Thermosensitized Effects of Adenovirus-mediated p53 (Adp53): Preclinical Study and a Phase II Clinical Trial in China

ZHANG SHAN-WEN*, XIAO SHAO-WEN, LU YOU-YONG

Deptment of Radiotherapy, Beijing University School of Oncology, Beijing 100036, P. R. China

Key words: adenovirus-mediated p53 gene; gastric carcinoma cell lines; thermosensitivity; hyperthermia

Introduction

Most previous investigators have reported that wild-type p53 gene plays a key role on cell cycle control and apoptosis, especially in stressed cell treated by irradiation, cytotoxicity agents or hyperthermia, and inhibits proliferation of tumor cell. Hyperthermia kills tumor cells via a way of inducing cell cycle arrest and apoptosis. Wild-type p53 promotes heat-inducing cell cycle arrest and apoptosis of tumor cells, therefore, enhances intrinsic thermosensitivity of tumor cells. While cells having mutant or vect p53 gene abrogate this response and increase resistance to heat stress 1, 2). p53 gene mutation occurring in more than 50% of all human tumors induces incurable to hyperthermia. Thus, adenovirus-mediated wild-type p53 (Adp53) restoring wild-type p53 gene into tumor cells which have p53 abnormality becomes a strategy in cancer gene therapy combined with radiotherapy or chemotherapy or hyperthermia 3, 4). Replication-deficient adenovirus are most widely used vectors in gene therapy. Because this vector can accept a large size of foreign gene (up to 7.5 kb) and high titers of viruses generally can be obtained with simple method to purify and concentrate adenovirus without loss of infectivity. The high efficacy of infection of most tumors cell lines, which leads to up to 100% gene transfer in cell's nuclear, hasn’t any heredity toxicity on host and makes adenovirus highly advantageous if compared with other transfection agents 5, 6). We have previously demonstrated that wild-type p53 promotes S arrest and apoptosis of tumor cells following hyperthermia, therefore, enhances intrinsic thermosensitivity of gastric carcinoma cells lines 7). Adenoviral p53 gene therapy promotes heat-inducing apoptosis in a nasopharyngeal carcinoma cell line 7). In the current study, wild-type p53 gene transfer into the two gastric carcinoma cell lines with different p53 genetic status was performed using the Adp53 to evaluate the effect of wild-type p53 on heat-induced apoptosis and thermosensitivity compared to that receive with hyperthermia alone in vitro and in vivo. In addition, we also conducted a phase II clinical trial on Adp53 (Gendicine®) combined with hyperthermia in Beijing Cancer Hospital.
Preclinical Study: adenovirus-mediated p53 gene transfer increases the thermosensitivity of human gastric carcinoma cell lines (in vitro and in vivo)

Materials and Methods

Adp53 generation, purification and concentration Recombinant E1-deleted adenovirus-mediated p53 gene (Adp53) was based on adenovirus serotype 5, expressing human wild-type p53 under the control of the cytomegalovirus promoter. Adp53 vector generates in 293 cells, a human embryo kidney cell line, which contain the adenoviral E1, that are permissive for adenovirus replication. Purifying and concentrating adenovirus performed in sequential centrifugation in CsCl step gradients. Adp53 vector were quantified according to their plaque-forming ability on 293 cells. Titors of adenovirus were quantified by plaque-forming units (pfu) / ml. Adp53 solution were prepared by purification and concentration up to $3 \times 10^{12}$ plaque-forming units (pfu) per ml for this experiment.

Cells and culture condition Two human gastric carcinoma cell lines (BGC823) with different p53 status, BGC823-wtp53 cell (abbreviate W) bearing the wilt-type p53 and BGC823-mutp53 cell (abbreviate M) bearing the mutant p53 throughly were used in this study and 293 cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, penicilin (50 units/ml), streptomycin (50 µg/ml) (abbreviate DMEM-10).

