




## Research Article

# CircFBXW7 Inhibits Proliferation, Migration, and Invasion of Nonsmall Cell Lung Cancer Cells by Regulating miR-492

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Received 6 June 2022; Accepted 29 July 2022; Published 8 September 2022

Academic Editor: Dong-Hua Yang

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**Background.** CircFBXW7 has been determined to be involved in various cancers; however, its role in nonsmall cell lung cancer (NSCLC) remains unclear. This study examined the function and potential mechanism of circFBXW7 in NSCLC. **Methods.** The structure of circFBXW7 was verified via RT-PCR and Sanger sequencing. The expression of circFBXW7 in NSCLC was determined by qRT-PCR. The effect of circFBXW7 overexpression on the proliferation, migration, and invasion of NSCLC cells was examined by CCK-8 and Transwell assays. Furthermore, a circFBXW7-miRNA network was established to explore their interaction. Predicted miRNA was determined by qRT-PCR. Moreover, the miRNA mimics were synthesized, wherein its effect on proliferation, migration, and invasion of NSCLC cells overexpressed circFBXW7 was assessed. **Results.** The circularity of circFBXW7 was verified. The expression of circFBXW7 was found to be downregulated in NSCLC cells compared with that in normal human lung epithelial BEAS-2B cells. Overexpression of circFBXW7 reduced cell proliferation, migration, and invasion. Furthermore, according to the circFBXW7-miRNA network prediction and qRT-PCR validation, miR-492 was identified to be the target of circFBXW7. The inhibitory effect of circFBXW7 overexpression on cell proliferation, migration, and invasion was reversed by miR-492 mimics. **Conclusion.** CircFBXW7 is downregulated in NSCLC. CircFBXW7 inhibits NSCLC cells proliferation, migration, and invasion by regulating miR-492.

## 1. Introduction

Lung cancer is the leading cause of cancer-related death, and nonsmall cell lung cancer (NSCLC) accounts for 85% of lung cancer mortality [1]. Although the survival of lung cancer has increased due to early diagnosis and improved treatment, its mortality remains high [2]. To improve the treatment of NSCLC, a better understanding of the molecules involved in NSCLC and their potential mechanism of action is needed to find new reliable biomarkers and therapeutic targets.

Circular RNAs (circRNAs) are a class of regulatory noncoding RNA with circular covalently bonded structures [3]. CircRNAs differ from linear RNAs as they are more stable with high tolerance to exonuclease digest, particularly in tissues and cells [1, 3]. Therefore, it is valuable to examine whether circRNAs are reliable molecules for cancer diagnosis and treatment [4]. Many circRNAs are conserved among eukaryotes. They localize in specific subcellular compartments and play different biological roles. Accumulating evidence shows that circRNAs regulate a diversity of cellular processes by acting as miRNA sponges, anchors

for circRNA binding proteins (cRBPs), transcriptional regulators, molecular scaffolds, and sources for translation of small proteins/peptides. The understanding of the biological functions of circRNAs has brought a new perspective to our appreciation of cellular physiology and disease pathogenesis. Recent studies have shown that the expression of circRNAs is tissue and cell type-specific and is regulated in development or disease progression, where they exert specific biological functions [5]. Furthermore, circRNAs have been reported to regulate cancer progression by acting as competing endogenous RNA (ceRNA) and sponges for microRNA (miRNA) to control mRNA expression at the posttranscriptional level [1, 6]. For instance, circGDI2 was reported to regulate the miR-454-3p/FOXF2 axis in the process of proliferation, migration, invasion, and apoptosis in oral squamous cell carcinoma [7]. Several studies have shown that circ-SATB2 and circ-FGFR1 regulated cell proliferation, migration, and invasion via the miR-326/FSCN1 and miR-381-3p/CXCR4 axes, respectively [1, 8]. In addition, many studies confirmed that circRNAs are differentially expressed in patients with different stages of lung cancer and affect tumor recurrence and metastasis by targeting downstream miRNAs. Recently, researchers observed that circHMGB2 drives immunosuppression and antiPD-1 resistance in lung adenocarcinomas and squamous cell carcinomas via the miR-181a-5p/CARM1 axis [9].

CircFBXW7 is derived from the parent gene of F-box and WD repeat domain containing 7 (FBXW7) [10], which participates in the progression of cancer via the ceRNA mechanism and can be translated into protein. Yang et al. observed that circFBXW7 encodes an FBXW7-185 protein of 21 kDa that acts as a tumor suppressor in the human brain [11]. Gao et al. revealed that overexpression of circFBXW7 inhibits glioma proliferation and metastasis via the miR-23a-3p/PTEN axis [12]. However, the role of circFBXW7 in NSCLC has not been reported.

