

## Original Article

# Hepatic arterial administration of sorafenib and iodized oil effectively attenuates tumor growth and intrahepatic metastasis in rabbit VX2 hepatocellular carcinoma model

Lin Zhang, Feng-Yong Liu, Jin-Xin Fu, Feng Duan, Qing-Sheng Fan, Mao-Qiang Wang

*Department of Interventional Radiology, Chinese PLA General Hospital, Beijing 100853, China*

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**Abstract:** Aim: To investigate the therapeutic effect of the hepatic arterial administration of sorafenib in rabbit VX-2 hepatocellular carcinoma (HCC) model. Methods: Rabbit VX-2 HCC models were established via implanting VX-2 tumors into the livers, and randomly divided into four groups, respectively treated with (1) The hepatic arterial administration of iodized oil alone (TACE-i), (2) The hepatic arterial administration of iodized oil and pharmorubicin (TACE-ip), (3) The hepatic arterial administration of iodized and cis-DDP (TACE-ic), (4) The hepatic arterial administration of iodized and sorafenib (TACE-is). The growth rate and intrahepatic metastasis of implanted VX-2 tumor in each rabbit were measured. Microvessel density (MVD) in the adjacent tissues of implanted VX-2 tumor were estimated by detecting the expression of CD34 and VEGF level in tumor adjacent tissues were also examined by Immunohistochemistry. Results: Compared with other groups, TACE-is treatment group presented a better effect on inhibiting tumor growth rate and intrahepatic metastasis in rabbit VX-2 HCC model. The angiogenesis (assessed by MVD) in the adjacent tissues were suppressed more dramatically in TACE-is treated group. Moreover, TACE-is treatment did not significantly increase the levels of alanine transaminase and creatinine compared to the group with TACE-i treatment. Conclusion: The hepatic arterial administration of sorafenib and iodized oil (TACE-is) effectively attenuates tumor growth and intrahepatic metastasis in rabbit VX-2 HCC model without obvious hepatic and renal toxicity. One of the related mechanisms may be due to the inhibition of angiogenesis in the adjacent tissues. Our data indicated that TACE-is may be a secure and effective treatment for HCC.

**Keywords:** Hepatocellular carcinoma, TACE, sorafenib, MVD, VEGF

## Introduction

Hepatocellular carcinoma (HCC) is fifth most common cancer worldwide and has become the third cause of cancer-related deaths [1]. As yet, numerous researches has been made to cure the disease, but the therapeutic effect of existing strategies for HCC patients are not satisfied [2, 3]. So new strategies for HCC treatment need to be further explored.

Transcatheter arterial chemoembolization (TACE) has become an efficient method for HCC patients, especially for the advanced ones [4-6]. TACE is manipulated by hepatic arterial injection of embolic agents and chemotherapeutic drugs to tumor tissues so as to inhibit tumor growth and metastasis [6]. TACE can not

only block the blood supply to tumor cells via embolic agents (such as iodized oil), but also kill tumor cells with chemotherapeutic drugs (such as pharmorubicin and cis-DDP). TACE treatment dramatically prolongs the survival rate of HCC patients in clinic [7-9]. However, there still exist some problems in TACE treatment, such as (1) the tumor tissues can reconstruct microvascular system via modulating angiogenesis, which recovers the blood supply to tumor cells and attenuates the therapeutic effectiveness of embolic agents; (2) the sensibilities of advanced HCC patients to traditional chemotherapeutic drugs in TACE are probably decreased, which descends the clinical curative effect; (3) the traditional chemotherapeutic drugs in TACE often leads to obvious side effects due to the lack of

tumor-specificity. Therefore, the strategies that inhibit angiogenesis or increase the sensibility and specificity of drugs may improve the therapeutic effectiveness of TACE in clinic.

