Original Article

CircPTN promotes angiogenesis via the MiR-595/LYRM5 signaling pathway in non-small cell lung cancer

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Background: Non-small cell lung carcinoma (NSCLC) is a highly malignant tumor with a poor prognosis worldwide. Some studies have demonstrated that circular pleiotrophin (circPTN) plays critical roles in tumorigenesis and tumor development. However, little is known about the role of circPTN in NSCLC.

Methods: The circPTN expression in human NSCLC tissues was measured via quantitative real-time polymerase chain reaction (qRT-PCR). The function and potential mechanisms of circPTN in NSCLC angiogenesis were also investigated. We aimed to explore the function and potential mechanisms and clinical significance of circPTN in NSCLC.

Results: We first found that circPTN was markedly upregulated in NSCLC tissues. A higher circPTN level was closely associated with angiogenesis and significantly shorter overall survival in patients with NSCLC. We then found that circPTN promoted angiogenesis in NSCLC. More importantly, we found that circPTN facilitated angiogenesis by regulating the expression of LYRM5 in NSCLC. Mechanistically, LYRM5 could be a direct target of microRNA-595 (miR-595). Additionally, we demonstrated that circPTN upregulated LYRM5 expression by sponging miR-595, which promoted NSCLC angiogenesis in NSCLC.

Conclusions: We found that circPTN serves as a competing endogenous ribonucleic acid that promotes angiogenesis via the miR-595/LYRM5 signaling pathway in NSCLC. Targeting circPTN might be a promising new therapeutic strategy for NSCLC.

Keywords: CircPTN; Angiogenesis; MiR-595; LYRM5; non-small cell lung cancer (NSCLC)

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Introduction

Lung carcinoma is a highly malignant tumor with a poor prognosis worldwide (1). It is divided into the following 2 subtypes: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC represents about 80% of all lung carcinoma cases (2,3). Angiogenesis plays a key role in most malignant tumors, including NSCLC (4). Abundant angiogenesis plays an important role in tumor malignant proliferation and metastasis (5-7). Thus, anti-angiogenic treatments efficiently prevent tumor growth and metastasis. However, the exact molecular mechanism associated with NSCLC angiogenesis is poorly defined.

Circular ribonucleic acids (circRNAs) are derived from the "back-splicing" of precursor messenger RNA (pre-

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mRNA) transcripts to form a covalently closed circular structure without a 3'polyA tail or 5' to 3' polarity (8-10). CircRNAs are more stable than linear RNAs, and provide resistance to degradation by ribonuclease R (11-13). There is emerging evidence that circRNAs are aberrantly expressed in a variety of human tumors, including gastric cancer (14), glioma (15), hepatocellular carcinoma (16), esophageal cancer (17), lung cancer (18), and breast cancer (19). CircRNAs play a vital role in many physiological and pathological processes (20). In tumors, circRNAs are involved in various aspects of malignant phenotypes, including apoptosis, the cell cycle, angiogenesis, invasion, and metastasis (21).

Circular pleiotrophin (circPTN) is derived from the pleiotrophin (PTN) gene. Recently, circPTN has been reported to sponge microRNA-330-5p/microRNA -145-5p to promote stemness and proliferation in glioma (22). CircPTN is highly expressed in hepatocellular cancer tissues, and promotes the growth of hepatocellular cancer by sponging miR-326 and inducing ErbB/PI3K expression (23). Additionally, circPTN inhibits glioma cell glycolysis, proliferation, and invasion by regulating the miR-432-5p/RAB10 axis (24). However, the roles of circPTN in the angiogenesis of NSCLC are largely unknown. Previous studies just reported circPTN have the promoting role in Glioma and hepatocellular carcinoma (22-24). Our team firstly confirmed circPTN promote NSCLC metastasis and via up-regulating LYRM5 protein expression, which is our study biggest innovation in the NSCLC

In the present study, we measured circPTN expression in human NSCLC tissues via quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, the function and potential mechanisms of circPTN in NSCLC angiogenesis were explored. We found that a higher circPTN level was closely related to angiogenesis, and significantly shorter overall survival in patients with NSCLC. Further, we found preliminary evidence that circPTN upregulates the expression of LYRM5 by sponging miR-595, which facilitates NSCLC angiogenesis.

We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-195/rc).

Methods

NSCLC tissues collection

In total, 80 pairs of NSCLC and matched normal tissues were obtained from the Department of Cardiothoracic Surgery, Zhuji People's Hospital from June 2019 to December 2019. The experimental protocol was reviewed and approved by the Research Ethics Committee of Zhuji People's Hospital (No. 2019056). A formal informed consent form was signed by each participant before sample collection. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture

The NSCLC cell lines [A549 (ATCC® CRM-CCL-185), H1299 (ATCC® CRL-5803), and H358 (ATCC® CRL-5807)] and normal human bronchial epithelial cell line (16HBE) were purchased from ATCC® (Maryland, USA). The NSCLC line PC-9 was gifted by the Shanghai Jiao Tong University.

