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Original Article

MMPs and VEGF were increased by irradiation combined with interventional treatment in the rabbit VX2 liver tumor

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Abstract: Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) play important roles in tumor aggressiveness. Transcatheter arterial chemoembolization (TACE) or sublethal irradiation stimulates upregulation of MMPs and VEGF. Changes in the biological behavior of residual viable hepatocellular carcinoma tissue after radiotherapy combined with TACE remain unclear. Our study was to explore the MMP-2, MMP-9 and VEGF expression of additional radiotherapy treated with lipiodol chemoembolization in residual surviving cancerous tissue in the rabbit VX2 liver tumor. 40 VX2 liver-tumor-bearing rabbits were randomly assigned to four groups, Group A (control group n=10) that had saline injected through hepatic artery; Group B (n=10) was treated with TACE; Group C (n=10) treated with radiotherapy; Group D n=10) treated with TACE and radiotherapy. MMP-2, MMP-9 and VEGF level were measured.We showed that VEGF and MMP-2,9 at both protein and mRNA levels were significantly higher in group B, C and D compared to Group A by immunohistochemistry,western blotting and Real-Time PCR ($P<0.05$). The level of VEGF and MMP-2,9 had no significance difference between group B, C and D ($P>0.05$). In summary, the overexpression of VEGF and MMP-2,9 levels were associated with TACE or/and radiation therapy.

Keywords: Ebolization, irradiation, matrix metalloproteinase, vascular endothelial growth factor, VX2 rabbit liver tumor

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide. Asians/Pacific Islanders had higher incidence and mortality rates than other racial/ethnic groups, Recent 1-year survival rates remained, however, less than 50% [1]. Although surgical resection is the treatment of choice, it is not always possible because of coexistent cirrhosis, multiple lesions, and other conditions not suitable for surgery [2]. Transarterial chemoembolization (TACE) using iodized oil (Lipiodol (®)) (Lp-TACE) as a carrier of chemotherapeutic agents has been routinely performed to control hepatocellular carcinomas [3]. The potent local antitumor effect of radiotherapy (RT) should be considered seriously as a part of the treatment strategy. Novel RT technologies have made it possible to deliver higher doses of radiation to the tumor while avoiding damage to critical normal tissues adjacent to the tumor [4]. Radiotherapy might play a significant role for the treatment of unresectable HCC, alone or combined with other locoregional treatment such as transarterial chemoembolisation (TACE) [5].

Extracellular proteolysis is an absolute requirement for new blood vessel formation; angiogenesis depend on tightly controlled interactions between cells and the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) is the most important relevant extracellular proteolytic enzymes. MMP-2 and MMP-9 are two critical members that play important roles in HCC invasion and metastasis by dgrading the basement membrane [6, 7]. In vivo evidence sug-