MiRNAs are short noncoding RNAs with about 22 nucleotides in length that are involved in the post-transcriptional regulation of gene expression [13]. MiRNAs play important roles in various biological processes, and abnormal miRNA expression is associated with diseases, including cancer [14]. Shi et al. reported that miR-19a promoted proliferation and migration of NSCLC cells by inhibiting PTEN expression [15]. Silencing miR-6515-3p has been shown to inhibit the proliferation of NSCLC cells [16]. Overexpression of miR-30c and miR-30d-3p inhibited the epithelial-to-mesenchymal transition (EMT) in NSCLC cells [17]. Besides, it has been reported that high expression of miR-492 was associated with poor prognosis of NSCLC patients. MiR-492 inhibitor inhibited the proliferation, migration, and invasion of tumor cells [18].

In this study, we aimed to examine the function and potential mechanism of action of circFBXW7 in NSCLC. Through bioinformatics analysis and validation, we identified miR-492 as the downstream target gene of circFBXW7 in NSCLC. The results of this study will provide potential use of circFBXW7 in the diagnosis and treatment of NSCLC, which will provide new ideas and targets for NSCLC research.

## 2. Methods

**2.1. Cell Culture.** Normal human lung epithelial BEAS-2B cells were obtained from Cellcook (Guangzhou, China) and were cultured in BEGM (#CM2010, Cellcook). NSCLC cells (HCC827, NCI-H358, NCI-H23, and NCI-H1299) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCC827, NCI-H358, NCI-H23, and NCI-H1299 were cultured in RPMI-1640 (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, GIBCO) at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>. Besides, NSCLC PC9 cells were obtained from Cellcook (Guangzhou, China) and were cultured in RPMI-1640. All cells were passaged three times before the experiments.

**2.2. Cell Transfection.** The miR-492 mimics, circFBXW7 overexpression plasmids, and negative control were provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). The mimics or plasmids were transfected into NCI-H23 and NCI-H1299 cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific).

**2.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-Time Quantitative-PCR (qRT-PCR).** RNA was extracted from cells using TRIzol (Thermo Fisher) reagents and was used to produce cDNA via a cDNA Synthesis Kit (Takara, Japan). For reverse transcription (RT)-PCR, divergent primers and convergent primers were used to amplify products for agarose gel electrophoresis using 2× Taq Master Mix (Vazyme, Nanjing, China). The products of divergent primers of circFBXW7 were then Sanger sequenced. For qRT-PCR, AceQ® Universal SYBR qPCR Master Mix (Vazyme) was used to test the gene expression on an ABI 7500 real-time PCR instrument. The primers used in this study are shown in Table 1. *GAPDH* was used as the internal control for circRNA and mRNA expression, and *U6* was used as the internal control for miRNA expression. The relative expression of genes was calculated using the  $2^{-\Delta\Delta Ct}$  method.

**2.4. Cell Counting Kit-8 (CCK-8) Assay.** After transfection for 48 h, cells were digested by trypsin (Gibco) and then were seeded into a 96-well plate at a density of  $4 \times 10^3$  cells per well. CCK-8 (Sigma) was added to each well after 24, 48, and 72 h. After 3 h, the light absorbance value at 450 nm was measured using a microplate reader (Beckman Coulter, USA).

**2.5. Colony Formation Assay.** After transfection for 48 h, cells were digested by trypsin (Gibco) and then seeded into a 6-well plate at a density of 600 per well. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 weeks. Then, cells were fixed for 30 min using formaldehyde and stained for 20 min using 0.1% crystal violet. After that, the plates were air-dried and the number of cell colonies was counted.

TABLE 1: The primer sequences in this study.

Name	Sequences (5'-3')
circFBXW7-CF	CCAGTAGTATTGTGGACCTGCC
circFBXW7-CR	CCCTCTGACCCAGTAACTCCACT
circFBXW7-LF	TGTGTCTGAAGGCAAGATTCTGT
circFBXW7-LR	CAGTTAATTTGGGAAATGCTGAGGT
GAPDH-F	GAGTCAACGGATTTGGTCGT
GAPDH-R	GACAAGCTTCCCCTTCAG
miR-492-F	AACAATAGGACCTGCGGGAC
miR-492-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGACTGGATACGACAAGA AT
Universe-R	GTGCAGGGTCCGAGGT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTACGAATTTGCGT

**2.6. Transwell Assay.** Cells were seeded into the upper chamber of a 24-Transwell system at a concentration of  $4 \times 10^4$  cells per well in a 200  $\mu$ L medium. Filters were precoated with Matrigel for testing cell invasion ability; 600  $\mu$ L RPMI-1640 medium plus 10% FBS was added into the bottom chamber. After 48 h, migrated cells were fixed for 30 min using formaldehyde and stained for 20 min using 0.1% crystal violet. An Olympus CKX53 inverted microscope (Olympus, Japan) was used to examine cell migration.

