without 5′ to 3′ polarity and polyadenylated tail, were identified forty years ago [4]. With the progression of high-throughput sequencing technology, researchers identify more and more circRNAs in various tissues. CircRNAs belong to endogenous non-coding RNAs and are expressed in a tissue-specific manner [5]. Further evidence indicated that circRNAs could serve as sponges to regulate gene expression via sequestering miRNAs [6]. Accumulating studies show that circRNAs exert essential functions, and might be potential biomarkers and targets in cancers [7]. For instance, Zhong et al. found that circC3P1 suppresses hepatocellular carcinoma growth and metastasis through miR-4641/PCK1 pathway [8]. Zhou et al. showed that downregulation of hsa_circ_0011946 suppresses the migration and invasion of the breast cancer cell line MCF-7 by targeting RFC3 [9]. Ma et al. identified that circMAN2B2 facilitates lung cancer cell proliferation and invasion via miR-1275/FOXK1 axis [10]. However, the functions of most circRNAs in tumor carcinogenesis are largely unknown.

In this study, we identified a novel circRNA hsa_circRNA_103809 with elevated expression in lung cancer tissues. hsa_circRNA_103809, with a length of 693 nucleotides, is located at chromosome 5p13.3 and consisted of 5 exons of ZFR gene. Kaplan-Meier survival analysis showed that hsa_circRNA_103809 could act as a prognostic marker in lung cancer. Moreover, hsa_circRNA_103809 knockdown significantly suppressed lung cancer

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cell proliferation and invasion. Mechanistically, we identified hsa_circRNA_103809 as a sponge of miR-4302 to promote ZNF121 expression, leading to elevated protein levels of MYC. In conclusion, our findings demonstrated the oncogenic role of hsa_circRNA_103809 and defined its functional mechanism, suggesting hsa_circRNA_103809 might be a potential therapeutic target in lung cancer.

2. Materials and methods

2.1. Patient samples

A total of 44 pairs of lung cancer tissues and adjacent normal tissues were collected from lung cancer patients who received treatment in Xuzhou Central Hospital. None of the patients have been received any chemotherapy or radiation before surgery. Written informed consent was obtained from all participants and the study was approved by the Board and Ethics Committee of Xuzhou Central Hospital. The tissues were immediately frozen in liquid nitrogen following surgery and stored at −80 °C until use.

2.2. Cell culture and transfection

The lung cancer cell lines (A549, H125, 95D, NCI-H292 and H1975) and the normal human bronchus epithelium cell line (HBE) were purchased from the American Type Culture Collection (ATCC, USA) and cultured in RPMI1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2. A549 and H1975 cells were transfected with siRNA targeting hsa_circRNA_103809, ZNF121 or MYC using siRNA-Mate (GenePharma, Shanghai, China). The overexpression vector of hsa_circRNA_103809 (pLCDH-hsa_circRNA_103809) was constructed by Geneseed Co., Ltd. (Guangzhou, China). miR-NC, miR-4302 mimic and miR-4302 inhibitor were all purchased from RiboBio Co., Ltd. (Guangzhou, China).

2.3. Cell Counting Kit-8 assay

Cell proliferation was detected by using the Cell Counting Kit-8 (7 sea biotech, Shanghai, China). Cells were grown in 96-well plates with 1×10^4 cells per well and incubated at 37 °C in 5% CO_2 until cell confluence reached 70%. After 48 h of plasmid transfection, cells were incubated for an additional 24, 48 or 72 h. A volume of 10 μL of CCK8 solution was added into each well. The absorbance at 450 nm was measured with the SUNRISE Microplate Reader (Tecan, Switzerland).

2.4. Colony formation assay

A549 and H1975 cells (1×10^3 cells per well) transfected with indicated siRNA were plated in 6-well culture plates and incubated at 37 °C for 14 days. Colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min, and the number of clones was manually counted.

2.5. Transwell invasion assay

Transwell invasion assay was evaluated using Matrigel invasion chambers (BD Biosciences, Franklin Lakes, New Jersey, USA) based on the manufacturer's specifications. Transfected cells (5×10^4 cells/well) suspended in serum-free RPMI-1640 medium was seeded into the upper chamber, while the complete RPMI-1640 medium with 5% FBS was placed into the lower chamber. After 24 h of incubation, cells remaining on the upper membrane were removed carefully, and adherent to underside of the membrane were fixed and stained with 0.5% crystal violet (Sigma, Santa Clara, CA, USA), followed by photographed and counted under a microscopy.

2.6. Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA from the tissue samples and cells were collected using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the ABI PRISM 7000 Fluorescent Quantitative PCR System according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 was measured as an internal control. The Ct-value for each sample was calculated with the ΔΔCt-method.

2.7. Xenograft assay

All experimental protocols were approved by the Animal Ethics Committee of Xuzhou Central Hospital. A total of 12 BALB/c nu/nu male mice (4 weeks old) were fed a regular standard diet and housed under controlled conditions with comfortable temperature and humidity. For the xenograft model, A549 cells were subcutaneously injected into the flanks of nude mice (4×10^6 cells per animal, 6 mice per group). Each mouse was intratumorally injected with siRNA or si-NC (10 nmol each time) once every 3 days. Tumor formation was monitored every 7 days by measuring tumor size. After 5 weeks, all mice were sacrificed, and the tumors were excised and weighed.

2.8. Luciferase reporter assay

Hsa_circRNA_103809-WT, hsa_circRNA_103809-mutant, ZNF121-WT and ZNF121-mutant were constructed into pGL3-Basic luciferase vector (Promega, Madison, WI, USA) to generate reporter plasmids. Cells transfected with indicated reporter plasmid were seeded in a 96-well plate and co-transfected with 50 nM miR-NC, or miR-4302 mimic using Lipofectamine 2000 (Life Technologies). Then dual-luciferase Reporter assay system (Promega) was used to detect luciferase activity in the cells lysates 48 h post-transfection.

2.9. Statistical analysis

All data are shown as mean ± standard deviation (SD). Student's t-test and one-way ANOVA analysis were used to estimate significant difference of different groups. Survival analysis was carried out by the Kaplan-Meier analysis, and statistical analysis was performed through log-rank test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. hsa_circRNA_103809 was highly expressed in lung cancer tissues

To investigate the correlation between circRNA and lung cancer progression, we analyzed an online-available dataset (GSE101586). According to this dataset, we found many differentially expressed circRNAs between lung cancer tissues and adjacent normal tissues (Fig. 1A). Among them, hsa_circRNA_103809 was the most upregulated circRNA in all 5 lung cancer tissues (Fig. 1A and B). To further validate it, we measured the expression of hsa_circRNA_103809 in 44 pairs of lung cancer tissues and adjacent normal tissues by qRT-PCR. The results indicated that hsa_circRNA_103809 expression was significantly increased in lung

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