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Original Article

Long noncoding RNA VPS9D1-AS1 promotes angiogenesis in colorectal cancer by regulating the VEGFA signalling pathway

Zheying Zhang[1*], Yifei Han[1*], Yang Yang[1], Xianglong Li[2], Yifan Han[3], Shuai Zhang[1], Yan Zou[1], Yu Zhang[1], Yitian Xie[1], Ying Sun[1], Jiateng Zhong[1], Baoshun Du[4], Shenglei Li[2], Na Li[1]

[1]Department of Pathology, Xinxiang Medical University, Xinxiang 453003, Henan, China; [2]Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China; [3]Beijing Emergency General Hospital Central Laboratory, Beijing 100005, China; [4]Department of Neurosurgery, Xinxiang Central Hospital, Xinxiang 453001, Henan, China. [*]Equal contributors.

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Abstract: To clarify the mechanism of long non-coding RNA VPS9D1-AS1 affecting angiogenesis in colorectal cancer (CRC). Western blot and qRT-PCR assays were performed to detect the expression of VPS9D1-AS1 in colorectal cancer. The effects of VPS9D1-AS1 regulating VEGFA and affecting the proliferation, migration and invasion of human umbilical vein endothelial cells (HUVECs) were examined using cell biology, in vitro tubeformation and Chorioallantoic membrane vascular assay. Chromatin Immunoprecipitation (ChIP) and dual luciferase assays were performed to verify the specific sites of transcription factor binding to the promoter region of VPS9D1-AS1. VPS9D1-AS1 is highly expressed in colorectal cancer. Interfering with VPS9D1-AS1 inhibited the proliferation, invasion and migration of HUVECs. Mechanistically, VPS9D1-AS1 can promote angiogenesis by upregulating VEGFA expression and activating the downstream PI3K/AKT pathway. In addition, CEBPB is a transcription factor of VPS9D1-AS1 predicted by database, and the results of ChIP experiments showed that CEBPB could directly bind to the VPS9D1-AS1 promoter region at the -698 bp to -794 bp site. The results of dual luciferase assay showed that CEBPB could enhance VPS9D1-AS1 promoter activity and promote its transcription. VPS9D1-AS1 can be activated by CEBPB transcription factor and target VEGFA to activate its downstream pathway to promote colorectal cancer angiogenesis, which may suggest that VPS9D1-AS1 is critical for regulating colorectal cancer angiogenesis.

Keywords: Colorectal cancer, VPS9D1-AS1, angiogenesis, VEGFA, CEBPB

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and it is the third leading cause of cancer globally [1]. Colorectal cancer has an insidious onset and most patients are already in advanced stages when diagnosed, and despite improved medical treatments, the disease is prone to recurrence and metastasis, resulting in poor patient survival [2], so it is important to study the mechanism of colorectal cancer development.

Angiogenesis is the process of growing new capillaries from existing blood vessels, and this process is critically associated linked to the growth and metastasis of colorectal cancer as well as many solid tumors [3]. One of the key steps in tumor progression and metastasis is the secretion of factors from tumor cells into the microenvironment, which then act on stromal cells and promote the growth of new blood vessels [4]. This abnormal regulation of angiogenesis is one of the causes of high tumor mortality and poor prognosis [5]. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a highly specific pro-vascular endothelial cell growth factor that promotes increased vascular permeability, extracellular matrix degeneration, vascular endothelial cell migration, proliferation and angiogenesis. VEGF is a family that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PGF). Typically, VEGF refers to

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VEGF-A. VEGFA is synonymous with vascular endothelial growth factor A. VEGFA protein is secreted by tumor cells, macrophages and fibroblasts, etc. It is widely distributed in many tissues ofthe human body, but the expression level is extremely low, and its role is primarily to maintain normal vascular density and basic permeability functions, ensuring nutrient supply. When tumor cells are present, its expression level generally increases significantly. It has been shown that VEGFA is associated with tumor aggressiveness and also with tumor susceptibility. High VEGF expression and increased tumor angiogenesis are closely associated with invasion, metastasis and poor prognosis in colorectal cancer as well as many other tumors [6, 7].

Approximately 75% of the human genome can be transcribed into RNA, of which only 3% encodes proteins [8], and there are RNAs that do not have the function of encoding proteins called ncRNAs. ncRNAs have been reported to play a crucial role in human malignancies, acting either as oncogenes or tumor suppressors to regulate cancer development and progression [9]. LncRNAs are non-coding RNAs, which are greater than 200 nucleotides in length. It has been widely reported in the literature that LncRNAs are important regulators of cancer-related disease phenotypes, for example, Wang et al. demonstrated that LncRNA HOXA-AS2 indirectly suppresses gene expression by competing for miRNA binding [10]; Cao H.L. et al. showed that lncRNA-RMRP promotes bladder cancer by targeting miR-206 [11]. Due to their unique molecular structure, lncRNAs can interact with macromolecules such as DNA, other RNAs, and proteins, performing powerful regulatory functions in cells. VPS9D1 antisense RNA 1 (VPS9D1-AS1) maps to chromosome 16q24.3 and the gene is 1,753 bp in length [12]. Previous studies have found that VPS9D1-AS1 is highly expressed in various cancers, for instance, Jiefeng Liu et al. demonstrated that LncRNA VPS9D1-AS1 promotes lung adenocarcinoma by targeting the miRNA-30a-5p/KIF11 axis [13]; Peng et al. showed that VPS9D1-AS1 promotes lung adenocarcinoma by enhancing proliferation, invasion, and epithelial-mesenchymal transition through the miR-377-3p/SGK1 axis in endometrial cancer [14]; VPS9D1-AS1 also promotes invasion and metastasis in colorectal cancer cells. For example, Liu et al. found that VPS9D1-AS1 promotes colon adenocarcinoma proliferation by sponging miR-1301-3p [15]; Liu H.R. et al. found that VPS9D1-AS1 promotes tumorigenicity of colorectal cancer cells by upregulating HMGA1 to suppress microRNA-525-5p [16].

Previous studies have demonstrated that VPS9D1-AS1 promotes proliferation, migration, and invasion in colorectal cancer, but its role in colorectal cancer angiogenesis remains unexplored. In the present study, we found that Lnc VPS9D1-AS1 was activated by CEBPB transcription factor and activated its downstream pathway to promote colorectal cancer angiogenesis through upregulation of VEGFA expression. This finding may contribute to the understanding of the molecular mechanisms involved in colorectal cancer angiogenesis and help in the diagnosis and treatment of this disease.

Materials and methods

Tissues and specimens

Twenty cases of human colorectal cancer tissues and paired paracancerous normal tissues were collected by relevant professional technicians from patients who underwent surgical resection of colorectal cancer in the Department of General Surgery of the Third Affiliated Hospital of Xinxiang Medical College, all of which were approved by the Ethics Committee of Xinxiang Medical College. Informed consent was obtained from all patients. Fresh colorectal cancer tissues and matched normal mucosal tissues (5 cm from the tumor margin) were collected, and the specimens were immediately placed in liquid nitrogen within 10 minutes of isolation and subsequently transferred to -80° ultra-low temperature refrigerator for long-term storage.

Cell lines and culture conditions

Human colorectal cancer cell lines SW480, LOVO, HCT-116, HCT-8, DLD1, CACO2, RKO were purchased from ATCC (American type culture); human normal colonic epithelial FHC were purchased from Wuhan Punosai Life Sciences Co. Ltd.; human umbilical vein endothelial cells HUVEC were obtained from the Department of Pathology, Xinxiang Medical College, Tumor The cells were stored in liquid nitrogen. Cells were cultured in DMEM contain-

ing 10% fetal bovine serum in a 37°C, 5% CO_2 incubator.

Conditioned medium

CRC cells (1×10^6) were cultured in 6-well plates overnight, and the culture medium was changed to fresh medium with or without fetal bovine serum (FBS) in each well. After 24 h, the conditioned medium was collected and subsequently used for CCK8, migration, scratch assay and tube formation assays on HUVECs. The procedures followed the methods described in the literature [17].

