

Apoptosis Induced by Adenovirus-mediated *p53* Gene Transfer in Human Glioma Correlates with Site-specific Phosphorylation¹

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ABSTRACT

Therapeutic replacement of the *p53* gene using an adenovirus vector (Ad-*p53*) may be an effective alternative to conventional therapies for the treatment of glioma. We have previously demonstrated that the introduction of Ad-*p53* into glioma cells containing mutant *p53* induces apoptosis, whereas glioma cells containing wild-type *p53* are resistant. However, Ad-*p53* will enhance the radiosensitivity of wild-type *p53* glioma cells by increasing their tendency for apoptosis. The mechanism underlying these different responses to Ad-*p53* has not been elucidated to date. Because phosphorylation of *p53* at serines 15, 20, and 392 may play a role in regulating *p53*-mediated apoptotic activity, we determined the phosphorylation status of exogenous *p53* in mutant and wild-type gliomas after Ad-*p53* transfer. Monolayer cultures of glioma cell lines expressing mutant *p53* (U251 and U373) or wild-type *p53* (U87 and D54) were infected with Ad-*p53* and analyzed by Western blotting. High levels of exogenous *p53* were detected in both cell lines after Ad-*p53* transfer. However, only apoptotic mutant *p53* cells expressed high levels of phospho-Ser15-*p53* and phospho-Ser20-*p53*. The levels of phospho-Ser15-*p53* and phospho-Ser20-*p53* were very low in wild-type *p53* cells after Ad-*p53* infection alone. When wild-type *p53* glioma cells were exposed to radiation after Ad-*p53* infection, phospho-Ser15-*p53* and phospho-Ser20-*p53* were detected at high levels, and the cells subsequently underwent apoptosis; no change in serine 392 was detected. The induction of apoptosis and the expression of phospho-Ser15 and phospho-Ser20 in these cells were also enhanced by the combination of Ad-*p53* and other DNA-damaging agents such as cisplatin and bichloroethyl nitrosourea. Furthermore, the expression of phospho-Ser15-*p53* and phospho-Ser20-*p53* correlated with the amount of apoptosis; the apoptotic activity of *p53* in glioma cells was partially inhibited by a mutation of *p53* at serine 15. These results suggest that phosphorylation of *p53* at serine 15 and serine 20 is critical for apoptosis induction in *p53* gene therapy for gliomas.

INTRODUCTION

Despite advances in conventional therapy over the past four decades (1), survival time for patients with glioblastoma multiforme, the most common malignant human glioma, has remained at <1 year. Because *p53* is frequently mutated or inactivated in human gliomas (2–8) and because it plays a critical role in cell cycle arrest and apoptosis (9, 10), replacement of the *p53* gene may be an effective treatment alternative for gliomas (11, 12). Several current studies using this approach have demonstrated that transfer of the *p53* gene using a replication-deficient type 5 adenovirus vector (Ad-*p53*) facilitates induction of high levels of apoptosis in glioma cells that contain a mutant *p53* allele (12–15). Based on these data, a Phase I clinical trial using Ad-*p53* to treat patients who have recurrent high-grade gliomas is currently under way (16).

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Although Ad-*p53* appears to be a promising therapy for gliomas containing mutant *p53*, studies have also shown that Ad-*p53* has little effect on gliomas that contain normal (wild-type) *p53* alleles. Gomez-Manzano *et al.* (13) were the first to demonstrate that unlike gliomas containing mutant *p53*, glioma cell lines harboring wild-type *p53* alleles do not undergo apoptosis after infection with Ad-*p53*. Thus these wild-type *p53* cells are resistant to the apoptosis-inducing effects of Ad-*p53* (13, 17). To overcome this resistance, we have combined Ad-*p53* with IR³ to induce apoptosis in wild-type *p53* glioma cell lines, resulting in radiosensitization of the glioma cells (18). This radiosensitizing action of Ad-*p53* on wild-type *p53* cell lines has recently been reproduced for other tumor types, including prostate and lung cancer (19, 20).

