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PVT1 Promotes Angiogenesis by Regulating miR-29c/Vascular Endothelial Growth Factor (VEGF) Signaling Pathway in Non-Small-Cell Lung Cancer (NSCLC)

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Lung cancer is a common tumor. Non-small-cell lung cancer (NSCLC) accounts for over 85% of lung cancer and has a high degree of malignancy. Angiogenesis plays an important role in NSCLC progression. Some studies have found that PVT1 can promote angiogenesis in tumor tissues, but the role of PVT1 in angiogenesis in NSCLC, as well as the underlying mechanism, is unclear.

Material/Methods: To explore the role of PVT1 in NSCLC, qRT-PCR, Western blot, luciferase reporter assay, and ELISA were carried out for detecting the relationship among PVT1, miR-29c, and VEGF. Tube formation assay was used to assess the role of PVT1 in angiogenesis in NSCLC.

Results: Our results showed that higher PVT1 was expressed in NSCLC and the elevated PVT1 was closely related to angiogenesis and poor prognosis in NSCLC. Further functional analysis showed that higher PVT1 expression could promote angiogenesis by regulating VEGF in NSCLC. Mechanistically, the luciferase reporter assay confirmed that VEGF was the targeted gene of miR-29c. In addition, we found that miR-29c is an inhibitory target of PVT1.

Conclusions: We found that PVT1 promotes angiogenesis through targeting the miR-29c/VEGF signaling pathway in NSCLC.

MeSH Keywords: Angiogenesis Inducing Agents • Carcinoma, Non-Small-Cell Lung • Vascular Endothelial Growth Factor A

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Background

Lung cancer is a common malignancy with extremely poor prognosis. Moreover, lung cancer is the main cause in cancer-related death. NSCLC, with a 5-year survival rate of <5%, is estimated to account for more than 85% of all lung cancer cases [1]. Notably, NSCLC shows quite poor survival due to high metastatic potential. More than two-thirds of patients suffering from NSCLC already have metastasized by the first diagnosis [2]. Metastasis is a quite complex process, and angiogenesis is necessary during the process. In NSCLC, tumor metastasis and prognosis are closely influenced by angiogenesis. Anti-angiogenic therapy has been considered to possibly be beneficial for the outcome of patients with NSCLC [3]. Therefore, exploring the molecular mechanism in angiogenesis in NSCLC is quite urgent.

Certain lncRNAs (long non-coding RNAs) can play quite important roles in angiogenesis [4]. For instance, PVT1 has been found to promote angiogenesis in gastric cancer [5]. In glioma, PVT1 is also confirmed to promote angiogenesis through affecting microvascular endothelial cells [6]. Actually, PVT1 is an oncogenic lncRNA is overexpressed in many cancers [5]. Studies have revealed the mechanism by which PVT1 facilitates cell proliferation [7], migration, and invasion [8], but the specific role of PVT1 in angiogenesis in NSCLC is unclear.

Vascular endothelial growth factor (VEGF, also referred to as VEGF-A) is universally recognized to play important roles in angiogenesis. VEGF was been identified as an endothelial cell-specific mitogen more than 25 years ago [9,10], and is involved in both developmental and pathological angiogenesis [11]. VEGF promotes new blood vessel formation through stimulating the proliferation and survival of endothelial cells [12]. Moreover, VEGF is overexpressed in many types of cancer and is closely associated with vascular density, metastasis, and prognosis [13]. Targeting VEGF effectively inhibits angiogenesis [14]. Bevacizumab is a humanized monoclonal antibody that targets circulating VEGF and subsequently prevents binding of VEGF to its receptors [15]. It is widely used clinically to block angiogenesis in various cancers [9,16–19], especially NSCLC [20,21]. Thus, the regulatory mechanisms of VEGF in angiogenesis are quite important for their potential as therapeutic targets. Although VEGF-mediated signaling has been studied intensely, much remains unknown. Herein, we attempted to identify the relevance of PVT1 and VEGF, as well as their effects on angiogenesis in NSCLC. We found that PVT1 promotes angiogenesis through regulating VEGF signaling in NSCLC.

Material and Methods

Clinical specimens

The study was approved by the Research Ethic Committee of Zhuji People's Hospital (20170165). We collected 120 pairs of NSCLC and corresponding normal controls from the Department of Cardiothoracic Surgery, Zhuji People's Hospital. Written informed consent were obtained from every patient.

