

Circ_0018534 Promotes Non-Small Cell Lung Cancer Progression by Upregulating BTBD7 via Sponging miR-153-3p

Haoran Bai

Shanghai University of Medicine Hospital

Lihua Zhu

Shanghai University of Medical Hospital

Hegen Li

Shanghai University of Medicine Hospital

Lei Zhou (✉ kozzmz@163.com)

Shanghai University of Medicine Hospital Shanghai: Jiading District Central Hospital Affiliated
Shanghai University of Medicine & Health Sciences

Research

Keywords: non-small cell lung cancer, circ_0018534, miR-153-3p, BTBD7

Posted Date: August 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-764297/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Non-small cell lung cancer (NSCLC) is the most common malignant tumor in lung. In this study, we aimed to explore the role and underlying mechanism of circular RNA 0018534 (circ_0018534) in NSCLC progression.

Methods: Real-time quantitative PCR (RT-qPCR) was used to detect the expression of circ_0018534, microRNA-153-3p (miR-153-3p) and BTB/POZ domain-containing protein 7 (BTBD7) in NSCLC tissues and cells. Protein expression was measured by western blot analysis. The morphological features of obtained NSCLC patient tissues were observed by haematoxylin and eosin staining assay. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 5-Ethynyl-29-deoxyuridine, flow cytometry analysis, and transwell migration and invasion assays were used to examine cell viability, proliferation, apoptosis, migration and invasion, respectively. Bioinformatics analysis and dual-luciferase reporter assay were carried out to determine the interaction among circ_0018534, miR-153-3p and BTBD7. Furthermore, lentivector for short hairpin RNA against circ_0018534 (sh-circ_0018534) was used to decrease circ_0018534 expression in an animal tumor model.

Results: Circ_0018534 and BTBD7 expression were significantly increased, whereas miR-153-3p expression was decreased in NSCLC tissues and cells. Circ_0018534 downregulation markedly alleviated cell proliferation, migration, invasion, and elevated cell apoptosis in NSCLC cells *in vitro*. MiR-153-3p inhibitors partially reversed the effects of circ_0018534 knockdown on cell proliferation, migration, invasion and apoptosis in NSCLC. Moreover, miR-153-3p could directly target BTBD7, and circ_0018534 promoted BTBD7 expression by targeting miR-153-3p. Besides, BTBD7 overexpression attenuated miR-153-3p mimics-mediated effects on NSCLC cell progression. Furthermore, circ_0018534 deletion suppressed NSCLC tumor growth *in vivo*.

Conclusion: Our results demonstrated that circ_0018534 might contribute to the progression of NSCLC by miR-153-3p/BTBD7 axis, providing a potential target for the treatment of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), a malignant tumor threatening human health and occupying more than 80% of all lung cancer cases, is regarded as the most common type of lung cancers ¹. Despite the remarkable progress of diagnosis and treatment of NSCLC has been made in recent years, the 5-year survival rate of NSCLC patients still remains dismal and just approximately 15%, while there was no significant change in the past few years ². The reason for the low 5-year survival rate of NSCLC patients might be lack of effective detection ways for NSCLC at an early stage. Therefore, a thorough understanding of the underlying molecular mechanism of the initiation and progression of NSCLC might provide new and effective diagnostic markers for early NSCLC patients.

Previous studies reported that there are at least 75% of the genomes with little protein-coding potential and less than 2% encoding proteins. Circular RNAs (circRNAs) are a type of the noncoding transcripts

with closed loop structure, whereas there are other small noncoding transcripts, such as piRNA, miRNA and snoRNA³. Many studies have demonstrated that circRNAs could regulate gene expression and act the oncogenic or suppressive roles involving in the initiation and progression of various cancers⁴. Accumulating evidences have reported that dysregulation of circRNAs is usually observed in a variety of cancers, including esophageal squamous cell carcinoma⁵, anaplastic thyroid carcinoma⁶, gallbladder cancer⁷ and also NSCLC⁸. Up to now, several studies have demonstrated that circRNAs contributed to the progression of NSCLC, such as circ_0016760⁹, circ_0000376¹⁰ and circ_0067934¹¹. Circ_0018534 is a circRNA and the underlying molecular mechanism of circ_0018534 in NSCLC remains unclear.

MicroRNAs (MiRNAs), a group of small endogenous non-coding RNAs consisting of about 22 nucleotides, are confirmed to regulate the downstream gene expression and serve as tumor regulatory genes in the initiation and development of cancers¹². Besides, accumulating evidences have indicated that a large number of miRNAs in NSCLC were studied. For instance, Jin and his colleagues detected miR-873 and GLI1 expression in PC9 and PC9/GR cells and discovered the inhibitory effects of miR-873 deletion on gefitinib resistance in NSCLC cells¹³. Jin *et al.* indicated that miR-1290 upregulation exerted promoting effect on cell growth *in vitro* and *in vivo*, and improved the invasive ability of NSCLC cells via degrading the expression of IRF2¹⁴. Moreover, few researchers observed the dysregulation of miR-153-3p in NSCLC, playing a suppressor role in the tumor progression of NSCLC¹⁵. However, the involvement of miR-153-3p in the biological processes of NSCLC is little known. Thus, we attempted to explore the functional effects and the relevant mechanism of miR-153-3p in NSCLC.

BTB/POZ domain-containing protein 7 (BTBD7) is a highly conserved protein, belonging to the BTB/POZ domain protein family¹⁶. Recently, emerging evidence suggested that BTBD7 represented the vital importance in protein degradation, cell metastasis and apoptosis¹⁶, thus exerted a critical role in the progression of cancers. It has been demonstrated that BTBD7 silencing significantly boosted E-cadherin in NSCLC cells and repressed cell migration ability, which was regarded as a tumor promoter in NSCLC¹⁷. The effects of BTBD7 on the biological behaviors in NSCLC were further revealed in this research.

In the present study, we detected the expression of circ_0018534, miR-153-3p and BTBD7 in NSCLC tissues and cell lines. And we investigated the interaction among circ_0018534, miR-153-3p and BTBD7. Furthermore, we aimed to explore the functional role of circ_0018534 on NSCLC cells' biological behaviors both *in vivo* and *in vitro*.

Materials And Methods

Tissue samples

Non-small cell lung cancer tissues (n=50) and the neighboring normal lung tissues (n=50) were collected from 50 NSCLC patients who underwent surgery at Shanghai University of Traditional Chinese Medicine hospital. All NSCLC patients involved in this study gave the signed informed consents. The experiments in the present research were approved by the Human Research Ethics Committee of Shanghai University

of Traditional Chinese Medicine hospital. None of the NSCLC patients participating in this research had undergone other therapy.

Cell lines

Two NSCLC cell lines (A549 and H1299) were obtained from American Type Culture Collection (Manassas, VA, USA), and the human bronchial epithelial cell line (16-HBE) used in this study was purchased from Shanghai Fuxiang Biological Technology Co. Ltd. (Shanghai, China). 16-HBE and NSCLC cells were cultured in modified Eagle's medium (MEM; Gibco, Rockville, MD, USA) and Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), and streptomycin/penicillin (100 U/mL; Invitrogen), respectively. All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Cell transfection

Small interfering RNA (siRNA) against circ_0018534 (si-circ_0018534) and the negative control (si-NC), miR-153-3p mimic (miR-153-3p) and the negative control (miR-NC), miR-153-3p inhibitor (anti-miR-153-3p) and its control (anti-miR-NC), the overexpression plasmid of BTBD7 (pcDNA-BTBD7) and the corresponding control (pcDNA), small hairpin RNA targeting circ_0018534 (sh-circ_0018534) and control (sh-NC) were built by Genepharma (Shanghai, China) and were transfected into cells using Lipofectamine 2000 Reagent (Invitrogen).