Cell transfection

When the cell confluence reached approximately 80%, the cells were ready for passaging. HCT-116 and SW480 cells in logarithmic growth phase were digested with 0.25% trypsin, washed with PBS, centrifuged, resuspended with serum-free medium and inoculated in 6-well plates. When the cell confluence rate reached 70%-80% on the following day, the medium in the six-well plate was aspirated and replaced with serum-free medium. Negative control (NC), si-VPS9D1-AS1-1, si-VPS9D-AS1-2, respectively, were mixed with transfection reagent Lipo8000 and added to the cells for incubation, and after 6-8 hours of incubation, the medium was replaced with serum-containing medium. Cells were then collected for subsequent studies. The sequence of VPS9D1-AS1 interference fragment 1 is: Forward 5'-GACACTGCTGTGCTAGCTTC-3', Reverse 5'-AGTGGTAGAGCCGACTGTGA-3'; The sequence of interference fragment 2 is: Forward 5'-CCCGAGGAAGGCTACTGATG-3', Reverse 5'-CTGCAGACTGTGTCTGAACCT-3'.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent, and the RNA concentration of each sample was determined. The total RNA of each sample was then reverse transcribed into cDNA by a reverse transcription kit. Polymerase chain reaction was performed using the SYBR Green dye method. Amplification conditions for VPS9D1-AS1 were as follow: pre-denaturation 95°C for 10 min, denaturation 95°C for 15 s, annealing/extension 60°C for 1 h, melting curve 95°C for 15 s, 60° for 1 min, 40 cycles were performed. GAPDH was used as the housekeeping gene, and the relative expression of the target gene was calculated using the $2^{-\Delta CT}$ (experimental group)/$2^{-\Delta CT}$ (control group). The primer sequences were as follows: GAPDH: Forward 5'-AATGCATCCTGCACCACCAA-3', Reverse 5'-GTAGCCATATTCATTGTCATA-3'; VPS9D1: Forward 5'-CCAGACAAGGACAGCTCGTT-3', Reverse 5'-CAGCCTGTCTACGGCATTGT-3'; VPS9D1-AS1: Forward 5'-TTTTCCCTGCAAGCCATG-3', Reverse 5'-CGACTGTGACCAGCCTTCTT-3'.

Cell counting kit-8 (CCK-8)

HUVEC cells were cultured in conditioned medium (CM) of two cell lines, HCT-116 and SW480, and inoculated in 96-well plates at a cell density of 5×10^3 cells/well. 10 μL of CCK-8 solution was added to each well the next day after cell seeding, and incubated at 37°C in an incubator containing 5% CO_2 for 1-4 h. The absorbance values at 450 nm were measured using an enzyme microplate reader. The cell proliferation rates were subsequently measured at 24 h, 48 h, 72 h and 96 h and cell growth curves were plotted. The horizontal axis represents the different time points and the vertical axis represents the absorbance value.

Scratch experiment

HUVECs were inoculated in 6-well plates at a density of 5×10^5 cells/well to reach 80% confluence on the second day. The endothelial cells were stimulated with conditioned medium for 2 days. Three replicate wells were set up by using a 200 μl pipette tip to make a straight line scratch in the middle of the six-well plate, three times per well in parallel, and incubated without serum for 24 h and 48 h, and then photographed under an inverted microscope to determine the migration ability of the cells.

Migration experiment

Conditioned medium of HCT-116 cells and SW480 cells were added to HUVEC cells for two days of co-culture. Two days later, the cells were digested and centrifuged, resuspended with serum-free medium, and cells were counted. 3×10^5 cells were inoculated in the upper chamber of a 24-well plate with approximately 200 μl. 550 μl of medium containing 10% fetal bovine serum was added to the lower chamber. After 24 h of incubation, the chamber was

removed and fixed with 4% paraformaldehyde and then stained with 1% crystal violet. The invaded HUVEC cells were imaged and counted under an inverted microscope.

In vitro tubule formation experiment

After stimulation with conditioned medium, HUVEC cells were starved overnight in advance, and the pre-chilled matrix gel, pipette tip, and 96-well plates were refrigerated at 4°C overnight. The next day, 50 μl/well of matrix gel was added to the 96-well plate (no air bubbles) and placed in the incubator for 30 min. HUVEC cells were digested with trypsin, resuspended with serum-free medium, and cells were counted and added to the matrix gel at 3×10^4/well cells, and observed and photographed under an inverted microscope after 4-6 h.

Chorioallantoic membrane vascular assay (CAM)

Fertilized eggs of 0 days embryonic age were collected, the shells were wiped with 1:1000 benzalkonium bromide solution and placed in a incubator at 37°C with the large head up to start incubation. At the 5th day, the eggs were illuminated with light in the dark to observe for air chambers and blood vessels, and the air chambers were drawn with a marker. On the 7th day, cell supernatants with 90%-100% cell confluence were collected, the eggs were carefully peeled off with forceps, the eggshell membrane was wetted with drops of saline, and the membrane was gently torn away. Place the area with a pre-autoclaved test ring and add 300 μl of conditioned medium dropwise to the test ring. Stick the upper gap with a hospital dressing patch (to ensure good air permeability and water resistance), place in an incubator at 37°C for 3 days, and observe and take pictures.

Chromatin immunoprecipitation (ChIP)

Add 37% formaldehyde crosslinking to the Petri dish, then add 1.1 ml of Glycine Solution, leave at room temperature for 5 minutes, discard the liquid, wash twice with precooled PBS, centrifuge the precipitate and then sonicate it so that the DNA size is 400-800 bp, centrifuge the precipitate after sonication, take 20 μl as input add NaCl and mix, heat, -20°C and store; After dilution of the remaining sonicated sample, add primary antibody, incubate overnight at 4°C; the next day, add 80 μl A/G beads, rotate at 4°C for 60 min, discard supernatant after magnetic separation, wash the beads; add 250 μl Elution buffer to the beads, vortex and shake, rotate and elute, magnetic separation to obtain supernatant, repeat once, add NACL after supernatant combination and mix, 65°C for 4 h The sample can be directly used for PCR amplification. Primer sequence of site one -1419 bp to -1539 bp: Forward 5'-TGGCTCATGCTTGTAATCCCAG-3', Reverse 5'-CAGCTCACGCCTCAGCCCTT-3'; Primer sequence of site two -698 bp to -794 bp primer sequences: Forward 5'-CATCCACAGGTGCCGCGCTA-3'; Reverse 5'-TTTCCTCGGCTTCTCCAACCTCC-3'.

Western blot

Protein samples were added to lysis solution for 30 min, 4°C, centrifuged, 12000 r, and protein quantification and concentration measurement. The samples were added into the electrophoresis tank for electrophoresis at 80 V for 40 min for the upper gel layer and 120 V for 60 min for the lower gel layer to separate the proteins with different molecular weights. After electrophoresis, the proteins were transferred to PVDF membrane for electrotransfer; the membrane was closed in milk for 2 h to remove the nonspecific bands; the primary antibody was incubated overnight; the membrane was washed; the secondary antibody was incubated for 1 h; the membrane was washed; and the exposure solution was developed.

Dual-luciferase reporter assay

The mutant and wild-type plasmids of VPS9D1-AS1 promoter region with loci from -664 bp to -844 bp were constructed by Nanjing Prime Tech Biotechnology Co. 239T cells in logarithmic growth phase were seeded into a 96-well plate protected from light, transfected the next day at the amount of cells transfected, and changed to normal medium after 6-8 h. 24-48 h later, the plate was removed and equilibrated at room temperature 10 min, add 100 μl of firefly detection reagent to each well, mix well, incubate at room temperature for 10 min, and assay on the machine. Add 100 μl of renilla luciferase assay working solution to each well, mix well, incubate at room temperature for 10 min, and test on the machine.

Statistical analysis

All data and figures were analyzed using GraphPad Prism 9 and ImageJ software. Differences between two groups were analyzed using the two-tailed t-test. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) was used to assess differences in group means. Subsequently, Tukey's honestly significant difference (HSD) test was applied for pairwise comparisons to analyze differences between individual groups. The statistical significance level was set at $P<0.05$.