Cell culture

Under the condition of 37°C and 5% CO_2, NSCLC cell line A549 (ATCC® CCL-185) was cultured with DMEM (HyClone) with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL).

RNA extraction and qRT-PCR (quantitative real-time PCR)

TRIzol (TakaRa, Japan) was applied for RNAs extraction. Then, reverse transcription was conducted with PrimeScript™ (TakaRa, Japan). qRT-PCR was executed on QuantStudio 6 Flex (Life, USA). All primers were designed by Sangon Biotech (China). The fold changes were calculated with $2^{-\Delta\Delta CT}$ method.

Western blot

Proteins were separated using SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). These membranes were then incubated in TBS buffer for about 60 min. At 4°C, primary antibody of VEGF (Abcam, UK) was incubated overnight. Then, at room temperature, the secondary antibodies were incubated with the membranes for 2 h. ECL was used for visualization.

Immunohistochemistry (IHC)

Antibodies against VEGF-A (ab1316) and CD31 (ab28364) were obtained from Abcam (Cambridge, UK). IHC was conducted on tumor tissues. At 4°C, primary antibodies were incubated overnight. At room temperature, the secondary antibody was incubated for 30 min. All images were taken via microscopy (Leica, Germany).

ELISA assay

Collected culture medium from NSCLC cells cultured with siRNA (Sangon Biotech, China) tested the concentration of VEGF with ELISA kit as shown in the manufacturers' protocols (Abcam, UK).

Tube formation experiment

We incubated 50 µL dissolved matrigel matrix (BD Biosciences, USA) in the well at 37°C for 0.5 h. Human umbilical vein

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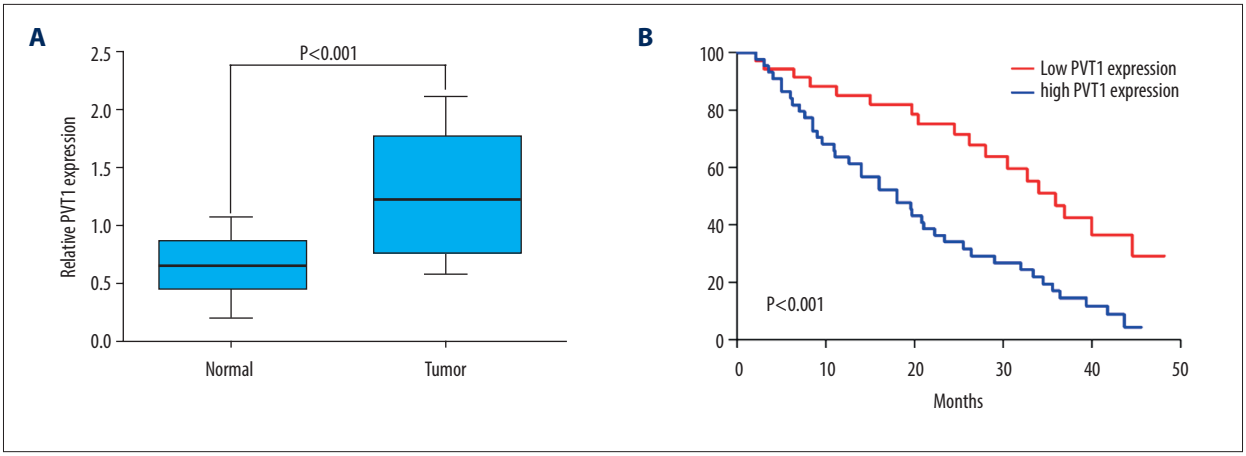

Figure 1. Effects of PVT1 on the prognosis in NSCLC. (**A**) RT-qPCR was used to detect PVT1 expression in tumor and corresponding normal lung tissues. ** *P*<0.001, n=120. (**B**) The patients with NSCLC survival curve on PVT1 expression. n=120.

endothelial cells (HUVECs) (2×10^4/well) were seeded in 96-well plates with dissolved matrigel. The structures (tube-like) were photographed under a microscope (Leica, Germany).

Dual-luciferase reporter experiment

A549 cells in 96-well plates were simultaneously transfected with reporter plasmids and miR-29c mimics (RiboBio, China). After 48 h of transfection, luciferase experiments were performed according to the Luciferase® Reporter Assay protocol (Promega, USA). The ratio of Renilla and Firefly luciferase activities was calculated.

