

# Effect of grape seed proanthocyanidins on tumor vasculogenic mimicry in liver cancer xenograft model

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**Abstract** *Objective:* As a novel blood supply pattern, vasculogenic mimicry (VM) has attracted increasingly attention in recent years, which may partly compensate for the absence of feeding and facilitate tumor perfusion. However, anti-angiogenic drugs have little effect on VM. The grape seed proanthocyanidins (GSPs), a kind of promising bioactive phytochemical, has shown anti-carcinogenesis and anti-angiogenic in several tumor models. However, GSPs regulation of VM and its possible mechanisms in a H22 hepatoma carcinoma model remain not clear. The aim of this study was to examine the effects of GSPs on proliferation and VM in a H22 hepatoma carcinoma model and to investigate the underlying mechanism. *Methods:* Seventy-five mice were divided into the control group and experimental groups treated with different concentration of GSPs. CD34-PAS dual staining was employed to identify the VM structure. The immunohistochemical staining for investigating the expression of VEGF, EphA2 and MMP-2 protein was performed. *Results:* Treatment of the H22 model with Endostar (4 mg/kg), 50, 100, 200 mg/kg of the GSPs resulted in 6.87%, 17.81%, 27.43%, 53.52% inhibition in tumor growth, respectively. The mean weight of tumors were significantly lower in GSPs (100 mg/kg) and GSPs (200 mg/kg) groups than in the control group (all  $P < 0.01$ ). Similarly, compared with the control group, the number of VM channels were significantly reduced in GSPs (100 mg/kg) and GSPs (200 mg/kg) groups (all  $P < 0.01$ ). Immunohistochemistry showed significant decreases in the expression levels of VEGF, EphA2 and MMP-2 protein in GSPs (100 mg/kg) and GSPs (200 mg/kg) groups when compared with control group (all  $P < 0.001$ ). *Conclusion:* This is the first report providing evidence that GSPs inhibit the VM structure by regulation of the VEGF/EphA2/MMPs signaling pathway. Therefore, we concluded that GSPs has the potential of being a clinical anti-VM inhibitor.

**Key words** vasculogenic mimicry; H22 hepatoma carcinoma model; grape seed proanthocyanidins; VEGF; EphA2; MMP-2

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated deaths in the United States. The incidence of HCC in China is significantly higher than that in the US [1–2]. The highly incidence rate may attribute, in large part, to chronic infection with hepatitis B virus (HBV) (50%–55%) or hepatitis C virus (HCV) (25%–30%) in China. Most HCC patients usually present at advanced stages at the time of diagnosis [3–4]. Although several advanced strategies are available for the treatment of liver cancer, including surgical resection, transarterial chemoembolization (TACE), transplantation, local ablation, radiotherapy, cytotoxic chemotherapy, and molecular targeted therapy, the five-year survival rate was disappointing, approximately at 3%–5%. Therefore, it is urgent to explore a novel therapy for the treatment of advanced HCC.

Hepatocellular carcinoma (HCC) is highly vascular-

ize tumor. Anti-angiogenic therapy, as a promising strategy, has been emerging over the last years. Traditionally, agents targeting the vascular endothelial growth factor (VEGF) may have satisfied therapeutic effects. Unfortunately, the benefits are at best transitory and may facilitate malignant growth and progression of tumors [5–8]. More recently, several groups have reported that vasculogenic mimicry (VM) has been suggested to be involved in the resistant to anti-VEGF therapy. Expect for traditional recognized tumor channels (angiogenesis, vasculogenesis), there exists another tubular network in highly aggressive tumors, which termed as VM. Vasculogenic mimicry has been observed in several aggressive tumors including breast cancer [9–11], gallbladder carcinomas [12] and hepatocellular carcinoma [13]. The newly vascular channels are transdifferentiated from highly malignant tumor cells without participation of endothelial cells [14]. Traditional antiangiogenic drugs, including endostatin and bevacizumab, have little effect on VM [7–8, 15]. Thus, it

makes sense to develop novel and accurate anti-vascular therapeutic agents targeting VM. To date, it is generally accepted that there existed several molecules or signaling pathways related to VM by aggressive malignant tumor, including VEGF, EphA2, MMPs, Ln-5 $\gamma$ 2, etc [12, 16–17]. Therefore, identification of new therapeutic drugs targeting VM and demonstration of molecular pathways of VM is of high priority in the treatment of patients with liver cancer.