The H358, H1299, and PC-9 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS). 16HBE and A549 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. These cell lines were cultured in an incubator at 37 ℃ with an atmosphere of 5% carbon dioxide.

Immunohistochemistry

The experiment was performed using methods described previously (25). Paraffin-embedded tissue samples were incubated overnight using a primary antibody against cluster of differentiation 31 (CD31) (ab24590). The antibody was purchased from Abcam (Cambridge, UK). Images were taken via microscopy.

qRT-PCR

Total RNA was extracted and reverse transcribed into complementary deoxyribonucleic acid (cDNA). SYBR-Green (TaKaRa, China) was used to perform the qRT-PCR. β-actin and U6 were used as the internal controls. The relative quantification $2^{-\Delta\Delta CT}$ was applied to assess the level of circPTN, miR-595, and LYRM5.

Tube formation assay

The 96-well plates were incubated with 50 μL of dissolved Matrigel matrix (BD Biosciences, USA) at 37 ℃ for 30 minutes. 2×10^4/well human umbilical vein endothelial

cells (HUVECs) were resuspended in 100 µL of serum-free medium, and seeded in the wells with dissolved Matrigel. Tube formation was observed under the microscope (Leica, Germany) after incubation at 37 ℃ for 6 to 8 hours. The total tubular length per well was assessed via ImageJ software.

Western blot

Western blot was performed as previously described (26) to test the level of LYRM5 protein. Briefly, 60 µg of protein was electrophoresed with 12.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). At 4 ℃, the primary antibody of LYRM5 (ab126379) (Abcam, UK) was incubated overnight. Band intensities were quantitated by enhanced chemiluminescence (ECL) (Beyotime, Shanghai, China).

Dual-luciferase reporter assay

The possible binding regions for miR-595 in circPTN and LYRM5 were predicted through a bioinformatics analysis. H1299 and A549 cells were transfected with miR-595-mimic and reporter plasmids. Reporter plasmids was purchased from Thermo Fisher Scientific. A dual-luciferase assay system (Promega, USA) was used to conduct the luciferase reporter assay.

Statistical analysis

The statistical analysis was performed by GraphPad Prism 8. The experiments were performed in triplicate. The data in this study are presented as the mean ± standard deviation. The correlation among the groups was explored via a Pearson correlation analysis. A Student's *t*-test or 1-way analysis of variance was used to compare quantitative variables. A P value <0.05 was considered statistically significant.

Results

CircPTN is upregulated and closely associated with angiogenesis in NSCLC

To investigate circPTN expression in NSCLC, we assessed its expression in 80 pairs of NSCLC tissues and matched normal tissues through qRT-PCR. We found that the circPTN level was higher in the NSCLC tissues than the corresponding normal tissues (see *Figure 1A*). Additionally, the Kaplan-Meier analysis showed that the overall survival of patients with higher circPTN expression was decreased (see *Figure 1B*). To further explore the effect of circPTN on angiogenesis in NSCLC, we detected the expression of CD31 in NSCLC tissues via immunohistochemistry. Increased CD31 expression was observed in high circPTN expression tumor tissues (see *Figure 1C*,*1D*). Additionally, the circPTN expression level was closely associated with microvascular density in NSCLC (see *Figure 1E*). These results suggest that circPTN is upregulated and closely associated with angiogenesis in NSCLC.

CircPTN promotes angiogenesis in NSCLC

To identify the effect of circPTN on the angiogenesis of NSCLC, circPTN expression was detected in NSCLC cell lines (H1299, H358, PC-9, and A549), and in normal bronchial epithelial cells (16HBE) by qRT-PCR. As *Figure 2A* shows, there was higher circPTN expression in the A549 and H1299 cell lines than the other 2 NSCLC cell lines. Next, A549 and H1299 cells were transfected with si-circPTN or si-NC, and si-circPTN transection efficiency was assessed through qRT-PCR (see *Figure 2B*). Conditioned medium from the A549 or H1299 cells transfected with si-circPTN or si-NC was collected, and HUVECs were stimulated with it. Compared to the si-NC group, decreased tube formation was detected in the si-circPTN group (see *Figure 2C*,*2D*). The results showed that circPTN promotes angiogenesis in NSCLC.