**2.7. Network Construction.** Potential miRNAs of circFBXW7 were predicted through miRanda software and miTarBase database, and Cytoscape software was performed to plot the circRNA-miRNA regulatory network. In addition, miR-492 was reported to regulate NSCLC progression [18–20].

**2.8. Bioinformatics Prediction and Dual-Luciferase Assay.** miRanda predicted the binding site of circFBXW7 and miR-492. CircFBXW7 sequences containing potential wild-type (WT) or mutant (MUT) binding sites were constructed into pmirGLO vectors (Promega Corporation, Fitchburg, WI, USA), and luciferase reporters were analyzed. Vectors, NC mimics, and miR-492 mimics were cotransfected into cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). After 48 h transfection, the luciferase activity was detected by dual-luciferase reporter assay (Promega Corporation).

**2.9. Statistical Analysis.** Data were analyzed using SPSS 15.0 software (IBM, USA) and presented as mean  $\pm$  standard deviation. The difference between the two groups was determined using Student's *t*-test. A *p* value of  $<0.05$  was identified as statistically significant.

### 3. Results

**3.1. Sequence and Structure of circFBXW7.** We first confirmed the origin and the structure of circFBXW7, which consisted of 620 nucleotides and was derived from exons 3 and 4 of *FBXW7* (Figure 1(a)). Divergent and convergent primers were designed to validate the structure of

circFBXW7; divergent primers amplify products from cDNA but not from genomic DNA (Figure 1(b)). Sanger sequencing of the amplified products verified the splice junction and the circular structure of circFBXW7 (Figure 1(c)). The sequence in circBase (<https://circrna.org/>) is shown in Figure 1(d). The above results indicated that circFBXW7 was a closed loop transcribed from exons 3 and 4 of *FBXW7*.

**3.2. Overexpression of circFBXW7 Reduced Cell Proliferation, Migration, and Invasion.** To examine the expression of circFBXW7 in NSCLC, we conducted qRT-PCR. As shown in Figure 2(a), expression of circFBXW7 was downregulated in NSCLC cells (HCC827, PC9, NCI-H23, and NCI-H1299) compared with that in normal human lung epithelial BEAS-2B cells. NCI-H23 and NCI-H1299 cells were determined to have the lowest expression of circFBXW7 and were selected for functional study. The overexpression plasmids were transfected into these cells for 48 h, followed by qRT-PCR to assess the efficiency of overexpression of circFBXW7. The results showed that the expression of circFBXW7 was significantly upregulated in the overexpression group, which was more than 3 times that of the control group. Following overexpression-circFBXW7 (oe-circFBXW7) transfection, the level of circFBXW7 was significantly increased compared with that in the oe-NC-treated group (Figure 2(b)). CCK-8 results showed that overexpression of circFBXW7 reduced cell proliferation (Figures 2(c) and 2(d)) and colony formation (Supplement Figure 1). In addition, overexpression of circFBXW7 reduced migration and invasion of NCI-H23 and NCI-H1299 cells in Transwell assays (Figures 2(e) and 2(f)). The above results indicated that circFBXW7 expression was downregulated in NSCLC and that overexpression of circFBXW7 reduced cell proliferation, migration, and invasion of NSCLC cells.

**3.3. Bioinformatics Analysis and Preliminary Verification of circFBXW7 Downstream Target miRNA.** To explore the potential mechanism of action of circFBXW7 in NSCLC, we predicted the potential miRNAs of circFBXW7 by miRanda software and miTarBase database and plotted circRNA-

