Clinical antiangiogenic effect of recombinant adenovirus-p53 combined with hyperthermia for advanced cancer

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Objective: To assess the safety and clinical antiangiogenic effect of recombinant adenovirus-p53 (rAd-p53) combined with hyperthermia plus or not plus radiotherapy in advanced cancer.

Methods: Expression of Vascular epithelial growth factor (VEGF) after intratumoral injection of rAd-p53 was assayed by immunohistochemistry (IHC) imaging. Forty-four patients with advanced cancer were enrolled into this clinical study. The patients were intratumorally injected with rAd-p53 (Gendicine) at a dose of 1×10^{12} vp once a week, with a total of 4-54 (mean 7.7) times. Total of 4-29 (mean 8.5) times of hyperthermia was given to the patients. Among the 44 patients, 30 patients were concurrently added with radiotherapy of a total dose 30-76 Gy/15-38 f/3-8 w (mean 58 Gy).

Results: Before and after intratumoral injection of rAd-p53, the VEGF IHC positive cell scores were 2.80 and 1.50, respectively (P=0.031). The treatment of rAd-p53 combined with hyperthermia plus or not plus radiotherapy in advanced cancer achieved CR rate of 13.60% (6/44), and PR rate of 29.6% (13/44), and thus the effective rate was 43.2%. In addition to 6 patients with CR, 19 patients (19/38, 50.0%) had low density area (LDA) of more than 50% area on CT image within tumor indicating tumor tissue necrosis.

Conclusions: Our data indicate that rAd-p53 inhibits VEGF expression and angiogenesis, and promotes tumor necrosis and shrinkage induced by hyperthermia plus or not plus radiotherapy in advanced cancer.

Keywords: Vascular epithelial growth factor (VEGF); recombinant adenovirus-p53 (rAd-p53); advanced cancer; hyperthermia; radiotherapy

Introduction

Tumor suppressor gene *p53*, well known as the genome guardian of cells, plays a key role in cell cycle control, apoptosis, and inhibition of tumor cell proliferation. *p53* gene also acts as a transcription factor and mediates cellular response to DNA damage induced by irradiation, hyperthermia, and cytotoxic agents (1,2).

Introduction of normal *p53* gene using viral vectors results in suppression and reversal of the malignant phenotype of tumors and induces thermosensitization or radiosensitization, which is a new strategy to convert a thermo- or radio-resistant phenotype into a thermo- or radio-sensitive one (3-5). Thus, recombinant adenovirus-p53 (rAd-p53) could act as a strong thermosensitizer or radiosensitizer for tumor therapy. These results support the combination use of *p53* gene therapy and hyperthermia or radiotherapy in antitumor treatment. Up to now, hyperthermia is not considered as a unitary clinical treatment method for cancer, because hyperthermia alone is negligible for late-stage cancer. Current hyperthermia alone has been an assistant method for cancer treatment. rAd-p53 acts as a thermosensitizer for hyperthermia, and upgrades hyperthermia to radical cure for patients with cancer.

rAd-p53 (trademarked as Gendicine) is an E1-substituted

replication-incompetent recombinant adenovirus encoding human *p53* gene. Gendicine is a *p53* gene therapy drug approved to market by China SFDA at October of 2003.

Vascular epithelial growth factor (VEGF) has been considered to induce angiogenesis, which is indispensable to tumorigenesis and progression. Introduction of wild-type *p53* gene into cancer cells with mutant *p53* gene markedly inhibited the expression of an angiogenic factor, VEGF, and increased the expression of a novel antiangiogenic factor, brain-specific angiogenesis inhibitor 1 (BAI 1), resulting in decrease in neovascularization *in vivo*. Microvessel density and microvessel counts were lower in xenografts from tumor cells containing wild-type *p53*. Overall data suggest that rAd-p53 is antiangiogenic, which may explain, in part, the mechanism of clinical antiangiogenic effect of *p53* gene combined with hyperthermia plus or not plus radiotherapy for advanced cancer, shown as tumor regression and obvious necrosis (6-8).

