Circular RNA sirtuin-1 restrains the malignant phenotype of non-small cell lung cancer cells via the microRNA-510-5p/SMAD family member 7 axis

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Circular RNA (circRNA) sirtuin-1 (SIRT1) is differentially expressed in non-small cell lung cancer (NSCLC), but its specific mechanism is still uncertain. The study was to figure out the latent molecular mechanism of circSIRT1 in NSCLC. The results clarified that circSIRT1 and SMAD family member 7 (SMAD7) were downregulated, but microRNA (miR)-510-5p was upregulated in NSCLC. Circ-SIRT1 expression was linked with tumor–node–metastasis staging and tumor size in NSCLC patients. Elevating circSIRT1 or suppressing miR-510-5p refrained NSCLC cell activities and glycolysis and inactivated the wnt/β-catenin pathway, while knockdown of circSIRT1 promoted the malignant behavior of NSCLC cells. Besides, inhibition of malignant behavior in NSCLC cells by elevating circSIRT1 was reversed by knockdown of SMAD7. circSIRT1 bound to miR-510-5p to target SMAD7. In short, circSIRT1 represses NSCLC cell malignant development via miR-510-5p to target SMAD7, making it a latent target for NSCLC treatment.

Keyword: Circular RNA sirtuin-1, non-small cell lung cancer, SMAD family member 7, MicroRNA-510-5p, proliferation

INTRODUCTION

Lung cancer (LC) takes up 17% of new cancer cases and 23% of cancer deaths (de Sousa et al., 2018). Owing to smoking and environmental pollution, the number of new cases of LC and relevant deaths in China is elevated (Cao et al., 2019). Non-small cell lung cancer (NSCLC) is the major kind of LC, taking up about 85% (Subramaniam et al., 2013). The cure for metastatic liver cancer has long been a challenge for clinicians and researchers, and the molecular mechanisms are not well understood. Circular RNAs (circRNAs) are a new class of ubiquitous endogenous RNA. Unlike linear RNA, circRNA forms a continuous ring with no 5’ or 3’ covalent closure (Meng et al., 2017; Yang et al., 2020). Abnormal expression of circRNAs is associated with the occurrence and development of tumors. For instance, circPTK2 is crucial in the growth and metastasis of colon cancer (CC), making it supposed to be a latent target for CC metastasis treatment, and a biomarker for early diagnosis of metastasis (Su et al., 2019). A study by Su et al. clarifies that circRNA Cdc16 promotes LC development (Qin et al., 2016). HSA_circ_0001649 expression is downregulated in LC tissues and is associated with tumor size and tumor embolism in hepatocellular carcinoma. While circ-ABCB10 motivates NSCLC cell proliferation, it refrains apoptosis via depressing KISS1 (Kong et al., 2019). Circ-sirtuin-1 (SIRT1) controls NF-κB activation through sequence-specific interactions and enhances SIRT1 expression via combining with microRNA (miR)-132/212 in vascular smooth muscle cells (Li et al., 2021). Meanwhile, circSIRT1 is discovered to be a tumor suppressor gene in gastric cancer (GC) (Sun et al., 2020). However, the function and mechanism of circSIRT1 in NSCLC have not been figured out yet.

The purpose of this study was to explore the potential molecular mechanism of circSIRT1 in NSCLC. It was assumed that circSIRT1 was a tumor suppressor gene in NSCLC. The potential downstream factor of circSIRT1 was identified by the dual luciferase reporting assay and bioinformatics website. In addition, in this work, it was confirmed that circSIRT1 blocks the activation of the wnt/β-catenin pathway in NSCLC cell lines.

MATERIALS AND METHODS

Clinical tissues obtaining

54 NSCLC patients were recruited and have signed the informed consent. No preoperative chemotherapy or radiotherapy was performed on the recruited patients. The study was approved by the Ethics Committee of Chinese Academy of Medical Sciences and Peking Union Medical College (Approval Number C201612M11). All clinicopathological factors of NSCLC patients were shown in Table 1. NSCLC tissues were confirmed by two histopathological experts. Cancer and adjacent nor-
mal tissues (>5 cm from the tumor) were harvested from 54 cases of NSCLC patients. Afterward, the tissues were immediately frozen in liquid nitrogen and stored in a refrigerator at –80°C.

