



Hepatitis B Virus preS2 Domain Promotes Angiogenesis in Hepatocellular Carcinoma via Transcriptional Activation of VEGFA Promoter

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Abstract

Angiogenesis is closely related to the development and progression of hepatocellular carcinoma (HCC). Angiogenic factors have been confirmed to be overexpressed in HCC. The hepatitis B virus preS2 domain is a transactivator that plays an important role in hepatitis B virus (HBV)-related HCC. Here, we aimed to investigate the potential of the preS2 domain in inducing angiogenesis in HCC. A total of 25 cases of pathologically confirmed HCC were screened. The levels of preS2, CD34, and vascular endothelial growth factor A (VEGFA) in HCC samples were evaluated by immunohistochemistry (IHC). The proliferation of vascular endothelial cells was detected by CCK-8. Besides, VEGFA was analyzed by Western blot in HCC cells. The effect of preS2 on the VEGFA promoter was measured by dual-luciferase reporter assays. We found that preS2 domain-positive HCCs had significantly higher microvessel density (MVD) and VEGFA expression than preS2 domain-negative HCCs. Overexpression of preS2 upregulated VEGFA expression in HepG2 and activated vascular endothelial cell proliferation. However, blocking preS2 expression reduced VEGFA expression in HepG2.2.15 and inhibited the proliferation of vascular endothelial cells. In addition, a dual-luciferase assay indicated that the preS2 domain could activate VEGFA promoter activity. In conclusion, we showed that the expression of the preS2 domain promotes angiogenesis by transactivating the VEGFA promoter in HCC.

Keywords: HBV preS2 Domain, Angiogenesis, VEGFA, Hepatocellular Carcinoma

1. Background

Hepatocellular carcinoma (HCC) is a primary type of liver cancer, which ranks as the fourth lethal tumor worldwide (1). The main cause of HCC is hepatitis B virus (HBV) infection, mostly in Asia and Sub-Saharan Africa (1). Hepatitis B virus surface proteins, also termed HBV surface antigens (HBsAg), are encoded by preS1, preS2, and S regions. Three viral surface proteins include the LHBs (large hepatitis B virus surface, coded by PreS1, PreS2, and S regions) protein, MHBs (middle hepatitis B virus surface, coded by PreS2 and S regions) protein, and SHBs (small hepatitis B virus surface, coded by the S region) protein (2). Amino acids 4 - 53 of the preS2 domain (amino acids 1 and 55), the minimal trans-activation functional unit of HBV surface protein, exist in MHBs and LHBs proteins (3, 4). These two HBV surface proteins may co-assemble with the SHBs protein and be secreted as subviral or viral particles or may be intracellularly retained as MHBst (3' terminally truncated MHBs pro-

tein) (2). Intracellular retention proteins include MHBst and overexpressed non-secreted LHBs (2-4). The HBV preS2 domain can translocate inside the nucleus (5). The nuclear translocation ability is closely associated with its ability to transactivate various genes, including hTERT, Foxp3, TAZ, AFP, and GPC3 (5-10). The trans-activation is important for preS2-promoted HCC development.

Hepatocellular carcinoma is also characterized as a hypervascular tumor, and tumor angiogenesis plays a critical role in HCC development, progression, and metastasis (11, 12). Various growth factors secreted by tumors can promote endothelial cell proliferation and induce neovascularization by supplying nutrients, resulting in tumor expansion (13). Among angiogenic factors, vascular endothelial growth factor A (VEGFA) plays a key role in the neovascularization of HCC. Overexpressed VEGFA is found in about 91% of advanced HCCs, and it has been recognized as a prognostic biomarker in patients with HCC (14, 15).

At present, it is unknown whether the preS2 domain is functional in angiogenesis of HBV-related HCC. Due to the high occurrence of preS2 expression in HBV-related HCC, it is reasonable to speculate that the HBV preS2 domain underscores a potentially important role through tumor angiogenesis during HCC development.

2. Objectives

In this work, we attempted to study the correlation of HBV preS2 domain with angiogenic factors in HCC tissues and human hepatoma cell lines.

3. Methods

3.1. Patients and Tissue Preparation

Twenty-five HCC tissue samples were screened from 86 patients in Shandong Provincial Hospital affiliated to Shandong First Medical University. None of the HBV-related HCC patients had occult HBV infection. All of them were adw sub-genotype and negative for the hepatitis C antigen or human immunodeficiency virus antigen. None of the patients consumed excessive quantities of alcohol. Before surgery, none of them had been treated with chemotherapy. The details of the patients involved in the study are listed in Appendix 1 Supplementary File. This study was approved by the Ethics Committee of Shandong First Medical University (code: NSFC:NO.2020-218).