Although the different responses of mutant *p53* and wild-type *p53* gliomas to Ad-*p53* alone have been known for some time, the mechanism underlying these differences has not been elucidated to date. The mechanism underlying the induction of apoptosis when Ad-*p53* is combined with radiation in wild-type *p53* gliomas is also unknown. It is well established that under normal physiological conditions, *p53* activity is regulated by multiple posttranslational modifications, including phosphorylation (10, 21). The *p53* protein is phosphorylated at several serine residues within the NH₂-terminal region (serines 6, 9, 15, 20, 33, and 37) and the COOH-terminal region (serines 315, 378, and 392) by a number of kinases. Of the multiple sites within *p53*, serine 15 was the first site shown to be inducibly phosphorylated (22–26). Both DNA-dependent PK and the ATM protein are capable of phosphorylating serine 15 in response to DNA damage, particularly after IR, and thereby inhibiting the ability of mdm-2, the primary negative regulator of *p53*, to complex with *p53* and target it for degradation. Likewise, phosphorylation of serine 20 is believed to be involved in the dissociation of *p53* from mdm-2 and to mediate the *p53* response to IR (24, 26). In contrast, serine 392 in the COOH terminus was shown to be phosphorylated by casein kinase II and to mediate responses to UV but not γ -radiation. Thus, current evidence suggests that phosphorylation of *p53* may play a role in regulating several *p53* functions, including its site-specific DNA binding, transcriptional activity, and transrepressional activity (22, 23, 25–29), and, significantly for our study, that phosphorylation may be critical in mediating *p53*-induced apoptosis (25).

Although *p53* phosphorylation has been shown to influence *p53* activity under physiological conditions, it has not been determined whether these posttranslational modifications affect the activity of supraphysiological levels of *p53*, such as those generated by adenovirus delivery. Defining the role of such posttranslational modifications could impact the effectiveness of Ad-*p53* gene therapy and also provide insight into the relative importance of these modifications for normal *p53* function. Therefore, we compared the phosphorylation status of exogenous *p53* in mutant *p53* and wild-type *p53* glioma cell lines after infection with Ad-*p53* alone and in combination with IR or

³ The abbreviations used are: IR, ionizing radiation; BCNU, bichloroethyl nitrosourea; PK, protein kinase; MEM, modified Eagle's medium; MOI, multiplicity of infection; PE, plating efficiency; SF, surviving fraction; mAb, monoclonal antibody; EGFP, enhanced green fluorescent protein; ATM, ataxia-telangiectasia mutant; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

DNA-damaging chemotherapeutic agents. We found that posttranslational phosphorylation of exogenous p53 on serines 15 and 20, but not on serine 392, is critical for the induction of apoptosis during p53 gene therapy and that the radio- and chemosensitizing action of p53 may be mediated at least in part through these posttranslational modifications.

MATERIALS AND METHODS

Human Glioma Cell Cultures. The cell lines U251, U87, and U373 were obtained from the American Type Culture Collection (Manassas, VA). D54 was generously provided by Dr. Darell Bigler (Duke University, Durham, NC). Cells were maintained in MEM supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air. U87 and D54 cells have homozygous wild-type p53 alleles based on sequence evaluations, whereas U251 and U373 cells contain a mutant p53 allele at codon 273 (CGT/CAT; Arg/His; Ref. 13).

Recombinant Adenovirus. The generation and features of the Ad-p53 construct have been reported previously (13, 17, 18). The E1A-deleted adenovirus vector (dl312) was used as a control.

Ad-p53 Infection Conditions. Cells (2.2×10^5) were plated in 10-cm dishes. Twenty-four h after plating, the cells were washed with PBS and incubated with purified virus in 2 ml of MEM without serum for 1 h at 37°C in a humidified atmosphere containing 5% CO₂/95% air, with brief agitation every 10 min. A MOI was based on the original cell number plated in all experiments and in all experiments was established at 100 plaque-forming units/cell. After 1 h, fresh MEM supplemented with 10% FBS was added to each dish.

Radiation Parameters. Two days after infection with Ad-p53, cell monolayers were irradiated with 9 Gy of radiation at room temperature using a cesium-137 source.

Chemotherapy Parameters. Cisplatin and BCNU were purchased from Bristol-Myers Squibb (Wallingford, CT). Two days after infection with Ad-p53, cell monolayers were treated with increasing doses of cisplatin or BCNU for 1 h. After 48 h, cells were analyzed for clonogenic survival.

Clonogenic Assay and Analysis. After irradiation, cells were trypsinized with 0.05% trypsin/1 mM EDTA solution and replated in specified numbers into 60-mm dishes for determination of colony-forming ability. After 14 days of incubation, dishes were stained with 0.5% crystal violet in absolute methanol, colonies with >50 cells were counted, and PE (number of surviving cells/number of cells plated) and SF (PE of experimental group/PE of control group) were determined. Chemotherapy survival curves were generated by combining data from three independent experiments and fitting the average survival levels by least-squares regression using the linear-quadratic model, as described by Fertil and Malaise (30).