Hyperthermia and FCM analysis In vitro, either W or M cells were cultured in DMEM-10 medium, and were infected with Adp53 solution at a viral multiplicity of infection of 100 (1 : 100MOI), 48hs later (cell-infected rate achieved to above 90% and the expressed p53 protein showed nuclear localization) followed by heating at 42°C for 2h or 43°C for 0.5h. Culture-flasks were heated by immersing in a water bath maintained within an error of ±0.05°C for treatment. Cell cycle redistribution and Sub-G1 peak (means apoptosis) of two human gastric carcinoma cell lines in 24h at 37°C after heat treatment were assayed to determin DNA content using flow cytometry a model FACS240. The collected data were processed by the computer program. All histograms of the G1, S and G2 phase are portrayed. The Sub-G1 peak represents cells that have lost DNA content as a prelude to apoptosis associated DNA degradation. Each FCM analysis represents the average of two flasks from two to three repeat experiments.

In vivo, one million either W or M cells were transplanted subcutaneously into right thigh of a nude mouse. When diameter of tumor reached to about 1cm diameter, tumors were intratumorally injected $1 \times 10^6$ pfu of Adp53. 48h later, the tumor-bearing leg was heated at 43°C for 0.5h in a hot water bath maintained within an error of ±0.05°C for treatment. From first heating day, two orthogonal diameters A and B were measured with a caliper two days to 12th day, and the tumor volume was calculated by the formula, $\pi / 6 \times (A + B) / 2$. If define the tumor volume at first heating day as 1, relative tumor volume growth curves related to following heating days were portrayed. Tumor response rate of combining treat group or either treat alone group were counted by comparison of tumor decrease of treat group to control group at 37°C. Analysis for each tumor response rate represent the average of four nude mouses from two repeat experiments. Thermo-enhancement ratio (TER) of Adp53 combined with heating at 43°C for 0.
5h as compared to heat alone were counted. The TER = tumor response rate at Adp53 + heating/ tumor response rate at heating only.

Results

Fig.1. showed the change of Adp53 alone, heating alone or Adp53 combined with heating on cell cycle distribution in W cells. A obvious increasing in the percentage of cells in G2 phase with decreasing in those in either the G1 or the S phase and a obvious sub-G1 peak (apoptotic peak) appeared 48h after infection of Adp53; A slightly increasing in the percentage of cells in G2 phase for heating at 42°C, 2h or 43°C, 0.5h and an obvious sub-G1 peak appeared; A more obvious increasing in the percentage of cells in G2 phase and more obvious Sub-G1 peak after infection of Adp53 combining with heating than either treatment alone were seen.

Fig.2. showed the change of Adp53 alone, heating alone or Adp53 combined with heating on cell cycle distribution in M cells. A obvious increasing in the percentage of cells in G2 phase with decreasing in those in either the G1 or the S phase and a obvious sub-G1 peak (apoptotic peak) appeared 48h after infection of Adp53; A slightly increasing in the percentage of cells in S phase for heating at 42°C, 2h or 43°C, 0.5h and an obvious sub-G1 peak appeared; A more obvious increasing in the percentage of cells in G2 phase and more obvious Sub-G1 peak after infection of Adp53 combining with heating than either treatment alone were seen.

Fig.1. Analysis of DNA content by FCM in the Adp53-infected W cell line, 48h later were heated at 42°C, 2h or 43°C, 0.5h, and showed that more prolongation of the G2/M phase and the induction of a Sub-G1 region meanted apoptosis than either treatment alone were seen.
Fig. 2. Analysis of DNA content by FCM in the Adp53-infected M cell line, 48h later were heated at 42°C, 2h or 43°C, 0.5h, and showed that more prolongation of the G2/M phase and the induction of a Sub-G1 region meant apoptosis than either treatment alone.

