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Laboratory Investigation

The Combined Effect of Gamma Knife Irradiation and p53 Gene Transfection in Human Malignant Glioma Cell Lines

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Objective : The purpose of this study is to elucidate in vitro responses to combined gamma knife irradiation and p53 gene transfection on human malignant glioma cell lines.
Methods : Two malignant human glioma cell lines, U87MG (p53-wild type) and U373MG (p53-mutant) were transfected with an adenoviral vector containing p53 (MOI of 50) before and after applying 20Gy of gamma irradiation. Various assessments were performed, including, cell viability by MTT assay; apoptosis by annexin assay; and cell cycle by flow cytometry, for the seven groups : mock, p53 only, gamma knife (GK) only, GK after LacZ, LacZ after GK, GK after p53, p53 after GK.
Results : Cell survival decreased especially, in the subgroup transfected with p53 after gamma irradiation. Apoptosis tended to increase in p53 transfected U373 MG after gamma irradiation (apoptotic rate, 38.9%). The G2-M phase cell cycle arrest markedly increased by transfecting with p53, 48 hours after gamma knife irradiation in U373 MG (G2-M phase, 90.8%).
Conclusion : These results suggest that the in vitro effects of combined gamma knife irradiation and p53 gene transfection is an augmentation of apoptosis and G2-M phase cell cycle arrest, which are more exaggerated in U373 MG with p53 transfection after gamma knife irradiation.

KEY WORDS : Adenovirus · Gamma knife · Gene therapy · Malignant glioma · *p53* · Radiation.

Introduction

Glioblastoma is the most malignant form of primary brain tumors and constitutes approximately 25% of primary adult brain tumors [20,27]. However, the average 5-year survival rate is only 9%[2], despite the use of combinations of surgery, radiotherapy and chemotherapy which are known to be the standard management of patients with malignant glioma [23,24]. One of the tumor biological characteristics different to malignant tumor in the other part of the body is that the distant metastasis is rare in malignant glioma. In this regard, loco-regional treatments, such as radiosurgery and gene therapy have a potential for the treatment of malignant gliomas. The authors previously reported a stereotactic device for gamma knife irradiation to cell lines[3] and in vitro biologic response of malignant glioma cell lines to gamma knife irradiation[11]. The in vitro biologic response of malignant glioma cell lines to gamma knife irradiation is the augmentation of apoptosis and G2-M phase cell cycle arrest[11], which might be in close relation to the *p53* transfection effect to malignant glioma cells.

Consecutively, the purposes of this study is finally to assess the *in vitro* interaction between gamma irradiation and *p53* gene transfection in the growth of human malignant glioma cell lines.

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Materials and Methods

Cell lines

Cell lines were obtained from the Korean Cell Line Bank. U87 MG contains a wild-type *p53* and homozygous deletion of p16. On the other hand, U373 MG has a mutant-type *p53* and retains exons 1 and 2 of the p16 gene, but does not contain exon 3[8]. These cells were cultured in 75cm^2 plastic tissue culture flasks (Corning, NY) containing Dulbecco's modified Eagles medium(DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Road Logan, Utah) and 10mg/ml antibiotic-antimycotic (Gibcor BRL, Gaithersburg, MD). Cell lines were maintained at 37 ℃, and in a humidified 5% CO_2 atmosphere, and passaged just prior to 100% confluence.

Gamma knife irradiation

Gamma irradiation was performed using gamma knife model 23004B2 (Elekta AB, Stockholm, Sweden). For dose planning, a Leksell Gamma Plan(Version 5.3) was used. An 18mm collimator was to condition the incident beam. Maximum doses of 20Gy was used for assays of combined gamma irradiation and *p53* transfection treatment. The isodose lines was the same as those in the previous report[11].

