

# Circular RNA ATXN7 is upregulated in non-small cell lung cancer and promotes disease progression

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**Abstract.** Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs that is generated from back-splicing, and is characterized by a covalent closed loop without 3' and 5' ends. Recently, the biological function of circRNAs has received increasing attention; however, studies on circRNAs in non-small cell lung cancer (NSCLC) have rarely been reported. In the present study, the expression profiles of circRNAs in NSCLC were investigated, and the association between the circular RNA ATXN7 (circATXN7) expression level and clinicopathological characteristics of patients with NSCLC was assessed. In addition, the effects of circATXN7 on cell proliferation and invasion were examined. The results revealed that circATXN7 was upregulated in 45 NSCLC tissues compared with its expression in non-tumor tissues. However, there was no marked difference between the expression level of circATXN7 and the majority of the examined clinicopathological characteristics. It was also observed that the survival time of patients with high circATXN7 levels was shorter compared with that of patients with low circATXN7 levels, although the difference was not statistically significant ( $P > 0.05$ ). Furthermore, silencing of circATXN7 by small interfering RNA inhibited the proliferation and invasion of NSCLC cells *in vitro*. Taken together, the present study was the first to identify that circATXN7 was upregulated in NSCLC tumor tissues. Furthermore, the downregulation of circATXN7 markedly inhibited the proliferation and invasion abilities of NSCLC cells.

## Introduction

Lung cancer is the most common malignancy and the leading cause of mortality among cancer-associated diseases worldwide (1). Non-small cell lung cancer (NSCLC) accounts for >85% of lung cancer cases (2). The pathologic types of NSCLC include large-cell carcinoma, squamous cell carcinoma and adenocarcinoma (3). In spite of recent advances in surgical resection, chemotherapy and radiotherapy for lung cancer, the survival rate of patients remains unsatisfactory, with a 5-year survival rate as low as 15%, as it is often diagnosed at an advanced stage (4,5). Therefore, there is an urgent need to identify effective molecular diagnostic markers for early detection of lung cancer (6).

Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs generated from back-splicing and characterized by a covalent closed loop without 3' and 5' ends (7). In the past two decades, circRNAs have been hypothesized to be non-functional due to errors in splicing (8). In 2011, Salmena *et al* (9) presented the competing endogenous RNA hypothesis, which suggests that mRNAs, transcribed pseudogenes and long non-coding RNAs can suppress microRNA (miRNA) expression and function by competitive binding to miRNA response elements. Furthermore, it was demonstrated that non-coding RNAs are involved in the initiation and progression of malignant tumors (10). As another class of non-coding RNAs, circRNAs are more stable than linear RNAs due to their covalently closed structures, and the sponge effect of circRNAs may be more efficient in comparison with that of linear RNAs (11). Recently, a number of circRNAs have been demonstrated to regulate the function of miRNAs, serving as miRNA sponges, as well as being involved in post-transcriptional regulation in cancer (12). For instance, Zhong *et al* (13) reported that circTCF25 was able to down-regulate miR-103a-3p and miR-107, increase cyclin-dependent kinase-6 expression, and promote the proliferation and migration of bladder cancer cells. However, as an emerging type of non-coding RNAs, the role of circRNAs in NSCLC has rarely been reported (14).

Our previous study investigated circRNA expression in NSCLC by ribosomal RNA depletion and RNA sequencing (15). Therefore, the present study was designed to analyze the expression profile of circATXN7 in NSCLC and

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examine its correlation with clinicopathological characteristics. In addition, the effect of circATXN7 on NSCLC cell proliferation and invasion was investigated.

## Materials and methods

**Patients and tissue samples.** A total of 57 pairs of NSCLC tissues and adjacent normal tissues ( $\geq 3$  cm away from the tumor) were obtained from patients who underwent surgery at the Department of Thoracic Surgery, Peking University People's Hospital (Beijing, China) between January 2012 and December 2013. These tissues were used for validation of circRNAs by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All surgical specimens were snap-frozen and stored in liquid nitrogen immediately following resection until the extraction of total RNA. The clinical and pathological characteristics of each patient were collected. The Tumor-Node-Metastasis (TNM) stage was determined according to The 8th Edition Lung Cancer Stage Classification (16). The present study was approved and supervised by the Ethics Committee of Peking University People's Hospital. Written informed consent was obtained from all the patients for research purposes.

