Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Hepatic arterial chemoembolization transcatheter (TACE) is one of the main treatment methods for liver cancer. However, the long-term therapeutic effect of HCC after TACE is still unsatisfactory, postoperative recurrence and metastasis rate is still very high. Furthermore, TACE operation due to liver cancer tissue ischemia and hypoxia will lead to up-regulation of vascular endothelial growth factor (VEGF) expression. In the current study, we investigated the effects of suppressed VEGF on HCC and its molecular mechanism provided a basis for targeting angiogenesis.

MATERIALS AND METHODS: We established rabbits primary HCC model by in situ embedding the VX2 subcutaneous transplantation tumor. Conventional Seldinger femoral artery and hepatic artery catheterization method were used to select the catheter over the tumor-bearing hepatic artery. The different groups were divided into TACE operation, and the experimental group was performed with the VEGF-siRNA molecular preparation in the catheter. 64-slice spiral CT were used to perfusion imaging of liver cancer model before and after TACE operation. We further assessed the efficiency of VEGF silencing and its influence on VX2 cells. The expression of VEGF mRNA and protein were detected by RT-PCR and Western blotting, respectively. Intratumoral microvessel density (MVD), VEGF and CD34 were evaluated by immunohistochemistry. We detected the cell apoptotic by immunofluorescence and flow cytometry.

RESULTS: Our findings indicated that VEGF-siRNA-2# could effectively suppress the expression of VEGF expression, inhibited the proliferation capability and promoted apoptosis of VX2 cells in vitro. Silencing of VEGF expression also suppress HCC tumor growth and reduce HCC angiogenesis in rabbits primary HCC model in vivo. Furthermore, We found that phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) activation were considerably reduced while inhibition VEGF expression in VX2 cells.

CONCLUSIONS: Our data demonstrated that VEGF silencing could suppress cells proliferation, promote cells apoptosis and reduce HCC angiogenesis through inactivation of VEGF/PI3K/AKT signaling pathway.

Key words: Hepatocellular carcinoma, Vascular endothelial growth factor, Hepatic artery perfusion, siRNA, Angiogenesis.

Introduction

Hepatocellular carcinoma (HCC) is a serious threat to life and health of people around the world. About 90 million people worldwide die from the disease every year, making HCC the third most common lethal malignancy tumor1,2. Currently, surgical resection is the most preferred treatment method. However, more than 80% of HCC cases are accompanied by cirrhosis3, which results in liver resection to a certain extent4. Also, tumours are adjacent to the first, second, or third hepatic hilum which causes great difficulties for the surgery. Therefore, surgery in HCC patients is efficient in less than 20%5.

Hepatic arterial infusion strategy is another main method to treat HCC. While its long-term efficacy is acceptable, the rates of recurrence and metastasization are still very high6. It was found that up-regulation of vascular endothelial growth factor (VEGF) due to ischemia and hypoxia within liver cancer tissue after hepatic artery surgery is the main reason for this low treatment effect7,8,9.
VEGF is the factor stimulating endothelial cell proliferation. It is also known as the strongest vascular penetrant, being 50,000 times more potent than histamine. VEGF increases permeability of capillaries, especially that of veins and small veins, and promotes blood vessel leakage. Therefore, expression of VEGF indirectly reflects proliferation, migration and angiogenesis of tumor vascular endothelial cells, and may indicate growth rate and metastasization tendency of the tumor. To reduce the recurrence and metastasis rate of hepatic arterial infusion, and to improve the survival rate, it is necessary to block the VEGF signal transduction pathway and down-regulate VEGF. This strategy can be combined with hepatic arterial infusion. One way to achieve this is by utilizing RNA interference (RNAi).