Recombinant adenoviruses

Recombinant adenoviral vector encoding human *p53* (Ad-*p53*) containing cytomegalovirus promoter, complementary DNAs of *p53* and the SV40 polyadenylation signal were constructed by inserting the expression cassette into the E1-deleted region of a modified Ad5 genome. Recombinant adenoviral vector encoding Escherichia coli β-gal gene (Ad-*lacZ*)was constructed in a similar manner. Viral stocks were propagated in 293 cells and purified using the $CsCl_2$ banding technique.

β-Galactosidase transduction assay

Ad-*lacZ* was used to determine the transduction efficiency. Monolayers of cell lines in 6-well plates (Becton Dickinson, Franklin Lakes, NJ) were treated with increasing doses of Ad-*lacZ*, with multiplicities of infection(MOI) ranging from 12.5 to 200. Culture medium was then added and incubation was continued for 48 hours at 37℃ Cells were washed with 1X phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde for 15min at 4℃, and incubated in a reaction mixture containing, 5mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2mM $MgCl_2$, and 1mg/ml 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal; Sigma, St Louis, MO) for 2hours at 37℃. The percentage of LacZ-positive cells (stained blue) on triplicate dishes were calculated by light microscopy. We chose an MOI that resulted in more than 90% transduction.

Western blot analysis

Tumor cells were seeded at a density of 2×10^6 in $25mm^2$ plastic tissue culture flasks (Corning, NY) 24hours before treatment. Cell monolayers were incubated with 1ml of serum-free DMEM containing recombinant adenovirus for 2hours at 37℃ Virus-containing medium was removed by aspiration, and cells were incubated in 5ml of fresh media for 48hours at 37℃ The medium was then removed, and the cell monolayers were washed with PBS at room temperature. The cells were then lysed, by adding 150ul of ice-cold lysis buffer (1% NP-40, 0.15M NaCl, 0.01M sodium phosphate pH 7.2, 2mM EDTA, 50mM sodium fluoride, 0.2mM sodium vanadate, and 100U/ml aprotinine), and incubated on ice for 1 hour before centrifuging at 15,000g for 20 min at 4℃ The resulting supernatant (total cell lysate) was stored at 80℃ The protein concentration was determined by Bradford assay. Forty ug of protein was electrophoretically separated in a 12% polyacrylamide-SDS gel and then transferred to a polyvinylidene difluoride membrane (Millipore Products Division, Bedford, MA). The membrane was probed with primary antibodies to *p53* (mouse anti-human monoclonal antibody [Santa Cruz Biotechnologies, Santa Cruz, CA]) and -actin (mouse anti-human monoclonal antibody as a loading control [Santa Cruz Biotechnologies, Santa Cruz, CA]). Horseradish peroxidase-conjugated goat anti-mouse (Amersham Pharmacia Biotech, UK) was used as a secondary antibody. Immunodetection was performed using an enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech, UK).

Assessment of cell growth inhibition

On day 0, cells in the exponential growth phase were harvested with 0.25% trypsin and resuspended to a final concentration of 8.0×10^3cells/ml in fresh medium containing 10% FBS and 10mg/ml of antibiotic-antimycotic. Cell suspensions (150ul) were then dispensed using a multi-channel pipette into the individual wells of a 96 well tissue culture plate with a lid (Falcon, Oxnard, CA). Each plate had a single control well-column containing medium alone. Cells were reincubated overnight to allow attachment.

Viability determination was based on the bioconversion of the tetrazolium compound, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT), into formazan, as determined by absorbance at 540nm using an enzyme-linked immunosorbent assay reader.

Seven cell groups were assessed; the mock group, the Ad-*LacZ* transfected group, the Ad-*p53* transfected group, the group transfected with Ad-*LacZ* 3 hours after gamma irradiation, the group with irradiated with gamma irradiation 3days after Ad-*LacZ* transfection, the group transfected with Ad-*p53* 3hours after gamma irradiation, and the group treated with gamma irradiation 3 days after Ad-*p53* transfection. Three hours after gamma knife irradiation and three days after *p53* gene transfection[11,12], viability assessment was performed.

