Treatment of ^{131}I-labeled anti-CD147 monoclonal antibody in VX2 carcinoma-induced liver tumors

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Abstract. Hepatocellular carcinoma (HCC) is a major health problem worldwide. CD147 has been reported to be overexpressed in HCC and blocking CD147 expression can decrease tumor growth. ^{131}I is often used in combination with other drugs to treat HCC and yields positive results. In this study, we combined the ^{131}I and CD147 monoclonal antibody to treat HCC in a rabbit VX2 animal model. In the ^{131}I-labeled CD147 antibody (^{131}I-CD147-Ab) treatment group, the animals lived considerably longer than the animals in the other treatment groups. Metastasis and tumor growth in the ^{131}I-CD147-Ab treatment group were also inhibited. MMP2 and CD31 expression were significantly lower in the treatment group, whereas TUNEL staining was overexpressed. These findings suggest that ^{131}I-CD147-Ab is a promising drug in the treatment of HCC, by inhibiting metastasis and growth and by decreasing the expression of MMP2 and CD31 or by inducing tumor necrosis. After testing the biochemical parameters, ^{131}I-CD147-Ab caused fewer side-effects in the animals.

Introduction

Hepatocellular carcinoma (HCC) is a major health problem in the world; it is the fifth most common cancer and the third cause of cancer-related mortality in the world (1). Moreover, HCC is often diagnosed at the advanced stage (2). Only a small proportion of patients are candidates for curative therapies, such as liver transplant or liver resection (3,4). In unresectable and non-transplantable HCC, chemoembolization with Lipiodol (iodized oil) is a common treatment. Although this method can reduce tumor growth, it does not significantly improve survival (5). Selective internal radiation therapy (SIRT) is another treatment option for non-resectable HCC. SIRT delivers the β ray radionuclides to the inner of the tumor and the β rays kill the tumor cells, without significantly affecting the normal hepatic cells. HCC patients have a high tolerance to this method compared to other treatments (6).

CD147 is a member of the immunoglobulin superfamily (7). It is a transmembrane glycoprotein that is categorized as an immunoglobulin superfamily of receptors (8). In the physiological status, CD147 expression is associated with MCT1 protein expression in retinal pigment epithelia and spermatogenesis (9). CD147 is also regarded as an inducer of matrix metalloproteinase which is related to various types of cancer (8,10-17). In HCC, the expression level of CD147 is elevated. When the expression of CD147 is blocked, HCC growth and metastasis are significantly inhibited (15,18-20).

Almost all blood supply of the tumor in the liver derives from the hepatic arterial system; thus, the transcatheter arterial (TA) procedure is suitable for delivering substances to treat HCC. Several studies have established its safety and efficacy with long-term survival rates comparable to those of surgical resection methods (21).

In this study we delivered the ^{131}I-labeled CD147 monoclonal antibody (^{131}I-CD147-Ab) to the tumor by TA in an established model of rabbit VX2 hepatic tumors and we studied the safety and antitumor effects of this new approach.

Materials and methods

Animal model and groups. This study was approved by the Institutional Animal Care and Use Committee of Zhengzhou University. New Zealand white rabbits (4 weeks of age, 2.5-3.5 kg) were used in the investigations. All rabbits were initially fed with standard food and water for a week during adaptation in the animal research facility. All rabbits were anesthetized using 3 mg/kg pentobarbital sodium (Alvetra,

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Abbreviations: HCC, hepatocellular carcinoma; TAI, transcatheter arterial infusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; BUN, blood urea nitrogen; Cr, creatinine; FT3, free triiodothyronine; FT4, free (unbound) thyroxin; TSH, thyrotropic-stimulating hormone; MMP2, matrix metalloproteinase-2; MVD, microvessel density; SPECT-CT, single-photon emission computed tomography

Key words: liver cancer, ^{131}I, CD147, transcatheter arterial infusion

Neumuenster, Germany) administered by ear vein injection prior to tumor implantation, interventional procedures and single-photon emission computed tomography (SPECT)-CT (Symbia T 16, Siemens, Germany) examination.

