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ORIGINAL ARTICLE

Circular RNA THBS1 promotes proliferation and apoptosis of non-small cell lung cancer cells by sponging miR-129-5p and regulating SOX4 expression

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Summary

Purpose: *To investigate the influence of circular ribonucleic acid thrombospondin-1 (circTHBS1) on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells by sponging miR-129-5p and regulating the expression of SRY-box transcription factor 4 (SOX4).*

Methods: *Carcinoma and para-carcinoma specimens were collected from 40 NSCLC patients, and 25 pairs of specimens were obtained from patients with metastatic and non-metastatic NSCLC. After NSCLC cells were cultured, the proliferation was detected via cell counting kit-8 (CCK-8) and 5-Ethynyl-2'- deoxyuridine (EdU) assays, and the cell cycle and apoptosis rate were analyzed through flow cytometry. Finally, the action targets of circTHBS1 were determined using dual-luciferase reporter gene assay, and Western blotting assay was applied to measure the changes in protein levels.*

Results: *The expression of circTHBS1 was markedly higher in NSCLC patients than that in control group, and it was increased in patients with metastatic NSCLC compared with that in patients with non-metastatic NSCLC. Moreover, the proliferative ability of the cells was weakened notably after transfection with small interfering (Si)-CircTHBS1, but it was enhanced remarkably after transfection with CircTHBS1-overexpression vector (OE). There were complementary sites in circTHBS1 for the 3'-UTR of miR-129-5p, and the fluorescence intensity of wild-type circTHBS1 declined evidently after interacting with miR-129-5p. Besides, there was a putative binding site between miR-129-5p and SOX4, and SOX4 expression was decreased obviously after overexpressing miR-129-5p but increased following overexpression of circTHBS1.*

Conclusions: *CircTHBS1 promotes the proliferation and inhibits the apoptosis of NSCLC cells through targeting miR-129-5p and regulating SOX4 expression.*

Key words: *circRNA THBS1, miR-129-5p, SOX4, non-small cell lung cancer*

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of mortality of lung cancer, accounting for 85% of all the lung cancer cases [1], and the early diagnosis and treatment of the disease are essential to improving the patient survival rate. However, the potential mechanism of lung cancer progression has not been completely elaborated. According to the latest progress in ribonucleic acid (RNA) research, non-coding RNAs (ncRNAs) have been found to be involved in multiple biological processes [2]. Previous studies revealed that micro RNAs (miRs) and long ncRNAs (lncRNAs) play critical roles in the development of lung cancer, especially controlling the proliferation, apoptosis and invasion [3-5]. Therefore, in-depth analysis of ncRNAs will help further clarify the mechanism related to cancers at the epigenetic level.

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Circular RNAs (circRNAs) are a category of newly discovered RNAs that are extensively expressed in eukaryotes [6]. The rapid advancement in high-throughput sequencing and bioinformatics in recent years has greatly enriched the knowledge of circRNAs. Most circRNAs reported so far belong to ncRNAs, some of which are capable of encoding polypeptides or proteins [7]. Formed by unconventional splicing, circRNAs are relatively resistant to degradation of exonucleases [8]. CircRNAs have close associations with the progression of various diseases, especially the occurrence and development of tumors [9,10], but their specific functions in NSCLC have not been well understood, which requires further exploration. In this research, therefore, the mechanism of circRNA thrombospondin-1 (circTHBS1) in regulating NSCLC progression was investigated, and the findings provide new clues regarding the identification of biomarkers of NSCLC.

Methods

Tissue samples and cell lines of NSCLC

A total of 40 cases of pulmonary puncture specimens were collected from NSCLC patients hospitalized from January 2017 to December 2018. All the participants signed the informed consent, and this research was approved by the Ethical Review Committee of the PLA Navy Anqing Hospital.