CircPTN facilitates angiogenesis by regulating LYRM5 in NSCLC

LYRM5 (also referred to as Ghiso) is a mitochondrial protein, and a member of the Complex1_LYR-like Superfamily (27,28). LYRM proteins play key roles in acetate metabolism, and in essential Fe-S cluster biogenesis (28). Mitochondria have been shown to be involved in the occurrence and development of carcinoma (29,30). To explore the role of circPTN in the expression of LYRM5, the LYRM5 protein and mRNA expression in the H1299 or A549 cells transfected with si-circPTN or si-NC were measured. We found that silenced circPTN suppressed LYRM5 mRNA and protein expression (see *Figure 3A*,*3B*). Next, tube formation assays were carried out using the HUVECs stimulated with conditioned medium from the A549 or

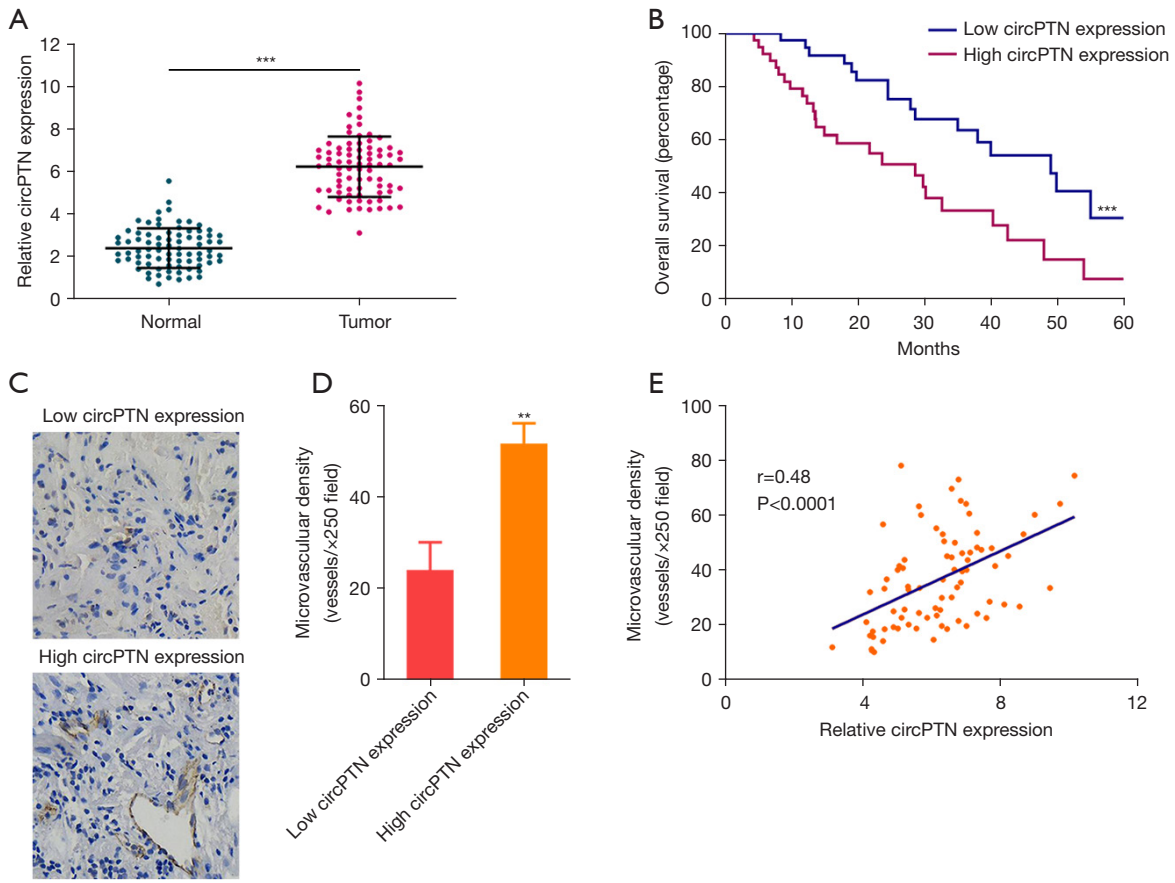

Figure 1 CircPTN (circular pleiotrophin) is upregulated and closely related to angiogenesis in non-small cell lung cancer (NSCLC). (A) QRT-PCR (quantitative real time polymerase chain reaction) was performed to measure the expression of circPTN in 80 pairs of NSCLC tissues and matched normal tissues. ***, P<0.001. (B) The overall survival in the circPTN low (N=40)/high (N=40) groups were investigated via a Kaplan-Meier survival analysis. ***, P<0.001. (C,D) CD31 level was measured in the circPTN low (N=40)/high (N=40) groups through immunohistochemistry. The microvascular density (CD31 staining) in the 2 groups was assessed (×100 magnification). **, P<0.01. (E) The correlation between microvascular density and circPTN in NSCLC tissues was investigated through a Pearson correlation analysis.