Haematoxylin and eosin (HE) staining

Tumor tissues from each group of collected NSCLC sufferer specimens were cut into the 5-µm sections, and immobilized with paraformaldehyde (Sigma, Billerica, MA, USA). Then, the section was dehydrated using with ethanol (Millipore, Bradford, MA, USA) and embedded in paraffin. HE (Beyotime, Shanghai, China) was employed to stain the tissues. The images were captured under a microscope (Nikon, Tokyo, Japan).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from NSCLC tissues and cells using Trizol reagent (Invitrogen). TaqMan™ Reverse Transcription reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transcribe RNA to complementary DNA. The expression of circ_0018534, miR-153-3p and BTBD7 was analyzed by an ABI 7500 Real-time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). U6 was used as the internal control for miR-153-3p, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as the internal load for circ_0018534 and BTBD7. We designed specific primers for miR-153-3p (F: 5'-ACACTCCAGCTGGGTTGCATAGTCACAAAA-3', R: 5'-TGGTGTCGTGGAGTCG-3'), circ_0018534 (F: 5'-AGGTGGTTTCTTTGGATT-3', R: 5'-CCATGTCAAGAGGGTAAG-3'), BTBD7 (F: 5'-TGCGAATGGTTAGAATGT-3', R: 5'-GAGACGAGGAACTGGAAT-3'), U6 (F: 5'-TCCGATCGTGAAGCGTTC-

3', R: 5'-GTGCAGGGTCCGAGGT-3'), GAPDH (F: 5'-TGAACGGGAAGCTCACTGG-3', R: 5'-TCCACCACCCTGTTGCTGT-3'). The data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein was extracted from NSCLC tissues and cells by using RIPA lysis buffer (Beyotime). Then 30 μ g total protein was applied and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein. After loading for 2 h, the protein was transferred to membranes (Sigma) and 5% milk was used to block the membranes. Then the membranes were incubated with the primary antibodies against BTBD7 (1:500, ab121006, Abcam, Cambridge, UK), matrix metalloprotein 9 (MMP9) (1:5000, ab76003, Abcam), Snail (1:1000, ab216347, Abcam), B-cell lymphoma-2 (Bcl-2) (1:2000, ab182858, Abcam), BCL2-associated x protein (Bax) (1:5000, ab32503, Abcam), proliferating cell nuclear antigen (PCNA) (1:1000, ab265609, Abcam) and β -actin (1:1000, ab8226, Abcam) overnight. Secondary antibodies included goat anti-rabbit IgG H&L (1:5000, ab6741, Abcam) and goat anti-mouse IgG H&L (1:2000, ab205719, Abcam). Finally, an enhanced chemiluminescence kit (GE Healthcare, Waukesha, WI, USA) was used to detect the immunoreactive proteins.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

The viability of NSCLC cells was measured by MTT assay. After transfection, NSCLC cells (5×10^3) in 96-well plates were cultured for 24 h, 48 h and 72 h. MTT reagent was added into the medium for 4 h. Subsequently, the medium was removed, and dimethyl sulfoxide was added to dissolve the blue crystal. Finally, the absorbance was detected at 450 nm by a microplate reader (Bio-Rad Laboratories).

5-Ethynyl-29-deoxyuridine (EdU) assay

The proliferation of A549 and H1299 cells was determined by EdU assay kit (Ribobio, Guangzhou, China). In brief, cells were grown in 12-well plates after transfected with plasmids or oligonucleotides. At 48 h after culture, cells were digested and seeded in 96-well plates. When the confluence of cells was about 30%, the EdU assay was carried out according to the instruction of manufacture. Immunostainings were visualized and captured with a fluorescence microscope (Olympus, Tokyo, Japan).

Cell apoptosis assay

Cell apoptosis was detected by flow cytometry analysis. The treated HSCLC cells were collected and centrifuged at 1000 rpm for 8 min. Then phosphate buffered solution (PBS) was used to wash the treated NSCLC cells, and the binding buffer was used to resuspend cells. Subsequently, cells were stained using Annexin V/Propidium Iodide (BestBio, Shanghai China) for 20 min in the dark. Cell apoptosis was measured by flow cytometry (Becton-Dickenson, Franklin Lakes, NJ, USA).

Transwell migration and invasion assay

The transwell chambers used in cell migration and invasion assays were purchased from Corning Incorporated (Big Flats, NY, USA). The chambers pre-coated or non-coated with Matrigel (BD Biosciences, San Jose, CA, USA) were used for cell invasion or migration assay, respectively. In the upper chamber, there were the cells mixed with serum-free medium, while DMEM containing 10% FBS was added into the lower chamber. Then cells not migrated or invaded were scraped, whereas the cells removed to the lower chamber were fixed, stained, and analyzed.

Dual-luciferase reporter assay

The circ_0018534 sequence and the 3'UTR fragment of BTBD7 containing the putative miR-153-3p binding sites were amplified by polymerase chain reaction and subsequently inserted into pGL3 promoter vector (Invitrogen), termed as circ_0018534-wild-type (circ_0018534-WT) and BTBD7-wild-type (BTBD7 3'UTR-WT). And the mutant sequences of circ_0018534 and BTBD7 were used to establish circ_0018534-mutant (circ_0018534-MUT) and BTBD7-mutant (BTBD7 3'UTR-MUT) reporter vectors. Then A549 and H1299 cells were co-transfected with reporter vectors with miR-153-3p mimics or miR-NC. The luciferase activity was assessed by the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Xenograft mouse model

BALB/c nude mice were purchased from the Beijing Laboratory Animal Center (Beijing, China). The mice were housed in a standard animal laboratory with a 12 h light-dark cycle, and free access to food and water. A549 cells (2×10^6) stably transfected with short hairpin RNA (shRNA) against circ_0018534 (sh-circ_0018534) or sh-NC were subcutaneously injected into the right flank of nude mice (5 per group). The transfected nude mice were classified into two groups: sh-NC nude mice group and circ_0018534 knockdown nude mice group. After 7 d, the tumor volume was detected for the first time after the tumors became visible. Subsequently, the volume in every group was measured using a caliper every 4 d. After injection for 28 d, all the mice were euthanized by asphyxia method. The mice were treated with the flow rate of CO₂ to displace 60% air of the chamber volume/min according to the current guideline of American Veterinary Medical Association (AVMA). The tumor weight was determined. The lung tissues were separated from the nude mice and stored at -80°C. The experiments were approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine hospital.

Statistical analysis

All data were displayed as the means \pm the standard deviations (SD). SPSS 18.0 software was used to analyze the data in the present study. Spearman correlation analysis was performed to analyze the correlation between variables. Student's *t*-test was used to analyze the statistical difference between groups. One-way analysis of variance (ANOVA) was compared the statistical difference among three or more groups. A value of $P < 0.05$ meant a statistically significant difference.

Results

Circ_0018534 expression is upregulated in NSCLC tissues and cells

To assess circ_0018534 expression in NSCLC tissues, the morphological features of NSCLC tissues and paracancerous normal lung tissues were observed by HE staining assay (Fig. 1A). We then determined the expression of circ_0018534 in NSCLC tissues. RT-qPCR indicated that circ_0018534 expression was higher in NSCLC tissues than in the corresponding normal tissues (Fig. 1B). Moreover, we found that circ_0018534 was overexpressed in A549 and H1299 cells compared with 16-HBE cells (Fig. 1C). The data suggested that circ_0018534 might play crucial roles in NSCLC.

Circ_0018534 deletion inhibits cell proliferation, migration, invasion, and promotes cell apoptosis in NSCLC

To investigate the functional role of circ_0018534 in NSCLC, si-circ_0018534 and si-NC were transfected into A549 and H1299 cells. RT-qPCR was used to evaluate the knockdown efficiency of si-circ_0018534. We found that knockdown of circ_0018534 remarkably decreased circ_0018534 expression in both A549 and H1299 cells (Fig. 2A). Then, we explored the effects of circ_0018534 on the biological behaviors in NSCLC. Results showed that circ_0018534 deletion inhibited cell viability and proliferation in both A549 and H1299 cells (Fig. 2B-E). Knockdown of circ_0018534 significantly elevated the apoptosis rate of NSCLC cells (Fig. 2F). Furthermore, transwell migration and invasion assays were used to measure the migratory and invasive abilities of NSCLC cells. The results showed that circ_0018534 deletion remarkably reduced the number of migrated and invaded cells (Fig. 2G-H). Meanwhile, we found that circ_0018534 silencing decreased the protein expression of PCNA, Bcl-2, Snail and MMP9, and increased Bax protein expression (Fig. 2I-J). These results demonstrated that circ_0018534 could promote the progression of NSCLC.

Circ_0018534 is associated with miR-153-3p in NSCLC cells

Circinteractome online database predicted that there were 6 miRNAs (miR-619-3p, miR-488-3p, miR-153-3p, miR-335-5p, miR-555 and miR-626) containing the putative binding sites of circ_0018534 (Fig. 3A). And miR-153-3p was employed as the supposed miRNA associated with circ_0018534 based on its downregulation in NSCLC tissues and the highest expression in NSCLC cells transfected with si-circ_0018534 (Fig. 3B-C). The binding sites between circ_0018534 and miR-153-3p were shown in Fig. 3D. Then, dual-luciferase reporter assay was performed to determine whether circ_0018534 could function as a molecular sponge to regulate miR-153-3p expression. Results firstly presented the high overexpression efficiency of miR-153-3p (Fig. 3E). Our data then indicated that the luciferase activity was significantly decreased in NSCLC cells co-transfected with miR-153-3p mimic and circ_0018534-WT, whereas there was no significant change when cells were co-transfected with circ_0018534-MUT and miR-153-3p mimic (Fig. 3F-G). Moreover, the expression of miR-153-3p was assessed in NSCLC tissues and cells. Results showed the expression of miR-153-3p was markedly decreased in NSCLC tissues and A549 and H1299 cells compared with normal lung tissues and 16-HBE cells, respectively (Fig. 3H-I). Furthermore, we found miR-153-3p expression was negatively related to circ_0018534 expression (Fig. 3J). These data demonstrated that circ_0018534 was associated with miR-153-3p.