**Cell culture and small interfering RNA (siRNA) transfection.** The NSCLC cell lines A549 and SPC-A1 were kindly provided by Professor Xu (Jiangsu Key Laboratory of Molecular and Translational Cancer Research, Nanjing Medical University Affiliated Cancer Hospital, Nanjing, China). A549 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), while SPC-A1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. For siRNA transfection, NSCLC cells were seeded in 6-well plates (6x10<sup>4</sup> cells/well) and cultured at 37°C until the cell confluence reached 60-70%. Subsequently, cells were transfected with specific siRNA (100 nM) or control siRNA (100 nM) using Lipofectamine<sup>®</sup> RNAi MAX according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequent experiments were performed 24 h after transfection. The following siRNA sequences were used: siRNA-1 for circATXN7 sense, 5'-GGAAGGGAGCGGAAA GAAUGU-3', and antisense, 5'-AUUCUUUCCGCUCUUUCCCG-3'; siRNA-2 for circATXN7 sense, 5'-GGGAAG GGAGCGGAAAGAAUG-3', and antisense, 5'-UUCUUU CCGCUCUUUCCCGA-3'.

**RNA extraction and RT-qPCR analyses.** Total RNA was extracted from the tumor tissues, adjacent normal tissues and cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Next, the RNA concentration was measured by Uv-vis spectrophotometry (Evolution 60S; Thermo Fisher Scientific, Inc.) and 1,000 ng total RNA was reverse transcribed in a final volume of 20  $\mu$ l using random primers under standard conditions with the PrimeScript RT Reagent kit with gDNA Eraser (cat. no. RR047A; Takara Biotechnology Co.,

Ltd., Dalian, China). qPCR was then performed using SYBR Select Master Mix (cat. no. 4472908; Applied Biosystems; Thermo Fisher Scientific, Inc.) with 0.5  $\mu$ l cDNA on a CFX96 Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol.  $\beta$ -actin was used as an internal control to determine the levels of circATXN7 expression. The primer sequences used were as follows:  $\beta$ -actin forward, 5'-GAAATCGTGCCTGAC ATTAA-3', and reverse, 5'-AAGGAAGGCTGGAAGAGT G-3'; circATXN7 forward, 5'-CCTAGGGACAGAATTGGA CGA-3', and reverse, 5'-GCCCCGCTCCGACATTCTT-3'. The qPCR reaction included an initial denaturation step in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. The relative levels of gene expression were normalized to  $\beta$ -actin and calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method to determine fold changes of circATXN7 expression (tumor vs. normal) (17). The PCR products were subjected to Sanger sequencing by TSINGKE Biological Technology Co., Ltd. (Beijing, China).

**Nucleic acid electrophoresis.** The cDNA and gDNA PCR products were investigated using 4% agarose gel electrophoresis with TBE running buffer. The primer sequences used were as follows: divergent forward, 5'-CCTAGGGACAGA ATTGGACGA-3', and reverse, 5'-GCCCCGCTCCGACATT CTT-3'; convergent forward, 5'-CTATCGTTTGCTGGGTTG CG-3', and reverse, 5'-ACTTTCGTCCAATTCTGTCCCT-3'.

**Cell proliferation assay.** Cell proliferation was detected using an MTT assay (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Briefly, the transfected cells were seeded into 96-well plates (3,000 cells/well), and the proliferation rates were measured at 0, 24, 48, 72 and 96 h after transfection. A total of 20  $\mu$ l MTT solution was added to each well and incubated for 2 h at 37°C. Next, the medium in each well was discarded, and 150  $\mu$ l dimethyl sulfoxide was added. Subsequent to shaking the plate for 15 min, the absorbance was measured spectrophotometrically at 490 nm. The data are representative of three individual experiments, where were conducted in triplicate.

For the EdU assays, at 24 h after transfection, 6x10<sup>4</sup> cells/well were seeded in a 24-well plate. After a further 24 h of incubation, EdU was added to a final concentration of 50  $\mu$ M per well. Following incubation with EdU for 2 h, the cells were fixed for 20 min with 150  $\mu$ l 4% formaldehyde solution in PBS. EdU labelling with the kFluor488-azide was performed using a kFluor488 Click-It EdU Cell Proliferation Assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). For excitation of kFluor488-azide, a 495-nm laser was used under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

**Clonogenic assay.** A total of 600 cells/well were transfected in a 6-well plate with 2 ml medium containing 10% FBS. RPMI-1640 medium and DMEM were used for A549 and SPC-A1 cells, respectively. The medium was changed every 3 days. After 10 days, the cells were immobilized using 4% paraformaldehyde and stained with 0.1% crystal violet for 15 min at room temperature. The visible colonies of each well were then manually counted (11).

