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In vivo comparison of transduction efficiency with recombinant adenovirus-mediated p53 in a human colon cancer mouse model by different delivery routes

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Abstract ***Objective:*** To evaluate transduction efficiency with recombinant adenovirus-mediated p53 (rAd/p53) therapy in a human colon cancer mouse model by intra-tumoral injection and intra-arterial delivery. ***Methods:*** The tumor pieces of human colon cancer SW480 were implanted in the livers of 45 nude mice. These mice were administrated with rAd/p53 by intratumoral injection and intra-arterial delivery. After 24 h, 48 h and 72 h rAd/p53 administration, 5 mice each group were killed with over anesthesia and their livers were removed. P53 expression and apoptosis of tumor and liver were assessed. ***Results:*** P53 expression and apoptosis of intratumoral administration group was higher than tail vein group and control group. Apoptosis and p53 expression of livers in three groups had no significant difference. ***Conclusion:*** p53 gene transduction efficiency and anticancer effect of rAd/p53 is much better by intra-tumoral injection than intra-arterial delivery.

Key words nude mouse; human colon cancer; hepatic allograft model; rAd/p53

In the treatment of carcinoma of preclinical study, Gene products are delivered into tumor by a variety of routes which include intratumoral injection [1–3], intraarterial gene delivery [4, 5], intraperitoneal injection [6] and intravenous administration [7–9], etc. However, because of different gene products and unit, it is difficult to evaluate gene transduction efficiency of these injection routes based on these studies. This study was designed to evaluation the p53 gene transduction efficiency and safe of rAd/p53 by intratumoral injection and intravenous administration. Animal experiments were performed in accordance with institutional guidelines and approved by the committee on the use and care on animal.

Materials and methods

Cell lines and cell culture

The human colon carcinoma cell line SW480 was purchased from Shanghai cell bank of chinese academy of Sciences. The cells were cultured in RPMI 1640 with 10% fetal calf serum, 1000 U/mL penicillin, and 1mg/mL streptomycin The cells were grown at 37 ℃ in a humidifield atmosphere of 5% CO_2 for 3–4 days until they reached the logarithmic phase of growth. Then the cells were enzymatically detached using 0.25% trypsin-EDTA mixture for 20 min. Trypan blue exclusion was used to determine viable cell counts. Cells were prepared for subcutaneous inoculation by re-suspension in 1×10^7 cells/1 mL of DBPS (Dulbecco's phosphate buffered saline).

Hepatic metastases model of colon cancer

BALB/c nude mice were obtained from the animal center of Sun Yat-sen university. Two female nude mice (6 weeks of age) were inoculated subcutaneously with 0.5×10^7 SW480 cells of each one. Ten days after tumor cell inoculation, the tumors in the subcutaneous tissue were excised and divided into small tissues of 1–2 mm^3. Laparotomy was performed on another anesthetized forty-six nude mice (5 to 6 weeks of ages, half female, half male),

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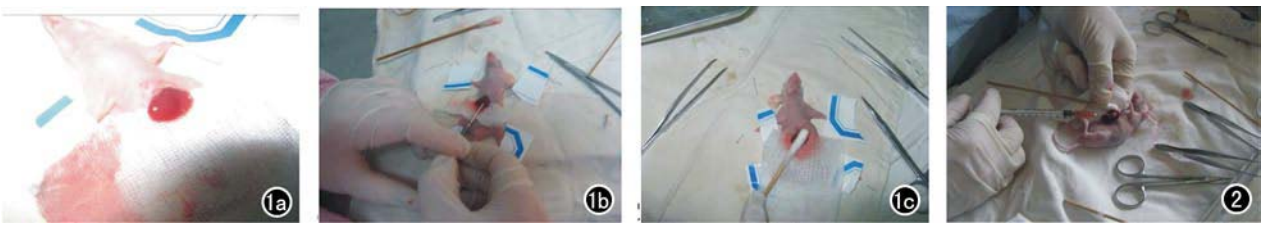

Fig. 1 The process of hepatic metastases model of colon cancer. (a) Exposure of mouse liver; (b) Injection of tumor tissue; (c) Oppression hemostasis with cotton stick

