

Hsa_circ_0033155: A potential novel biomarker for non-small cell lung cancer

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Abstract. Circular RNAs (circRNAs) are a novel type of endogenous RNAs and increasing evidence have uncovered the important role of circRNA in tumor progression; however, the function of circRNAs in non-small cell lung cancer (NSCLC) remains largely unclear. In the present study, it was demonstrated that the expression level of hsa_circ_0033155 was significantly downregulated in NSCLC tissue and the expression of hsa_circ_0033155 correlated with lymphatic metastasis. In order to further investigate the possible role of hsa_circ_0033155 in NSCLC progression, circRNA was overexpressed in NSCLC cells and it was observed that the overexpression of hsa_circ_0033155 significantly decreased cell proliferation, colony formation and migration, and elevated the level of phosphatase and tensin homolog deleted on chromosome 10, a tumor suppressor in many types of tumor. In conclusion, hsa_circ_0033155 may serve as a prospective biomarker for detection and a promising therapeutic target for NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide and >80% cases are non-small cell lung cancer (NSCLC) (1). Despite consistent advances in the diagnosis and molecular targeted therapy techniques for effective control of lung cancer, the clinical outcomes remain unsatisfactory (2). The number of mortalities due to lung cancer is estimated to be 154,050 in 2018, accounting for 25.27% of all cancer-associated mortalities (1), and the overall 5-year survival rate of patients with lung cancer remains poor at <20% (3). These poor statistics are primarily due to the fact that the majority of patients

present with advanced disease stage at the time of diagnosis, at which point, the optimal time to perform surgery has passed (4). Therefore, improving the rate of early diagnosis is particularly important in increasing the opportunities for surgical intervention of patients with NSCLC, and identification of promising diagnostic markers for early detection is urgent.

Circular RNA (circRNA), which widely exists in mammalian cells, has received increasing focus in endogenous noncoding RNA research (5). One important characteristic of circRNA is the high level of stability that provides great possibilities as a biological marker (5,6). Additionally, emerging studies have suggested the aberrant expression of circRNAs in various diseases, including esophageal squamous cell carcinoma, gastric cancer and pancreatic ductal adenocarcinoma (7,8). It has been suggested that circRNAs act as microRNA (miRNA) sponges, regulating gene expression, and serving important roles in tumorigenesis and tumor progression (7,8). The aforementioned evidence indicates the potential value of circRNAs as novel biomarkers and therapeutic targets for cancer diagnosis and treatment. At present, the roles of circRNAs in the progression of NSCLC remain unclear (9,10). The majority of valuable circRNAs, including hsa_circ_0000190 in gastric cancer (11), are potential biomarkers with an increased sensitivity and specificity compared with classic biomarkers for the diagnosis of malignancies and includes carcinoembryonic antigen and CA19-9. circRNAs have not been comprehensively investigated in the field of heterogeneous NSCLC (12).

In the present study the circRNA, hsa_circ_0033155 was investigated, which was demonstrated to be downregulated in NSCLC tissues according to our previous circRNA microarray analysis (unpublished data). To the best of our knowledge, the present study revealed for the first time that the expression level of hsa_circ_0033155 was significantly downregulated in NSCLC tissues. Subsequently, the association between hsa_circ_0033155 expression and clinicopathological parameters of NSCLC was analyzed. In order to study the role of hsa_circ_0033155 in NSCLC progression, the biological functions of hsa_circ_0033155 were investigated. In addition, the levels of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a modulator of cell survival, which has been identified as a tumor suppressor in various tumor types (13), were evaluated.