In recent years, a new molecular targeted drug sorafenib has been developed for advanced HCC patients and presents a considerable curative effect [10-13]. Sorafenib can inhibit several tyrosine protein kinases (such as VEGFR and PDGFR) and certain signaling pathways (such as RAF/MEK/ERK pathway) [11]. Previous studies have confirmed that sorafenib can not only repress angiogenesis of HCC tissues, but also suppress proliferation and induce apoptosis in HCC cells [14-16]. The oral administration of sorafenib has been widely used for HCC patients since it's approved by FDA in 2007, but the research about the local administration of sorafenib in HCC tumors has been limited until now [17, 18]. The local drug delivery (such as TACE) can enrich drugs in specific tumor tissues, which could enhance the curative effect and reduce the side effects. Theoretically, the local delivery of sorafenib to HCC tissues could be a feasible and efficacious strategy for HCC patients.

In the present study, the hepatic arterial administration of sorafenib and embolic agent (iodized oil) was investigated in rabbit VX-2 hepatocellular carcinoma model. Meanwhile, the hepatic arterial administrations of iodized oil alone, pharmorubicin and iodized oil, cis-DDP and iodized oil were also used in the model as controls. The data in our study demonstrates that the hepatic arterial administration of sorafenib and iodized oil (defined as TACE-is) presents a better effect on inhibiting tumor growth rate and intrahepatic metastasis compared to other groups. The results indicate that TACE-is may be a secure and effective treatment for HCC patients in clinic.

### Materials and methods

#### *Animal model*

The Japanese rabbits in the study were provided by the general hospital of Chinese People's Liberation Army and were approved by the ethics committee of the Chinese PLA General Hospital (2011-X7-10). Thirty-two male rabbits (2.5-3.0 kg) were used in this experiment. The rabbit VX2 hepatocellular carcinoma model was established as follows: (1) VX2 tumor cells

were implanted in the femoribus internus subcutaneous of the rabbit for proliferation. After two weeks, the auxetic VX2 tumor (almost 2 cm in diameter) was separated under sterile conditions and is made into  $1 \times 1 \times 1$  mm<sup>3</sup> pieces of tumor tissue. The pieces were stored in physiological saline. (2) The rabbit was operated in the abdominal median incision and then the left of hepatic central lobe was selected as an area for the microinjection of VX2 tissue pieces. Subsequently, five pieces of VX2 tissue were implanted in the candidate hepatic area and the puncture was covered by gelatin sponge. Then the abdominal cavity was sterilized with gentamicin and the incision was sutured. (3) All of the rabbits were given intramuscular injection of penicillin for three days after the implantation of VX2 tumors.