Results

VPS9D1-AS1 is highly expressed in colorectal cancer

First, we analyzed the expression profiles of LncRNAs in human colorectal cancer tissues and paracancerous tissues using the TCGA database and plotted heat maps and volcano maps. The top 50 LncRNAs with upregulated and downregulated expression in colorectal cancer were listed, among which VPS9D1-AS1 showed a significant difference in expression in colorectal cancer ($P<2.19e-22$) (**Figure 1A** and **1B**). Previous studies have shown that VPS9D1-AS1 functions as a LncRNA primarily by sponging miRNAs. However, its additional mechanisms of action in colorectal cancer remain unclear. Therefore, we selected VPS9D1-AS1 for further investigation in this study. To assess the expression of VPS9D1-AS1 in colorectal cancer, we analyzed its expression in colorectal cancer tissues compared to adjacent normal tissues using the GEPIA database (http://gepia.cancer-pku.cn/). The results revealed that VPS9D1-AS1 expression was significantly upregulated ($P<0.05$) (**Figure 1C**). Subsequently, the expression of VPS9D1-AS1 was quantified in normal colorectal epithelial cells and colorectal cancer cells using real-time fluorescence, and the results showed that VPS9D1-AS1 was highly expressed in colorectal cancer cells (**Figure 1D**). Real-time quantitative PCR analysis of VPS9D1-AS1 expression in eight colorectal cancer tissues and matched adjacent normal tissues revealed upregulation in cancer tissues (**Figure 1E**).

VPS9D1-AS1 promotes the proliferation and migration of HUVEC under the influence of colorectal cancer cells and angiogenesis in vitro and in vivo

It has been documented that VPS9D1-AS1 is associated with tumor proliferation and migration, but whether VPS9D1-AS1 also affects other processes of tumor malignant progression remains unknown. First, we predicted the correlation between VPS9D1-AS1 and key proteins in cell autophagy, iron death and angiogenesis using GEPIA database, and the results showed that VPS9D1-AS1 was not significantly correlated with key proteins involved in cell autophagy (ATG2B, ATG3, ULK1) and key proteins associated with ferroptosis (ACSL4, SLC7A11, TP53) (**Figure 2A**). We further analyzed the correlation between VPS9D1-AS1 and angiogenesis-related proteins (VEGFA, VEGFR) and microvessel density (MVD), a key measure of tumor angiogenesis. The results indicated significant correlations between VPS9D1-AS1 and VEGFA, VEGFR, and MVD ($P<0.05$) (**Figure 2B**). To investigate whether VPS9D1-AS1 could affect angiogenesis in colorectal cancer, we selected two highly expressed HCT-116 and SW480 cells for correlation assays. First, we transiently transfected two distinct VPS9D1-AS1-specific siRNA fragments targeting different loci into HCT116 and SW480 cells and confirmed their interference efficiency (**Figure 2C**). The effect of VPS9D1-AS1 on vascular endothelial cells was examined by scratch assay, CCK8, transwell, and in vitro tubule formation assay after stimulation of HUVEC cells with conditioned medium transfected with si-VPS9D1-AS1 in HCT-116 and SW480 cells. The results of CCK8 showed that the proliferation of HUVEC cells slowed down after knocking out VPS9D1-AS1 (**Figure 2D**). Scratch assay and Transwell results demonstrated that HUVEC cells treated with conditioned medium (CM) from VPS9D1-AS1-silenced cells exhibited significantly reduced migration and invasion compared to the control group (**Figure 2E** and **2F**). The results of in vitro tubule formation assay showed that the tubule formation ability of HUVEC cells treated with CM that silenced VPS9D1-AS1 was significantly reduced ($P<0.05$) (**Figure 2G**), suggesting that knockdown of VPS9D1-AS1 reduced the formation of blood vessels by HUVEC cells. We confirmed these

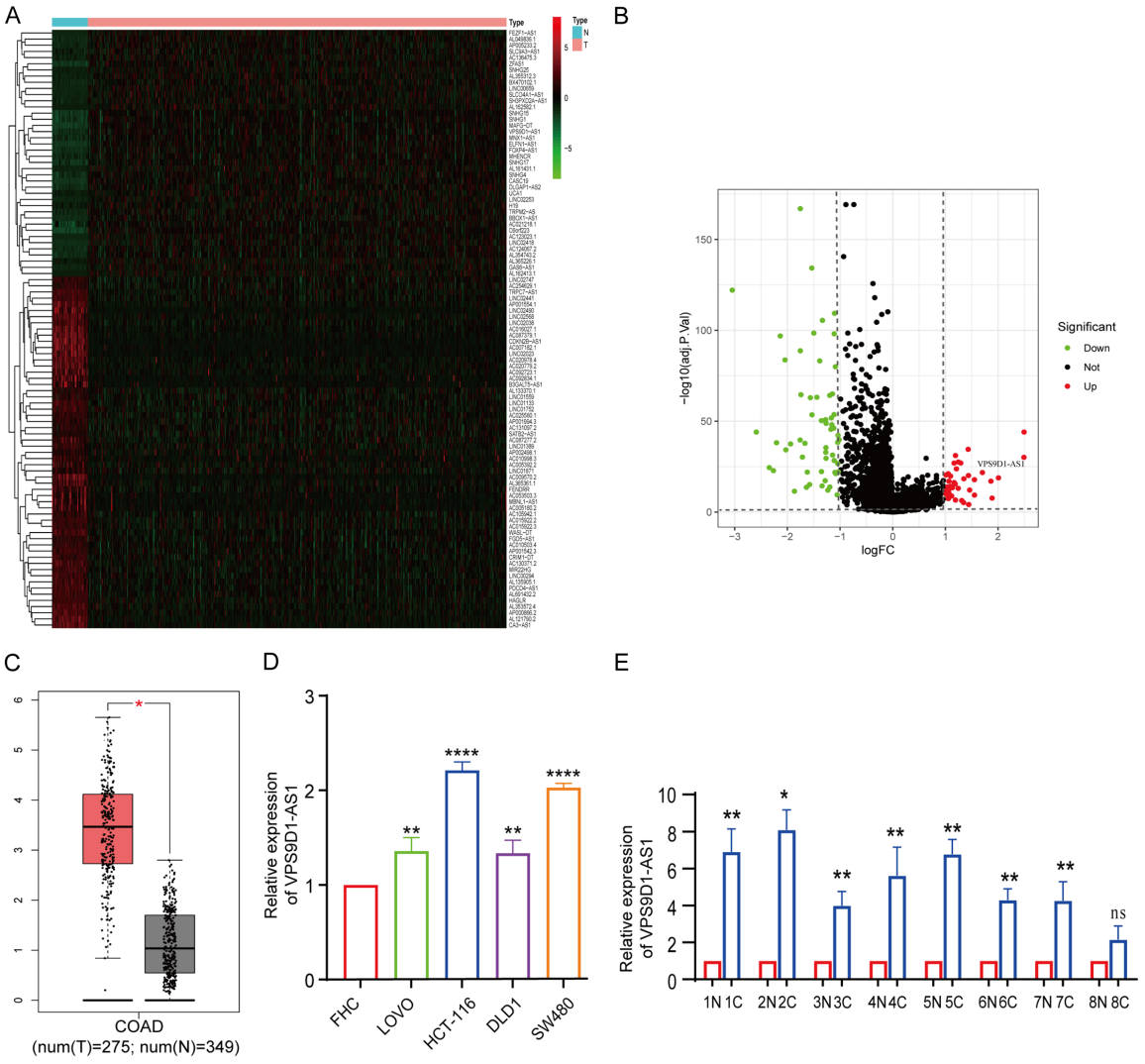
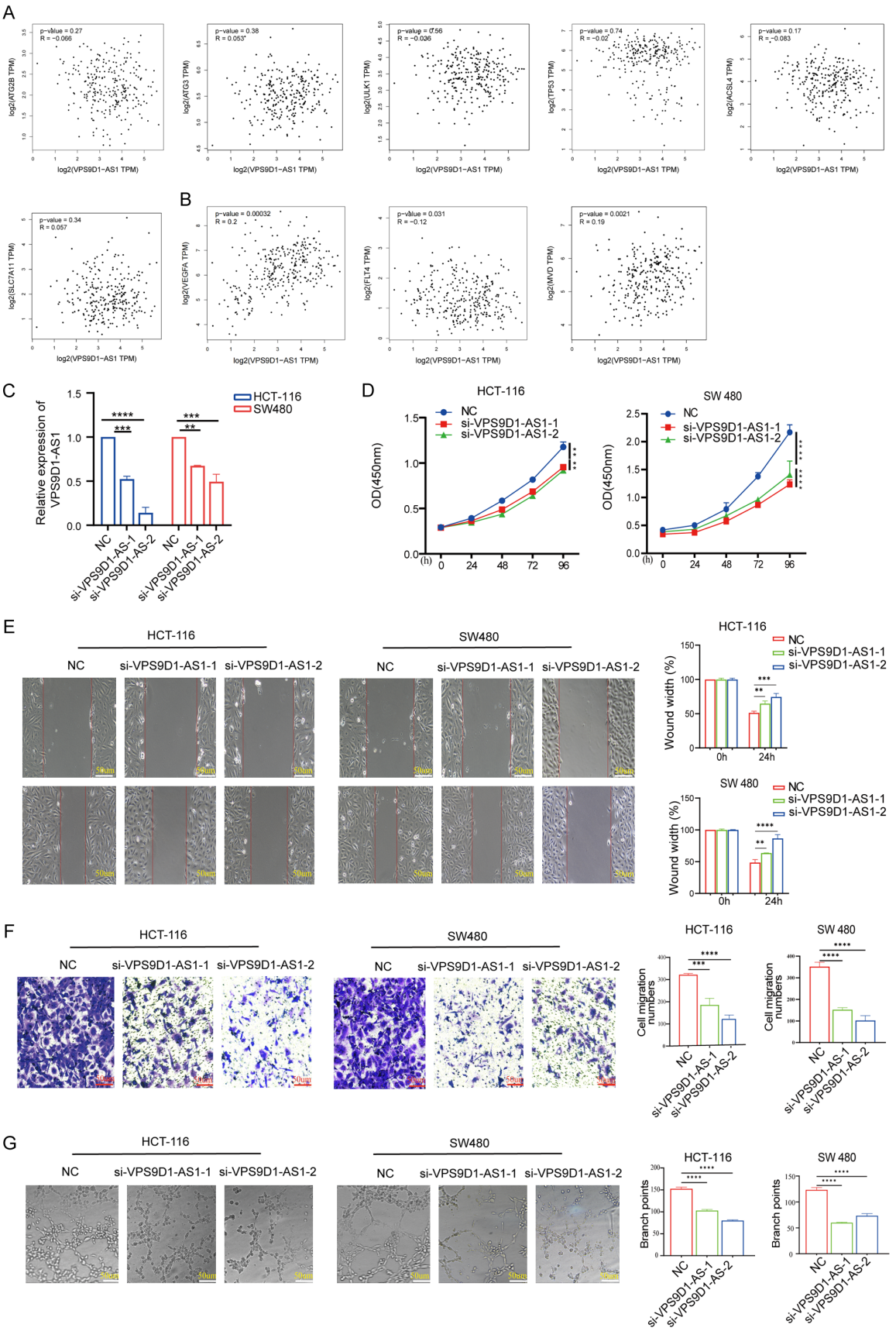


