Int J Clin Exp Pathol 2015;8(11):13945-13956
www.ijcep.com /ISSN:1936-2625/IJCEP0014151

*Original Article*

# Inflammation and cancer: inhibiting the progression of residual hepatic VX2 carcinoma by anti-inflammatory drug after incomplete radiofrequency ablation

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Received August 8, 2015; Accepted September 21, 2015; Epub November 1, 2015; Published November 15, 2015

**Abstract:** Background: Accelerated progression of residual hepatocellular carcinoma (HCC) after incomplete radiofrequency ablation (RFA) has been reported more frequently. Recent data have redefined the concept of inflammation as a critical component of tumor progression. However, there has been little understanding regarding the relationship between progression of residual HCC and the inflammation induced by thermal destruction of the tumor after RFA. The present study was designed to determine whether inflammation facilitates rapid progression of residual hepatic VX2 carcinoma and to clarify the possible underlying mechanisms. Methods: Forty-eight rabbits were each implanted with two VX2 hepatic tumors *via* supraumbilical median laparotomy. One of the tumors in two different lobes was ablated by RFA. All the rabbits were then randomly divided into four groups (12 rabbits in each group) receiving anti-inflammatory treatment with different doses of aspirin: control group, AS-L group (aspirin, 5 mg/kg/d), AS-M group (aspirin, 20 mg/kg/d), and AS-H group (aspirin, 100 mg/kg/d). The levels of serum interleukin-6 (IL-6), high sensitivity C-reactive protein (hs-CRP), and tumor necrosis factor-α (TNF-α) were detected to evaluate the effect of the anti-inflammation. Tumor growth, lung and kidney metastasis, and survival were assessed. The expression of proliferating cell nuclear antigen (PCNA), matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF), and cysteinyl aspartate specific proteinase 3 (caspase-3) in residual tumor was examined by immunohistochemistry and Western-blotting. Results: The levels of serum IL-6, hs-CRP, and TNF-α in the AS-H group decreased significantly in comparison with those of the control group ($P<0.05$). The focal tumor volume and lung and kidney metastases of rabbits in the AS-H group were less significant compared with those of the control group ($P<0.05$). The expression of PCNA, MMP-9, and VEGF in the AS-H group decreased significantly compared with the control group ($P<0.05$). Finally, the survival time of the AS-H group was longer than that of the control group ($P<0.05$). Conclusions: Inflammation induced by thermal destruction of the tumor following RFA could be an important cause of rapid progression of residual hepatic VX2 carcinoma. The anti-inflammation effect of aspirin can inhibit proliferation, invasion, and metastasis of residual tumor cells, and aspirin may be a good candidate drug as an adjuvant therapy with RFA for treating HCC.

**Keywords:** Hepatocellular carcinoma, RFA, radiofrequency ablation, IL-6, interleukin-6

## Introduction

Each year, more than half a million people worldwide receive a diagnosis of hepatocellular carcinoma (HCC), and HCC related to hepatitis C virus (HCV) is the fastest-rising cause of U.S. cancer-related deaths. Worldwide, HCC is the fifth most common malignancy and the third most common cause of cancer-related mortality [1]. Although surgical-treatments (transplantation/resection) provide the best outcomes, most patients present with advanced tumor stages and are not candidates for these options. As a result, a variety of locoregional therapies, including radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT), transarterial chemoembolization (TACE) and others, have

been developed to destroy cancer cells in situ [2-4]. Among these techniques, RFA is currently the most widely used treatment, both for curative and palliative purposes. This minimally invasive technique can serve both as a treatment for patients who are not surgical candidates, as well as an adjunct to surgery, facilitating resection or in combination with surgery to achieve total tumor burden control [5, 6]. However, one of the major challenges with RFA is residual tumor tissue and local recurrence after local treatment, especially in case when the tumor diameter is >3 cm or the tumor is located near the intrahepatic vasculature [7].

Recently, residual tumor progression after insufficient RFA has been reported and some possible mechanisms have also been proposed [8-12]. It has been increasingly recognized that inflammation is strongly associated with the development, progression, and prognosis of most human cancers. Indeed, some researchers call inflammation a driving force speeding cancer metastasis [13]. Inflammation is a part of the host's response to either internal or external environmental stimuli. Thermal destruction of liver carcinoma by RFA also leads to the body's inflammatory response to restore tissue, including the formation of fibrous tissue, leukocyte infiltration, angiogenesis factors, and cytokines networks [14]. Based on analysis of the aforementioned research, we hypothesized that inflammation induced by the thermal destruction of RFA at the target sites might play an important role in facilitating rapid progression of residual HCC tumor. The present study was designed to test this hypothesis and to clarify the possible underlying mechanisms.

## Material and methods

### *Ethics statement*

The experiments were approved by the Animal Care Committee of Capital Medical University, Beijing, China and were performed in accordance with the institutional guidelines.

### *Animal housing and tumor implantation*

Forty-eight New Zealand white rabbits (weighing 2.5-3.0 kg, average 2.7±0.17 kg) were selected randomly from the experimental animal center of our university. The animals were housed under 12/12 h light-dark cycles and were allowed food and water ad libitum between the various procedures. VX2 carcinoma cells were donated by the Department of Hepatobiliary Surgery, Beijing Chao-yang Hospital.