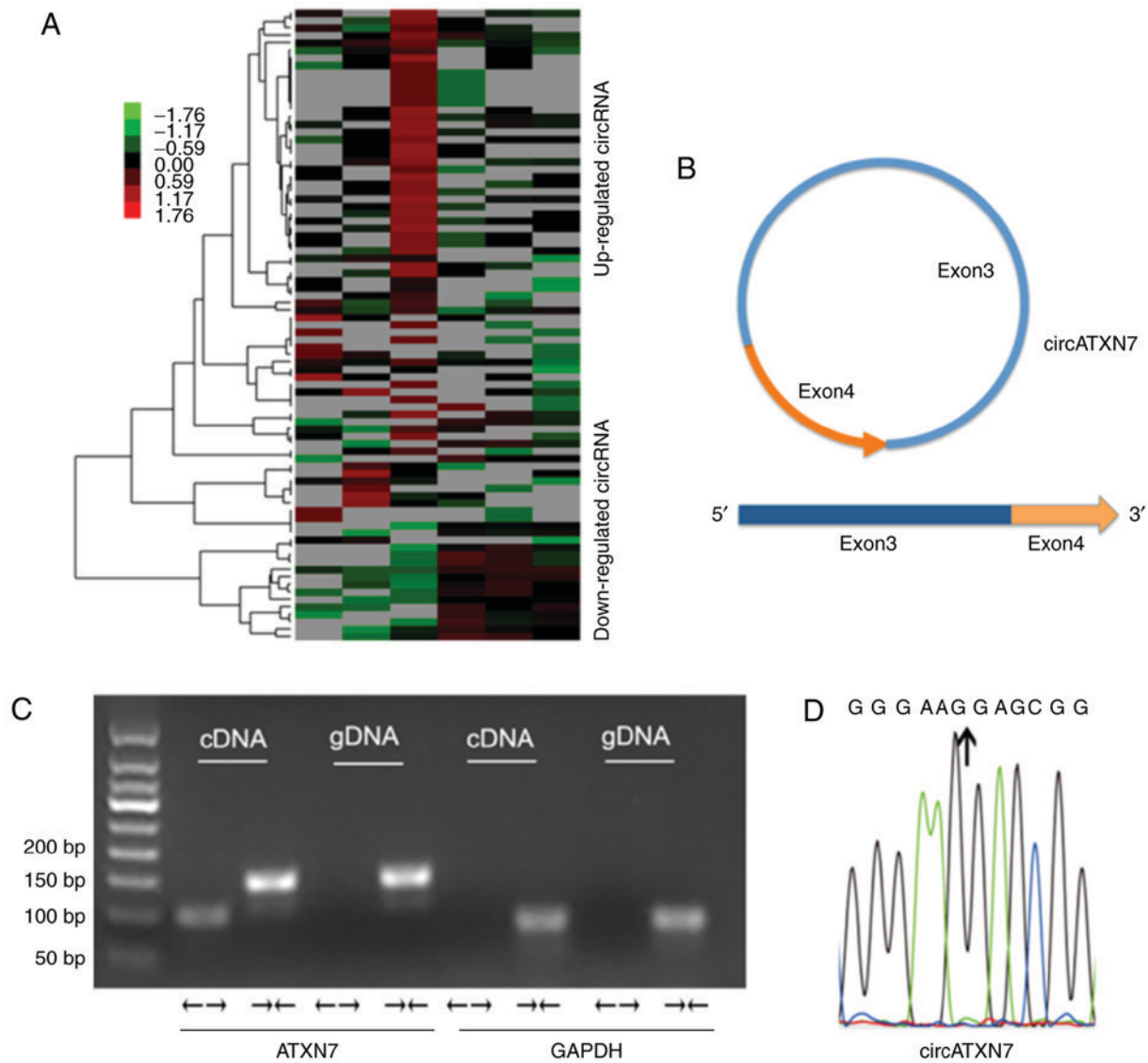


Figure 1. Expression profiles of circRNAs in NSCLC tissues compared with the adjacent normal tissues. (A) Heat map demonstrating the differential expression and hierarchical clustering of circRNAs between NSCLC and adjacent normal tissues. (B) circATXN7 is back-spliced by exons 3 and 4 of ATXN7. (C) Divergent primers detected circRNAs in cDNA, but not gDNA. The arrows indicate the direction of the primers: ←→ indicates divergent primers and →← indicates convergent primers. (D) Sanger sequencing of circATXN7 indicated the back-splice junction. The different colors represent different DNA bases. circRNA, circular RNA; NSCLC, non-small cell lung cancer.