3.2. Immunohistochemical Staining

Immunohistochemical (IHC) staining of 25 HCC tissues was performed using anti-preS2 (mouse monoclonal, epitope: aa positions 131 - 139 of HBsAg, adw subtype), anti-CD34, and anti-VEGFA (Abcam, USA) at a dilution of 1:100. The HRP labeled secondary antibody was used at a dilution of 1:2000. Besides, preS2 and VEGFA staining was scored according to staining intensity and the number of stained cells (10). The CD34 marking for Microvascular Density (MVD) was reported according to a correction method (16). For each specimen, we selected the three most active areas of neovascularization and counted microvessels per 400 × field. The average value in the three areas was regarded as the MVD value.

3.3. Plasmids

The HBV preS2 domain (1-55aa) expression vector with HA tag pcS2-HA was kindly provided by the School of Basic Medical Science, Shandong University. The human VEGFA full-length promoter (-989 to + 52) and core promoter (-88

to + 52) were amplified by PCR from human genomic DNA using forward (989-F: TAGGTACCACTCCACAGTGCATACGT, 88-F: TAGGTACCCGGGGCGGGCCGGGGCGGGGT) and reverse (989-R:5' TAAAGCTTTAGCCCCAGCGCCACGA, 88-R: TAAAGCTTTAGCCCCAGCGCCACGA) primers. The PCR products were inserted into KpnI and HindIII cleavage sites of pGL3-Basic (Promega, Beijing, China) to construct plasmids pGL3B-989 and pGL3B-88. The numbers indicate positions relative to the transcriptional initiation site.

3.4. Knockdown of PreS2 by Antisense Oligonucleotide

Antisense oligonucleotide sequences completely complementary to the preS2 domain in the initial translation region (3203 ~ 3219 bp) were designed. The sequences were synthesized (Boshang, Jinan, China) as follows: PS-asODNs/preS2: 5'-CCACTGCATGGCCTGAG-3' (17-mer), PS-RODNs: 5'-TTGCCGAGCGGGTA-3' (15-mer). An RT-PCR was used to assay the blocking efficiency.

3.5. Reverse Transcription-Polymerase Chain Reaction

The preS2 mRNA levels were determined using reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed according to the HBV (C Genotype, adr subtype, GI:59404) genome sequence in GenBank. Forward and reverse primers were as follows: preS2, 5'CCACCATGCAGTGGAACTC3', 5'TGTGTTCTCCATGTTCCGGTG3'; β -actin, 5'GGCATCGTGATGGACTCCG-3', 5'-GCTGGAAGGTGGACAGCGA-3'. Amplification was done at 95°C for one minute, 55°C (β -actin) and 65°C (preS2) for one minute, and 72°C for one minute, with a final extension for five minutes at 72°C.

3.6. Western Blot Analyses

Protein extracts were obtained from 10⁶ HCC cells. HepG2 cells were transfected with pcS2-HA or pcDNA3 plasmids (Invitrogen, Beijing, China). HepG2.2.15 cells were transfected with PS-asODNs/preS2 or PS-RODNs. Immunoblotting was performed with anti-human VEGFA (Abcam, USA), HA (Covance, USA), or β -actin (Santa Cruz, USA).

3.7. Cell Culture and Proliferation Analysis

The HCC cell lines HepG2 and HepG2.2.15 were cultured in the minimum essential medium (MEM). The HepG2.2.15 cells harbored four copies of HBV-DNA. Human umbilical vein endothelial cells (HUVEC) were incubated in the endothelial cell growth medium with glutamine and heparin (PELO Biotech, Germany) (17). The medium was supplemented with 10% fetal calf serum for hepatocellular carcinoma cell lines and 20% for HUVEC. Then, HCC cells (the under chamber) and HUVEC cells (the upper chamber) were

co-cultured in transwell chambers. Cells were cultured at 37°C under 5% CO₂. Finally, CCK-8 (Dojindo Molecular Technologies, Shanghai, China) was used to measure cell proliferation at A450 with an enzyme-labeled minireader (BioRad, Tokyo, Japan).