Continuously, a combination of rAd-p53 and hyperthermia was adopted in this study. This study aimed to further confirm that the effects and mechanism of combination of rAd-p53 and hyperthermia in 44 patients with advanced cancer.

Materials and methods

Immunohistochemistry (IHC) imaging

After intratumoral injection of rAd-p53, the adenoviral particle infects targeted tumor cells and delivers the adenoviral genome carrying the therapeutic *p53* gene to the cell nucleus for transcription. Biopsies before injection and 48 h after the first intratumoral injection of rAd-p53 were assessed for P53 protein and P53-targeted genes *p21*, *Bax*, and *VEGF*, the downstream P53-transactivated genes, by IHC staining in 50 couples of available samples of 25 patients. IHC staining of paraffin-embedded tissues was performed according to standard methods. Sections in which more than 75% of cells had definitive nuclear reactivity were scored 4; 50% to 75%, 3; 25% to 50%, 2; 5% to 25%, 1; and fewer than 5%, 0.

Patients' characteristics

This is a clinical observation protocol only, because the enrolled patients were in advanced stage including recurrent cases failing in surgery, radiotherapy or chemotherapy, and all enrolled tumors were refractory and uncurable. Antitumor effects of rAd-p53 alone was not confirmed in

clinical study before, so there were no patients receiving rAd-p53 alone used as control in this study. Our previous preclinical *in vitro* study demonstrated that after wild-type *p53* mediated by adenovirus was transferred into four kinds of human gastric carcinoma cell lines with different *p53* status, the expression of P53 protein in cell nucleus, increased radiation-inducing G2/M arrest and apoptosis, and increased radiosensitivity were found. So in clinic, it does not need to detect patient's *p53* gene status beforehand (1-4).

Details about patient approval to participate

Inclusion criteria

Patients should be 18 to 80 years old, and had a histological diagnosis of malignant tumor in advanced stage with measurable disease and no distant metastasis. The patients were clinically staged according to the fifth edition of the International Union against Cancer (UICC) TNM staging systems [1997]. Patients must have a projected life expectancy of at least three months and a Karnofsky performance score of at least 70%. Patients were required to have adequate bone marrow function (white blood cell count $\geq 4.0 \times 10^9$/L, hemoglobin ≥ 7 g/L, platelet count $\geq 70 \times 10^9$/L) and adequate liver and renal function [aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine (Cr) <1.5 times of the upper limit).

Exclusion criteria

Pregnant or nursing women, patients with uncontrolled serious infection, or patients with serious heart and lung failure were excluded.

Study protocol

This is a non-randomized controlled clinical study, and was approved by the Ethics Committee of Beijing Cancer Hospital at Sep 12, 2001.

In this study, we used intratumoral injection of rAd-p53 combined with hyperthermia plus or not plus radiotherapy for treatment of advanced cancer. Between October 2001 and December 2009, 44 patients, including 33 males and 11 females with a median age of 54.3 years old (range of 23-80 years old), were enrolled. All these patients were diagnosed as advanced cancer by pathohistological examination. Among the 44 patients, 21 patients had squamous cell carcinoma (9 in nasopharynx, 3 in larynx, *et al*.), 6 patients had adenocarcinoma (1 adenoid cystic carcinom, 1 thyroid adenocarcinoma, *et al*.), and 17 patients

had soft tissue sarcoma (4 liposarcoma, 2 malignant neurinoma, *et al*.). There were 38 patients with recurrent tumors and 6 patients with primary tumor receiving no treatment before they were enrolled. Of these 38 patients with recurrent tumors, 34 person-times failed in surgery, 30 person-times failed in radiotherapy, and 21 person-times failed in chemotherapy. Most of the 38 patients failed in combination of three types of treatment, 12 patients failed in two types of treatment, and 5 patients failed in one type of treatment.

Treatment options

All patients were intratumorally injected with rAd-p53 at multiple points, at a dose of up to 1×10^{12} viral particles (vp) once a week, with a total of 4-54 (mean 7.7) times. Intratumoral injection was done directly, or guided by ultrasound for neck node, abdominal, pelvic deep-seated tumor, or guided by computed tomography (CT) for lung tumor. Hyperthermia was given weekly 2 d after rAd-p53 injection at 43-44 ℃ using a 915 MHz microwave machine for 1 h for superficial tumor, and at 42-43 ℃ using a 41 MHz radiofrequency machine for 1 h for deep-seated tumor. Total of 4-29 (mean 8.5) times of hyperthermia was given to all patients.