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Materials and methods

Specimens. All clinical samples were collected from the respiratory department of Shanghai Jiaotong University Affiliated Sixth People's Hospital (Shanghai, China) between April 2013 and June 2016. NSCLC tissues and their matched adjacent non-tumorous tissues 5 cm from the edge of the tumor were obtained from 40 surgical patients, (males, 23; females, 17; age range, 36-57 years). All tissue specimens were immediately preserved in RNA-fixer reagent (Biotek Corporation, Beijing, China) following resection and stored at -80°C prior to use in subsequent experiments. No patients received radiotherapy, chemotherapy or targeted therapy prior to surgery. Tumor size was calculated using the widest diameter determined on computerized tomography (CT) images. Magnetic resonance imaging (MRI) and CT findings were used to diagnose lymphatic metastasis and to evaluate tumor differentiation, respectively. Tumor histological grading and staging were performed according to the World Health Organization classification criteria and the Tumor Node Metastasis system of the International Union Against Cancer (7th edition). The present study was approved by the Human Research Ethics Committee of Shanghai Jiaotong University and written informed consent was obtained from all patients.

Cell culture. NSCLC cell lines HCC827 and H1975 were purchased from American Type Culture Collection (Manassas, VA, USA) and normal human bronchus epithelium cell line BEAS-2B and NSCLC cell lines PC9 and H1650 were from Shanghai Suer Biological Technology Co., Ltd. (Shanghai, China). The cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were incubated at 37°C with 5% CO_2 .

Transient transfection. PC9 and H1650 were cultured in the above mentioned medium, seeded in 6-well plates at a density of 4.0×10^5 cells/well and incubated at 37°C in 5% CO_2 . The following day, cells were transfected with the overexpression vector (14 μg) for hsa_circ_0033155 (pLCDH-ciR-hsa_circ_0033155; Genesee Biotech Co., Ltd., Guangzhou, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Negative control (NC) groups were established by transfection with the control vector (pLCDH-ciR; Genesee Biotech Co., Ltd.). Cells were cultured for 24 h following transfection. For selection of the successfully transfected clones, cells were seeded in 6-well plates (4,000 cells/well) and treated with 400 $\mu\text{g}/\text{ml}$ G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 10 days. Medium containing G418 was replaced every 3 days. Positive clones were verified using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Total RNA extraction and RT-qPCR. Total RNA from paired NSCLC and adjacent non-tumorous tissues as well as cell lines were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Then, ReverTra Ace (Toyobo Life Science, Osaka, Japan) was applied to reverse transcribe total RNA into single-stranded cDNA following the manufacturer's protocol. qPCR was performed using the SYBR Premix Ex Taq™ II

kit (Takara Biotechnology Co., Ltd., Dalian, China) in a final volume of 10 μl containing 0.5 μl cDNA, 0.5 μl each primer and 5 μl SYBR Green. Primers were synthesized by Sangon Biotech Co., Ltd., (Shanghai, China) and their sequences were as follows: GAPDH, forward 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse 5'-GGCTGTTGTCATACTTCT CATGG-3'; hsa_circ_0033155, forward 5'-GGGGTCAGG AAAGAAACTGC-3' and reverse 5'-TGTCGTCTTCTTGCA TCTGG-3'; PTEN, forward 5'-TGGATTGACTTAGACTT GACCT-3' and reverse 5'-GGTGGGTTATGGTCTTCAAAA GG-3'. Thermal cycling was as follows: 94°C for 5 min, then 42 cycles at 94°C for 5 sec and 60°C for 1 min. Data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method (14), whereby a higher ΔCt values indicates a lower expression of hsa_circ_0033155.

Cell proliferation assay. Cells in NC and hsa_circ_0033155 groups were seeded in 96-well plates at a density of 3.5×10^3 cells/well and incubated for 1-7 days, under the culture conditions stated above. A total of 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) was subsequently added to the wells and cultured at 37°C for 3 h, then the supernatant was discarded and dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added. Subsequently, the absorbance was detected at 492 nm to determine the proliferation rate. Cell proliferation was evaluated using the following equation: Proliferation rate = $A(\text{sample})/A(\text{control})$; where A indicates the absorption measured at 492 nm and the control describes the sample analyzed at day 0.

