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Adenovirus-Mediated *p53* Tumor Suppressor Gene Therapy against Subcutaneous HuH7 Hepatoma Cell Line Nodule of Nude Mice

Mutations of the tumor-suppressor gene *p53* have been found in 30-50% cases of hepatocellular carcinoma (HCC). In this study, E1-negative adenoviral vector encoding wild-type *p53* under the control of the human cytomegalovirus promoter (*AdCMV-p53w*) was constructed to evaluate its therapeutic efficacy against tumor nodules developing after injection of HuH7 cell lines in ten nude mice. When each nodule had reached 10 mm in perpendicular diameter, 1.5×10^8 pfu of *AdCMV-p53w* per session was injected intratumorally as follows: In group I (n=3), five sessions were injected every other day. In group II (n=3), only one session. Group III (n=4) as negative controls. The mice were sacrificed at 28 days post *AdCMV-p53w* injection. Tumor growth was significantly suppressed and delayed in group I and II compared to group III as compared by tumor volume at the end of observation. These results suggest that *AdCMV-p53w* may not only be effective in treating HCCs expressing mutant p53, but also useful as a local injectable gene therapy.

Key Words: *Gene, p53; Gene, tumor suppressor; Adenoviridae; Gene therapy; Carcinoma, hepatocellular; HuH7*

Jong Young Choi, Young Min Park, Byun Hun Byun, Boo Sung Kim, Eun-Gyeong Hong*, Deug Yong Shin*, Young Rim Seong*, Dong-Soo Im*

Department of Internal Medicine, Kangnam St. Mary's Hospital, College of Medicine, and WHO Collaborating Center for Reference and Research on Viral Hepatitis, and Molecular Hepatology Laboratory, Institute of Human Molecular Genetics, The Catholic University of Korea, Seoul, Korea
*Korea Research Institute of Bioscience & Biotechnology, Yusong, Taejon, Korea

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Address for correspondence
Young Min Park, M.D.
Department of Internal Medicine, Kangnam St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 505, Banpo-dong, Seocho-gu, Seoul 137-040, Korea
Tel: +82.2-590-2388, 2384, Fax: +82.2-536-9559
E-mail: ympark@cmc.cuk.ac.kr

INTRODUCTION

Hepatocellular carcinoma is a common malignancy which causes an estimated one million deaths per year worldwide (1). Various therapeutic approaches are available for the treatment of hepatocellular carcinoma, which include surgical resection, cryosurgery, percutaneous ethanol injection therapy, arterial chemoembolization, hepatic dearterialization, systemic chemotherapy, radiation therapy, and liver transplantation (2, 3). However, each of these treatment modalities has shown to be appropriate only for a minority of patients because of the extent of the tumor and associated cirrhosis at the time of diagnosis.

Gene therapy for cancer diseases is usually aimed at treating cancer cells selectively by means of transferring genes containing wild-type gene to the cancer cells containing mutant-type gene, or transferring genes that induce apoptosis of the cells such as HSV-tk/Ganciclovir. In cases involving *p53* gene therapy, normal p53 protein, produced from transferred gene, acts more dominant than mutant p53 protein, resulting in apoptotic cell death, even if the mutation itself is not corrected. So, a promising method for the treatment of hepatocellular carcinoma is *p53* gene therapy.

Among several gene delivery systems, a recombinant human adenovirus, which is E1-negative and noncytopathic, has been suggested as being useful in delivering the genes into the eukaryotic cells (4, 5). This viral vector can infect both dividing and quiescent cells, replicate without integrating into the host cell genome (6), and generate stable high-titer viruses for the efficient infection and subsequent gene expression in target cells (7). It has also been reported that the introduction of wild-type *p53* gene by adenoviral-mediated delivery can suppress growth in some human cancer mouse models (8, 9). In the present study, a recombinant E1-negative adenoviral vector containing wild-type *p53* gene, expressed under the control of human cytomegalovirus promoter (*AdCMV-p53w*) was constructed and its therapeutic efficacy was evaluated against hepatocellular carcinoma nodules generated in nude mice by subcuta-

neously injecting a hepatoma cell line with mutant *p53* gene, HuH7.

MATERIALS AND METHODS

The human osteosarcoma cell line, saos-2, and hepatocellular carcinoma cell line, HuH7, were grown in minimal essential media containing 10% fetal calf serum, 200 mM glutamine, and 0.05% gentamycin. The human embryonic kidney cell line 293 (ATCC), which was maintained in Kaighns media supplemented with 15% fetal calf serum, was used for the propagation of recombinant virus.