Among the 44 patients, 30 patients were concurrently added with radiotherapy using the 6 or 8 MV linear accelerator X-ray with the conventional fractionation 2 Gy once a day from Monday to Friday to a total each week at a total dose of 30-76 Gy/15-38 f/3-8 w (mean 58 Gy).

Response assessment and adverse events

One longest diameter of each tumor lesion was measured according to CT or magnetic resonance imaging (MRI) findings of tumor. Tumor shrinkage rate of rAd-p53-injected tumor after 2-month treatment was counted by comparison to the pre-treatment tumor size. The tumor shrinkage rate was graded using Response Evaluation Criteria in Solid Tumor (RECIST) Guidelines (one-dimension): complete response (CR), disappearance of lesions; partial response (PR), more than a 30% decrease; stable disease (SD), neither PR nor progressive disease (PD); and PD, more than a 20% increase (9). The tumor density measurement (Hounsfield unit, HU) on CT images is a good indicator and provides a reliable quantitative means of monitoring the tumor. After treatment, low-density area [low density area (LDA), CT value <25 HU] on CT images

appeared and expanded within the tumor that did not regress completely. Histopathologic examinations showed the LDA to be massive coagulation necrosis. A greater extent of LDA indicated the improvement of local tumor control and survival of patients (10-12).

The patients were monitored for adverse events. Toxic and adverse events were assessed as light (grade 1), mild (grade 2), serious (grade 3), and life-threatening (grade 4) according to the WHO's evaluation standard for adverse events. Particular attention was paid to body temperature.

Statistical analysis

Overall survival (OS) was calculated according to the Kaplan-Meier method from the first date of treatment to the date of death. If a patient was not dead, then survival was censored at the time of the last visit. Version 11.0 of the SPSS statistical program (SPSS Inc., Chicago, IL, USA) was used for analysis. $P<0.05$ was considered statistically significant.

Results

Treatment and efficacy

The research showed that rAd-p53-specific *p53* mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis in the 16 tissue samples out of 17 (94.1%) assessable samples taken at 48 h after intratumoral injection of rAd-p53. The expression of *p53* gene and *p53* downstream genes in the tumor samples taken at 48 h after intratumoral injection of rAd-p53 were assayed by IHC. Up-regulation of cell cycle relative gene *p21* and apoptosis relative gene *Bax*, and down-regulation of *VEGF* were observed in tumor tissues after injection of rAd-p53. Immunostaining was scored semiquantitatively. The positive cell scores before and after injection were 1.44 and 2.48 for P53 (P=0.050), 0.32 and 0.88 for P21 (P=0.015), 0.92 and 1.63 for Bax (P=0.088), and 2.80 and 1.50 for VEGF (P=0.031), respectively (5).

As shown as *Figure 1*, the VEGF immunostaining in the tumor tissues from two patients before and after intratumoral injection of rAd-p53 showed VEGF positive in 75% of cells before intratumoral injection of rAd-p53 and negative in all cells after injection of rAd-p53.