Cell culture
NSCLC cell lines (A549, H1975, H1650, and HCC827) and non-cancerous bronchial epithelial cell lines (BEAS-2B) (American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific, Waltham, MA, USA) consisting of 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific).

Actinomycin D and RNase R treatments
To verify the stability of circSIRT1 in A549 cells, actinomycin D and RNase R assays were conducted (Chen et al., 2020). For actinomycin D treatment, A549 cells were cultured in a complete medium supplemented with 2 μg/mL actinomycin D (Sigma, St Louis, MO, USA) or dimethyl sulfoxide (Sigma). For RNA detachment, total RNA from A549 cells was incubated with 3 U/μg RNase R (Geneseed, Guangzhou, China) or diethyl pyrocarbonate-treated water (Sigma). CircSIRT1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Cell transfection
siRNA targeting circSIRT1 and SMAD family member 7 (SMAD7) (si-circSIRT1: AGTTTGAAGAAGATACCTTCT; si-SMAD7: CTCCAGATACCGGATG-GATTTC), or pcDNA3.1 (pcDNA3.1-circSIRT1), si-negative control (NC) and pcDNA3.1 NC were purchased from GenePharma (Shanghai, China), and miR-510-5p mimic (UCCUGGGGGCCUGUGUCUUAGGC)/inhibitor (GCCUAAGACAGCGGCCAGGAG) and mimic/inhibitor NC were purchased from Sangon (Shanghai). At 80% confluence, cells were transfected in a 6-well plate using lipofectamine 2000 (Invitrogen). Cells were then collected for subsequent experiments.

Colonies formation assay
Transfected NSCLC cells (5×10^2/well) were seeded in a 6-well plate (Thermo Fisher) containing FBS. After incubation at 37°C with 5% CO_2 for 10 days, the colonies were stained with 0.1% crystal violet solution (Sigma). Colonies (over 50 cells) were then counted under a microscope (Nikon Eclipse E600, Nikon Instruments, Melville, NY, USA) (Li et al., 2020).

5-ethyl-2'-deoxyuridine (EdU) analysis
A549 and H1650 cells (1×10^4) were seeded in a 96-well plate. Each well was added with 10 μM EdU solution (Genecopoeia, USA). After incubation, the cells were fixed with 4% paraformaldehyde, incubated with glycine, and then washed with phosphate-buffered saline consisting of 0.5% TritonX-100. The cells were incubated with Andy Fluor™ 555 azide (A004, Genecopoeia, USA) or 4',6-diamidino-2-phenylindole. EdU-positive cells were observed under a fluorescence microscope (XSP-BM13C, Shanghai CSOIF. Co., China). Cell proliferation rate = the number of proliferating cells/total number of cells ×100% (Zhu et al., 2021).

Transwell detection of invasion and migration
For invasion detection, Matrigel (BD Biosciences, San Jose, CA, USA) was coated in the Transwell chamber (BD Biosciences). The lower chamber was added with 600 μL RPMI1640 medium consisting of 10% FBS (Thermo Fisher Scientific), and the upper chamber was supplemented with 200 μL serum-free medium consisting of 1×10^5 cells. Cells not passing through the membrane were removed, and the remaining cells were fixed with methanol, stained with crystal violet, and observed under a microscope. Five fields of view were randomly selected to calculate the average number of cells. A Transwell chamber not coated with matrigel was used in the migration assay, and the remaining steps were the same as the invasion (Tang et al., 2021).

Glycolysis test
In line with the manufacturer's instructions, a glucose measurement kit and a lactic acid measurement kit

| Table 1. CircSIRT1 is implicated in TNM staging and tumor size of NSCLC patients |
|-----------------|-----------------|-----------------|
| Features        | Group           | Cases           | Circ SIRT1 expression | P                  |
| Age (years)     | Cases           | Elevation (n=27) | Reduction (n=27)      |
| <60             | 18              | 7               | 11                  | 0.2482             |
| ≥60             | 26              | 20              |                     |
| Gender          | Male            | 38              | 17                  | 0.2332             |
| Female          | 16              | 10              | 6                   |
| Tumor size (cm) | <3              | 29              | 21                  | 0.0004             |
| ≥3              | 25              | 19              |                     |
| Lymph node metastasis | No | 23              | 13                  | 0.4090             |
|                 | Yes             | 31              | 14                  | 0.0021             |
| Tumor, node, and metastasis stage | I + II | 16              | 5                   |
|                 | III-IV          | 33              | 11                  |                     |
| Differentiation degree | Good | 15              | 8                   | 0.7613             |
|                 | Medium/bad      | 39              | 19                  |                     |