Human NSCLC cell lines A549 and SPC-A1 were offered by Chinese Academy of Sciences (Shanghai, China) and cultured with Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Hyclone Laboratories Inc., South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 1% penicillin and 5% streptomycin in a cell incubator at 37°C under 5% CO_2 and 95% air.

Analyses of circRNA expression profiles and circRNA-miRNA-messenger RNA (mRNA) co-expression network

The gene expression profile GSE101586 of NSCLC was obtained from the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo). The differentially expressed circRNAs in GSE101586 were analyzed using on-line tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/).

Cytoscape software (Version 3.4.0) was employed to construct the circRNA-miR-mRNA co-expression network of circTHBS1 based on the prediction of target miRs and genes. The primer sequences are shown in Table 1.

Cell proliferation assay

The cell proliferation was determined by means of cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The cells were inoculated into 96-well plates at an initial density of 2×10^4 cells/mL. Then, CCK-8 solution (10 μL/well) was added at 12, 24, 48 or 72 h to measure the cell viability. After incubation at 37°C under 5% CO_2 for 2 h, the absorbance at 450 nm in each well was determined by flow cytometry (Beckmam Coulter, Brea, USA).

Detection of apoptosis via flow cytometry

Cells treated differently in each group were cultured separately, then harvested and washed with phosphate-buffered saline (PBS) (Beyotime, Shanghai, China) in accordance with the instructions of flow cytometry kit. After digestion, the cells were prepared into suspension at a concentration of 1×10^6/mL, followed by centrifugation and discarding of the supernatant. Later, the cells were added with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 10 μL of propidium iodide (PI), gently mixed and incubated for 20 min. Subsequently, the apoptosis rate was detected using flow cytometer & cell sorter system. Finally, the cells were analyzed via flow cytometry (FACScan, BD Biosciences, San Diego, CA, USA) equipped with CellQuest software (BD Biosciences, San Diego, CA, USA) for three times.

Plasmid construction and determination of dual luciferase activity

Wild-type (WT) and mutant-type (Mut) fragments of circRNA carrying the putative binding sites of miR-129-5p were inserted into pGL3 vectors (Promega, Madison, WI, USA), so as to construct the luciferase reporter gene recombinant plasmids named as pGL3-circRNA WT and pGL3-circRNA Mut. Subsequently, NSCLC cells were seeded into 96-well plates (1×10^4 cells/well) and cultured for dual-luciferase reporter gene assay.

After incubation at 37°C for 16 h, the cells were co-transfected with pGL3-circRNA WT and miR inhibitor. Then, A549 cells were seeded into 96-well plates (1×10^4 cells/well) and cultured at 37°C for 16 h, followed by co-transfection with pGL3-circRNA WT and miR mimic. Finally, the activity of firefly and Renilla luciferase was examined by virtue of a dual luciferase assay system (Promega, Madison, WI, USA), and the activity of Renilla luciferase was normalized to that of firefly luciferase.

Statistics

Statistical analyses were performed using SPSS 22.0 software (IBM, Armonk, NY, USA). Data were presented

Table 1. Primer sequences

Gene	*Forward Primer Sequence*	*Reverse Primer Sequence*
CircTHBS1	5'-ATGTGGCCTACCCAGCTCAAG-3'	5'-GCTGTTCCGATGGTGTCTTT-3'
miR-129-5p	5'-CTGGTCGTGGAATTCAGTTGA-3'	5'-CTGGCTCCTCACTTGGAGGC-3'
U6	5'-AAGTACTCTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

as mean ± standard deviation (SD). Comparison between multiple groups was done using one-way ANOVA test followed by *Post Hoc* Test (Least Significant Difference). P<0.05 suggested significant differences.

Results

Microarray

GSE101586 for NSCLC samples and normal samples was applied to identify related genes involved in NSCLC (Figure 1A). CircTHBS1 was confirmed as one of the leading 38 abnormally regulated circRNAs in GSE101586 (Figure 1B). The data from UCSC Genome Browser indicated that circTHBS1 originated from THBS1 gene.