The rabbits were administered general anesthesia by intravenous injection of 3% sodium pentobarbital (30 mg/kg body weight). VX2 tumors were first grown for two weeks on the hind legs of carrier rabbits and then were harvested after they reached a size of 1.5-2 cm. The tumors were placed into saline solution and stripped to obtain fresh hoary fish meat-like tissue. Then the necrotic tissue, the surrounding connective tissue and the fat were removed. The harvested tumors were cut into cubes of 1 $mm^3$. The abdomens of the recipient rabbits were shaved and prepared with povidone iodine, and a midline subxiphoid incision was made. The anterior surface of the liver was exposed and two of the prepared cubes of tumor tissue were implanted in the right and left lobes of the liver using a 21-gauge angioneedle. There were two inoculation sites in each liver. This method allowed the growth of two solitary, well-demarcated tumors in different lobes of the liver [8]. Proper aseptic technique was rigorously observed during each inoculation. The laparotomy incision was closed by suturing with non-dissolving stitches. After surgery, the animals were returned to their cages, kept warm, and monitored in the animal laboratory until they recovered from anesthesia. The animals were observed by sonography (Acuson Corporation, Mountain View, CA, USA) until both the tumors of the different liver lobes reached 1.0 cm in diameter, and they were then used for experimentation. Ultrasonography was performed by the same operator with a 7v3c probe at a frequency of 7.0 MHz. The period of time for tumors to reach the size of 1.0 cm ranged from 10 to 14 days. All inoculations were performed by the same individual investigator, who inoculated specimens of the same tumor into all rabbits to minimize inter-animal variations in tumor growth rate.

### *Model of residual hepatic VX2 carcinoma following RFA*

RFA was performed using the same anesthesia protocol as for carcinoma implantation. Two grounding pads were applied to the animal's flank before RFA. The abdomens of the experi-

mental rabbits were shaved and prepared with povidone iodine, and a midline subxiphoid incision was made. One of the tumors in the two different lobes was ablated by RFA. Before RFA, it was ascertained that the residual tumor did not adhere to the other one. An RF current generator (Model 1500 Generator; RITA Medical Systems, Manchester, GA, USA) was used to generate RF energy. To deliver the RF energy, we used a 14-gauge expandable RF needle electrode (StarBurst™ XL; RITA Medical Systems), 10 cm in length. Each ablation cycle lasted for 5 min.

*Inhibiting the rabbit inflammatory response induced by RFA*

The 48 rabbits were randomly divided into one of four groups (n=12 each) for the next procedure of inhibiting the inflammatory response after RFA. Assignment to the groups was done by an observer blinded to the purpose and the treatment options of the study. The control group consisted of animals receiving normal saline orally, whereas animals of the AS-L group received low-dose aspirin (Aspirin Effervescent Tablets obtained from AstraZeneca, Wuxi, China) orally once daily at a dose of 5 mg/kg body weight). The AS-M group received a middle dose of aspirin, 20 mg/kg body weight/day, and the AS-H group received high-dose aspirin at a dose of 100 mg/kg body weight/day. The drugs were dissolved in water and given directly into the oropharyngeal cavity at a volume of 1 mL/kg, seven days a week in the morning after RFA. The above aspirin dose regimens were used because they correspond, in a human adult of 60 kg, to daily aspirin dosages of 300 mg, 1200 mg and 6000 mg respectively.

*Monitoring of clinical evolution and serum markers of systemic inflammation*

All animals underwent clinical evaluation to assess their disease evolution using objective parameters of post-surgical recuperation, such as resumption of feeding and activity. Effects on inflammation were assessed by biochemical analysis of serum markers, including interleukin-6 (IL-6), high-sensitivity C-reactive protein (hs-CRP), and tumor necrosis factor-α (TNF-α). At days 1, 3, and 7 after RFA, blood (1-2 mL) was withdrawn from animals of each group in non-siliconized glass tubes and allowed to clot for 60 min at 37°C. Serum was separated by centrifugation (1000× *g* for 15 min), and aliquots were stored frozen at -20°C until measurement by enzyme-linked immunosorbent assay (ELISA) kits (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China).

*Gross pathological analysis*

On day 7 post-RFA, six rabbits of each group were sacrificed by intravenous anesthesia overdose. The liver, lungs and kidneys were carefully dissected and excised. The number and size of masses were noted. Tumor sizes were measured and tumor volumes calculated according to the formula: $V = ab^2/2$, where is the longest and b the smallest diameter of the tumor in vivo [15]. The pathological findings could thus be compared directly using the tumor numbers, volumes, and locations. Quantitative evaluation of the lung and kidney metastatic nodules was made by two pathologists using the following procedures: macroscopic study by stereoscopic magnifying glass and counting the metastatic nodules on the pleural surface of all the lobules [12]. The variation between the pathologists' findings was <5%. The rest of the animals in the four groups were observed and they were sacrificed when they became moribund. The tumor, liver, lungs and kidneys were carefully dissected and examined.