Note: The relationship between circSIRT1 and clincopathological features of NSCLC was examined using Spearman correlation analysis.
**Table 2. RT-qPCR primer sequence**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’– 3’)</th>
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| GAPDH | Forward: 5’- ATCTTCAGGGAGGAGATCCC-3’  
Reverse: 5’- TGAGCTCTCCACGATACAA-3’ |
| U6    | Forward: 5’- CTCGCTGCAGCAATCC-3’  
Reverse: 5’- AAGCTTCTACACGCTTCTGGA-3’ |
| CircSIRT1 | Forward: 5’- AGGATTTGTTTTTCTTGAAGAC-3’  
Reverse: 5’- GAGGGTTTCTAGAGTGCTG-3’ |
| miR-510-5p | Forward: 5’- GCCATGATTTGTTTCTTGAAGAC-3’  
Reverse: 5’- GCATCATGGCCAGCATTACA-3’ |
| SMAD7  | Forward: 5’- GGTTGAGAAAATCCCTAGGGG-3’  
Reverse: 5’- GGTTGAGAAAATCCCTAGGGG-3’ |

Flow cytometry detection of apoptosis

Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) was utilized for analysis of cell apoptosis (Huang et al., 2020). NSCLC cells were resuspended in a 1× binding buffer. After staining with Annexin V-FITC and propidium iodide, cells were evaluated to analyze the apoptosis rate on a flow cytometer (BD Biosciences).

RT-qPCR

RNA was extracted from tissues and cells using the RNeasy Mini Kit (Qiagen, Duesseldorf, Germany). RNase-free treatment was used to avoid RNA contamination. RNA was quantified using a NanoDrop ND-1000 device (Thermo Fisher Scientific). mRNA was reverse-transcribed into complementary DNA (cDNA) using M-MLV Reverse Transcriptase Kit (Invitrogen). After mixing cDNA with SYBR GreenER qPCR SuperMix Universal (Invitrogen), RT-qPCR was performed in the PCR system (Applied Biosystems PCR Thermal Cyclers, thermofisher). GAPDH or U6 was used as a control gene for mRNA and miRNA, respectively. The 2*ΔΔCT method was applied for data analysis. Primer sequences were manifested in Table 2.

Western blot

Total proteins were extracted from tissues and cells using Radio-Immunoprecipitation assay lysis buffers containing protease inhibitors. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and 30 μg sample was electro-blotted onto a polyvinylidene fluoride membrane (Millipore), blocked with 5% skimmed milk, and incubated with primary antibodies and the secondary antibody. Primary antibodies were SMAD7 (25840-1-AP, Proteintech, 1:1000), HK-2 (22029-1-AP, Proteintech,1:1000), GLS1 (ab156876, Abcam, 1:1000), β-catenin (ab32072, Abcam, 1:1000), GAPDH (ab8245, Abcam, 1:1000), e-myc (M4439, Sigma, 1:1000), with goat anti-rabbit Immunoglobulin G (7076, Cell Signaling Technology) as the secondary antibody. Protein signals were detected by the BeyoECL Star ECL kit (Beyotime) and analyzed by ImageJ software (National Institutes of Health, Maryland, USA).

Luciferase activity assay

circSIRT1 and SMAD7 wild-type (WT) and mutant (MUT) luciferase reporter vectors (circSIRT1/SMAD7 WT/MUT) consisting of putative binding sites of miR-510-5p were purchased from Promega (Madison, Wisconsin, USA). A549 and H1650 cells were seeded in a 48-well plate (4.5×10^4/well) and cultured to 70% confluence. Co-transfection of the above vectors was done with miR-510-5p mimic or mimic NC in A549 and H1650 cells using lipofectamine 2000. Luciferase activities in NSCLC cells were measured in the dual luciferase reporter gene detection system (Promega).

**RESULTS**

**CircSIRT1 is reduced in NSCLC and is associated with NSCLC clinicopathological features**

In order to clarify the potential characteristics of circSIRT1 in NSCLC, circSIRT1 expression was examined in NSCLC. CircSIRT1 in NSCLC tissues and the four NSCLC cell lines were reduced compared with normal tissues and the bronchial epithelial cell line BEAS-2B (Fig. 1A, B). Subsequently, the circular structure of circSIRT1 in A549 cells was examined by actinomycin D and RNase R assay. As shown in Fig. 1C and 1D, circSIRT1 had a longer half-life period and was not degraded by RNase R compared to linear GAPDH mRNA.