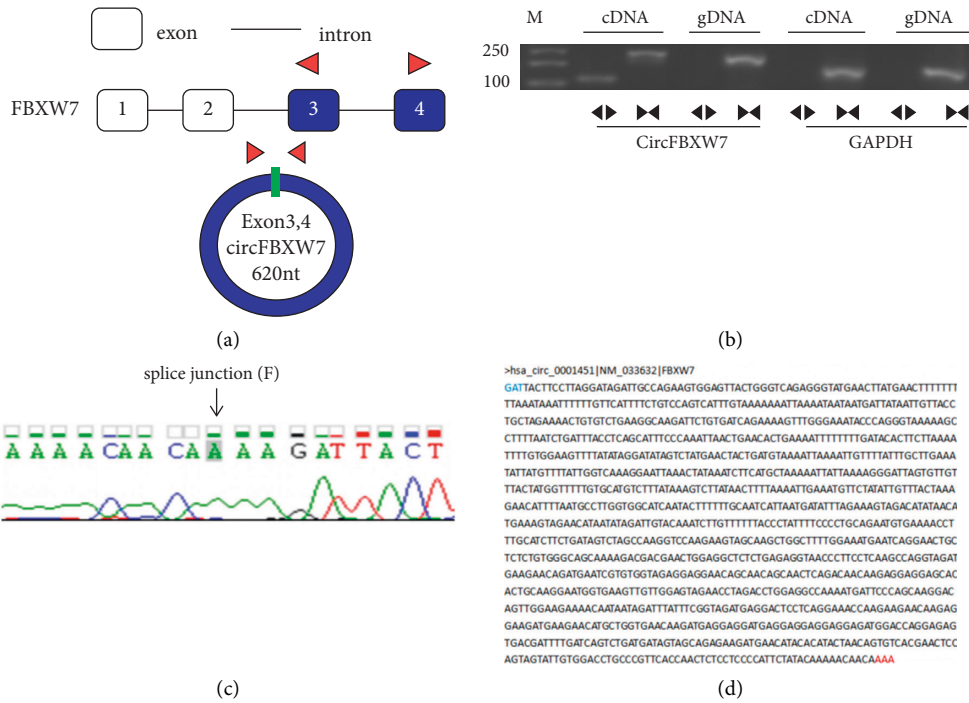


FIGURE 1: The sequence and structure of circFBXW7. (a) circFBXW7 contains 620 nucleotides and was derived from exons 3 and 4 of *FBXW7*. (b) Divergent and convergent primers designed for PCR to validate the structure of circFBXW7. *GAPDH* as control. (c) The splice junction of circFBXW7. The products of divergent primers were collected for Sanger sequencing. (d) The sequence of circFBXW7 in circBase (<https://circrna.org/>). *FBXW7*, F-box and WD repeat domain containing 7; M, DNA marker; cDNA, complementary DNA; *GAPDH*, glyceraldehyde-3phosphate dehydrogenase.

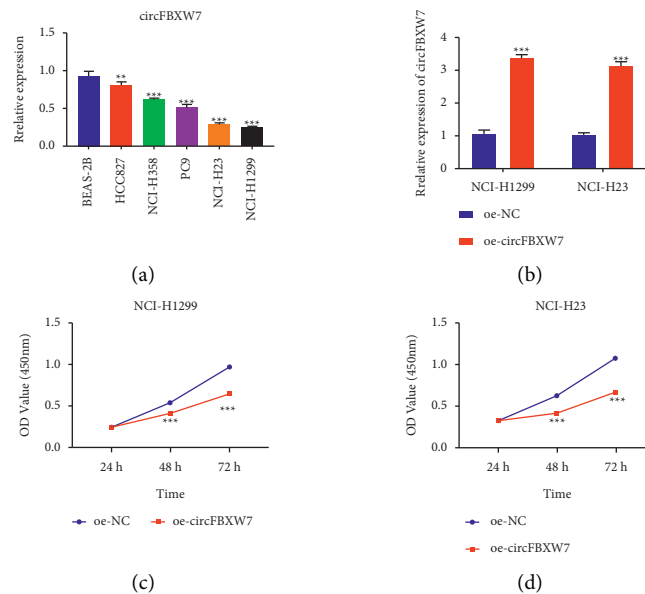


FIGURE 2: Continued.