*Immunohistochemical analysis*

The streptavidin-peroxidase two-step method was used for immunohistochemical detection of matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA) and cysteinyl aspartate specific proteinase 3 (caspase-3). Representative 5 μm tissue sections were cut from paraffin-embedded specimens. The sections were washed three times for 3 min with phosphate-buffered saline (PBS), and blocked with a solution of 30 ml/L hydrogen peroxide in ethanol for 10 min at room temperature. They were immersed in 30 ml/L normal horse serum for 10 min at room temperature. The sections were incubated for 16 h at 4°C with primary antibodies (mouse monoclonal antibodies; Abcam, Cambridge, UK) specific to MMP-9 (dilution 1:50), VEGF (dilution 1:50), or PCNA (dilution 1:1). Negative controls consisted of tissue sections incubated with PBS instead of the primary antibody. The immunoreactivity was then

**Table 1.** Serum IL-6, hs-CRP and TNF-α levels in the four groups after RFA (means [SD])

| Items | Time | Control group (n=12) | AS-L group (n=12) | AS-M group (n=12) | AS-H group (n=12) |
|---|---|---|---|---|---|
| IL-6 (pg/ml) | 1st day | 159.41 [20.40] | 150.56 [14.36] | 147.29 [18.82] | 102.02 [14.24]* |
| | 3rd day | 48.12 [8.16] | 46.19 [6.84] | 42.25 [9.02] | 42.70 [6.55] |
| | 7th day | 44.85 [7.29] | 44.18 [4.77] | 42.85 [3.82] | 39.80 [5.09] |
| hs-CRP (mg/L) | 1st day | 6.28 [0.34] | 6.25 [0.38] | 6.02 [0.22] | 5.29 [0.25]* |
| | 3rd day | 6.69 [0.42] | 6.58 [0.37] | 6.22 [0.27]** | 5.66 [0.18]** |
| | 7th day | 3.97 [0.31] | 4.03 [0.49] | 4.01 [0.41] | 3.95 [0.39] |
| TNF-α (ng/L) | 1st day | 263.84 [8.48] | 263.59 [4.54] | 255.63 [6.90] | 233.79 [9.55]* |
| | 3rd day | 266.37 [5.83] | 260.70 [5.83] | 258.05 [4.17] | 236.94 [6.95]** |
| | 7th day | 208.96 [6.75] | 208.24 [5.83] | 206.79 [4.25] | 209.81 [4.91] |

IL-6: interleukin-6, hs-CRP: high-sensitivity C-reactive protein, TNF-α: tumor necrosis factor-α. *: Compared with Control group on the 1st day after RFA by one-way ANOVA, P<0.05; **: Compared with Control group on the 3rd day after RFA by one-way ANOVA, P<0.05.

**Table 2.** Tumor volume and metastasis on the 7th day after RFA

| Group | Tumor volume [SD] ($cm^3$) | Lung metastasis (number) |
|---|---|---|
| Control group | 3.541 [0.687] | 7 (in 2 rabbits) |
| AS-L group | 3.331 [0.455] | 5 (in 1 rabbit) |
| AS-M group | 2.910 [0.620] | 0 |
| AS-H group | 1.625 [0.495]* | 0 |

*Compared with Control group by one-way ANOVA, P<0.05.

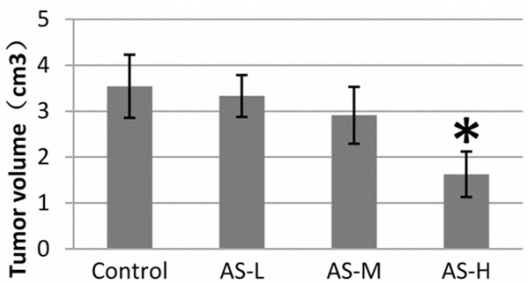


**Figure 1.** Growth of residual hepatic VX2 carcinoma after RFA by inhibiting the systemic inflammation. Data were expressed as means ± SD of four groups. (*Compared with control group, *P*<0.05).

visualized by incubating the samples in 3,3'-diaminobenzidine. Finally, the slides were counterstained with hematoxylin. To evaluate the expression of MMP-9, VEGF, PCNA, and caspase-3, all slides were examined and scored by two independent pathologists who were blinded to the animal data. A few cases with discrepant scores were reevaluated to reach a final agreement. Staining of tissue specimens was scored using a semi quantitative scoring system that takes into account tumor heterogeneity and that is subject to statistical analysis [16]. Using the 10× objective lens, staining intensity and distribution in each field was scored as absent (0), weak (1), moderate (2), or strong (3). All 10× fields in a given specimen were individually scored, the percentage of fields at each intensity was determined, and scores were added to yield an average staining intensity score ($I_s$) for the entire specimen. $I_s = (0\times F_0) + (1\times F_1) + (2\times F_2) + (3\times F_3)$, where F is the percent 10× fields score of the different intensities.

*Western blotting*

Proteins for western blotting were isolated from fresh-frozen tissue using T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's recommendations. The supernatants were frozen at -80°C until use. The proteins were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer onto nitrocellulose filters (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The filters were blocked at 4°C overnight with a blocking buffer (pH 7.6) that contained 5% non-fat dry milk. The filters were incubated with a primary monoclonal antibodies to MMP-9 (1:200; Abcam), VEGF (1:200; Abcam), PCNA (1:100; Beijing Biosynthesis Biotechnology Co., Ltd.), and caspase-3 (1:100; Abcam) and a secondary anti-mouse horseradish peroxidase (HRP) -antibody (1:1000; Saier Biotechnology, Tianjin, China) for 2 h at room temperature. Immunoreactive bands were visualized using Piere™ Enhanced Chemi-