Colorimetric Assay and Analysis. Cells were seeded in 96-well plates (300–900 cells/well) and treated 24 h later with Ad-p53, control vector, or medium at the appropriate MOI in 50 μ l of serum-free medium for 1 h as described above. After 1 h, 150 μ l of medium with 10% FBS were added to each well. After 2 days, cells were irradiated with increasing doses as described above. Ten days later, cell survival was determined using a colorimetric MTT assay as described previously (31), and 0.25 mg of MTT (50 μ l of 5 mg/ μ l MTT) was added to each well and incubated for 4 h at 37°C. Medium was then aspirated, and formazan crystals were dissolved in 150 μ l of DMSO in all experiments. The absorbance was read at 540 nm using an automated microplate reader. SF (absorbance of experimental group/absorbance of control group) was determined for each treatment.

Plasmids and Transfections. Human wild-type p53 cDNA and mutant p53 cDNA (Ser¹⁵ to Ala) constructs were generously provided by Dr. Guillermina Lozano (The University of Texas M. D. Anderson Cancer Center). The cDNA constructs were cloned into the EcoRV site of the internal ribosome entry site-EGFP plasmid (pIRES-EGFP; Clontech, San Diego, CA) vector. For transfection, cells were plated at 10⁵ cells/6-cm dish. Twenty-four h after plating, cells were transfected with 1 μ g of plasmid mixtures and preincubated for 15 min with 6 μ l of FuGENE6 transfection reagent (Roche Diagnostics Corp., Mannheim, Germany).

Western Blot Analysis. The p53 protein and phospho-p53 (serine 15, 20, and 392) levels were determined by Western blotting as described previously (18). Briefly, cells were collected at the indicated time points, and cell lysates

were prepared in 10 mM Tris (pH 7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP40, 5 mM NaF, 0.5 mM sodium vanadate, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Proteins were separated by means of 8% SDS-Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk, 0.05% Tween 20, 150 mM NaCl, and 50 mM Tris (pH 7.5) and incubated with the primary antibodies [mouse antihuman p53 mAb (DO-7; PharMingen, San Diego, CA), mouse antihuman α -tubulin mAb (Oncogene Research Products, Cambridge, MA), mouse antihuman phospho-p53 mAb (Ser15; Cell Signaling Technology, Beverly, MA), and rabbit antihuman phospho-p53 polyclonal antibody (Ser20 and Ser392; Cell Signaling Technology)]. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody and developed according to the manufacturer's instructions.

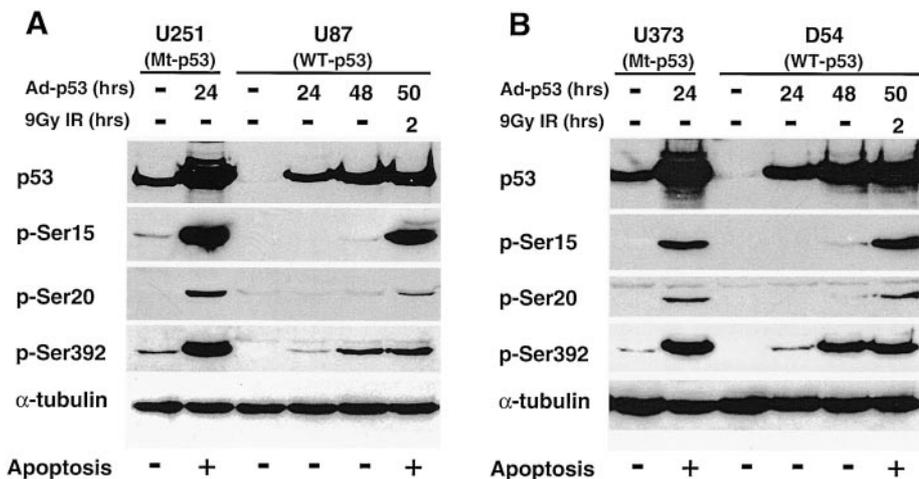
Annexin V-PE Assay and Flow Cytometry. Annexin V-PE assay (PharMingen) was performed according to the manufacturer's instructions. Briefly, cells were collected and washed twice with cold PBS and resuspended in 1 \times binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂]. Annexin V-PE (5 μ l) was added to 100 μ l of solution (1×10^5 cells), and the mixture was incubated in the dark for 15 min. After the addition of 400 μ l of 1 \times binding buffer, the specimens were analyzed with the aid of flow cytometry. An analysis region was set based on the negative controls, and the percentage of labeled cells was calculated from this region.