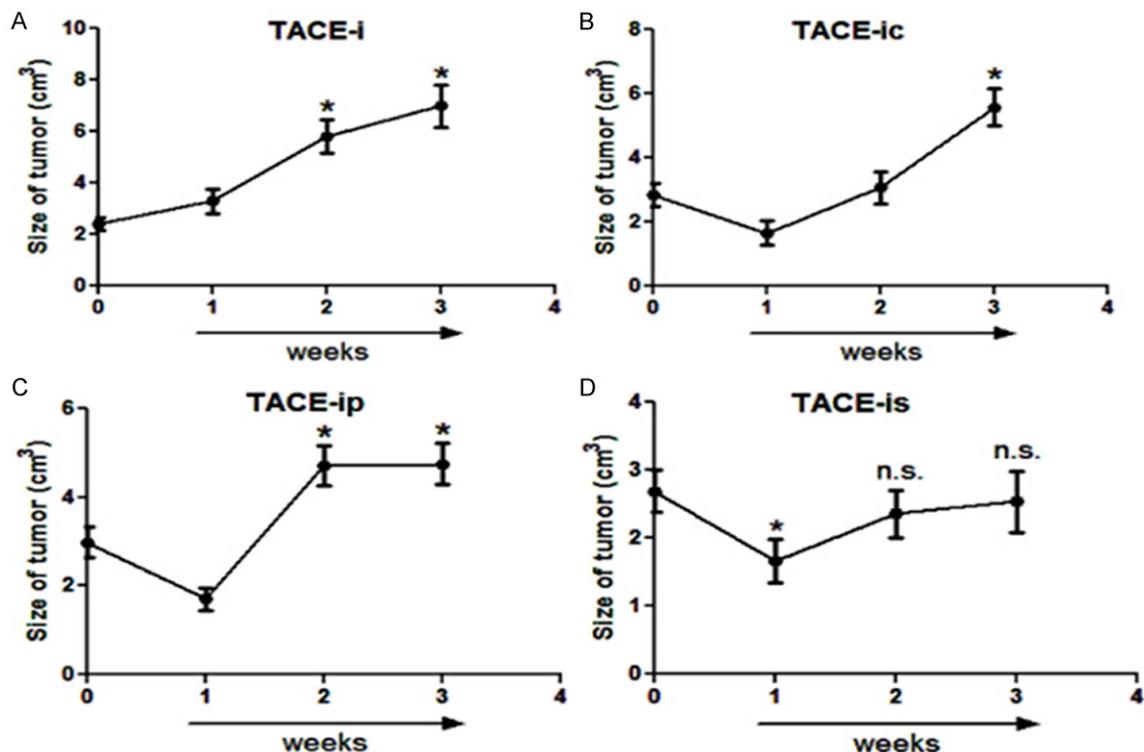
#### *Procedure of TACE treatments*

The rabbits of VX2 hepatocellular carcinoma models were treated under general anesthesia, and then the arteria cruralis of each rabbit was dissected bluntly. Subsequently, a catheter guide wire was moved to the hepatic tumor feeding artery from arteria cruralis under the guidance of DSA. Then different drugs were separately injected into the feeding artery of the rabbits in four groups, which were respectively named as TACE-i, TACE-ip, TACE-ic and TACE-is. TACE-i group: the mixture of 1 ml 0.9% physiological saline solution and 5 ml iodized oil. TACE-ip group: the mixture of 20 mg pharmorubicin, 1 ml 0.9% physiological saline solution and 5 ml iodized oil. TACE-ic group: the mixture of 50 mg cis-DDP, 1 ml 0.9% physiological saline solution and 5 ml iodized oil. TACE-is group: the mixture of 100 mg sorafenib, 1 ml 0.9% physiological saline solution and 5 ml iodized oil. All the mixtures were made into suspension with ultrasonic concussion for 20 min and injected into the VX2 tumors of the rabbits via hepatic tumor feeding artery (0.15 ml/Kg for each rabbit). Finally, the arteria cruralis of each rabbit was sutured and all of the rabbits were given intramuscular injection of penicillin for three days.

#### *Measurement of tumor size*

The perfusion CT was used to estimate the tumor size as described previously [19]. The maximum diameter (A) and transverse diameter (B) of tumor were separately recorded and the tumor size (V) was calculated as  $V = A \times B^2 / 2$ .

## Effect of sorafenib on rabbit VX-2 hepatocellular carcinoma



**Figure 1.** TACE-is treatment inhibited the growth of primary tumor in rabbit VX2 hepatocellular carcinoma model. Thirty-two rabbits were implanted with VX-2 tumors and randomly divided into four groups (TACE-i, TACE-ip, TACE-ic and TACE-is). The sizes of implanted tumors (also named as primary tumor) in four groups were detected by Perfusion CT on Day 3 before TACE treatments and the values were presented in (A-D) at “0” time point as a control for TACE treatment. Subsequently, the rabbits in four groups were separately treated with TACE-i, TACE-ip, TACE-ic and TACE-is. TACE-i: the hepatic arterial administration of iodized oil alone; TACE-ip: the hepatic arterial administration of iodized oil and pharmorubicin; TACE-ic: the hepatic arterial administration of iodized and cis-DDP; TACE-is: the hepatic arterial administration of iodized and sorafenib. Then the sizes of primary tumors in each group were measured by perfusion CT on days 7, 14 and 21 after TACE treatments. n.s.: no significance; \*:  $P < 0.05$ .