CircTHBS1 was highly expressed in NSCLC patients and NSCLC cells

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to detect the expression of circTHBS1 in the carcinoma and para-carcinoma tissues of 40 NSCLC patients as well as 25 patients with metastatic and non-metastatic NSCLC. The results manifested that the expression of circTHBS1 was markedly higher in NSCLC patients than that in the control group (p<0.05, Figure 2A), and it was increased in patients with metastatic NSCLC compared with non-metastatic NSCLC (p<0.01, Figure 2B), suggesting that circTHBS1 may be a potential risk factor for NSCLC.

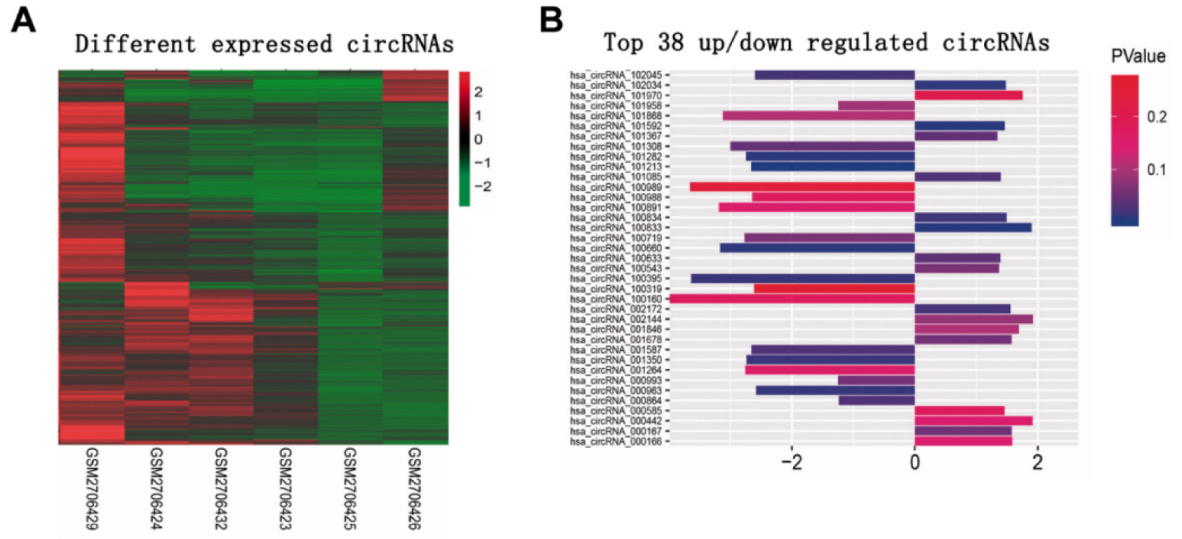

Figure 1. CircTHBS1 is confirmed as a NSCLC-related circRNA. **A:** Hierarchical cluster analysis of differentially expressed circRNAs in GSE101586 for NSCLC tissue and normal tissue. **B:** First 38 differentially expressed circRNAs in GSE101586.