An orthotopic model of hepatocarcinoma was created using VX2 carcinoma as previously described (22). All rabbit models were randomly assigned to the 3 groups (n=14 each group) according to treatment; saline (group A), ^{131}I (group B), ^{131}I-CD147-Ab (group C). The treatments were performed by TA at 14 days after the animal models were established. Five rabbits in each group were sacrificed for histopathological examination at Day 7 after TA infusion (TAI) in all groups, and the rest of the animals were kept for survival study.

Treatment procedure and tumor response measurement. TAI was performed 14 days after tumor implantation using a digital subtraction angiography system (Allura Xper FD20, Philips Medical Systems, Best, The Netherlands) according to a method previously described (23). Briefly, a 4F vascular sheath was inserted into the right femoral artery of the anesthetized rabbits (1% sodium pentobarbital at 3 mg/kg intravenous infusion) (Terumo, Tokyo, Japan). Selective catheterization of the proper hepatic artery feeding the VX2 carcinoma was carried out by a 3F microcatheter (GP, Terumo), which was coaxially inserted through a 4F Cobra catheter (Cook Inc., Bloomington, IN, USA). Hepatic angiography was obtained with hand injection of 3 ml of contrast medium (Omnipaque 300; Ansheng Pharmaceutical Co., Shanghai, China) at a rate of ~0.5 ml/sec. The ^{131}I-CD147-Ab solution was infused carefully through the 3F microcatheter into the tumor-feeding artery. Then, the femoral artery was ligated and the wound was closed. All procedures were performed under DSA monitor and guaranteed the ^{131}I-CD147-Ab flow through the liver tumor.

The animals were scanned by SPECT-CT at Days 1, 7 and 14 following TAI. Images were acquired 300 sec for static images and at the speed of 25 sec per image for tomo-images.

The objective responses to treatment were evaluated according to the criteria by Therasse *et al* (24) including complete response (CR), a partial response (PR), stable disease (SD) and progressive disease (PD) as judged by the longest dimension or the sum of the longest dimensions of all measured target lesions or any new lesion. All SPECT-CT images were analyzed by two radiologists who were blinded to the experimental design.

^{131}I-labeled CD147 antibody. CD147-Ab was labeled according to Mather's method (25), Briefly, we dissolved 10 mg CD147 Ab powder in phosphate-buffered saline (0.1 mol/l, pH 7.4); antibody solution was added with 1700 MBq ^{131}I sodium iodine solution which was based on the weight of the rabbits (27 MBq/kg). N-bromosuccinimide (2 mg) was dissolved in 2 ml of 0.1 M PBS. NBS (200 μl) was added to the antibody/iodine mixture. The vial was gently swirled and the reaction was quenched by 10 mg/ml of human serum albumin (HSA) in 0.5 ml of 0.1 ml PBS. The reaction mixture was purified on a Sephadex-G25 column according to the manufacturer's instructions. The final production was taken into the sterile penicillin vial for the research. The iodine of the final production was >95% bound as demonstrated by high-pressure liquid chromatography (HPLC) and its immunoreactivity was >40%.

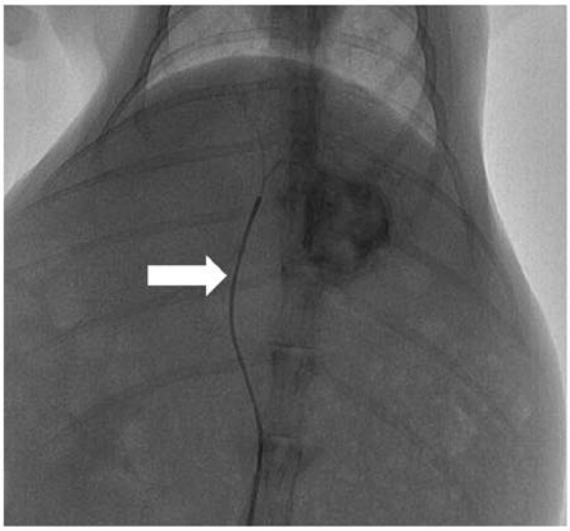

Figure 1. Representative rabbit hepatic angiographic images. Selective placement of 3F microcatheter (white arrow) and injection of ^{131}I-CD147-Ab in the proper left hepatic artery revealed ill-defined hypervascularity tumor staining in the liver.