**Invasion assay.** A Transwell assay was performed to investigate the migration and invasion of human lung cancer SPC-A1 and A549 cells. Briefly, the cells were transfected with 100 nM siRNA-circATXN7 or siRNA-NC for 24 h, and then plated at a density of  $1 \times 10^5$  cells/well in the upper Transwell chamber (pore size,  $8 \mu\text{m}$ ; Corning, Inc., Corning, NY, USA), which contained 200  $\mu\text{l}$  of the corresponding serum-free medium. RPMI-1640 medium or DMEM containing 10% FBS was added to the lower chamber for A549 and SPC-A1 cells, respectively. After 48 h of incubation at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ , the migrated cells on the filter surface were fixed with methanol and stained with 0.01% crystal violet for 15 min at room temperature, while the cells remaining in the upper membrane were discarded. The cell numbers were calculated in five random fields under a microscope (CTR6000; Leica Microsystems GmbH, Wetzlar, Germany). Each experiment was performed in triplicate.

**Statistical analysis.** All statistical analyses in the present study were performed using the SPSS software package (version 20.0; IBM Corporation, Armonk, NY, USA) and GraphPad Prism software (version 7.0; GraphPad software Inc., La Jolla, CA, USA). All quantitative data are presented as the mean  $\pm$  standard deviation from at least three independent experiments. Two-tailed Student's t-test was used for the analysis of continuous variables. Differences among the three groups were analyzed by analysis of variance and Scheffe's test. The prognostic value of circATXN7 expression was further analyzed using the Kaplan-Meier method.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**circRNAs in NSCLC.** In our previous study, a number of circRNAs were detected to be overexpressed in NSCLC (15)

Table I. Associations between circATXN7 level and clinicopathological characteristics of patients with non-small cell lung cancer.

Characteristic	circATXN7		P-value
	Low expression (n=12)	High expression (n=45)	
Sex			0.577
Male	8	26	
Female	4	19	
Age, years			0.177
≥60	9	24	
<60	3	21	
TNM stage			0.058
I-II	7	13	
III-IV	5	32	
Lymph node metastasis			0.533
No	6	18	
Yes	6	27	

TMN, Tumor-Node-Metastasis; circATXN7, circular RNA ATXN7.

(Fig. 1A). In particular, one of the circRNAs was significantly overexpressed, which was subsequently defined as circATXN7, since it is derived from the ATXN7 gene. circATXN7 is back-spliced by exons 3 and 4 of ATXN7 (Fig. 1B). To characterize the circular form of circATXN7, convergent primers that amplify the linear transcript of ATXN7 and divergent primers that amplify circATXN7 were designed. The genomic DNA (gDNA) and complementary DNA (cDNA) of A549 cells were amplified by divergent and convergent primers, respectively. As indicated, the cDNA of the PCR products was amplified by the divergent primers, while both cDNA and gDNA of the PCR products were amplified by the convergent primers. GAPDH served as a negative control (Fig. 1C). Sanger sequencing confirmed the splicing junction site (Fig. 1D).

**Aberrant expression of circATXN7 in NSCLC.** To investigate the expression of circATXN7 in NSCLC, RT-qPCR analysis was performed in 57 pairs of NSCLC tissues and matched adjacent non-tumor tissues. It was observed that circATXN7 was upregulated in 45 of the 57 NSCLC tissues as compared with its expression in the matched non-tumor tissues (Fig. 2A). However, no significant associations were detected between the expression level of circATXN7 and the clinicopathological characteristics, including age, sex, lymph node metastasis or TNM stage (Table I) (18). The prognostic value of circATXN7 expression was demonstrated by the Kaplan-Meier survival curves. It was observed that the survival time of patients with high levels of circATXN7 was shorter compared with that of patients with low levels of circATXN7, although the association was not statistically significant ( $P>0.05$ ; Fig. 2B).