After treatment by intratumoral rAd-p53 injection combined with hyperthermia, the CR rate was 13.60% (6/44), the PR rate was 29.6% (13/44), the SD rate was 52.3% (23/44) and the PD rate was 4.5% (2/44). The
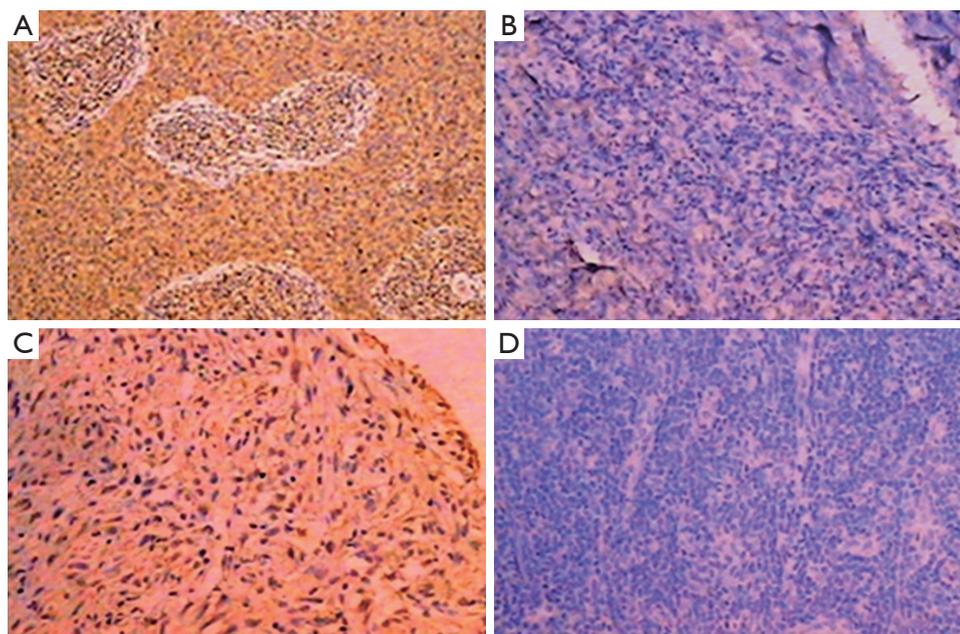
Figure 1 Tumor tissue sections of two patients before and after treatment. (A,B) One patient with nasopharyngeal cancer, before treatment (A), Vascular epithelial growth factor (VEGF) immunohistochemistry (IHC) is positive (+75%), and after intratumoral injection of rAd-p53 (B), VEGF IHC is negative; (C,D) The other patient with leiomyosarcoma, before treatment (C), VEGF IHC is positive (+75%), and after intratumoral injection of rAd-p53 (D), VEGF IHC is negative.

responsible rate (CR + PR) was 43.2% and non-responsible rate (SD + PD) was 56.8%. Three patients in the CR patients were considered a pathologic complete response (PCR). There were 19 patients' CT scans showed clear LDA and their CT value decreased to <30 HU. At 2-month after treatment, the samples from the LDA were almost complete coagulation or colliquation necrosis and no tumor cells appeared (*Figures 2,3*).

Figure 2 shows the CT scans and histopathologic examination from a typical case: 46-year-old man with recurrent mucoid liposarcoma, 6 cm in diameter, in retroperitoneum. CT value was 86 HU before treatment. The patient received intratumoral injection of rAd-p53, at a dose of 1×10^{12} vp once a week, for 7 times combined with 11 times of deep-seated hyperthermia and 56 Gy of irradiation. At 1-month after treatment, there was no change in tumor size but CT value went down to 25 HU. Obvious liquefaction necrosis was observed on CT image. Histopathologic examination showed that the sarcoma cells disappeared and a lot of lymphocyte cells were full of resected tissue.

Figure 3 shows the data of another typical case: 28-year-old man with recurrent spindle type sarcoma,

7.0 cm in diameter, in submaxilla. The tumor involved in right thyroid cartilage and was unresectable. The patient was treated with nine times of intratumoral injection of rAd-p53, nine times of superficial hyperthermia and 40 Gy of radiotherapy. The tumor size reduced to 4%, and CT value was reduced from 58 HU before treatment to 27 HU. Three months later, the tumor was completely removed and obvious necrosis could be observed in tumor tissue. Above 70% tumor cells were disaggregation under microscope.

OS was calculated using Kaplan-Meier method. The date of the last follow-up was December 2009 and the median follow-up time was 11.5 (range 3-76) months. The median survival time (MST) was 12.0±1 months [95% confidence interval (95% CI): 10-14 months] for the 44 patients. The 1-, 2-, 3-, 4- and 5-year OS was 43.7%, 27.2%, 21.2%, 14.1%, and 7.1% , respectively.