Histopathological and immunohistochemical evaluation. The animals were sacrificed by intravenous injection of an overdose of sodium pentobarbital at Day 14 following TAI. The whole liver and lung were resected and fixed in 10% formalin and the specimens were cut into 4-μm sections in the axial positions corresponding to the section of the SPECT-CT scan. Hematoxylin and eosin (H&E)-stained sections were examined microscopically.

To evaluate TUNEL and MMP2 expression, the slides were stained by a TUNEL or MMP2 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions. Ten random non-necrotic areas (x200) from each specimen were evaluated. TUNEL and MMP2 expression were semiquantitatively evaluated at three levels: positive staining in <10% was regarded as negative (-), positive staining in 10-50% as weakly positive (±) and positive staining in ≥50% as positive (+) (26).

For determination of microvessel density (MVD), the paraffin-embedded sections were stained using anti-CD31 rabbit monoclonal antibody (Dako Corp., Carpinteria, CA, USA) following a standard SABC procedure, according to a method previously described (27). The MVD was determined by CD31 staining densities.

Briefly, five fields of 'vascular hot spots' with a 200-fold magnification in each tumor section obtained at 7 days after TAI were examined and the mean MVD value was recorded in a blinded fashion. The percentage of the necrotic area in the entire tumor area was calculated from H&E sections according to a previously described method (28).

Biochemical studies. Peripheral blood samples (2.0 ml) were collected for biochemical examination at Days 0, 1, 3, 7, 10, 14 and 21 after treatment. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), serum creatinine (Cr) and total bilirubin (TBIL) levels were measured using a biochemical autoanalyzer (Model LX 20; Beckman, CA, USA). Free triiodothyronine (FT3), free