**circATXN7 promotes the proliferation and invasion abilities of NSCLC cells.** SPC-A1 and A549 were selected as the

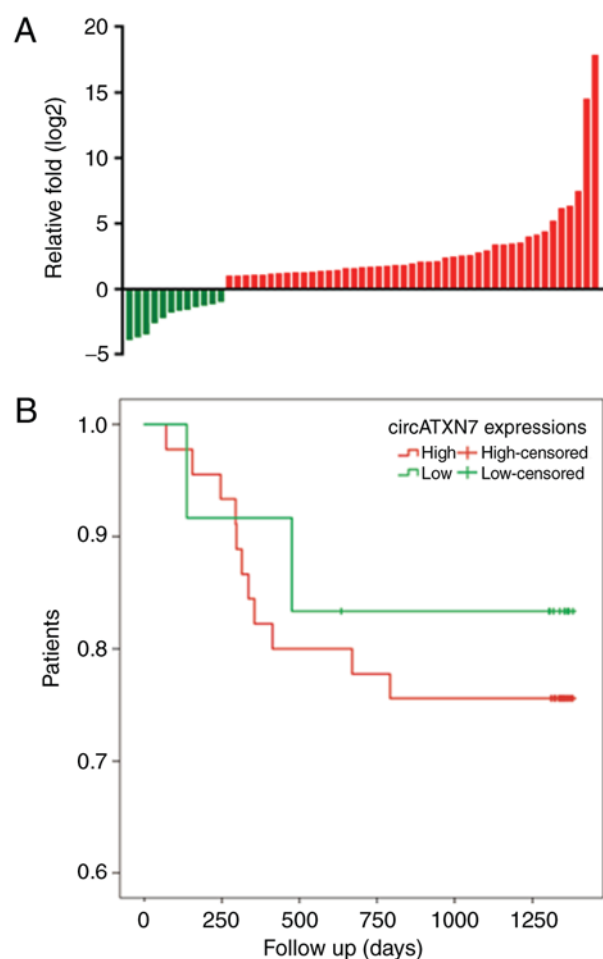


Figure 2. (A) circATXN7 expression was upregulated in 45 NSCLC tissues in comparison with the paired normal tissues, as determined by reverse transcription-quantitative polymerase chain reaction. (B) Correlation of NSCLC patient survival with the level of circATXN7 expression. NSCLC, non-small cell lung cancer.

experimental cell lines in the current study. Two siRNAs that specifically target the junction of the covalently joined 3' and 5' ends were designed to inhibit circATXN7 expression and investigate its biological function *in vitro*, and the circATXN7 silencing in A549 and SPC-A1 cells was successfully induced by transfection (Fig. 3A). Next, the results of the MTT assay indicated that the silencing of circATXN7 significantly suppressed the proliferative ability compared with that in cells transfected with negative control (NC) siRNA (Fig. 3B). Furthermore, the clonogenic assay demonstrated that silencing circATXN7 significantly decreased the number of clones in NSCLC cells (Fig. 3C). The EdU assay also suggested that circATXN7 silencing inhibited the proliferative ability of A549 cells (Fig. 4). Thus, these results revealed that circATXN7 silencing suppressed the proliferation of NSCLC cells.

The effect of circATXN7 on the invasive ability of NSCLC cells was then further analyzed. The Transwell assay confirmed that the silencing of circATXN7 markedly inhibited the invasive ability of A549 and SPC-A1 cells (Fig. 5). Taken together, the results indicated that circATXN7 silencing was able to inhibit the proliferation and invasion of lung cancer cells *in vitro*.