H1299 cells transfected with si-LYRM5 or si-Control. The silencing of LYRM5 resulted in the significant inhibition of angiogenesis (see *Figure 3C,3D*). Our observations indicated that circPTN facilitates angiogenesis by regulating LYRM5 in NSCLC.

MiR-595 is an inhibitory target of circPTN

There is accumulating evidence that circRNAs are involved in the development and progression of cancer by serving as competing endogenous RNAs (ceRNAs), and competitively reduce miRNA activity (31-33). To further explore the molecular mechanisms associated with circPTN-induced LYRM5 expression, bioinformatics databases (RNAhybrid, TargetScan, and miRanda) were used to predict the potential target miRNAs. There was a potential binding site between circPTN and MiR-595 (see *Figure 4A*). The interaction between them was confirmed by dual-luciferase reporter assays (see *Figure 4B*). Additionally, the silencing of circPTN resulted in an obvious increase in miR-595 expression in A549 and H1299 cells (see *Figure 4C*). In NSCLC tissues, an inverse correlation between miR-595 and circPTN was verified via a Pearson correlation analysis (see *Figure 4D*). These data showed that miR-595 can be

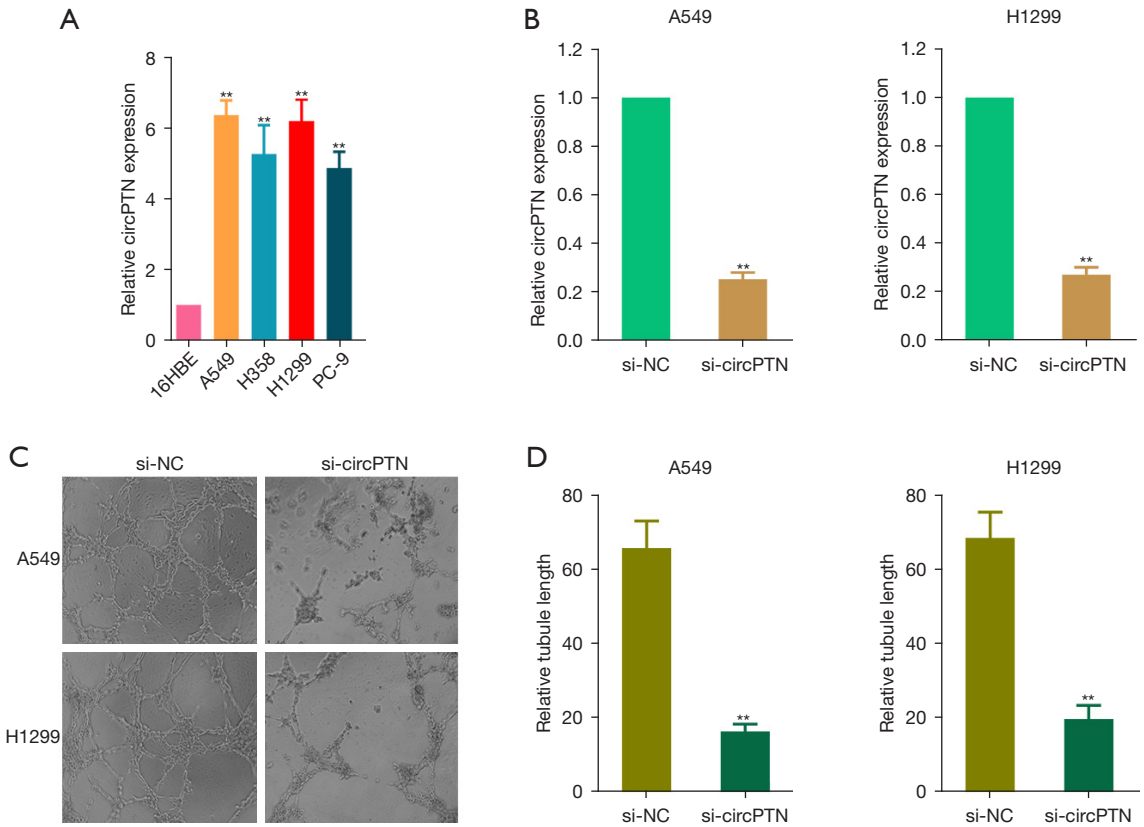

Figure 2 CircPTN (circular pleiotrophin) promotes angiogenesis in non-small cell lung cancer (NSCLC). (A) CircPTN expression was detected in NSCLC cell lines (H1299, H358, PC-9, and A549) and normal bronchial epithelial cells (16HBE) through qRT-PCR (quantitative real time polymerase chain reaction). **, P<0.01. (B) CircPTN expression was assessed in A549 and H1299 cells transfected with negative control siRNA (si-NC) or circPTN siRNA (si-circPTN). **, P<0.01. (C,D) Conditioned medium from A549 or H1299 cells transfected with si-circPTN or si-NC was collected, and human umbilical vein endothelial cells (HUVECs) were stimulated with it. Tube formation assays were performed in HUVECs. The pictures were scanned at ×100 magnification. Tube formation assays were photographed digitally at five randomly selected fields using an inverted microscope (Leica Microsystems, Shanghai, China). Tubule length was assessed using the ImageJ software. **, P<0.01.

sponged by circPTN in NSCLC.