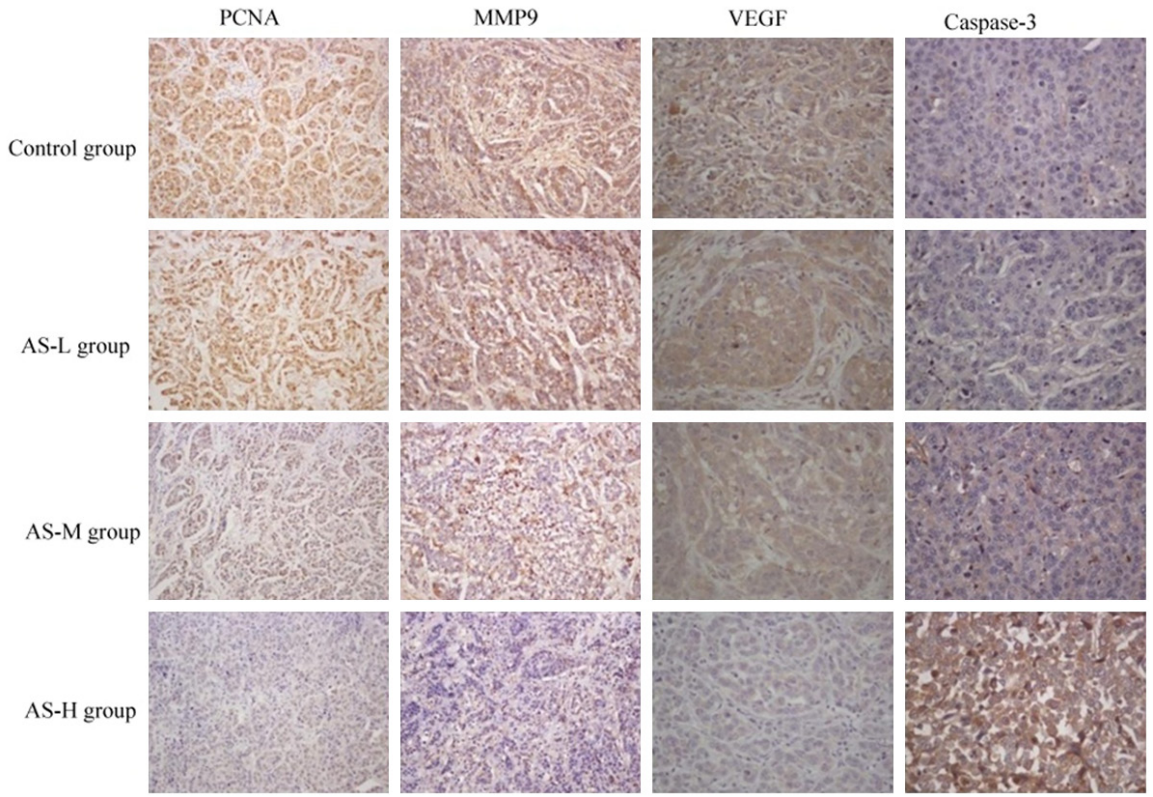


**Figure 2.** Immunohistochemical staining for PCNA, MMP9, VEGF and Caspase-3 in residual hepatic VX2 carcinoma tissues. Original magnifications: PCNA and MMP9, ×200; VEGF and Caspase-3, ×400.

**Table 3.** Immunohistochemical results of PCNA, MMP9, VEGF and Caspase-3

| Group | PCNA | MMP9 | VEGF | Caspase-3 |
|---|---|---|---|---|
| Control | 2.102±0.283 | 2.162±0.305 | 0.684±0.186 | 0.430±0.097 |
| AS-L | 2.080±0.359 | 2.120±0.313 | 0.660±0.146 | 0.484±0.161 |
| AS-M | 1.892±0.188 | 1.932±0.231 | 0.394±0.127* | 0.530±0.188 |
| AS-H | 1.008±0.207* | 1.168±0.176* | 0.290±0.087* | 0.888±0.211* |

*Compared with control group by one-way ANOVA, P<0.05.

luminescence (ECL) Western Blotting Substrate (Thermo, Fisher Scientific, Inc., Waltham, MA, USA).

*Statistical analysis*

Data were presented as the means ± standard deviation (SD) for the indicated number of separate experiments. All statistical calculations were performed with the SPSS statistical package (version 19.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test and Dunnett's C test, the chi-squared test and Fisher's exact test were used to evaluate statistical significance. The Kaplan-Meier method was used for survival analysis. A value of $P<0.05$ was considered significant.

## Results

*Aspirin inhibited the systemic inflammatory reaction of animals after RFA*

The levels of serum IL-6 in the AS-H group significantly decreased in comparison with those of the control group on the first day after RFA ($P<0.05$). The level of serum TNF-α in the AS-H group also decreased in comparison with those in the control group on the first day and third day after RFA ($P<0.05$, $P<0.05$). Similarly, the levels of serum hs-CRP in the AS-H group significantly decreased in comparison with those of the control group on the first day and third day after RFA ($P<0.05$, $P<0.05$). Finally, the serum hs-CRP in the AS-M group showed lower

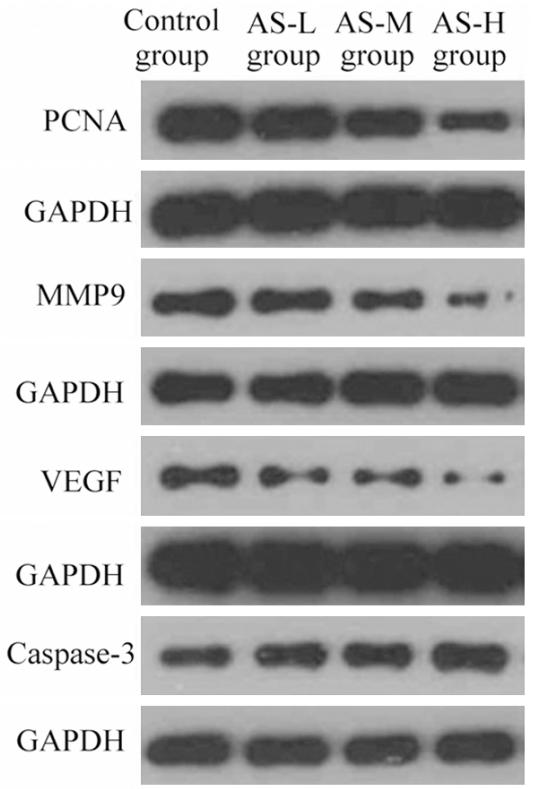


**Figure 3**. PCNA, MMP9, VEGF and Caspase-3 expression in residual hepatic VX2 carcinoma tissues.

levels than those of the control group on the third day after RFA (**Table 1**).