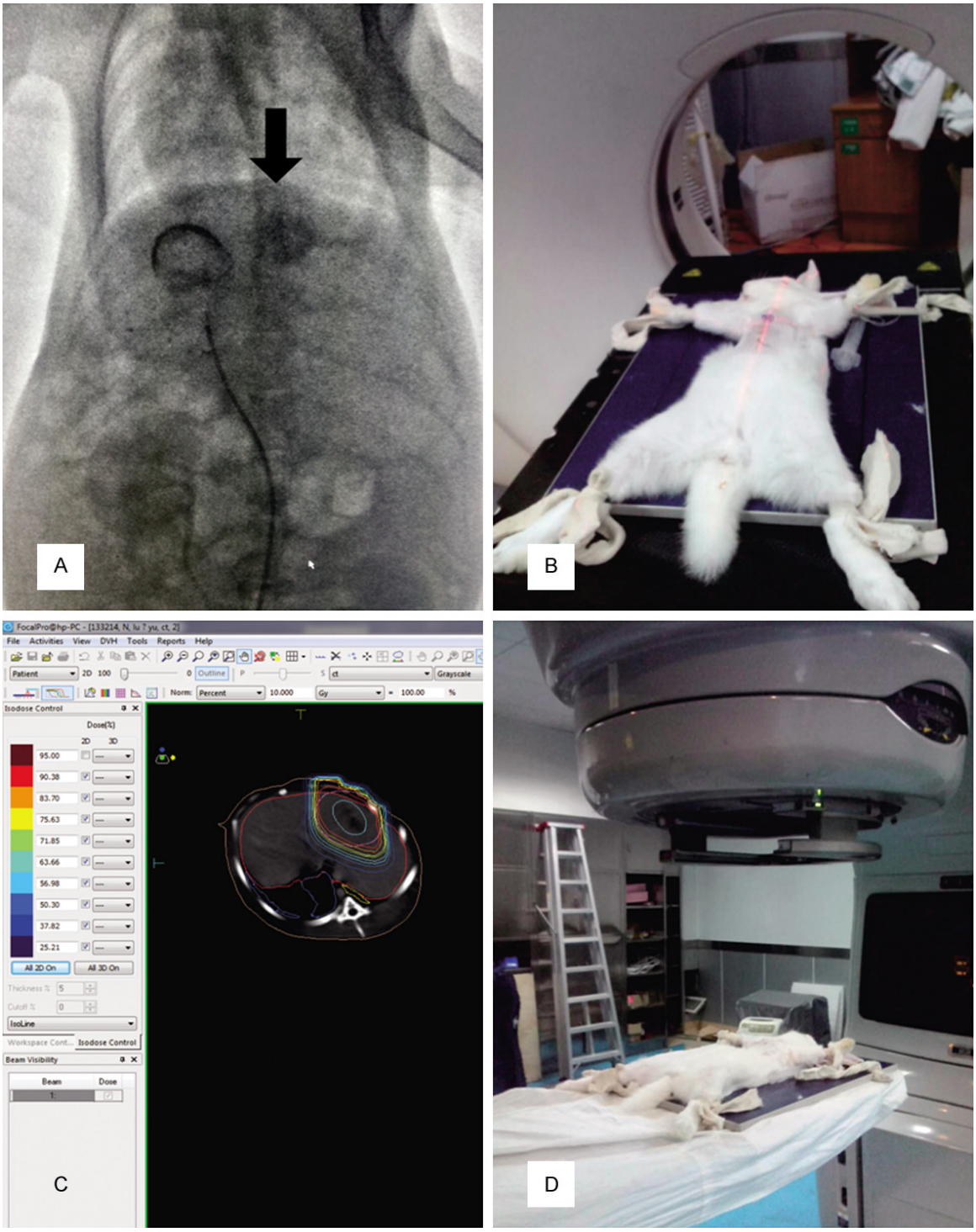

Figure 1. A. 3F microcatheter and injection of lipiodol in the left hepatic artery revealed ill-defined hypervascularity tumor staining in the left lobe. B. CT scans for radiotherapy planning. C. Treatment plan for liver irradiation: The inner circle corresponds to the 90% isodose lines. D. A rabbit in radiotherapeutic set-up was irradiated with a linear accelerator.

gests MMP-9 plays an important role in ascites formation in this model by increasing the bioavailability of VEGF [8]. VEGF high expression is associated with recurrence rates and poor survival in patients with HCC [9]. Expression of VEGF was found in the hepatocytes and HCC

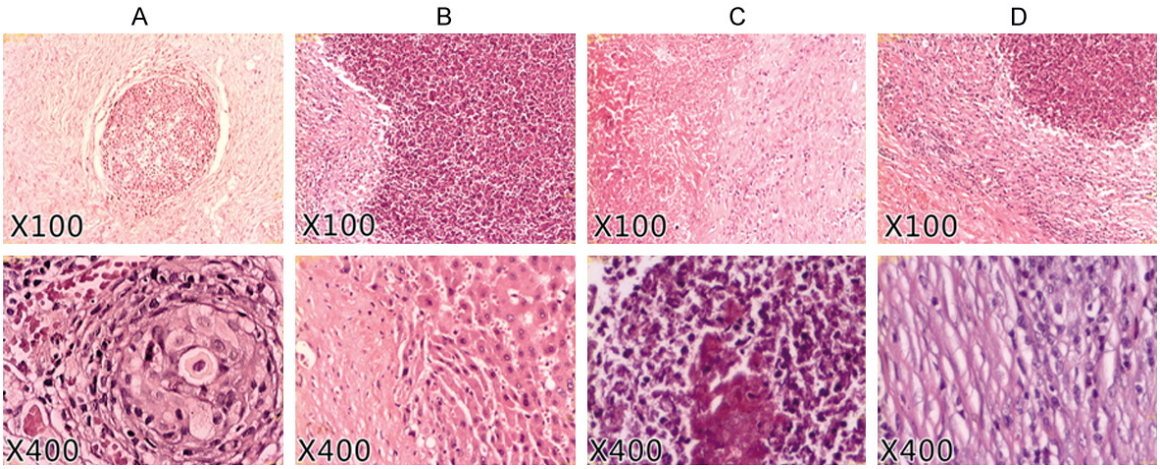

Figure 2. Histologic sections of VX2 liver tumors after treatment in all group (hematoxylin and eosin staining, original magnification ×100 and ×400).