LYRM5 is a direct target of miR-595

To identify the role of miR-595 in LYRM5 expression induced by circPTN, the possible targets of miR-595 were predicted via a bioinformatic analysis using miRDB and TargetScan. A binding site for miR-595 in LYRM5 was identified (see *Figure 5A*). Subsequently, the interactions of miR-595 and LYRM5 was demonstrated through luciferase reporter assays (see *Figure 5B*). Additionally, our results showed that the protein and mRNA levels of LYRM5 were dramatically more reduced in the miR-595-mimic group than the negative control (NC)-mimic group (see *Figure 5C,5D*). A negative relationship between LYRM5 and miR-595 in NSCLC tissues was confirmed via a Pearson correlation analysis (see *Figure 5E*). More importantly, we proved that the inhibitory effect of silenced circPTN on the angiogenesis of HUVECs cells was attenuated by miR-595 (see *Figure 5F*). Above all, our findings revealed that LYRM5 is a direct target of miR-595 in NSCLC.

Discussion

It is estimated that deaths due to malignant lung cancer represent roughly 1/5 of all deaths worldwide (34,35). NSCLC represents about 80% of all lung carcinoma cases among the common subtypes of lung carcinoma (36). Until

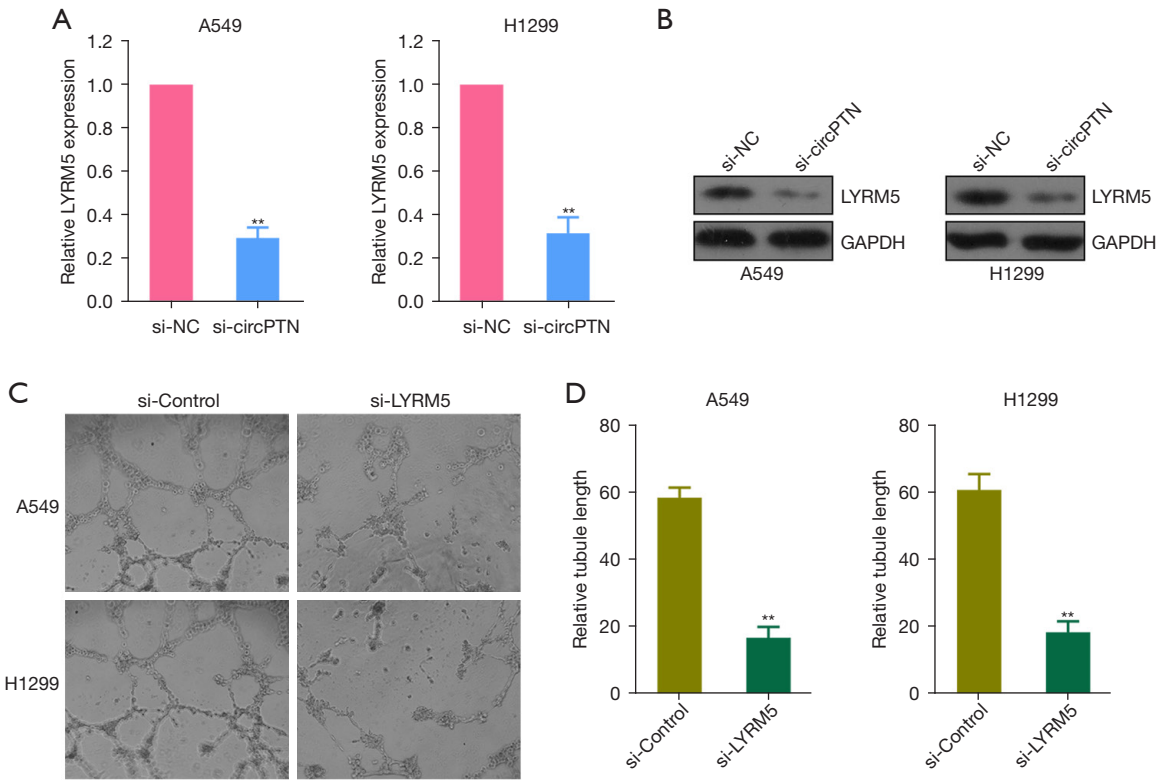

Figure 3 CircPTN (circular pleiotrophin) facilitates angiogenesis by regulating LYRM5 in non-small cell lung cancer (NSCLC). (A,B) A549 or H1299 cells were transfected with circPTN siRNA (si-circPTN) or negative control siRNA (si-NC), and the protein and mRNA levels of LYRM5 were measured through Western blot and qRT-PCR (quantitative real time polymerase chain reaction). **, P<0.01. (C,D) Human umbilical vein endothelial cells (HUVECs) were stimulated with conditioned medium from A549 or H1299 cells transfected with LYRM5 siRNA (si-LYRM5) or control siRNA (si-Control). HUVECs were used to perform the tube formation assay. Tube formation assays were photographed digitally at five randomly selected fields using an inverted microscope (Leica Microsystems, Shanghai, China). Tubule length was assessed using the ImageJ software. **, P<0.01.