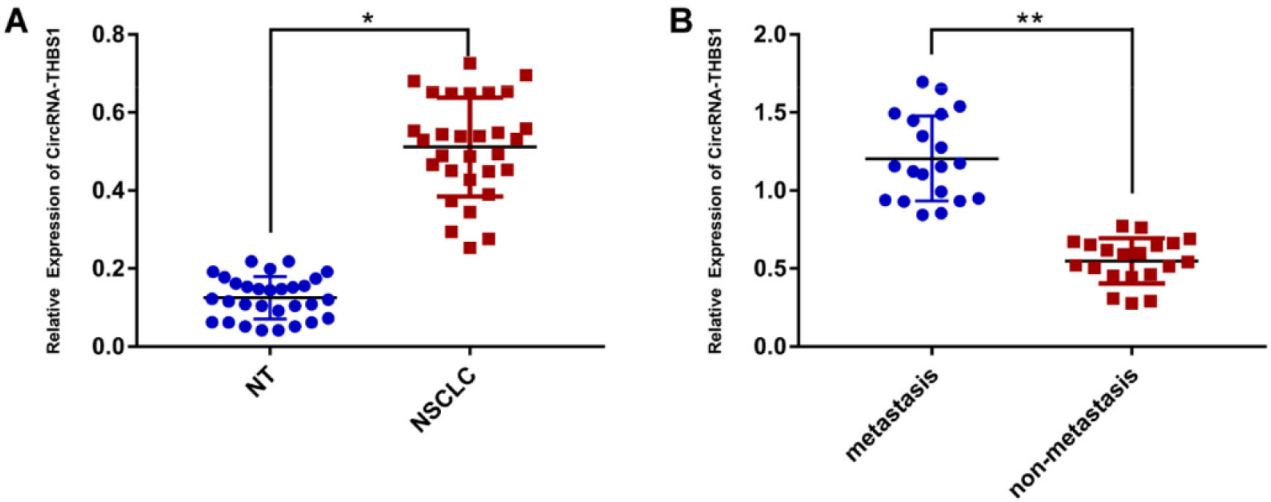

Figure 2. A: Expression of circTHBS1 in carcinoma and para-carcinoma tissues of 40 NSCLC patients detected via qRT-PCR. **B:** Expression of circTHBS1 in patients with metastatic and non-metastatic NSCLC detected via qRT-PCR. *p<0.05, **p<0.01.

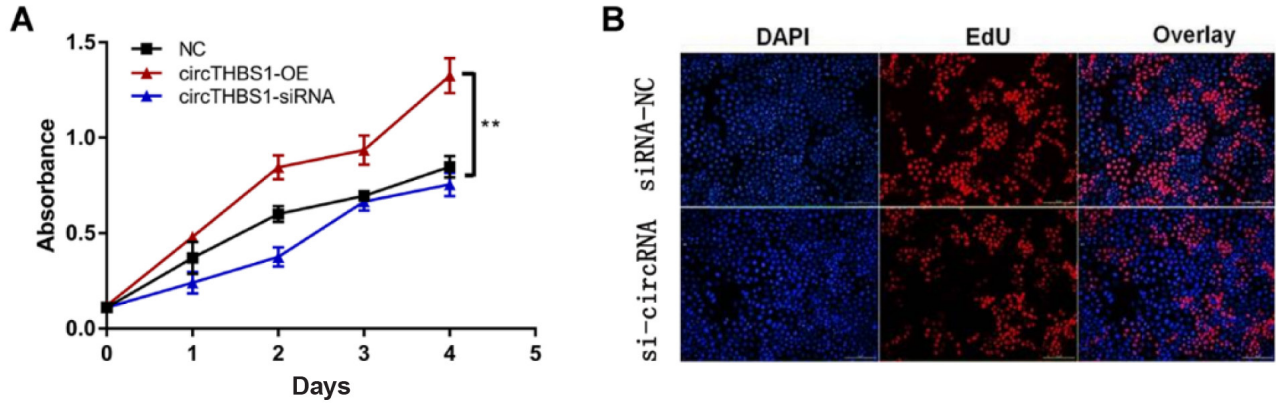

Figure 3. Proliferative ability of cells examined through CCK-8 assay **(A)** and EdU assay (magnification 200×) **(B)**. The proliferative ability of the cells transfected with Si-CircTHBS1 is evidently attenuated, but that of cells transfected with CircTHBS1-OE is distinctly enhanced (**p<0.01).