### Assessment of intrahepatic metastasis

The rabbits in four groups were sacrificed and anatomized on day 21 after TACE treatments. Then the size of metastatic tumors in livers was measured macroscopically.

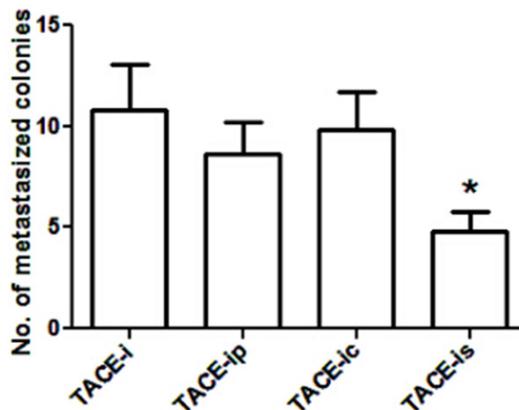
### Analysis of MVD

MVD was evaluated using CD34 staining as described previously (Hussein 2007). Briefly, the rabbits in four groups were sacrificed using overdose of pentobarbital on day 21 after TACE treatments. Then the tumor adjacent tissue was obtained and made into paraffin sections. The sections were deparaffinized and rehydrated. Next the sections were sequentially incubated with monoclonal anti-CD34 antibody for 30 min at room temperature and a catalyzed signal amplification system. The sections were counterstained with Harris haematoxylin and

were dehydrated, dipped in xylene and mounted with DPX. Then the sections were treated with peroxidase-labeled streptavidin for 30 min and incubated with 14-diamino-benzidine and  $H_2O_2$  for 10 min. Subsequently, the sections were scanned at low magnifications ( $40\times$ ) so as to identify 5 areas with the most dermis vessels (hotspot areas). Then the amount of CD34-positive vessels in the areas were counted at high magnifications ( $400\times$ ). Every immunolabeled cell or cell cluster separated from adjacent or other connective tissue microvessel was defined as a single. The mean value of microvessels from 5 areas was defined as MVD.

### Evaluation of VEGF expression

The VEGF staining by immunohistochemistry was manipulated as CD34 staining. The VEGF



**Figure 2.** TACE-is treatment suppressed intrahepatic metastasis of VX2 tumor compared to TACE-i group. The rabbits with implanted VX-2 tumors were treated with TACE-i, TACE-ip, TACE-ic and TACE-is. Then the VX2 tumor rabbits were sacrificed and anatomized on day 21 after TACE treatments and the amount of visual metastatic tumors in livers were counted. \*:  $P < 0.05$ .

expression was evaluated semiquantitatively by calculating the sum of the scores from dyeing range and intensity. The scores of dyeing range followed the criteria: 1+, 0~10% dyeing cells; 2+, 10%~50% dyeing cells; 3+, 50%~75% dyeing cells; 4+, 75~100% dyeing cells. The scores of dyeing intensity were calculated as follows: 1+, mild (the dyeing intensity in the cells was weak and even defect); 2+ moderate (the amount of cells evenly stained bronzing particles were less than that of 10% total cells in the area); 3+, serious (the amount of cells evenly stained bronzing particles were more than that of 10% total cells in the area).

#### Detection of alanine transaminase and creatinine levels

The auricular vein blood of each rabbit was separately obtained on day 3 before TACE treatment and day 3, 7, 14 and 21 after TACE treatment. Then the levels of alanine transaminase (ALT) and creatinine (SCr) were respectively detected by Cobas 8000 (Roche, Germany).

#### Statistical analysis

The data in the present study were showed as mean  $\pm$  SD. A two-way ANOVA was used to analyze the variance in different groups using GraphPad Prism 5.0 software.  $P < 0.05$  was considered significant.

