

# Mir-455-3p Inhibits Hepatocellular Carcinoma Tumorigenesis, Lymphangiogenesis and Angiogenesis via Regulating Vascular Endothelial Growth Factor C as well as Vascular Endothelial Growth Factor Receptor-2

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## Research Article

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# Abstract

**Background:** MicroRNAs (miRNAs), functioning as tumor suppressors or oncogenes, exert a considerable regulative influence in biological processes. Previous studies showed that miR-455-3p was significantly down-regulated in hepatocellular carcinoma (HCC), while the specific role of miR-455-3p in HCC is unclear.

**Methods:** QRT-PCR was used to detect miR-455-3p expression levels in HCC cells and tissues. We adopted colony formation, EdU, CCK-8, transwell assays and wound-healing to identify the influences of miR-455-3p on proliferation, invasion and migration in HHCC and HuH-7 cells. Western blot analysis, angiogenesis assays, dual-luciferase reporter gene assay and bioinformatics analysis were also adopted to investigate the underlying molecular mechanisms.

**Results:** MiR-455-3p was down-regulated in HCC tissues and cells. In vitro experiments identified miR-455-3p overexpression inhibited HCC cell proliferation, migration and invasion. Interestingly, it was identified that vascular endothelial growth factor C (VEGFC) was the downstream target gene of miR-455-3p. Additionally, overexpression of miR-455-3p in HCC cells and human umbilical vein endothelial cells (HUVECs) cells led to decreased expression of VEGFC, vascular endothelial growth factor receptor (VEGFR)-2, and subsequently decreased phosphatidylinositol 3-kinase (AKT) signaling pathway. Furthermore, in vivo restoration of miR-455-3p significantly suppressed tumorigenicity of HuH-7 cells in nude mice and inhibited both angiogenesis and lymphangiogenesis of tumor xenografts.

**Conclusions:** Our findings suggest that miR-455-3p could play a role in HCC tumorigenesis at least in part by modulation of angiogenesis and lymphangiogenesis through targeting VEGFC, and could simultaneously block AKT signaling pathways.

## Background

As the sixth most ubiquitous cancer worldwide, liver cancer is placed the fourth prior reason of all cancer-related deaths, with nearly one million cases reported in 2016[1, 2]. Survival rates for patients with hepatocellular carcinoma are shockingly low, killing more than 700,000 people a year and leaving only 10 per cent alive two years after diagnosis in the UK[3]. According to statistics, 27,000 liver cancer patients died in the United States in 2016, and there's only a 15% 5-year survival rate[4]. Concerning the development of HCC, chronic hepatitis B and C virus infection were placed the most common risk factors[5]. While for most patients there isn't any curative treatment[6].

MicroRNAs (miRNAs), about 19–24 nt in length, are endogenous non-coding small RNA exerting important influences in animals and plants, which is through targeting the target gene mRNA at its 3-terminal non-coding region thus causing transcriptional repression or regulating mRNA degradation[7, 8]. Numbers of studies have shown that miRNAs exert a considerable regulative influence in the progress of various diseases, especially tumors[9, 10]. Some miRNAs were found to be tumor suppressors in HCC, including miR-17-5p[11], miR-20a[12], miR-202[13], and microRNA-214[14]. In addition, HCC is a typical

hypervascular tumor, whose growth and metastasis depend on angiogenesis[15]. However, miRNAs function as key modulators of angiogenesis and lymphangiogenesis[16, 17]. Therefore, we studied the pathogenesis of HCC from the perspective of miRNAs, which is helpful to find targets for early diagnosis and treatment.

Wentao Jiang and his colleagues analyzed the GSE98269 chip and found through further studies that in hepatocellular carcinoma, miR-455-3p was significantly down-regulated and could be used as a biomarker of HCC[18]. Lan et al. also anticipated low level of miR-455-3p with poor prognosis in HCC[19]. And investigations have demonstrated that miR-455-3p can inhibit the progression of a variety of tumors. For example, in prostate cancer microRNA-455-3p targets eIF4E, thus works as a tumor suppressor[20]. By targeting FAM83F, miR-455-3p also inhibits the proliferation and invasion of esophageal squamous cell carcinoma, as a prognostic marker[21]. Via Targeting TAZ, in pancreatic cancer cells miR-455-3p down-regulation correlates with proliferation and drug resistance[22]. However, the pathogenesis of miR-455-3p involvement in HCC remains unclear.

Central regulators of vasculogenesis, angiogenesis and lymphangiogenesis includes VEGFs (VEGFE, VEGFD, VEGFC, VEGFB, VEGFA, and PlGF, or placental growth factor) as well as their endothelial tyrosine kinase receptors[23]. Mature VEGFC binds VEGFR-3 and VEGFR-2 respectively, which is mostly located on lymphatic and vascular endothelial cells, after VEGFC undergoing extensive proteolytic processing[24–26]. On tumor cells, including leukemic, skin, and gastric cells, VEGFC and its cognate receptors are expressed, and through autocrine signaling facilitates tumor progression partly[27, 28].

It was identified in this study that in HCC tissues and cells miR-455-3p was down-regulated and miR-455-3p works as a tumor suppressive miRNA by targeting VEGFC. Beside VEGFC and VEGFR-2 expression, ectopic expression of miR-455-3p led to inhibition of the cell invasion, migration and proliferation. Additionally, in HCC xenograft treatment model miR-455-3p overexpression curbed tumor growth, angiogenesis and lymphangiogenesis. In brief, we discovered the function of miR-455-3p in HCC, likely supplying a new sight on therapy for HCC patients.

## Methods

### Human HCC tissue samples

From August 2016 to September 2018, 40 paired HCC tissues as well as adjoining normal tissues were harvested from diagnosed patients in our hospital. All included patients had been diagnosed, and they didn't receive treatment of radiotherapy or chemotherapy before. All included subjects inscribed an informed consent and the investigation got approval from Ethics Committee of Affiliated Hospital of Youjiang Medical University for Nationalities. We maintained these tissues in liquid nitrogen.