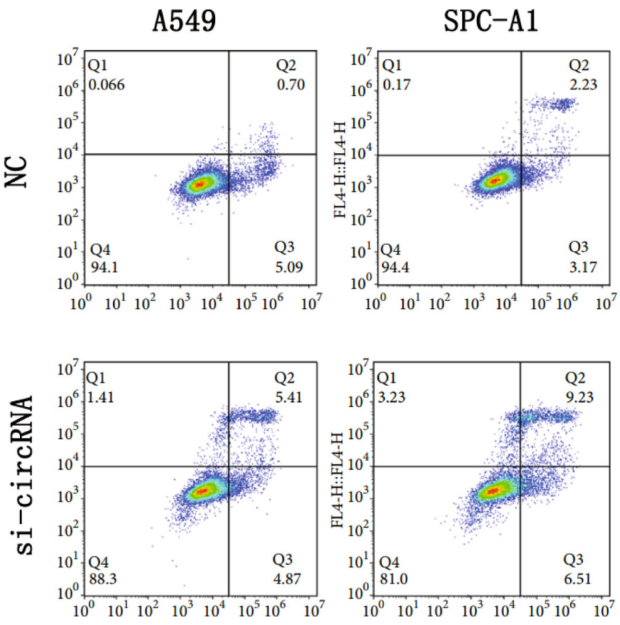

Figure 4. Apoptosis rate detected via flow cytometry. In A549 cells, the apoptosis rate is 10.21±0.32% and 6.3±1.34% in the Si-CircTHBS1 group and control group, respectively. In SPC-A1 cells, the apoptosis rate in the Si-CircTHBS1 group and control group is 15.24±1.32% and 5.41±1.41%, respectively (p<0.05).

CircTHBS1 promoted proliferation of NSCLC cells

The proliferative ability of NSCLC cells was examined after transfection with small interfering (Si)-CircTHBS1 and CircTHBS1-overexpression vector (OE). It was found that the proliferative ability of the cells was weakened notably after transfection with Si-CircTHBS1, but it was enhanced remarkably after transfection with CircTHBS1-OE (p<0.01, Figure 3A). Furthermore, similar results were obtained through 5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay (p<0.01, Figure 3B), illustrating that Si-CircTHBS1 can facilitates cell proliferation.

CircTHBS1 inhibited NSCLC cell apoptosis

After overexpressing and repressing the expression of circTHBS1, the apoptosis rate was determined using a flow cytometer. The apoptosis rates of A549 cells were 10.21±0.32% and 6.3±1.34% in the Si-CircTHBS1 group and control group, respectively, while the apoptosis rates of SPC-A1 cells in the two groups were 15.24±1.32% and 5.41±1.41%, respectively, with statistically significant differences (p<0.05) (Figure 4).

CircTHBS1 bound to miR-129-5p (luciferase reporter gene assay)

The targets of circTHBS1 were predicted by bioinformatics methods, and it was shown that there were complementary sites in circTHBS1 for the 3'-UTR of miR-129-5p (Figure 5A).

Following mutation of the binding sites of circTHBS1, only the fluorescence intensity of wild-type circTHBS1 declined evidently after interacting with miR-129-5p according to the luciferase reporter gene assay (p<0.05) (Figure 5B).

MiR-129-5p targeted SRY-box transcription factor 4 (SOX4) gene

In order to verify the target genes of miR-129-5p, it was discovered in the competing endogenous RNA (ceRNA) network of circTHBS1 in miR-129-5p that miR-129-5p could target multiple critical oncogenes. Based on the bioinformatics analysis, there were putative binding sites between miR-129-5p and SOX4, which were conserved among species (Figure 6A).

Moreover, Western blotting analysis revealed that SOX4 expression was weakened prominently after the overexpression of miR-129-5p, but it was enhanced by overexpressing circTHBS1 (Figure 6B).