*Early effects of anti-inflammation on growth of residual hepatic VX2 carcinoma*

Receiving aspirin treatment after RFA, none of animals showed any complications and deaths in the post-RFA period in any treatment group. After seven days of treatment of inhibiting the inflammation, the tumor volumes in the AS-H group were significantly less than those in the control group significantly ($P<0.05$). A few lung metastases appeared, only among some of the animals of the control group and the AS-L group (**Table 2**; **Figure 1**).

*Immunohistochemistry and western-blotting assay*

PCNA, MMP-9, VEGF, and caspase-3 were found to be mainly expressed in cancerous lesions, but also in some normal tissues (**Figure 2**). The anti-PCNA and anti-MMP-9 antibodies were used to assess the proliferation and invasiveness of carcinoma cells, while the angiogenesis and apoptosis of the cancer cells were evaluated using anti-VEGF and anti-caspase-3 antibodies, respectively. The percentage of positive PCNA, MMP-9, and VEGF tumor cells in the AS-H group were markedly lower than those in the control group (**Table 3**, $P<0.001$). The expression of VEGF in the AS-M group was also lower than that in the control group ($P<0.001$). Compared with the control group, the AS-L group, and the AS-M group, the percentage of positive caspase-3 tumor cells in the AS-H group was higher ($P<0.001$).

The expression of PCNA, MMP-9, VEGF, and caspase-3 in tumor tissues with western blotting showed similar trend to the immunohistochemical assays. Due to inhibition of the inflammation after RFA with high-dose aspirin, the expression levels of PCNA, MMP-9, and VEGF in residual hepatic VX2 carcinoma tissues were lower than those in other groups. In addition, the expression of caspase-3 in tumor tissues was markedly increased in the AS-H group (**Figure 3**).

*Long-term effect of anti-inflammation on growth of residual hepatic VX2 carcinoma*

Due to tumor peritoneal metastasis and adhesion to the abdominal organs, the volumes of tumors could not be measured. The tumor metastases were carefully observed individually. It was found that all groups appeared to manifest lung metastases, and the number of lung metastases in the AS-H group was less than that in the control group (**Figures 4** and **5**; **Table 4**). A few kidney metastases were detected in the control group, the AS-L group and the AS-M group (**Table 4**).

The animals in the control group, AS-L group, AS-M group and AS-H group survived for 29, 30, 32 and 37 days, respectively, after receiving the VX2 tumor implantation. The mean survival times of animals in the AS-H group was longer compared to that in the control group ($P=0.001$). The Kaplan-Meier curves revealed that the higher dose of aspirin was inhibiting the inflammation, the longer was the survival time of the animals (**Figure 6**).

## Discussion

At present, a growing number of clinical studies have identified the rapid progression of residu-