### Cell culture

Five HCC cell lines (HB611, HHCC, H-97, HuH-7, Li-7), normal liver cell line (LO2) and human umbilical vein endothelial cells (HUVECs) were acquired from Shang Hai Ze Ye Biotechnology Co., Ltd. (Shang Hai, China) and American Type Culture Collection (Manassas, VA, USA). We verified the authenticity of each cell line with short tandem repeat analysis. We preserved all cell lines in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), with 1% penicillin-streptomycin and 10% fetal bovine sera (FBS; Gibco, USA), and stored them in a 5% CO<sub>2</sub> humidified incubator at 37°C.

### **QRT-PCR (Quantitative reverse transcriptase-polymerase chain reaction)**

For total RNA extraction we used RNeasy Mini Kit (Qiagen, Germany) and for reversed transcription Quantitect Reverse Transcription Kit (Qiagen, Germany) was used, during which we referred to the manufacturer's instructions. We performed amplification on a Lightcycler 480 platform (Roche, Germany) for each sample in triplicate. According to GAPDH levels, we normalized relative expression levels. Here followed primer sequences:

MiR-455-3p, 5'-ACACTCCAGCTGGGCACAUAUACGGGUAC-3' (F),

5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCGTCAGGT-3'(R);

GAPDH, 5'-ATGGGGAAGGTGAAGGT-3'(F),

5'-AAGCTTCCCGTTCTCAG-3'(R).

### **Western blot analysis**

We prepared Protein lysates with RIPA buffer (Thermo Scientific Inc., Waltham, MA, USA) which contains 1% protease inhibitor and performed western blotting in triplicate experiments. We subjected protein lysates to electrophoresis on a 4% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and electrotransferred proteins to polyvinylidene fluoride membranes (Millipore, USA). With 5% non-fat dry milk in TBS, we incubated membranes and probed them with anti-VEGFC and anti-VEGFR-2 (sc-1881, sc-6251 respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pAKT, anti-AKT and anti-β-actin (#4056, #9272, #4970, respectively; Cell Signaling Technology, USA) in TBST (0.1% Tween 20 in TBS). For detection of immunoreactive proteins, we used Horseradish peroxidase-conjugated anti-rabbit (or mouse) IgG (Cell Signaling Technology, USA) by chemiluminescence (Pierce<sup>®</sup> ECL kit)

### **Cell proliferation assay**

Via the Cell Counting Kit-8 assay (CCK-8, Dojindo, Japan), we referred to the manufacturer's protocol and performed cell proliferation assays. We seeded a sum of 1×10<sup>3</sup> cells per well and cultured them for 1, 2, 3, 4 and 5 days. We also added ten microlitres of the CCK-8 solution to each well, and incubated sample at 37°C for 4 h. We read absorbance at 450 nm on a microplate reader (MultiSkan Spectrum).

For EDU assay, we used EDU labeled solution (KeyGen Biotech, Nanjing, China) for cell culture for 2 h. As recommended by the manufacturer we performed EdU staining, after 15 min of cell fixation which was in 4% paraformaldehyde.

For colony-formation assays, with trypsin–collagenase we treated transfected HCC cells for digesting them into a single cell suspension, and we seeded them at a rate of 400 cells/well into 12-well plates before culturing them at 37°C for 14 days in a 5% CO<sub>2</sub> incubator. We then use PBS to wash the cells twice and for 15 min stained them with 2% crystal violet, then at room temperature we dried the plate. We counted the clone number which were formed in the plate.

We performed all experiments thrice, consequently calculated the average of the results.

### **Wound-healing assay**

We seeded cells in six-well plates and cultured them. After they reached confluence, we scratched confluent monolayer cells and washed them with PBS for three times for clearing suspension cells and cell debris. We added fresh serum-free medium and under normal conditions allowed the cells to close the wound for 24h. At the same position of the wound, with a computer-assisted microscope (Nikon) we took photographs.

### **Transwell assay**

Using a Transwell chamber (8- $\mu$ m pore size; Corning, USA), we performed cell invasion experiments. In the upper chamber we seeded HCC cells at a density of 10<sup>5</sup> cells per well, in which we also added DMEM containing 1% FBS. We precoated the upper chamber with Matrigel (Corning, USA, dilution ratio: 1:6) while in the lower chamber there was 600 $\mu$ L DMEM which contains 10% FBS. We fixed cells with 4% methanol, which were migrated or invaded to the lower surface of membrane, after incubation at 37°C for 24 hours then we strained them with crystal violet. We selected 5 random microscopic fields and counted the number of cells in each field. Each experiment was carried out three times.

### **Generation of stable cell lines expressing miR-455-3p**

From LO2 cell line genomic DNA, a DNA fragment which contains the miR-455-3p was amplified then into the pcDNA-copGFP vector (System Biosciences, USA) we cloned it. Name LV-miR-455-3p was given to the lentivirus vector expressing miR-455-3p. Via Lipofectamine2000 (Invitrogen, CA, USA) Lentiviral packaging plasmids and LV-miR-NC (as a negative control) were co-transfected following the manufacturer's instruction in 293FT packaging cells. To infect HCC cells and HUVECs, we collected, filtered and used the lentivirus in the supernatant at the time forty-eight hours after transfection. Using qRT-PCR, we obtained stable clones and confirmed the expression of mature miR-455-3p after two weeks of antibiotic selection.

### **Tumor xenograft treatment model**

We got approval of animal experimental procedures from the Institutional Animal Care and Use Committee of Affiliated Hospital of Youjiang Medical University for Nationalities. Of each BALB/c nude mouse (5–6 weeks of age, 20–22 g) we injected a suspension of HuH-7 cells ( $1 \times 10^7$ ) into the right flank subcutaneously. From the Laboratory Animal Center (Shanghai, China), we purchased all mice and maintained them under specific pathogen-free conditions in laminar flow cabinets. On day 7 post-implantation, when tumors formed, we randomly divided mice into two treatment groups: one is Lv-miR-455-3p group which was treated with lentivirus encoding miR-455-3p; another is Lv-NC which was treated with negative control lentivirus. After treatment with calipers two major axes, we measured tumor volumes every 7 days, and we calculated with the formula:  $V = 0.5 \times L \text{ (length)} \times W^2 \text{ (width)}$ . With all the experiments approval from Affiliated Hospital of Youjiang Medical University for Nationalities, we cared all animals with humane care referring to the criteria by the National Academy of Sciences and the National Institutes of Health, which outlines in the “Guide for the Care and Use of Laboratory Animals”.