Adverse events

Total data showed that all the values in blood, urine and stool examination, and liver and renal function remained within normal range before and after treatment in the group receiving rAd-p53 combined with hyperthermia. Lung
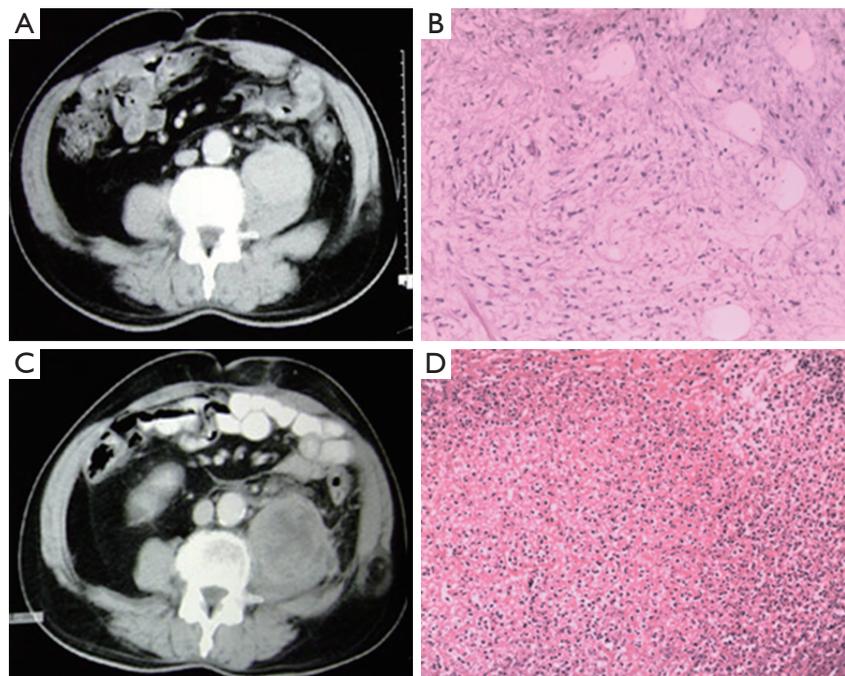
Figure 2 CT scans and histopathologic examination of a patient before and after treatment. (A) A 6 cm diameter tumor in retroperitoneum, CT value 86 HU; (B) Histologically diagnosed as mucoid liposarcoma before treatment; (C) Almost no change of tumor volume but CT value down to 25 HU and obvious liquefaction necrosis in tumor after treatment; (D) Disappearance of sarcoma cells and a lot of lymphocyte cell being full of resected tissue after treatment.
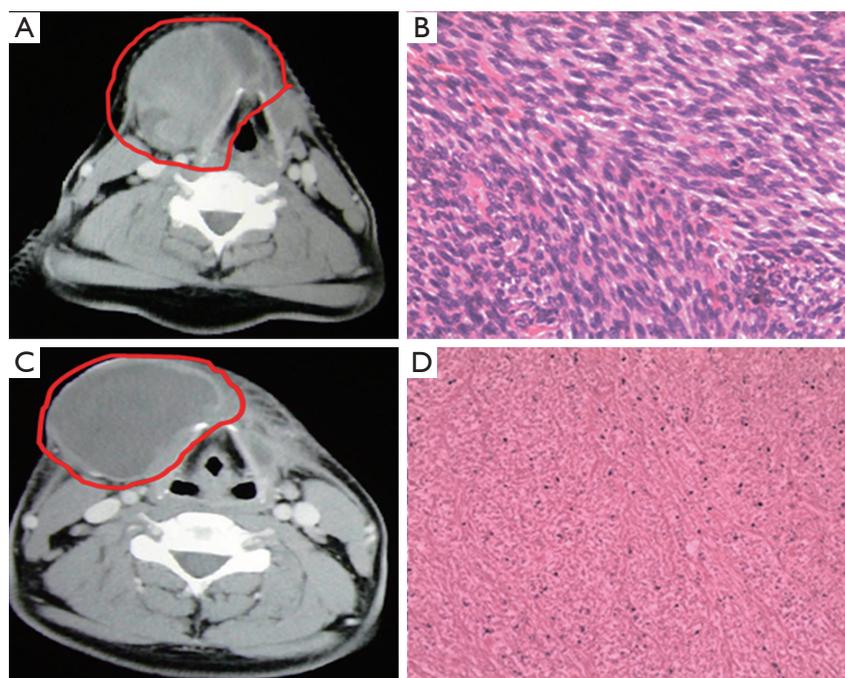
Figure 3 CT scans and histopathologic examination of a patient before and after treatment. (A) A 7 cm diameter tumor in submaxilla, CT value 58 HU; (B) Histologically diagnosed as spindle type sarcoma before treatment; (C) Almost no change of tumor volume but CT value down to 27 HU after treatment; (D) Spindle type sarcoma cells degeneration and necroses ratio >75% in resected sample after treatment.