The grape seed proanthocyanidins (GSPs), which is derived from grape pip, is a kind of promising botanical agents. GSPs is used to anti-carcinogenesis and anti-angiogenesis *in vitro* and *in vivo*, involved in skin, prostate, breast and liver cancer models [18–21]. However, we do not determine whether GSPs would also inhibit the development of VM. In this study, H22 bearing mice have been employed to examine the inhibition efficacy and molecular mechanism of GSPs on VM.

## Materials and methods

### Drugs and reagents

The GSPs was kindly provided by our laboratory, which contained approximately 90% proanthocyanidins [20, 22]. Both the primary antibodies specific for VEGF, EphA2, MMP-2 and peroxidase-conjugated secondary antibodies were purchased from Boster, Wuhan, China. Endostar was donated by Shandong Simcere-Medgenn Bio-Pharmaceutical Co., Ltd (China).

### Animals

In the present analysis, healthy Kunming mice ( $n = 75$ ) used as the tumor-bearing mice model should satisfy following eligibility criteria: aged from 4 to 6 weeks, weighing 18–22 g, half male and half female. Kunming mice (Animal license SCXK Lu20080002) were purchased from the lab center of Shandong Lukang medicines Co., Ltd., Shandong, China, which were maintained in a specific pathogen-free conditions ( $23 \pm 2$  °C, humidity of  $55 \pm 5\%$ , 12 h light/dark cycle). Besides, standard laboratory pellet diet and water were provided during experiments. Mice were acclimatized for at least one week prior to experiments. All the procedures were approved by Animal Ethical Council of Qingdao University.

### Establishment of H22 xenograft model

We purchased H22 hepatoma ascites tumor of mouse from the Shandong experimental animal center, which passaged three sequentially in the abdominal cavity. Seven days after inoculation, we collected aseptically ascite cells of mice and adjusted to a concentration of  $1.0 \times 10^7$  cells/mL with physiological saline. 0.2 mL suspension ( $2 \times 10^6$  H22 cells/mouse) was injected subcutaneously at the left armpit of the mouse. After 24 h of tumor implanta-

tion, 75 Kunming mice were randomly divided into 5 groups (15 mice per group), and disposal methods were as follows: group A, the mice were intragastrically treated with 0.1 mL/10 g body weight of normal saline every day as control; group B, mice were injected Endostar (4 mg/kg) via intraperitoneal injection daily; GSPs suspended in normal saline was orally administered once a day at a dose of 50, 100, 200 mg/kg for group C, D, E respectively. All administrations were repeated for 10 days. Then, all the mice were sacrificed by cervical dislocation and the tumors implanted were excised and weighted immediately. They were fixed with buffered formalin and processed for paraffin sectioning. The tumor inhibition ratio were calculated as follows:  $(W_{\text{control}} - W_{\text{treated}}) / W_{\text{control}} \times 100\%$ .  $W_{\text{control}}$  and  $W_{\text{treated}}$  referred to average tumor weights of control and treated mice, respectively.

### CD34-PAS dual staining

CD34-PAS dual staining was used to detect the presence of VM channels (CD34-negative and PAS-positive vessels). Firstly, CD34 (1:80, provided by Boster, Wuhan, China) immunohistochemical staining was performed. Then, 5  $\mu\text{m}$  thickness sections were treated with 0.5% periodate acid solution for 10 min, followed by distilled water for 2 min, then incubated with Schiff solution for 15 min in a dark chamber. After another distilled water rinsing, sections were counterstained with hematoxylin for 1 min and covered slipped. The dual staining sections were observed and counted at  $\times 400$  magnification. The mean value of VM channels in ten fields per section was the final outcome.