It has been shown that siRNA can block or down-regulate expression of a target gene. Therefore, siRNA are likely to become the next generation of therapeutic drugs with broad prospects. It has been demonstrated that tumor angiogenesis can be inhibited in ovarian cancer, malignant melanoma, colon cancer, and stomach cancer by modulating the expression of VEGF by RNAi. So far, the majority of these studies are done in cell culture. There are fewer reports on using RNAi in vivo models, and such studies are still limited to surface organs or tumours are targeted by local injections. Moreover, it has been reported that siRNA can target the tumours in experimental animal if administered via intravenous injection, but this approach is marred by drug toxicity and low drug concentration in the target tissue.

To overcome these shortcomings, we tested an interventional therapy by super-selecting artery with a catheter and directly administering siRNA targeting VEGF. We further combined the anti-angiogenesis and anti-VEGF therapy with hepatic arterial infusion to decrease VEGF expression after the surgery and interrupt the tumour angiogenesis. This approach has a potential of reducing the rate of cancer recurrence and metastasis and eventually improving the survival of patients with HCC.

Materials and Methods

Cell culture and supplies

The VX2 cells were purchased from the ATCC. Dulbecco's Modified Eagle Medium (DMEM) culture medium, trypsin-EDTA, and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and DMSO was obtained from Hyclone (Logan, UT, USA). The anti-CD34 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), and anti-human VEGF antibody was from Abcam (Cambridge, MA, USA).

Specific siRNA targeting VEGF

We designed a 19nt target sequence in accordance to siRNA design principles using the VEGF sequence in Genebank. siRNA sequences was as follows: VEGF-siRNA#1, sense: UCGAGACCUCGGUGGACAUdTdT, antisense: AUGUCCACCAAAGGUCUCGAdTdT; VEGF-siRNA#2, sense: GGAUGUACCUGUGAGAUCdTdT, antisense: GAUCUCAUGGUACUCCdTdT; VEGF-siRNA#3, sense: UGAUGAAAGCCUGGAGUGCdTdT, antisense: GCACUCCAGGU-UUCAUCdTdT. The negative control siRNA had the following sequence: sense: UUCUCCGAACGUGUCACGUdTdT, antisense: ACGUGACACGUUCCGAGAdTdT. To ensure stability of these siRNAs in blood, the end of each siRNA sequence was modified by methylation.

Establishment of NOD-SCID mice model expressing VX2

The VX2 cells were cultured in DMEM containing 10% fetal bovine serum. When the cells grew to confluence, they were Lifted with 0.25% trypsin supplemented 0.02% EDTA. Detached cells were collected into a centrifuge tube and centrifuged for 5 min at 1000 rpm. After removal of the supernatant, a single cell suspension of VX2 cells was prepared at the cell density of 1.5×10⁷/mL. Then, 2 × 10⁶ VX2 cells were resuspended in 50 μL PBS and subcutaneously injected into the left part of the abdomen of non obese diabetic-severe combined immunodeficiency (NOD-SCID) mice.

Establishment of VX2 transplantation tumour in a rabbit

The VX2 cells were inoculated into an inguinal muscle gap of experimental rabbits. Then, the rabbits were kept in the cages and fed normally for 2-3 weeks. At that point, a substantial tumour of about 3 cm in size formed at the vaccination site on rabbit legs. Using 1% pentobarbital sodium (30 mg/kg), rabbits were anaesthetized, and tumour tissue was collected and washed 3 times with normal saline. The tissue was cut into 1 × 1 × 1 mm blocks and placed in a saline solution.
We also established a rabbit VX2 transplantation liver tumour model. Experimental rabbits were fasted for 12 hours on the operation day but were allowed to consume water. The hair was removed in the xiphoid and upper abdomen area, and disinfected with ultraviolet for 30 min. Then, rabbits were anesthetized by ketamine, placed in supine position on a home-made platform, with limbs fixed, and disinfected in the subxyphoid area with iodophor. A 1.5-cm incision was made on partial left subxiphoid and blunt dissected to expose the liver. The liver was gently pulled out from the body, while moistened with gauze soaked with physiological saline. Then, a thicker sinus about 1 cm long was established in the left lobe of the liver, a tumor block was built in the sinus, and the liver was confirmed to have no bleeding. The wound was sutured and disinfected. After 10 days, conventional CT plain scan and enhanced scan were performed (Figure 4B). A tumour lesion was confirmed in the lobe of the liver.