A recombinant human adenovirus encoding wild-type human *p53* under the control of the human CMV immediate early gene promoter was constructed by co-transfecting an adenovirus transfer plasmid, encoding the *p53* gene and *pJM17* into 293 cells. The methods used for adenovirus preparation are modified from Graham and Prevec (10). Briefly, after 293 cells were infected by adenoviral vector encoding wild *p53* and were harvested, they were purified by CsCl method. By plaque assay on 293 cell monolayer, viral titer was measured.

Infection of the saos-2 cells was carried out by dilution of viral stock to a certain concentration, addition of viral solutions (0.5 mL per 60-mm dish) to cell monolayers, and incubation at room temperature for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to a 37℃ incubator. To detect DNA ladders, the infected cells were collected after 24 hr of infection and prepared for DNA ladder assay according to the protocol previously described (11).

The infected cells were lysed in RIPA buffer, 20 μg of protein from each lysates was separated on 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (MEN Life Science Products, Boston, MA, U.S.A.). Immunoblot analysis was performed as described previously (12) by using the ECL western blotting detection system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.). Monoclonal antibodies were purchased: DO-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) for p53; Ab1 (ONCOGENE Research Products, Cambridge, MA, U.S.A.) for p21.

HuH7 cells, containing *p53* mutations at codon 220 (A:T→G:C), were plated in 96 well plates and allowed to adhere overnight at 37℃. The cells were then infected at various MOI units of virus/cell with *AdCMV-p53w* for an additional 70, 120, 216 hr of incubation at 37℃. The cells were stained with 0.1% crystal violet for 20 min, washed in distilled water, and the stain was extracted by the use of 200 μL of Sorenson's buffer.

In a defined pathogen-free environment, 5×10^6 HuH7 cells per 400 μL of phosphate buffered saline (PBS) were injected subcutaneously into the back of 15 nude mice using a 28-gauge needle. By visual inspection, tumor nodules could be identified initially in 75% (15/20) of the mice, approximately 10 days after the injection. When emerging tumor nodules had reached 10 mm in perpendicular diameter, 1.5×10^8 pfu of *AdCMV-p53w* per session were injected intratumorally using a 26-gauge needle. Once a needle punctured the skin covering the tumor, *AdCMV-p53w* solution was injected at each of four different sites within the tumor. They were randomly assigned to three groups (5 mice in each group) by treatment: 5 sessions in alternate days, on day 0, 2, 4, 6, and 8 (Group I), only I session treatment at day 0 (Group II), and no treatment as negative controls (Group III). During observation, 5 mice, including 2, 2, and 1, from group I, II, and III, respectively, died before the end of the experiment. The remaining 10 mice were sacrificed at 28 days after initial treatment. Tumor diameters, the longest one (a) and shortest one (b), were measured daily with a linear caliper, and the tumor volume was calculated as follows: volume = $a\times b^2\times0.4$ (13). Mean values of tumor volumes were compared among 3 groups at the end of the experiment.

Tissue samples were fixed with 10% formalin for 24 hr and tissue sections were deparaffinized. Tissue sections were treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed using monoclonal antibody to p53 (DAKO, Carpinteria, CA, U.S.A.) with avidine-peroxidase technique (DAKO). The primary antibody was a mouse anti-human p53 monoclonal antibody DO7 (DAKO), and the secondary antibody was a biotinylated goat anti-mouse IgG (DAKO). An avidin and biotinylated horseradish peroxidase macromolecular complex reagent was used to detect the antigen-antibody complex. These tissue sections were stained with 3,3'-diaminobenzidine tetrahydrochloride and then counterstained with hematoxylin.

Detection of apoptotic cells in situ was performed using ApoTag (Oncor Inc., Gaithersburg, MD, U.S.A.) on tissues obtained 28 days after the first injection of *AdCMV-p53*. Briefly, after proteinase K digestion and inactivation of endogeneous peroxidase, the specimens were incubated at 37℃ for 1 hr in a solution containing terminal deoxynucleotidyl transferase, digoxigenin-labeled deoxyuridine triphosphate and deoxyadenosine triphosphate. In addition, the specimens were reacted with peroxidase-labeled anti-digoxigenin antibody at room temperature for 30 min. Color reaction was performed with 3,3'-diaminobenzidine-tetrahydrochloride solution, and then the specimens were counterstained with methyl green.