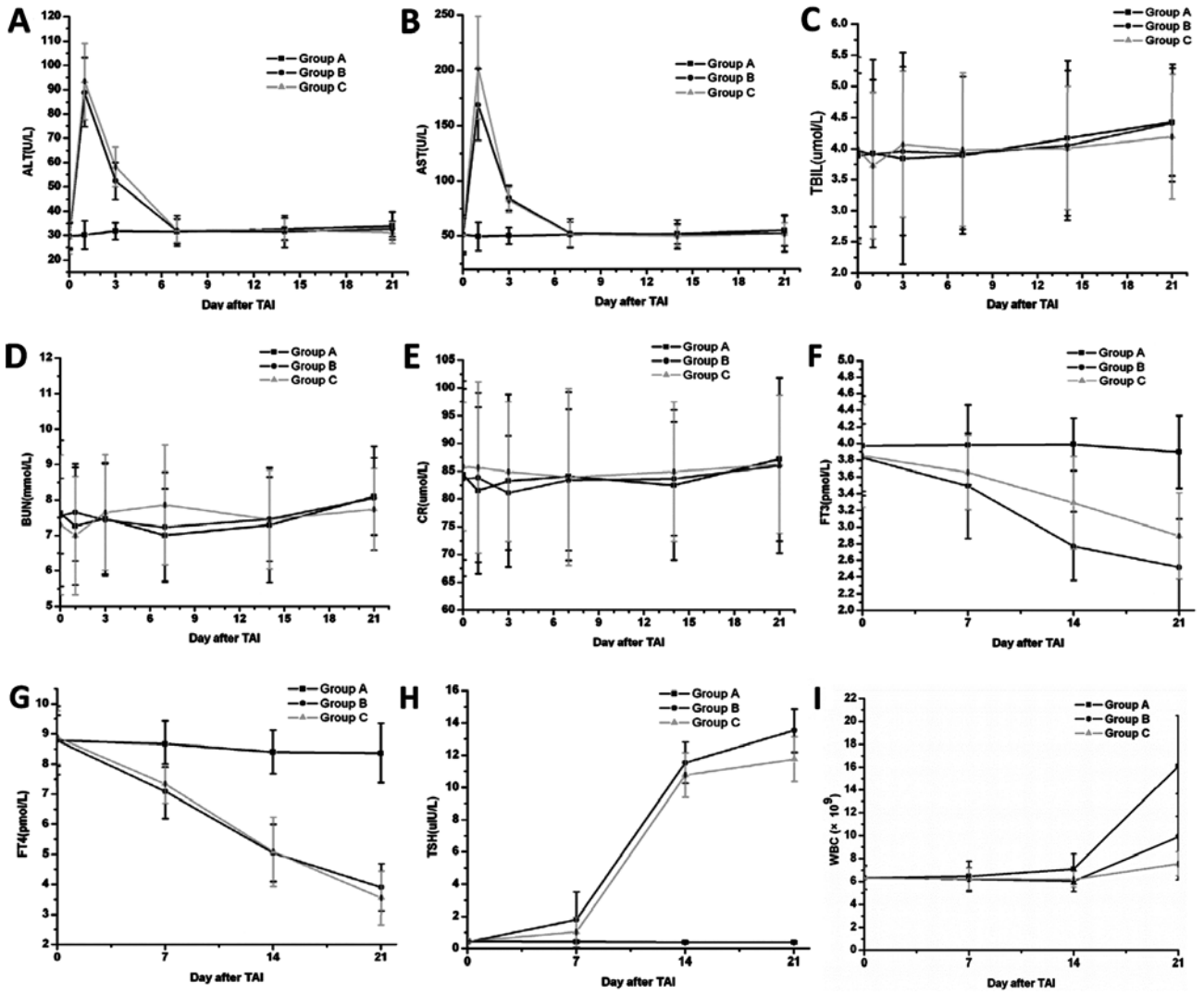

Figure 2. Biochemical test results. (A-C) Liver functions. The renal function test, the plasma BUN and Cr values were detected. (D and E) No statistically significant differences were found among the groups. (F-H) Thyroid gland functions changing during treatment. (I) WBC count did not change significantly until Day 21.

(unbound) thyroxin (FT4), thyrotropic-stimulating hormone (TSH) and white blood cells (WBCs) were also measured. Each sample was measured in triplicate.

Statistical analysis. Statistical evaluation was performed using SPSS software (ver.13.0; PSS Inc., Chicago, IL, USA). Numerical data were expressed as the means ± SD. $P<0.05$ was considered to indicate a statistically significant difference. The Kruskal-Wallis and Mann-Whitney U tests were performed among groups. Survival rates were assessed using the Kaplan-Meier method.

Results

One animal in group B died 10 days after the tumor implantation. This animal was excluded from further analysis. The remaining surviving animals all successfully underwent TAI (Fig. 1) and SPECT-CT procedures.

Biochemical tests. Liver function tests showed that AST and ALT levels increased transiently 1 day after intra-arterial infusion with ^{131}I or ^{131}I-CD147-Ab and lasted ~7 days, before returning to normal levels. The elevation of AST and ALT levels was significantly different ($P<0.05$) at Day 1 in the treatment groups compared with the saline group (Fig. 2A and B). The plasma TBIL (Fig. 2C) remained apparently unaltered in the three groups. For the renal functions, BUN (Fig. 2D) and Cr (Fig. 2E) levels did not significantly change in any group at any day.

To test the thyroid functions, we measured the FT3, FT4 and TSH levels in all groups at Days 0, 7, 14 and 21 after TAI. The value of FT3 and FT4 tended to decrease, whereas TSH increased; significant differences were observed only at Days 14 and 21 ($P<0.05$), and not at Day 7 ($P>0.05$) (Fig. 2F-H). The WBC did not change significantly until Day 21 following treatment (Fig. 2I).

Lung and liver tumor measurement. The gross size of the tumor measured by the ruler in group C was significantly smaller than in groups A and B. Intrahepatic metastasis was the least extensive in group C, as was lung metastasis (Fig. 3), with the fewest nodes observed in group C.