**Statistical analysis**

Statistical analysis was performed by GraphPad Prism software (La Jolla, CA, USA). Data were shown as mean ± standard deviation (S.D.). Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was utilized to analyze two or more groups. P<0.05 emphasized obvious statistical meaning. The relationship between circSIRT1 and the clinical features of patients was determined using Spearman correlation tests. All experiments were carried out with at least three biological replicates (N=3).
To determine whether circSIRT1 was associated with clinicopathological features of NSCLC patients, patients were divided into circSIRT1 low expression group and circSIRT1 high expression group according to the median expression of circSIRT1, and Spearman correlation analysis was performed. CircSIRT1 was associated with tumor–node–metastasis (TNM) staging and tumor size of NSCLC patients (Table 1). These results indicated that circSIRT1 expression was reduced in NSCLC and was supposed to be related to NSCLC development. CircSIRT1 performs as a key gene to suppress the malignant phenotype of NSCLC

As circSIRT1 expression levels were the lowest in A549 and H1650 cells, these two cell lines were selected for subsequent experiments. Subsequently, whether circSIRT1 was associated with the malignant phenotype of NSCLC was explored. circSIRT1 was upregulated and inhibited in A549 and H1650 cells, respectively, by transfection of circSIRT1-targeted overexpression plasmid and siRNA (Fig. 2A). Functional tests clarified that up-regulating circSIRT1 was available to repress A549 and H1650 cell proliferation, invasion, migration and DNA replication, but elevated the apoptosis rate (Fig. 2B–E). Cancer cells usually require a lot of energy to survive. Next, it was explored whether circSIRT1 controls the glycolysis capacity of NSCLC cells. Up-regulation of circSIRT1 suppressed glucose consumption and lactate production in A549 and H1650 cells (Fig. 2F, G). Meanwhile, upregulating circSIRT1 also repressed glycolytic rate-limiting enzyme HK-2 and glutamine hydrolase GLS1 in A549 and H1650 cells (Fig. 2H). However, after the elimination of circSIRT1, the exact opposite result was shown (Fig. 2B–H). Overall, circSIRT1 performed as a tumor suppressor gene in NSCLC cells.
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CircSIRT1 binds to miR-510-5p to target SMAD7

CircSIRT1 can perform as a sponge of miRNA to modulate protein expression and impact disease development. Subsequently, downstream miRNAs absorbed by circSIRT1 were analyzed. It was found that circSIRT1 had a potential binding site with miR-510-5p through bioinformatics website query (Fig. 3A). Subsequently, a dual luciferase reporting assay was carried out. The results showed that wild-type circSIRT1 lowered the luciferase activity in the miR-510-5p mimic group, while mutant circSIRT1 had no effect on the luciferase activity in the miR-510-5p mimic group (Fig. 3B). After elevation or knockdown of circSIRT1, miR-510-5p expression in A549 and H1650 cells was enhanced or repressed, respectively (Fig. 3C). This indicated that miR-510-5p might be a downstream target of circSIRT1.

Potential binding sites between SMAD7 and miR-510-5p were found on the bioinformatics website (Fig. 3D). WT SMAD7 could reduce the luciferase activity of the miR-510-5p mimic group (Fig. 3E). Former studies have noted that SMAD7 is under-expressed in various cancers, including LC. Consistent results were also gained in this study (Fig. 3F, G). Meanwhile, SMAD7 was promoted or inhibited in A549 and H1650 cells with downregulated or upregulated miR-510-5p, respectively (Fig. 3H). Therefore, SMAD7 was supposed to be a target gene of miR-510-5p.