MiR-153-3p inhibitors reverses the effects of circ_0018534 deletion on the progression of NSCLC

To further investigate the relationship between circ_0018534 and miR-153-3p in NSCLC cells, the expression of miR-153-3p was measured in A549 and H1299 cells transfected with anti-miR-NC or anti-miR-153-3p. As described in Fig. 4A, the expression level of miR-153-3p was reduced by anti-miR-153-3p. And results showed that downregulation of circ_0018534 suppressed the cell viability and proliferation, whereas miR-153-3p inhibitors reversed these effects in A549 and H1299 cells (Fig. 4B-E). Our data also noted that miR-153-3p inhibitors significantly reversed the promotion effect of circ_0018534 deletion on cell apoptosis (Fig. 4F). In transwell migration assay, we found that the inhibitory effect of circ_0018534 deletion on cell migration ability was markedly attenuated by miR-153-3p inhibitors (Fig. 4G). A similar phenomenon was also discovered in invasion assay, in which miR-153-3p inhibitors reversed the suppressive effect of circ_0018534 downregulation on the invasive ability of A549 and H1299 cells (Fig. 4H). Furthermore, si-circ_0018534 mediated effects on the protein expression of PCNA, Bax, Bcl-2, Snail and MMP9 were also attenuated after miR-153-3p downregulation (Fig. 4I-J). Taken together, circ_0018534 deletion significantly suppressed proliferation, migration, invasion, and promoted apoptosis of A549 and H1299 cells by binding to miR-153-3p.

MiR-153-3p targets BTBD7 in A549 and H1299 cells

Starbase online database predicted that BTBD7 possessed the complementary binding sites of miR-153-3p (Fig. 5A). Then the reporter plasmids containing the predicted miR-153-3p binding site (BTBD7 3'UTR-WT) and the mutant-type sequence (BTBD7 3'UTR-MUT) were constructed. Our data suggested that the luciferase activity was significantly suppressed by miR-153-3p in A549 and H1299 cells transfected with BTBD7 3'UTR-WT; however, for cells transfected with BTBD7 3'UTR-MUT, the luciferase activity was not changed (Fig. 5B-C). Subsequently, results demonstrated that BTBD7 expression at the mRNA and protein levels was markedly increased by anti-miR-153-3p, and was decreased by miR-153-3p in both A549 and H1299 cells (Fig. 5D-E). The mRNA and protein expression of BTBD7 were upregulated in NSCLC tissues and cells relative to normal tissues and cells (Fig. 5F-I). Furthermore, a negative linear relationship between BTBD7 and miR-153-3p expression was observed (Fig. 5J). Overall, miR-153-3p targeted BTBD7 in NSCLC cells.

MiR-153-3p mimics represses NSCLC cell progression by targeting BTBD7

Then, we explored the functional effects between miR-153-3p and BTBD7 on the biological processes in NSCLC. We firstly detected the expression of BTBD7 in A549 and H1299 cells transfected with pcDNA or pcDNA-BTBD7. The data suggested that the mRNA and protein expression of BTBD7 were highly expressed in A549 and H1299 cells transfected with pcDNA-BTBD7 compared with control groups (Fig. 6A-B). Data indicated that miR-153-3p mimics significantly suppressed cell viability and proliferation in NSCLC cells, whereas these effects were reversed by BTBD7 overexpression (Fig. 6C-F). Then the apoptosis rate of NSCLC cells was measured by flow cytometry, and we discovered that cell apoptosis was remarkably elevated by miR-153-3p mimics, which was attenuated after BTBD7 overexpression (Fig. 6G). Subsequently, transwell migration and invasion assays were performed. The results indicated that

upregulation of BTBD7 significantly attenuated miR-153-3p mimics-mediated repression on the migratory and invasive abilities of NSCLC cells (Fig. 6H-I). MiR-153-3p mimics-mediated effects on the protein expression of PCNA, Bax, Bcl-2, Snail and MMP9 were also impaired by BTBD7 overexpression (Fig. 6J). All these data demonstrated that miR-153-3p regulated NSCLC cell progression by targeting BTBD7.

Circ_0018534 regulates BTBD7 expression by interacting with miR-153-3p

The interaction among circ_0018534, miR-153-3p and BTBD7 was continued to be revealed. Results firstly showed BTBD7 expression was positively correlated with circ_0018534 expression (Fig. 7A). Subsequently, we found that circ_0018534 silencing decreased the mRNA and protein expression of BTBD7, which was reversed by miR-153-3p inhibitors (Fig. 7B-C). These evidences suggested circ_0018534 could modulate BTBD7 expression by interacting with miR-153-3p.

Inhibition of circ_0018534 suppresses NSCLC tumor growth *in vivo*

Then, we established a xenograft model to investigate the functional role of circ_0018534 *in vivo*. A549 cells stably transfected with sh-circ_0018534 or sh-NC were injected subcutaneously into nude mice. As shown in Fig. 8A-B, the volume and weight of the tumors were significantly suppressed by decreased circ_0018534. Subsequently, we measured the expression of circ_0018534, miR-153-3p and BTBD7 in the tumors of nude mice. The results suggested that the expression of circ_0018534 and the mRNA and protein levels of BTBD7 was markedly suppressed, whereas the expression of miR-153-3p was significantly increased in the tumors from nude mice (Fig. 8C-F). Taken together, our results demonstrated that circ_0018534 could significantly elevate NSCLC tumor growth *in vivo*.

Discussion

NSCLC, the most common malignant lung cancer, ranks one of the lethal diseases. The previous studies reported the poor diagnosis methods at an early stage of NSCLC and the poor prognosis caused by the occurrence of tumor metastasis and high mortality¹⁸. Accumulating evidences indicated that some circRNAs, as well as miRNAs, were involved in the progression of types of cancers^{19,20}. Recently, emerging evidences have suggested that many circRNAs could sponge miRNAs to exert functional effects in diseases²¹. Moreover, dysregulation of circRNAs was observed in NSCLC in several researches²²⁻²⁴. Thus, circRNAs might be novel therapeutic targets for clinical treatment for NSCLC.

In NSCLC progression, multiple circRNAs were involved. For example, circ_0067934 was indicated to promote cell proliferation and metastasis as well as be associated with poor prognosis of NSCLC cases¹¹. Circ_0002483 was revealed to enhance the sensitivity of NSCLC to Taxol by binding to miR-182-5p²⁵. Additionally, Ding *et al.* found that circ_001569 could promote cell proliferation by modulating Wnt/ β -catenin pathway in NSCLC²⁶. In this study, circ_0018534 was revealed to regulate NSCLC progression for the first time. We found that the expression of circ_0018534 was higher in NSCLC tissues and cells than the expression in their corresponding normal groups. The downregulation of the expression of

circ_0018534 was used to figure out the functional effects of circ_0018534 in NSCLC *in vitro*, as well as *in vivo*. Here, we discovered that downregulated circ_0018534 could attenuate cell proliferation, enhance the apoptotic rate, as well as decrease the number of migrated and invaded cells in NSCLC cells *in vitro*. Besides, we also found the inhibition of tumor growth via downregulating circ_0018534 in the nude mice model *in vivo*. And circ_0018534 was identified to directly target miR-153-3p. Considering all these data, we guessed that circ_0018534 might exert the oncogenic effects in NSCLC by targeting miR-153-3p.

The involvement of miR-153-3p in various types of cancer cells has been well-documented in the previous researches, such as colorectal cancer cells²⁷, ovarian cancer cells²⁸, and breast cancer cells²⁹. Besides, miR-153-3p bound by NEAT1 was the regulatory mechanism for NSCLC progression according to the exploration from Zhao and his colleagues¹⁵. In this paper, we found that miR-153-3p was lowly expressed in NSCLC tissues and cells, and negatively regulated by circ_0018534. MiR-153-3p inhibitors also reversed circ_0018534 silencing-mediated NSCLC development. Additionally, we observed that BTBD7 was a novel target of miR-153-3p, and increased BTBD7 was discovered in NSCLC tissues and cells relative to the normal groups. In this study, the effects of BTBD7 on the biological behaviors were similar to circ_0018534 in NSCLC cells, and the effects of BTBD7 overexpression, including promotion of cell viability and cell metastasis, repression of cell apoptosis were observed in NSCLC cells in the present research. Subsequently, we performed the reverse experiments and found that overexpression of BTBD7 reversed the effects of miR-153-3p on cell growth, apoptosis and metastasis in NSCLC cells. Furthermore, rescue experiments also demonstrated that circ_0018534 regulated BTBD7 expression by interacting with miR-153-3p.

In summary, we found that the expression of circ_0018534 and BTBD7 were significantly boosted, and miR-153-3p expression was decreased in NSCLC tissues and cells. Functionally, circ_0018534 downregulation could suppress cell growth and metastasis, and exert a significant promotion effect on cell apoptosis in NSCLC *in vitro*. Additionally, circ_0018534 deletion also had a suppressive effect on tumor growth *in vivo*. And circ_0018534 could regulate BTBD7 expression via targeting miR-153-3p. More importantly, we discovered a novel regulatory mechanism in NSCLC that circ_0018534 promoted the progression of NSCLC via miR-153-3p/BTBD7 axis. Our data indicated that circ_0018534 might be a potential target for NSCLC treatment.