3.8. Transfection and Luciferase Reporter Assays

HepG2 cells were transiently transfected with pcS2-HA, and HepG2.2.15 cells were transiently transfected with as RNA using LipofectamineTM2000 (Invitrogen, Beijing, China). Cells were harvested 48 hours later.

For analyzing the transactivation of the HBV preS2 domain on the VEGFA promoter, co-transfections into HepG2 cells were performed with lipofectamineTM2000; 0.5 mg VEGF promoter-reporter plasmids and 0.5 mg preS2-domain expression plasmids were co-transfected, and 20 ng of SV40-Renilla reporter plasmids (Promega, Beijing, China) were used to standardize the transfection efficiency in each well. After 48 hours, a Dual-Luciferase Reporter Assay system (Promega, Beijing, China) was used for performing luciferase assays.

3.9. Statistical Analysis

Data were statistically analyzed by GraphPad Prism 5.0 Software (San Diego, CA). Spearman analysis was performed to assess the relationship between preS2, CD34, and VEGFA. A student-*t*-test (for quantitative comparison) or Mann-Whitney U-test (for semi-quantitative comparison) was used for determining the differences between the two groups. A *P* < 0.05 was considered statistically significant, and all *p* values were two-tailed (95% confidence interval).

4. Results

4.1. There Is a Higher Potential for Inducing Angiogenesis in PreS2 Domain-positive HCC Patients

Detection of HBV preS2 domain was positive in 18 HBV-related HCC samples while it was negative in the rest seven HCC tumors by IHC staining. According to the expression of the preS2 domain, we divided the 25 HCC samples into two groups. The CD34 expression levels were also semi-quantitatively analyzed by IHC staining. The average MVD counts in preS2 domain-positive HCC samples and preS2 domain-negative HCC samples were 63.22 ± 8.90 and 48.14 ± 6.74 , respectively. As presented in [Figure 1A](#) and [B](#), higher levels of MVD were detected in preS2 domain-positive HCC samples than in preS2-domain negative HCC samples (*U* = 13.00, *P* < 0.01).

4.2. Difference of VEGFA Expression Between PreS2 Domain-positive HCC Samples and PreS2 Domain-negative HCC Samples

In the same way, we semi-quantitatively analyzed the expression of VEGFA by IHC staining. The average results of VEGFA expression were 5.33 ± 2.25 and 2.29 ± 2.14 in HBV preS2 domain-positive HCC samples and HBV preS2 domain-negative HCC samples, respectively. As presented in [Figure 1C](#) and [D](#), higher levels of VEGFA were detected in preS2 domain-positive HCC samples than in preS2 domain-negative HCC samples (*U* = 24.0, *P* < 0.05).

4.3. Correlation Between MVD, VEGFA, and HBV PreS2 Domain

Spearman analysis was performed to analyze the correlation between MVD, VEGFA, and HBV preS2 domain in HCC samples. The results confirmed that the MVD count was positively related to the expression of HBV preS2 domain (*r* = 0.6707, *P* < 0.01) ([Figure 2A](#)). Statistical analysis also showed a positive correlation between MVD and VEGFA expression (*r* = 0.5543, *P* < 0.05) in preS2 domain-positive HCC ([Figure 2C](#)). But, there was no significant correlation between HBV preS2 domain and VEGFA (*r* = 0.3646, *P* > 0.05) ([Figure 2B](#)).

4.4. PreS2 Overexpression Up-Regulates VEGFA and Promotes Proliferation of Vascular Endothelial Cells

To investigate the effects of preS2 on angiogenesis, pcS2-HA or pcDNA3 was transfected into HepG2 cells. Then, HUVEC cells were co-cultured with HepG2 cells after transfection. The protein levels of VEGFA and preS2 were determined by western blot, and the proliferation of vascular endothelial cells was analyzed by CCK-8 48 hours later. The results showed that preS2 overexpression increases the VEGFA levels ([Figure 3A](#)) in HCC cells and promotes the proliferation of co-cultured HUVEC cells ([Figure 3B](#)).

4.5. PreS2 Knockdown Downregulates VEGFA and Inhibits the Proliferation of Vascular Endothelial Cells

As expected, the suppression of preS2 expression by antisense RNA decreased preS2 mRNA and VEGFA expression in HepG2.2.15 cells ([Figure 3C](#)). Meanwhile, the proliferation of co-cultured HUVEC cells was obviously reduced ([Figure 3D](#)).