### **Dual-luciferase reporter gene assay**

We cloned the 3'-UTR regions of human VEGFC mRNA, which is downstream of the luciferase reporter gene. We first amplified it by PCR and then transferred it into the pGL3-basic vector (Promega, Madison, WI, USA). The construct as Wt (wild-type) and named it VEGFC Wt. Using PCR and the site-directed mutagenesis kit (Takara), which acted as template, the mutated 3'-UTR was generated. Into the luciferase reporter we inserted the mutated sequence and named it VEGFC Mut. Then the Wt or Mut of VEGFC, firefly luciferase reporter and mock LV-miR-455-3p vector or miR-NC were co-transfected into HHCC and HuH-7 cells. 48 h after, we harvested cells and treated them with the Dual Luciferase Assay kit I.

### **In vitro angiogenesis assays**

By tube formation assay, we determined the in vitro angiogenic activity of HUVECs, for a significant angiogenic property of HUVECs is capillary tube formation on Matrigel. After transfection, we serum-starved HUVECs in endothelial basal medium (EBM; Clonetics, CA, USA) with 0.2% BSA for 24 h by incubation. We harvested HUVECs and in a 12-well plate seeded  $8 \times 10^4$  cells, which is coated with Matrigel basement membrane matrix (BD Biosciences, USA), after serum starvation. With a computer-assisted microscope (Nikon), we observed tube formation after 8 h of incubation. We defined tube formation as a tube-like structure with a length four times its width. We selected 10 random microscopic fields and took images of tube morphology at 100 magnification per sample. Using LAS software (Leica), we measured the number of tubes.

### **Statistical analyses**

Using SPSS statistical software (16.0 for Windows), we performed statistical analyses. We displayed experimental data as means  $\pm$  standard deviation (SD). Through Student's t-test and one-way analysis of variance (ANOVA), we analysed the differences between groups. We performed all statistical tests two sided.  $P < 0.05$  were considered significant statistically. We performed all experiments three times or more.

# Results

## In human HCC, miR-455-3p expression is down-regulated

The GSE98269 chip was analyzed by Wen et al., and the fact that miR-455-3p was remarkably down-regulated in HCC was identified in further studies[18]. Using qRT-PCR, we assessed miR-455-3p level in 40 matched non-tumoral tissues and corresponding HCC specimens for determination whether miR-455-3p involves in the regulation of human HCC progress. Compared with non-tumoral tissues, miR-455-3p was remarkably down-regulated in tumor tissues as shown in **Figure. 1A**. Additionally, in human HCC cell lines (HB611, HHCC, H-97, HuH-7 and Li-7) and normal liver cell line (LO2), we also examined the miR-455-3p expression. In comparison with the LO2 cell line, miR-455-3p expression was examined remarkably lower in human HCC cell lines (**Figure. 1B**).

## Overexpression of miR-455-3p suppressed the proliferation, migration and invasion of HCC cells

In HHCC and HuH-7 cells, we enhanced miR-455-3p expression to probe into the function of miR-455-3p, then observed the effects on the cell invasion, migration and proliferation. We displayed the transfection efficiency in **Figure. 2A**. Via CCK-8, EdU, and colony-formation assays, we examined cell proliferation. The growth rate of HHCC and HuH-7 cells was identified to remarkably decrease by miR-455-3p overexpression (**Figure. 2B-D**). We also adopted wound-healing assay for investigating the influence of miR-455-3p on cell migration. In comparison with miR-NC cells, miR-455-3p-overexpressing cells showed remarkably slower migration as shown in **Figure. 2E**. Besides, the invasiveness of miR-455-3p overexpressed cells was remarkably lower than that of the control cells, by transwell assay (**Figure. 2F**).

## miR-455-3p directly targets VEGFC gene

Using mirdb and Starbase, we searched candidate target genes of miR-455-3p and identified that VEGFC acted as a possible target of miR-455-3p. We displayed binding sequences of miR-455-3p and VEGFC in **Figure. 3A**. Then, in HHCC and HuH-7 cells we used dual-luciferase reporter gene assay for verifying the targeted binding relationship (**Figure. 3B**). The relative fluorescence activity of miR-455-3p mimic was remarkably decreased in VEGFC Wt group. However, co-transfection group with VEGFC Mut did not have this effect. Additionally, in miR-455-3p-overexpressing HCC cells the protein level of VEGFC was significantly inhibited, showed by western blot analysis (**Figure. 3C**). MiR-455-3p could target VEGFC was indicated by these results.

Investigations have suggested that VEGFC could perform various biological actions so that facilitate tumor progression, including exerting autocrine stimulation influences on VEGFR-2 expression, vital for tumor angiogenesis and lymphangiogenesis[29, 30]. Thus, whether miR-455-3p mediated VEGFC inhibition could exert influence in expression of VEGFR-2 in HCC was analyzed. Compared with miR-NC cells the protein expressions of VEGFR-2 in miR-455-3p-overexpressing HCC cells were remarkably reduced (**Figure. 3C**). The phosphatidylinositol 3-kinase-AKT pathway, critical for cellular survival and growth, was activated by VEGFRs both in cancer cells and in endothelial cells. Thus, whether this

pathway involves in miR-455-3p-caused growth suppression was furtherly examined. In HCC cells the phosphorylation of AKT was remarkably decreased by ectopic expression of miR-455-3p (**Figure. 3D**).

### **MiR-455-3p inhibited tumor growth, angiogenesis and lymphangiogenesis in tumor xenografts**

With HuH-7 cells, we performed a xenograft experiment in vivo to probe into whether the expression of miR-455-3p have an effect on tumor growth. We displayed xenograft tumors in **Figure. 4A**. Mice treated with Lv-miR-455-3p were inhibited compared with that treated with Lv-NC in the tumor volume and weight (**Figure. 4B, C**). Next, in Lv-miR-455-3p-treated groups, VEGFC protein level were remarkably lower than in Lv-NC groups, which is showed by western blot analysis (**Figure. 4D**). And in Lv-miR-455-3p-treated groups the Ki-67 level were remarkably lower than that in Lv-NC groups, which is indicated by immunohistochemical staining (**Figure. 4E**).