Abbreviations

NSCLC

Non-small cell lung cancer; circ_0018534:circular RNA 0018534; (RT-qPCR):Real-time quantitative PCR; miR-153-3p:microRNA-153-3p; BTBD7:BTB/POZ domain-containing protein 7; sh-circ_0018534:short hairpin RNA against circ_0018534

Declarations

Acknowledgement

None

Availability of data and materials

Not applicable

Disclosure of interest

The authors declare that they have no financial conflicts of interest

Author's contribution

All authors have been involved in the management of the patient and in the conception of the manuscript. Haoran Bai and Lei Zhou have been involved in the drafting of the manuscript or its critical revision for important intellectual content. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of Shanghai University of Traditional Chinese Medicine Hospital.

Funding

This work was supported by:

1. National Thirteenth Five-Year Science and Technology Major Special Project for New Drug Innovation and Development: The construction of a demonstration technology platform for the clinical evaluation of new drugs for malignant tumor and other diseases (2017ZX09304001)
2. Construction of Shanghai's most important clinical medical center and key disciplines, Name: TCM Chronic Diseases (Malignant Tumor, Bone Degenerative Diseases) Prevention and Treatment Center 2017ZZ01010

References

1. Ettinger DS, Akerley W, Borghaei H, et al. Non-small cell lung cancer, version 2.2013. Journal of the National Comprehensive Cancer Network: JNCCN. 2013;11(6):645–53. quiz 653.

2. Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong KK. Non-small-cell lung cancers: a heterogeneous set of diseases. *Nature reviews Cancer*. 2014;14(8):535–46.
3. Chen LL, Yang L. Regulation of circRNA biogenesis. *RNA Biol*. 2015;12(4):381–8.
4. Arnaiz E, Sole C, Manterola L, Iparraguirre L, Otaegui D, Lawrie CH. CircRNAs and cancer: Biomarkers and master regulators. *Semin Cancer Biol*. 2019;58:90–9.
5. Cao S, Chen G, Yan L, Li L, Huang X. Contribution of dysregulated circRNA_100876 to proliferation and metastasis of esophageal squamous cell carcinoma. *Onco Targets Ther*. 2018;11:7385–94.
6. Liu F, Zhang J, Qin L, et al. Circular RNA EIF6 (Hsa_circ_0060060) sponges miR-144-3p to promote the cisplatin-resistance of human thyroid carcinoma cells by autophagy regulation. *Aging (Albany NY)*. 2018;10(12):3806–20.
7. Wang S, Zhang Y, Cai Q, et al. Circular RNA FOXP1 promotes tumor progression and Warburg effect in gallbladder cancer by regulating PKLR expression. *Mol Cancer*. 2019;18(1):145.
8. Gao P, Wang Z, Hu Z, Jiao X, Yao Y. Circular RNA circ_0074027 indicates a poor prognosis for NSCLC patients and modulates cell proliferation, apoptosis, and invasion via miR-185-3p mediated BRD4/MADD activation. *J Cell Biochem*. 2020;121(3):2632–42.
9. Hao Y, Xi J, Peng Y, et al. Circular RNA Circ_0016760 Modulates Non-Small-Cell Lung Cancer Growth Through the miR-577/ZBTB7A Axis. *Cancer Manag Res*. 2020;12:5561–74.
10. Sun H, Chen Y, Fang YY, et al. Circ_0000376 enhances the proliferation, metastasis, and chemoresistance of NSCLC cells via repressing miR-384. *Cancer Biomark*. 2020;29(4):463–73.
11. Wang J, Li H. CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC. *Eur Rev Med Pharmacol Sci*. 2018;22(10):3053–60.
12. Plones T, Elze M, Kayser G, Pfeifer D, Burger M, Zissel G. mRNA and miRNA analyses in cytologically positive endobronchial ultrasound-guided transbronchial needle aspiration: implications for molecular staging in lung cancer patients. *Cancer cytopathology*. 2014;122(4):292–8.
13. Jin S, He J, Li J, Guo R, Shu Y, Liu P. MiR-873 inhibition enhances gefitinib resistance in non-small cell lung cancer cells by targeting glioma-associated oncogene homolog 1. *Thoracic cancer*. 2018;9(10):1262–70.
14. Jin JJ, Liu YH, Si JM, Ni R, Wang J. Overexpression of miR-1290 contributes to cell proliferation and invasion of non small cell lung cancer by targeting interferon regulatory factor 2. *The international journal of biochemistry cell biology*. 2018;95:113–20.
15. Zhao L, Bi M, Zhang H, Shi M. Downregulation of NEAT1 Suppresses Cell Proliferation, Migration, and Invasion in NSCLC Via Sponging miR-153-3p. *Cancer Biother Radiopharm*. 2020;35(5):362–70.
16. Cheng D, Qian W, Meng M, Wang Y, Peng J, Xia Q. Identification and Expression Profiling of the BTB Domain-Containing Protein Gene Family in the Silkworm, *Bombyx mori*. *Int J Genomics*. 2014;2014:865065.

17. Fan C, Miao Y, Zhang X, et al. Btbd7 contributes to reduced E-cadherin expression and predicts poor prognosis in non-small cell lung cancer. *BMC Cancer*. 2014;14:704–4.
18. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer research*. 1990;50(19):6130–8.
19. Liang HF, Zhang XZ, Liu BG, Jia GT, Li WL. Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271. *Am J Cancer Res*. 2017;7(7):1566–76.
20. Han J, Zhao G, Ma X, et al. CircRNA circ-BANP-mediated miR-503/LARP1 signaling contributes to lung cancer progression. *Biochem Biophys Res Commun*. 2018;503(4):2429–35.
21. Qi X, Zhang DH, Wu N, Xiao JH, Wang X, Ma W. ceRNA in cancer: possible functions and clinical implications. *Journal of medical genetics*. 2015;52(10):710–8.
22. Liu C, Zhang Z, Qi D. Circular RNA hsa_circ_0023404 promotes proliferation, migration and invasion in non-small cell lung cancer by regulating miR-217/ZEB1 axis. *Onco Targets Ther*. 2019;12:6181–9.
23. Hong W, Xue M, Jiang J, Zhang Y, Gao X. Circular RNA circ-CPA4/ let-7 miRNA/PD-L1 axis regulates cell growth, stemness, drug resistance and immune evasion in non-small cell lung cancer (NSCLC). *J Exp Clin Cancer Res*. 2020;39(1):149.
24. Zhang H, Wang X, Hu B, Zhang F, Wei H, Li L. Circular. RNA ZFR accelerates non-small cell lung cancer progression by acting as a miR-101-3p sponge to enhance CUL4B expression. *Artif Cells Nanomed Biotechnol*. 2019;47(1):3410–6.
25. Li X, Yang B, Ren H, et al. Hsa_circ_0002483 inhibited the progression and enhanced the Taxol sensitivity of non-small cell lung cancer by targeting miR-182-5p. *Cell Death Dis*. 2019;10(12):953.
26. Ding L, Yao W, Lu J, Gong J, Zhang X. Upregulation of circ_001569 predicts poor prognosis and promotes cell proliferation in non-small cell lung cancer by regulating the Wnt/ β -catenin pathway. *Oncol Lett*. 2018;16(1):453–8.
27. Liu JX, Li W, Li JT, Liu F, Zhou L. Screening key long non-coding RNAs in early-stage colon adenocarcinoma by RNA-sequencing. *Epigenomics*. 2018;10(9):1215–28.
28. Li C, Zhang Y, Zhao W, Cui S, Song Y. miR-153-3p regulates progression of ovarian carcinoma in vitro and in vivo by targeting MCL1 gene. *J Cell Biochem*. 2019;120(11):19147–58.
29. Yu L, Xu Q, Yu W, Duan J, Dai G. LncRNA cancer susceptibility candidate 15 accelerates the breast cancer cells progression via miR-153-3p/KLF5 positive feedback loop. *Biochem Biophys Res Commun*. 2018;506(4):819–25.