RESULTS

Expression of Phosphorylated p53 after Ad-p53 Infection in Mutant p53 and Wild-type p53 Glioma Cell Lines. To investigate whether posttranslational phosphorylation of exogenous p53 plays a role in the different responses of gliomas containing mutant p53 and those with wild-type p53 to treatment with Ad-p53 alone, we compared the phosphorylation status of exogenous p53 after Ad-p53 infection in U251 cells (which contain mutant p53) with that of exogenous p53 in U87 cells (which contain wild-type p53). Because previous studies suggested that phosphorylation of serines 15 and 20 is important in p53-mediated apoptosis under physiological conditions, we analyzed these sites using phosphorylation-specific antibodies. The phosphorylation status of serine 392 was also determined to gain insight into the significance of posttranslational modifications in the COOH terminus of adenovirus-delivered p53.

Monolayer cultures were treated with Ad-p53 (MOI 100) or PBS, and cells were collected 24–48 h after infection and analyzed by Western blotting (Fig. 1A). As seen in Fig. 1, uninfected U251 cells (which contain a mutant p53 allele) had readily detectable levels of endogenous p53, consistent with the increased half-life of the mutated p53 protein (32–34). In contrast, in uninfected U87 cells (which contain wild-type p53), little or no endogenous p53 protein was detected. Infection of both cell lines with Ad-p53 resulted in high levels of exogenous p53 protein expression in each, indicating similar levels of Ad-p53 infectivity and p53 expression in the mutant p53 and wild-type p53 cell lines. However, phosphorylated p53 was detected only in the mutant p53 cell line U251 after Ad-p53 infection. Significant increases in phosphorylation at serine 15 (phospho-Ser15-p53), serine 20 (phospho-Ser20-p53), and serine 392 (phospho-Ser392-p53) were evident in U251 cells 24 h after infection. In contrast, there was low or undetectable expression of phospho-Ser15-p53 and phospho-Ser20-p53 in U87 cells at both 24 and 48 h after Ad-p53 infection. Phosphorylation of p53 at serine 392 was evident in U87 cells at 24 h after Ad-p53 infection and increased further by 48 h after infection. Identical results were obtained in U373 cells (which contain mutant p53) and D54 cells (which contain wild-type p53), indicating that the effect was evident in other mutant and wild-type p53 cell lines (Fig. 1B). Because only the U251 and U373 cell lines undergo apoptosis after Ad-p53 infection (13, 17, 18), and there was no evidence of apoptosis in U87 or D54 cells even at 48 h after Ad-p53 treatment (13, 18), the induction of apoptosis in the mutant p53 cell lines clearly

Fig. 1. Western blot analysis of glioma cells (A, U251 and U87; B, U373 and D54) for p53 and phospho-p53 after treatment with Ad-p53 (100 MOI). Cells (2.2×10^5) were plated as monolayers and treated 24 h later with Ad-p53. At the indicated times, whole cell lysates were prepared. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes, and sequentially analyzed with antibody to p53 (DO-7), phospho-Ser15-p53, phospho-Ser20-p53, phospho-Ser392-p53, or α -tubulin. In addition, 48 h after infecting wild-type p53 glioma cells (U87 and D54) with Ad-p53, cells were exposed to 9 Gy of radiation. After 2 h, cell lysates were prepared and analyzed as described.



correlates with phosphorylation of exogenous p53. Specifically, it was the phosphorylation of serines 15 and 20 but not serine 392 that was associated with apoptosis induction.

Expression and Phosphorylation of p53 after Ad-p53 plus IR in Wild-type p53 Gliomas. We have previously shown that Ad-p53 sensitizes the U87 glioma cell line (which contains wild-type *p53* alleles) to IR and that this radiosensitization is associated with an increase in apoptosis (18). To determine whether the sensitizing action of Ad-p53 observed in U87 cells occurs in other glioma cell lines containing wild-type *p53*, we infected the glioma cell line D54 with Ad-p53 (MOI 100), vector control (dl312), or medium alone, and 2 days later, cultures were irradiated (see "Materials and Methods"). Infection of D54 cells with Ad-p53 alone had no measurable effect on the survival of D54 cells (Fig. 2A); however, Ad-p53 significantly enhanced the radiosensitivity (Fig. 2B). Moreover, similar to U87 cells, this Ad-p53 radiosensitization in D54 cells was associated with an increase in apoptotic frequency (Fig. 2C). Thus, Ad-p53-mediated radiosensitization is not unique to U87 glioma cells.