Figure 1. VPS9D1-AS1 is highly expressed in colorectal cancer. A. Data were downloaded from the TCGA database and collated and analyzed for differentially expressed LncRNAs in colorectal cancer. B. Volcano plot analysis of the screened LncRNA expression trends. The horizontal axis represents ploidy changes. The vertical axis represents the *p*-value of the difference. Green, down-regulated genes; red, up-regulated genes. C. Expression of Lnc VPS9D1-AS1 was predicted in 275 colorectal tissues and 349 normal tissues adjacent to cancer using the GEPIA database. *P<0.05. D. qRT-PCR assay to detect the expression of VPS9D1-AS1 in five cell lines of colorectal cancer. E. qRT-PCR assay to detect VPS9D1-AS1 expression in 8 matched pairs of colorectal cancer clinical tissues.

findings using the chick chorioallantoic membrane (CAM) assay, demonstrating that interference with VPS9D1-AS1 significantly inhibited angiogenesis (P<0.05) (**Figure 2H**). We confirmed that VPS9D1-AS1 can promote angiogenesis in colorectal cancer as shown by in vitro functional assays and in vivo experiments.

VPS9D1-AS1 promotes angiogenesis in colorectal cancer via VEGFA

To investigate the mechanism by which VPS9D1-AS1 affects angiogenesis, we previously predicted the correlation between VPS9D1-AS1 and VEGFA using the GEPIA database, and the difference was significant. We further predicted the relationship between VPS9D1-AS1 and other angiogenesis-related factors, including VEGFB, VEGFC, and VEGFD, and the results showed no significant correlation (**Figure 3A**), so we hypothesized that VPS9D1-AS1 affects colorectal cancer angiogenesis through VEGFA. Next, we knocked down VPS9D1-AS1 in HUVECs, a type of vascular endothelial cell, and the results showed that knockdown of VPS9D1-AS1 significantly


A
p-value = 0.27
R = -0.066
log2(ATG2B TPM)
p-value = 0.38
R = 0.053
log2(ATG3 TPM)
p-value = 0.56
R = -0.036
log2(ULK1 TPM)
p-value = 0.74
R = -0.02
log2(TP53 TPM)
p-value = 0.17
R = -0.083
log2(ACSL4 TPM)
log2(VPS9D1-AS1 TPM)
B
p-value = 0.34
R = 0.057
log2(SLC7A11 TPM)
p-value = 0.00032
R = 0.2
log2(VEGFA TPM)
p-value = 0.031
R = -0.12
log2(FLT4 TPM)
p-value = 0.0021
R = 0.19
log2(MVD TPM)
C
Relative expression of VPS9D1-AS1
HCT-116
SW480
NC
si-VPS9D1-AS-1
si-VPS9D1-AS-2
D
HCT-116
SW 480
NC
si-VPS9D1-AS1-1
si-VPS9D1-AS1-2
OD(450nm)
(h)
E
HCT-116
SW480
NC
si-VPS9D1-AS1-1
si-VPS9D1-AS1-2
Wound width (%)
0h
24h
F
Cell migration numbers
G
Branch points

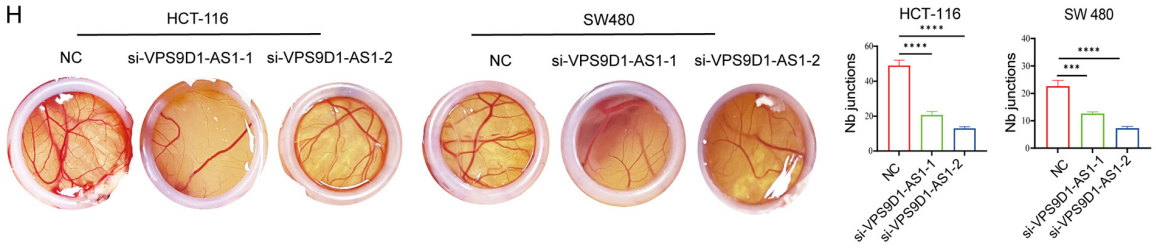


Figure 2. VPS9D1-AS1 promotes proliferation, migration, and angiogenesis in Colorectal Cancer. A. GEPIA database predicted the correlation of VPS9D1-AS1 with ATG2B, ATG3, ULK1, SLC7A11, TP53, ACSL4. B. GEPIA database predicted the correlation of VPS9D1-AS1 with VEGFA, MVD, VEGFR, *P<0.05. C. VPS9D1-AS1 siRNA was transfected into HCT-116 and SW480, and the expression of VPS9D1-AS1 was detected (n=3); *P<0.05. D. Two siRNAs of VPS9D1-AS1 with different interference sites were transfected into HCT116 and SW480 cells, and supernatants and HUVEC were co-cultured after 48 h. cck8 detection of the effect of VPS9D1-AS1 on the proliferation of HUVEC cells (n=3); *P<0.05. E. Two siRNAs of VPS9D1-AS1 with different interference sites were transfected into HCT116 and SW480 cells, and supernatants and HUVEC were co-cultured after 48 h. Scratch experiment detection of the effect of VPS9D1-AS1 on the proliferation of HUVEC cells (n=3); *P<0.05. F. Two different interfering sites of siRNA of VPS9D1-AS1 were transfected into HCT-116 and SW480 cells, and the supernatants were co-cultured with HUVEC after 48 h. Transwell assays were performed to evaluate the effect of VPS9D1-AS1 on HUVEC cell invasion, and compared with NC group (n=3); *P<0.05. G, H. In vitro tubule, chick embryo chorioallantoic membrane assay to detect angiogenesis after three co-culture supernatant treatments of NC, si-VPS9D1-AS1-1, and si-VPS9D1-AS1-2, and the branching nodes of blood vessels were reduced compared with the NC group (n=3); *P<0.05.