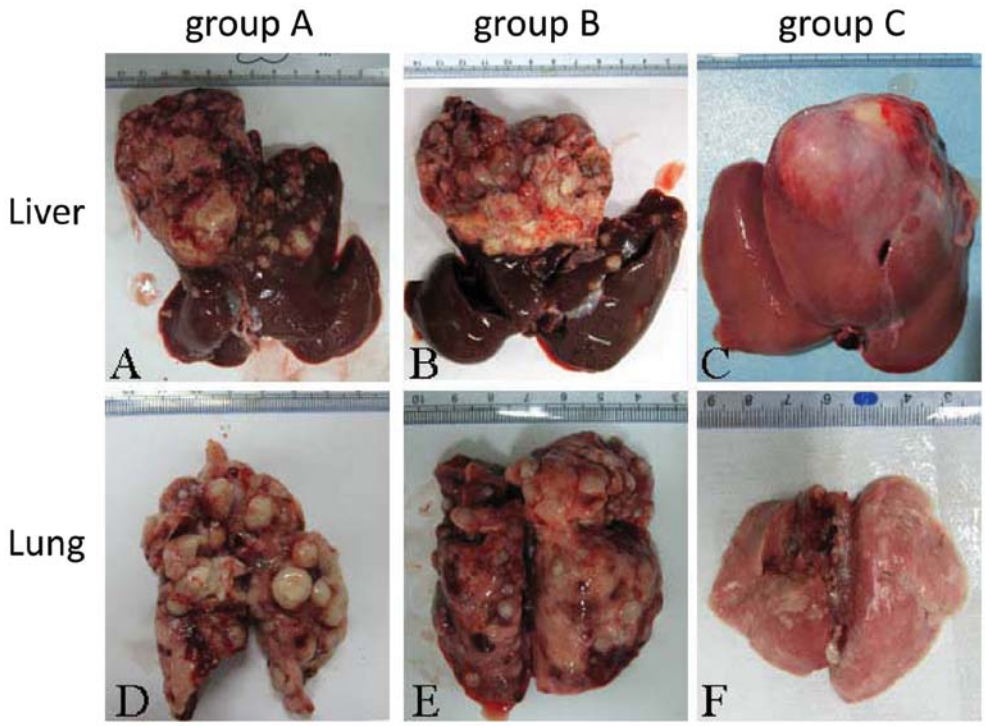

Figure 3. Representative pathologic specimens of liver (A-C) and lung (D-F). The size of the tumor was much smaller in group C than in groups A and B at Day 14 following TAI. The metastasis of the tumor was less extensive in group C (C and F), compared to groups A and B, and group B (B and E) presented less metastasis than group A (A and D).