4.6. PreS2 Transactivates VEGFA Promoter in a Dose-dependent Manner

The identification of VEGFA core and full-length promoter activity is shown in [Figure 4A](#). The identification

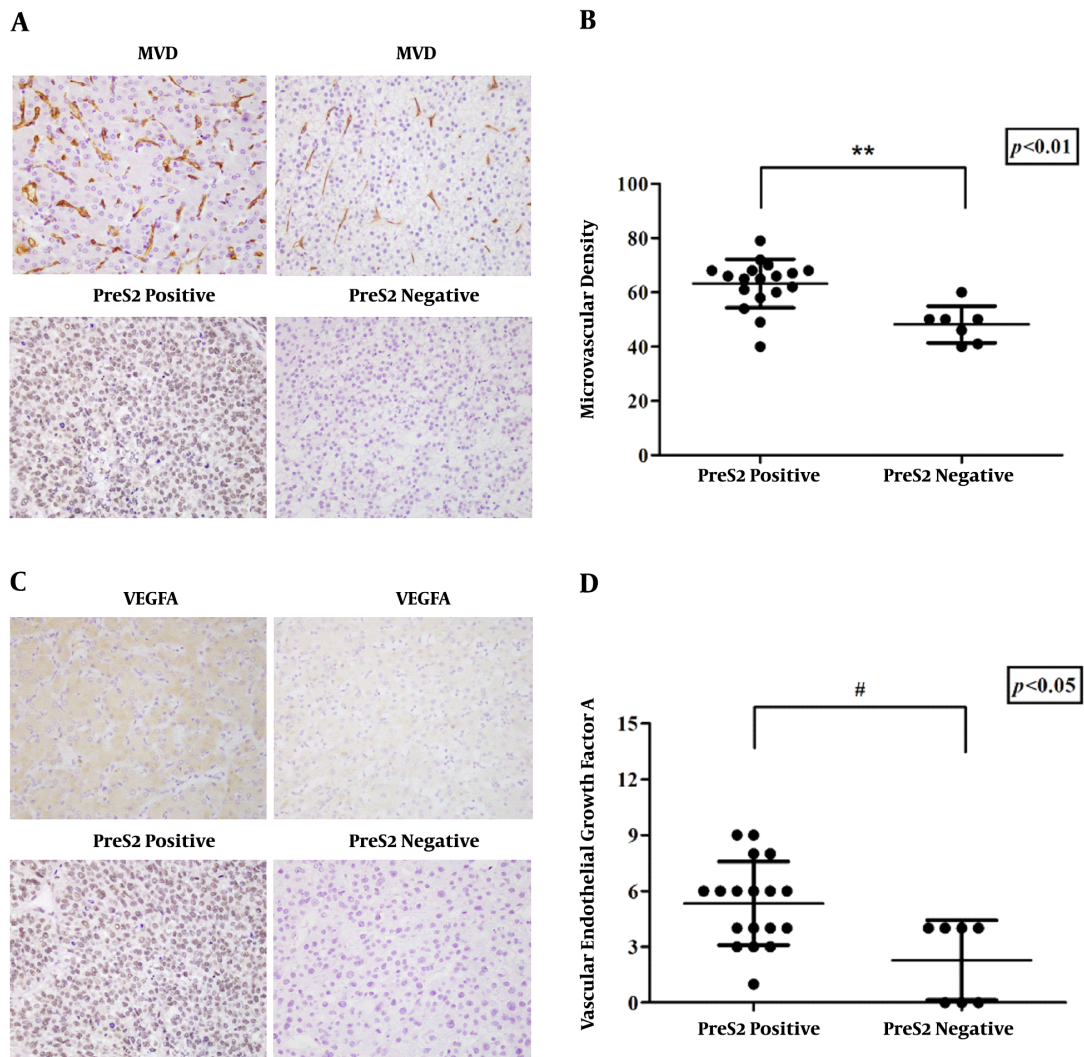


Figure 1. The difference in MVD counting and VEGFA expression between preS2 domain-positive HCC samples and preS2 domain-negative HCC samples. A, Immunohistochemical staining of CD34 for MVD (left upper, 400 \times) and preS2 (left middle, 400 \times) in cancer biopsies from two of 25 HCC samples; B, twenty-five HCC specimens were divided into two groups by IHC of the preS2 domain. A significant difference in MVD counting was determined by the Mann-Whitney U test (**, $P < 0.01$); C, immunohistochemical staining of VEGFA (right upper, 400 \times) and preS2 (right middle, 400 \times) in cancer biopsies from two of 25 HCC samples; D, twenty-five HCC specimens were divided into two groups by IHC of the preS2 domain. A significant difference in VEGFA levels was determined by the Mann-Whitney U test (#, $P < 0.05$).