Statistical analysis

GraphPad Prism 6 was applied for data analysis. SD (mean ± standard deviation) is shown for normally distributed data. *P*<0.05 was considered statistical significance (unpaired *t* test).

Results

The elevated PVT1 is closely related to poor prognosis in NSCLC

PVT1 expression was higher in NSCLC than in normal tissues [22]. Moreover, upregulated PVT1 is associated with poor prognosis in colorectal cancer [23]. However, there have been no studies about the relationship between PVT1 and NSCLC prognosis; therefore, in the present study we explored the role of PVT1 in NSCLC prognosis. We first collected 120 tumor tissues of NSCLC and corresponding normal lung tissues, and then measured PVT1 expression. The results showed that PVT1 expression was obviously higher in tumor tissues than in corresponding normal lung tissues (Figure 1A). Next, we determined whether higher PVT1 expression was associated with clinical response. As shown in Figure 1B, in NSCLC, the patients with low PVT1 expression lived much longer than patients with high PVT1 expression (*P*<0.001). These results indicated that PVT1 is highly expressed in NSCLC, and higher PVT1 was related to relatively poor prognosis in NSCLC.

PVT1 expression is positively correlated with angiogenesis in NSCLC

PVT1 has been reported to promote angiogenesis in gastric cancer [5]. To further exploration of the influence of PVT1 on angiogenesis in NSCLC, we first tested PVT1 expression in NSCLC tumor tissues, and then detected VEGF and CD31 expression in NSCLC tumor tissues with unequal PVT1 expression. We found increased VEGF and CD31 expression in tumor tissues with high PVT1 expression compared to tumor tissues with low PVT1 expression (Figure 2A). We further explored the relation between PVT1 expression and microvascular density, and found that microvascular density was obviously upregulated in tumor tissues with high PVT1 expression (Figure 2B). Importantly, microvascular density was positively correlated with PVT1 expression level (Figure 2C). These results showed that PVT1 expression is positively correlated with angiogenesis in NSCLC.

PVT1 can promote angiogenesis by regulating VEGF in NSCLC

Although PVT1 and VEGF participate in angiogenesis separately, limited research was reported on the interaction of PVT1 and VEGF in NSCLC. Here, we measured the expression PVT1 in normal bronchial epithelial cells (16HBE) and NSCLC cells (H358, H157, PC-9, and A549). As shown in Figure 3A, PVT1 expression obviously increased in A549, PC-9, and H157 cells than in 16HBE cells. The highest expression of PVT1 was in A549 cells. Next, we treated A549 cells with PVT1-targeted siRNA and