reduced the expression of VEGFA (**Figure 3B**). We transiently transfected the interfering fragment of VPS9D1-AS1 in colorectal cancer HCT-116 and SW480 cells and transfected plasmids overexpressing VEGFA to verify its efficiency (**Figure 3C**). HUVECs were stimulated with conditioned medium from 116 and 480 cells transfected with the interfering fragment of VPS9D1-AS1 and overexpressing VEGFA, and the effect of VPS9D1-AS1 on vascular endothelial cells was examined by scratch assay, CCK8, Transwell, and in vitro tubule formation assay. The results of the CCK8 assay showed that, after knocking down VPS9D1-AS1 in CM culture, the proliferation of HUVECs cultured with disrupted VPS9D1-AS1 and overexpressed VEGFA was significantly restored compared to the control group (**Figure 3D**). The scratch assay and transwell results showed that CM treatment of HUVECs silenced with VPS9D1-AS1 significantly inhibited the migration and invasion of HUVEC cells compared to the control group. In contrast, HUVEC cells cultured with CM that interfered with VPS9D1-AS1 and overexpressed VEGFA recovered their migratory and invasive abilities (P<0.05) (**Figures 3E** and **4A**). The results of in vitro tubule formation assay showed that the tubule formation ability of HUVECs treated with CM that silenced VPS9D1-AS1 was significantly reduced. In contrast, the lumen formation ability of HUVECs cultured with CM interfering with VPS9D1-AS1 and overexpressing VEGFA was increased, P<0.05 (**Figure 4B**). We verified using the chorioallantoic membrane vascular assay and showed that disrupting VPS9D1-AS1 and overexpressing VEGFA increased angiogenesis, P<0.05 (**Figure 4C**), and the difference was statistically significant. As shown by cell biological function experiments and in vivo experiments, we confirmed that VPS9D1-AS1 affects angiogenesis through VEGFA in colorectal cancer. According to the literature, antisense lncRNAs can bind DNA or histone modifying enzymes to regulate the epigenetics of the site of the gene in which they are located, thus affecting the expression of genes on the opposite strand. We knocked down VPS9D1-AS1 in HCT-116 and SW480 cells and used qRT-PCR assays to detect the expression of VPS9D1 encoded by the opposite strand in both cells and to observe whether VPS9D1-AS1 regulates its opposite strand-encoded VPS9D1, and the results showed that VPS9D1-AS1 does not regulate its opposite strand-encoded VPS9D1 (**Figure 4D**). To explore the mechanism by which VPS9D1-AS1 further affects colorectal cancer angiogenesis, we predicted the correlation between VPS9D1-AS1 and key molecules in the VEGFA downstream pathway using the GEPIA database. The results showed that: VPS9D1-AS1 was significantly associated with the AKT signaling pathway (**Figure 4E**). After incubating human umbilical vein endothelial cells (HUVEC) with superna-

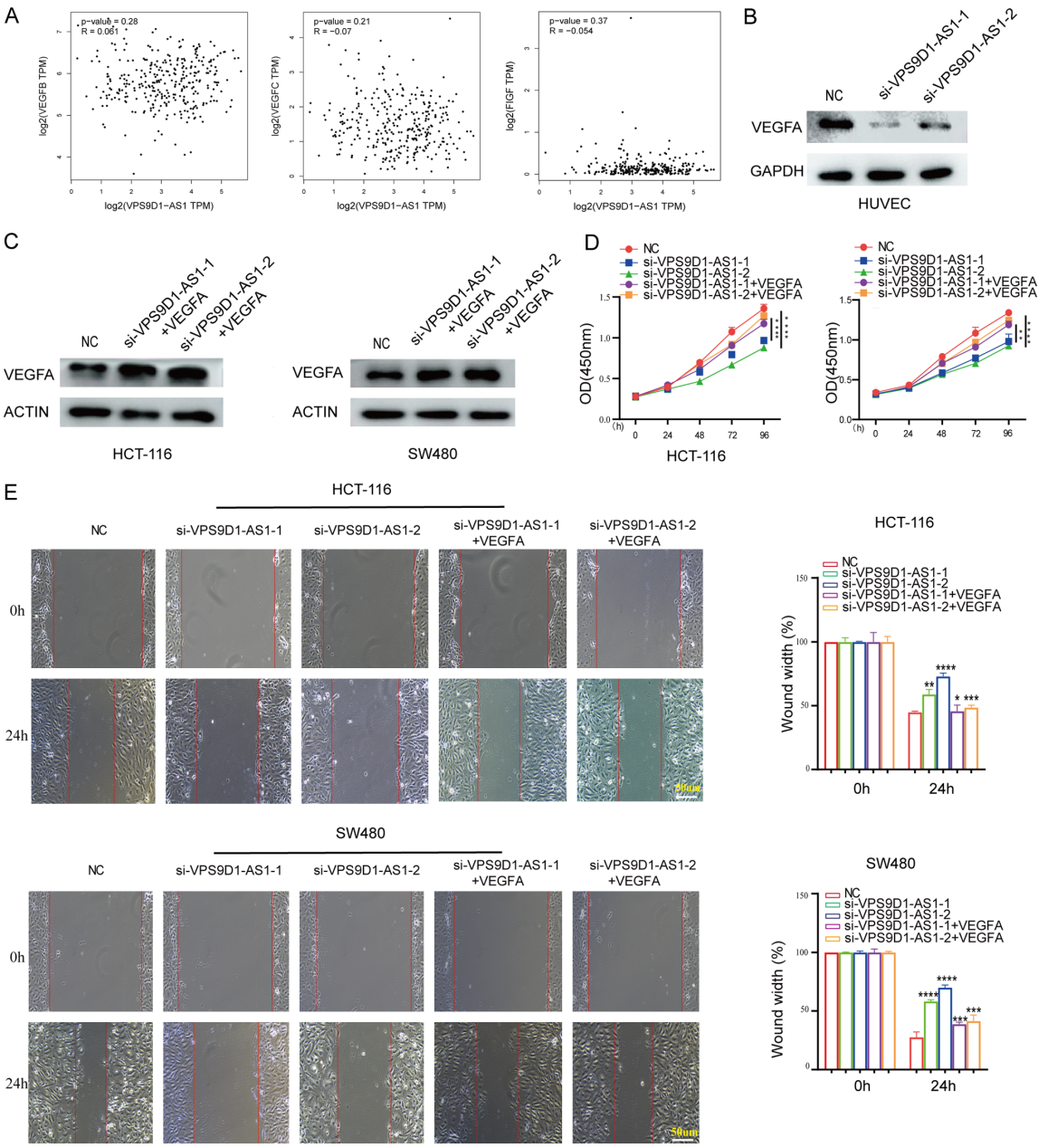
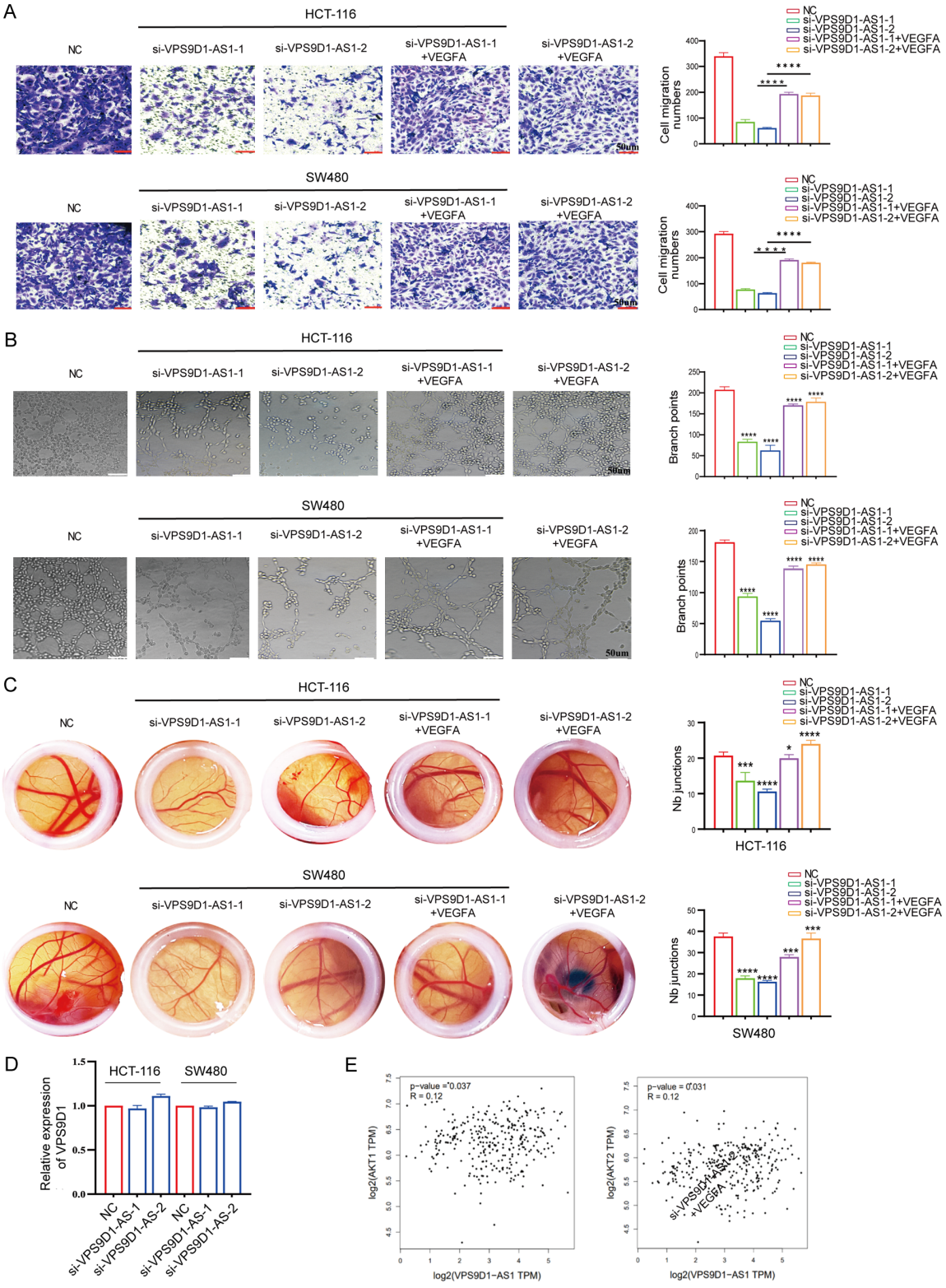