| Table I. Cell cycle phase and Sub-G1 rate of Adp53-infected W cells following heating |
|---------------------------------|-------|-------|-------|-------|-------|
| condition                      | G1 (%) | S (%)  | G2/M (%) | Sub-G1 (%) | TER  |
| Control at 37°C                | 38.7±2.6 | 39.3±7.3 | 22.0±1.6 | 2.6±1.7 |
| Adp53                          | 38.5±2.4 | 26.8±2.7 | 34.7±1.0 | 8.5±0.7 |
| 42°C, 2h                       | 36.8±5.0 | 37.8±3.9 | 25.3±6.0 | 6.5±4.8 |
| 43°C, 0.5h                     | 40.2±7.1 | 34.0±4.7 | 24.0±4.3 | 14.2±2.2 |
| Adp53+42°C, 2h                 | 44.8±5.1 | 21.8±6.7 | 34.0±2.9 | 21.4±9.8 |
| Adp53+43°C, 0.5h               | 35.8±2.1 | 40.5±7.6 | 23.8±7.1 | 22.5±12.2 |

* Thermo-enhancement rate (TER) = apoptosis ratio at Adp53+heating/ apoptosis ratio at heating only.

| Table II. Cell cycle phase and Sub-G1 rate of Adp53-infected M cells following heating |
|---------------------------------|-------|-------|-------|-------|-------|
| condition                      | G1 (%) | S (%)  | G2/M (%) | Sub-G1 (%) | TER  |
| Control at 37°C                | 45.8±1.5 | 34.8±0.5 | 20.0±0.8 | 2.2±1.5 |
| Adp53                          | 41.3±2.4 | 33.5±6.2 | 25.8±7.8 | 8.2±5.2 |
| 42°C, 2h                       | 41.3±5.0 | 36.5±12.2 | 22.0±5.9 | 4.7±1.5 |
| 43°C, 0.5h                     | 43.8±3.9 | 38.5±4.7 | 17.5±5.1 | 7.5±2.2 |
| Adp53+42°C, 2h                 | 42.5±10.8 | 32.0±3.3 | 25.3±13.4 | 9.8±2.2 |
| Adp53+43°C, 0.5h               | 41.5±2.5 | 36.3±9.9 | 22.5±12.5 | 13.3±0.5 |

(14)
Both Table I and Table II showed that both W cells and M cells 48h after infection with Adp53 had strong arrest in G2 (34.7% of original population for W cells and 25.8% of original population for M cells) and a increasing apoptotic response (apoptosis rate 8.5% for W cells and 8.2% for M cells). Only heating at 42°C, 2h or 43°C, 0.5h, a slightly increasing in the percentage of cells in G2 phase for W cells and a slightly increasing in the percentage of cells in S phase for M cells and a obvious apoptotic rate in W cells than M cells were seen. The effect of heating toxicity was enhanced by Adp53, that produced a strong arrest in G2 (34.0% or 23.8% of original population for W cells at 42°C, 2h or 43°C, 0.5h and 25.3% or 22.5% of original population for M cells at 42°C, 2h or 43°C, 0.5h, respectively). If evaluating heat-biologic efficacy by apoptosis rate, the effect of heating toxicity was enhanced by Adp53, produced a 230% or 60% increasing of apoptosis rate for W cells than only 42°C, 2h or 43°C, 0.5h and a 110% or
80% increasing of apoptosis rate for M cells than only 42°C, 2h or 43°C, 0.5h respectively.