### Immunohistochemical studies

In order to investigate the role of VEGF, EphA2, MMP-2 protein in VM information, we performed immunohistochemistry analysis for the expression levels of these proteins from sections of xenograft specimens, as previously reported [17, 23–24]. In brief, paraffin-embedded and formalin-fixed tissues were cut into 5  $\mu\text{m}$  sections. Subsequently, the sections were deparaffinized, rehydrated through graded alcohol into water for 10 min each and rinsed with PBS. After rehydrating, the endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 50% methanol for 20 min at room temperature. Then, the sections were treated with citrate buffer (0.01 M citric acid, pH 6.0) for 20 min at 100 °C in a microwave oven. After rinsing with PBS, nonspecific binding sites were blocked by normal goat serum for 20 min at 37 °C. Then, the sections were incubated overnight at 4 °C exposure to primary antibody for the anti-VEGF, anti-EphA2 and anti-MMP-2 antibodies (1:100, provided by Boster, Wuhan, China). After washing in PBS for three times, the peroxidase-conjugated secondary antibodies were added in sequence for 30 min at 37 °C. Visualization was per-

Table 1 Effect of GSPs on tumor growth in hepatoma H22 bearing mice

Groups	Dosage (mg/kg)	Average tumor weight (g) (means $\pm$ S.D. <sup>a</sup> )	Tumor inhibition rate (%)
Control	–	2.257 $\pm$ 0.773	–
Endostar	4	2.102 $\pm$ 0.580	6.87
GSPs 50 mg/kg	50	1.855 $\pm$ 0.525	17.81
GSPs 100 mg/kg	100	1.638 $\pm$ 0.589 <sup>Δ</sup>	27.43
GSPs 200 mg/kg	200	1.049 $\pm$ 0.258 <sup>Δ</sup>	53.52

Data was expressed as mean  $\pm$  S.D. ( $n = 15$ ). <sup>a</sup> S.D.: Standard deviation;  $\Delta P < 0.01$  comparison with the control group. GSPs: The grape seed proanthocyanidins

formed using a DAB Kit under microscope, then counter-stained with hematoxylin.

### Quantification of determination of the positive ratio for VEGF, EphA2, MMP-2

The expression levels of VEGF, EphA2, MMP-2 proteins were calculated in this study. Positive staining was indicated by brownish-yellow granules in the cytoplasm for VEGF, EphA2, MMP-2. The staining intensity was classified as 0 for negative, 1 for weak, 2 for intermediate, 3 for strong. Meanwhile, the number of positive cells was stratified as follows: 0 for  $< 10\%$  positive cells, 1 for  $< 25\%$  positive cells, 2 for  $< 50\%$  positive cells, 3 for  $> 50\%$  positive cells. The sum of both staining intensity and percentage of positive cells was used to determine the expression of proteins. Positive cells were counted in ten different fields per section under  $\times 400$  magnification. Tissues scored more than 2 were in positive group. In contrast, a score  $< 2$  was in negative group. The expression levels of VEGF, EphA2, MMP-2 proteins were calculated by three independent observers without knowledge of the outcome.

### Statistical analysis

All data were expressed as the mean  $\pm$  standard deviation (SD). Statistical software SPSS 22.0 was used in the analysis. Differences of the mean weight of tumors and counts of VM channels were compared using the one-way ANOVA. Meanwhile, the chi-square test was performed to determine the protein expression differences between the treated and the control groups. A value of  $P < 0.05$  was considered as statistically significant.

## Results

### Anti-tumor effects of GSPs in H22 bearing mice

Seven days after H22 cells inoculation, the engrafted masses were detected clearly in subcutaneous area of left axilback and more or less. During the 10 day duration experiment, the tumor volumes of the mice in the treatment groups had increased relatively slowly compared with the control group. As shown in Table 1, compared with the control group, the mean weight of tumors in GSPs (100 mg/kg) ( $P = 0.000$ ) and GSPs (200 mg/kg) ( $P = 0.004$ ) groups were significantly reduced. Although the mean weight of tumors in GSPs (50 mg/kg) and Endo-

star (4 mg/kg) groups were lower than the control group, no significant difference were found between them. Additionally, the tumor inhibition rates for the groups receiving Endostar, GSPs at a dosage of 50, 100, 200 mg/kg were 6.87%, 17.81%, 27.43%, 53.52%, respectively. Besides, mice in the GSPs groups showed relative normal behaviors, which indicated that GSPs did not show such adverse effects with the test concentrations used. Accordingly, these results showed that GSPs effectively suppress the H22 tumor growth in a dose-dependent manner.