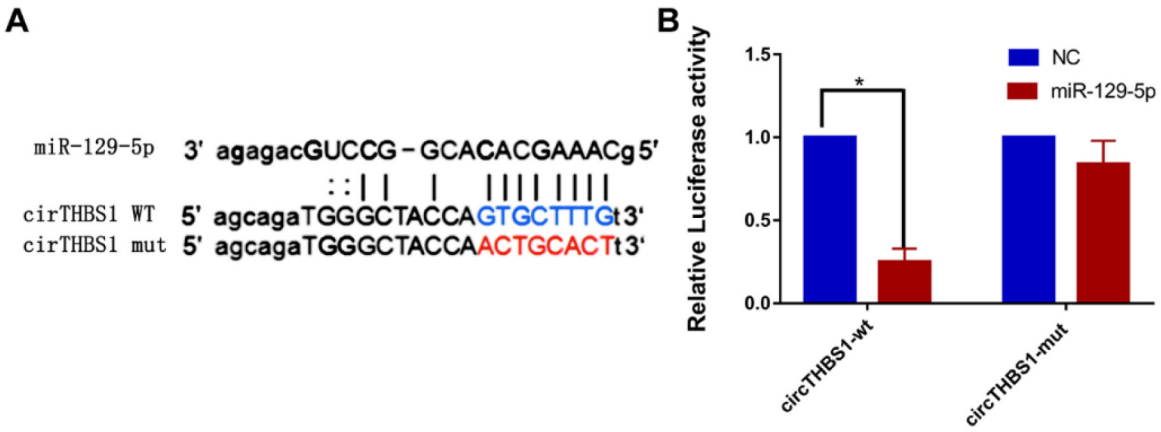

Figure 5. A: Targets of circTHBS1 predicted by bioinformatics methods. **B:** Luciferase reporter gene assay: the fluorescence intensity of wild-type circTHBS1 is reduced notably after interaction with miR-129-5p (*p<0.05).

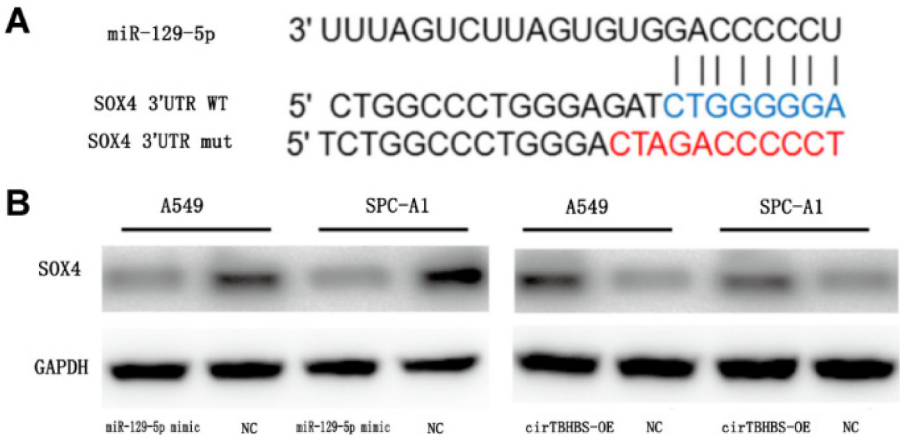

Figure 6. A: Bioinformatics analysis: There are putative binding sites between miR-129-5p and SOX4. **B:** Western blotting analysis: SOX4 expression is weakened prominently after the overexpression of miR-129-5p, but it is enhanced by overexpressing circTHBS1.

Discussion

Recent studies have demonstrated that many miRs, circRNAs and lncRNAs play vital roles in regulating the occurrence and development of tumors [11,12], but the mechanisms of circRNAs in the incidence and progression of cancers remain unclear.