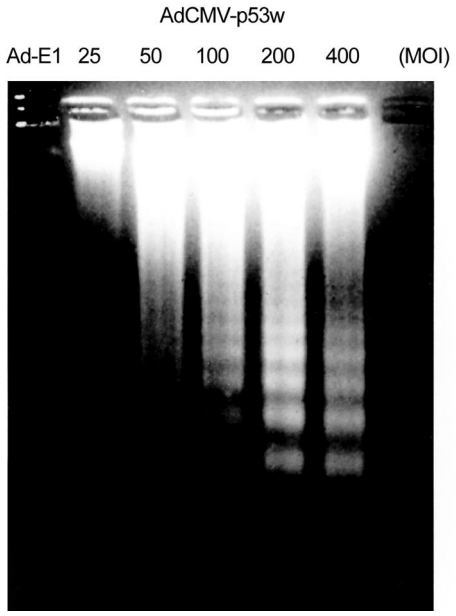

Fig. 1. Induction of apoptosis by adenoviral-mediated *p53* expression. DNA fragmentation was detected on agarose gel. Cells (saos-2 cell) were infected at various MOI.

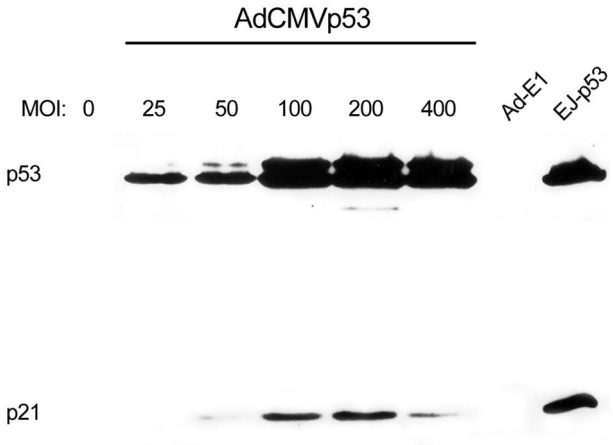

Fig. 2. Expression of p21 protein detected by western blotting. Saos-2 cells were infected with *AdCMV-p53w* at MOI including 25, 50, 100, 200, and 400, and after 48 hr, protein lysate was isolated. A immunoblot was performed using a human p53 and p21-specific antibody on separated proteins. EJ-p53: bladder carcinoma cell line transfected with *p53w*.

Fisher's exact test and unpaired t-test were performed to compare differences of the tumor volume among the groups studied.

RESULTS

DNA fragmentation assay showed typical DNA ladder pattern in cell infected at various MOI (Fig. 1). In *p21* induction assay by *Ad5CMV-p53w* vector, *AdCMV-p53w* showed increasing p21 protein in a dose-dependent manner in infected saos-2 cell line (Fig. 2).

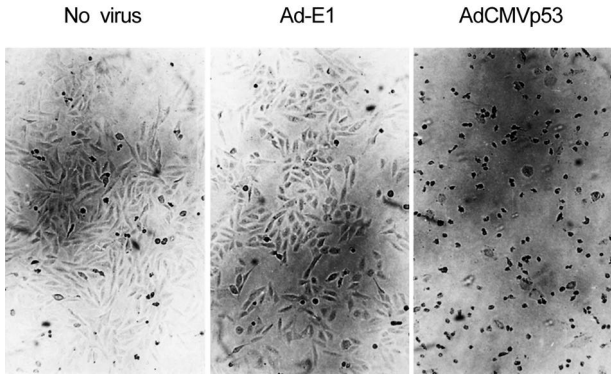

Fig. 3. Morphology of HuH7 cells after infection by no virus or Ad-E1 vector, *AdCMV-p53w*. When cells were infected with *AdCMV-p53w*, the growth of tumor cell was suppressed significantly.

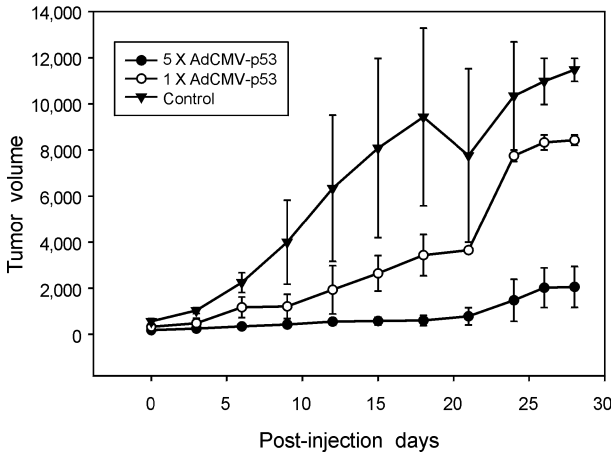

Fig. 4. Local subcutaneous injection of *AdCMV-p53w* into tumor nodules of nude mice. Tumor volume (μL) was measured on a daily basis. Untreated control mice inoculated with 5×10^6 HuH7 hepatoma cells; five times *AdCMV-p53w*-treated mice bearing tumors (n=3); one time *AdCMV-p53w*-treated mice bearing tumors (n=3). The difference of tumor volume among groups was shown in the later stage.