Figures

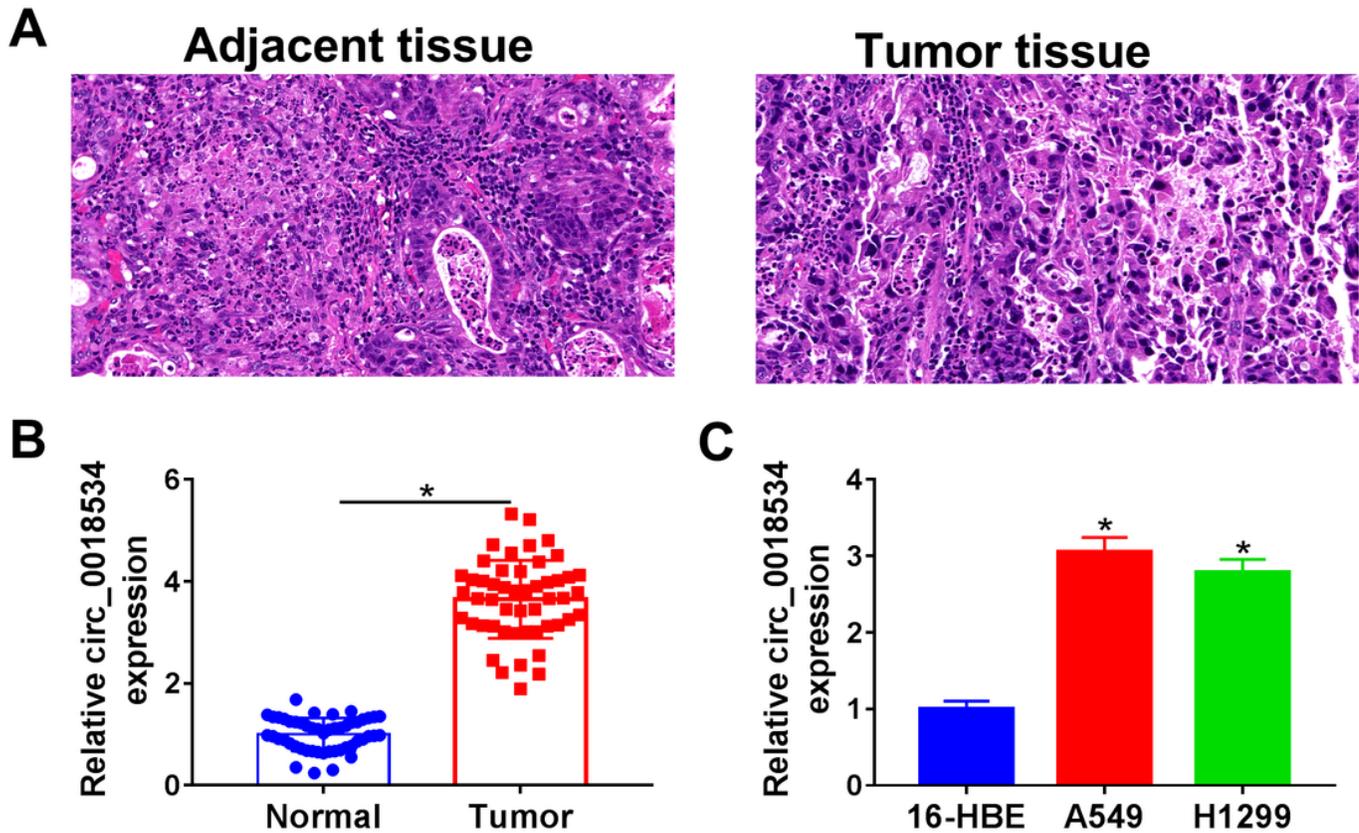


Figure 1

Circ_0018534 is upregulated in NSCLC tissues and cells. (A) The morphological features of collected NSCLC patient tissues and paracancerous normal tissues were observed by HE staining assay. (B and C) The expression of circ_0018534 was detected by RT-qPCR in 50 pairs of normal lung tissues and NSCLC tissues as well as 16-HBE, A549 and H1299 cells. *P < 0.05.

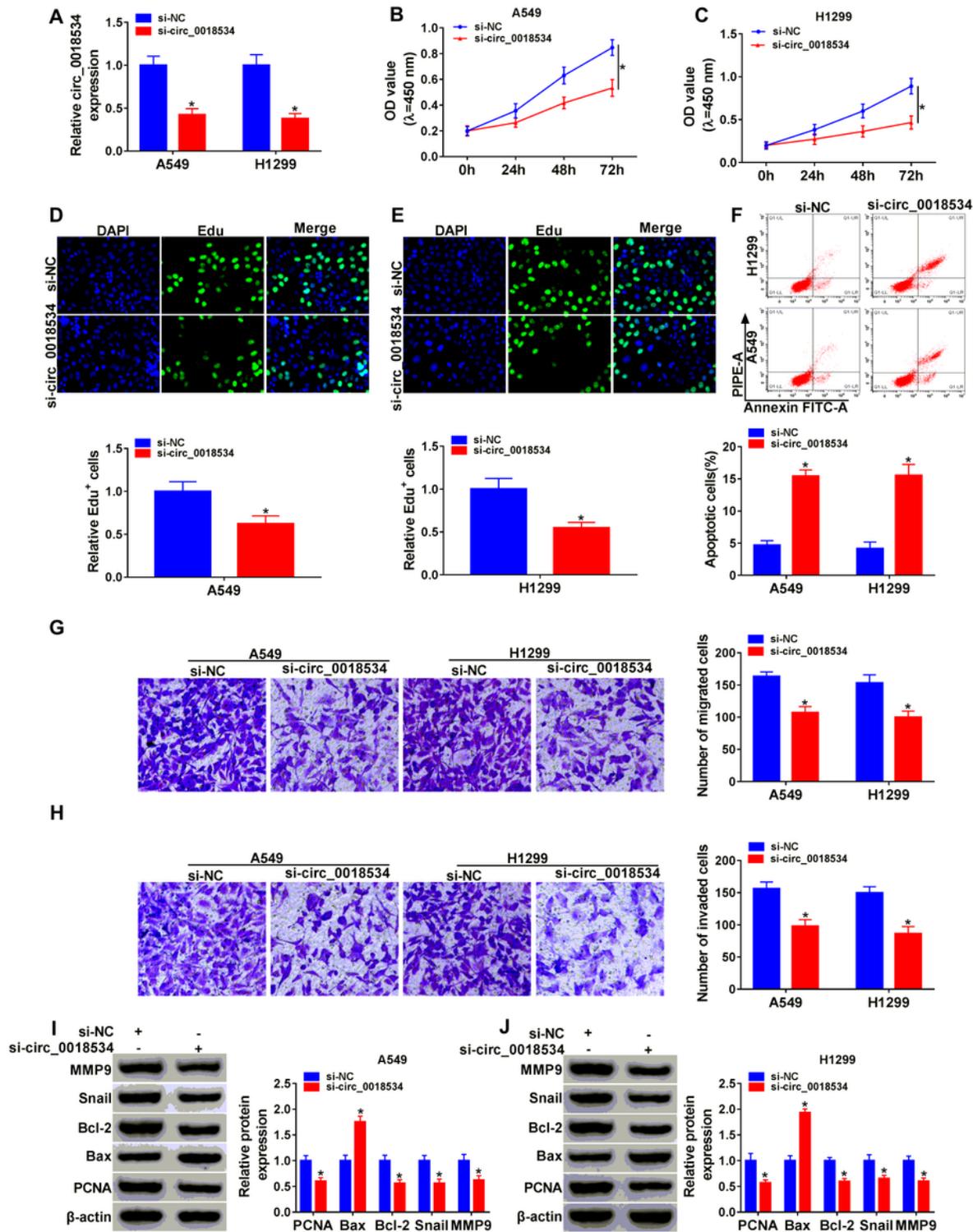


Figure 2

Circ_0018534 deletion inhibits cell proliferation, migration, invasion, and promotes cell apoptosis in NSCLC. (A) The expression of circ_0018534 in A549 and H1299 cells transfected with si-circ_0018534 was detected by RT-qPCR. (B-E) The viability and proliferation of A549 and H1299 cells was determined by MTT and Edu assays, respectively. (F) The apoptosis of A549 and H1299 cells was measured by flow cytometry analysis (G-H) Transwell migration and invasion assays were carried out to detect the

migratory and invasive abilities of NSCLC cells. (I and J) Western blot analysis was performed to detect the effects of circ_0018534 silencing on the protein expression of PCNA (proliferation-related protein), apoptosis-related Bax and Bcl-2 and metastasis-linked Snail and MMP9 in A549 and H1299 cells. *P < 0.05.