To determine whether Ad-p53-mediated radiosensitization of wild-type p53 gliomas is associated with phosphorylation of exogenous p53, we treated U87 (Fig. 1A) and D54 (Fig. 1B) cells with IR (9 Gy) 48 h after infection with Ad-p53 and analyzed them for phosphorylated p53 at serines 15, 20, and 392. Only slight increases in p53 levels could be detected after the addition of Ad-p53 plus IR compared with the addition of Ad-p53 alone. However, significant levels of phospho-Ser15-p53 and phospho-Ser20-p53 were detected after IR. In contrast,

there was no significant difference in phospho-Ser392-p53 expression after Ad-p53 with or without 9 Gy of radiation. As shown in Fig. 3, increases in phospho-Ser15-p53 and phospho-Ser20-p53 after treatment with Ad-p53 and IR occurred within 30 min after radiation, were maximal at 4 h, and had diminished by 48 h. Treatment of U87 cells with control vector (dl312) and IR did not result in significant apoptotic cell death, nor did treatment with dl312 and IR induce significant p53 phosphorylation (Fig. 3). Therefore, whereas Ad-p53 or IR alone does not induce phosphorylation of p53 at serines 15 or 20, and these individual treatments do not induce apoptosis, the combination of Ad-p53 and radiation resulted in serine 15 and serine 20 phosphorylation and increased the frequency of apoptosis. Taken together with the results of Ad-p53-mediated apoptosis in mutant p53 cells, these findings support the notion that phosphorylation is critical for apoptosis induction during *p53* gene therapy.

Apoptotic Activity and p53 Phosphorylation Induced by Ad-p53 plus Chemotherapeutic DNA-damaging Agents. Because IR induces DNA damage, we sought to determine whether Ad-p53 sensitizes glioma cells to the DNA-damaging agents cisplatin and BCNU. Monolayer cultures of U87 cells were treated with cisplatin (0–55 μ M for 1 h) or BCNU (0–40 μ M for 1 h) at 48 h after infection with either Ad-p53, control viral vector (dl312), or medium alone. We then assayed them for clonogenic survival. As shown in Fig. 4A at each delivered dose of cisplatin, transfection of the wild-type *p53* gene resulted in sensitization of the wild-type p53 U87 cell line. Similar sensitization was found for BCNU (data not shown).

Fig. 2. Effect of Ad-p53 and irradiation on wild-type p53 cell line D54. A, effects of Ad-p53 alone on clonogenic survival. D54 cells (10^5) were seeded in 6-well plates (300–900 cells/well) and treated 24 h later with medium, control vector (dl312; MOI 100), or Ad-p53 (MOI 100). After 10 days, cell survival was determined using a colorimetric MTT assay as described in "Materials and Methods." SF (absorbance of experimental group/absorbance of control group) was determined for each treatment. The values represent the mean \pm SE of three independent experiments. B, survival of D54 cells after irradiation and Ad-p53. D54 cells were plated in 6-well plates and treated with medium (MOI 0) or Ad-p53 (MOI 100). After 48 h, the cultures were irradiated with 2- or 4-Gy doses, and survival was determined using a colorimetric MTT assay. The values represent the mean \pm SE of four independent experiments. C, effects of Ad-p53 on radiation-induced apoptosis in D54 cells. Cells were irradiated at 9 Gy 48 h after treatment with medium or Ad-p53 (MOI 100). After irradiation, cells were incubated for 48 h and then subjected to annexin assay for quantitation of apoptotic cells as described in "Materials and Methods." Representative graph from three separate experiments is shown.