Assessment of apoptosis

To independently assess necrosis and apoptosis, annexin binding in early-staged apoptotic cells was evaluated using an Annexin V-FITC Apoptosis Detection kit I (Pharmingen, San Diego, CA). Cells were plated at 2×10^5/well in two wells of six well-plates. After treatment, cells were harvested by centrifugation at 700rpm for 5min. Cell pellets were resuspended in

200ul of Annexin V buffer, 5ul of Annexin V-FITC and 10ul of propidium iodide (PI, 50ug/ml). Annexin V-positive and PI-positive cells were taken to be apoptotic.

Forty eight hours after gamma knife irradiation at a dose of 20Gy, and three days after *p53* gene transfection, annexin assays were also performed for different treated groups; i.e., the mock group, the Ad-*p53* treated group, the Ad-*lacZ* treated group, the group transfected with Ad-*LacZ* 48 hours after gamma irradiation, the group transfected with Ad-*p53* 48hours after gamma irradiation, the group treated with gamma irradiation 3days after Ad-*LacZ* transfection, the group treated with gamma irradiation 3days after Ad-*p53* transfection.

Cell cycle analysis

Tumor cells were seeded at a density of 4×10^5/well in two wells of six well-plates 24hours before treatment. Cells were harvested by centrifugation at 1200rpm for 5min, washed twice in ice-cold PBS, fixed in 70% ethanol, stored at 4 ℃ for a minimum of 1hour, washed with ice-cold PBS, and resuspended in a mixture of 200ul PBS, 20ul RNase (10mg/ml [Promega, Madison, WI]), and 2ul PI (25mg/ml[Sigma, St Louis, MO]). The cell suspension was passed through a 40um pore filter to remove undesirable particles and aggregates. FACS (Fluorescence-Activated Cell Sorting) was performed using a Becton Dickinson FACScan using an excitation laser set at 480nm and a detection wavelength of 575nm. A minimum of 10,000 events/sample was analyzed.

Forty eight hours after gamma irradiation at a maximum dose of 20Gy and three days after *p53* gene transfection, the mock group, the group treated with gamma irradiation at 20Gy only, the group transfected with Ad-*LacZ* 48hours after gamma irradiation, the group treated with gamma irradiation 3days after Ad-*LacZ* transfection, the group transfected with Ad-*p53* 48hours after gamma irradiation, and the group treated with gamma irradiation 3days after Ad-*p53* transfection were subjected to FACS analysis.

Statistical analysis

SPSS (version 11.0, SPSS Inc., Chicago, IL) was used for analyzing the MTT test. Optical densities of the treated groups were compared over more than three groups using the Kruskal-Wallis test and between two groups using the Mann-Whitney test. Differences were deemed significant at $p<0.05$.

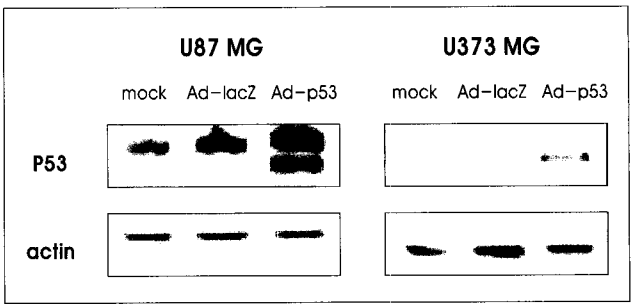

Fig. 1. Western blot analysis. Confirmation of *p53* expression by adenoviral vectors in U87 MG (left) and U373 MG cells (right) by Western blotting. The relative expression of exogenous wild-type *p53* is lower than that of endogenous *p53* in U87 MG cells. Actin is used as an internal loading control.