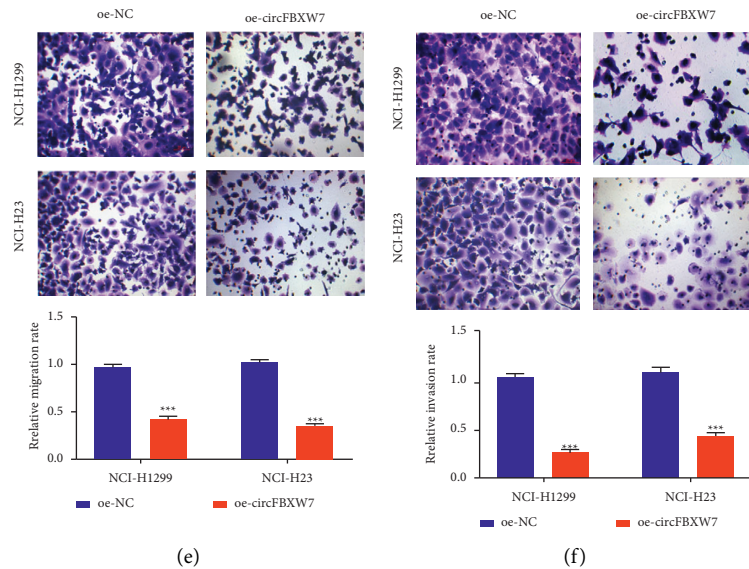


FIGURE 2: Overexpression of circFBXW7 reduced cell proliferation, migration, and invasion. (a) circFBXW7 downregulated in NSCLC cells, including HCC827, PC9, NCI-H23, and NCI-H1299 cells. qRT-PCR was used to test the expression of circFBXW7. (b) circFBXW7 expression upregulated by oe-circFBXW7 transfection for 48 h in NCI-H23 and NCI-H1299 cells. ((c) and (d)) Overexpression of circFBXW7 decreased proliferation in NCI-H1299 and NCI-H23 cells; CCK-8 was used to assess cell proliferation. ((e) and (f)) Overexpression of circFBXW7 reduced the migration and invasion ability of NCI-H1299 and NCI-H23 cells; Transwell assays were used to assess cell migration and invasion (40x). FBXW7, F-box and WD repeat domain containing 7; oe-NC, circFBXW7 overexpression negative control; oe-circFBXW7, circFBXW7 overexpression plasmids; qRT-PCR, real-time quantitative polymerase chain reaction; CCK-8, cell counting kit-8; \*\*\*  $p < 0.001$ .

miRNA interaction using Cytoscape software (Figure 3(a)). Among the miRNAs, miR-492 has been reported to regulate NSCLC progression [18–20]. Then, qRT-PCR detection showed that miR-492 expression was significantly downregulated in the oe-circFBXW7 group (Figure 3(b)). Figure 3(c) shows the binding site of circFBXW7 and miR-492. Dual-luciferase detection revealed that the fluorescence activity of the circFBXW7 WT plasmid group was significantly downregulated, confirming the targeted binding sites between circFBXW7 and miR-492 (Figure 3(d)).

**3.4. CircFBXW7 Reduced Cell Proliferation, Migration, and Invasion through Binding to miR-492.** To confirm whether the inhibitory effect of circFBXW7 overexpression on proliferation, migration, and invasion was regulated by miR-492, we synthesized miR-492 mimics and verified its effect via qRT-PCR. As shown in Figure 4(a), the expression of miR-492 was repressed when circFBXW7 was overexpressed, whereas this was reversed by miR-492 mimics in NCI-H23 and NCI-H1299 cells, indicating that the synthesized mimics effectively promoted the expression of miR-492. The proliferation of NCI-H23 and NCI-H1299 cells was promoted in the oe-circFBXW7-treated group in the presence of the miR-492 mimics compared with that in the oe-circFBXW7-treated group (Figures 4(b) and 4(c)). In addition, transfection of miR-492 mimics rescued the inhibition of circFBXW7 overexpression on cell migration and invasion (Figures 4(d) and 4(e)). These results showed that circFBXW7 could reduce cell proliferation, migration, and invasion by regulating miR-492.

## 4. Discussion

NSCLC is a common type of lung cancer characterized by poor prognosis [21]. CircRNA plays an important role in the occurrence and development of NSCLC, but its pathogenesis in NSCLC has not been fully clarified. In this study, we showed that circFBXW7 was expressed at a low level in NSCLC cells, particularly in NCI-H23 and NCI-H1299 cells. Overexpression of circFBXW7 in NCI-H23 and NCI-H1299 cells suppressed cell proliferation, migration, and invasion. However, following the overexpression of circFBXW7, the expression of miR-942 was downregulated in NCI-H23 and NCI-H1299 cells. MiR-942 mimics reversed the effect of circFBXW7 overexpression on cell proliferation, migration, and invasion in oe-circFBXW7-treated NCI-H23 and NCI-H1299 cells.