the last decade, the 5-year overall survival rate of patients with metastatic NSCLC was <5% (36). An improved understanding of the biology of NSCLC has resulted in the development of new biomarker-targeted therapies, and led to improvements in overall survival for patients with metastatic or advanced NSCLC. For many tumors, including NSCLC, angiogenesis has been taken as an essential therapeutic target. Angiogenesis is involved in the process of primary tumor growth, proliferation, and metastasis (4,6,7,37). In the present study, we verified that circPTN upregulated LYRM5 expression by sponging miR-595, which promoted NSCLC angiogenesis.

CircRNAs are derived from the "back-splicing" of pre-mRNA transcripts to form a covalently closed circular structure without a 3' polyA tail or 5' to 3' polarity (8-10). It is well established that circRNAs are aberrantly expressed in a variety of human tumors, and involved in various aspects of malignant phenotypes, including apoptosis, cell cycle, angiogenesis, and invasion, and metastasis, as an RNA sponge (8,10,21,38,39). CircPTN is derived from the PTN gene. Recently, circPTN has been reported to sponge miR-145-5p/miR-330-5p to promote proliferation and stemness in glioma (22). CircPTN is highly expressed in hepatocellular cancer tissues, and promotes the growth of hepatocellular cancer by sponging miR-326 and inducing ErbB/PI3K expression (23). Additionally, circPTN inhibits glioma cell glycolysis, proliferation, and invasion by regulating the miR-432-5p/RAB10 signaling pathway (24).

Numerous studies have confirmed that circRNAs are involved in tumor angiogenesis (40-42). For example, circ-CCAC1 levels are increased in cancerous bile-resident extracellular vesicles and cholangiocarcinoma tissues, and circ-