Results

Expression of β-galactosidase activity

At a virus titer of 100MOI, more than 90% of the cells infected with Ad-*LacZ* were found to express high levels of β-ga-lactosidase (Data not shown), which was localized to the cytoplasm and nucleus by X-gal staining.

Western blot analysis

Forty-eight hours after *p53* transfection, protein extracts were obtained. The transfection of Ad-*p53* induced the expression of *p53*. Endogenous *p53* expression was observed in U87MG cells but not in U373MG cells. As shown in Fig. 1, the relative molecular weight of the exogenous *p53* was lower than that of the wild-type *p53* gene in U87MG cells.

Assessment of cell growth inhibition by MTT assay

The results of the MTT assays for combined treatment with gamma irradiation and *p53* gene transfection are shown in Fig. 2. No significant difference in cell survival was observed between the *p53*-treated and *p53*-nontreated groups (p=0.706)

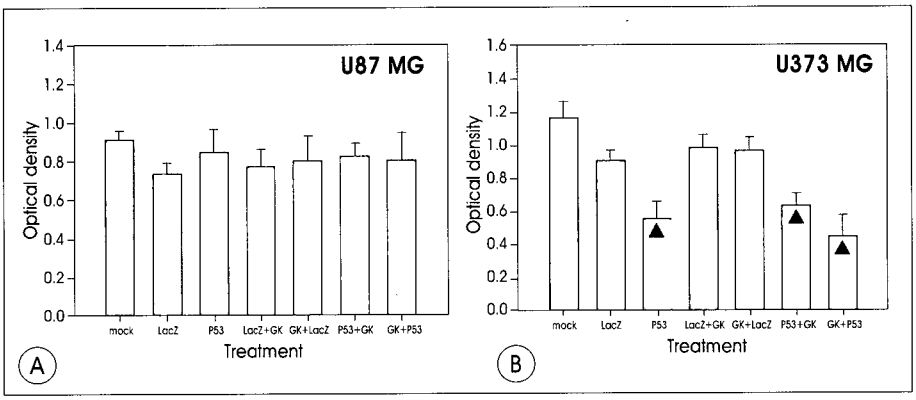

Fig. 2. MTT assay. Cell survival is lower, especially in group with *p53* transfection after gamma irradiation, on U373 MG. The difference is statistically significant (black triangle). A : U87 MG; B : U373 MG.