Fig. 2 Intratumoral injection of rAd/p53

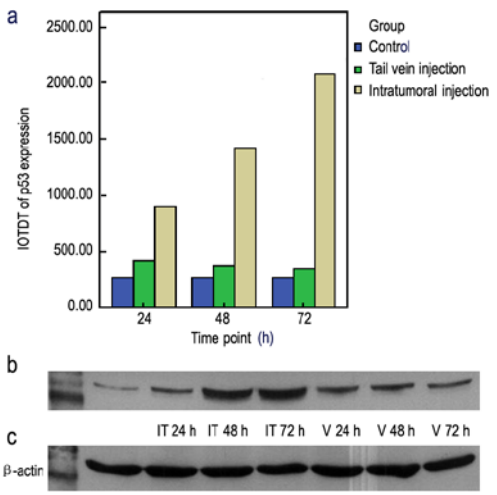

Fig. 3 p53 protein expression of tumor tissues in three groups. (a) The IOTDT of p53 expression; (b) Western blot analysis of p53 expression; (c) Control group. IT, intratumoral administration group; V, tail vein administration group

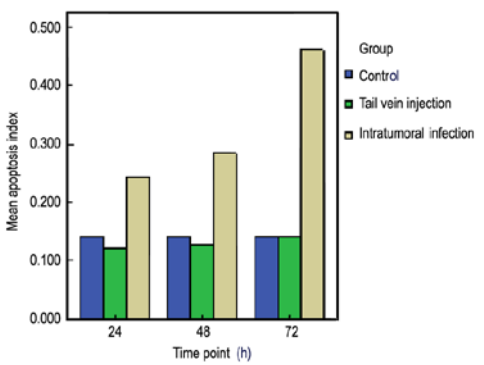

Fig. 4 The percentage of apoptosis of tumor tissues in three groups

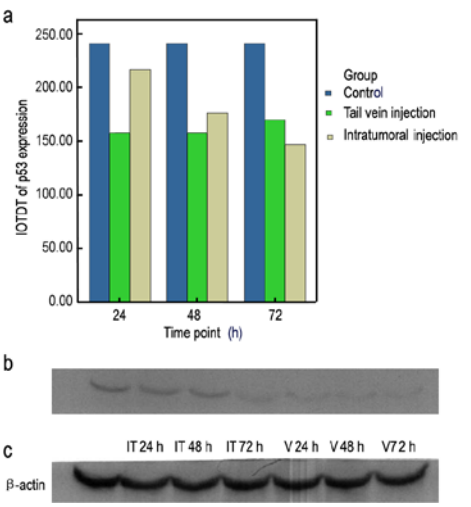

Fig. 5 p53 protein expression of mice liver in three groups. (a) The IOTDT of p53 expression. (b) Western blot analysis of p53 expression

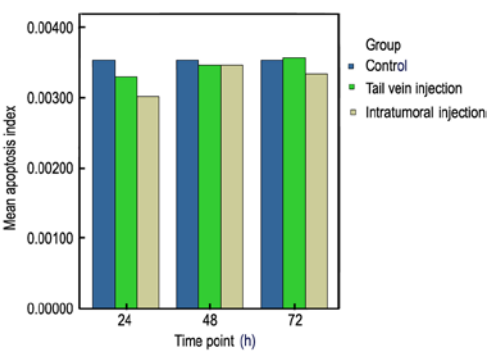

Fig. 6 The percentage of apoptosis of mice liver in three groups

and the small tumor tissues were injected into their livers, the peritoneal wall and skin were sutured (Fig. 1). One mouse with abnormal color of its liver was excluded.

Forty-two days after tumors were implanted, mice were random divided into three groups (15 mice/group; intratumoral injection group, tail vein injection group and control group). rAd/p53 (Gendicine, Sibiono GeneTech Co., Ltd, Shenzhen, China) were administrated intratumorally in anesthetized animals (Fig. 2) or through the tail vein. Immediately before rAd/p53 intratumoral injection, the tumor size was measured and the volume (v) was calculated according to $v= \pi/6abc$ [10]. The tumor diameter was regarded as 15 mm for mice through the tail vein. The rAd/p53 dose administered was 1×10^6 VP/mm^3 for intratumoral injection group, 2×10^6 VP/mm^3 for the tail vein group. The actual dose of tail vein injection was as 3.3–7.5 times as that of intratumoral injection.