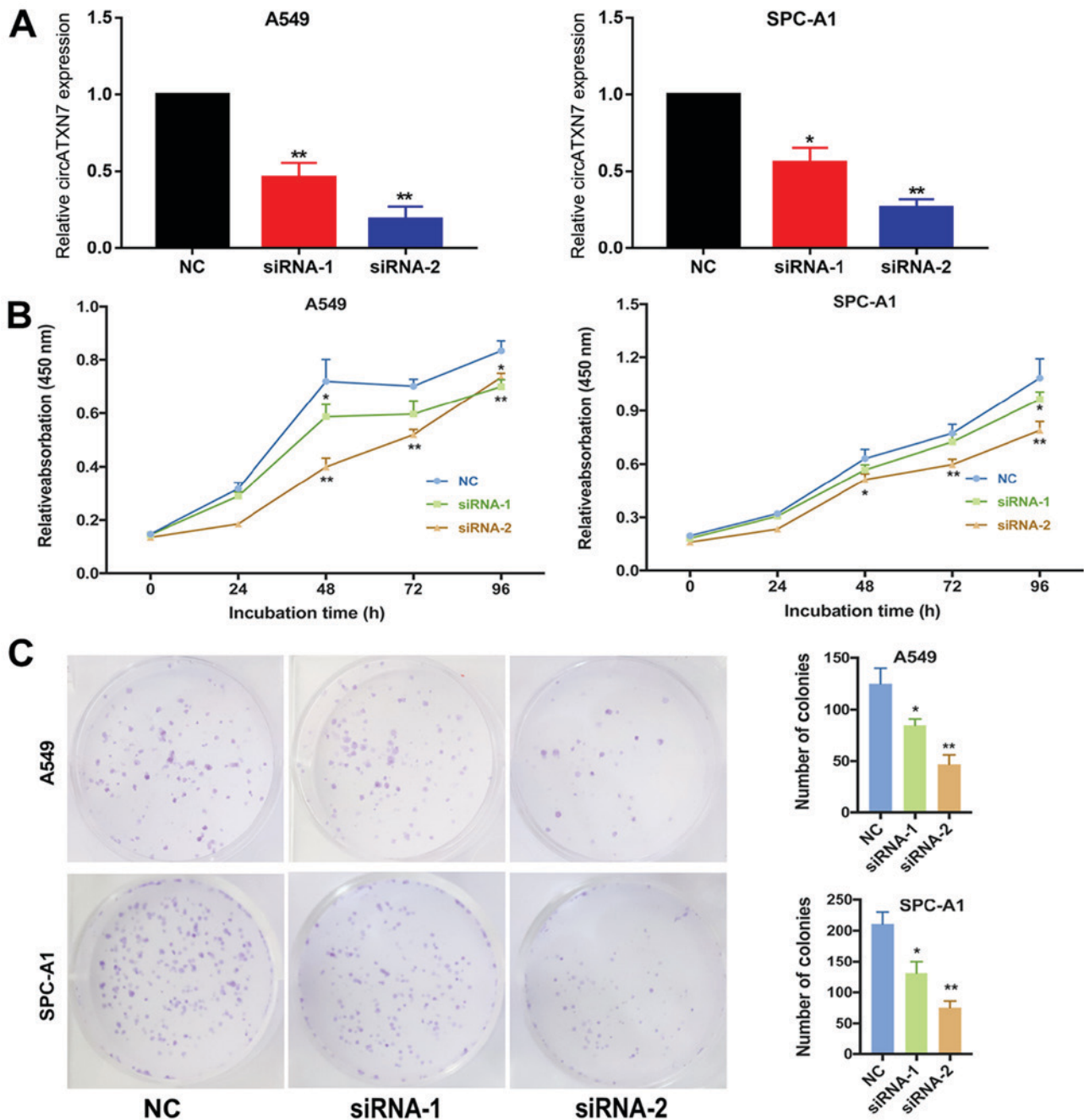


Figure 3. circATXN7 promotes the proliferation of two non-small cell lung cancer cell lines *in vitro*. (A) Reverse transcription-quantitative polymerase chain reaction analysis of circATXN7 following transfection with two siRNAs. (B) MTT assay indicates that siRNA transfection inhibited the proliferation of A549 cells and SPC-A1 cells. (C) Clonogenic assay, indicating the same results as the MTT assay. \* $P < 0.05$  and \*\* $P < 0.01$  vs. NC group. siRNA, small interfering RNA; NC, negative control.

## Discussion

circRNAs are a type of non-protein coding RNAs that are characterized by a covalently closed loop, and have been detected for decades in viruses, plants and animals (10). To date, the role and mechanism of circRNAs in the progression of malignancies remain to be identified (19). circRNAs are characterized by highly conserved sequences and have a specific covalently closed circular construction (20). They may serve considerable roles in the initiation and development of cancer, and may be candidate biomarkers for the diagnosis of diseases, such as cancer (21,22).

The tumorigenesis of NSCLC is a complex dynamic biological process that involves multiple genes (23). However, a limited number of studies have examined the association between circRNAs and NSCLC. Previously, we demonstrated that a novel circular RNA, circPRKCI, functions as a sponge for both miR-545 and miR-589 in NSCLC and inhibits their suppression of the pro-tumorigenic transcription factor E2F7 (14).