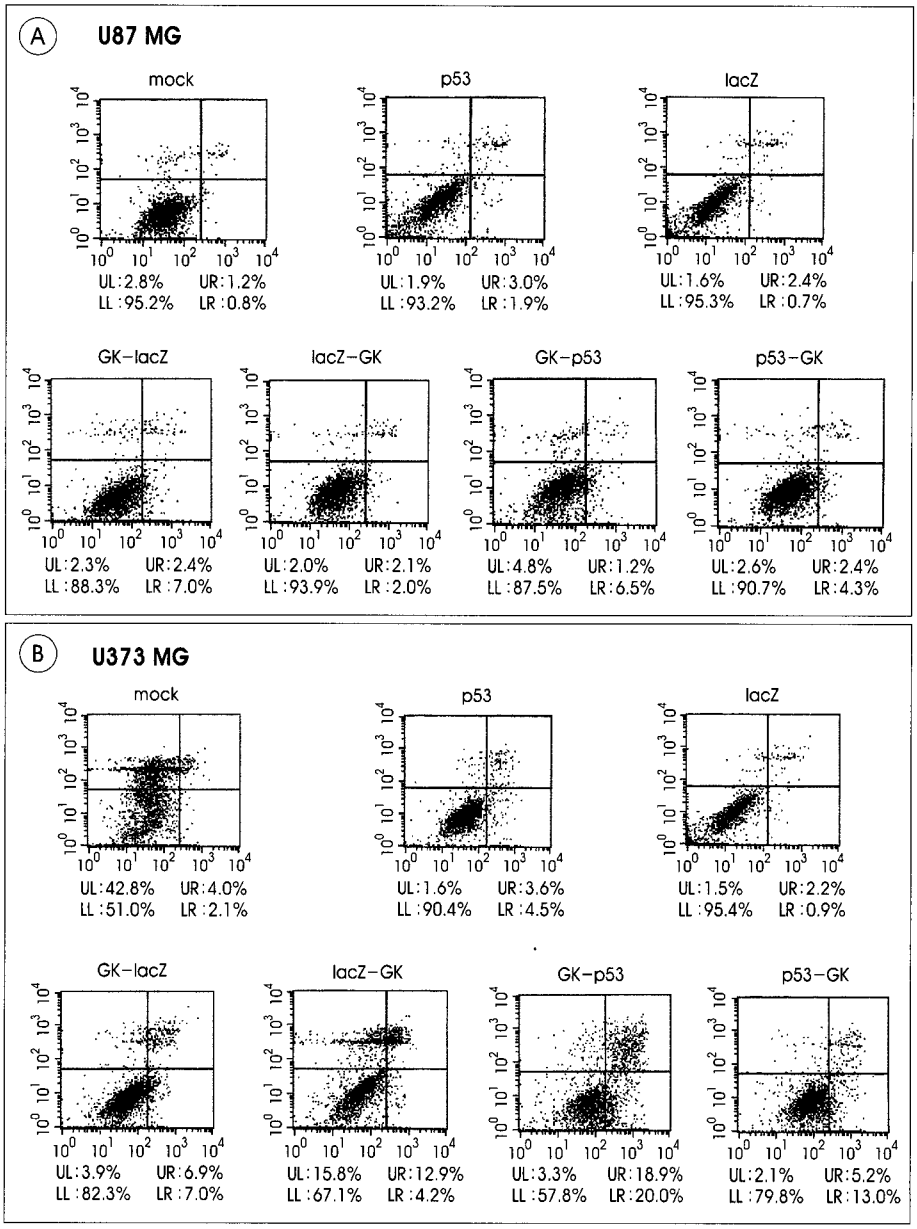

Fig. 3. Annexin assay. The assay shows that apoptosis tended to increase by transfecting p53 after gamma irradiation on U373 MG. A : U87 MG; B : U373 MG.

in U87MG. On the contrary, U373MG showed a definite difference between the *p53*-treated and *p53*-nontreated groups (p=0.000). In addition, of the gamma irradiation pretreated groups, least cell survival was shown among the *p53*-treated groups (p=0.010).

Assessment of apoptosis by annexin assay

More apoptotic changes were observed for *p53* transfection after gamma irradiation for U373MG cells. However, U373 MG cells treated with gamma knife irradiation after *p53* transfection were 18.2% positive for apoptosis, whereas cells transfected with *p53* after irradiation were 38.9% positive (Fig. 3).

Cell cycle analysis by FACS assay

No significant changes were observed between the *p53*-treated and *p53*-nontreated groups. Moreover, U373 MG cells treated with *p53* transfection after gamma irradiation were prominently arrested in the G2-M phase of the cell cycle : 90.8% of U373 MG cells transfected with *p53* after gamma irradiation were found in the G2-M phase of the cell cycle, whereas cells treated with gamma irradiation after *p53* transfection were 35.9% positive for G2-M phase (Fig. 4).

Discussion

The *p53* gene is located on chromosome 17p13.1 and contains 11 exons within a domain of 20kb. Loss of *p53* function occurs in 35 to 60% of glioblastomas and appears to be an early event in tumor progression[7,26]. It is now understood that *p53* causes cell cycle arrest at G1 and the induction of apoptosis[14,16]. The overexpression of *p53* prevents cell cycle progression from the G1 to the S phase by the transcriptional activation of *p21*, an inhibitor of cyclin-dependent kinases (CDKs)[5,6]. *p53* also induces apoptosis through either transcriptional activation of the Bax or Fas genes or via transcription-independent pathways[10,18,19,22].