results of VEGFA promoter constructs by enzyme digestion and sequencing are shown in the supplementary material. Co-transfection and dual-luciferase assays demonstrated the effective transactivation of the preS2 domain on the VEGFA core and full-length promoter activity in HepG2 cells (Figure 4B). The upregulation of the preS2 domain on VEGFA full-length promoter activity also occurred in a dose-dependent manner in HepG2 cells (Figure 4C).

5. Discussion

Hepatocellular carcinoma (HCC) is a malignant solid tumor enriched with blood vessels. Hepatitis B virus is closely related to the occurrence of HCC, and clinical studies have found that 63.2% of HCC patients are HBsAg-positive, and HBV preS2 domain is positively expressed in HBsAg-positive HCC (10). As reported previously, enhanced or blocked preS2 expression in vitro and in vivo could only promote or inhibit HCC cell proliferation for a certain period and to a certain extent (7). As known, cultured tumor

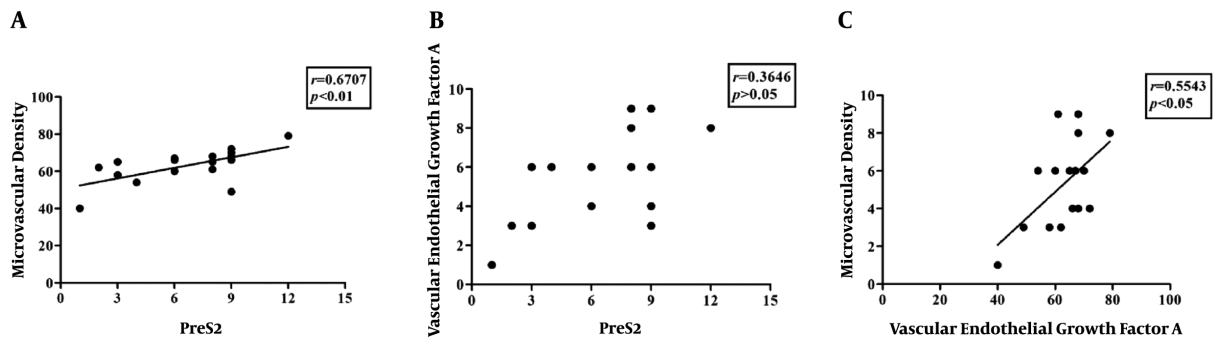


Figure 2. Correlation among MVD, VEGFA, and preS2 expression in HCC samples. A, A positive correlation between MVD and preS2 was determined by spearman analysis ($r = 0.6707$, $P < 0.01$); B, no obvious correlation between VEGFA and preS2 ($r = 0.3646$, $P > 0.05$); C, a positive correlation between MVD and VEGFA was determined by spearman analysis ($r = 0.5543$, $P < 0.05$).