**Experimental grouping, siRNA administration, tumour growth index determination, and hepatic artery perfusion**

Rabbits were randomly divided into three groups, each group consisting of 5 rabbits. The Group A was treated with 100 μL PBS via hepatic artery perfusion, injected every other day, 3 times a week, with a total of 9 injections. The Group B was treated with 100 μL negative control siRNA via hepatic artery perfusion, starting from the day 14 after tumour inoculation. Negative control siRNA was injected every other day, 3 times a week for a total of 9 injections. The Group B was treated with VEGF-siRNA-2#; the injection of VEGF-siRNA-2# (100 μM) was done via hepatic artery perfusion starting from the day 14 after tumour inoculation, with a frequency similar to negative control siRNA.

Expressions of VEGF, CD34 and Bax mRNAs in tumour tissue were determined by qPCR. Total RNA was extracted using Trizol (Sigma, Saint Louis, MO, USA). Then, PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan) was used for reverse transcription. qPCR was used to quantify expression of genes of interest using the CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The GAPDH mRNA expression served as endogenous control.

Then, the intratumoral MVD was quantitatively detected in 10 high power fields. Finally, expression of VEGF and CD34 protein were analyzed by immunohistochemistry in tumour tissues from different rabbit models. Tumour volume and tumour weight were detected according to the formula: V = a b²/2, where “a” represents the length and “b” the width of the tumour. The tumour growth curve and the inhibition rate of the tumour were also calculated. Animals from each group were sacrificed on the day 17 after administration, and tumours were weighed.

Hepatic artery perfusion was done as described in prior publications.

**Immunohistochemical staining procedure**

Immunohistochemical staining procedure according to the requirements of the operating instructions. Dewaxed paraffin with water; antigen retrieval, each movie three times, each two minutes. PBS washed 5 minutes for 3 times, 3% hydrogen peroxide at room temperature for 15 minutes, to remove the endogenous peroxidase activity; then PBS wash 5 minutes for 3 times, 5% goat serum blocking solution treatment at room temperature 30 minutes. Remove excess liquid, and added primary antibody, CD31 (1:200), VEGF (1:100), incubated overnight at 4° C in the refrigerator; 37° C incubated for 1 hour. Subsequently, PBS wash 5 minutes for 3 times, adding universal secondary antibody treatment 30 minutes; PBS wash 5 minutes for 3 times, adding horse radish peroxidase (HRP) streptavidin working solution for 30 minutes; PBS wash 5 minutes for 3 times, diaminobenzidine (DAB) reagent color, color side view in the edge under a microscope. washing 5-15 minutes to terminate the color reaction; hematoxylin, dehydrated and transparent, were mounted.

**Quantification of the intratumoral MVD**

Tumour tissue was fixed with 10% formalin and paraffin-embedded. Then, the tumour tissue was cut into 4 μM sections. Sections were de-paraffinized in xylene and rehydrated in ethanol. Then, antigen retrieval was carried out, and slides were incubated either with anti-VEGF or anti-CD31 antibodies (both from Abcam) overnight at 4° C in a humidified chamber. The DAB system was used to visualize the signal on tissue slide counterstained stained with hematoxylin. One investigator has then blindly quantified tumour angiogenesis.

**Cell proliferation assay**

The cell proliferative potential was evaluated by Cell Titer Blue assay according to manufacturer’s procedure. At first, 1 × 10⁶ cells were in-
fected with Ad-VEGF-siRNA, Ad-Control-siRNA and PBS, respectively. After infected with 1-7 days, 10 μL Cell Titer Blue/well was added to each plate and were cultured for another 2h, the absorbance at 450 nm was read by multi-well spectrophotometer (Bio-Rad).