At 24 h, 48 h, 72 h after rAd/p53 administration, 5 mice each group were kill with over anesthesia and their

Table 1 Tumor volumes in mice liver of three groups ($\bar{\chi} \pm s$, mm^3)

Time (h)	Control	Tail vein	Intratumoral
24	1241.14 ± 691.13	1103.19 ± 711.07	1124.18 ± 765.22
48	1212.27 ± 798.31	1287.72 ± 820.02	1137.19 ± 831.91
72	1093.18 ± 773.72	1219.35 ± 810.39	1116.82 ± 789.63

livers were removed. Tumors in the livers were divided equal half parts, one part was frozen in –30 ℃ refrigerate for western blot, another was fixed in PBS (pH = 7.3) containing 4% formaldehyde and 0.2% glutaraldehyde, embedded with paraffin, sectioned and H&E stained.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay

The in situ TUNEL assay was performed on paraffin-embedded tissue sections using an Apoptosis in situ detection kit (KeygenBiotech Co., Ltd, Nanjing, China) according to the manufacturer's instructions. Briefly, after deparaffinization, sections were incubated with 20 mL proteinase K for 15 min at room temperature and then washed with dH_2O. Endogenous peroxidase was inactivated by covering the sections with 2% H_2O_2 for 5 min at room temperature. The sections were rinsed with PBS and immersed in equilibration buffer for at least 10 sec. A working strength TdT enzyme was incubated in a humid chamber at 37 ℃ for 1 h. The reaction was terminated by transferring the slides to sop/wash buffer for 10 min and rinsing with PBS. Anti-digoxigenin peroxidase conjugate was applied to the slides for 30 min and rinsed with PBS. The sections were covered with peroxidase substrate (DAB) for 3 min and washed with dH_2O. Then, counterstain was done using 0.5% methyl green for 10 min at room temperature and washed with dH_2O. After washing with 100% n-butanol and mounting, apoptosis was evaluated. Apoptosis index was calculated as the percentage of positively stained nuclei and significant morphological change cells.

Western blot analysis

Western blot analysis was done to evaluate the effect of adenovirus-mediated wild-type p53 expression as described in instruction manual. Cell extracts were made from frozen tumor tissues. An immunoblot analysis was performed using anti-p53 monoclonal antibody (Santa Cruz Biotechnology, USA). Subsequent protein detection was performed using the enhanced chemiluminescence (ECL) detection system (Hitachi, Japan). The bands of p53 expression were scanned into computer as JPG formation and analyzed with imagepro-plus 6.0 soft to automatically get IOTDT of bands intensities.

Statistical analysis

SPSS 13.1 statistical package (SPSS, Chicago, USA) was used for all calculations. Tumor response of three groups were compared using log rank test and between groups using wilcoxon test which had a significance level of 0.05.

Results

Tumor volumes in mice liver of three groups

In order to confirm that tumor size among three groups are homogenous and random, tumor volumes in mice liver of three groups were compared (Table 1). Log-rank test show no significant difference among three groups (χ^2 & P at 24 h, 48 h, 72 h, respectively were 24, 0.548; 20, 0.350; 18, 0.139).

Expression of exogenous p53 protein in SW480 tumor tissue of three groups

A Western blot analysis was performed to examine the protein expression level of p53 in tumor tissue of three groups. A small amount of p53 was expressed at 24 h, 48 h and 72 h in tail vein group and control group (Fig. 3). A high level of p53 protein expression was observed at 48h and 72 h in intratumoral group (Fig. 3). The IOTDT of p53 expression of intratumoral administration group was increased at 24 h, 48 h and 72 h, and higher than tail vein group (Wilcoxon test, W statistics & *P* at 24 h, 48 h, 72 h respectively were 24, 0.548; 16, 0.032; 15.5, 0.016.) and control group (W statistics & P at 24 h, 48 h, 72 h respectively were 16, 0.016; 16, 0.016; 15, 0.008). The IOTDT of p53 expression between tail vein group and control group had no significant difference.