Figure 3. MiR-510-5p accelerates the malignant behavior of NSCLC

(A, B) RT-qPCR detection of miR-510-5p expression in NSCLC tissue and adjacent normal tissue, NSCLC cell lines A549, H1975, H1650, HCC827 and non-cancerous bronchial epithelial cell line BEAS-2B; (C) RT-qPCR detection of miR-510-5p expression; (D) Colony formation assay detection of cell proliferation; (E) EdU assay detection of DNA replication; (F) Flow cytometry detection of apoptosis; (G) Transwell detection of cell invasion and migration; (H) Glycolytic ability of cells; (J) Western blot detection of HK-2 and GLS1 expression; C–J, in A549 and H1650 cells after transfection with miR-510-5p inhibitor. The data were manifested as mean ± S.D. (N=3); *P<0.05.
MiR-510-5p motivates the malignant behavior of NSCLC cells

Former studies have confirmed the promoting effect of miR-510-5p on renal cell carcinoma and thyroid cancer development (Liu et al., 2019; Zhan et al., 2020). miR-510-5p expression was evaluated in NSCLC. As manifested in Fig. 4A, B, higher miR-510-5p was presented in NSCLC patients and cell lines. Subsequently, miR-510-5p was knocked down in A549 and H1650 cells after transfecting with miR-510-5p inhibitor (Fig. 4C). Downregulating miR-510-5p repressed A549 and H1650 cell proliferation, DNA replication, migration and invasion, elevated apoptosis rate, and reduced glucose consumption, lactate production, HK-2 and GLS1 protein expression (Fig. 4D-J). Briefly, miR-510-5p motivated the malignant behavior of NSCLC cells.

CircSIRT1 represses the malignant behavior of NSCLC cells by controlling the miR-510-5p/SMAD7 axis

Next, the researchers explored whether circSIRT1 influences the malignant behavior of NSCLC through the miR-510-5p/SMAD7 axis. pcDNA 3.1-circSIRT1 and si-SMAD7 were co-transfected into A549 and H1650 cells. It came out that pcDNA 3.1-circSIRT1 elevated SMAD7 expression, and after co-transfection with si-SMAD7, SMAD7 expression was repressed (Fig. 5A). The suppressive effects of upregulating circSIRT1 on A549 and H1650 cell proliferation, DNA replication, migration, invasion, and glycolysis, as well as the promoting effect on apoptosis, were turned around after knocking down SMAD7 (Fig. 5B–H). In short, circSIRT1 repressed the malignant behavior of NSCLC cells by controlling the miR-510-5p/SMAD7 axis.

DISCUSSION

LC is a very familiar tumor in clinical practice, and it is also the malignant tumor with the highest mortality rate in the world. NSCLC is characterized by a high degree of malignancy and a very low 5-year survival rate. This is mainly due to a lack of understanding of the basic biology of NSCLC, which in turn leads to a...
lack of reliable biomarker tests and effective therapeutic drugs (Tang et al., 2016). The prognosis of patients with advanced or metastatic LC is quite unpleasing (Gupta et al., 2006). Hence, understanding the pathogenesis of LC is essential for the development of therapeutic targets. The research of circRNA in LC has attracted much attention recently. At present, the key characteristics and latent functions of circRNAs are not well understood (Qu et al., 2015; Hansen et al., 2013). Certain circRNAs are crucial in cancer. For instance, hsa_circRNA_102958 expression in GC tissues is up-regulated and is positively linked with TNM staging (Wei et al., 2020). Hsa_circRNA_102034 motivates LC progression via activating NR2F6 (Wang et al., 2019); In NSCLC, a former study has discovered that hsa_circRNA_012515 expression in NSCLC tissues is overexpressed and is closely implicated in the lymph node metastasis and TNM staging of NSCLC. In the meantime, NSCLC patients with elevated hsa_circRNA_012515 have a clearly shorter survival time (Fu et al., 2020). In the research, it was discovered that circSIRT1 overexpression was available to repress NSCLC proliferation, invasion, migration and glycolysis, but promotes apoptosis.

Subsequently, we further clarified the function of circSIRT1 from the molecular mechanism. CircRNA frequently serves as a ceRNA for miRNA. A former study has clarified that miR-510-5p induces renal cell carcinoma and thyroid cancer cell proliferation, invasion and migration (Wang et al., 2021; Zhang et al., 2020). In this study, it was discovered that miR-510-5p, as a down-
CONCLUSION

Overall, circSIRT1 blocks wnt/β-catenin signal activation and NSCLC malignant behaviors via miR-510-5p/SMAD7. These findings offer favorable data support for further understanding of the characteristics of circRNAs in LC and offer a probable therapeutic target for the latter treatment of NSCLC.

Declarations

Acknowledgments. Not applicable.

Declaration of Conflicting Interests. Authors declared no conflict of interest.

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