Colony formation assay. Cells in NC and hsa_circ_0033155 groups were seeded in 6-well plates (6.0×10^2 cells/well) and incubated at 37°C for 14 days. The medium was replaced every 2 days during the culture. Subsequently, the plates were washed with PBS and the colonies were fixed with 100% methanol at room temperature (RT) for 15 min, then stained with 1% crystal violet at RT for 30 min. Finally, stained colonies with >50 cells were counted under a light microscope at x200 magnification.

Migration assay. The migration assay was performed in cells from NC and hsa_circ_0033155 groups using 24-well Transwell inserts (8- μm pore size; EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. A total of 1.0×10^4 cells were suspended in serum-free RPMI-1640 and seeded in the upper chamber, whereas RPMI-1640 containing 10% FBS was added to the lower chamber. Following incubation at 37°C for 24 h, cells in the upper surface were removed and cells remaining on the bottom surface were fixed with 100% methanol at RT for 15 min, followed by staining with 1% crystal violet at RT for 30 min. Finally, stained cells were photographed under a light microscope at x200 magnification and counted in at least five randomly selected fields.

Western blot analysis. NC and hsa_circ_0033155 cell lysates were prepared using cell lysis buffer (Sigma-Aldrich; Merck KGaA) and then centrifuged ($13,800 \times g$; 5 min; 4°C). Total protein concentration was detected using Bradford protein assay kit from Hangzhou MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China) following the manufacturer's protocol. Protein (20 μg) was separated on 12% SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride membranes. The membranes were incubated with the

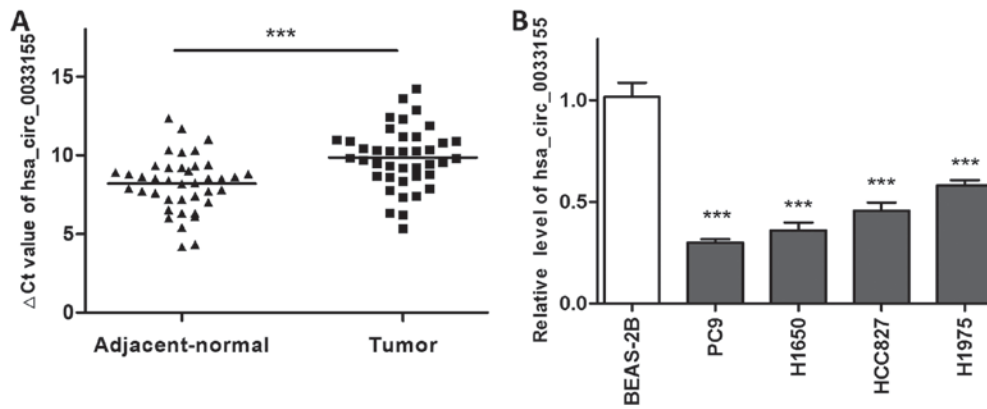


Figure 1. Hsa_circ_0033155 was downregulated in NSCLC tissues and cell lines. (A) Relative Δ Cq value of hsa_circ_0033155 in 40 pairs of NSCLC tissues and the non-tumorous tissues. *** $P < 0.001$. (B) The level of hsa_circ_0033155 in NSCLC cells. *** $P < 0.001$ vs. BEAS-2B. NSCLC, non-small cell lung cancer.

corresponding monoclonal antibody at 4°C overnight followed by incubation with a secondary antibody at RT for 1 h. GAPDH was used as the loading control. Primary antibodies against PTEN (#9188; 1:1,000), GAPDH (#5174; 1:1,000) and horseradish peroxidase-conjugated secondary antibody (#7074; 1:2,000) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Bands were visualized with an enhanced chemiluminescence kit from Pierce Biotechnology, Inc. (Thermo Fisher Scientific, Inc.).