Then, we analyzed angiogenesis and lymphangiogenesis of tumors via immunohistochemical staining with anti-LYVE-1 antibodies and anti-CD34. Compared with Lv-NC, Lv-miR-455-3p treated groups showed remarkable reductions in the lymph and blood vessels, which is identified by quantitative analysis (**Figure. 4F**). These results indicated that miR-455-3p expression remarkably inhibited the tumor progression in vivo, and miR-455-3p seems to regulate tumorigenesis by inhibiting VEGFC-mediated angiogenesis and lymphangiogenesis.

### **Overexpression of miR-455-3p inhibited tube formation**

For the reason that a significant angiogenic property of HUVECs is capillary tube formation on Matrigel, whether miR-455-3p inducing VEGFC up-regulation affected tube formation was furtherly investigated. After transfection with LV-miR-455-3p or LV-miR-NC for 48 h, we serum-starved HUVECs for 24 h before on a Matrigel-coated 12-well plate serum-starved cells the following day for 8 h, well-organized capillary-like structures was formed by LV-miR-NC-transfected HUVECs as seen in **Figure. 5A**. However, tube-forming activity was significantly impaired as a result of transfection with miR-455-3p. Our studies illustrated that in angiogenesis of HUVECs, miR-455-3p exerts negative influences. Before we examined whether miR-455-3p affects AKT phosphorylation, it is known that AKT works as a vital downstream pathway of VEGF/VEGFR signals. And in miR-455-3p-overexpressing HUVECs, VEGFC and his receptors (VEGFR-2) were negatively regulated (**Figure. 5B**). Compared with miR-NC-transfected HUVECs, AKT phosphorylation was significantly reduced in miR-455-3p-transfected HUVECs as displayed in **Figure. 5C** in miR-455-3p-transfected HUVECs. These results pointed out that an induced of VEGFC protein by miR-455-3p suppresses VEGFR-induced activation of the AKT signaling pathway as evident by reduction in AKT phosphorylation.

As presented in **Figure. 6**, down-regulated of miR-455-3p promoted the level of VEGFC, VEGFR-2, and AKT phosphorylation. Led to an increase of the cell proliferation, migration, invasion, angiogenesis and lymphangiogenesis.

## **Discussion**

Nowadays, increasing evidences have demonstrated that miRNAs work as regulators of the tumor phenotype via regulating the expression of signaling pathways and critical genes, which involves in tumorigenesis and other malignant processes[31, 32]. It was showed in our study that in HCC cell lines and tissues miR-455-3p expression was remarkably down-regulated. However, only a small amount of patient tissues was included in our study, and it is necessary to conduct further study with larger amount to confirm its clinical importance in HCC.

Lentivirus-mediated miR-455-3p-overexpression HHCC and HuH-7 cells were established to research the functions of miR-455-3p in HCC, then we conducted a series of experiments in vivo and in vitro. It was demonstrated clearly in our results that HCC cell proliferation, colony formation, migration and invasion was remarkably suppressed by miR-455-3p in vitro, as well in nude mice HCC xenograft, tumor growth was suppressed. MiR-455-3p was implied by these results as an inhibitor of HCC tumorigenesis.

Through bioinformatics analysis, western blot assay and dual-luciferase reporter gene assay, our indicated that miR-455-3p could target the 3'UTR of VEGFC. In addition, VEGFC, VEGFR-2 and the phosphorylation of AKT, as well as angiogenesis and lymphangiogenesis were decreased by miR-455-3p overexpression in HCC cells. All data provided a basis for new anti-HCC therapies, among which anti-angiogenesis and anti-lymphangiogenesis approaches are significant.

## **Conclusion**

In conclusion, it was showed that in HCC, miR-455-3p was down-regulated, and it worked as a tumor suppressor, directly targeting VEGFC. MiR-455-3p ectopic expression inhibited tumor angiogenesis, progression and lymphangiogenesis, and could simultaneously inhibit AKT signal pathway. In a word, these conclusions may supply a tactic for targeting the interaction of miR-455-3p/VEGFC, as well as a new treatment for HCC.

## **Declarations**

### **Ethics approval and consent to participate**

This study was conducted according to the World Medical Association Declaration of Helsinki and approved by the Medical Ethic Committee of Affiliated Hospital of Youjiang Medical University for Nationalities. All participants provided written informed consent prior to their inclusion in the study. All animal experiments were approved by the Institutional Animal Care and Use Committee of Affiliated Hospital of Youjiang Medical University for Nationalities.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

**Sufang Zhou** designed the research and revised the manuscript; **Xiaoyun Bin** and **Jianchu Wang** performed the experiments and drafted the manuscript; **Libai Lu** and **Guanbin Ye** collected the data and did the analysis; All authors read and approved the final manuscript.