Both Fig.3. and Fig.4. showed that the growth of tumors of both W and M cells was significantly delayed by hyperthermia combined with Adp53 as compared to tumors receiving either treatment alone. As shown as Fig. 3., tumor suppress rate were 15.8%, 21.8% and 27.2% for 43°C, 0.5h, Adp53 and Adp53 + 43°C, 0.5h, respectively. TER of Adp53 at 43°C, 0.5h was 1.72 for W cell tumor. As shown as Fig.4., tumor suppress rate were 24.2%, 31.0%, and 37.8% for 43°C, 0.5h, Adp53 and Adp53 + 43°C, 0.5h, respectively. TER of Adp53 at 43°C, 0.5h was 1.56 for M cell tumor°C

Discussion

Wild-type p53 functions as a transcriptional factor to control the cell cycle and apoptosis, especially in stressed cells treated by irradiation, cytotoxicity agents or hyperthermia, and inhibits proliferation of tumor cell. Wild-type p53 enhances and prolongs cell cycle arrest heat-induced, and stimulates the apoptosis inducing by heat stress, whereas mutated p53 abrogates it extremely. The p53 gene is at least a factor for determining cellular thermosensitivity and wild-type p53 contributes to thermosensitization resulting by heat-induced apoptosis. About 50% of all human tumors contain p53 mutation and mutation rate is up to 50-70% in stomach cancers, colorectal cancers, breast cancers and other common tumors. A high incidence of local-regional failure and distant metastasis contributes to the poor overall survival rate of around 40% for patients with cancer. Stomach cancer is one of leading causer of cancer in China and showed resistance and incurable to radiotherapy, chemotherapy and hyperthermia, a poor survival rate of around 20~30%. Thus, replacing wild-type p53 gene into tumor cells which have p53 abnormality becomes a new strategy in cancer gene therapy. In this study, a novel approach was evaluated of combining of Adp53 gene therapy with hyperthermia. This study demonstrated that culture-infected Adp53 transfered exogenous wild-type p53 into W and M gastric cancer cells and increased cellular thermosensitivity via a way of G2 phase arrest and apoptosis heat-induced in vitro, and that intratumorally injected Adp53 resulted in an increasing in W and M cell tumor thermosensitivity in vivo. Results showed that thermosensitized effects of Adp53 were independent on tumor cellular intrinsic p53 status either in vitro or in vivo. These results support the combination of p53 gene therapy with hyperthermia in clinical trials.

Clinical Trial:

recombinant adenovirus— p53 (Gendicin®) combined with hyperthermia in advanced cancer (a reporter of 7 cases)

p53 Gene Drug.

Gendicine®, a recombinant adenoviral-p53 anticancer agent developed by Shenzhen SiBiono GeneTech Co. Ltd, was approved for human clinical trials by the FDA (The State Food and Drug Administration of China) in 1998. Gendicine® is an E1 substituted replication-incompetent recombinant adenovirus encoding the human p53 gene. The protocol used in this study was approved by the FDA. Gene transfer vector, recombinant adenovirus-p53 (Gendicin®) was supplied by Shenzhen Sibiono Genetech Co. Ltd and stored at -20°C at concentrations of 1 × 10^12 vp/ml (vp: virus particle).
Intratumoral injection of Gendicin® solution was thawed and diluted moderately in 0.9% physio-saline solution according to tumor size, within 0.5h of use. Results of randomly controlled study demonstrated that wild-type p53 gene therapy induced significantly radio-enhancemental effect in patients with HNSCC (P < 0.05). Results of the randomized controlled study showed that wild-type p53 gene radiosensitized enhancement rate 1.47 at 40Gy in HNSCC. The CR rate for HNSCC patients treated by Gendicin® combined radiotherapy increased by nearly 205% than radiotherapy alone. 53 patients with HNSCC received multiple intratumoral injection of Gendicine®, no dose-limiting toxicity and adverse events were noted, except transient fever was the most common finding.

**Gendicine® Combined with Hyperthermia**

Meantime 7 patients with advanced cancer confirmed by patho-histological examination were intratumorally injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia alone for 3 patients, and plus radiotherapy for 4 patients. Two largest orthogonal diameters A and B of tumors were measured according to CT scan of tumors. The tumor size was calculated by the formula, $A \times B$. Tumor shrinkage rate of Gendicine®-injected tumor after treatment were counted by comparison to pre-treatmental tumor size. Results were monitored for tumor shrinkage rate to evaluate by CR (complete response), PR (partial response), SD (stable disease), PD (progressive disease) according to WHO’s evaluation standard of solid tumor treatment.