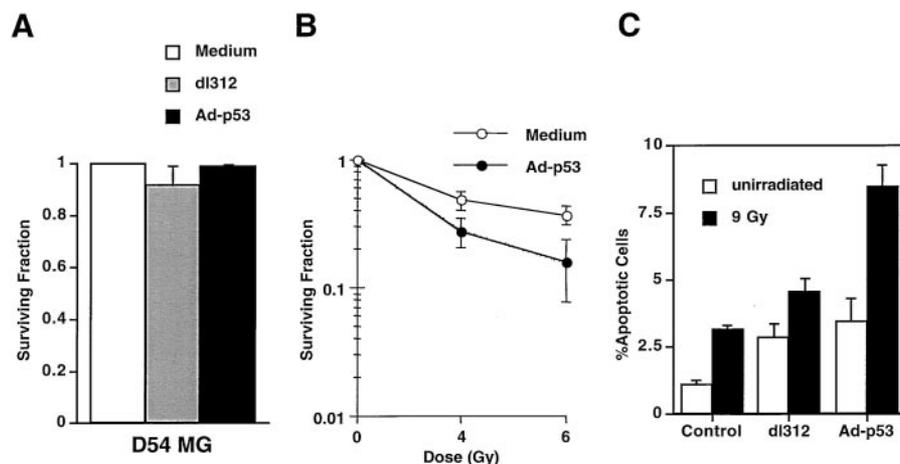
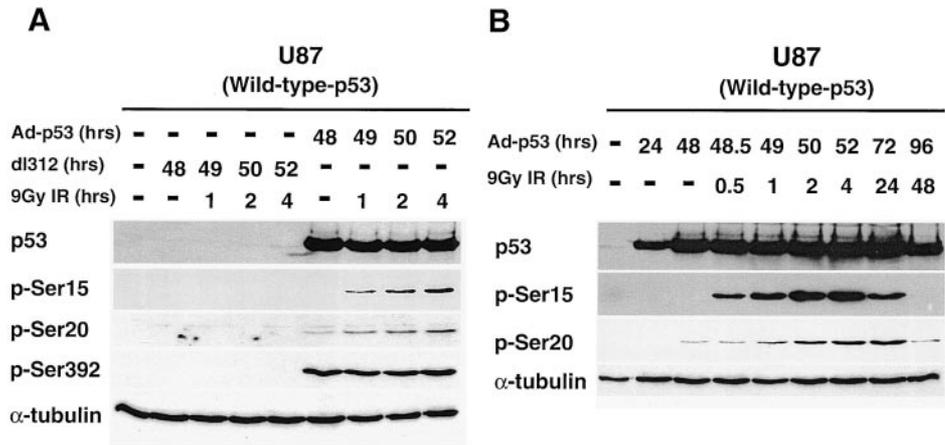


Fig. 3. *A*, Western blot analysis of U87 (wild-type p53) cells showing the time course of p53 protein and phosphorylated p53 expression after treatment with Ad-p53 or control vector (dl312) and 9 Gy of radiation. Cells (2.2×10^5) were plated as monolayers and treated 24 h later with Ad-p53 or dl312 (MOI 100). After 48 h, cells were exposed to 9 Gy of radiation, and whole cell lysates were prepared and analyzed as described in the Fig. 1 legend. *B*, Western blot analysis of U87 cells showing the extended time course of p53 and phosphorylated p53 expression after treatment with Ad-p53. Cells were treated as described in *A*.



To determine whether this Ad-p53-mediated chemosensitization is associated with an increase in the frequency of apoptosis, we calculated the amount of apoptosis using annexin V-PE assay and flow cytometry. U87 cells were treated with Ad-p53 or vector control (dl312), and after 48 h they were exposed to either cisplatin ($30 \mu\text{M}$) or BCNU ($40 \mu\text{M}$). Whereas treatment with control vector (dl312) in combination with each of these agents resulted in minimal apoptosis, the combination of Ad-p53 plus either cisplatin or BCNU resulted in a significant increase in apoptosis, as shown in Fig. 4*B*.

To investigate the relationship between the chemosensitizing action of Ad-p53 and phosphorylation of exogenous p53, we examined the expression of p53 protein, phospho-Ser15-p53, and phospho-Ser20-p53 in U87 cells after treatment with the combination of Ad-p53 and cisplatin. Whereas treatment with control vector plus cisplatin had no effect, treatment with Ad-p53 plus cisplatin resulted in significant phosphorylation of exogenous p53 at serines 15 and 20 and was maximal at 24 h after treatment with $30 \mu\text{M}$ cisplatin (Fig. 5*A*). The combination of Ad-p53 and $40 \mu\text{M}$ BCNU also enhanced the expression of phospho-Ser15-p53 and phospho-Ser20-p53 in U87 cells, with

maximal expression also occurring 24 h after BCNU treatment (see Fig. 5, time course not shown).