### Inhibitory effects of GSPs on VM channels

The CD34-PAS dual staining was simultaneously used to detect the structure of vasculogenic mimicry (VM). The VM channel was lined by PAS-positive base membrane, but not composed of CD34-positive endothelial cell. We found that 9 out of 15 samples (60%) exhibited VM structures in the control group (group A). Meanwhile, the positive rates of VM channels in group B, C, D, E were as follows: 8/15 (53.3%), 5/15 (33.3%), 5/15 (33.3%), 4/15 (26.7%). As shown in Fig. 1, cells around the VM channels were negative for CD34 and positive for PAS, which confirmed that VM channels were not lined by endotheliums but by liver cancer cells. The number of VM channels was significantly lower in the GSPs (100 mg/kg) ( $P = 0.000$ ) and GSPs (200 mg/kg) ( $P = 0.000$ ) groups than that of the control group. However, in the GSPs (50 mg/kg) and Endostar (4 mg/kg) groups, though the number of VM were reduced than those in the control group, no significant differences were found between them. The comparison of mean numbers of the VM channels among different groups was shown in Fig. 1.

### Inhibitory effects of GSPs on the protein expressions of VEGF, EphA2 and MMP-2

In order to further explore the molecular mechanism by which GSPs inhibit VM, we examined VM signaling-related proteins VEGF, EphA2, MMP-2 proteins through immunohistochemical staining. Fig. 2 shows that the positive expression site of VEGF, EphA2, MMP-2 proteins were located in the cytoplasm. Expression of VEGF, EphA2 and MMP-2 protein of H22 xenografts in GSPs (100 mg/kg) or GSPs (200 mg/kg) group was lower significantly than that of control group (Fig. 2; all  $P < 0.001$ ); and no difference on expression of these VM signaling-related proteins was observed between the rest groups.

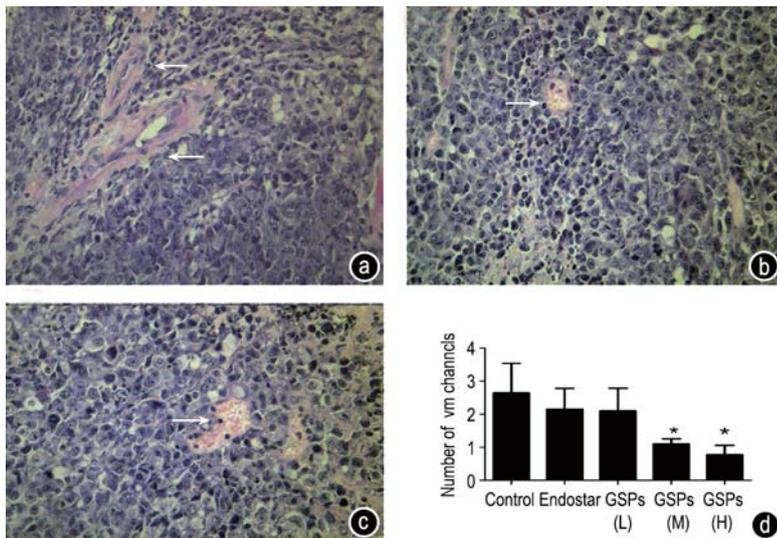


Fig. 1 Effects of GSPs on the VM channel in a H22 mouse hepatocellular carcinoma model. VM channel was positive for PAS staining and negative for CD34 staining in sections (x 400). The arrows indicated VM, which stained as light pink by PAS staining. (a) vehicle control; (b) 100 mg/kg GSPs; (c) 200 mg/kg GSPs; (d) comparison of VM channels in group A, B, C, D, E. GSPs (L): 50 mg/kg GSPs; GSPs (M): 100 mg/kg GSPs; GSPs (H): 200 mg/kg GSPs. \*  $P < 0.01$  vs. control group