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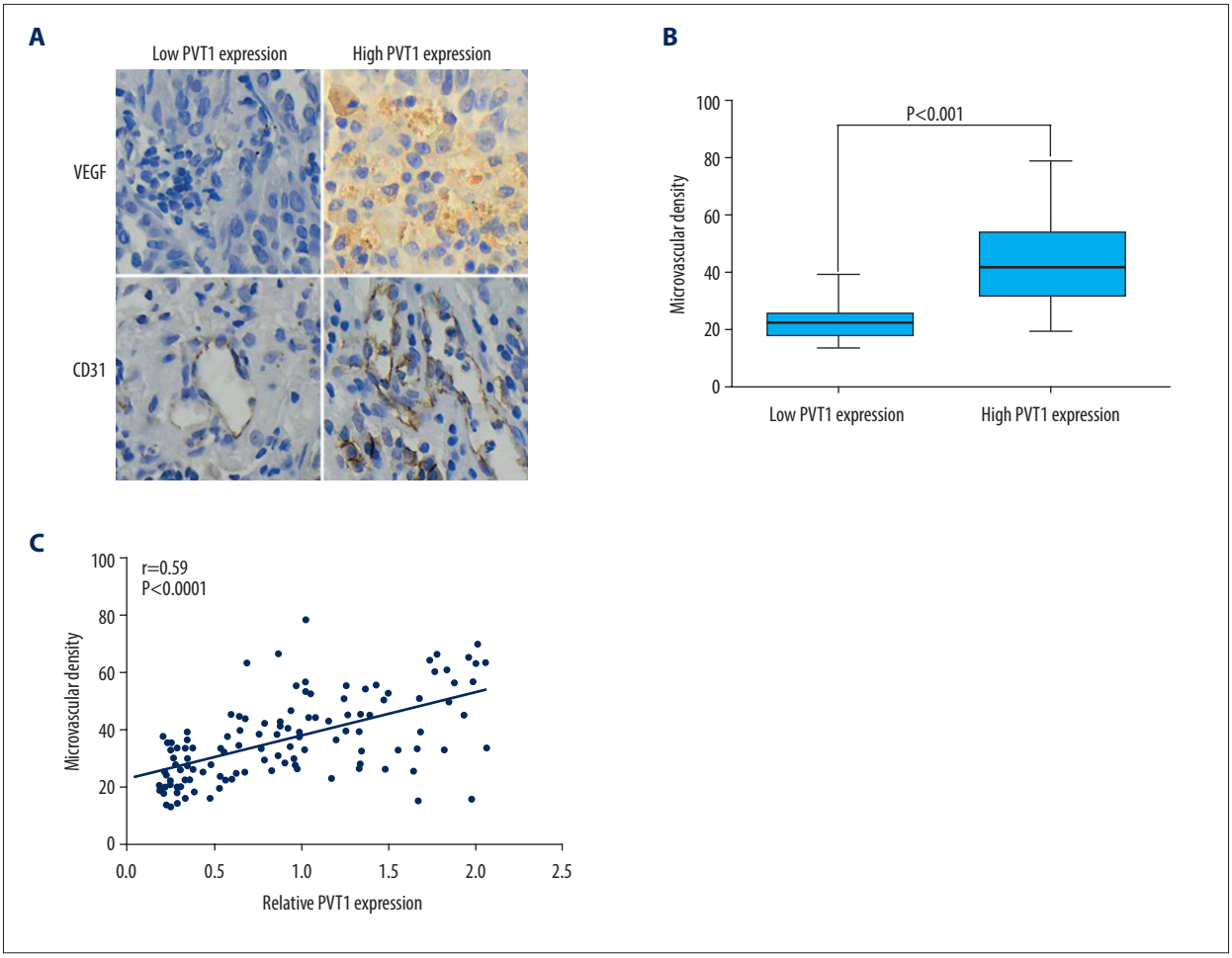

Figure 2. Positive correlation between PVT1 and angiogenesis in NSCLC. (**A**) Immunohistochemistry was used to assess VEGF and CD31 expression tumor tissues of NSCLC. (**B, C**) Statistical analysis of the interaction of PVT1 expression and microvascular density. CD31 staining was used for analysis of microvascular density (MVD). Six fields of view per slide were examined for the MVD.

measured the mRNA and protein expression of VEGF. As shown in Figure 3B, PVT1 upregulated the mRNA expression of VEGF in A549 cells. Similar results were observed in VEGF protein level (Figure 3D). VEGF is secreted by tumor cells and stromal cells, and then released out of the cells affect endothelial cells, causing angiogenesis. So, to investigate the influence of VEGF secretion by PVT1 in NSCLC cells, we collected the A549-cell culture medium treated with PVT1-targeted siRNA to measure VEGF concentration. The results indicated that the concentration of VEGF was upregulated by PVT1 (Figure 3C). Then, the A549 cell-conditioned medium treated with PVT1-targeted siRNA was applied to HUVECs, which was subsequently treated with or without VEGF antibody. Next, we tested tube formation abilities of HUVECs using tube formation assay. The potential for tube formation of HUVECs was lower in the group with PVT1-targeted siRNA compared to the control group. Moreover, VEGF antibody decreased the tube formation of HUVECs compared with IgG (Figure 3E, 3F). Our results show that PVT1 increases the expression and secretion of VEGF in NSCLC cells, and PVT1 promotes angiogenesis in NSCLC through upregulating VEGF.

VEGF is the targeted gene of miR-29c

TargetScanHuman software (*http://www.targetscan.org/*) predicated that there was a miR-29c-binding locus in 3'-UTR of VEGF (Figure 4A). Accordingly, we cloned the miR-29c-binding sequences in 3'-UTR of VEGF wildtype (WT) and mutant counterparts (MUT) into reporter plasmids, then assessed the relative luciferase activities. The results showed that the relative luciferase activities declined in 3'-UTR of VEGF (WT) when miR-29c mimics were exposed in A549 cells (Figure 4B). However, the activities had no changes in the mutant counterparts (MUT) (Figure 4C). Furthermore, we treated A549 with miR-29c mimics, then we examined the mRNA and protein expression of VEGF. Our results demonstrated that higher miR-29c downregulated both the mRNA (Figure 4D) and protein (Figure 4F) expression of VEGF in A549. Then, to investigate the influence