**Western blotting**

Total proteins were separated by SDS-PAGE on an 8-10% gel. After electrophoresis, gels were transferred onto 0.45 μm PVDF membrane (Millipore Corp, Darmstadt, Germany) and blocked with 2% BSA for 2-4h at room temperature. The membranes were incubated with the following primary antibodies, VEGF (Abcam), p-PI3K and p-AKT (Santa Cruz), total-PI3K (Abcam), total-AKT (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs, Bar Harbor, ME, USA). The bound antibody was detected by chemo-fluorescence detection kit (Amersham, Piscataway, NJ, USA) according to the manufacturer’s instructions. GAPDH was used as a loading control and the representative images were shown.

**Cell apoptosis analysis by flow cytometry**

The VX2 cells were treated with VEGF-siRNA-2#, negative control siRNA, or PBS. For this, 5 × 10^5 VX-2 cells were harvested after the treatment for 24 hours and double-stained with fluorescein APC-labeled Annexin-V and PI (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of apoptotic cells were detected by flow cytometry (Becton Dickinson) after staining. Analysis of the apoptotic rates in different groups and the representative images were shown.

**Statistical Analysis**

Results were presented as mean ± SD. Statistical significance was determined using SAS9.0 or SPSS15.0 statistical programme, and ANOVA test. The p < 0.05 was considered as indicating a statistically significant difference.

**Results**

**Effectively inhibit VEGF expression by siRNA in VX2 cells or VX2 cells culture supernatant**

In order to analyze the role of VEGF in rabbit HCC tumorigenesis, at first, we investigated the biological effects of VEGF on VX2 cells. Firstly, we designed three 19nt target sequences corresponding to siRNA design principles and the sequence of rabbit VEGF in Genbank. Then, the siRNAs were transfected into VX2 cells, and the inhibition efficiency of siRNAs was detected by RT-PCR and Western blotting assays.

The data showed that the transfection efficiency of siRNAs was efficient (Figure 1A). Our results also indicated that expression of VEGF mRNA (Figure 1B) and protein (Figure 1C) were markedly decreased in siRNA-1# group, siRNA-2# group, siRNA-3# group, compared with untreated group and/or NC-siRNA group. In the three siRNA groups, especially siRNA-2# group exhibited the most significant inhibition effect. In addition, the secretion of VEGF protein in VX2 cell culture supernatant was detected by ELISA.
assay. The data attested that secretion of VEGF in VX2 cells culture supernatant was significantly inhibited by siRNA-2#, compared with siRNA-1# group, siRNA-3# group, untreated group and/or NC-siRNA group (Figure 2).

VEGF silencing efficiently inhibited the growth of VX2 cells in vitro and in NOD-SCID mice model

To explore that anti-tumor effects of VEGF siRNA on VX2 cells in vitro, we treated VX2 cells with VEGF-siRNA-2#, NC-siRNA and/or untreated, respectively. CCK-8 assay was applied to detected the proliferation ability of VX2 cells. The results were indicated that the proliferative potential of VX2 cells was markedly inhibited in VEGF-siRNA-2# group compared with untreated group and/or NC-siRNA group (Figure 3A). To further investigated the effects of VEGF silencing on VX2 cells in vivo, VX2 cells were applied to generate a xenograft HCC model in NOD-SCID mice. The NOD-SCID mice were randomly divided into three groups, and each group contains 5 NOD-SCID mice. Group A was treated with VEGF-siRNA-2# via hepatic artery perfusion, Group B and C were treated with PBS and NC-siRNA from hepatic artery perfusion, respectively. Figure.3B indicated that inhibition of VEGF expression could significantly reduced the growth of tumor in NOD-SCID mice. In addition, the tumor volume and tumor weight were obviously lower in VEGF-siRNA-2# group compared with PBS group and/or NC-siRNA group at day 26 (Figure 3C, 3D).