Despite recent advances in *p53* biology, the relationship between *p53* gene expression and cellular radiosensitivity remains unclear. Reports of increased, decreased, and no apparent differences in radiosensitivities have all been associated with *p53* function[17,24,25]. In a normal cell, irradiation or some other DNA-damaging agents can induce a *p53*-dependent cell cycle arrest, which presumably allows time for genome repair. Alternatively, if DNA damage is beyond repair, *p53*-dependent apoptosis be triggered to prevent the transfer of new mutations

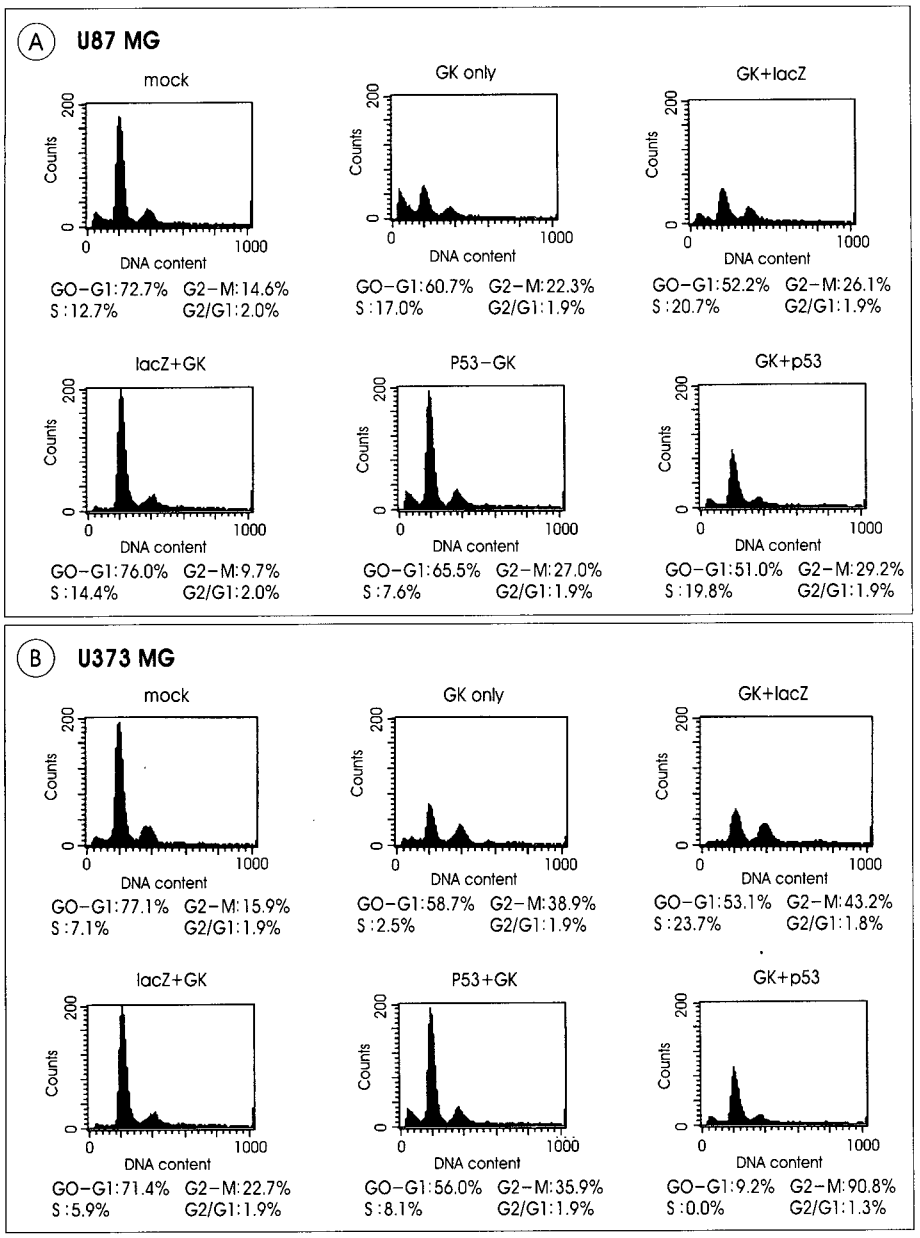

Fig. 4. FACS assay. Cells transfected with p53 after gamma irradiation are arrested more at the G2-M phase of the cell cycle, especially in U373 MG. A : U87 MG; B : U373 MG.

to daughter cells. In a tumor cell bearing wild-type *p53* ($p53^{wt}$), however, such a response could provoke both beneficial and harmful results. After DNA damage have been inflicted by chemotherapy or radiotherapy, *p53*-dependent G1 arrest could confer therapeutic resistance by providing tumor cells with enough time to undergo DNA repair before cell division. Conversely, *p53*-dependent apoptosis could confer therapeutic sensitivity upon the tumor cells. In a tumor cell bearing mutant-type *p53* ($p53^{mut}$), the opposite may be true : cells may become sensitive by not arresting in G1 for repair or may become resistant by not inducing the apoptosis provoked by radiotherapy or chemotherapy. The overexpression of *p53* by gene therapy may, therefore, have both beneficial and harmful therapeutic consequences, which may depend upon the cell type and endogenous nature of *p53* and other cell cycle and apoptotic regulatory genes[9].

In the present study, U373MG cells containing $p53^{mut}$ showed Ad-*p53* induced apoptosis within 48hours of viral exposure, which is consistent with previous reports[1,8]. Although gamma irradiation alone did not induce apoptosis within this time frame, it did result in G2-M arrest, confirming the lack of *p53* function in the G1-S checkpoint in this $p53^{mut}$ cell line. The finding that the G2-M phase fraction was 35.9% in U373MG treated with gamma irradiation after *p53* transfection