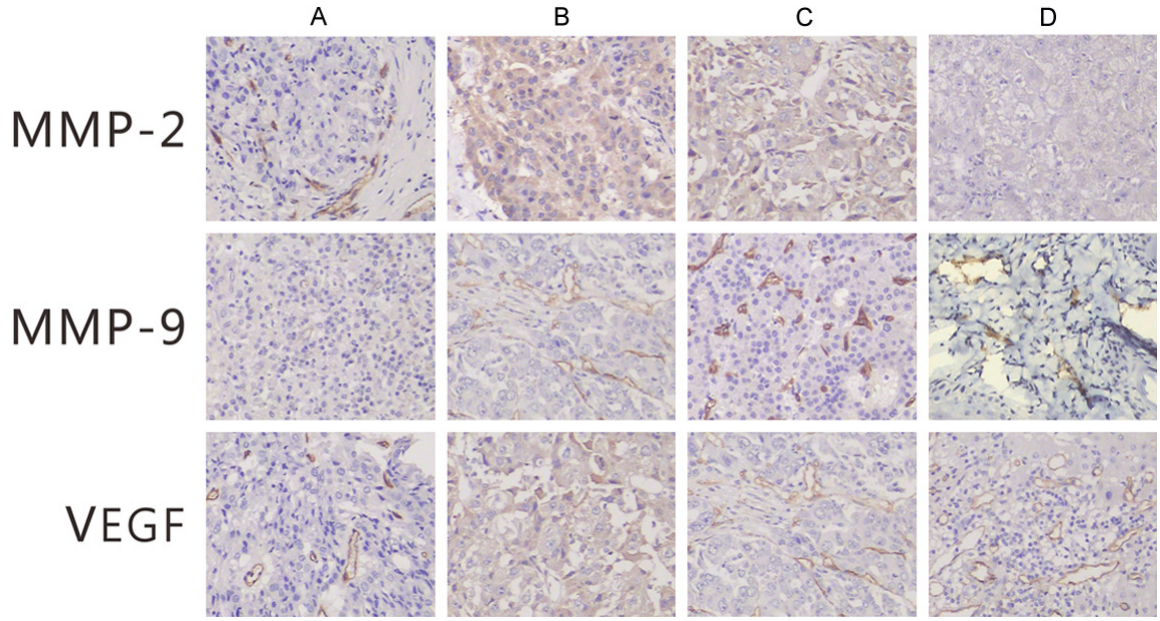

Figure 3. The protein expression level of MMP-2, MMP-9 and VEGF were detected in all groups by immunohistochemistry. MMP-2, MMP-9 and VEGF in B-D group were significantly increased compared to A group.

cells. The degree of VEGF expression was correlated with angiogenesis and cell proliferation activity [10]. The residual surviving cancerous tissue in HCC after TACE has a rich vascularity. TACE increases VEGF expression in the residual surviving cancerous tissue [11]. The increased expression of VEGF and MMP-9 in residual VX2 Rabbit Liver Tumour cells and tumor angiogenesis post-embolization would be responsible for the increased metastatic potentiality and invasiveness of these cells [12]. Recent evidences have shown that irradiation can promote the invasiveness of hepatocellular carcinoma cells with MMP-9, VEGF, MMP-2 expression [13, 14].

The purpose of the present study was to learn whether the combination of TACE and radiation therapy will be in generating a effect on the expressions of MMPs and VEGF reflcting tumor invasion and metastasis in an experimentally induced rabbit VX2 liver tumor model.