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## Figures

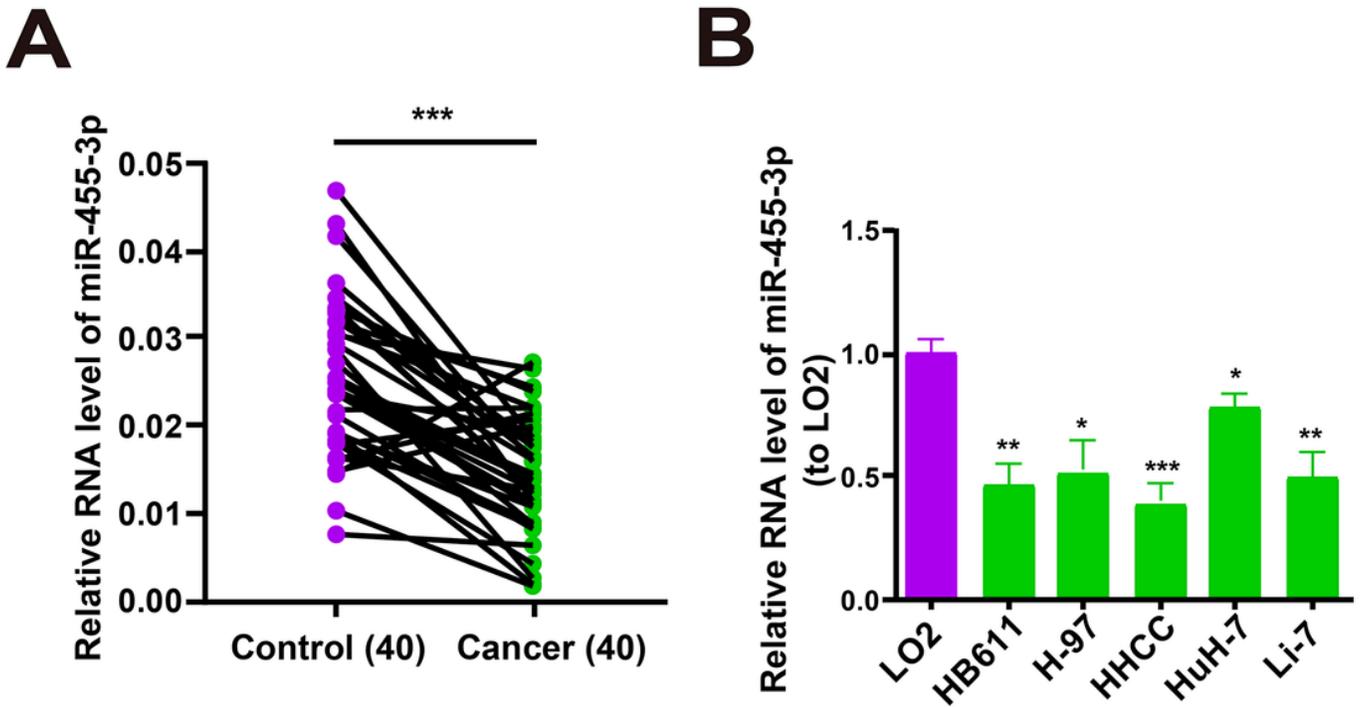
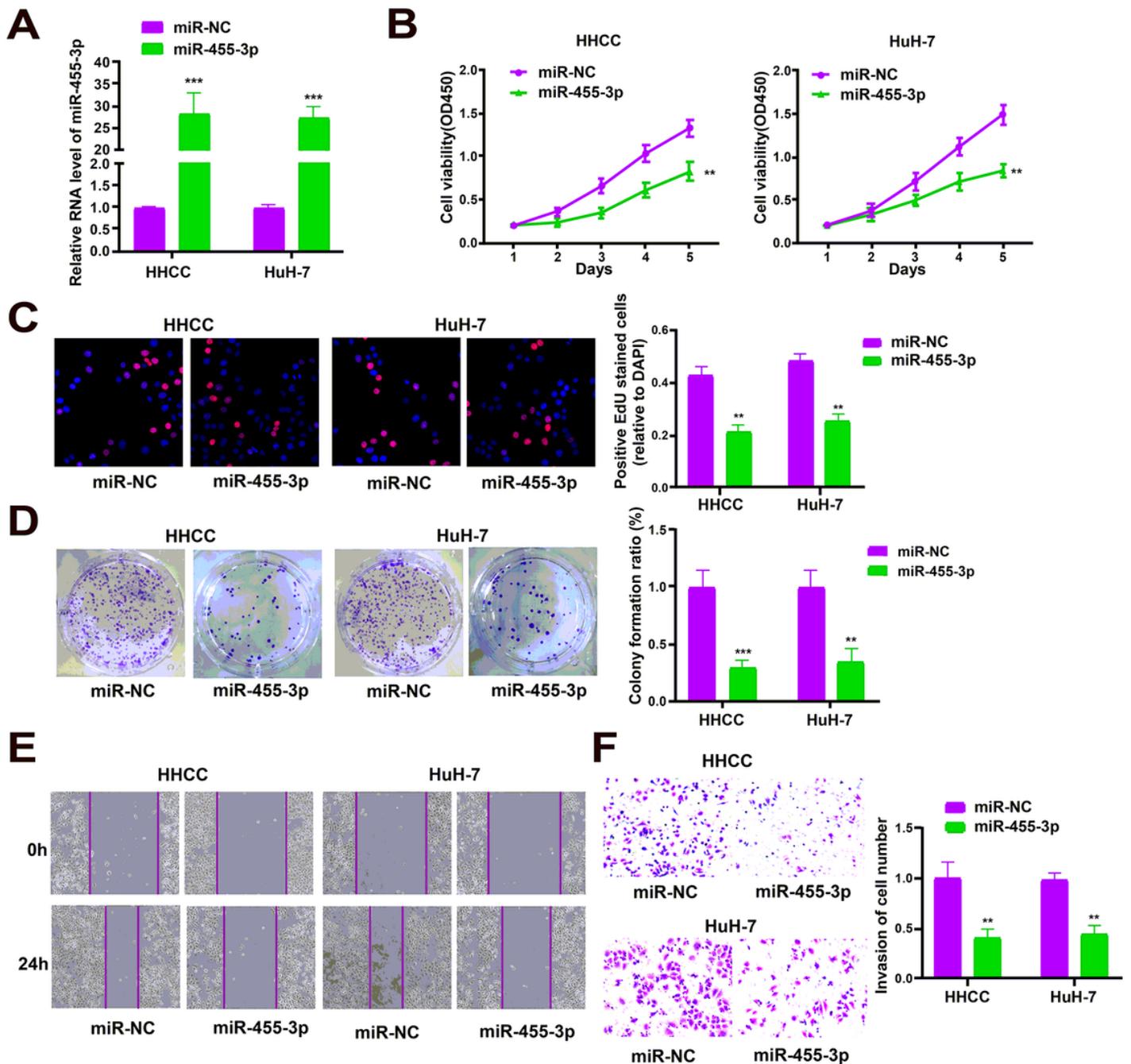