Inhibition of VEGF expression could promote the apoptosis of VX2 cells

To further elucidate that whether VEGF plays an important role in VX2 cells via induced cell apoptosis. Bax is a pivotal pro-apoptotic gene; therefore, we detected the expression of Bax by RT-PCR. The results demonstrated that expression of Bax mRNA were significantly up-regulated in VEGF-siRNA-2# group compared with PBS group and NC siRNA group (Figure 5A, p<0.001). Allophycocyanin-Annexin V/propidium iodide (APC-Annexin V/PI) double-labeled was also used to detected the cell apoptotic proportion in VX2 cells. The results displayed that the apoptotic rates of VX2 cells were increased after hepatic artery perfusion with VEGF-siRNA-2#, compared with PBS group and/or NC siRNA group (Figure 5B). The representative images of cell apoptotic were shown in Figure 5C. These data implied that VEGF silencing could suppress the cell survival of VX2 cells by inducing cell apoptosis.

Inhibition of VEGF expression effectively restrain HCC orthotopic xenografted angiogenesis in rabbits model

Our study was shown that silencing of VEGF expression could effectually suppressed the growth of VX2 in rabbits model. Subsequently, we further inquired into the factors which caused inhibited the growth of VX2 cells. Angiogenesis is a pivotal factor in promoting the growth of VX2 cells. Microvessel density (MVD) is a key indicator for assessing tumor angiogenesis; therefore, we evaluated the effect of VEGF silencing on VX2 cells angiogenesis in rabbits model. VEGF and CD34 were used to label the vascular endothelial cells. The results indicated that expression of VEGF (Figure 6A, p<0.001) and CD34 (Figure 6B, p<0.001) were also decreased in VEGF-siRNA-2# group compared with PBS group and/or NC siRNA group. The data also displayed that VEGF-siRNA-2# could decrease the counts of intratumoral MVD compared with PBS group and/or NC siRNA group (Figure 6C, p<0.001; Table I). The representative images were shown in Figure 6D. All together, these results demonstrated that angiogenesis was inhibited distinctly in suppression of VEGF expression.
Inhibition of VEGF expression could suppress activation of VEGF/PI3K/AKT signaling pathway in VX2 cells

VEGF was one of the pivotal regulators in tumor angiogenesis, playing an important role in tumor cells survival. The PI3K/AKT signaling pathway was the cardinal downstream of VEGF, participating in differently cellular biological processes. In the current study, we firstly detected the expression of VEGF in VX2 cells after treated with VEGF-siRNA-2#, NC-siRNA or PBS. Expression of VEGF protein was remarkably suppressed in VEGF-siRNA-2# group. In VX2 cells, we further detected the expression of total-PI3K, total-AKT, p-PI3K, and p-AKT by western blotting. Treatment of VX2 cells with VEGF-siRNA-2# was no significant effect the expression of total-PI3K or total-AKT. However, phosphorylation of PI3K and AKT were markedly decreased in VX2 cells compared with PBS group and/or NC-siRNA group (Figure 7). Therefore, our results indicated that effects of inhibition of VEGF expression on VX2 cells proliferation and angiogenesis was closely related with inactivation of VEGF/PI3K/AKT signaling pathway.

Discussion

Regional infusion chemotherapy can increase the concentration of chemotherapy drugs and/or decrease the drug distribution in peripheral blood as well as other organs, which is one of the most important strategy to synergistic attenuate toxicity of solid tumor\cite{22}. Owing to hepatic
arterial infusion has pharmacokinetic advantages, it often used as a common method to fight hepatocellular carcinoma (HCC)\textsuperscript{23}. HCC is a typically malignant tumor that has rich blood supply, and its occurrence, development and metastasis is closely correlated to tumor angiogenesis\textsuperscript{24}. Tumor angiogenesis is a prerequisite for the growth and metastasis of HCC. If there is no new vascular support, tumor will not grow to more than 1-2 mm. While the prognosis of HCC is closely related to angiogenesis regulatory factor and MVD\textsuperscript{25}.