Figure 3. VPS9D1-AS1 promotes angiogenesis through VEGFA in colorectal cancer. A. Correlation of VPS9D1-AS1 with VEGFB, VEGFC and VEGFD was predicted by GEPIA database. B. siRNA of VPS9D1-AS1 was transfected in HUVEC cells and VEGFA expression was detected using western blot. C. VEGFA was overexpressed in HCT-116 and SW480 transfected with si-VPS9D1-AS1, and western blot was used to detect the expression of VEGFA. D, E. The siRNA of VPS9D1-AS1 was cotransfected with VEGFA into HCT-116 and SW480 cells, and the supernatant and HUVEC were co-cultured after 48 h. cck8, scratch assay to detect the effect of VPS9D1-AS1 on the proliferation and migration of HUVEC cells. si-VPS9D1-AS1-1 and si-VPS9D1-AS1-2 were compared with NC group (n=3); *P<0.05. si-VPS9D1-AS1-1+VEGFA and si-VPS9D1-AS1-2+VEGFA were compared with si-VPS9D1-AS1-1 and si-VPS9D1-AS1-2 groups, respectively (n=3); *P<0.05.

tants of the indicated cells for 48 h, western blotting was used to detect the corresponding signaling pathway activity in endothelial cells. Compared with control cells, the key regulators of HUVEC, p-PI3K and P-AKT, were significantly reduced in colorectal cancer cells co-cultured with knockdown of VPS9D1-AS1. In contrast, the levels of key signaling molecules were


A
HCT-116
NC
si-VPS9D1-AS1-1
si-VPS9D1-AS1-2
si-VPS9D1-AS1-1 +VEGFA
si-VPS9D1-AS1-2 +VEGFA
50um
Cell migration numbers
SW480
B
Branch points
C
Nb junctions
HCT-116
SW480
D
Relative expression of VPS9D1
E
p-value =0.037
R = 0.12
p-value = 0.031
log2(AKT1 TPM)
log2(AKT2 TPM)
log2(VPS9D1-AS1 TPM)

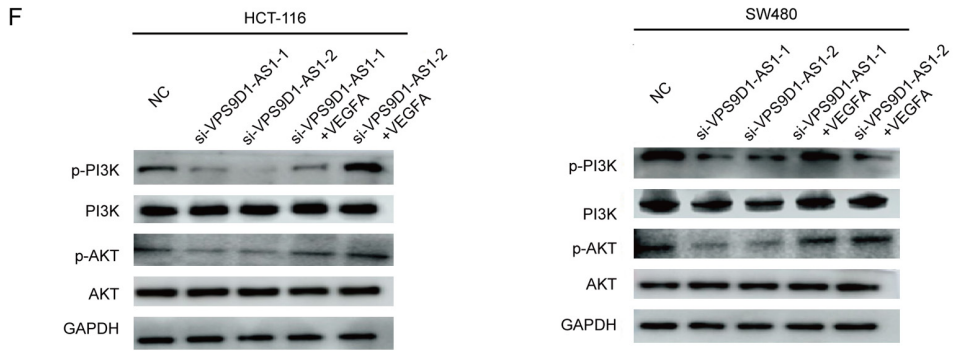


Figure 4. VPS9D1-AS1 promotes angiogenesis through VEGFA in colorectal cancer. A. The siRNA of VPS9D1-AS1 was cotransfected with VEGFA into HCT116 and SW480 cells, and the supernatant and HUVEC were co-cultured after 48 h. The effect of VPS9D1-AS1 on HUVEC cell invasion was detected by Transwell. si-VPS9D1-AS1-1 and si-VPS9D1-AS1-2 were compared with NC group (n=3); *P<0.05. B, C. In vitro tubule and chick embryo chorioallantoic membrane assays were performed to detect the effects of NC, si VPS9D1-AS1-1+VEGFA, si-VPS9D1-AS1-2+VEGFA after three co-culture supernatant treatments, and the branching nodes of blood vessels were reduced compared with si-VPS9D1-AS1-1 and si-VPS9D1-AS1-2 groups, respectively, (n=3); *P<0.05. D. qRT-PCR assay to detect VPS9D1 expression in HCT-116 and SW480 cells with knockdown of VPS9D1-AS1. E. Correlation analysis of GEPIA database predicted VPS9D1-AS1 with AKT1 and AKT2. F. Western blot detection of p-PI3K, p-AKT and VEGFA expression in HUVEC cells after incubation with supernatants of the indicated cells for 48 h.

increased in HUVEC co-cultured with knockdown of VPS9D1-AS1 and overexpression of VEGFA cell supernatant (**Figure 4F**).