Statistical analysis. SPSS 16.0 (IBM Corp., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) were used for data processing. Data are presented as the mean \pm standard deviation, and each experiment was performed at least three times. Paired and unpaired Student's t-tests were used to analyze significant differences between two groups in tissue samples and cultured cells, respectively. Comparisons of data among multiple groups were performed by one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hsa_circ_0033155 is downregulated in NSCLC tissues and cell lines. The expression level of hsa_circ_0033155 was initially detected in NSCLC and the matched adjacent non-tumorous tissues using RT-qPCR. As presented in Fig. 1A, the Δ Cq values in the NSCLC tissues were significantly higher compared with those in the adjacent non-tumorous tissues, suggesting that the expression of hsa_circ_0033155 in the tumor samples was downregulated. To validate this, the expression level of hsa_circ_0033155 was then measured in four NSCLC cell lines. qPCR results indicated that the levels of circRNA in PC9, H1650, HCC827 and H1975 cells were significantly lower compared with that in BEAS-2B, a normal human bronchus epithelium cell line (Fig. 1B).

Clinical diagnostic value of hsa_circ_0033155 in NSCLC. Subsequently, the association between hsa_circ_0033155 expression levels and clinicopathological characteristics of patients with NSCLC was evaluated. As presented in Table I, the aberrant expression of hsa_circ_0033155 in NSCLC tissues was significantly correlated with lymphatic metastasis

Table I. Associations between hsa_circ_0033155 expression levels and clinical pathological characteristics of patients with NSCLC.

Characteristic	Cases, n (%)	Δ Cq (mean \pm SD)	P-value
Age, years			
≤ 60	24 (60.0)	9.86 \pm 1.47	0.973
> 60	16 (40.0)	9.83 \pm 2.56	
Gender			
Male	22 (55.0)	9.51 \pm 2.22	0.229
Female	18 (45.0)	10.26 \pm 1.51	
Tumor size, cm			
≤ 5	21 (52.5)	9.48 \pm 1.72	0.213
> 5	19 (47.5)	10.25 \pm 2.14	
Lymphatic metastasis			
Yes	12 (30.0)	10.90 \pm 2.27	0.024
No	28 (70.0)	9.40 \pm 1.63	
TNM stage			
\leq II	18 (45.0)	9.83 \pm 1.78	0.971
$>$ II	22 (55.0)	9.86 \pm 2.11	
Tumor differentiation			
Well	13 (32.5)	10.01 \pm 1.85	0.722
Moderate	16 (40.0)	9.91 \pm 1.95	
Poor	11 (27.5)	9.57 \pm 2.20	

Δ Cq = Cq(hsa_circ_0033155) - Cq(GAPDH). NSCLC, non-small cell lung cancer; SD, standard deviation; TNM, Tumor Node Metastasis.

($P = 0.0237$), whereas no significant association was detected between hsa_circ_0033155 expression and other clinicopathological characteristics.

Overexpression of hsa_circ_0033155 decreases cell proliferation. The aforementioned results suggested that downregulated hsa_circ_0033155 may serve a role in NSCLC progression. Therefore, the effects of hsa_circ_0033155 on tumor cell phenotypes were investigated. As relatively lower