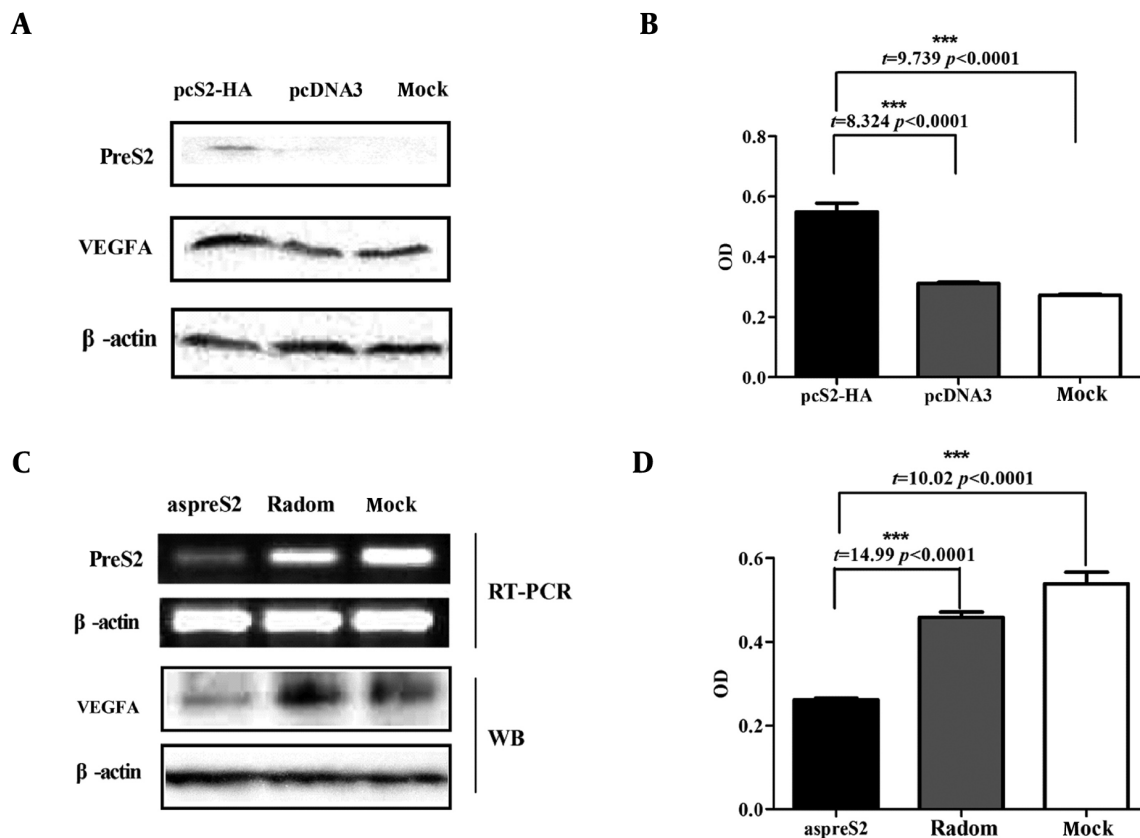


Figure 3. Effect of preS2 on VEGFA expression in HCC cells and cell proliferation of HUVEC cells. A, Western blot was used to assay preS2 and VEGFA expression; B, the proliferation of HUVEC cells was promoted compared to the PS-rODNs group and mock group by using the CCK-8 assay (***, $P < 0.0001$); C, RT-PCR was used to assay preS2 mRNA expression, and western blot was used to assay VEGFA expression; D, the proliferation of HUVEC cells was reduced compared to the PS-rODNs group and mock group by using the CCK-8 assay (***, $P < 0.0001$).

cells cannot grow beyond a certain size due to hypoxia and lack of essential nutrients. Angiogenesis is necessary for transformation from a small and harmless cell cluster to a

large and malignant solid tumor, and the tumor shows exponential growth mostly dependent on angiogenesis (18).

There are two sources of microvessels in tumor tissues.

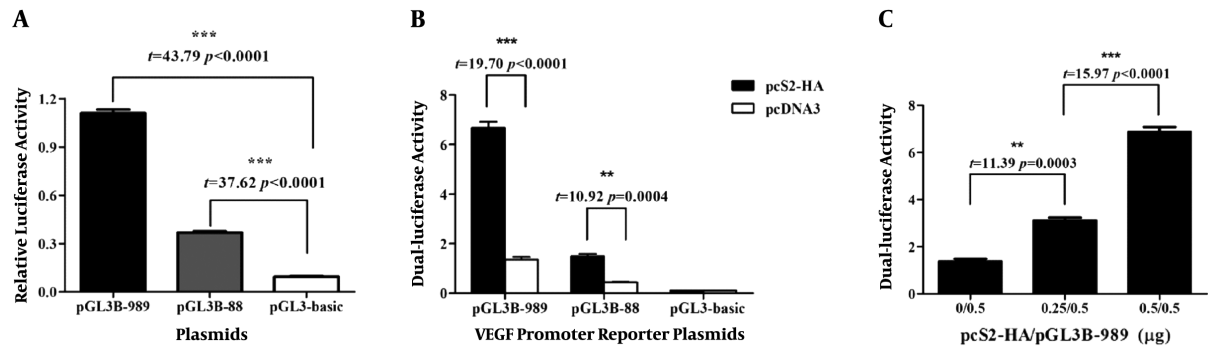


Figure 4. Transcriptional activation of preS2 on VEGFA promoter. A, Promoter activities of pGL3B-989 and pGL3B88 plasmids compared to pGL3-basic; B, promoter activities of pGL3B-989 and pGL3B-88 were increased by pcS2-HA; C, dose-dependent transactivation of preS2 on VEGFA promoter. 0, 0.25, and 0.5 mg pcS2-HA plasmids were co-transfected with 0.5 mg pGL3B-989 and 20 ng pRL-TK plasmids into HepG2 (means \pm SD represents three separate experiments, **, $P < 0.0001$, ***, $P < 0.0001$).