CEBPB can bind to the promoter of VPS9D1-AS1 and activate its transcription

To explore how the transcription of VPS9D1-AS1 is regulated, we used bioinformatics methods in PROMO (*https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?*) and JASPAR (*https://jaspar. genereg.net/*) databases to predict and intersect the transcription factors that may regulate VPS9D1-AS1 expression, respectively, and found that the following five transcription factors may be involved in its regulation: IRF1, STAT4, FOXP3, NF1, CEBPB. Based on the above two databases, the number of times these five transcription factors bind to VPS9D1-AS1 and the binding sequences were analyzed and compared, and the combined scores revealed that CEBPB is most likely to be a transcription factor for VPS9D1-AS1.We further predicted the correlation between VPS9D1-AS1 and five transcription factors using the GEPIA database (**Figure 5A**), and found that CEBPB had the strongest correlation with VPS9D1-AS1, with the most significant difference (R=0.22, *P*=0.00017). Therefore, we hypothesized that CEBPB may regulate the transcription of VPS9D1-AS1. CCAAT enhancer-binding protein beta (CEBPB) is a basic region leucine zipper transcription factor that plays a key role in cell proliferation, cell differentiation, and tumorigenesis [18]. Many studies have shown that CEBPB plays an important role in the development of various cancers, including gastric cancer, liver cancer, prostate cancer, and breast cancer. Recently, CEBPB has also been suggested to promote the development of human colorectal cancer [19, 20]. However, the regulatory mechanism of CEBPB in colon cancer has not been extensively explored. Using the CPTAC (*https://gdc.cancer.gov*) database, the expression of CEBPB was predicted in 100 colorectal cancer tissues, and the results showed that the expression of CEBPB was higher in colorectal cancer tissues than in normal tissues adjacent to the cancer, *P*<0.0001 (**Figure 5B**). The PROMO and JADPAR databases were used to synthetically predict two sites where CEBPB might bind to the VPS9D1-AS1 promoter region (**Figure 5C**). The results of CHIP-PCR experiments showed that CEBPB could bind to the site region from -698 bp to -794 bp of VPS9D1-AS1 promoter, while there was no binding at the site from -1419 bp to -1539 bp (**Figure 5D**). Next, we cotransfected the CEBPB overexpression plasmid in 293T cells with the PLR-TK sea kidney internal reference reporter gene plasmid and the dual luciferase reporter gene plasmid (PGL3-VPS9D1-AS1-WT/MT-Luc), which contains the promoter of VPS9D1-AS1 -698 bp to -794 bp. We con-

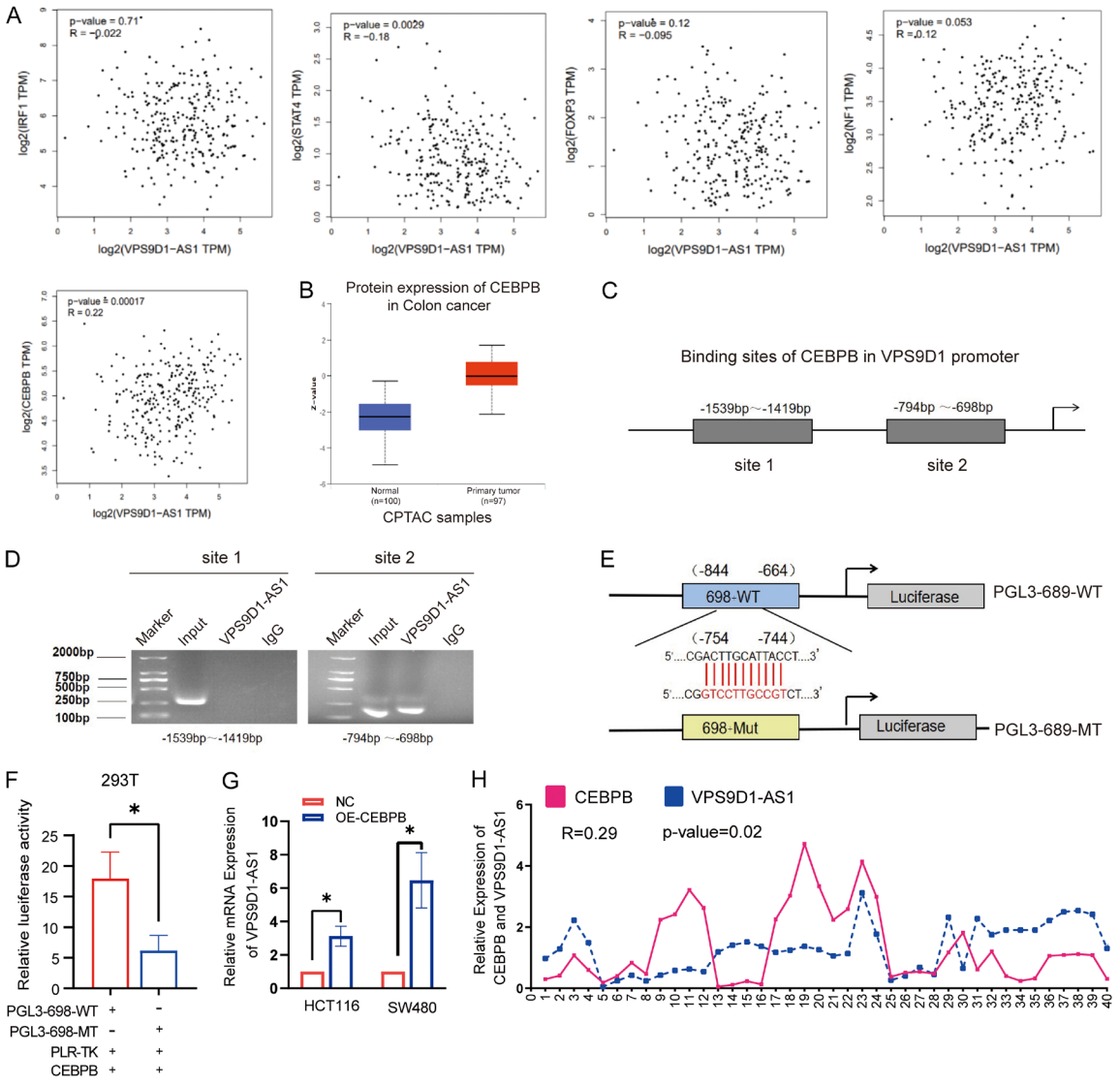


Figure 5. CEBPB binds to the promoter of VPS9D1-AS1 and activates its transcription. A. Correlation of molecular VPS9D1-AS1 with transcription factors in the GEPIA database. B. Predicted protein expression of CEBPB in 100 colorectal cancer tissues and 97 paracancerous normal colorectal cancer tissues from the CPTAC database. C. Predicted promoter sites of CEBPB binding to VPS9D1-AS1 according to the database. D. Chip-PCR detected no binding at the -1419 bp to -1539 bp site and binding at -698 bp to -794 bp. E. Construction of dual luciferase reporter gene plasmids with wild-type and mutant sequences of the promoter of VPS9D1-AS1 inserted before Luciferase. F. Co-transfection of CEBPB, PRL-TK, PGL3-698-WT/MT plasmids to detect dual luciferase activity (Firely/Renilla) MT group < WT group (n=3); *P<0.05. G. Detection of mRNA expression level of VPS9D1-AS1 after overexpression of CEBPB by qRT-PCR. H. qRT-PCR to detect the expression of CEBPB and VPS9D1-AS1 in 40 colorectal cancer tissues (n=3); *P<0.05.

structed wild-type and mutant plasmids of the VPS9D1-AS1 promoter region as shown in **Figure 5E**. The dual luciferase reporter gene results showed that CEBPB could increase the transcriptional activity of wild-type VPS9D1-AS1, with which the transcriptional activity of mutant VPS9D1-AS1 was repressed compared to it (**Figure 5F**). Further overexpression of CEBPB was found to upregulate VPS9D1-AS1 expression (**Figure 5G**). Subsequently, we verified the correlation between CEBPB and VPS9D1-AS1 in colorectal cancer tissues, as shown in the figure, CEBPB was positively correlated with VPS9D1-AS1 and the difference was significant (**Figure 5H**). We also examined the correlation between VPS9D1-AS1 and

VEGFA, and the results show that there is a significant correlation between VPS9D1-AS1 and VEGFA (Supplementary Figure 1). These studies show that CEBPB can directly bind to the promoter region of VPS9D1-AS1 and regulate the expression of VPS9D1-AS1.