Apoptosis in SW480 tumor tissue of three groups

The induction of apoptotic cell death in SW480 tumor tissue of three groups was examined by TUNNEL staining. Either significant morphological change or TUNNEL-positive cells was observed in tumor tissue of three groups. The percentage of Apoptosis of intratumoral administration group was increased at 24 h, 48 h and 72 h, and bigger than tail vein group (Wilcoxon test, W statistics & *P* at 24 h, 48 h, 72 h respectively were 15,0.008; 15, 0.008; 15, 0.008) and control group (W statistics & p at 24 h, 48 h, 72 h respectively were 15, 0.008; 15, 0.008; 15, 0.008). The percentage of apoptosis between tail vein group and control group had no significant difference (Fig. 4).

Expression of exogenous p53 protein in liver of three groups

A small amount of p53 was expressed at 24 h, 48 h and 72 h in three groups (Fig. 5). The IOTDT of p53 expression among three groups had no significant difference (Log-rank test, χ^2 & *P* at 24 h, 48 h, 72 h, respectively were 4.769, 0.094; 1.292, 0.571; 0.907, 0.806).

Apoptosis in liver of three groups

Either significant morphological change or TUNNEL-positive cells was observed in livers of three groups. The percentage of Apoptosis of livers in three groups had no significant difference (Log-rank test, χ^2 & P at 24 h, 48 h, 72 h, respectively were 4.5, 0.104; 0.5, 0.815; 1.6538, 0.4803) (Fig. 6).

Discussion

Experiments showed that tumor cells transduced with the wt-p53 gene can inhibit *in vivo* tumor growth [1–3, 11, 12]. How to transfect exogenous p53 gene in high transduction efficiency into tumor cells is a key in the treatment of cancer with rAd/p53. In clinical practice, gene delivery routes directly affect gene transduction efficiency.

Intra-tumoral injection is most common gene delivery routes in animal experiment. Baek *et al* [1] treat syngeneic rat model for colorectal cancer with Ad5CMV, or Ad5CMV-p53 (2×10^{11} VP) or PBS through intra-tumoral injection at three divided dose every other day, tumor growth was significantly suppressed by intra-tumoral Ad5CMV-p53 therapy, more apparent apoptotic cells in Ad5CMV-p53-treated tumors than in other groups. In a Tamm *et al* study [2], treatment of mice with subcutaneously transplanted tumors or liver metastases of colorectal cancer cells with AdCMV. P16 via intra-tumoral injection resulted in significantly reduced tumor volume and prolonged survival. The Ad vector dose administrated was adjusted to the tumor size assuming 10^6 cells/mm^3. Another preclinical study of Adenoviral p53 gene therapy for esophageal cancer in vivo by intra-tumoral injection in the does of 2×10^{10} PFU showed that significant tumor growth suppression [3].

Shimada *et al* [13] investigated the therapeutic efficacy of phase I/II adenovirus-mediated p53 gene transfer in patients with chemoradiation resistant advanced esophageal carcinoma. On a 28-day cycle, intra-tumoral injections of Ad5CMV-p53 were administered on days 1 and 3 at four dose levels (10×10^{11} particles to 25×10^{11} particles) and treated for up to five cycles. Using PCR analyses, gene transfer and p53 specific transgene expression were detected in tumor biopsy tissue. mRNA levels of p53, p21 and MDM2 increased in all but one case. Three patients showed absence of disease upon repeat biopsies. Guan *et al* [14] treat 68 patients with advanced hepatic carcinoma via intra-tumoral injection of rAd/p53 (1×10^{12} VP per week for four weeks) at 48 h–72 h before transcatheter arterial embolization (TAE). The rate of tumor regression was higher than patients given TAE only.