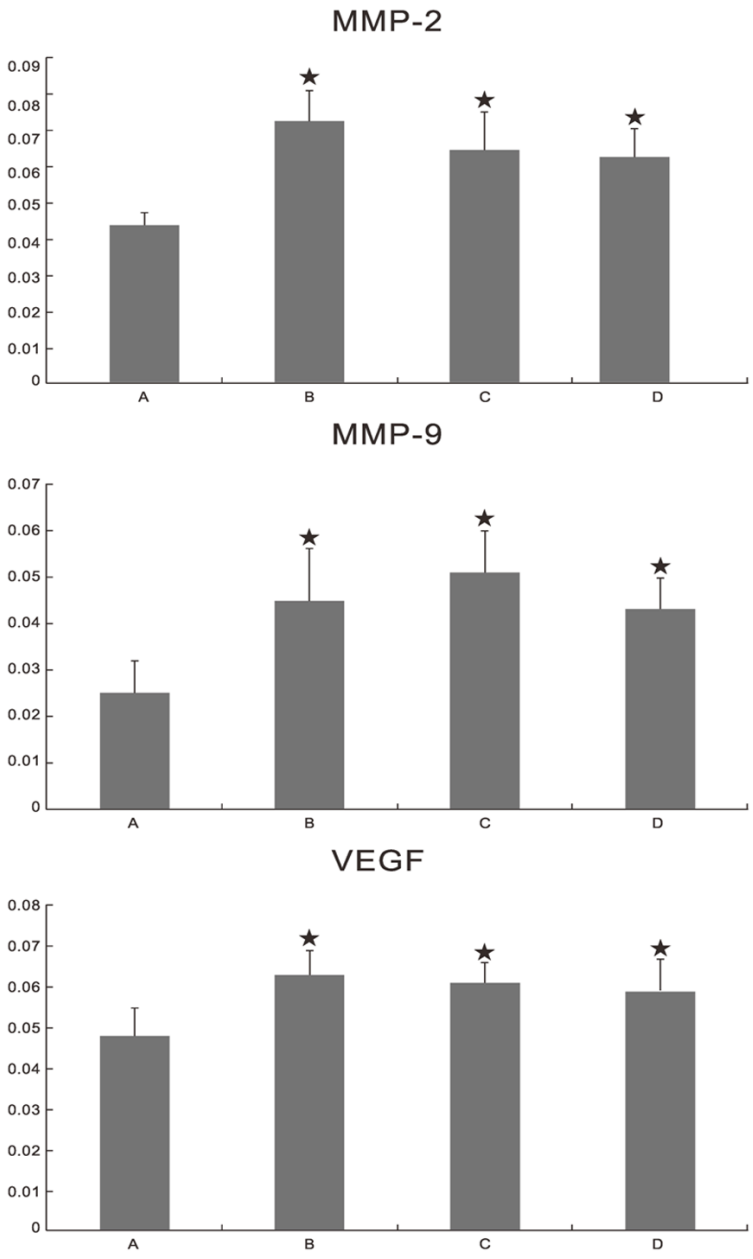

Figure 4. Immunohistochemistry quantitative analysis of MMP-2, MMP-9 and VEGF in different experimental groups was performed. The data are expressed as mean ± SD. Compare to A group, ★P<0.05.

Material and methods

Animals and treatment

The initial weight was median 4.6 kg (range 4.2-5.5 kg). For treatment and follow-up, Rabbits were anesthetized with 25 mg/kg sodium pentobarbital through ear vein and immobilized on a surgical table. The VX2 cells were implanted into subcutaneous tissues of the limb of a carrier rabbit and finally VX2 tumors developed. Then, the donor tumor was excised and divided into 1 mm pieces. a midline abdominal incision was made to expose the liver in recipient rabbits. Tumor tissue was inserted approximately 1.0 cm into the left lobe. A single dose of penicillin was intravenously injected via an auricular vein was used to prevent infection.

Experiments were carried out 3 weeks later which is a period required for tumor cells growth, when the tumors were anticipated to be 15-25 mm in diameter. CT scans of the liver were performed on all animals before treatment. A total of 40 tumor-bearing rabbits were randomly divided into four groups, with ten rabbits in each group (n=10/group). In group A (control) 0.5 ml of 0.9% saline was injected; Group B 1 ml of lipiodol was injected into the hepatic artery; Group C with radiotherapy; While the Group D received radiotherapy afer TACE one week later.