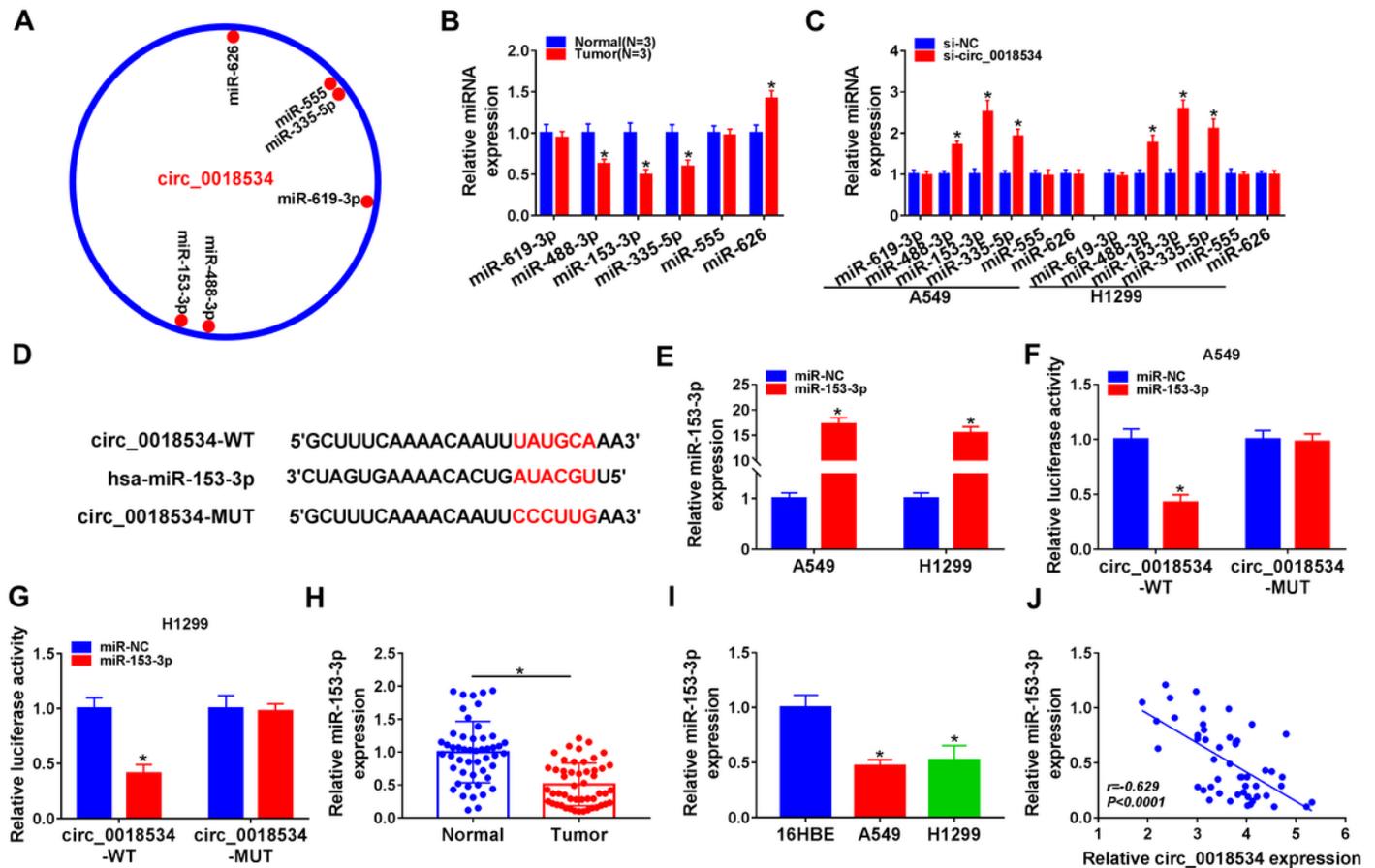


Figure 3

Circ_0018534 is associated with miR-153-3p in NSCLC cells. (A) Circinteractome online database was employed to predict the miRNAs containing the binding sites of circ_0018534. (B) The expression of miR-619-3p, miR-488-3p, miR-153-3p, miR-335-5p, miR-555 and miR-626 was detected by RT-qPCR in NSCLC tissues (N=3) and normal lung tissues (N=3). (C) The expression of miR-619-3p, miR-488-3p, miR-153-3p, miR-335-5p, miR-555 and miR-626 was detected by RT-qPCR in A549 and H1299 cells transfected with si-circ_0018534 or si-NC. (D) Circinteractome online database predicted the putative binding sites between circ_0018534 and miR-153-3p. (E) MiR-153-3p expression was determined by RT-qPCR in A549 and H1299 cells transfected with miR-153-3p or miR-NC. (F-G) Dual-luciferase reporter assay was carried out to detect the luciferase activity in A549 and H1299 cells co-transfected with miR-NC or miR-153-3p and circ_0018534-WT or circ_0018534-MUT. (H and I) The expression of miR-153-3p was detected by RT-qPCR in 50 pairs of normal lung tissues and NSCLC tissues as well as 16-HBE, A549 and H1299 cells. (J) Spearman correlation analysis was employed to assess the linear relationship between circ_0018534 and miR-153-3p expression. *P < 0.05.

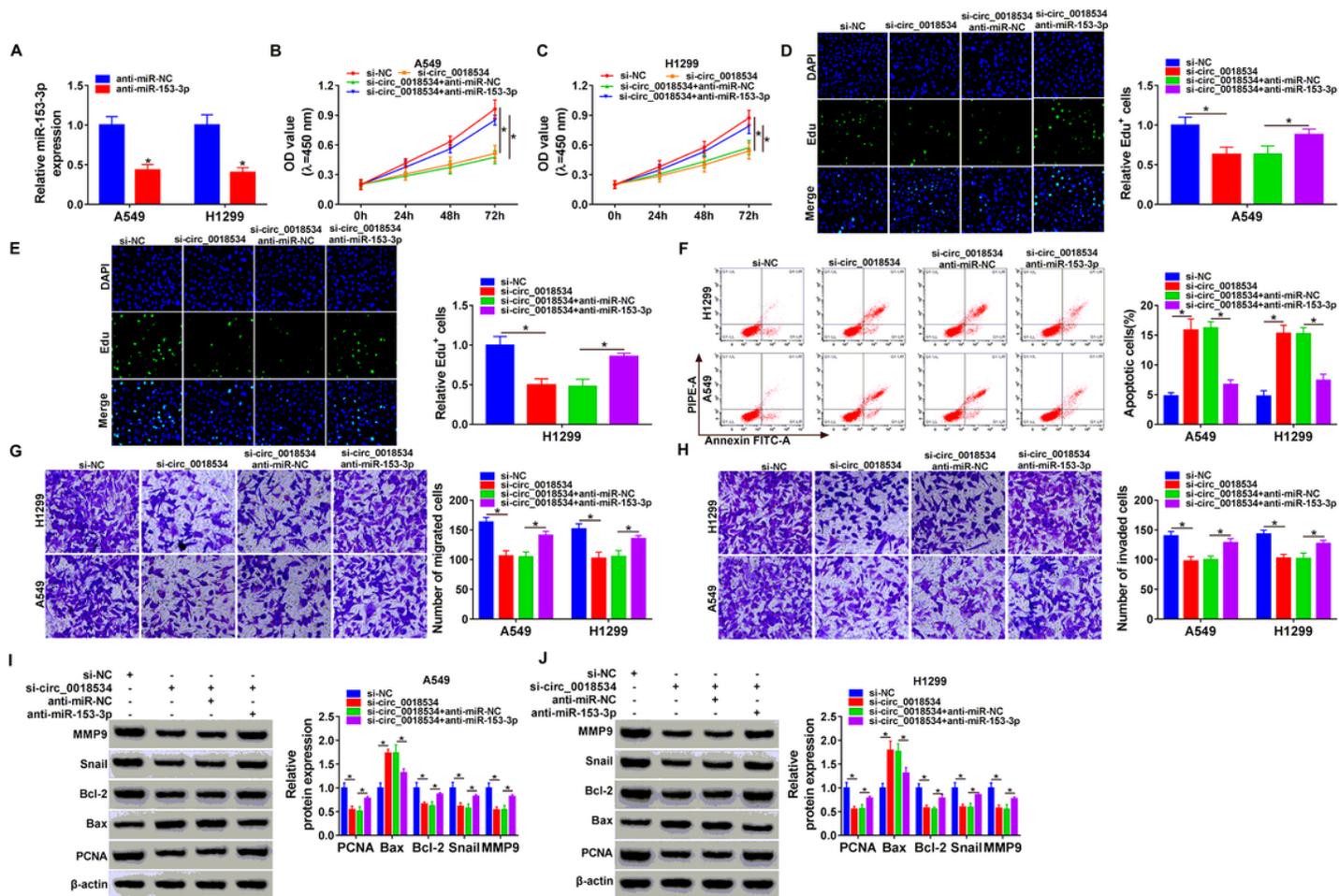


Figure 4

MiR-153-3p inhibitors reverses the effects of circ_0018534 deletion on the progression of NSCLC. (A) MiR-153-3p expression was detected by RT-qPCR in A549 and H1299 cells transfected with anti-miR-NC or anti-miR-153-3p. (B-J) A549 and H1299 cells were transfected with si-circ_0018534, si-NC, si-circ_0018534 + anti-miR-NC or si-circ_0018534 + anti-miR-153-3p. (B-E) Cell viability and proliferation in A549 and H1299 cells was evaluated by MTT and Edu assays, respectively. (F) Cell apoptosis in A549 and H1299 cells was detected by flow cytometry. (G-H) Cell migration and invasion in A549 and H1299 cells were measured by transwell migration and invasion assays. (I and J) Western blot analysis was performed to detect the protein expression of PCNA, Bax, Bcl-2, Snail and MMP9. *P < 0.05.