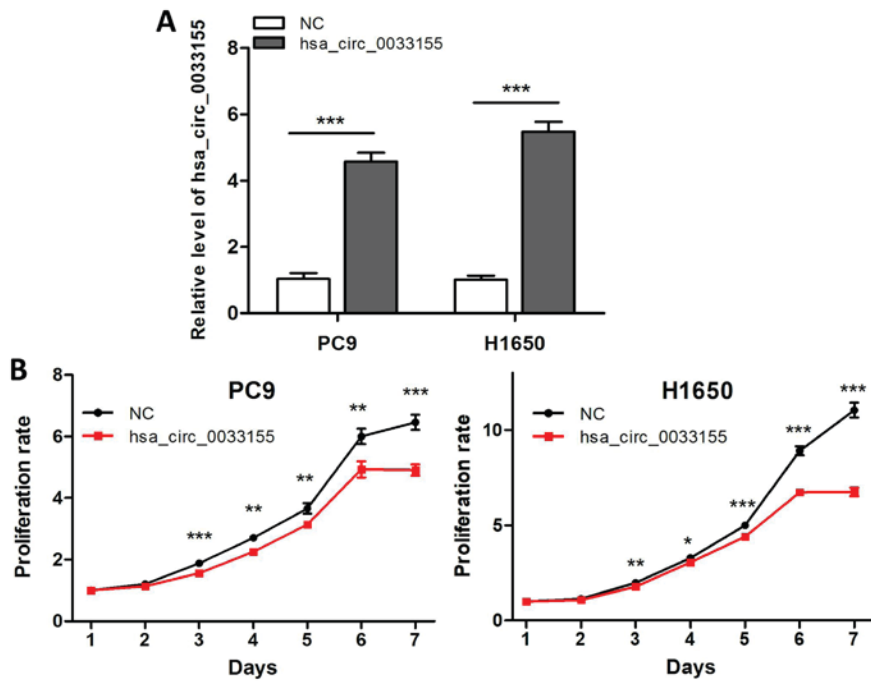


Figure 2. Overexpression of hsa_circ_0033155 decreased cell proliferation. (A) The level of hsa_circ_0033155 in hsa_circ_0033155 overexpressed cells. ***P<0.001. (B) The effect of hsa_circ_0033155 on cell proliferation. Cells were cultured for 7 days and a MTT assay was applied to detect cell viability daily. *P<0.05, **P<0.01, ***P<0.001 vs. hsa_circ_0033155. NC, negative control.

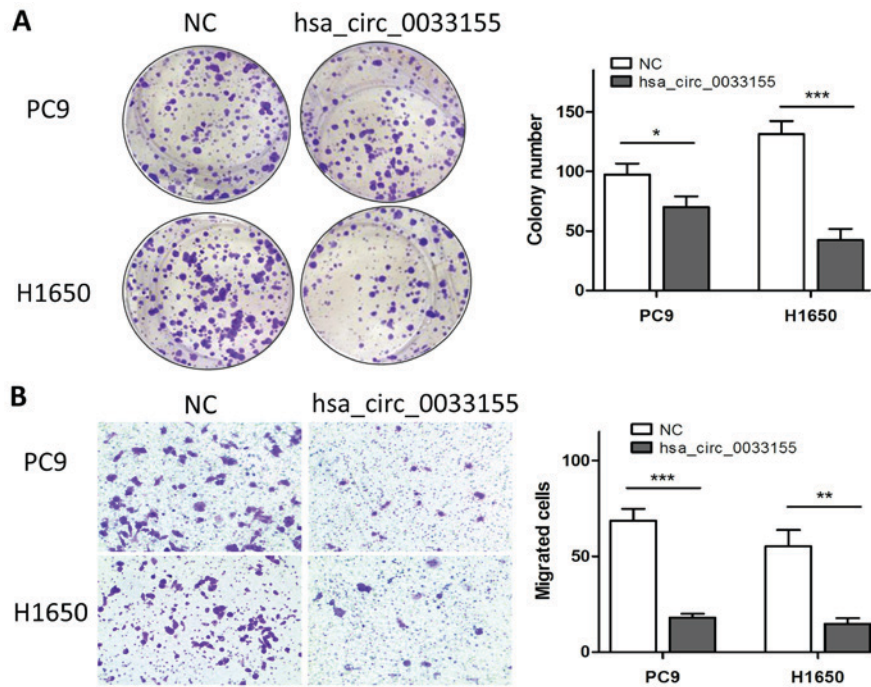


Figure 3. Overexpression of hsa_circ_0033155 inhibited colony formation and migration. (A) Colony formation ability of hsa_circ_0033155-overexpressed NSCLC cells. Cells were cultured for 14 days, then stained, numbered. (B) Migration ability of hsa_circ_0033155-overexpressed NSCLC cells. Cells were cultured for 24 h in a Transwell chamber, then stained and counted (magnification, x200). *P<0.05, **P<0.01, ***P<0.001. NC, negative control.

levels of hsa_circ_0033155 were observed in PC9 and H1650 cells compared with in HCC827 and H1975 cells (Fig. 1B), the former two cell lines were selected to overexpress hsa_circ_0033155. The results of the qPCR assay in Fig. 2A suggested that hsa_circ_0033155 was successfully transfected into the two cell lines. The MTT assay indicated that the overexpression of hsa_circ_0033155 exhibited a significant

decrease in cell proliferation following incubation for >2 days (Fig. 2B).