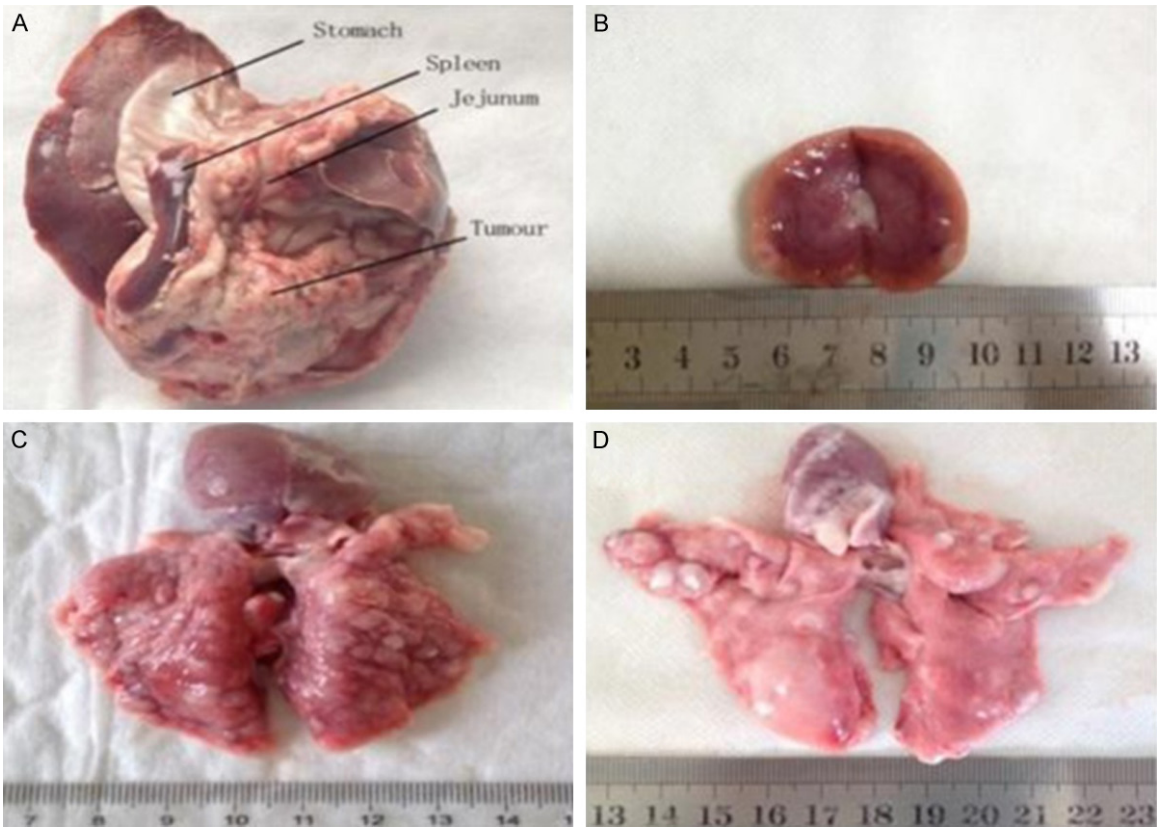


**Figure 4.** Gross features of tumors and tumor metastasis. A. The liver tumor had invaded serosa and adhered to the surrounding organs. B. The kidney metastasis was mainly located in renal cortex. C. Lung metastasis in control group. D. Lung metastasis in AS-H group.

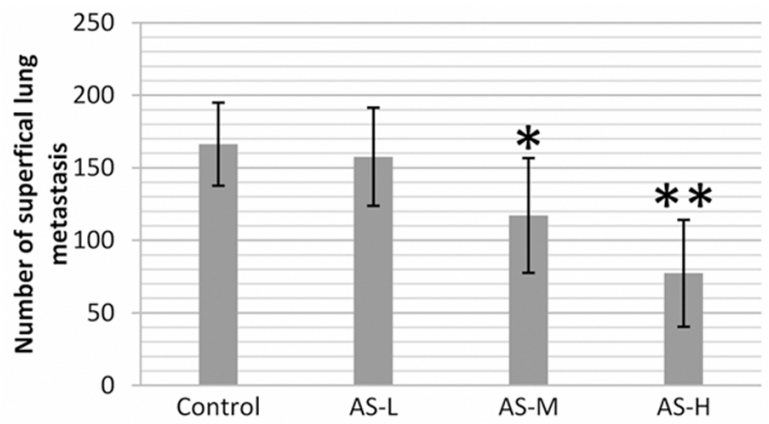


**Figure 5.** Frequency of pulmonary metastatic nodules in the control group and groups treated with aspirin. Data were expressed as means ± SD of four independent experiments. *Compared with control group by one-way ANOVA, $P<0.05$. **$P<0.001$.

al cancer after incomplete RFA in treating HCC [8-12]. Previous research had provided several potential mechanisms that might help explain these findings. Hyperthermia may play an important role in the rapid growth of residual HCC after RFA by promoting angiogenesis of residual HCC via hypoxia-inducible factor 1α (HIF-1α)/VEGF-A [10]. Another study demonstrated that suboptimal RFA accelerated HCC growth and spread by transiently inducing an epithelial-mesenchymal transition (EMT)-like and more aggressive cellular phenotype [17]. Recent data have expanded the concept that inflammation is a critical component of tumor progression. Inflammation is strongly associated with the development, progression and prognosis of most human cancers [18]. Thus, we are particularly concerned with inflammation promoting the progression of residual hepatic tumors. It should be noted that

**Table 4.** Tumor metastasis at the time when animal being moribund [SD]

| Group | Lung metastasis (number) | Kidney metastasis (cases) |
|---|---|---|
| Control group | 166.33 [28.61] | 5/6 |
| AS-L group | 157.67 [33.86] | 3/6 |
| AS-M group | 117.17 [39.53]* | 4/6 |
| AS-H group | 77.33 [36.85]** | 0/6# |

*Compared with control group by one-way ANOVA, P<0.05. **P<0.001. #Compared with control group by Chi-square test, P<0.05.

inflammation, especially in the wound healing process, has many similarities to tumor formation. The inflammation in the process of wound healing involves the formation of granulation tissues, the components' building of the stromal cells, and the process of angiogenesis [19]. In clinical settings, many patients with hepatic tumors present with a period of fever and/or local pain after receiving the treatment of RFA. The inflammatory response induced by the thermal destruction of liver tumor involves the formation of fibrous tissue, leukocyte infiltration, angiogenesis factors, and cytokine networks to "heal" the afflicted tissue [14]. In the present study, we introduced the orthotopic rabbit HCC model with two hepatic tumors in different lobes. During the treatment of one of the cancer nodules with RFA, another nodule would be affected just by the following inflammation and not influenced by the heat energy generated by RFA. That is similar to the clinical situation in which patients with huge hepatic tumors and/or multiple tumors could not be treated by RFA one time.