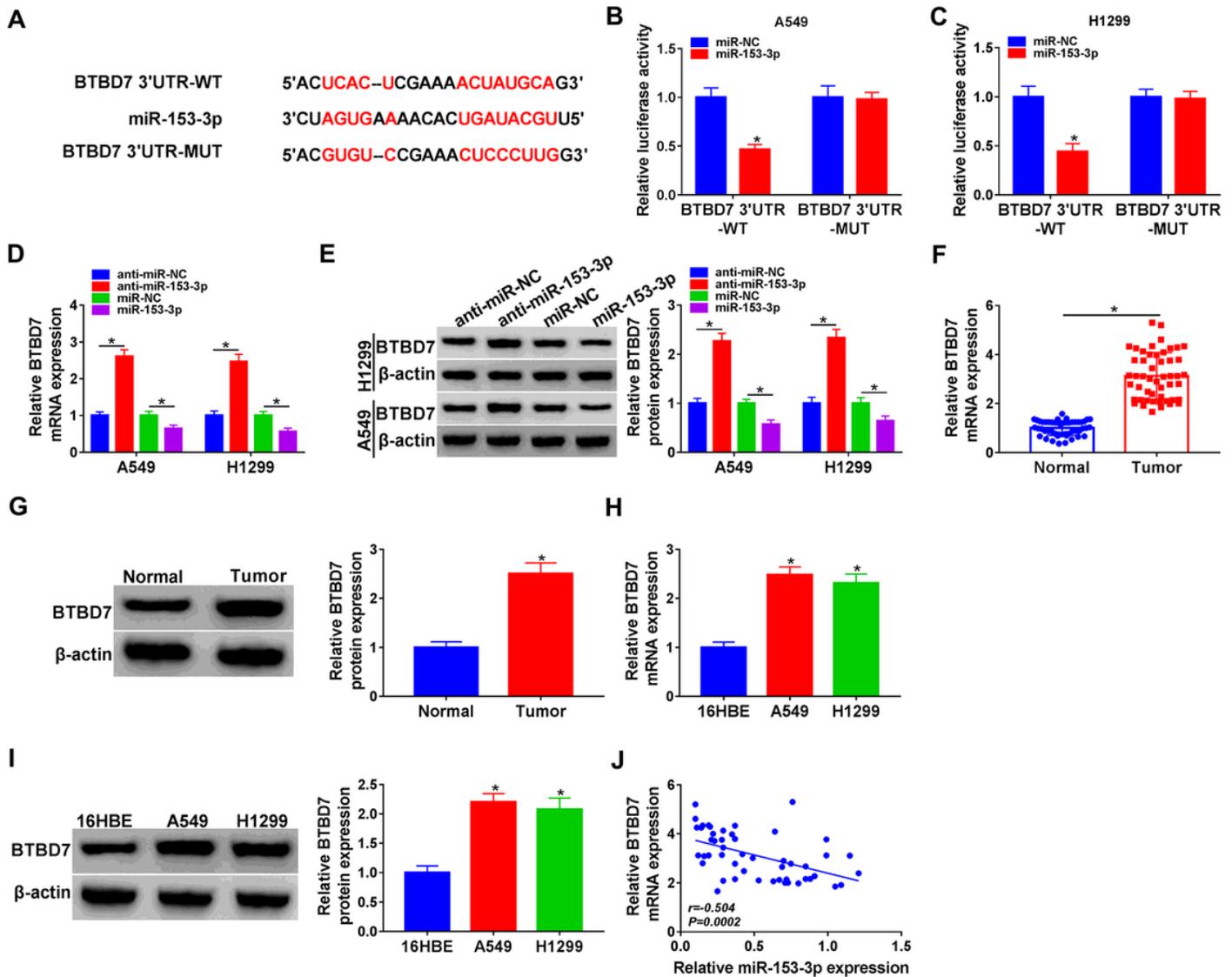


Figure 5

MiR-153-3p targets BTBD7 in A549 and H1299 cells. (A) Starbase predicted that the BTBD7 mRNA 3'UTR sequence contained the binding sites of miR-153-3p. (B-C) The 3'UTR of wild type BTBD7 (BTBD7 3'UTR-WT) or 3'UTR of mutant BTBD7 (BTBD7 3'UTR-MUT) reporter plasmid was co-transfected into A549 and H1299 cells with miR-NC or miR-153-3p. The luciferase activity was detected by dual-luciferase reporter assay. (D-E) The mRNA and protein expression of BTBD7 in A549 and H1299 cells transfected with anti-miR-NC, anti-miR-153-3p, miR-NC or miR-153-3p were detected by western blot. (F-I) The mRNA and protein expression of BTBD7 was severely detected by RT-qPCR or western blot analysis in 50 pairs of normal lung tissues and NSCLC tissues as well as 16-HBE, A549 and H1299 cells. (J) Spearman correlation analysis was carried out to determine the linear relationship between BTBD7 and miR-153-3p expression.*P < 0.05.

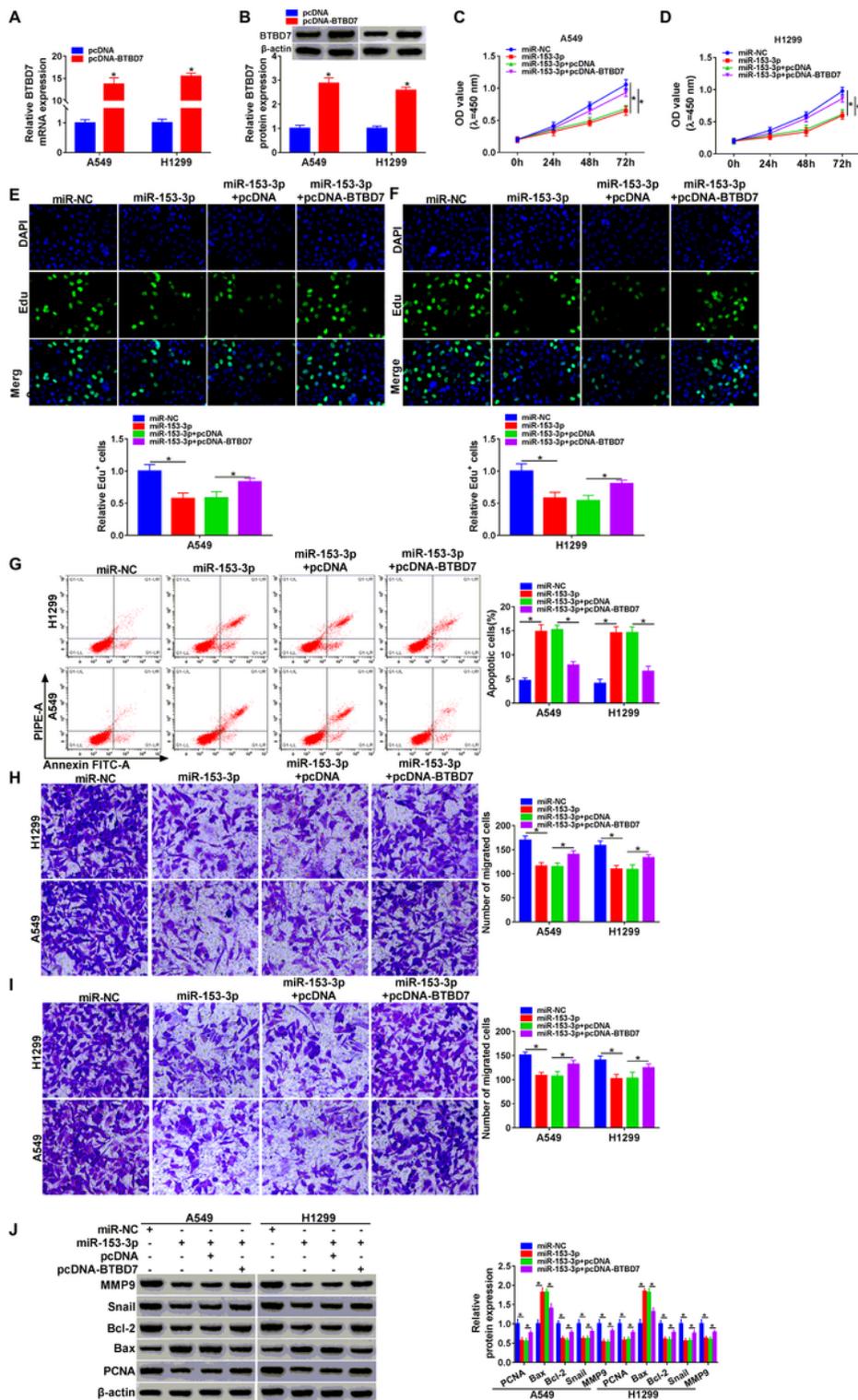


Figure 6

MiR-153-3p regulates NSCLC cell processes by binding to BTBD7. (A-B) BTBD7 mRNA and protein expression in A549 and H1299 cells transfected with pcDNA or pcDNA-BTBD7 were detected by RT-qPCR and western blot, respectively. (B-J) A549 and H1299 cells were transfected with miR-NC, miR-153-3p, miR-153-3p + pcDNA or miR-153-3p + pcDNA-BTBD7. (C-F) Cell viability and proliferation was evaluated by MTT and EdU assays, respectively, in A549 and H1299 cells. (G) Cell apoptosis was detected by flow

cytometry in A549 and H1299 cells. (H-I) Cell migration and invasion were measured by transwell migration and invasion assays in A549 and H1299 cells. (J) Western blot analysis was performed to detect the protein expression of PCNA, Bax, Bcl-2, Snail and MMP9. *P < 0.05.