FBXW7 is the parent gene of circFBXW7, which has been reported to be involved in cell division, growth, migration, invasion, and drug resistance [22–24]. CircFBXW7 is transcribed from exons 3 and 4 of FBXW7 and has been reported to be vital in various biological processes. For instance, the expression of circFBXW7 was reported to be downregulated in tumor tissues, and overexpression of circFBXW7 significantly inhibited the proliferation, migration, and invasion ability of triple-negative breast cancer and glioma [12, 25]. It was reported that circFBXW7 attenuated the malignant progression of lung adenocarcinoma by sponging miR-942-5p [26]. Here, we observed that circFBXW7 was expressed at a low level in NSCLC cells. Overexpression of circFBXW7 decreased NSCLC cell proliferation, migration, and invasion. This is the first report on

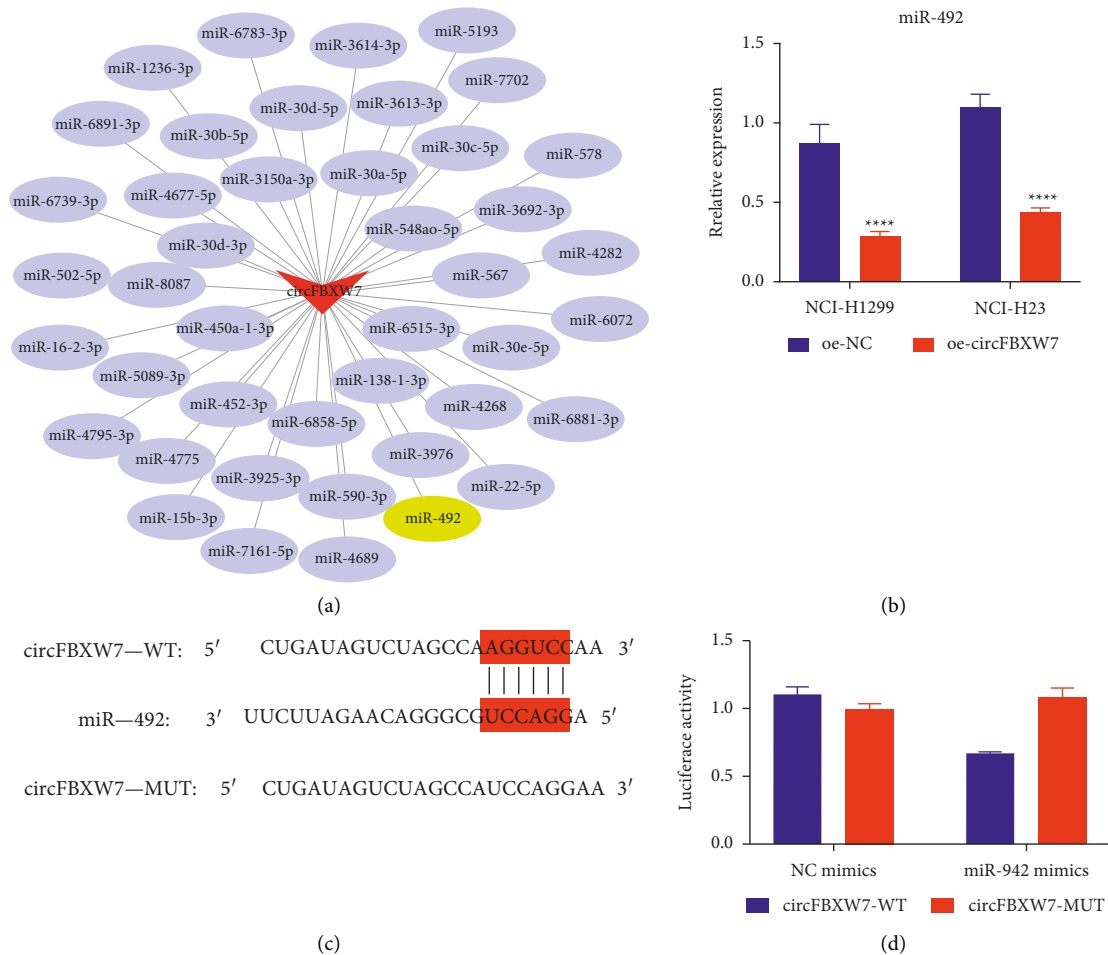


FIGURE 3: CircFBXW7 targeted miR-492. (a) A regulatory network of circFBXW7-miRNAs constructed by Cytoscape. Potential miRNAs of circFBXW7 were predicted by miRanda software and miTarBase database. (b) The expression of miR-492 downregulated after circFBXW7 overexpression in both NCI-H23 and NCI-H1299 cells; qRT-PCR validated miR-492 expression. (c) The binding sites of circFBXW7 and miR-492. (d) The targeted binding relationship between circFBXW7 and miR-492 verified by a dual-luciferase reporter assay. FBXW7, F-box and WD repeat domain containing 7; oe-NC, circFBXW7 overexpression negative control; oe-circFBXW7, circFBXW7 overexpression plasmids; qRT-PCR, real-time quantitative polymerase chain reaction; \*\*\*\*  $p < 0.001$ .