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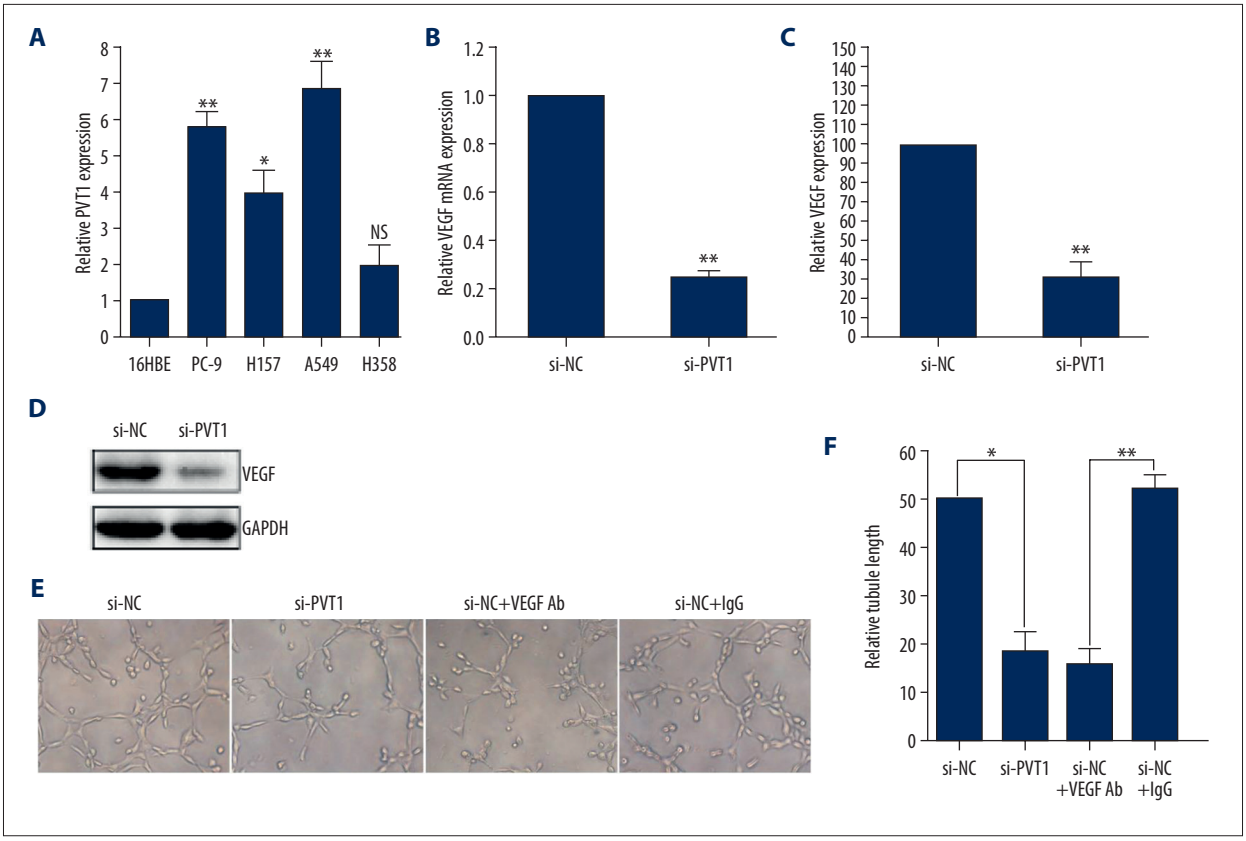

Figure 3. PVT1 promotes angiogenesis by regulating VEGF in NSCLC cells. (**A**) qRT-PCR was used to detect PVT1 expression in multiple NSCLC cell lines. * $P<0.05$, ** $P<0.01$ *vs.* 16HBE group. NS – no significant. (**B–D**) NSCLC A549 cells were treated with PVT1-targeted siRNA. qRT-PCR for detecting VEGF mRNA expression. Western blot analysis was used to assess VEGF protein expression. ELISA was used for detecting VEGF secretion. ** $P<0.01$. (**E, F**) HUVECs were cultured with A549 cell-conditioned medium, which was pretreated with PVT1-targeted siRNA. VEGF antibody was applied to HUVECs. The tube formation of HUVECs was observed. * $P<0.05$, ** $P<0.01$.