HuH7 hepatoma cells were infected with increasing concentrations of *AdCMV-p53w*, Ad-E1, and no virus. The control Ad-E1 vector had no effect on cell growth at any MOI tested. In contrast, *AdCMV-p53w* reduced cell growth (Fig. 3). The volume of the tumor was lower in the *AdCMV-p53w* treated group (Group I, 2,051$\pm$1,531 μL; Group II, 8,426$\pm$319 μL) than in control group (Group III, 11,476$\pm$707 μL) at 28 days after injection ($p<0.01$) (Fig. 4, 5). Tumors from the *p53-*

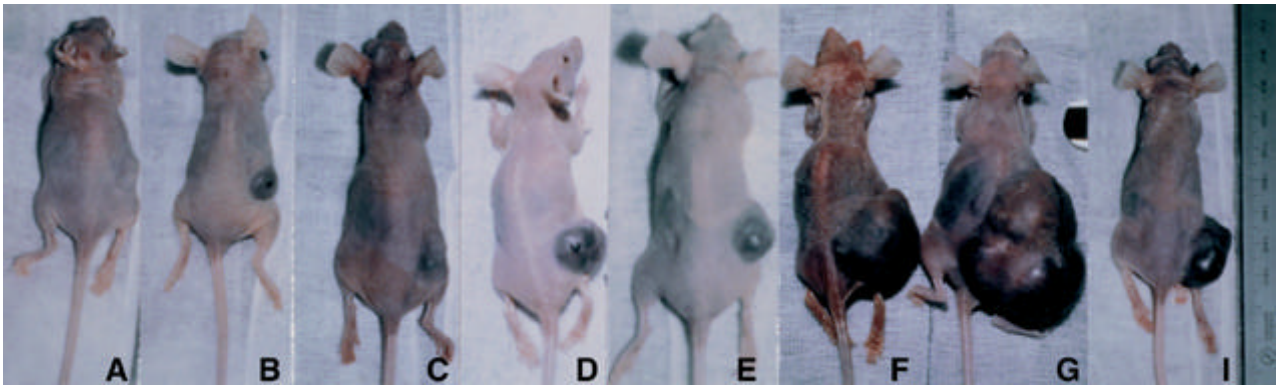

Fig. 5. Representative feature of human hepatoma nodules following injections with *AdCMV-p53w*; five injections of *AdCMV-p53w* (A, B, C); one injection of *AdCMV-p53w* (D, E); not injected control (F, G, I).