## Results

### *TACE-is treatment inhibited the growth of primary tumor in rabbit VX2 hepatocellular carcinoma model*

Firstly, thirty-two rabbits with implanted VX-2 tumors were randomly divided into four groups (TACE-i, TACE-ip, TACE-ic and TACE-is). Next the implanted VX2 tumors in the rabbits were detected by perfusion CT on Day 3 before TACE treatments. The sizes of implanted tumors (also named as primary tumor) in four groups were calculated and the values were presented in **Figure 1A-D** at "0" time point as a control for TACE treatment. Subsequently, the rabbits in four groups were separately treated with TACE-i, TACE-ip, TACE-ic and TACE-is. The sizes of primary tumors in each group were measured by perfusion CT on day 7, 14 and 21 after TACE treatments. The results showed that the growth of VX2 primary tumors was suppressed on day 7 by all of the four TACE treatments, but only inhibited on day 14 by TACE-ic and TACE-is and on day 21 by TACE-is (**Figure 1A-D**). Taken together, TACE-is treatment presented a more dramatic inhibition of VX2 primary tumor growth compared to other groups.

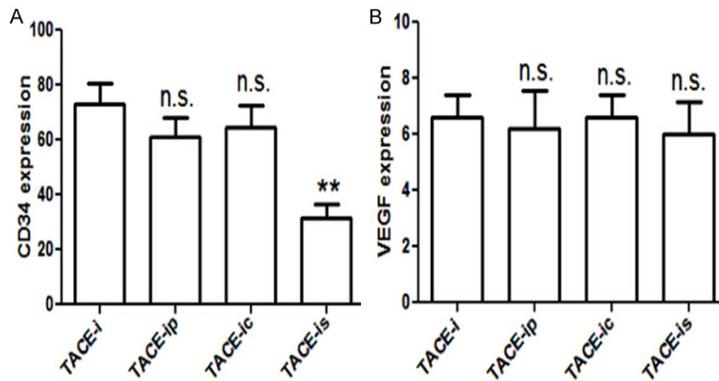
### *TACE-is treatment suppressed intrahepatic metastasis of VX2 tumor compared to TACE-i group*

The VX2 tumor rabbits were sacrificed and anatomized on day 21 after TACE treatments. The amount of visual metastatic tumors in livers was counted. The data indicated that TACE-is treatment (not TACE-ic and TACE-ip treatments) inhibited intrahepatic metastasis of VX2 tumor compared to TACE-i group (**Figure 2**).

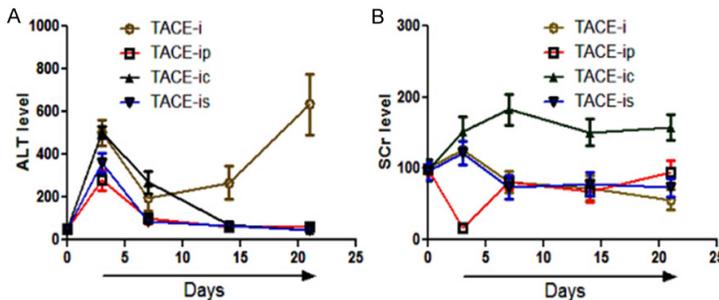
### *TACE-is treatment depressed tumor angiogenesis, but did not change the level of VEGF expression*

We further investigated the angiogenesis of tumor adjacent tissue via detecting the microvessel density (MVD) in the VX2 tumor rabbits. MVD was assessed by CD34 expression. As shown in **Figure 3A**, CD34 expression in TACE-is group (not in TACE-ic or TACE-ip group) was remarkably decreased compared with TACE-i group. The data indicated that the hepatic arterial administration of sorafenib presented an effective inhibition of angiogenesis. In addition, the expression of vascular endothelial

## Effect of sorafenib on rabbit VX-2 hepatocellular carcinoma



**Figure 3.** TACE-is treatment depressed tumor angiogenesis, but didn't change the level of VEGF expression. The rabbits with implanted VX-2 tumors were treated with TACE-i, TACE-ip, TACE-ic and TACE-is. Then the VX2 tumor rabbits were sacrificed and anatomized on day 21 after TACE treatments and the expression of CD34 and VEGF were detected by immunohistochemistry. n.s.: no significance; \*\*:  $P < 0.01$ .