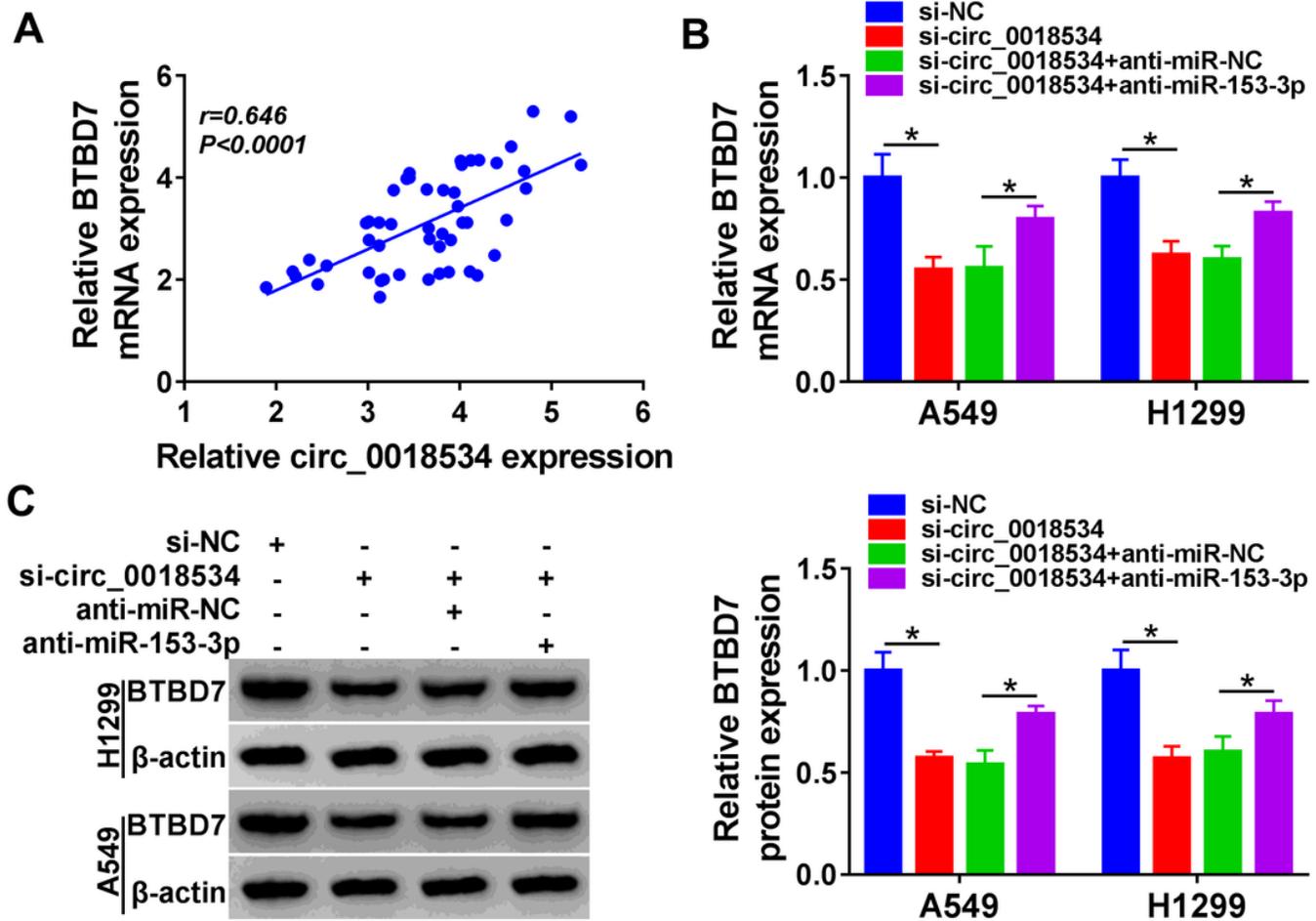


Figure 7

Circ_0018534 silencing downregulates BTBD7 expression by binding to miR-153-3p. (A) The linear relationship between circ_0018534 expression and BTBD7 expression was revealed by Spearman correlation analysis. (B and C) The effects between circ_0018534 silencing and miR-153-3p inhibitors on the mRNA and protein expression of BTBD7 were demonstrated by RT-qPCR and western blot, respectively. *P < 0.05.

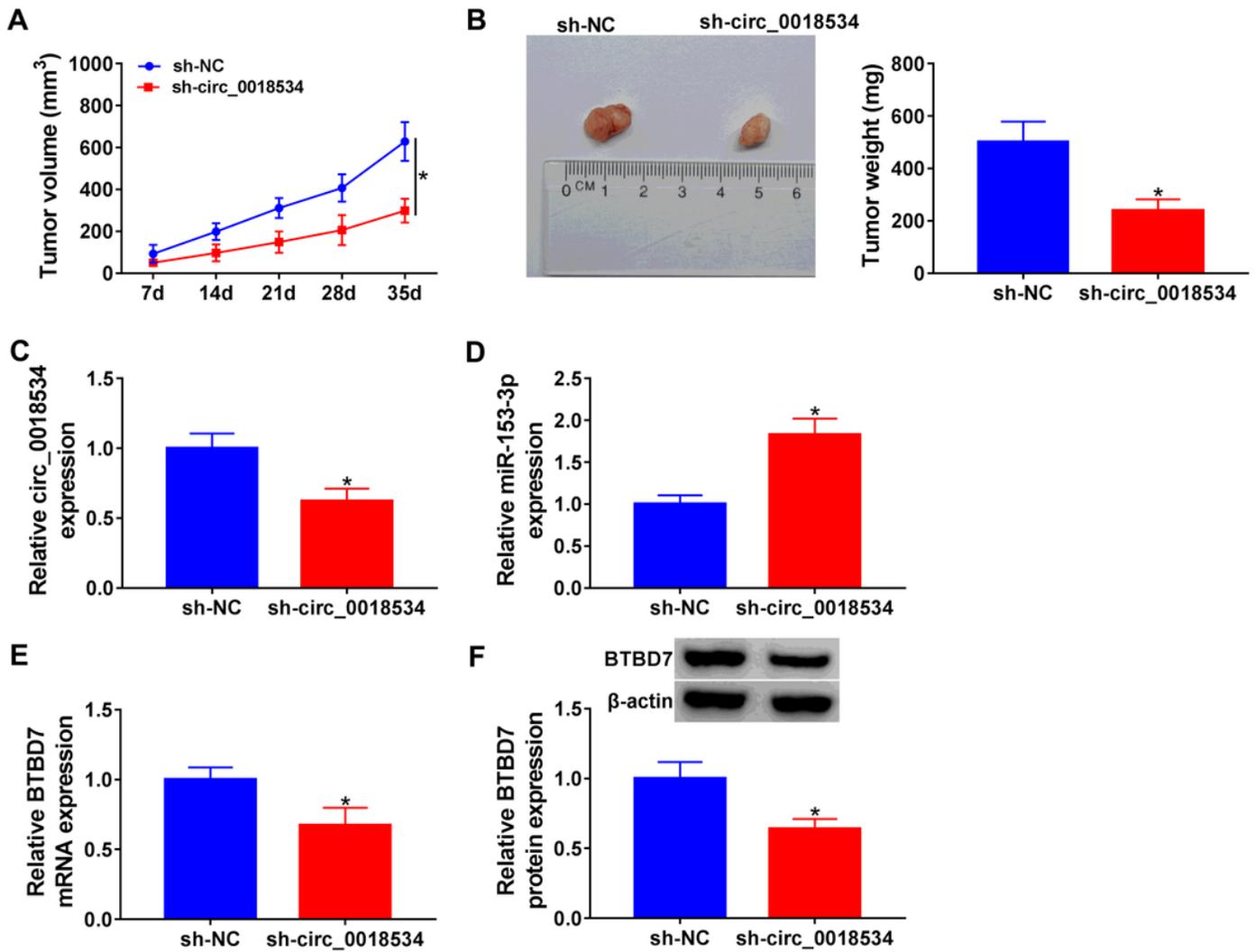


Figure 8

Inhibition of circ_0018534 suppresses NSCLC tumor growth in vivo. (A) Transfection of sh-circ_0018534 suppressed the tumor volume of nude mice. (B) Transfection of sh-circ_0018534 reduced the weight of nude mice tumor. (C-D) RT-qPCR was used to detect the expression of circ_0018534 and miR-153-3p in tumor tissues of nude mice in response to circ_0018534 deletion. (E-F) The mRNA and protein expression of BTBD7 in tumor tissues of nude mice in response to circ_0018534 deletion were measured by RT-qPCR and western blot, respectively. *P < 0.05.