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RAPID COMMUNICATION

Trans-arterial gene therapy for hepatocellular carcinoma in a rabbit model

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Abstract

AIM: To study the effect of adenovirus (Ad)-*p*53 gene therapy on hepatocellular carcinoma (HCC) in a rabbit model.

METHODS: VX2 tumor was grown in the liver of 24 rabbits. Animals were divided into four groups: group A receiving trans-arterial gene therapy (Ad-*p*53) only, group B receiving combined Ad-*p*53 therapy and trans-arterial embolization (lipiodol), group C receiving trans-arterial chemoembolization (lipiodol + mitomycin C), control group (D) receiving sodium chloride. Tumor volume (V1) was measured by using MRI (d 13). Interventional procedure was applied (d 14).Tumor volume (V2) was assessed by MRI (d 21) and the mean ratio (V2/V1) was calculated. After the second MRI, specimens of the liver were abstained and examined immunohistochemically using mutant-type *p*53 antibody. The positive expression was scored.

RESULTS: Compared with control group ($\bar{x}$ = 3.14 ± 0.64), therapeutic groups all showed a significant decrease in the tumor growth ratio ($P < 0.05$) . A slight difference was found between group A ($\bar{x}$ =2.35 ± 0.59) and group B ($\bar{x}$ = 1.75 ± 0.28) ($P = 0.048$). No statistically significant difference was observed between group B and group C ($\bar{x}$ = 2.00 ± 0.44). The positive expression rate of mutant-type *p*53 was the lowest in group B and significantly different between group A and group C ($P < 0.05$).Compared to the control subjects, groups A and C both showed a decrease in the expression of mutant-type *p*53, but there was no significant difference between them.

CONCLUSION: Trans-arterial Ad-*p*53 gene therapy can reduce tumor growth of HCC in rabbit model.

Key words: Liver hepatocellular carcinoma; Trans-arterial chemoembolization; Gene therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, responsible for an estimated 1 million deaths annually and a poor prognosis due to its rapid infiltrating growth and complicating liver cirrhosis[1]. To date, surgical approaches including liver resection and liver transplantation are regarded potentially as curative treatments for HCC, particularly in patients with small and non-invasive tumors[2]. However, only a small number of patients are suitable for surgical treatment due to multicentric tumors, extrahepatic metastases, early vascular invasion, shortage of donor organs, high complication rates and comorbidities[3-5].

Local methods of tumor ablation including trans-arterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation (RFA)[6-8], microwave coagulation therapy (MCT) and laser-induced thermotherapy (LITT), are promising additional tumor therapies, especially in patients with poor liver function and unresectable or multifocal tumors[9]. Since TACE was introduced as a palliative treatment in patients with unresectable HCC, it has become one of the most common forms of interventional proceduces[10-12]. TACE has been shown to reduce systemic toxicity, enhance local effects and thus improve the therapeutic results[11]. Its therapeutic effect is limited due to the lack of appropriate and reliable embolic agents in patients when the tumor is infiltrative in nature or hypovascular[13].

In the past years, it was proved that *p*53 gene absence is correlated with the occurrence of HCC[14]. Wild-type *p*53 tumor suppressor gene is praised as a gene guardian and the loss of wild-type *p*53 is thought to be responsible for the lack of apoptotic signals in tumor cells and thus for their uncontrolled proliferation and recurrence[15]. Many human tumors carry mutations in the *p*53 and mutant or absent *p*53 status has been associated with resistance to radiation therapy and to apoptosis-inducing chemotherapy[16]. Gendicine, a recombinant human Ad-*p*53 injection (Shenzhen Sibiono Bentech, China) has obtained a

drug license from the State Food and Drug Administration of China (SFDA; Beijing, China) and become the first commercially-licensed gene therapy drug in the world. Gendicine consists of advenovirus vectors and normal wild-type *p*53 tumor suppressor gene. Gendicine was used in clinical trial on patients with late-stage head and neck squamous cell carcinoma (HNSCC), showing that complete and partial regression of the tumor can be achieved in 64% and 32% of patients, respectively[17]. A similar study by Sze *et al*[18] also showed that hepatic arterial infusion of *p*53 gene in combination with chemotherapy results in tumor regression. The purpose of our study was to compare the effects of the gene therapy and other therapies on HCC in rabbit model.