Discussion

It is well known that a large number of non-coding RNAs are present in the human and mammalian genomes [21]. Many lncRNAs have been reported to play important roles in cell biology and regulate tumor progression [22, 23], and act as oncogenes or tumor suppressors during carcinogenesis. Therefore, we predicted LncRNAs with differential expression in colorectal cancer in the TCGA database, among which the differential expression of VPS9D1-AS1 was significant. VPS9D1-AS1 is a novel non-coding RNA that has been rarely reported in colorectal cancer and its effect on colorectal cancer angiogenesis has not been reported. In this study, we verified the expression of VPS9D1-AS1 in colorectal cancer tissues and normal tissues adjacent to cancer, and the results showed that VPS9D1-AS1 showed a trend of high expression. In this study, we performed VPS9D1-AS1 gene silencing to assess its biological function. First, we conducted preliminary bioinformatics screening to explore potential associations between VPS9D1-AS1 expression and common tumor phenotypes including autophagy, ferroptosis, and angiogenesis. According to the results, autophagy and ferroptosis did not show significant correlations with VPS9D1-AS1. However, we identified a significant correlation between VPS9D1-AS1 and angiogenesis, prompting us to focus our subsequent investigations on this critical biological process. We observed that inhibition of VPS9D1-AS1 inhibited the proliferation and metastatic ability of HUVECs. Furthermore, we found that inhibition of VPS9D1-AS1 significantly inhibited angiogenesis in colorectal cancer using in vitro angiogenesis and chick embryo chorioallantoic membrane assays. Our findings demonstrate that VPS9D1-AS1 exhibits oncogenic properties in colorectal cancer by promoting the proliferation and metastasis of vascular endothelial cells. However, the specific molecular mechanisms through which VPS9D1-AS1 facilitates angiogenesis in colorectal cancer remain to be elucidated.

Recent studies have shown that lncRNAs can interact with proteins to perform their functions [24, 25]. Therefore, we speculate that the role of VPS9D1-AS1 in affecting angiogenesis in colorectal cancer may also be regulated by such a mechanism. The progression of solid tumors is closely associated with angiogenesis stimulation, with vascular endothelial growth factor-A (VEGFA) serving as one of the primary mediators driving tumor vascular bed expansion [26]. Numerous studies have demonstrated that various molecular regulators can modulate angiogenesis by targeting VEGFA in multiple tumor types. Wang et al. [27] demonstrated that B7-H3 induces VEGFA expression through NF-κB pathway activation, promoting angiogenesis in colorectal cancer, while Chen et al. [28] reported that the circRNA 001971/miR-29c-3p axis regulates colorectal cancer growth, metastasis, and angiogenesis via VEGFA. To investigate this, we correlated VPS9D1-AS1 with VEGFA, and the results showed a positive and statistically significant correlation. We found a significant decrease in VEGFA expression after knocking down VPS9D1-AS1 in HUVEC cells. Through CCK8, Transwell, scratch wound, and in vitro tubule formation assays, we demonstrated that simultaneous VPS9D1-AS1 knockdown and VEGFA overexpression could rescue VPS9D1-AS1-mediated promotion of colorectal cancer angiogenesis. Therefore, we further speculate that VPS9D1-AS1 may also target VEGFA to activate its downstream pathway.

PI3K serves as a crucial regulator of angiogenesis [29, 30], and VEGFA activates PI3K through multiple mechanisms, including FAK [31], SHB [32], GAB1 [33], IQGAP1 [34], TSAd/Src/Axl [35], as well as direct binding to pY1175 in VEGFR2 [36, 37]. PI3K is associated with vascular endothelial cell vessel formation, proliferation, survival and vascular permeability [29]. AKT is an intermediate of the PI3K downstream signaling pathway, and plays a critical role in vascular endothelial growth factor-regulated HUVECs. In endothelial cells, Akt activation leads to tumor vasodilation and hyperpermeability. The Akt isoforms have emerged as promising therapeutic targets for angiogenesis-related diseases, including ischemic injury and cancer [38]. AKT is present in endothelial cells as three isoforms Akt1-3 and is activated by PI3K via phosphatidylinositol-dependent kinase 1 (PDK1). Activated Akt is associated

with angiogenesis through endothelial cell survival, proliferation, vascular permeability, synthesis and release of matrix metalloproteinases (MMPs), and properties of inflammatory endothelial cells [39, 40]. Using the GEPIA database, we identified a significant relationship between VPS9D1-AS1 and AKT, revealing that VPS9D1-AS1 is strongly associated with the PI3K/AKT signaling pathway. We detected the key genes within the pathway, P-PI3K and P-AKT, and further confirmed that the downstream pathway was activated after VPS9D1-AS1 targeting VEGFA. These findings suggest that VPS9D1-AS1 may target VEGFA to activate the PI3K/AKT signaling pathway, thereby promoting angiogenesis. We predicted in our database that a transcription factor CEBPB is likely to regulate the expression of VPS9D1-AS1 gene. By CHIP-qPCR assay and dual luciferase reporter gene we found that CEBPB binds to VPS9D1-AS1 promoter region at sites -698 bp - -764 bp. We validated the correlation between CEBPB and VPS9D1-AS1 in colorectal cancer tissues, demonstrating a significant positive relationship. These results suggest that CEBPB binds to the promoter region of the VPS9D1-AS1 gene and activates its transcription.

In summary, we identified a novel lncRNA, VPS9D1-AS1, as a crucial regulator of angiogenesis in colorectal cancer that promotes the proliferation and migration of vascular endothelial cells both in vitro and in vivo. LncRNA VPS9D1-AS1 can be activated by CEBPB transcription factor and promotes colorectal cancer angiogenesis by upregulating VEGFA expression. These novel findings may provide new insights into the molecular mechanisms by which the CEBPB/VPS9D1-AS1/VEGFA axis promotes malignant behavior in colorectal cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Shenglei Li, Department of Pathology, The First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Erqi District, Zhengzhou 450052, Henan, P. R. China. E-mail: lishenglei@zzu.edu.cn; Na Li, Department of Pathology, Xinxiang Medical University, No. 601, Jinsui Road, Xinxiang 453003, Henan, P. R. China. E-mail: yaoyao-lina@163.com

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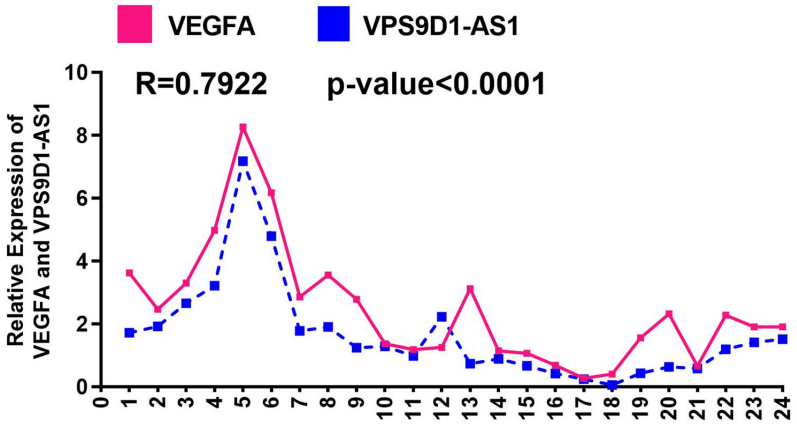

Supplementary Figure 1. The correlation between VPS9D1-AS1 and VEGFA.