However, the feature of multi-lesions for patients with advanced cancer leads to partial death of tumor cells in cancer lesions with intra-tumoral injection. Therapy gene can be injected into liver by a variety of routes. Intra-arterial gene delivery is another important route, especially for cancer in the deep organs or tissues. Intra-arterial pCMV-luc+ gene used as a reporter gene delivery in rabbit hepatic tumors was performed by selective catheterization of the hepatic artery, and selective gene expression in tumor cells was confirmed by means of immunohistochemical analysis for luciferase [4]. High volume hydrodynamic injection of plasmid DNA via the hepatic artery results in a high level of pCMV-SPORT-beta-galactosidase in rat hepatocellular carcinoma induced by diethylnitrosamine [15]. Hannay *et al* [5] delivered AdFlag p53 ($1 \times$, $2 \times$, 3×10^{11} VP) into human leiomyosarcoma xenografts by isolated limb perfusion (ILP). Seventy-two hours after delivery, Flag p53 expression was confirmed. These results showed that therapeutic gene can be successfully delivered by intra-arterial injection. In addition, intratumoral injection in combination with intra-arterial injection is also a delivery route in clinical study [16].

Intravnous injection which is convenient, minimally invasive and high repeatable is a common route of gene delivery in animal and clinical studies. Ohashi *et al* [7] treated liver cancer of mice with 2×10^9 PFU of Adv-AFP-E1AdB from tail vein, significant tumor-growth suppression was observed in tumor burden mice. 3×10^7 PFU Ad-CMV-CD were injected into the tail vein of hepatic metastases model of colon cancer, tumor growth in hepatic metastases reduced significantly [8]. In a study of intravenous administration of ALVAC-p53 at total does of $1 \times 10^{7.5}$ $CCID_{50}$ in advanced colorectal cancer patients, patients received ALVAC-p53 showed stable disease, patients of control group showed progressive disease [17].

It is difficult to compare and evaluate gene transduction efficiency via a variety of delivery routes as stated above, because of different kind and biological activity of therapeutic gene product. Therefore, in present study, p53 gene transduction efficacy with recombinant adenovirus-mediated p53 (rAd/p53) therapy in a human colon cancer mouse model by intratumoral injection and intravenous delivery was evaluated. The rAd/p53 dose administrated was adjusted to the tumor size assuming 10^6 cells/mm^3. The dose of rAd/p53 via intravenous route was as 3.3–7.5 times as that of intratumoral injection. Exogenous wt-p53 gene is delivered into tumor and succeeded in transcription in tumor cell which leads to wt-p53 gene expression [18, 19]. High expression of exogenous p53 protein induces tumor cell arrest at the G1 stage of the cell cycle and apoptosis by inhibiting proliferation of cells through several biologic pathways [18, 19]. In this study, p53 gene transduction efficiency was assessed with p53 protein expression and apoptosis of tumor cells by western blot and TUNNEL at 24 h, 48 h, 72 h after rAd/p53 delivery.

We herein reported the findings of a preclinical study which reveal escalation of p53 protein expression and apoptosis of tumor cells after both intratumoral and in-

travenous injection rAd/p53 from 24 h to 72 h. p53 protein expression and apoptosis of tumor cells had no significant difference between intravenous injection group and control group. P53 protein expression and apoptosis of tumor cells in intratumoral injection group was significantly higher than that in control group from 24 h to 72 h, and significantly higher than that of intravenous injection group at 48 h and 72 h. These findings showed that the anticancer effect of rAd/p53 via intratumoral injection was much better than intravenous injection. When p53 products are injected intravenously, barriers such as the endothelial lining of tumor vasculature impair the efficiency of adenoviral vectors for gene delivery into tumor [20, 21]. In addition, we think dilution in blood and reduced biological activity of transportation in blood may be related factors. This study also showed that p53 protein expression was very low in liver tissue, and apoptosis of mice liver cells was less than 1%, which suggested rAd/p53 cannot substantially increase transduction of mouse liver and is safe to liver cell.

A clear limitation of the present study is lack of intra-arterial delivery group because we could not succeed in catheter hepatic artery of mouse. Increased intra-arterial delivery group to further evaluate the p53 transduction efficacy through a variety of delivery routes common used in clinic is necessary.

In summery, rAd/p53 can increase transduction of p53 into colon cancer cells either via intratumoral or intravenous injection. However, even with rAd/p53 does as 3.3–7.5 times as that of intratumoral injection, intravenous injection had much lower p53 transduction efficacy and poorer anticancer effects than intratumoral injection. rAd/p53 is safe to liver with both gene delivery.

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