MATERIALS AND METHODS

Tumor implantation

A total of 30 adult New Zealand white rabbits (3.0-3.5 kg) were used. The study was approved by the Animal Care Committee of Shandong University School of Medicine, and experiments were performed in accordance with the institutional guidelines. VX2 tumors were first grown for 2 wk on the hind legs of each of six carrier rabbits. Each carrier rabbit was used to supply tumor cells for implantation in four separate rabbits. The carrier rabbit was then killed under deep anesthesia by intravenous injection of 100 mg/kg pentobarbital. All animals were anesthetized with a mixture of promethazine (2.5 mg/kg) and ketamine hydrochloride (44 mg/kg) administered intramuscularly. Intravenous access was gained *via* a marginal ear vein and pentobarbital was given intravenously to maintain anesthesia. VX2 tumor tissue was excised from the carrier rabbit and placed in Hanks solution. Abdomens of the recipient rabbits were shaved and prepared with povidone iodine, and then a midline subxyphoid incision was made. The anterior surface of the liver was exposed and the harvested tumor cells (0.1-0.2 cm^3) were implanted onto the left lobe of the liver using a 21-gauge angioneedle. This method allowed the growth of a solitary, well-demarcated tumor. The abdomen was closed in two layers. Proper aseptic technique was rigorously observed during each implantation. After surgery, the animals were returned to their cages, kept warm, and monitored in the animal laboratory until they recovered from anesthesia. Buprenorphine (0.01 mg) was administered for analgesia when the animals showed pain or physical distress. The tumors were allowed to grow for another 12 d when they reached a mean diameter size of 1.0-2.0 cm. No animal died during that phase of the protocol.

Interventional therapy

After 2-wk implantation of VX2 tumors in the rabbit livers, transcather hepatic arterial embolization was performed. A total of 24 animals were treated in such a manner. The animals were brought to the angiography table. Surgical incision was done in order to gain access to the right common femoral artery, into which a 3-F sheath was placed. A specially manufactured 3-F catheter with a tip in the shape of a hockey stick (Terumo, Japan) was manipulated into the celiac axis, after which celiac arteriography was performed to delineate the blood supply to the liver and confirm the location of the tumor. In all cases, the tumor could be readily visualized as a region of hypervascular blush located on the left side, near the gastric fundus. The left hepatic artery, which nearly exclusively provides flow to the tumor, was selectively catheterized off the common hepatic artery. When the catheter was adequately positioned within the left hepatic artery, the mixture of drugs was infused into the artery. Group A received Ad-*p*53 (1×10^{11} VP/10 mL), group B Ad-*p*53 (1×10^{11} VP/10 mL) and 0.6-0.8 mL lipiodol, group C 0.5mg/kg mitomycin C and 0.6-0.8 mL lipiodol, group D 10 mL sodium chloride, respectively. Digital spot images obtained after interventional therapy demonstrated intense staining of the tumor consistent with excellent distribution of the mixture in the tumor. The catheter was then removed and the common femoral artery was ligated using resorbable suture material to obtain hemostasis. The procedure was successfully performed in all animals. Proper aseptic techniques were observed throughout the procedure. The animals were monitored after the procedure and given analgesics if they showed any sign of physical distress.

MR imaging

All studies were performed with a 3.0-T GE EXCITE Ⅱ MR scanner (Milwankee, Wisconsin, USA) using a knee coil before (d 13) and after therapy (d 21). T1-weighted (FSPGR: TR/TE, 120/2.1 ms) and T2-weighted (FSE:TR/TE, 3000/89.2 ms) transverse images with a section thickness of 3 mm, 0.5 mm gap and 128 × 128 matrix were acquired. All images were interpreted regarding tumor size, tumor necrosis and exhepatic metastatic lesions by two radiologists, respectively. No contrast medium was administered. The tumor volume was evaluated and calculated on T2-weighted image according to the following formula[19]: tumor volume (m^3) = largest diameter (mm) [smallest diameter (mm)]^2/2.

Histologic analysis

After completion of the MR imaging experiments, the liver was carefully dissected and excised. The liver containing tumor nodules, which were easily identifiable as hard white areas, was prepared for histologic examination. The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using mutant-type *p*53 antibody BAO521 at dilution: 1:100 (Boster Co., Wuhan, China). Positive controls were selected cases known to be positive for primary antibody (supplied by Boster Co.). Negative controls were stained with a nonspecific IgG (normal goat IgG) and Tris-buffered saline. Brown-yellow staining in nuclei of cancer cells was found in *p*53 positive cells. All slides were reviewed with JD morphology image analysis system (JD801, TSJEDA Technology, Inc) and percentage of positive expression was scored by two independent observers in blind. A few cases with discrepant scores were reevaluated to reach a final agreement.

Statistical analysis

The mean growth ratio of V2/V1 and the *p*53 protein expression were analyzed by using Dunnett's test (SPSS, USA) comparing the effect of each therapeutic group. $P < 0.05$ was considered statistically significant.