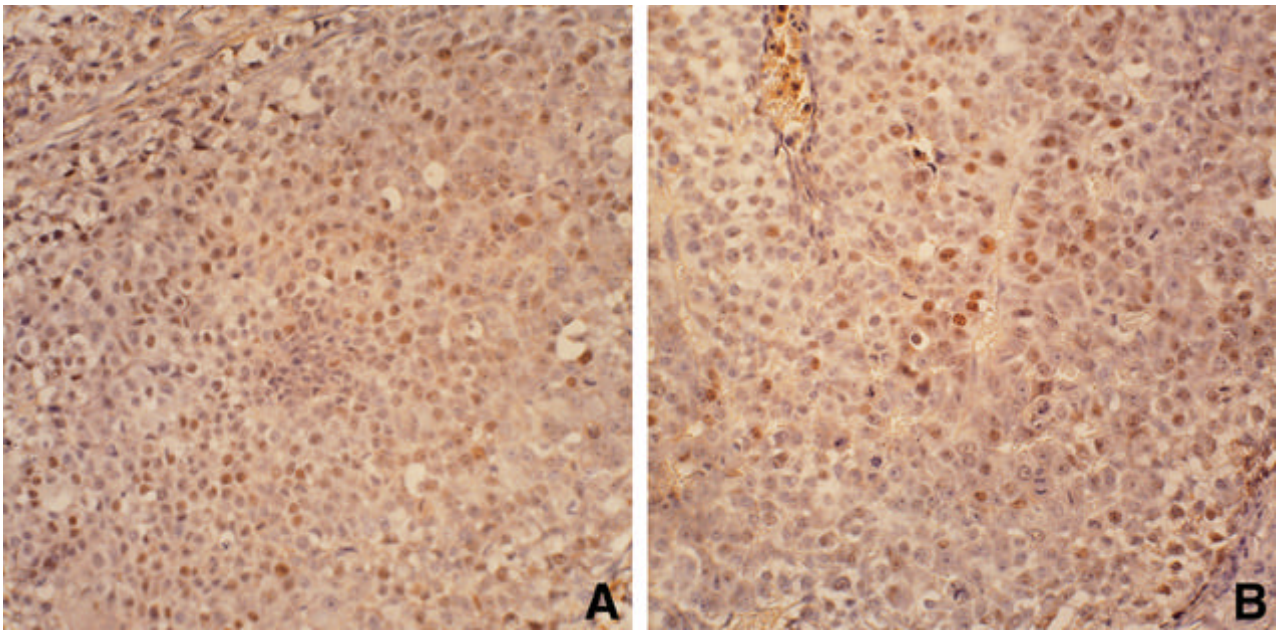

Fig. 6. Histologic examination and induction of apoptosis in the primary tumors of *AdCMV-p53w*-treated mice 28 days following the first injection. Histological findings of tumor tissue from *p53*-treated group versus control do not reveal contrasting morphology (performed in a blind fashion). The immunohistochemical stain of p53 shows intranuclear staining on both groups (A: *p53* treated, B: control, ×200).

treated mice did not reveal differences in histology as compared to tumors of the control mice. The immunohistochemical staining of p53 showed intranuclear staining in three groups (Fig. 6). Apoptotic cells were not detected in *p53* treated groups and controls.

DISCUSSION

The tumor suppressor gene *p53* plays an important role in determining two different pathways of cells at the G1/S transition phase. It can maintain normal cell cycle or kill the severely damaged cells through a self-destruct mechanism, apoptosis. When DNA is damaged by X-ray or drugs, p53 normally blocks the progression of the cell cycle at the G1 phase and transactivates other genes related to its functions (14, 15). However, if *p53* gene is mutated, cell cycle is uncontrollably activated, damaged DNA can not be recovered, and finally, cells containing mutant *p53* gene become malignant.

Clinical applications of *p53* gene therapy showed positive responses, including complete responses in one third (3/9) of patients with lung cancers with *p53* mutations (16). A test use of wild-type *p53* gene on patient with

unresectable hepatocellular carcinoma also produced good results (17). The mutations of *p53* gene are commonly found, in 20-60% cases of hepatocellular carcinoma. This sacrifies the validity of the *p53* gene therapy.

In the first part of this study, we confirmed that p53 protein produced by *AdCMV-p53w* acts normally and induces apoptosis via the expression of the p21 protein. And in the second part, we proved that *AdCMV-p53w* induces confluent apoptosis of HuH7 hepatoma cell line grown in culture media, while adenoviral vector, Ad-E1, without *p53* gene does not.

Other experimental models for *p53* gene therapy, including cervial cancer, mammary cancer, and prostate cancer, used *AdCMV-p53w* before visible nodule formation in the injection sites (13, 18-20). Those protocols proved that the wild-type *p53* may prevent the development of tumor nodules, when used in the early stages.

In the present study, *AdCMV-p53w* was used against established tumor nodules with approximately 1 cm in diameter, and injected directly into the tumors to evaluate the therapeutic efficacy of *AdCMV-p53w*. These showed that the therapeutic efficacy of our *AdCMV-p53w* construct significantly suppressed tumor cell growth, although none of the nodules disappeared.

Regarding to the failure of complete remission in our experiment, two possibilities are suggested: one is that the amount of *AdCMV-p53w* used was not enough for an 1 cm-sized tumor nodule, and the other is that *AdCMV-p53w* injected intratumorally might not be distributed throughout the whole nodule. It was also considered that tumor cells transplanted into the tissue might be more resistant to the p53 function than those grown in a culture condition.

Overall, these results indicate that adenovirus-mediated delivery of *p53* may be useful for the treatment of hepatocellular carcinoma. Although it is not clear at the moment whether *AdCMV-p53w* may be advantageous in cases with non-resectable hepatocellular carcinoma or in cases accompanied by decompensated cirrhosis. This data suggests that the intra-tumoral injection of *AdCMV-p53w* can have a substantial effect on tumor growth in vivo.

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