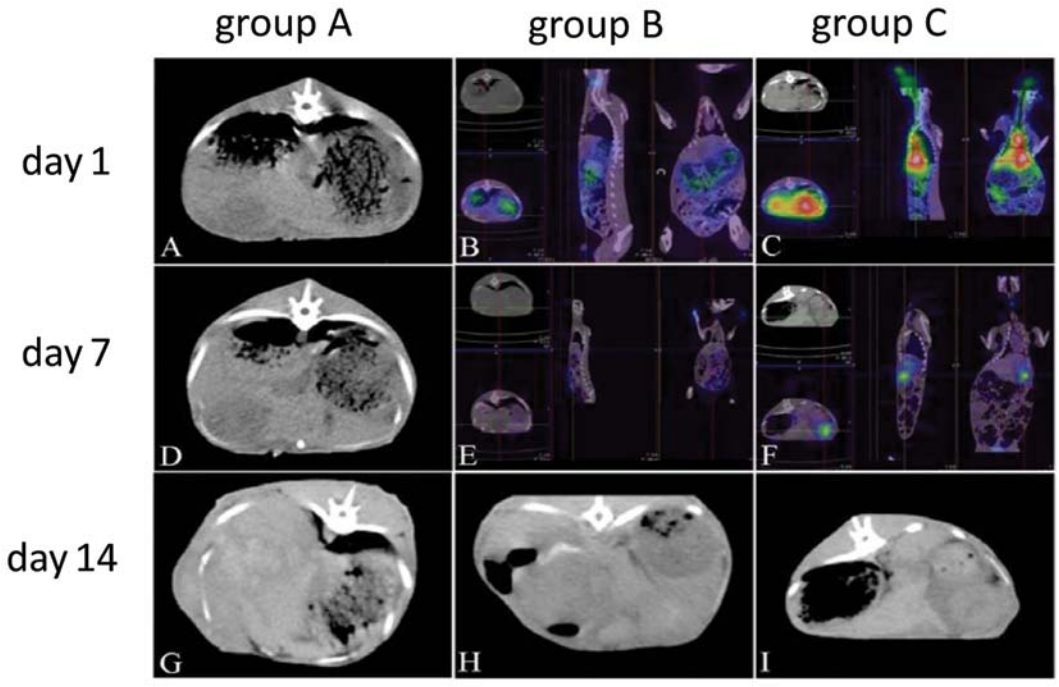

Figure 4. SPECT-CT imaging following treatment in all groups. The radionuclide uptakes of SPECT-CT in group C were much higher than in groups A and B (there was no ^{131}I in group A, therefore they cannot be imaged on SPECT) at Days 1 and 7 after TAI (A-F). The radionuclide uptakes disappeared at Day 14 following TAI in all groups. In the groups A and B the liver tumor increased faster than in group C.

SPECT-CT inspection. The liver tumors increased quickly in groups A and B, but not in group C (Fig. 4). The radionuclide uptake was markedly higher in group C than in B at Days 1 and 7 after treatment (Fig. 4B, C, E and F). This suggested that ^{131}I-CD147-Ab in group C binds to the tumor cell more effectively than the ^{131}I alone. At Day 14, the radioactivity had almost disappeared in all groups.

Treatment-associated tumor response. The tumor-inhibited effect as evaluated by the Response Evaluation Criteria in

Table I. Tumor response by RECIST criteria.

Group	Tumor response		
	Complete response (%)	Partial response (%)	Stable disease (%)
Group A	0	0	0
Group B	0	3 (37.5)	4 (50)
Group C	0	8 (88.9)	1 (11.1)

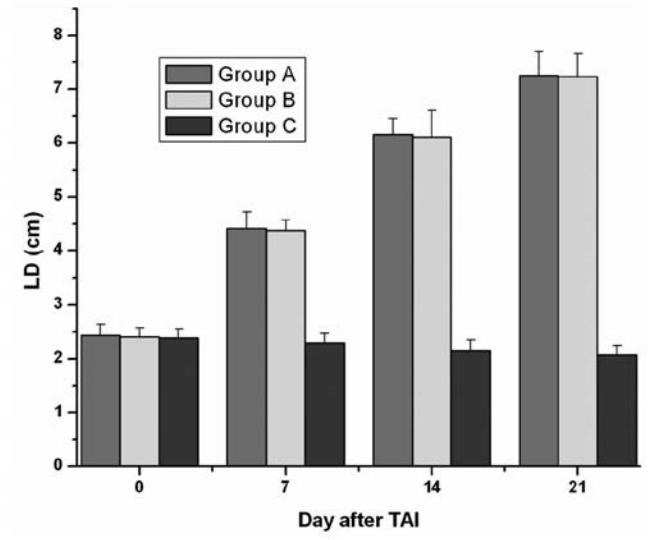

Figure 5. Antitumor effects evaluated by the tumor long-axis dimensions in the three groups. During the experimental period, the mean LD decreased significantly in group C compared to the other two groups.

Solid Tumors (RECIST) methodology at Day 14 is shown in Table I. A gradual decrease of the tumor's LD on SPECT-CT scanning was observed during the following 2 weeks in all group C rabbits treated by ^{131}I-CD147-Ab. The mean LD in group C at 1 and 2 weeks post-treatment was significantly smaller than in the other two groups (P<0.05, each; Fig. 5).

Histological findings

H&E staining. The necrosis of the tumor was confirmed by H&E staining. The necrosis of group C was much larger than in the other two groups (Fig. 6). The overall necrosis rate of group C was the highest (P<0.01) (Table II).

TUNEL, CD31 and MMP2 expression. The TUNEL expression was markedly high in group C compared to the other 2 groups (Fig. 7A-C), while CD31 (Fig. 7D-F) and MMP2 (Fig. 7G-I) expression were much lower than in the other 2 groups; there were no significant differences between groups A and B.

Survival. All rabbits died of hepatic and/or respiratory failure secondary to extensive tumor burden within 73 days. The animals treated by ^{131}I-CD147-Ab lived significantly longer than the animals in the other two groups (Kaplan-Meier method with log-rank test, P<0.001) (Fig. 8).

Table II. Mean tumor necrosis ratios in the different groups.