VEGF is known to the strongest pro-angiogenic factor, and generally considered that tumor cells can self-secrete VEGF to improve the formation of its own vascular system\textsuperscript{26,27}. The formation of new blood vessels is required for malignant tumour growth, invasion and metastasis, but also are important indicator of tumor malignancy degree. Chen et al\textsuperscript{28,29} reported VEGF is highly expressed in HCC cells, which is negatively related to migration and prognosis of HCC. Moreover, Oda et al\textsuperscript{30} found that the level of VEGF is directly related to tumor MVD, suggesting that tumor angiogenesis induced by over-expression of VEGF will lead to invasion, metastasis and recurrence of HCC. The MVD and/or microvessel count were used to determine tumor invasion, and to assay the treatment sensitivity and prognosis.

Moreover, VEGF secreted by tumor cells may be a crucially indicating factor in the tumors initiation, migration and metastasis\textsuperscript{31}. In the current study, our founding demonstrated that the microvessel density or microvessel count were fewer in VEGF-siRNA-2\# group than NC-siRNA group and/or PBS group, suggested that MVD and VEGF had a strong positive correlation. Our results also indicated that tumor microvascular usually distributed to the irregular edges of HCC, and this distribution of microvascular is conducive to tumor invasion and metastasis. In addition, our findings also confirmed that there were no significant correlation between MVD and age, onset time or histological subtype in HCC (data not shown).

MVD can reflect the angiogenesis of HCC, and CD34 is the most sensitive tumor marker currently\textsuperscript{32}. In this study, we detected the MVD in tumor tissues via using CD34 to mark vascular endothe-
Our data showed that the MVD was relatively lower in VEGF-siRNA-2# group, compared to control group and NC-siRNA group, and the difference was statistically significant (p < 0.05), while there was no significant difference between control group and NC-siRNA group (p > 0.05). These results indicated that combination hepatic artery infusion with siRNA could effectively reduce the MVD in HCC tissues, while it is not significant to decrease the MVD of HCC by hepatic artery infusion combined with siRNA chemotherapy can effectively reduce the blood vessel density of HCC tissues, and it is not significant to reduce the microvessel density of hepatocellular carcinoma by hepatic artery infusion therapy alone. In summary, VEGF-siRNA can effectively inhibit the expression of VEGF in HCC, reduce tumor MVD, and inhibit tumor angiogenesis.

Figure 5. Silencing of VEGF expression promote apoptosis of VX2 cells. A, Expression of Bax mRNA was detected by RT-PCR in VX2 cells, ***p < 0.001. B, The cell apoptotic rate was calculated by flow-cytometry (FCM) in VX2 cells after treated with VEGF-siRNA-2# and for 24 hours, PBS and/or NC-siRNA group cells were considered as normalization. C, The cell apoptotic was measured by immunofluorescence and the representative images were shown.
Furthermore, we further explored the molecular mechanism of promoted cell apoptosis in HCC cells via inhibiting VEGF, and measured the impact of VEGF silencing on PI3K/AKT signaling pathway. Our findings indicated that p-PI3K and p-AKT was subsequently decreased, accompanied by down-regulation of VEGF in VX2 cells after treated with VEGF-siRNA-2#. However, total-PI3K and total-AKT remained relatively constant, which implies that VEGF may affect the occurrence and development of HCC by regulating PI3K/AKT signaling pathway.

**Conclusions**

Altogether, these results will provide a therapeutic strategy for targeting HCC angiogenesis.

**Table I.** VEGF levels and MVD value in rabbit VX2 model

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<thead>
<tr>
<th>Groups</th>
<th>VEGF</th>
<th>MVD</th>
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<tbody>
<tr>
<td>VEGF-siRNA-2#</td>
<td>26743.21 ± 128421.28</td>
<td>5.24 ± 0.31</td>
</tr>
<tr>
<td>Negative control siRNA</td>
<td>2193742.38 ± 453849.39</td>
<td>13.41 ± 1.26</td>
</tr>
<tr>
<td>PBS</td>
<td>2494674.94 ± 384944.39</td>
<td>14.84 ± 1.47</td>
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Data are mean ± SD
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Conflict of Interest
The authors declare that there are no conflict of interest.

References
Inhibition of human hepatocellular carcinoma tumor angiogenesis by siRNA silencing of VEGF