Overexpression of hsa_circ_0033155 inhibits colony formation and migration. The effects of hsa_circ_0033155 on colony formation were then determined following incubation for 2 weeks. As presented in Fig. 3A, the overexpression of

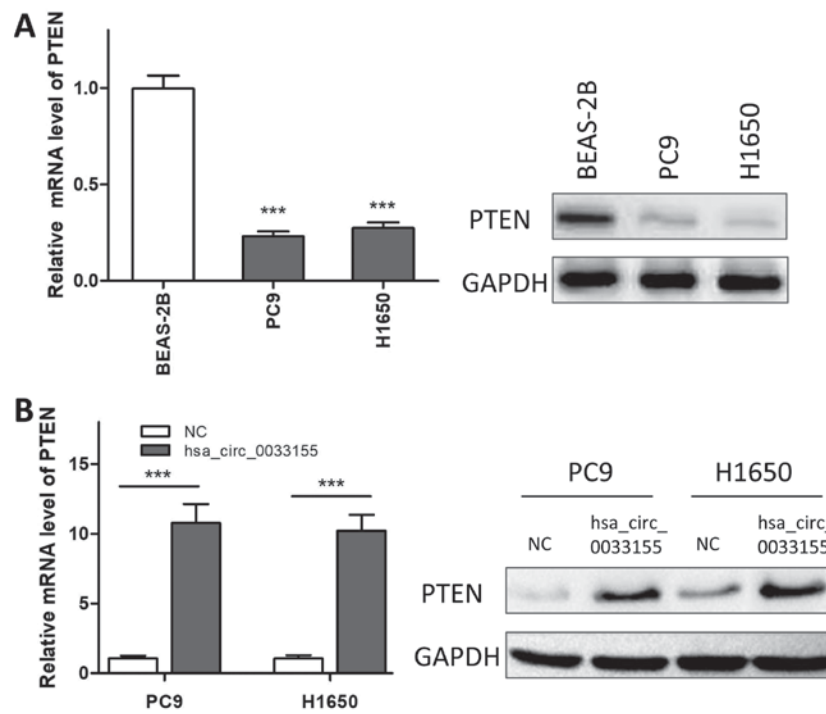


Figure 4. Overexpression of hsa_circ_0033155 upregulated the expression level of PTEN. (A) The mRNA and protein levels of PTEN in BEAS-2B and NSCLC cells. (B) The mRNA and protein level of PTEN in hsa_circ_0033155-overexpressed NSCLC cells. ***P<0.001. PTEN, phosphatase and tensin homolog deleted on chromosome 10; NC, negative control.

hsa_circ_0033155 significantly inhibited the colony formation ability of the two cell lines. The effect of hsa_circ_0033155 on H1650 cells was greater than that on PC9 cells. The effects of hsa_circ_0033155 on the migration ability of cells were also assessed. The number of cells that migrated was significantly inhibited following overexpression of hsa_circ_0033155 (Fig. 3B).

Overexpression of hsa_circ_0033155 upregulates the level of PTEN. As presented in Fig. 4, the mRNA expression of PTEN in PC9 and H1650 cells was significantly decreased compared with that in BEAS-2B cells, which was consistent with the western blotting results (Fig. 4A). The expression level of PTEN was then analyzed in hsa_circ_0033155-overexpressed cells. The results revealed that overexpression of hsa_circ_0033155 significantly increased the expression of PTEN at the mRNA and protein levels (Fig. 4B).