Group	Mean necrotic area ratios (%) (mean ± SD)
Group A	45.09±13.72[a,b]
Group B	47.64±7.55[c]
Group C	83.56±5.38

Mann-Whitney U test. [a]Group A vs. group B, P>0.05. [b]Group A vs. group C, P<0.001. [c]Group B vs. group C, P<0.001.

Discussion

Although transcatheter arterial chemoembolization (TACE) can extend the survival time of HCC patients, the overall survival rate of these patients remains poor (29,30). Xu *et al* used the Licartin (^{131}I labeled human CD147 monoclonal Ab) to treat HCC patients following liver transplantation (19). They found that Licartin decreases the recurrence rate and improves the survival rate. Although the survival time and metastasis of the ^{131}I-CD147-Ab treatment group were better than the other groups in our experiment, all the rabbits eventually died. However, the animals in the ^{131}I-CD147-Ab treatment group lived significantly longer than the animals in the other two groups, as we only gave one treatment to the animals in our study. If ^{131}I-CD147-Ab was given 2 or 3 times, as Xu *et al* reported in the clinic, the survival time would be much longer.

SPECT-CT and tumor size resected from the animals confirmed the inhibition of metastasis and growth in the treatment group. From the SPECT-CT examination, we found that the ^{131}I-CD147-Ab in the treatment group remained significantly longer in the tumor than groups A and B, indicating that the CD147 monoclonal antibody can combine its Ag effectively by TAI, thus, the ^{131}I and the CD147 monoclonal antibody can work collaboratively. Administration of the ^{131}I-CD147-Ab via TA was suitable for the delivery of this drug without any side-effects. ^{131}I-CD147-Ab decreases the MVD and MMP2 expression in the tumor, partly since the Ab blocked the expression of CD147 which can affect its downstream signaling. Also, the ^{131}I can kill tumor cells. All these work together to inhibit the tumor growth and metastasis. In this experiment, delivering the ^{131}I-CD147-Ab by TA was more specific for the tumor and caused fewer side-effects in the surrounding normal liver. This method is suitable treatment for unresectable HCC patients, with fewer side-effects.

Several groups have found that CD147 is related to tumor invasion and metastasis (12,13,15,20,31-33). Our data also showed that ^{131}I-CD147-Ab was closely associated with multiple processes of HCC invasion and metastasis. We found that the expression levels of CD31 and MMP2 were significantly decreased following treatment compared to the other two groups, as reported by previous studies (34-36). This may be one of the mechanisms by which ^{131}I-CD147-Ab prolongs survival time and decreases the tumor size in the animal model.

This report is the first to demonstrate that ^{131}I-CD147-Ab inhibits the tumor growth and metastasis in the animal model

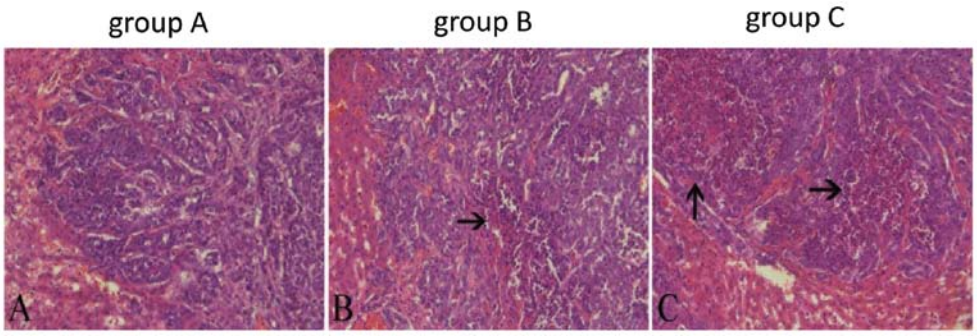

Figure 6. Representative H&E staining images. The necrosis areas of the three different groups are shown. Arrows point to the necrosis areas. The necrosis areas in group C were much larger (C) than those in group B (B) and in group A (A).