Nonsteroidal anti-inflammatory drugs (NSAIDs), especially aspirin, are the most well recognized drugs worldwide for the treatment of pain, inflammation, and fever. NSAIDs are commonly administered for the treatment of inflammatory diseases, rheumatoid arthritis, osteoarthritis, dysmenorrhea, and ischemic cerebrovascular disorders. These drugs inhibit prostaglandin biosynthesis and produce their therapeutic effects [20]. In our study, we chose aspirin as the anti-inflammatory drug to inhibit the local and systemic inflammation induced by the thermal destruction of the liver tumors. A significant decrease was observed in the levels of serum IL-6, hs-CRP and TNF-α, the laboratory biomarkers of inflammation, in the AS-H group. Therefore, the results suggest both that aspirin could effectively inhibit the inflammatory response and that the effect of anti-inflammation was dose-dependent. When the tumor and tissue damage are in one body, the inflammation of the tissue damage interacts with the tumor. The interaction depends on the distance between them. If the tumor is far from the wound, the interaction is mainly affected by the inflammatory factors present in the serum. Inflammation in the process of damage healing under the body's normal regulation could be in the form of cytokines or inflammatory factors in the serum delivered to the tumor. In contrast, if the tumor is close to the wound, the tumor would be affected both by the inflammatory factors and various inflammatory cells recruited owing to the wound. In the present study, the two nodules existed in one organ but in different lobes. Therefore, the residual VX2 nodule might be affected by both local and systemic inflammation after the other hepatic nodule was destroyed by RFA.

In our study, the proliferation, invasion and metastasis of HCC were evaluated by the expression of PCNA, MMP-9, caspase-3, and VEGF. PCNA is a nuclear protein that plays a key role in DNA-synthesis and repair, cell cycle regulation, chromatin remodeling, and apoptosis. [21]. The temporal pattern of PCNA expression makes it a useful tool to study cell proliferation. It starts to accumulate in the G1 phase of the cell cycle, reaches its highest level during the S phase, and decreases during the G2/M phase. PCNA was found to be valuable in studying the proliferative activity in different tumors including HCC [22]. Caspase-3 is a well-known downstream effector caspase of the caspase cascade, which is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [23]. The cell disassembly intrinsic to apoptosis is largely mediated by caspase-3, which targets structural substrates including nuclear laminins, focal adhesion sites, and cell -cell adherence junctions [24]. Cell invasion is a major component of the complex multistep process of tumor metastasis. Invasion of malignant tumor cells requires destruction of basement membranes and proteolysis of extracellular matrix (ECM). MMPs, a family of closely related enzymes that degrade the extracellular matrix, are considered to be important in facilitating tumor invasion and

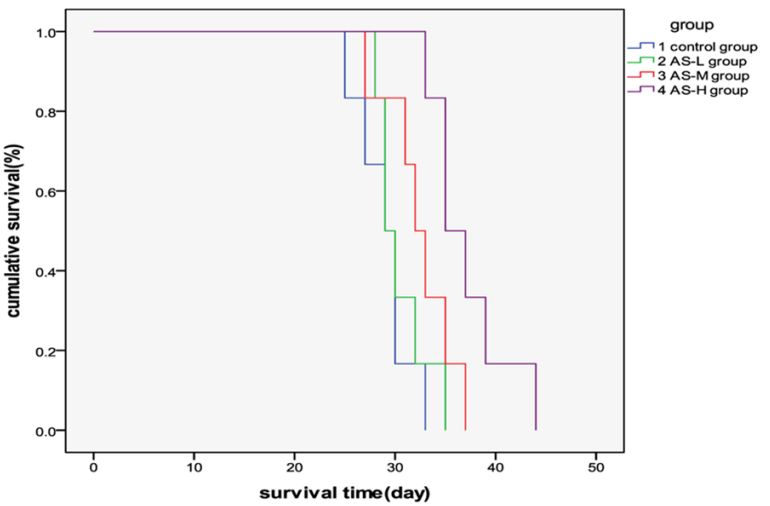


**Figure 6.** Kaplan-Meier survival curves and survival rates of rabbits with VX2 carcinoma receiving incomplete RFA in the four groups. The survival time of animals in AS-H group was significantly longer than that of control group (P = 0.001).

spread [25]. Some studies have suggested a major role for MMP-9 (92-kDa gelatinase/type IV collagenase) in the digestion of basement membrane type IV collagen, as an important mechanism for vessel invasion and metastasis [26, 27]. Angiogenesis, the formation of new blood vessels from existing vasculature, is an important process in many malignancies including HCC. Studies have shown that growth of liver tumors is dependent on angiogenesis. The proliferation of vascular endothelial cells is induced when the tumor size is greater than 0.5 mm [28, 29]. VEGF is a critical pro-angiogenic factor in cancer. VEGF binding to its receptor can facilitate the healing of vascular endothelium, angiogenesis, and increased vascular wall permeability. Thus, VEGF may be associated with tumor recurrence, metastasis and prognosis [30]. In the present study, it was shown that expression of PCNA, MMP-9, and VEGF, in tumor tissues in groups that received aspirin to inhibit the inflammation after incomplete RFA, decreased remarkably. Meanwhile, the expression of caspase-3 increased significantly compared to that in the control group. Furthermore, it seemed that the expression levels of PCNA, MMP-9, VEGF and caspase-3 were associated with the dose of aspirin, as well as the degree of inhibition of inflammation. These data suggest that the inflammation induced by RFA facilitates the proliferation, invasion, and metastasis of residual hepatic tumor cells.