of VEGF secretion by miR-29c, we further collected the culture medium of A549 cells treated with miR-29c mimics to measure VEGF concentration (Figure 4E). The results showed that miR-29c downregulated VEGF concentration. Together, these results suggest that VEGF is the targeted gene of miR-29c.

miR-29c is an inhibitory target of PVT1

Starbase software (*http://www.targetscan.org/*) predicated that there was a binding site of miR-29c in PVT1 (Figure 5A). Then, we cloned the miR-29c-binding sequences in the PVT1 wildtype (WT) and mutant counterparts (MUT) into the reporter plasmids. The relative luciferase activities were assessed, showing decreased PVT1 (WT) when treated with miR-29c mimics in A549 cells (Figure 5B), but no changes in the mutant counterparts (MUT) (Figure 5C). Furthermore, higher miR-29c expression was observed in A549 cells treated with si-PVT1 compared to the si-NC group (Figure 5D). Then, the conditioned medium (CM) from A549 cells treated with miR-29c mimics was collected for the application for HUVECs for exploring the interaction of PVT1 and miR-29c as well as their contributions in angiogenesis in NSCLC. Next, tube formation assay was used to test tube formation abilities of HUVECs. We found lower potential for tube formation of HUVECs in the group treated with miR-29c mimics than in the control group (Figure 5E, 5F). We also examined the expression of miR-29c in NSCLC and corresponding normal tissues, and found that miR-29c expression was decreased in NSCLC compared to normal tissues (Figure 5G). Notably, miR-29c expression was negatively correlated with PVT1 expression (Figure 5H). Taken together, these results show that PVT1 promotes angiogenesis through regulating the miR-29c/VEGF signaling pathway in NSCLC.

Discussion

NSCLC has a high rate of metastasis and is the most lethal cancer worldwide. To promote proliferation and metastasis, NSCLC has to rely on angiogenesis, or new vessels formation. Therefore, anti-angiogenic therapy is an important strategy in

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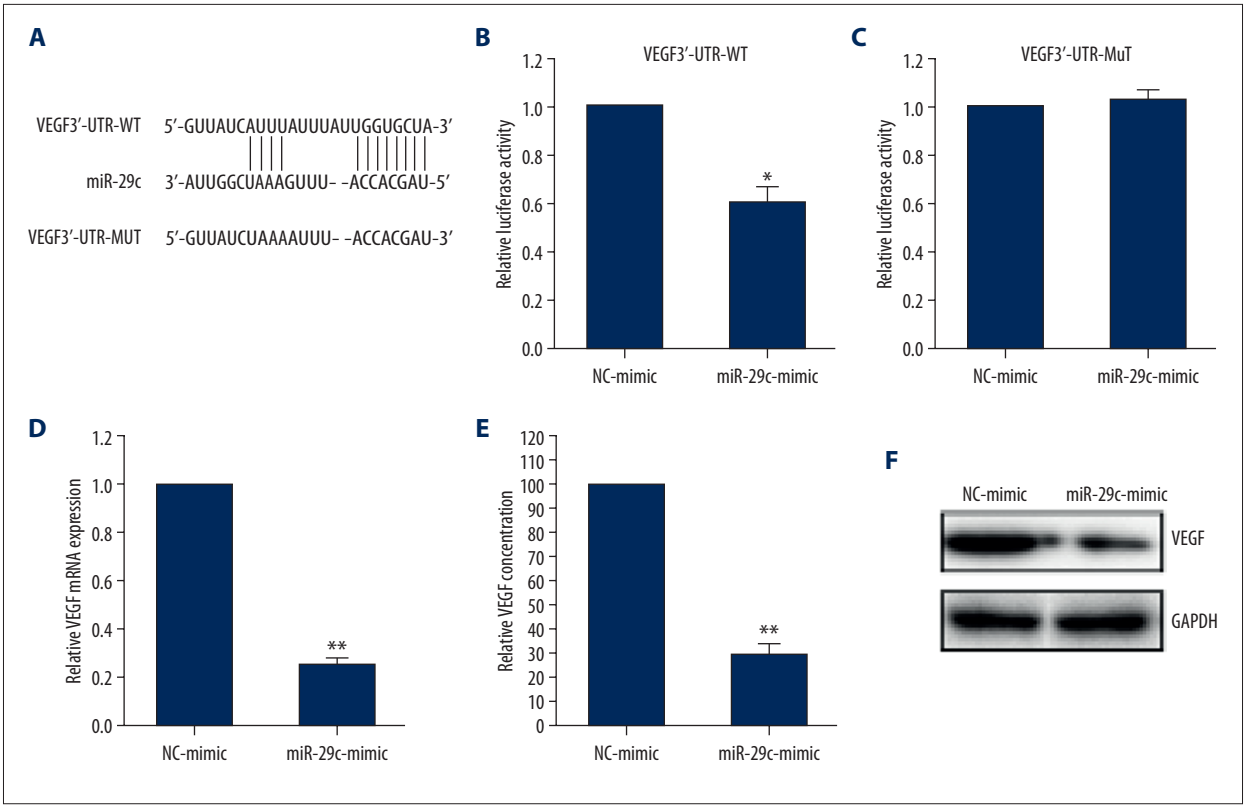