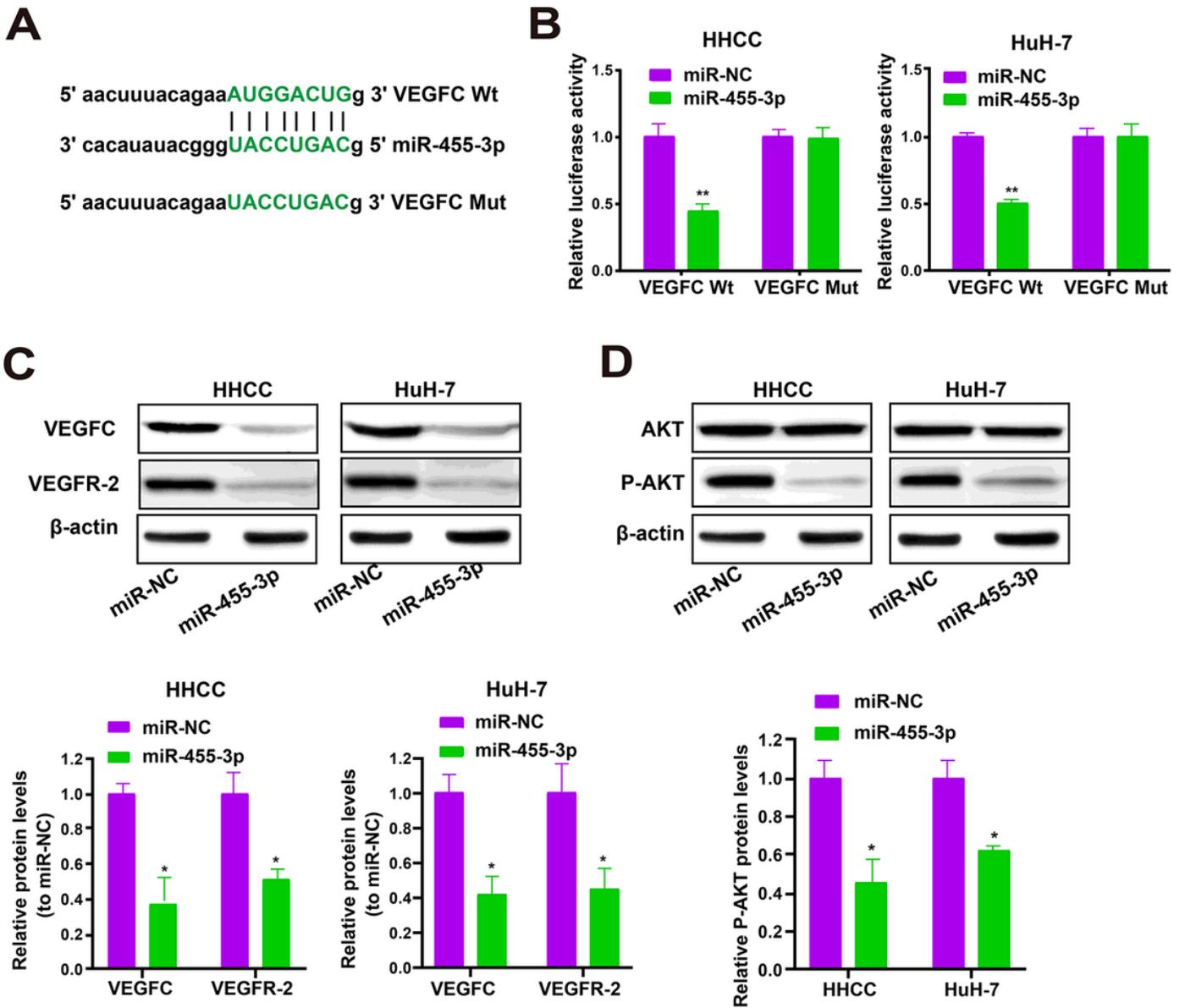
Figure 1

Low expression of miR-455-3p in HCC tissues and cells (A) Relative expression of miR-455-3p in HCC tissues in comparison with corresponding non-tumor colon tissues. (B) Relative expression of miR-455-3p in HCC cell lines comparison with the human normal liver cell line LO2. Data are shown as the means  $\pm$  SEM of three experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