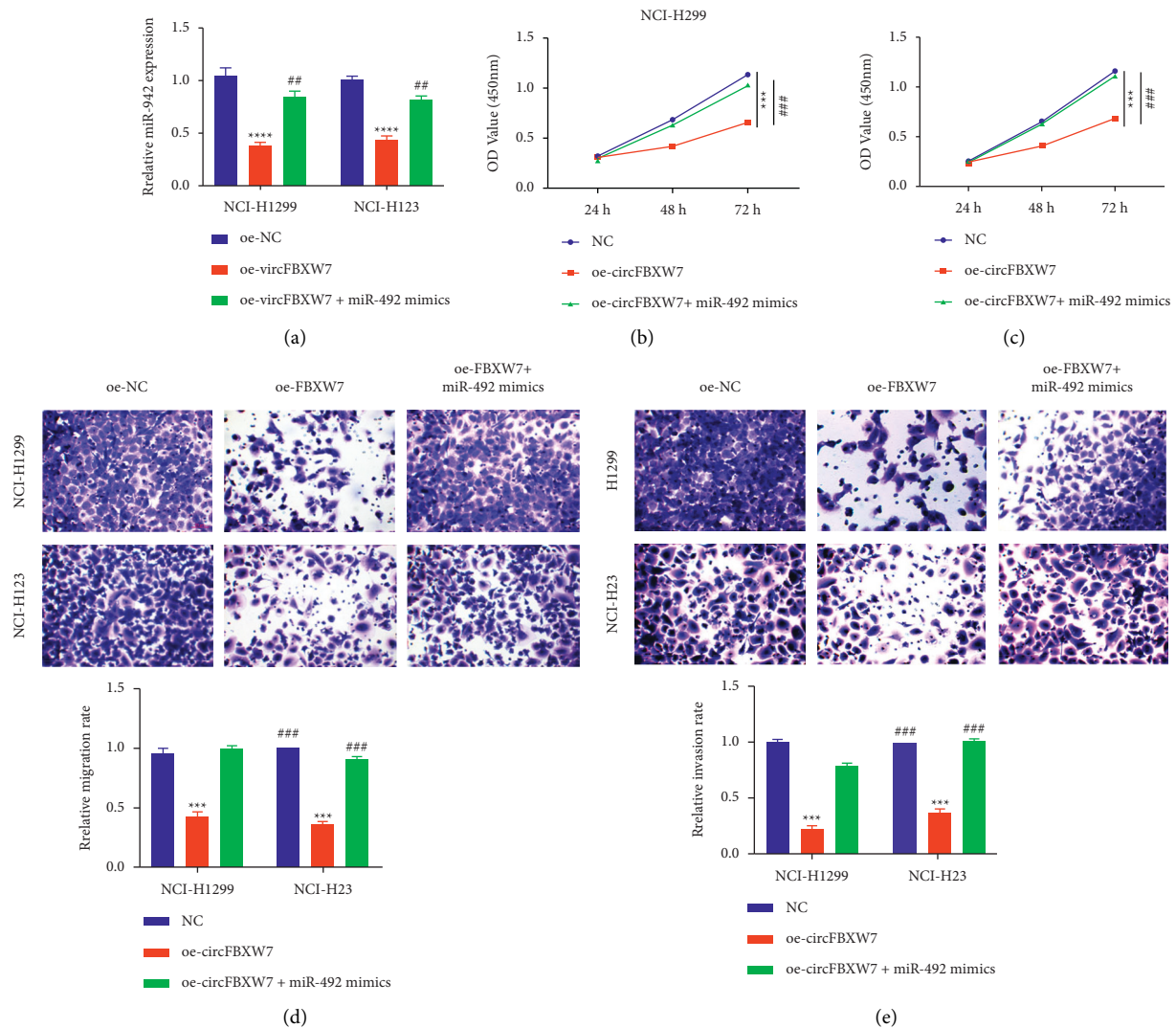
the role of circFBXW7 in NSCLC. This finding suggests that circFBXW7 could be a marker for the diagnosis of NSCLC.