In the present study, a novel circRNA, namely circATXN7, was identified in NSCLC. The potential association of the expression of circATXN7 with clinical factors and its prognostic value were investigated. Next, the potential function of

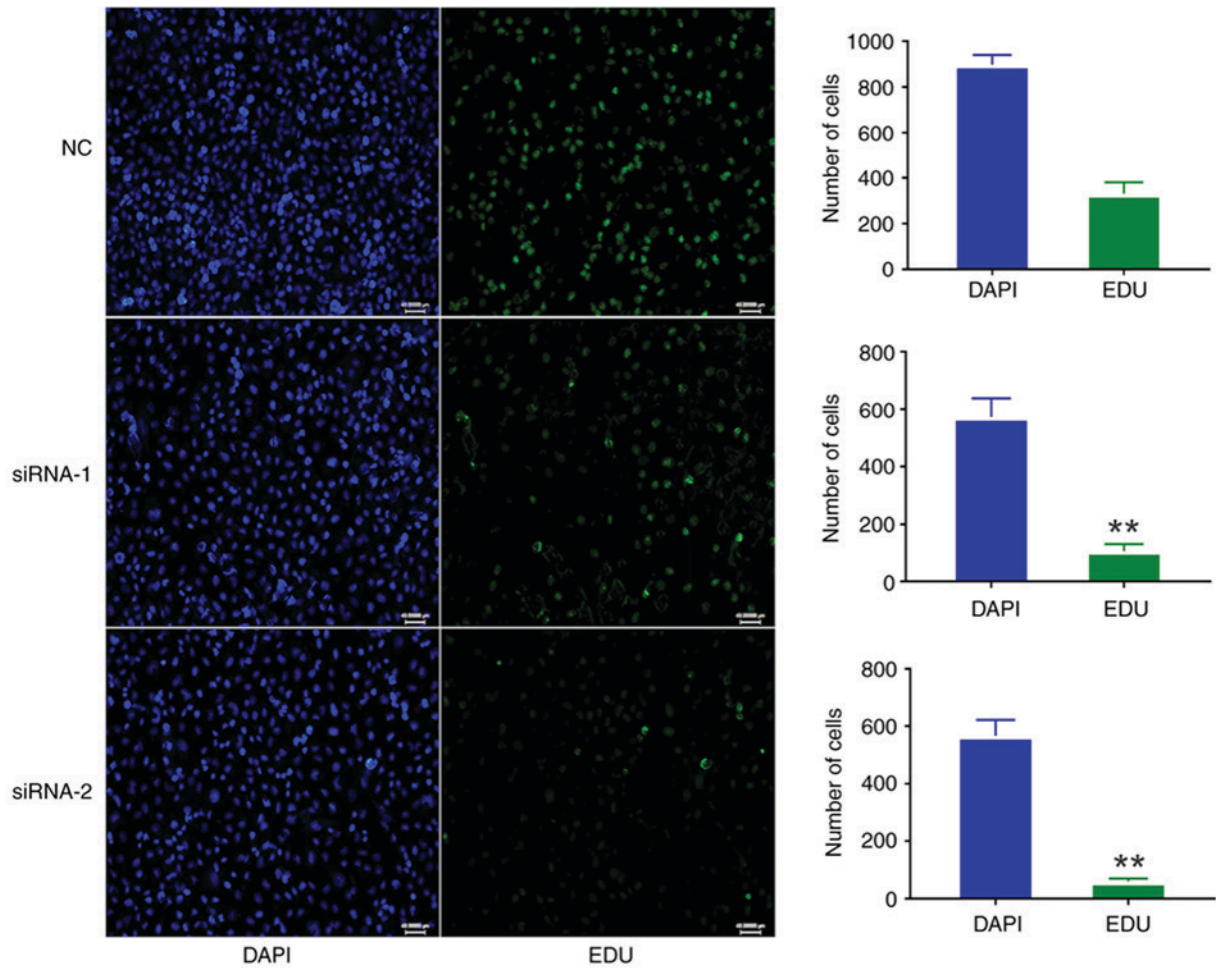


Figure 4. EDU assay demonstrated that transfection with siRNA targeting circATXN7 inhibited the proliferation of A549 cells. \*\* $P < 0.01$  vs. DAPI group. siRNA, small interfering RNA; NC, negative control.