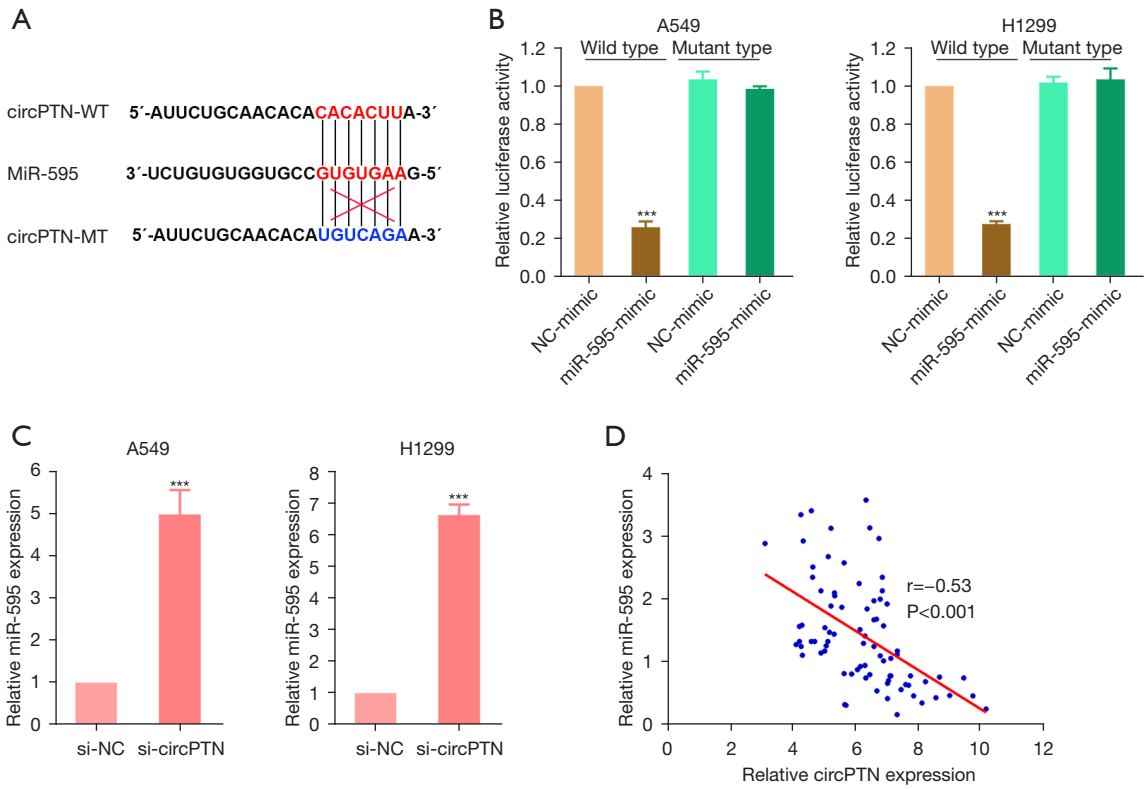

Figure 4 MiR-595 is an inhibitory target of circPTN (circular pleiotrophin). (A) Bioinformatics databases (miRanda, TargetScan and RNAhybrid) were used to predict the potential interactions between MiR-595 and circPTN. (B) A549 and H1299 cells were transfected with NC-mimic or miR-595-mimic, and luciferase reporter assays were carried out. ***, P<0.001. (C) MiR-595 levels were assessed via qRT-PCR (quantitative real time polymerase chain reaction) in A549 and H1299 cells transfected with circPTN siRNA (si-circPTN) or negative control siRNA (si-NC). ***, P<0.001. (D) The correlation between miR-595 and circPTN in non-small cell lung cancer (NSCLC) tissues were assessed via a Pearson correlation analysis.

CCAC1 from CCA-derived extracellular vesicles is transferred to endothelial monolayer cells, disrupting endothelial barrier integrity and inducing angiogenesis (40). SRSF10 facilitates circ-ATXN1 biogenesis and subsequently increases its binding with miR-526b-3p to induce metallopeptidase and vascular endothelia growth factor A (VEGFA), thus regulating glioma angiogenesis (41). Circ-001971 promotes the proliferation, invasion and angiogenesis of colorectal cancer via the miR-29c-3p/VEGFA axis (42). Similar to previous results, we found that circPTN was highly expressed in NSCLC tissues. A higher circPTN level was closely associated with angiogenesis and short overall survival in patients with NSCLC. With the development of small molecule drugs, the well-designed circPTN interference peptide is a novel useful therapeutic target to treat NSCLC patients with the highly circPTN expression. Moreover, the circPTN levels could be employed to predict the prognosis of NSCLC patients. Moreover, circPTN facilitated angiogenesis by regulating LYRM5 in NSCLC. Currently, just the miRNA-based drugs have been used in clinical trials (NCT03713320, NCT03837457). In comparison with miRNA, circRNA has the more complex circular conformation and sequence overlap with linear mRNA counterparts (43). In addition, circRNA is limited by drug chemistry, drug formulations and different routes of administration. All in all, circRNA is unable to be applied in the medical field. With the development of technology, the circRNA will be used in the future clinical trial.

The mechanisms associated with the functional roles of circRNAs in regulating tumorigenesis are still unclear. However, there is accumulating evidence that circRNAs regulate the expression of oncogenic or tumor-suppressive genes by acting as miRNA sponges and forming the circRNA-miRNA-mRNA signaling pathway (13). Chen