Angiogenic factors produced by tumor cells induce the formation of microvessels in the tumor. Besides, host vessels remaining in the tumor gradually become tumor vessels, which is named as tumorigenesis of host vessels (15). Among the numerous angiogenic factors, VEGF is a key regulator and the only mitogen that can specifically act on endothelial cells. Besides, VEGF-A, as the main member, regulates most of the endothelial responses (14). In this manuscript, we supplied the following evidence to support the positive regulation of HBV preS2 domain on angiogenesis via VEGF-A in HCC. First, as demonstrated by IHC staining, HBV preS2 domain-positive HCC samples show higher MVD counting and VEGFA expression compared to HBV preS2 domain-negative HCC samples. Second, preS2 overexpression increases VEGF-A expression in HepG2 cells and promotes the proliferation of co-cultured HUVEC cells, while preS2 knockdown decreases VEGF-A expression in HepG2.2.15 cells and reduces the proliferation of co-cultured HUVEC cells. Third, the HBV preS2 domain transactivates the VEGFA promoter. The above studies additionally confirm the important role of HBV-encoded transcription activator preS2 in HCC development. The HBV preS2 domain promotes the growth of hepatocyte tumors, which is accompanied by changes in tumor vascular density.

It is reported that VEGF might be a prognostic indicator of HBV-related HCC. The expression of VEGF-A is positively correlated with the recurrence rate of HCC after curative resection (19). In our study, correlation analysis of clinical specimens demonstrated no relationship between HBV preS2 domain and VEGFA expression, which may be partly due to the small amount of HCC samples limited by anti-preS2 (adv subtype). However, the dual-luciferase assay in

vitro showed the transactivation of preS2 on the VEGF promoter, which suggested the indirect interaction between the preS2 domain and VEGFA. Hypoxia inducible factor (HIF) is a helix transcription factor participating in tumor growth through the regulation of genes involved in angiogenesis (20). Previous studies reported that HBX induces VEGF expression and promotes angiogenesis by increasing the stability and transcriptional activity of the HIF-1 protein (21). Our study showed that the preS2 domain upregulated the VEGFA promoter activity dose-dependently. As described previously, an SHBs protein with an N-terminal truncation leaving a fragment of aa 102 - 226 without any preS2 domain had DNA-binding properties (22). Given all the above studies, we speculate that the HBV preS2 domain may activate the VEGFA promoter by enhancing HIF-1 stability or expression levels. However, the mechanisms need further study.

At present, studies on the angiogenesis mechanism of HBV-related HCC mainly focus on the HBX protein. There is no report on the effect of HBV preS2 domain on angiogenesis in HBV-related HCC. The proliferation of vascular endothelial cells is the basis of tumor angiogenesis. This study initially explored the effects of the preS2 domain on the proliferation activity of vascular endothelial cells and the expression of VEGF-A. The implementation and completion of this study will help further reveal the role of the preS2 domain in HCC and provide a reliable experimental basis for clinical intervention.

5.1. Conclusions

In summary, we demonstrated that the expression of HBV preS2 domain is positively correlated with MVD expression in HCC, potentially because the preS2 domain can

transcriptionally activate VEGFA and participate in the proliferation of vascular endothelial cells. Further studies are required to investigate other factors possibly involved in neovascularization in HBV-related HCC.

Supplementary Material

Supplementary material(s) is available [here](#) [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Acknowledgments

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Footnotes

Authors' Contribution: FL and YW conceived and designed the study, and wrote the paper. FL, BL, and XG performed research. FL and BL analyzed data.

Clinical Trial Registration Code: The clinical trial registration code was NSFC:NO.2020-218.

Conflict of Interests: The authors declare that they have no conflict of interest.

Ethical Approval: This study was initiated with the approval of the Shandong First Medical University Ethics Committee as per the Declaration of Helsinki (code: NSFC:NO.2020-218).

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Informed Consent: In this study, we retrospective screened 25 HCC patients. Because we are Teaching hospital, the patients have been told that their samples would be used for research when they are in hospital.

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