Currently, only a minority of circRNAs have been confirmed to exert crucial effects in NSCLC [13]. As a novel category of ncRNAs, circRNAs usually manifest tissue-specific or time-specific expressions in mammals, but their biological functions have not been elucidated yet [13]. Besides, circRNAs are mainly characterized by high conservation and extreme stability [14], so they are considered as promising biomarkers of tumors in precision medicine due to such features. Large quantities of studies have illustrated that circRNAs are closely associated with the pathogenesis of a variety of human diseases, including Alzheimer's disease, type 2 diabetes mellitus and diverse cancers [15-17], implying that circRNAs have potential functions as molecular diagnostic biomarkers. For example, a latest research on 10 pairs of NSCLC and normal samples revealed that the expression of circRNA phenylalanyl-tRNA synthetase subunit alpha (circFARSA) is raised in the NSCLC tissues and plasma, demonstrating that circFARSA probably serves as a latent non-invasive biomarker of NSCLC [18]. In addition, it was confirmed in a study that circRNA fibroblast growth factor receptor 1 (circFGFR1) is a key circRNA that is highly expressed in NSCLC tissues. CircFGFR1 expression is positively correlated with the clinicopathologic characteristics of NSCLC with poor prognosis, such as tumor size, lymph node metastasis and poor differentiation [19]. It was also verified through a study that circFGFR1 facilitates the progression of NSCLC and enhances the drug resistance to anti-PD-1 therapy by up-regulating CXCR4 expression [20].

In this research, the circRNA microarray was primarily applied to detect the circRNA expression

profiles in NSCLC samples and paired samples, so as to better characterize the relationship between circRNA expression and NSCLC and provide preliminary theoretical bases for the search of early diagnostic biomarkers of NSCLC. It was testified that NSCLC tissues exhibited increasing expression of circTHBS1 in comparison with para-carcinoma tissues, implying that circTHBS1 plays a role in the pathogenesis of NSCLC. Next, the effect of circTHBS1 in tumor progression in the case of NSCLC *in vitro* was investigated after circTHBS1 was confirmed to be up-regulated in NSCLC tissues and cell lines. The results of this research manifest that circTHBS1 may be a promising diagnostic and prognostic biomarker of NSCLC patients. However, the potential diagnostic value of circTHBS1 in NSCLC needs verification by more clinical samples. Additionally, a single circRNA probably does not have very high accuracy of diagnosis. Therefore, it was argued that more microarray data should be analyzed to obtain a group of circRNAs from the biomarkers of NSCLC.

It has been reported that miR-129-5p can modulate tumor progression in various cancers. For instance, miR-129-5p is capable of promoting cell death *in vitro* and is correlated with poor prognosis of tumors in the case of bladder cancer. It has also been discovered in 17 cases of lung cancer that miR-129-5p induces G1 phase arrest by down-regulating CDK6 expression, thereby regulating the cycle of proliferating cells [21]. Furthermore, miR-129-5p can strengthen the *in vivo* and *in vitro* chemotherapy of 5-fluorouracil in tumors by repressing B-cell lymphoma-2, a critical anti-apoptotic protein [22].

Moreover, the latent regulatory mechanism of circTHBS1 in NSCLC cells was explored in this research. CircTHBS1 is located at chr17: 35800000-35810000, with a length of 250 kb in gene THBS1. As a newly identified circRNA, circTHBS1 has not been reported in any study so far. Nevertheless, there is growing evidence that circRNAs act as target miR sponge to exert their effects in NSCLC progression by regulating corresponding oncogenes or anti-oncogenes. For example, Yang reported that the promoting effect of circABCB10 on the *in vivo* and *in vitro* progression of NSCLC is mediated by the miR-1252/FOXR2 axis [23]. In this research, the target miRs of circTHBS1 were predicted by means of bioinformatics analysis, and the circTHBS1/miR-129-5p axis was confirmed to be able to control the expression of SOX4 in NSCLC, providing a theoretical support for ceRNA regulation.

Conclusions

In conclusion, the findings in this research demonstrate that circTHBS1 facilitates NSCLC process through stimulating miR-129-5p sponge to release SOX4, thereby providing diverse therapeutic targets and diagnostic biomarkers for NSCLC patients.

Conflict of interests

The authors declare no conflict of interests.

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