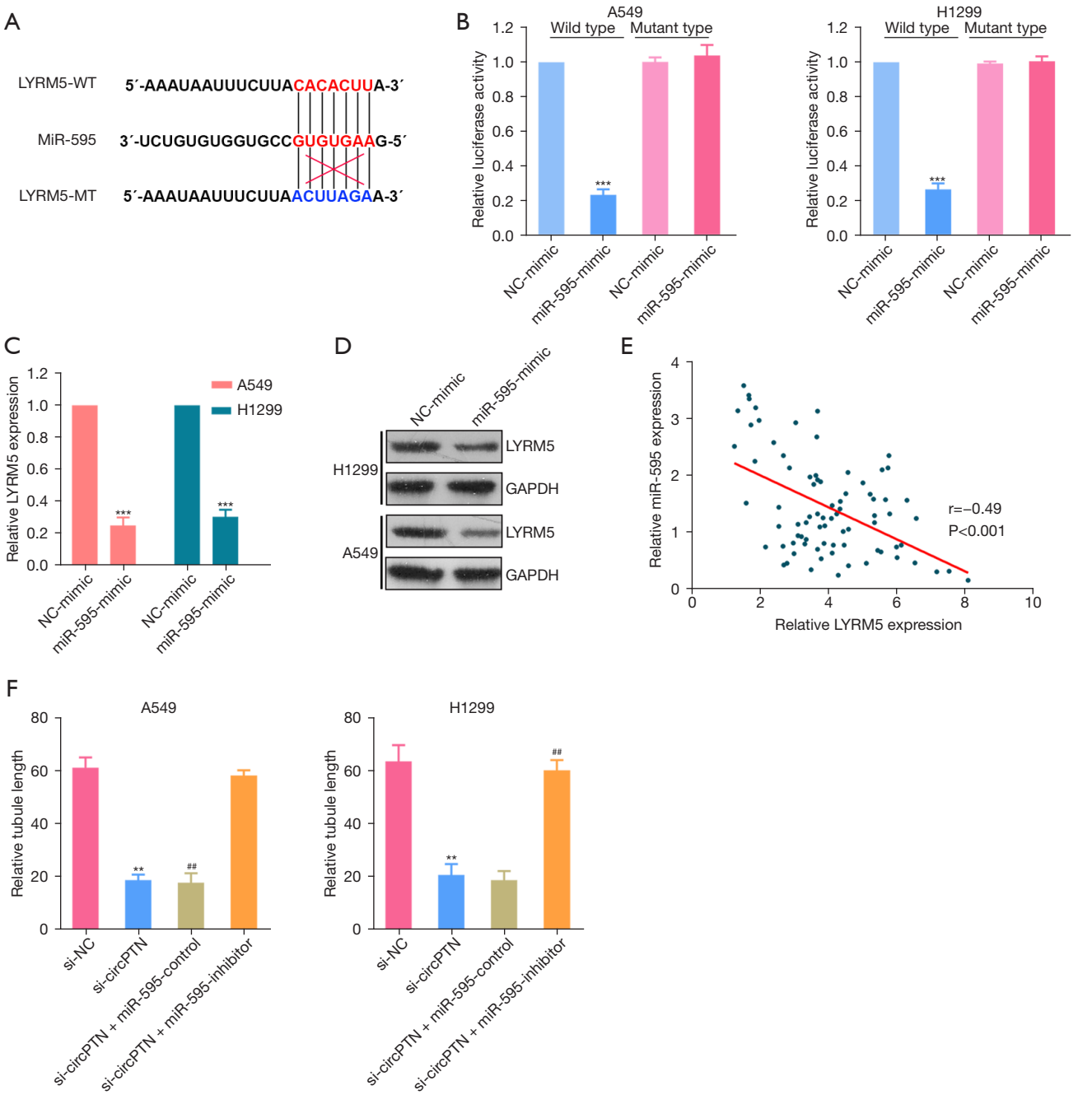

Figure 5 LYRM5 is a direct target of miR-595. (A) The possible targets of miR-595 were predicted via bioinformatic analysis using TargetScan and miRDB. (B) A549 and H1299 cells were transfected with reporter plasmids containing either the mutant LYRM5 3′-UTR with miR-595 binding sequences or the wild-type counterparts. Luciferase reporter assays were performed. ***, P<0.001. (C,D) The protein and mRNA expression of LYRM5 in A549 and H1299 cells were determined through Western blotting and qRT-PCR. ***, P<0.001. (E) A Pearson correlation analysis was used to investigate the correlation between miR-595 and LYRM5 in non-small cell lung cancer (NSCLC) tissues. (F) Human umbilical vein endothelial cells (HUVECs) were stimulated with conditioned medium from A549 or H1299 cells transfected with a miR-595 inhibitor and a si-circPTN (circPTN siRNA). Tube formation assays were carried out. ##, P<0.01 *vs.* miR-595-inhibitor group + si-circPTN. **, P<0.01 *vs.* si-NC group.

J teams have verified that circPTN sponges miR-145-5p/miR-330-5p to promote proliferation and stemness in glioma (22). In hepatocellular carcinoma, circPTN promotes the growth of hepatocellular carcinoma by sponging miR-326 and inducing ErbB/PI3K expression (23). Additionally, circPTN suppresses glioma cell glycolysis, proliferation, and invasion by the miR-432-5p/RAB10 axis (24). In the present study, our results showed that LYRM5 could be a direct target of miR-595. Further, we found that circPTN upregulated LYRM5 expression by sponging miR-595, which facilitated NSCLC angiogenesis.

Our study proved for the first time that circPTN was highly expressed in NSCLC tissues. A higher circPTN level was closely associated with angiogenesis and significantly shorter overall survival in patients with NSCLC. Additionally, circPTN facilitated angiogenesis by regulating LYRM5 in NSCLC. Further, we found that circPTN upregulated LYRM5 expression by sponging miR-595, which promoted angiogenesis in NSCLC. Overall, circPTN serves as a ceRNA to promote NSCLC angiogenesis via the miR-595/LYRM5 signaling pathway. Our findings provide potential targets for the treatment of patients with NSCLC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-195/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-195/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-195/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experimental protocol was reviewed and approved by the Research Ethics Committee of Zhuji People's Hospital (No. 2019056). A formal informed consent form was signed by each participant before sample collection. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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