Figure 4. VEGF is the targeted gene of miR-29c. (**A**) The predicted miR-29c-binding site in 3′-UTR of VEGF. (**B, C**) The relative luciferase activity on 3′-UTR-WT and MUT counterparts were measured. * $P<0.05$. (**D–F**) NSCLC cells A549 was treated with miR-29c mimics. qRT-PCR for detecting VEGF mRNA expression. Western blot analysis of VEGF protein expression. ELISA was used to detect VEGF secretion. ** $P<0.01$.

NSCLC treatment [24]. In our study, we found that PVT1 promotes angiogenesis through targeting the miR-29c/VEGF signaling pathway in NSCLC.

PVT1 is a lncRNA in the 8q24 gene desert [25]. As an oncogene, PVT1 has been found to be overexpressed in many types of cancer, such as NSCLC [22], bladder cancer [26], and thyroid cancer [27]. Inhibiting PVT1 expression represses cancer cell viability and conversely promotes cancer cell apoptosis. Furthermore, PVT1, which is positively correlated with pathologic phase and tumor size, has also been confirmed to stimulate cell proliferation and invasion in NSCLC [28]. Combined with our results, these studies have broadened our understanding of the effects of PVT1 on NSCLC, which can promote proliferation, invasion, angiogenesis, and metastasis. In terms of mechanisms, lncRNAs, with more than 200 nucleotides, serve as the gene regulator in the tumor microenvironment. lncRNAs have effects on many cellular pathways through regulating numerous genes [29]. In our study, we found that PVT1 directly targeted miR-29c to stimulate angiogenesis. miR-29c has been reported to suppress the angiogenesis in NSCLC, consistent with our study. Furthermore, in lung cancer, miR-29c has been confirmed to play suppressive roles, and can suppress tumor proliferation, invasion, and metastasis [30]. In addition, VEGFA is confirmed to be the functional target gene of miR-29c. Downregulation of VEGFA by miR-29c affects HUVEC tube formation and inhibits angiogenesis in NSCLC [31]. VEGFA belongs to the VEGF family. Angiogenesis is regulated by the balance between pro-angiogenic and anti-angiogenic factors, among which VEGF is one of the most pro-angiogenic factors. VEGF binds to its receptors on blood vessel endothelial cells to regulate endothelial cell migration, proliferation, and survival [32]. Moreover, decreased VEGF expression can inhibit angiogenesis. Therefore, cancer treatment with drugs targeting VEGF, such as sorafenib and bevacizumab, is promising [33]. Bevacizumab was confirmed to benefit patients with metastatic colorectal cancer, and subsequent clinical studies have extended to these findings to NSCLC [9]. Our results showed that VEGF promotes angiogenesis, which was regulated by PVT1/miR-29c. In conclusion, PVT1 promotes angiogenesis through targeting the miR-29c/VEGF signaling pathway in NSCLC, which might be a potential therapy for NSCLC.