Characteristics and results of the 7 patients with advanced cancer as follows.

**Case 1** patient, female, 69 years, with recurrent thyroid cancer. Post-operation 9 months later, neck local recurrent tumor failed in radio-chemo-hyperthermia was US-guided injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia alone at 42~43°C using 915MHz machine over to eight. After treatment front-neck scab was exfoliated and severe dyspnea was almost relieved. Pre-treatment the neck tumor area 22.5cm² were shrinked to 11.7cm², decreased to 48%.

**Case 2** patient, female, 68 years, with recurrent liposarcoma in post-peritoneum failed in fifth operation and neutron irradiation. Recurrent tumor in left lumbar vertebra 3 level was US-guided injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia alone at 42~43°C using 40MHz radiofrequency machine over to eight. Pre-treatment the tumor area in post peritoneum 29.0 cm² were shrinked to 17.5cm², decreased to 40%. Obvious low-density area (LDA) on CT images in the tumor mean necrosis was seen.

**Case 3** patient, male, 36 years, with nasopharyx carcinoma. After radiotherapy 3 years later, metastasis in right-upper neck lymph node failed in X-knife irradiation. The right-upper neck lymph node was US-guided injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia alone at 42~43°C using 915MHz machine over to tight, twice a week. Pre-treatment the tumor area 26.6 cm² were shrinked to 19.2cm², decreased to 28%. LDA’s percentage area in the tumor was more than 50%.

**Case 4** patient, male, 50 years, with recurrent liposarcoma in post-peritoneum failed in second operation. Great recurrent tumor in pelvic cavity was US-guided injected with Gendicine® $2 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia at 42~43°C using...
40MHz radiofrequency machine over to fourteen, twice a week, and plus irradiation to 60 Gy. Pretreatment the tumor area in pelvic cavity 132.9 cm² were not shrunk but LDA’s proportion in the tumor was more than 50%. Obvious necrosis in the tumor was seen. During treatment severe intestinal obstruction was almost relieved.

Case 5 patient, male, 73 years, with retro-tongue malignant neurinoma, to be not removed. The tumor directly was injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia at 42~43°C using 915MHz machine over to eight, and plus irradiation to 70 Gy. Pre-treatment the tumor area 10.4 cm² were shrunk to 2.6 cm², decreased to 75%.

Case 6 patient, female, 69 years, with ovarian cancer, post-operation one year later recurrent tumor in pelvic cavity, failed in chemotherapy. Great recurrent tumor in pelvic cavity was US-guided injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to eight, concurrently 3 days later combined with hyperthermia at 42~43°C using 40 MHz radiofrequency machine over to eight, once a week, and plus irradiation to 60 Gy. Pre-treatment the tumor area 95.0 cm² were shrunk to 11.5 cm², decreased to 88%. Obvious necrosis in the tumor was seen. During treatment severe difficult defecation was almost relieved.

Case 7 patient, male, 68, with perineum squamous carcinoma injured bladder and rectum. The great tumor directly was injected with Gendicine® $1 \times 10^{12}$ vp/ml once a week over to ten, concurrently 3 days later combined with hyperthermia at 42~43°C using 915 MHz machine over to four, and plus irradiation to 70 Gy. Pre-treatment the tumor area 80.0 cm² were nearly disappeared.

Conclusion

7 patients with advanced cancer injected by Adp53 (Gendicine®) combined hyperthermia alone, or plus radiotherapy achieved CR 1, PR 2 and SD 4, and results of necrosis in tumors were seen. P53 gene therapy is a thermosensitized potential in advanced cancer.

Reference


Foundation item: This work was supported by the National Natural Foundation of China (No. 39670234)