where it was 90.8% in the same cells transfected with *p53* after gamma irradiation also supports this idea. Thus, treatment of U373MG cells with *p53* transfection after gamma irradiation induced more apoptosis than the reverse treatment. This observation indicates either that *p53* cannot act as a radiosensitizer in this type of cell line or that the gamma irradiation might cause synchronous cell cycle arrest in most of the tumor cells, and hence causes the tumor cells to be more susceptible to the apoptosis induced by the *p53* transfection.

It has been proposed that *p53* overexpression by viral vectors induces both growth inhibition and programmed cell death in culture and in tumors regardless of whether the tumor cells express $p53^{wt}$ or $p53^{mut}$ [4,15,21]. However, some authors have reported that U87MG cells showed least response to Ad-*p53* transfection, probably due to a lack of apoptosis and weak G1 arrest[8,13]. This lack of a response in $p53^{wt}$ tumors is supported by the results of the present study, i.e., of no significant differences between U87MG cells harboring $p53^{wt}$ exposed to combined gamma irradiation and *p53* transfection in terms of

cell survival and apoptosis. In U87MG cells, the apoptotic pathways might already be saturated due to *p53* expression.

In addition, our data showed that the G2-M phase cell cycle arrest was more exaggerated in the groups treated by *p53* transfection after gamma knife irradiation than in those irradiated by gamma knife after *p53* transfection. This may be explained by the synchronous cell cycle arrangement in most of the tumor cells by gamma knife irradiation, which is more susceptible to the cell cycle arrest by successive *p53* transfection, as described above.

Conclusion

The response to combined *p53* transfection and gamma irradiation may be variable among gliomas. This combined treatment may not be an effective treatment for $p53^{wt}$-expressing malignant gliomas, and its efficacy on $p53^{mut}$ tumors may be limited not only by transduction efficiency *in vivo* but also by the activation of other cell cycle regulatory or apoptotic machinery.

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