and heart function was normal before and after treatment. This indicates that rAd-p53 was safe and well-tolerated in the patients with advanced cancer. Additionally, rAd-p53 administration did not appear to increase the adverse effects caused by hyperthermia and radiation treatment.

All fever events were grade 1 (less than 38 ℃) and grade 2 (38 to 40 ℃), and were transient and self-limited. The incidence of fever among the 44 patients receiving rAd-p53 injections was 81.0% (36 of 44), including grade 1 fever (43.0%) and grade 2 fever (38.0%). Development of fever was observed as early as approximately 3 h after injection, lasting about 4 h and then disappearing spontaneously. Only slight pain and discomfort at local injection sites were felt from the repeat injections.

Discussion

rAd-p53 seems to act synergistically with conventional treatments such as chemotherapy and/or radiotherapy. In addition, this apparent synergy still exists in patients who were resistant to chemotherapy and radiotherapy. rAd-p53 therapy has been demonstrated to be safe, feasible, and efficient, including local transgenic expression and evidence of local tumor regression for patients with head and neck squamous cell carcinoma (HNSCC) (5,13,14), esophageal cancer (15), lung cancer (16,17), ovarian cancer (18), bladder cancer (19), and so on.

In this study, the enrolled patients were in advanced stage including recurrent cases failing in surgery, radiotherapy or chemotherapy, and were refractory and uncurable. After the treatment, the CR rate was 13.60% (6/44), and the PR rate was 29.6% (13/44). The responsible rate (CR + PR) was 43.2%. The MST was 12.0±1 months (95% CI: 10-14 months) for the 44 patients. The 1-, 2-, 3-, 4- and 5-year OS was 43.7%, 27.2%, 21.2%, 14.1%, and 7.1%, respectively. In addition to 6 patients with CR, 19 patients (19/38, 50.0%) had LDA of more than 50% area on CT image within tumor, indicating tumor tissue necrosis. These results demonstrated that rAd-p53 combined with hyperthermia plus or not plus radiotherapy could increase local tumor control and improve OS of patients with advanced cancer (20). Our previous study demonstrated that down-regulation of VEGF was observed in tumor tissue treated by intratumoral injection of rAd-p53 (5). Obvious necrosis was found in tumor tissues of the patients who received intratumoral injections of rAd-p53, which may be correlated with rAd-p53-induced down-regulation of VEGF. Other study showed that the overexpressed P53

protein also stimulates the expression of genes that encode proteins involved in suppressing angiogenesis (blood vessel formation). Angiogenesis is a process required for solid tumor formation and progression (21). Inhibition of the VEGF/VEGF receptor pathway is of special interest in targeting therapy for cancers. The antiangiogenesis effect of P53 protein appears to involve three possible mechanisms: (I) interfering with central regulators of hypoxia that mediates angiogenesis; (II) inhibiting production of pro-angiogenic factors; and (III) directly increasing the production of endogenous angiogenesis inhibitors. Recently, P53 has been shown to inhibit hypoxia-inducible factor-1 (HIF-1) activity, which induces angiogenic factors in response to hypoxia (22). Activation of the P53 pathway inhibits angiogenesis and suppresses tumor growth (23). rAd-p53 inhibits VEGF expression and angiogenesis, and then induces tumor necrosis in the clinical course, especially in *p53* gene intratumoral injection combined with hyperthermia-based treatment.

Acknowledgements

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