Transcatheter arterial chemoembolization (TACE): General anesthesia was induced and the right femoral artery was exposed. A 3-F microcatheter (Cook, Bloomington, Ind), was inserted into the celiac artery. Celiac angiography was performed to identify the hepatic arterial anatomy and the feeder artery of the tumor, under a DSA angiographic unit (AlluraXper, Philips Healthcare, Nederland). A dose of 1 ml of lipiodol (Guerbert Co., France) or 0.9% saline was injected carefully to avoid effluxion of the embolic materials out of the artery via the hyperselective microcatheter (**Figure 1A**). The catheter was then removed and the femoral artery was ligated. Immediately after TACE to obtain hemostasis. CT (Light Speed Ultra, GE Medical Systems, and

Table 1. The expressions of proteins in rabbit liver tumor by immunohistochemistry and quantitation by using image-pro plus 6.0

Group	N	MMP-2	MMP-9	VEGF
A	10	0.045±0.004	0.025±0.007	0.048±0.007
B	10	0.075±0.009*	0.045±0.011*	0.063±0.006*
C	10	0.067±0.011*	0.051±0.009*	0.061±0.005*
D	10	0.065±0.008*	0.043±0.007*	0.059±0.008*
F value		20.58	15.29	9.61
P		0.000	0.000	0.000

Values are means ± SE. Compare to group A, *P<0.05.

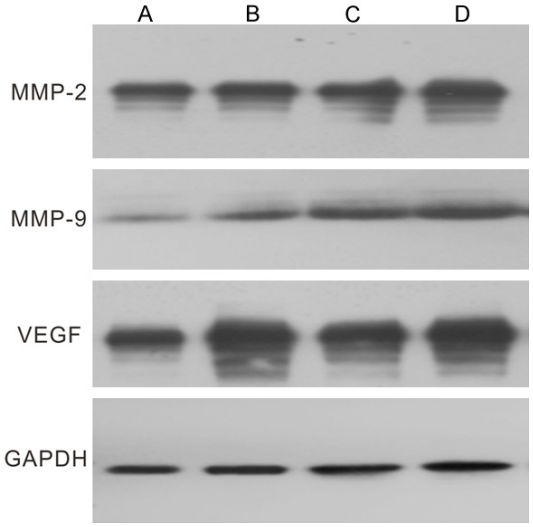

Figure 5. The protein expression level of MMP-2, MMP-9 and VEGF of rabbit liver tumor were detected in all groups by western blotting. The levels of MMP-2, MMP-9 VEGF in B-D group were significantly higher than those in A group. Protein expression levels were normalized to GADPH.

New Berlin, USA) scans were obtained to evaluate the distribution of of iodized oil.

Radiotherapy: The rabbits were fastened on the wooden base plate. CT (Light Speed Ultra, GE Medical Systems, New Berlin, USA) scans for radiotherapy planning were done with rabbits placed in a supine position (**Figure 1B**). The data of the enhanced series were immediately sent to a treatment planning station and the rabbit remained on the wooden base plate.The three-dimensional CT-based planning system (XIO-Release 4.80, Elekta, Ltd., Stockholm, Sweden) was used to cover adequately the tumor target with 0.8 cm margins which covered the entire tumor and decrease the irradiated bowel, the normal liver and kidneys (**Figure 1C**). The liver tumors were irradiated with 6MeV external electron beam (**Figure 1D**) at a dose of 15 Gy one day using a linear accelerator (23 EX, Varian Medical Systmes, Palo Alto, USA, dose rate of 4 Gy/min).

Animals were sacrificed by an intravenous dose of sodium pentobarbital one week after therapy mercifully. Tumor tissue was collected. The experimental protocols were approved by the Nanhai Affiliated Hospital of Southern Medical University Animal Care and Use Committee and were performed in accordance with the Southern Medical University Guidelines for the Care and Use of Laboratory Animals.

Immunohistochemistry

The EnVision two-step method was done according to the manufacturer's instructions. Consecutive sections were deparaffinised with xylene, dehydrated by a series of ethanol solutions (100, 95, 80 and 70%). The sections were washed with distilled water, washed three times with PBS for 5 min each, inactivated endogenous peroxidase using 3% hydrogen peroxide for 10 min, inactivated endogenous peroxidase using 3% hydrogen peroxide for 15 min. The sections were rinsed in PBS three times for 10 min each and were blocked with 5% normal goat serum for 30 min. MMP-2 antibody (ab 2462, ABGENT, Abcam, UK), MMP-9 antibody (ab73734, ABGENT, Abcam, UK) and VEGF antibody VEGF antibody (ab1316, ABGENT, Abcam, UK) were employed for the detection of the respective proteins, with anti-rabbit Envison-PO (DAKO) secondary antibodies. Binding was visualised with 3,3'-diaminobenzidine (DAB) and slides were examined after counterstaining with hematoxylin. It was considered to be positive result when brown precipitate localized in the cytoplasm. Pictures were captured and pixels were counted for quantification, using Image Pro Plus Version 6.0 software (Media Cybernetics Inc., Bethesda, MD).