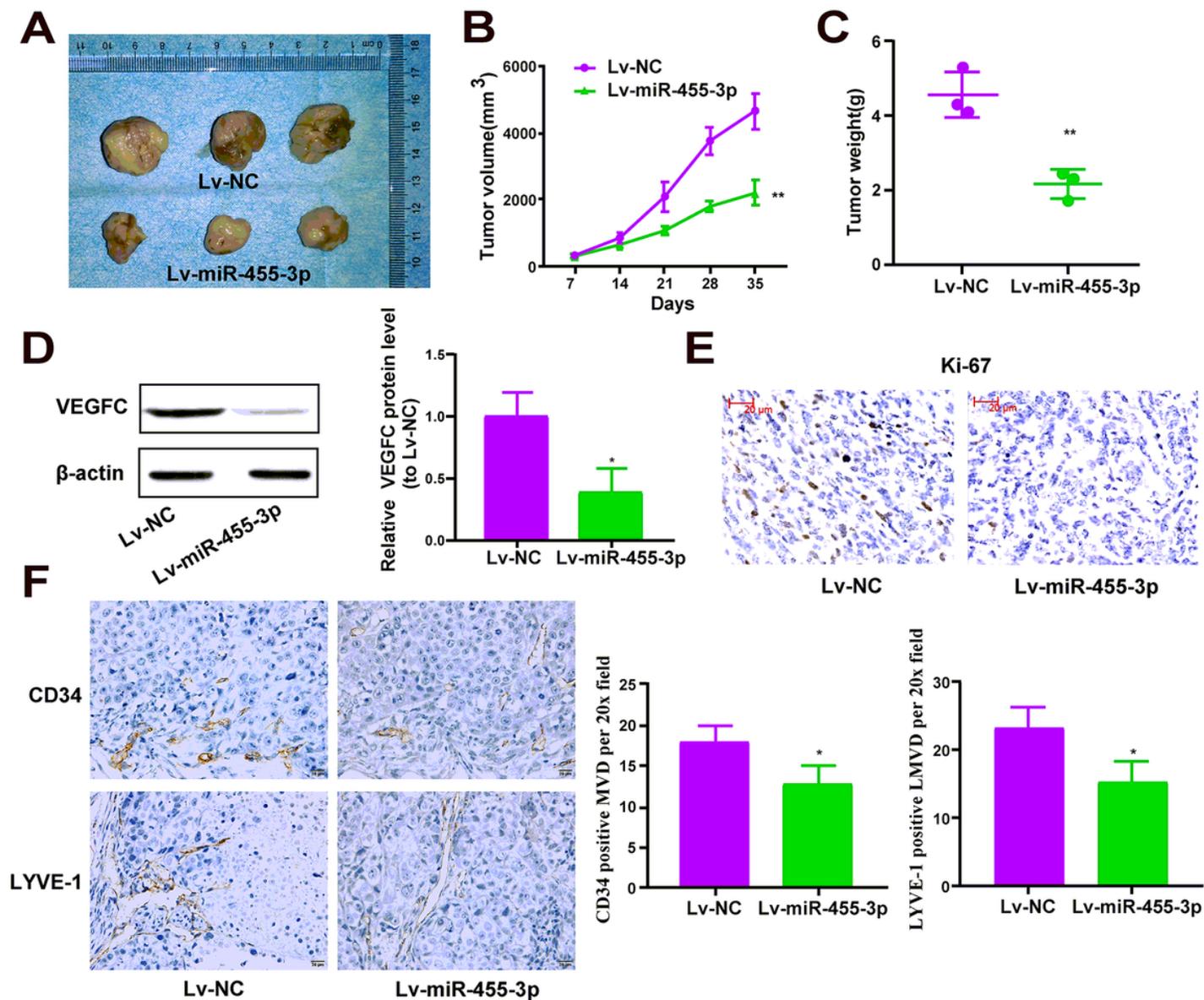
**Figure 2**

Ectopic miR-455-3p expression inhibited the proliferation, migration and invasion of HCC cells (A) Stable cell lines expressing miR-455-3p were examined by qRT-PCR. (B) CCK8 assay was performed to determine the proliferation of cells at 1, 2, 3, 4 and 5 days after transfection. (C) Representative results of EdU of HCC cells stably transfected with miR-455-3p or miR-NC. (D) Representative results of colony formation of HCC cells stably transfected with miR-455-3p or miR-NC. (E) Wound-healing assay was performed to determine the effect of miR-455-3p on cell migration. (F) Cell invasion of HCC cells stably transfected with miR-455-3p or miR-NC. Data are representative of three independent experiments. Data are shown as the means  $\pm$  SEM of three experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