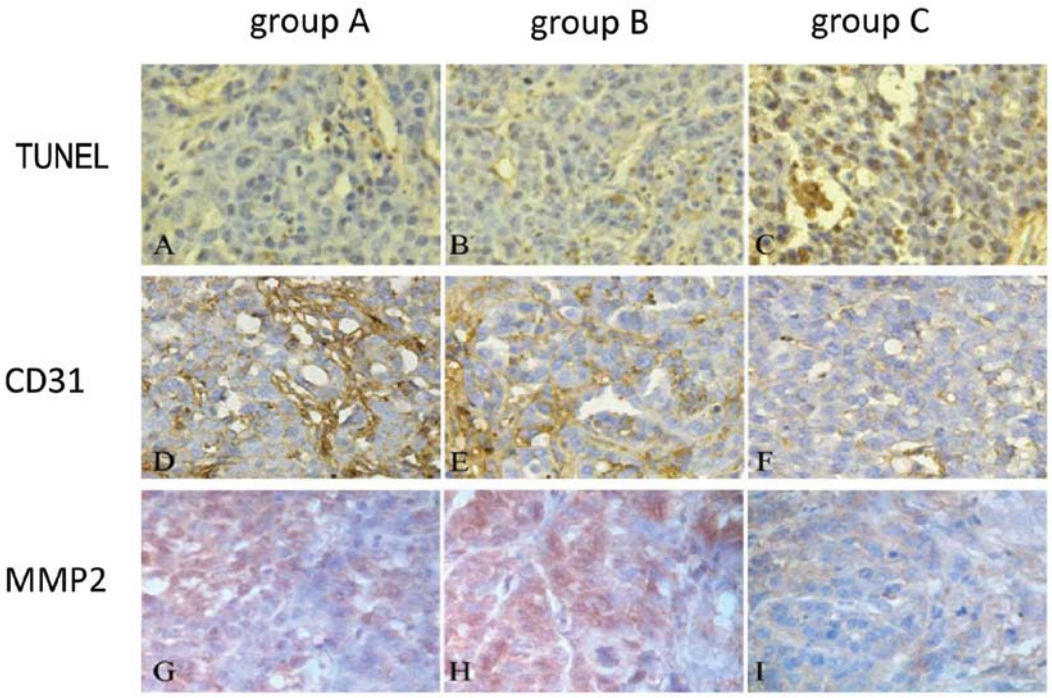

Figure 7. Representative immunohistochemical findings of TUNEL, CD31 and MMP2 in VX2 liver tumors in each group. Necrosis and MVD were stained by dark brown (original magnification, x200). Increased necrosis can be seen in group C (C) compared to group A (A) and group B (B). However, for the MVD, a marked reduction was observed in group C (F). The MMP2 was stained red brown. The density of MMP2 expression in group C (I) was much fainter than in the other two groups (G and H).

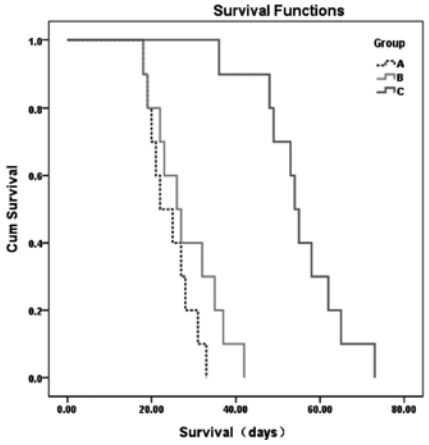

Figure 8. Kaplan-Meier survival analysis of liver tumor-bearing rabbits. Graph of the Kaplan-Meier method with the log-rank test shows a significant survival benefit for the animals treated with ^{131}I-CD147-Ab (group C) compared with those in groups A and B.

of VX2 carcinoma, a suitable model for the study of HCC. Although we obtained some encouraging results in our preliminary study, a lot remains to be elucidated and further studies into the treatment of HCC are required. However, our study has some limitations; first, we did not compare the therapeutic effects of ^{131}I-CD147-Ab with those of other chemotherapy drugs or ^{131}I-Lipiodol treatment which is already used for liver tumors. We used 14 rabbits per group in our study; future studies should include larger groups. Also, although ^{131}I-CD147-Ab can decrease tumor growth and metastasis, the specific mechanisms were not fully investigated; further studies into the underlying mechanisms are warranted.

Despite these limitations, this preliminary study demonstrated that ^{131}I-CD147 is a promising drug for HCC and may ultimately be used for clinical practice in the future.

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