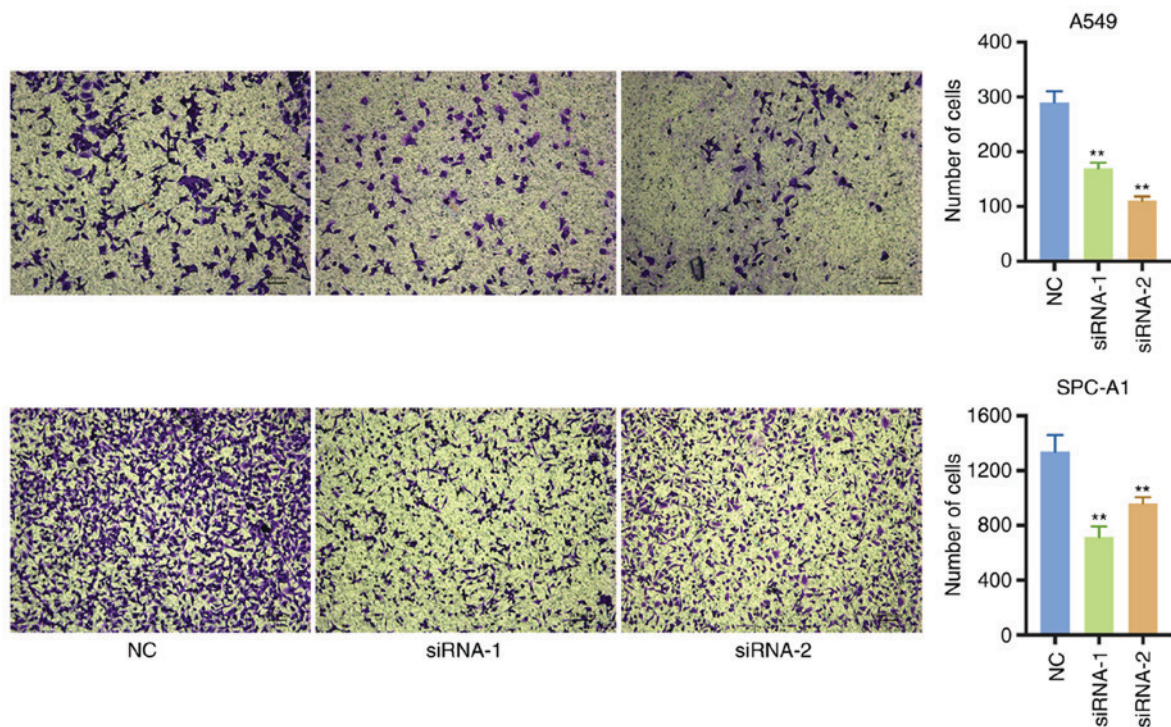


Figure 5. Transwell assay indicated that transfection with siRNA targeting circATXN7 inhibited the invasion ability of A549 and SPC-A1 cells. \*\* $P < 0.01$  vs. NC group. siRNA, small interfering RNA; NC, negative control.

circATXN7 was investigated by siRNA-mediated silencing. The findings of the current study indicated that the prognosis of patients with upregulation of circATXN7 was poorer compared with patients with low circATXN7 levels; however, the association was not statistically significant. Furthermore, there was no marked association between circATXN7 expression level and the majority of clinicopathological characteristics, including the patient age, cancer location, lymph node metastasis, tumor size, tumor differentiation, T stage or TNM stage. Overall, the association between circATXN7 expression level and the overall survival of patients with NSCLC requires further research.

Silencing of circATXN7 by siRNA was subsequently conducted in the current study to investigate its biological functions in NSCLC cells. The results indicated that circATXN7 silencing inhibited the proliferation and invasion of NSCLC cells, suggesting the oncogenic role of circATXN7 in NSCLC. Hsu and Coca-Prados (24) detected circRNAs in eukaryote cells using electron microscopy in 1979, and circRNAs have since been hypothesized to be promising biomarkers in numerous diseases, particularly in cancer, owing to sequence conservation and biological stability (25,26). circRNAs may harbor numerous miRNA binding sites, functioning as a huge 'sponges' that can consume target miRNAs (27). circHIPK3, which is derived from exon 2 of the HIPK3 gene, was reported to sponge 9 miRNAs with 18 potential binding sites (28). Therefore, further specific studies are required to analyze whether circATXN7 serves as a 'sponge' for miRNAs.

In conclusion, the present study is the first to identify that circATXN7 was upregulated in NSCLC tumor tissues. Furthermore, the downregulation of circATXN7 inhibited the proliferation and invasion abilities of NSCLC cells.

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#### Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

MQ, JW and QH designed experiments. QH, MQ, SW, XL, FY, CF and KZ performed the experiments. MQ and QH performed the data analysis and wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All procedures in the current study involving human participants were performed in accordance with the standards of the Ethical Committee of Peking University People's Hospital, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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