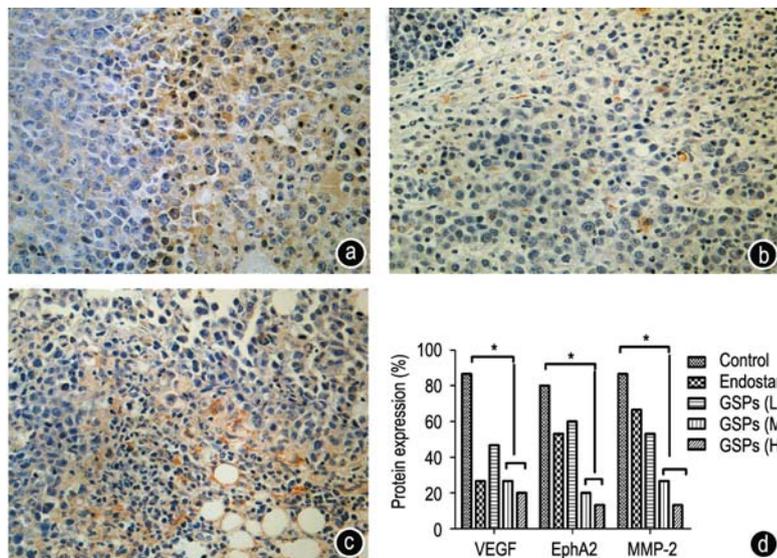


Fig. 2 Inhibitory effects of GSPs on the proteins expression of VEGF, EphA2 and MMP-2 in H22 bearing mice (immunohistochemical staining x 400). Immunohistochemical staining results of VEGF, EphA2 and MMP-2 protein in GSPs(H) group (A–C). (a) VEGF expression; (b) EphA2 expression; (c) MMP-2 expression. The positive positions of the tumor cells were located in cytoplasm, which stained as dark brown. As shown in photograph, (d) expression of VEGF, EphA2 and MMP-2 protein of H22 xenografts in GSPs (M) or GSPs (H) group was decreased significantly when compared with control group (\* all  $P < 0.001$ )

Concomitant with the diminish the presence of tubular network channels was an inhibition of VEGF, EphA2, MMP-2 proteins expression. The results demonstrated that GSPs inhibits the VM channels of H22 bearing mice by suppression of the VEGF/EphA2/MMPs signaling pathways.

### Discussion

Patients with HCC generally have a high risk of invasion, migration, recurrence. These clinical features of HCC have been, in largely part, related to abundant blood vessels. Angiogenesis inhibitors may have theoretically produced satisfied therapeutic effects. However, the benefits are at best transitory, followed by malignant growth and progression of tumors. Previously studies

have described that liver cancer microcirculation consists of endothelial-lined vessels and VM channels. As a new tumor microcirculation pattern, Patterned matrix VM was distinct from endothelium-dependent vessels in which tumor cells mimic endothelial cells to form vasculogenic networks<sup>[13]</sup>. Therefore, we deduced that the existence of VM is likely responsible for low effectiveness of antiangiogenesis drugs. Traditional antiangiogenic drugs have limited effect on VM, for example endostatin and bevacizumab. Many researchers have been engaged in seeking new avenues for anti-VM formation. In the present study, the most significant findings are that dietary administration of GSPs resulted in inhibition of VM formation in H22 tumor model.

GSPs, a dietary botanical agent, has been shown to have anti-carcinogenic effects and devoid of major side effects in some tumor models. In the present study, we

further examined the anti-VM effect of GSPs as a VM inhibitor. Moreover, our previously work found that dietary administration of GSPs could inhibit tumor growth and reduce angiogenic vessels density in H22 xenograph model [25]. Our data demonstrated that the average quantity of VM in GSPs treatment group was significantly decreased in dose-dependent manner compared with that in the control and Endostar group.

Although VM has been shown to associate with poor prognosis in HCC, the molecular events involved in VM have not been defined. Therefore, understanding of mechanisms underlying VM would provide potential targets for new therapies of liver cancer. Previous studies have revealed that several molecules or signaling pathways related to VM by aggressive malignant tumor, including VEGF, EphA2, MMPs, Ln-5γ2, etc [16]. In order to demonstrate whether the declined information of VM in GSPs-treated groups correlates with VEGF, EphA2 and MMPs proteins, we assessed the expression of VEGF, EphA2, MMPs by immunohistochemistry. In the previous study, VEGF is the upstream molecular factor of EphA2, then phosphorylation EphA2 regulate activity of MMPs, specifically MMP-2, which promoted cleavage of laminin into fragments resulting in increasing migration, invasion, and VM. We observed low expression of VEGF, EphA2, MMP-2 in GSPs-treated groups compared to vehicle-treated group in a dose-dependent manner. What's more, the downregulation of VEGF, EphA2 and MMP-2 paralleled the lack of VM after treatment with various concentration GSPs. Therefore, we concluded that GSPs suppressed VM by downregulating VEGF/EphA2/MMPs signaling pathways, thereby inhibiting tumor growth. Taken together, our findings provide a novel rationale to HCC VM information. More importantly, we showed for the first time that GSPs could serve as a potential anti-VM agent in the treatment of HCC with VM.

### Conflicts of interest

The authors indicated no potential conflicts of interest.

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