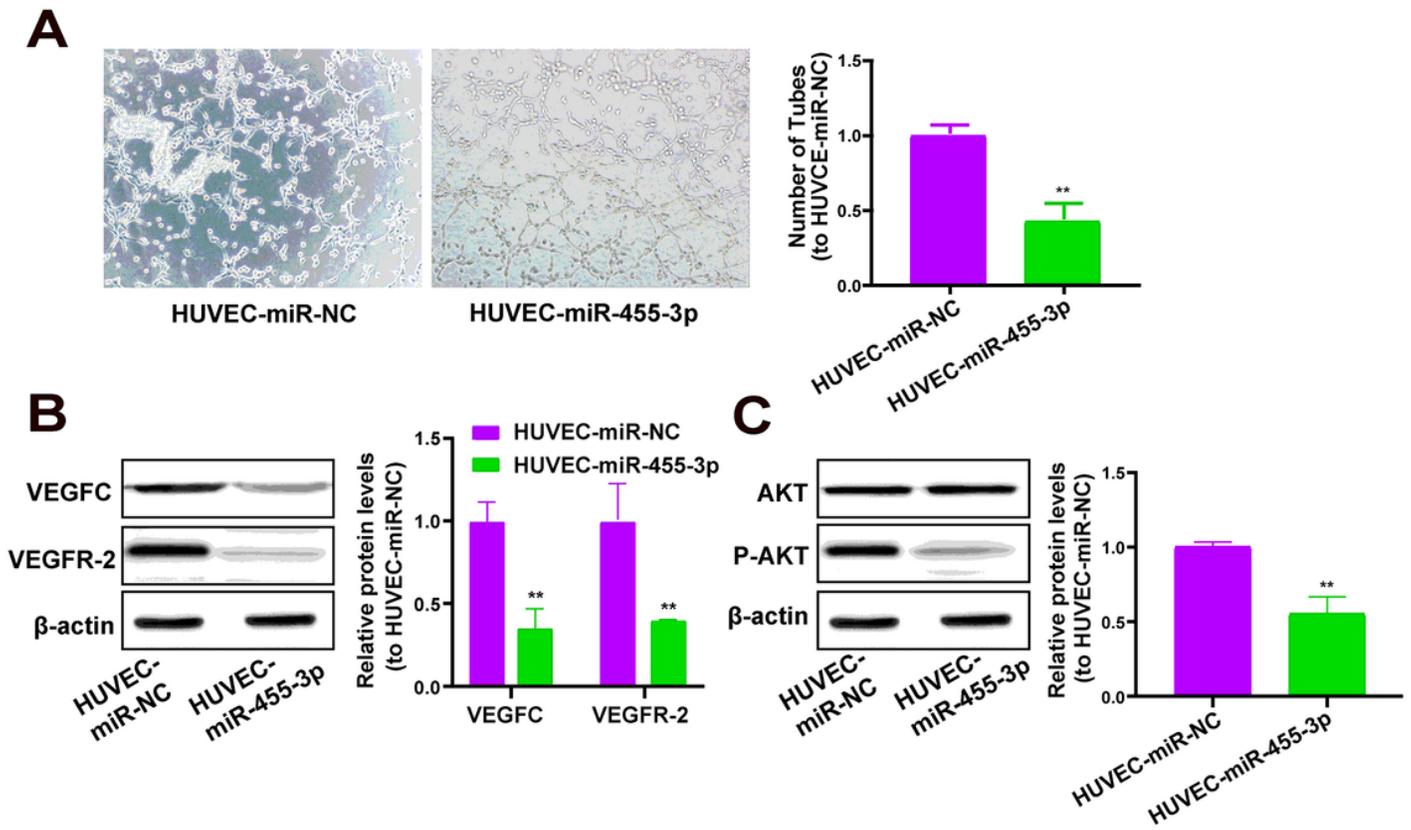
**Figure 3**

miR-455-3p directly targets VEGFC (A) The sequence of miR-455-3p matches VEGFC 3'-UTR. (B) Dual-luciferase reporter gene assay in HHCC and HuH-7 cells. (C) Western blot analysis of the expression levels of VEGFC, VEGFR-2 in cells. (D) Total and phosphorylated AKT was analysed by Western blotting. Data are shown as the means  $\pm$  SEM of three experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .



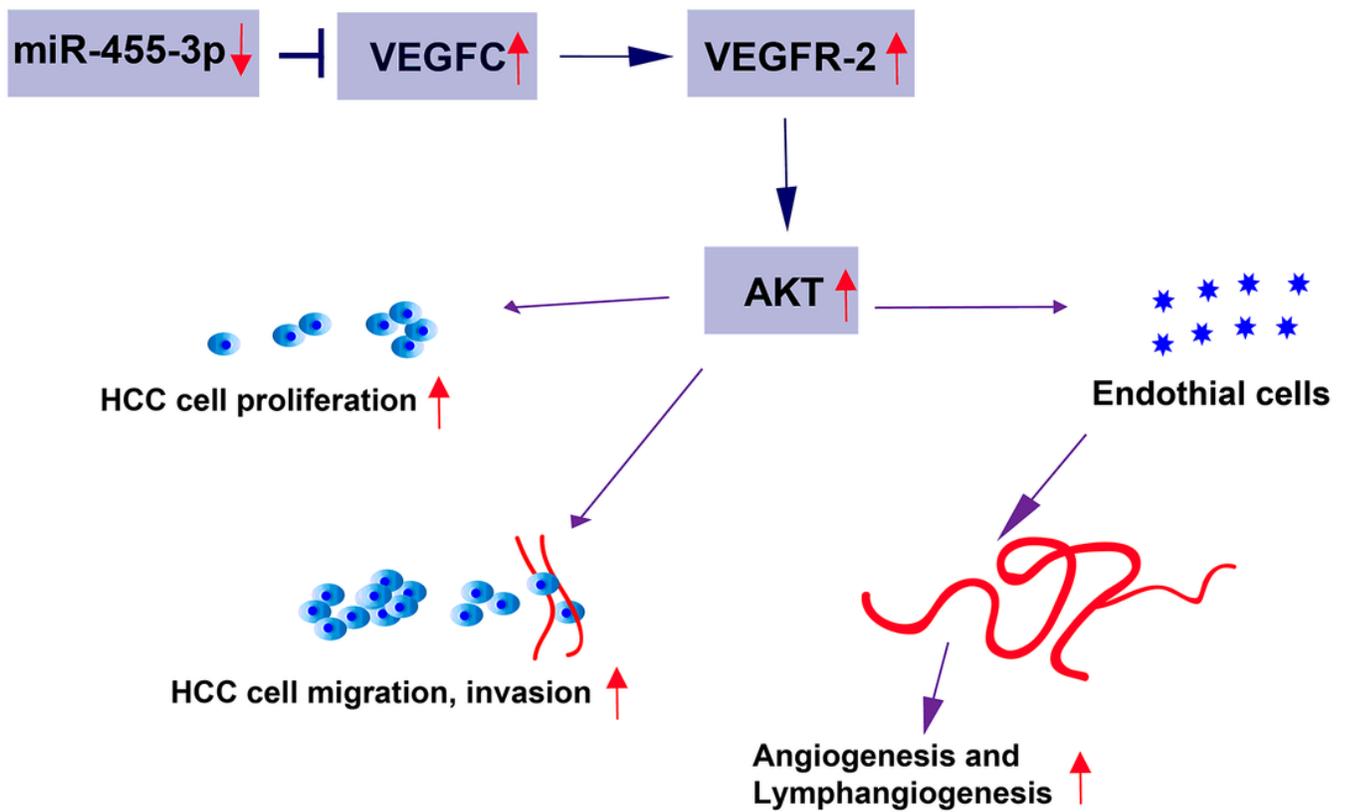
**Figure 4**

Therapeutic effects of miR-455-3p on human HCC xenografts (A) Xenograft tumors. (B) The volume and (C) weight of tumors were measured. (D) VEGFC protein level in tumor tissue was detected by Western blotting. (E) Immunohistochemical staining for Ki-67. (F) Tumor tissues from miR-455-3p-treated mice showed significantly reduced microvessel densities (MVD) and lymphatic microvessel densities (LMVD) by immunohistochemical analysis. Data are shown as the means  $\pm$  SEM of three experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 5**

Overexpression of miR-455-3p inhibits tube formation ability of HUVECs (A) Micrographs of capillary-like structures formed by HUVECs transfected with miR-455-3p or miR-NC. The number of tubes was measured in three photographic fields using LAS software (Leica). (B) Western blot analysis of the expression levels of VEGFC and VEGFR-2 in HUVEC cells. (C) Total and phospho-AKT in HUVEC cells were analysed by Western blotting. Data are shown as the means  $\pm$  SEM of three experiments. \*\*P < 0.01.



**Figure 6**

Hypothetical model of miR-455-3p function in HCC cells and endothelial cells. MiR-455-3p expression level is decreased in HCC tissues and cell lines. And VEGFC is directly targeted by miR-455-3p. In addition, miR-455-3p negatively regulates the expression of VEGFC, VEGFR-2 and the phosphorylation of AKT. In a word, down-regulation of miR-455-3p increases the expression of VEGFC and VEGFR-2, also increases the phosphorylation of AKT, and ultimately promote NSCLC progression, angiogenesis and lymphangiogenesis.