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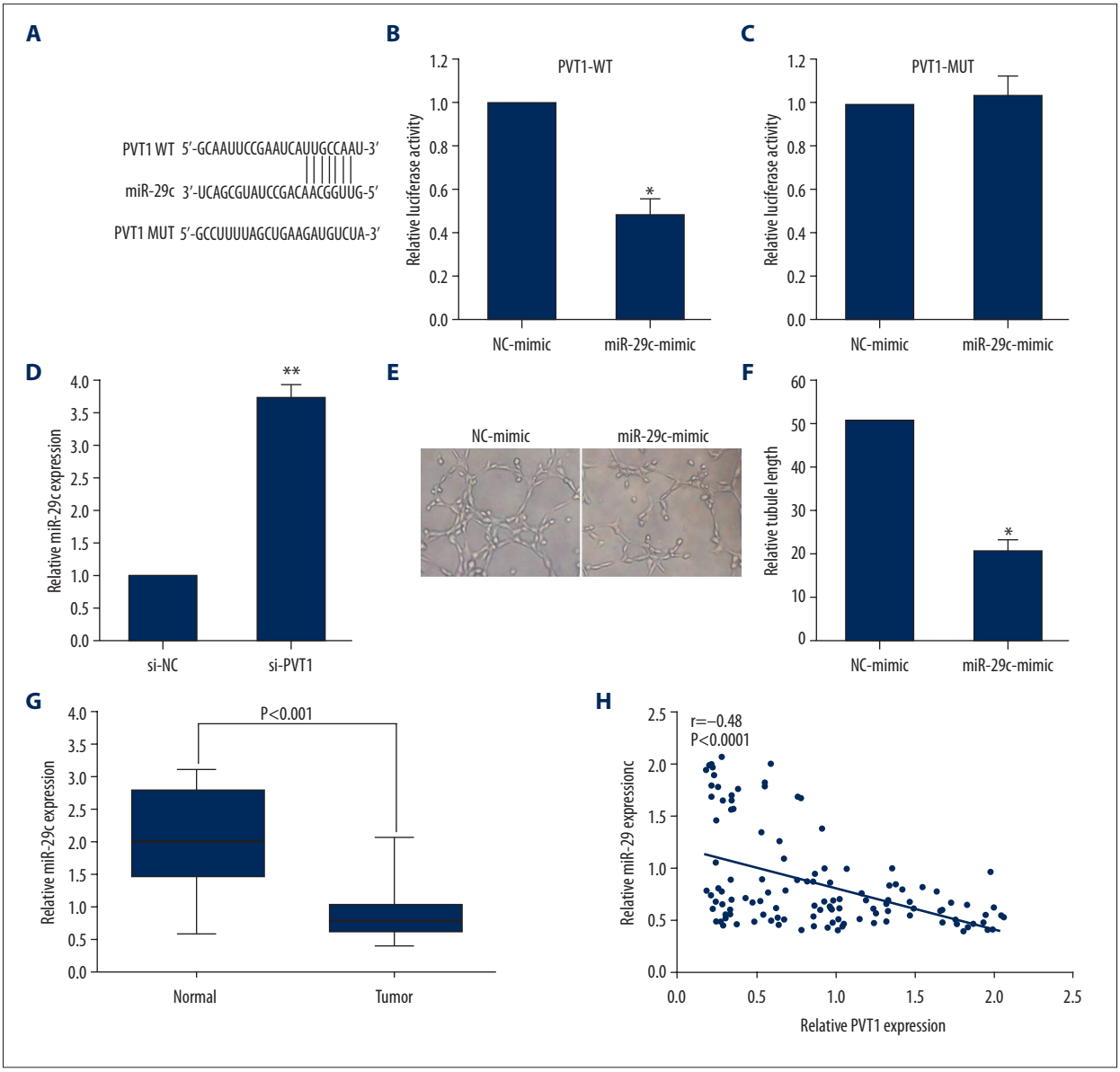

Figure 5. miR-29c is targeted by PVT1. (**A**) Schematic diagrams of the predicted binding site of miR-29c in PVT1. (**B, C**) The relative luciferase activity of PVT1 WT and MUT counterparts were measured. * *P*<0.05. (**D**) The expression of miR-29c in A549 cells treated with si-NC and si-PVT1 was measured by qRT-PCR. ** *P*<0.01. (**E, F**) HUVECs were cultured with A549 cell conditioned medium, which was pretreated with miR-29c mimics. The tube formation of HUVECs was observed. * *P*<0.05. (**G**) qRT-PCR for detecting the expression of miR-29c in 120 NSCLC and corresponding normal tissues. (**H**) Statistical analysis of the correlation between miR-29c and PVT1 expression in 120 NSCLC tissues.

Conclusions

Our results indicate that PVT1 is overexpressed in NSCLC and the elevated PVT1 is closely related to the angiogenesis and poor prognosis found in NSCLC. We also found that PVT1 promotes angiogenesis in NSCLC and PVT1 upregulates VEGF expression and secretion by directly targeting miR-29c. In conclusion, PVT1 promotes angiogenesis through targeting the miR-29c/VEGF signaling pathway in NSCLC.

Conflict of interests

None.

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