Table 1 Tumor volume (mm^3) and the mean ratio in different groups

No.	Group A *p*53			Group B *p*53 + TAE			Group C TACE			Group D		
	V1	V2	V2/V1	V1	V2	V2/V1	V1	V2	V2/V1	V1	V2	V2/V1
1	847	1296	1.53	650	908	1.40	1714	2267	1.32	580	1912	3.30
2	527	1008	1.91	196	320	1.63	879	2001	2.28	908	3402	3.75
3	405	936	2.31	612	1150	1.88	370	886	2.39	445	1089	2.45
4	900	2268	2.52	445	962	2.16	1470	3402	2.31	582	1538	2.64
5	385	1250	3.25	847	1296	1.53	786	1328	1.69	908	2468	2.72
6	446	1152	2.58	527	1008	1.91	668	1524	2.28	850	3402	4.00

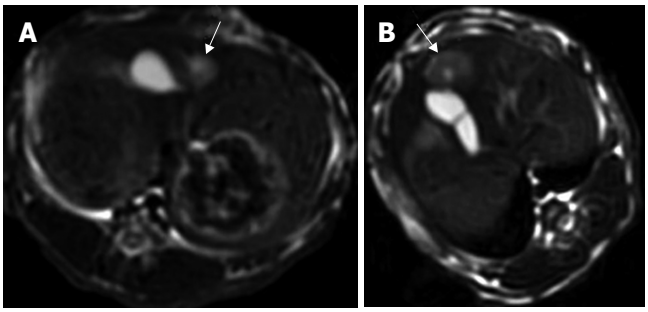

Figure 1 Images in a VX2 rabbit with a solid HCC in group B. **A**: Pretherapeutic unenhanced T2-weighted axial MR imaging with FSE sequence (3000/89.2). The hyperintense lesion with a size of 9 mm×10 mm is well discernible from the surrounding liver tissue. **B**: Post-therapeutic unenhanced T2-weighted axial MR imaging with FSE sequence (3000/89.2) showing the hyperintense lesion (15 mm × 11 mm) with central hypointense area corresponding to the intratumoral necrosis.

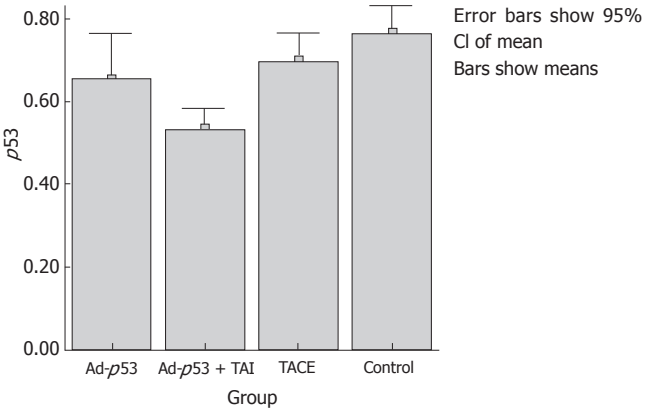

Figure 3 Positive cell rate of mutant-type *p*53 protein in different groups.

RESULTS

Tumor implantation

The rate of tumor implantation reached 100%. None of the animals died during implantation or interventional therapy. All the 24 VX2 tumors were seen with no enhanced MR imaging (100%) before treatment.

MR imaging

The mean ratio of V2/V1 was 2.35 ± 0.59 in group A, 1.75 ± 0.28 in group B, 2.00 ± 0.44 in group C and 3.14 ± 0.64 in group D. Compared to group D, the other groups all showed a significant decrease in the tumor growth ratio

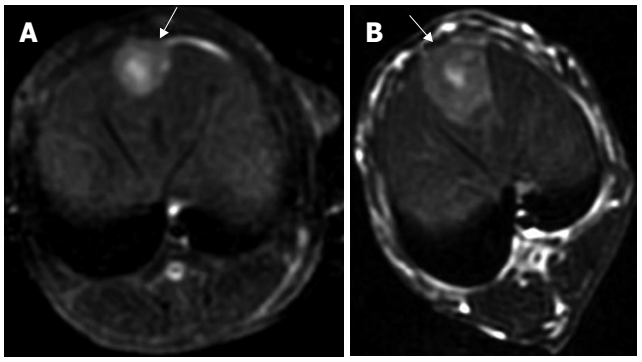

Figure 2 Images in a VX2 rabbit with a solid HCC in group D. **A**: Pretherapeutic unenhanced T2-weighted axial MR imaging with FSE sequence (3000/89.2). The hyperintense lesion with a size of 12 mm × 13 mm is well discernible from the surrounding liver tissue; **B**: Post-therapeutic unenhanced T2-weighted axial MR imaging with FSE sequence (3000/89.2) showing an inhomogeneous area corresponding to the intrahepatic metastasis. The tumor with a size of 23 mm × 27 mm had a rapid growth compared with that before therapy.