Total RNA extraction and real-time PCR assays

Quantitative real-time PCR was performed to examine VEGF and MMPs mRNA expression

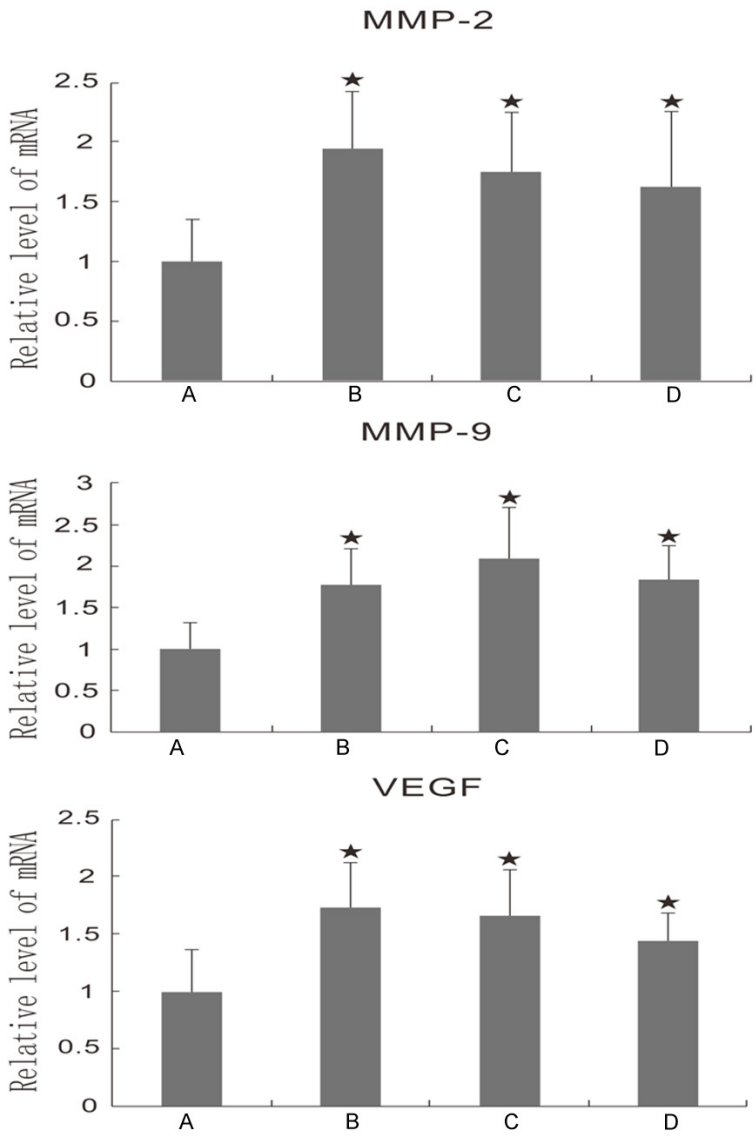

Figure 6. The transcription levels of MMP-2, MMP-9 and VEGF mRNA of rabbit liver tumor were analyzed by Realtime PCR. Real-time PCR examination of MMP-2, MMP-9 and VEGF in A-D groups. The mRNA expression levels are presented as the increasing fold compared with A group and were normalized to GAPDH. Bars represent the SEM. Compare to A group, ★P<0.05.

changes of different groups in rabbit liver tumor. Total RNA was extracted from tissues using TRIzol according to the manufacturer's instructions (Invitrogen). 2 μg RNA was converted to cDNA using a Revert Aid first-strand cDNA synthesis kit (Bestar qPCR RT Kit). PCR using the following primers: VEGF (fwd 5'-CTTGCTGCTCTACCTCCACCAT-3', rev 5'-CTTTGGTCTGCATTCACATTTG-3'), MMP-2 (fwd 5'-ATGGAGGCGCTAGGGGCC-3', rev 5'-CAGCTGGTGTCTTTATTCACA-3'), MMP-9 (fwd 5'-TCACCATGAGCCCCAGACA -3', rev 5'-AAACGTTAAAGAATCAACTTTATTTAGAAAC-3') and β-actin (fwd 5'-CGAGATCGTGCGGGACAT-3', rev 5'-CAGGAAGGAGGGCTGGAAC-3'). Total RNA was transcribed to cDNA with primer and was amplified by SYBR Green real-time PCR reaction system. The relative expression was calculated using the comparative 2-ΔΔCt method. 2-ΔΔCt >3 or <1/3 was deemed statistically significant.