It is important to clarify the underlying mechanisms of the manner in which inflammation facilitates the rapid progression of residual HCC after RFA, to optimize the therapeutic principles and strategies of RFA. It has been increasingly recognized that an inflammatory component is present and contributes to tumor proliferation, angiogenesis and metastasis. In response to tissue injury, a multifactorial network of chemical signals initiates and maintains a host response designed to "heal" the afflicted tissue. This involves activation and directed migration of leukocytes (neutrophils, monocytes, and eosinophils) from the venous system to sites of damage, and tissue mast cells also have a significant role [18]. Various kinds of proinflammatory cytokines are secreted by the activated leukocytes, as well as from platelets, to initiate wound healing and recruit ECM components. Some inflammatory cytokines are also common in the cytokine network of tumor tissue. TNF, for example, is a major mediator of inflammation, with actions directed toward both tissue destruction and recovery. TNF stimulates fibroblast growth and destroys blood vessels but also induces angiogenic factors [31]. TNF has also been implicated in carcinogenesis due to its participation in chronic inflammatory diseases. This cytokine may act as an endogenous tumor promoter, contributing to the tissue remodeling and stromal development necessary for tumor growth and spread [32]. IL-6 is a multifunctional cytokine with pivotal roles in the inflammatory response in many tissues. The transient expression of IL-6 contributes to host defenses against infections and tissue injuries by stimulating the acute-phase immune response and hematopoiesis. When tissue homeostasis is restored, the synthesis of IL-6 ceases [33]. IL-6 is another important inflammatory cytokine linking inflammation and cancer. The role of IL-6 in accelerating tumorigenesis is becoming clear as exogenous administration of IL-6 to mice during tumor initiation resulted in an increase in tumor burden and multiplicity. IL-6

also enhances tumor proliferation in tumor-initiating intestinal epithelial cells (IECs) through the nuclear factor (NF)-κB-IL-6-signal transducer and activator of transcription (STAT3) cascade and can act as an inducer of EMT in breast cancer cells characterized by suppressing E-cadherin expression and inducing vimentin, N-cadherin, Snail, and Twist [13].

Cyclooxygenase-2 (COX-2) converts arachidonic acid to prostaglandins, which in turn induce inflammatory reactions in damaged tissues [34]. COX-2 is also involved in carcinogenesis pathways in many organs. It has been reported that COX-2 expression is correlated with angiogenesis, invasion, relapse, chemoresistance, and tumorigenesis in HCC [35]. Besides, a significant correlation between COX-2 expression and active inflammation in the adjacent noncancerous liver is associated with shorter disease-free survival in HCC patients [36]. Prostaglandin E2 (PGE2), the major product of COX-2, stimulates proliferation, migration, and invasion in hepatoma cells by activating β-catenin and Akt signaling [37]. NSAIDs are widely consumed as analgesics to relieve minor aches and pains, as antipyretics to reduce fever, and as anti-inflammatory medications. Most NSAIDs, such as aspirin for example, are nonselective inhibitors of COX. A number of epidemiological studies have linked the long-term use of some NSAIDs, especially aspirin, with reduced cancer incidence and most significantly, with reduced cancer mortality [38]. The ability of NSAIDs to inhibit COX underlies their mechanism(s) of chemoprevention. Aspirin is nonselective in its inhibition of platelet function by acetylating and irreversibly inactivating both COX-1 and COX-2. Inactivation prevents platelet synthesis of prostaglandins, endoperoxides and thromboxane A2 (TAX2) [18]. Recently, increasing numbers of studies have demonstrated the anticancer effects of NSAIDs in HCC. Lu et al. reported that aspirin could minimize the pro-metastasis effect of sorafenib by up-regulating the tumor suppressor HIV-1 TAT-interacting protein (HTATIP2) in HCC, mediated through inhibition of COX-2 [39]. Sahasrabuddhe VV et al. showed that aspirin use was associated with reduced risk of developing HCC and of death due to chronic liver disease (CLD) whereas nonaspirin NSAID use was only associated with reduced risk of death due to CLD [40]. Aspirin inhibited hepatocyte growth factor (HGF)-induced invasiveness of HepG2 cells by inhibiting the kinase activity of extracellular signal-regulated kinase (ERK) 1/2, resulting in the suppression of transcriptional activity of Elk-1 as well as of NF-kB and activator protein-1 (AP-1) [41]. In chemically induced HCC, aspirin suppressed lung metastasis by down-regulating intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) or by inhibiting NF-kB signaling [41, 42]. The present study demonstrates that aspirin inhibiting the inflammation induced by thermal destruction of liver carcinoma could decrease the progression of residual hepatic VX2 cancer. Besides, it seemed that the lower the degree of inflammation after RFA was, the more blunt were the local proliferation and distant metastasis (e.g., to the lungs and kidneys) of the tumor. It should also be mentioned that although the mean survival times of animals in the AS-H groups were longer compared to those in the control group, this result might not reflect the real fact. The reasons for some animals being moribund were not liver failure or lung metastasis but obstruction of the digestive tract due to the tumor adhering to the surrounding organs. The result should be confirmed by additional study.

## Conclusions

In conclusion, the current study has proven that inflammation could be an important reason for rapid progression of residual hepatic VX2 carcinoma. In addition, anti-inflammation by aspirin can inhibit proliferation, invasion and metastasis of residual tumor cells. Aspirin shows high potential for use as an adjuvant therapy with RFA for treating HCC. Future clinical studies are necessary to validate the therapeutic potential of aspirin for HCC.

## Acknowledgements

This work was supported by Beijing Municipal Commission of Education Fund (Grant No. KM201110025026), Projects of State Commission of Science Technology of China (Grant No. 2012BAI06B01), Capital Development Fund of Medicine (Grant No. 2005-2034), the Capital Health Development Special Funds (Grant No. 2011-2018-03), Beijing Municipal Health Bureau (Grant No. 2011-2-18) and Organ Transplantation Research Fund from the Ministry of Health (Grant No. RHECC08-2012-08).

## Disclosure of conflict of interest

None.

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