($P < 0.05$) using Dunnnett's *t* test. A slight difference in the tumor growth ratio was found between groups A and B ($P = 0.048$). Group A showed a little increase compared to group C ($P > 0.05$). There was no statistically significant difference between groups B and C (Table 1).

One intrahepatic metastasis lesion in group A and two intrahepatic foci in group D were detected respectively at T2 weighted imaging. Hyper-dense areas on the T2-weighted images, probably due to intratumoral necrosis with bleeding , occurred in three of six rabbits in groups B and C, respectively (Figures 1 and 2).

Pathologic analysis

The mean expression of mutant-type *p*53 was 0.66 ± 0.11 in group A, 0.53 ± 0.05 in group B, 0.70 ± 0.06 in group C and 0.77 ± 0.07 in group D. The positive cell rate of mutant-type *p*53 was the lowest in group B, and differed significantly from that in groups A and C ($P < 0.05$). Compared to the control group, groups A and C showed a decrease in the expression of mutant-type *p*53, but there was no significant difference between them (Figures 3 and 4).

DISCUSSION

Rabbit VX2 tumor model has been well established for many years. Because its vascularization is similar to that of human liver tumor, it has been used in clinical research on liver tumor imaging, chemotherapy and tumor etiology[20].

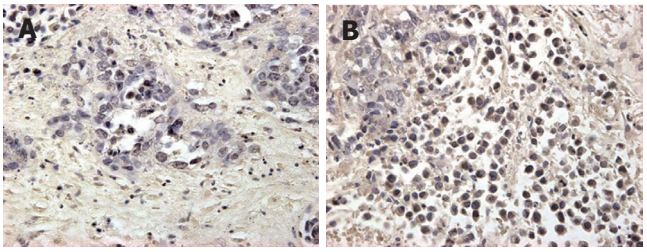

Figure 4 Histological examination of VX2 tumor in different groups. **A**: immunohistochemical stain showing the low expression of mutant-type *p*53 gene high in VX2 cells in group B, magnification × 400, **B**: The same stain showing the expression in group D, (× 400).

VX2 tumor is hypervascular, and cellular necrosis is more easy to occur at its early stage, which may influence the outcome evaluation of therapy. So this study applied the method of performing MR examination one week after interventional therapy. Because the tumor volume is different before therapy, we took the ratio of V2/V1 as the evaluation index for tumor growth.

The wild-type *p*53 gene is a tumor suppressor gene involved in the control of cell proliferation. Loss of wild-type *p*53 function is associated with the uncontrolled growth of many types of human cancer. The reintroduction and expression of wild-type *p*53 into *p*53-altered tumor could suppress tumor growth or induce apoptosis both *in vitro* and *in vivo*. In the present study, mutant-type *p*53 protein was detected in all VX2 tumors by immunohistochemical method. Ad-*p*53 is a recombinant adenoviral vector containing the wild-type human *p*53 gene. The wild-type *p*53 protein brings specific anti-tumor cells into effect by inducing apoptosis or necrosis, immune response, or by regulating cell cycle. Recently some studies have used Ad-*p*53 to suppress tumor growth in liver[17-18]. In our study, Ad-*p*53 therapy also suppressed the tumor growth in liver. Histological examination revealed the low expression rate of mutant-type *p*53 protein in group A, as shown in TACE therapy. Wild-type *p*53 gene can express normal *p*53 protein. However, normal *p*53 protein stays for a short time in blood and tissue and degrades quickly, making it hard to be detected. On the contrary, mutant-type *p*53 protein produced by mutant-type *p*53 tumor cells is easy to be found in tissue. We took the mutant-type *p*53 protein to reflect the effective of tumor reduction indirectly. Combined Ad-*p*53 therapy with trans-arterial embolization could inhibit tumor growth, suggesting that tumor growth is related with the local concentration of Ad-*p*53. Because of high-velocity blood stream, Ad-*p*53 gene drug may stay in the tumor for a short time and cannot exert its antiproliferative effects on liver tumor cells. Iodized oil could embolize the local vessels to slow the washout of local drug, and significantly enhance the anticancer effect of Ad-*p*53 gene drug.

TACE has become one of the most common interventional therapies. It has been shown to result in regression of HCC and reduction of systemic toxicity, thereby improving the therapeutic effects[21]. Some patients with unresectable HCC cannot endure the effect of chemotherapeutics, and some patients are resistent to the chemotherapy. However, they may receive the treatment with trans-arterial Ad-*p*53 and TAE instead of TACE. We should pay attention to the toxicity of Ad-*p*53.

In summary, combined TAE and Ad-*p*53 can suppress the growth of VX2 tumors and can be used in the treatment of HCC. However, more accurate and specific evaluation methods are required.

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