Protein isolation and western blot analysis

Proteins from tissues were resolved by SDS-PAGE and immunoblotting. Briefly, 15 mg of total protein lysates were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting. Antigen-antibody complexes were visualized by a chemiluminescence kit (BCATM Protein Assay Kit). The following primary antibodies were used: VEGF antibody (ab1316, ABGENT, Abcam, UK), MMP-2 antibody (ab2462, ABGENT, Abcam, UK), MMP-9 antibody (ab73734, ABGENT, Abcam, UK).

Statistical analysis

Data are shown as the mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA, and the difference between two groups was analyzed by Bonferroni test. Statistical analysis was performed using SPSS for Windows version 16.0. *P*-values of <0.05 were considered to be statistically significant.

Results

General observation and histological analysis of residual carcinoma

Photomicrographs of the tumors are shown in **Figure 2**. The untreated tumor consisted of via-

ble VX2 cells with bright and large nucle. After transcatheter lipiodol embolization was given through the hepatic artery, we found that lipiodol was mainly penetrated in the periphery of tumors. But only a few lipiodol deposited in the central region where was poorly vascularized. A few viable VX2 cells, inflammatory cell infiltration and necrosis were seen in all the treatment groups. Necrosis area was different from the pretreatment showing as hard, pale and dull shape. After irradiation at a dose of 15 Gy, the number of viable tumor cells was markedly decreased. The VX2 cells were replaced by the fibrous tissue.

The expression of VEGF, MMP-2, MMP-9 in tumor cells

The location of VEGF, MMP-2, MMP-9 in tumor tissue was displayed in all groups by immunohistochemical staining. They were mainly expressed in the cytoplasm of tumor cells. Quantitation of the protein content of VEGF, MMP-2 and MMP-9 were assessed by computerized planimetry in the carcinoma cells in immunohistochemically stained slides by using Image-Pro Plus 6.0. In B, C and D group, the levels of VEGF, MMP-2 and MMP-9 were significantly increased compared to A group ($P<0.05$). However, The levels of VEGF and MMP-2,9 were showed no significant difference among B, C and D group ($P>0.05$; **Figure 3**). The data are shown in **Figure 4** and **Table 1**.

Western blot analysis was also performed to measure the expressions of VEGF, MMP-2, MMP-9 (**Figure 5**). The expression of VEGF, MMP-2 and MMP-9 were also increased in B, C and D group compared to A group.

We compared the the transcription levels of VEGF, MMP-2 and MMP-9 mRNA of residual tumor cells by real time PCR. According to Ct values from the results of quantitative real-time PCR, the difference between A group and treatment groups (D, B and C group) was statistically significant for VEGF, MMP-2 and MMP-9 ($P<0.05$), but there were no significances between B, C and D group ($P>0.05$; **Figure 6**).