Discussion

circRNAs are a type of novel untranslated RNA molecule that lacks 5'-3' UTRs and a poly A tail, covalently forming closed loops, which were previously considered to be functionless products of errors in splicing (7). Notably, it is the unique circular structure that protects them from the degradation of exonuclease RNase R and confers these RNAs excellent stability (15). In addition, the expression level of circRNAs, including circular *ANRIL*, is higher compared with that of linear RNAs (16,17). In addition, their expression level and function are independent of linear RNA isomers (16,17). The aforementioned advantages indicate that, circRNAs may serve as ideal diagnostic biomarkers for cancer, and be superior to

other non-coding RNAs such as long non-coding RNAs and miRNAs (18,19).

In the present study, the expression of hsa_circ_0033155 was detected in 40 pairs of NSCLC tissues and the adjacent non-tumorous tissues with qPCR for the first time, to the best of our knowledge. Furthermore, the level of hsa_circ_0033155 was validated in NSCLC cell lines. The results demonstrated that the levels of hsa_circ_0033155 were significantly down-regulated in both NSCLC tissues and NSCLC cell lines compared with their corresponding controls. The dysregulated expression was significantly associated with lymphatic metastasis, suggesting the potential value of hsa_circ_0033155 as a biomarker for NSCLC diagnosis. Coincidentally, circRNA_100876 was found to be associated with lymphatic metastasis in NSCLC (20). Therefore, circRNAs including hsa_circ_0033155 from NSCLC biopsy samples may be combined with previously reported circ-ITCH and circRNA_100876, which were correlated with tumor staging, to increase positive diagnosis rate (21).

An increasing number of studies have revealed that the aberrant expression of circRNAs is associated with tumor progression. circRNA_100290 is markedly upregulated in oral squamous cell carcinoma tissue, and the knockdown of circRNA_100290 significantly inhibits the proliferative ability of cells (22). In lung cancer, interference of upregulated hsa_circ_0000064 markedly blocked cell cycle progression, promoted cell apoptosis, and decreased migration and invasive activities (23). Therefore, hsa_circ_0033155 was overexpressed in NSCLC cells with lower levels of the circRNA to detect whether the biological functions were attenuated. The results indicated that overexpression of hsa_circ_0033155 significantly decreased cell proliferation and colony formation. Furthermore,

the migration ability of cells, an important determinant of malignancy progression and metastasis, was also significantly inhibited. These results indicated that hsa_circ_0033155 may serve a cancer-suppressive role in NSCLC progression.

PTEN, a modulator of cell survival and cell cycle progression, has been identified as a tumor suppressor that is downregulated and mutated in various cancers, including hepatocarcinoma, glioblastoma, ovarian and prostate cancer (13,24,25). Therefore, aberrant hsa_circ_0033155 may be associated with the regulation of PTEN, thus regulating cell biological functions. It was also demonstrated that the level of PTEN in NSCLC cell lines was markedly decreased, and that the overexpression of hsa_circ_0033155 significantly enhanced the level of PTEN, revealing the regulatory role of hsa_circ_0033155 on PTEN expression. Emerging studies have demonstrated that circRNA functions as miRNA sponges, removing the inhibitory effect of miRNA on its target genes, and further regulating the expression of target genes, including PTEN (26,27). The miRNA that is regulated by hsa_circ_0033155, subsequently regulating the level of PTEN remains to be investigated.

In conclusion, downregulated hsa_circ_0033155 is associated with lymphatic metastasis in NSCLC and overexpression of hsa_circ_0033155 significantly decreased the proliferation, colony formation and migration abilities of NSCLC cells. Additionally, PTEN may serve an important role in these regulations. Overall, hsa_circ_0033155 may serve as a prospective biomarker and a promising target for NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

GW and XF worked on the conception and design of the study, and analyzed and interpreted the data. XG and HS collected and assembled data. All authors contributed to preparation of the manuscript and approved the final version.

Ethics approval and consent to participate

The present study was approved by the Human Research Ethics Committee of Shanghai Jiaotong University (Shanghai, China) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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