**Figure 4.** TACE-is treatment presented little toxicity of liver and kidney. The levels of serum alanine transaminase (ALT) and creatinine (SCr) in the rabbits with implanted VX-2 tumors were separately measured on Day 3 before TACE treatments and on day 3, 7, 14 and 21 after TACE treatments.

growth factor (VEGF, an important factor for angiogenesis) was little of difference among the four groups (Figure 3B).

### *TACE-is treatment presented little toxicity of liver and kidney*

To assess the toxicity of TACE-is treatment on liver and kidney, the levels of serum alanine transaminase (ALT) and creatinine (SCr) in the rabbits were separately measured. As shown in Figure 4A and 4B, TACE-is treatment didn't significantly change the levels of ALT and SCr, which suggested that TACE-is treatment may be a safe strategy without obvious hepatic and renal toxicity.

### Discussion

In the present study, we evaluated the availability of TACE-is treatment (The hepatic arterial

administration of iodized and sorafenib) in rabbit VX2 hepatocellular carcinoma model. The data demonstrated that TACE-is treatment can effectively attenuates tumor growth and intrahepatic metastasis of VX2 tumor without obvious hepatic and renal toxicity. Moreover, the anti-tumor effect of TACE-is treatment may be potently better than the traditional TACE treatments (such as TACE-i, TACE-ic and TACE-ip). One of the relevant antitumor mechanism may be associated with the inhibition of angiogenesis in VX2 tumors of TACE-is treated rabbits.

The blood vessels of tumor can not only promote the proliferation of tumor cells via blood supplement, but also mediate hematogenous metastasis of tumor cells [20-24]. Therefore, targeting tumor vessels could be a promising anti-tumor strategy [25-27]. Previous studies have verified that the oral administration of sorafenib can efficaciously inhibit tumor growth through the anti-angiogenesis role. Here we found that the hepatic arterial administration of sorafenib also dramatically inhibit angiogenesis of im-

planted VX2 tumors compared to pharmorubicin and cis-DDP, which may be a anti-tumor mechanism of TACE-is treatment. VEGF is an important factor of angiogenesis and the level of VEGF often increases in the tumor area. Downregulation of VEGF could suppress tumor growth [28]. It has been reported that sorafenib can decrease the expression of VEGF in certain conditions [29]. However, in our present study TACE-is treatment didn't significantly change VEGF level compared with other TACE treatments. As sorafenib could antagonize VEGFR (Vascular Endothelial Growth Factor Receptor), the role of VEGF/VEGFR pathway in the inhibition of angiogenesis can't be excluded in VX2 tumor rabbits with TACE-is treatment [30, 31]. More studies need to be explored the detail mechanisms.

The hepatic or renal toxicity is a common side effect of anti-tumor drugs, which often reduces

the therapeutic effect and even directly leads to death in clinic [32]. So evaluating the hepatic or renal toxicity of TACE-is treatment is indispensable for its further clinical application. In the present study we found that TACE-is treatment didn't induce obvious hepatic and renal toxicity, which may indicates a safe drug dosage for the rabbit VX2 hepatocellular carcinoma model. Of course, more potential side effects (such as drug rash) need to be further studied.

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#### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Feng-Yong Liu, Department of Interventional Radiology, Chinese PLA General Hospital, 28 Fuxing Road, Haidian District, Beijing 100853, China. Tel: +86-10-66939340; Fax: +86-10-66875311; E-mail: fengyongliu@aliyun.com

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## Effect of sorafenib on rabbit VX-2 hepatocellular carcinoma

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