Discussion

Metastasis is an exceedingly complex process that includes cell adhesion to the extracellular matrix (ECM), protease secretion, ECM degradation and tumor cell migration [15]. Matrix metalloproteinases (MMPs), a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all ECM components,apparently play an important role in all of these aspects of tumour development [16]. MMPs play important roles in facilitating the metastasis of tumor cells,Therapies designed to interfere with MMP actions may be useful in the control of metastatic disease. Matrix metalloproteinases (MMPs)-2 (gelatinase A) and -9 (gelatinase B) are cancer-associated, secreted, zinc-dependent endopeptidases. Gelatinases cleave many different targets (extracellular matrix, cytokines, growth factors, chemokines and cytokine/growth factor receptors) that in turn regulate key signaling pathways in cell growth, migration, invasion, inflammation and angiogenesis [17]. MMP-2 and MMP-9 over expressions in HCC tissues were correlated with liver cirrhosis, capsular invasion, presence of intrahepatic metastasis, vascular invasion and higher TNM stage, up-regulated MMP-2 and MMP-9 mRNA levels were correlated with later TNM stage and metastasis in HCC [18]. Release of VEGF by MMP-9 correlates with an angiogenic switch, which promotes tumor progression [19]. Proteolysis of the ECM releases bound VEGF, increasing its bioavailability, and MMP-9 and possibly other MMPs are important for mobilizing VEGF from the ECM [20]. Vascular endothelial growth factor (VEGF) led to a marked increase in expression of MMP-2 [21]. Animal experiments and clinical studies showed that the VEGF signaling pathway plays an important role in endothelial cell differentiation, vascular permeability and promotion of new vessel growth. The expression of VEGF and MMP-1 mRNA in the tumor tissue of the Hepatic arterial ligation group which was used to block the hepatic arterial blood supply a simulate TAE increased significantly compared with that of the control groups [22]. In HCC patients undergoing TACE prior to partial hepatectomy, tissue expression level of VEGF and bFGF increase in comparison to patients with surgery only, suggesting that hypoxia due to arterial embolization upregulates these angiogenic factors, TACE of HCC can up-regulate the expression of VEGF and bFGF in HCC tissues possibly due to anoxia and ischemia [23]. When TACE is not totally effective, it may induce a significant neoangiogenetic reaction, suggesting an increase in VEGF

and β-FGF following treatment which affects patient survival [24].

Recurrence and metastasis are frequently observed after radiotherapy for hepatocellular carcinoma (HCC), upregulation of matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) induced by radiation has been claimed to be involved [25]. Radiation may increase invasion ability through activating MMP proteolytic system, simultaneous administration of the MMP inhibitor during radiotherapy suppresses the radiation-enhanced invasion from latent type to active type [26]. Sublethal irradiation of rat 9 L glioma cells results in the formation of a greater number of tumor satellites in the rat brain in vivo concomitant with enhanced MMP-2 and reduced tissue inhibitor of metalloproteinases-2 expression [27].

VX2 rabbit liver tumor is suitable for experimental research of transcatheter arterial chemoembolization (TACE) and radiation therapy [28, 29]. However, few articles have targeted the expressions of the angiogenic factors MMPs and VEGF associated with combination of TACE/TAE and irradiation. In our study, we used rabbit VX2 hepatic neoplasm model to investigate whether there were changes in the expression of VEGF and MMPs in residual hepatic tumor cells after embolization with lipiodol and/or irradiation. We hope to find out the molecular mechanisms responsible for the recurrence and metastasis of HCC after TACE and/or irradiation.

In this study, we showed for the first time that VEGF, MMP-2,9 expression were significantly increased in the group TACE, irradiation and TACE plus irradiation compared with the control group. The expression VEGF, MMP-2,9 slightly were decreased in TACE plus radiation group compared to monotherapy. However there was no significant difference between the rabbits treated with TACE/irradiation alone and TACE plus radiation therapy. This puzzling phenomenon may be attributed to the synergistic antitumor effect of TACE and irradiation.

Radiobiology research found that the occurrences of radiation-induced injury are related to the radiation dose, dose division, radiation field range, and whether or not the use of other therapy during the treatment. The normal liver tissue radiation tolerance dose for TD5/5 is 25 Gy, TD50/5 is 40 Gy. But the radiological reaction occurs after 20-25 Gy/2-3 weeks combined with chemotherapy in the human liver.In the initial stage of this experiment, we have chosen 25 Gy dose for the liver tumor irradiation, but some experimental animals died. It is possible that the radiological radiation and TAE reaction occurs in rabbit normal liver tissues. Therefore we changed to 15 Gy dose for the irradiation in the latter part of the experiment. So no more serious reaction have been observed.

To sum up, the VEGF, MMP-2 and MMP-9 are over-expressed in residual tumor cells after embolization and/or irradiation. This could further enhance the invasive potentiality of residual tumor cells to form new neoplasms and metastases of hepatic tumors after treatment. Our findings indicates that some related metastatic genes change in residual tumor cells after embolization and/or irradiation. The related molecular mechanism leading to disease recurrence and metastasis of residual HCC after embolization and/or irradiation may partly result from the expression changes of metastatic genes in residual tumor cells such as MMPs and VEGF. This may have significant implications for the treatment of HCC, will provide new clues to suppress the post-treatment disseminations and metastasis, thereby improve the prognosis of HCC patients.

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Disclosure of conflict of interest

None.

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