CircRNAs have been reported to act as sponges for miRNAs to regulate mRNA expression and translation [27]. Therefore, we screened potential target miRNAs as predicted by miRanda software and miTarBase database combined with the results from previous studies. MiR-942 was selected for further study [18–20]. Zhang et al. reported that overexpression of hsa\_circ\_0005567 promoted polarization of M2-type macrophages through the miR-492/SOCS2 axis and inhibited the progression of osteoarthritis [28]. In addition, circ\_0001368 inhibited the growth and invasion of renal cell carcinoma by inhibiting miR-492 [29]. Hsa\_circ\_0072309 inhibited the proliferation and invasion of breast cancer cells by targeting miR-492 [30]. Ye et al. suggested that overexpression of circFBXW7 inhibited triple-negative breast cancer progression via sponging of

miR-197-3p [25]. In our study, we observed that circFBXW7 targeted miR-492. We verified that overexpression of circFBXW7 decreased the expression of miR-492 in both NCI-H23 and NCI-H1299 cells. In addition, the inhibitory effect of circFBXW7 overexpression on cell proliferation, migration, and invasion was reversed by miR-492 mimics. This finding suggests that circFBXW7 plays an important role in NSCLC by regulating miR-492.

However, there are some limitations in this study. More works are needed to verify the role of circFBXW7 in NSCLC, such as examining the expression of circFBXW7, miR-942 in NSCLC tissues, and validating the relationship between circFBXW7 and miR-942 in vivo.

Taken together, this study suggests that overexpression of circFBXW7 inhibits miR-942 expression, which, in turn, inhibits the progression of NSCLC. CircFBXW7 could be a promising biomarker for lung cancer patients for predicting tumor prognosis and recurrence.



**FIGURE 4: CircFBXW7 reduced cell proliferation, migration, and invasion by regulating miR-492.** (a) The expression of miR-492 was downregulated by circFBXW7 overexpression, whereas this effect was reversed by miR-492 mimics in NCI-H23 and NCI-H1299 cells; the expression of miR-492 was determined by qRT-PCR after transfection for 48 h. ((b) and (c)) Proliferation was reduced by circFBXW7 overexpression, whereas this effect was reversed by miR-492 mimics in NCI-H23 and NCI-H1299 cells; the proliferation of NCI-H23 and NCI-H1299 cells was detected at 24, 48, and 72 h by CCK-8 assay after transfection for 48 h. ((d) and (e)) migration and invasion were decreased by circFBXW7 overexpression, whereas this effect was reversed by miR-492 mimics in NCI-H23 and NCI-H1299 cells. Migration and invasion were detected via Transwell assay after transfection for 48 h (40x). FBXW7, F-box and WD repeat domain containing 7; oe-NC, circFBXW7 overexpression negative control; oe-circFBXW7, circFBXW7 overexpression plasmids; qRT-PCR: real-time quantitative polymerase chain reaction; CCK-8: cell counting kit-8; \*\*\*  $p < 0.001$  vs. the NC group, \*\*\*  $p < 0.001$  vs. the oe-circFBXW7 group.

## Data Availability

The sequence of hsa\_circ\_0001451 comes from circBase, and the products of divergent primers were used for Sanger sequencing to validate the circularity of circRNA. The datasets generated and/or analyzed during the current study are available in the circBase repository.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Zu-Xiong Zhang, Shen-Yu Zhu, Liang Gu, and Xiao-Yuan Tang conceptualized and designed the study and wrote the first draft of the manuscript. Cheng-Peng Sang, Ding-Yu Rao, Yao Xia, Mao-Yan Si, Hua-Qiu Shi, Jie Li, Shu-Lin Li, and Chun-Fa Xie prepared materials, collected and analyzed data, and organized the figures. Xiao-Liang Yuan and Zhi-Xian Tang acquired fund and critically revised the manuscript for important intellectual content. All authors have read and approved the final version. Shen-Yu Zhu, Zu-

Xiong Zhang, and Liang Gu contributed equally to this work.

## Acknowledgments

The authors acknowledge the expert assistance and kind help of researchers from Forevergen Biosciences Co., Ltd. This work was supported by the National Natural Science Foundation of China (81860337, 81960326, and 82060384), the Natural Science Foundation of Jiangxi Province (20202ACBL206014, 20192BAB205009, and 2020BABL206116), the Science and Technology Programs of Jiangxi Health Commission (20201080 and 202130660), the National Health Commission Science and Technology Development Research Center (2019ZH-07E-003), the general projects of Jiangxi Traditional Chinese Medicine Science and Technology (2020B0214), The Ganzhou Key Laboratory of Deep Hypothermic Circulatory Arrest Brain Protection (20210180), and the Science and Technology Research Project of Jiangxi Provincial Education Department (GJJ190804).

## Supplementary Materials

Supplement Figure 1. circFBXW7 reduced cell colony formation. FBXW7, F-box and WD repeat domain containing 7; oe-NC, circFBXW7 overexpression negative control; oe-circFBXW7, circFBXW7 overexpression plasmids; \*\*  $p < 0.01$ . (Supplementary Materials)

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