Based on the above-mentioned observations, we sought to determine whether the amount of apoptosis correlated with the level of p53 phosphorylation. As shown in Fig. 4*B* for U87 cells, the frequency of apoptosis was greatest when Ad-p53 was combined with $30 \mu\text{M}$ cisplatin, was least when Ad-p53 was combined with $40 \mu\text{M}$ BCNU, and was intermediate when Ad-p53 was combined with IR (9 Gy). To compare the expression of phospho-p53 in each combination therapy, U87 cells from the same experiments in which apoptosis was measured were collected simultaneously and subjected to Western blotting. As shown in Fig. 5, *B* and *C*, the highest level of expression of phospho-Ser15-p53 and phospho-Ser20-p53 occurred after Ad-p53 was combined with $30 \mu\text{M}$ cisplatin, whereas the lowest expression levels occurred after $40 \mu\text{M}$ BCNU. These data suggest that the level of p53 phosphorylation correlates with frequency of apoptosis.

Apoptotic Activity Induced by p53 Transfection is Partially Blocked by Mutating Serine 15. Several studies have suggested that phosphorylation of serine 15 is critical to apoptosis induction after DNA damage. Although our studies provide a correlation between apoptosis and site-specific p53 phosphorylation, in an attempt to establish a causal relationship we used a site-specific mutant *p53* construct (kindly provided by Dr. Guillermina Lozano) in which the serine at position 15 was replaced with alanine. Because of the potential biological hazard of constructing adenoviruses with mutant *p53*, we inserted this mutant *p53* construct or the wild-type *p53* construct into the plasmid pIRES-EGFP, which contains the EGFP next to the insert site. Thus, cells expressing EGFP also express p53, which allows for preferential selection of cells expressing high levels of p53 protein. To determine the relationship between phosphorylation at this site and apoptosis, U87 cells were transfected with *p-p53*-(ser15)-EGFP or *p-p53*-(ala15)-EGFP, treated with $30 \mu\text{M}$ cisplatin 48 h later, and subjected to Western blotting after selection for EGFP-positive cells by flow cytometry. As shown in Fig. 6*A*, treatment with $30 \mu\text{M}$ cisplatin alone or empty plasmid plus cisplatin resulted in a minimal increase in endogenous p53 expression and low levels of phospho-Ser15-p53 expression (Lanes 2 and 4). However, the level of exogenous p53 protein expression after transfection with *p-p53*-(ser15)-EGFP alone increased significantly and was the same as that after transfection with *p-p53*-(ala15)-EGFP alone (Lanes 5 and 7). Moreover, treatment with *p-p53*-(ser15)-EGFP plasmid plus $30 \mu\text{M}$ cisplatin significantly enhanced the expression of phospho-Ser15-p53 (Lane 6 versus Lane 5). Importantly, the combination of *p-p53*-(ala15)-EGFP and cisplatin abrogated the serine 15 phosphorylation.

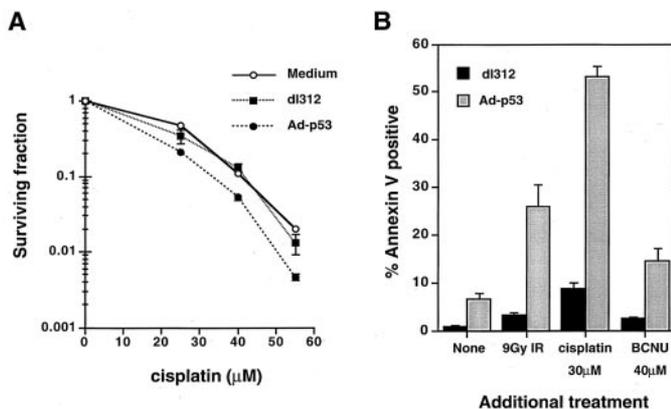


Fig. 4. *A*, effects of Ad-p53 combined with antineoplastic agent cisplatin on the survival of U87 cells (wild-type p53). Cells were plated on T25 flasks and treated 24 h later with medium (DMEM), control vector (dl312; MOI 100), or Ad-p53 (MOI 100) for 1 h. Cells were treated 48 h later with increasing concentrations of BCNU or cisplatin for 1 h, trypsinized, and replated in specified numbers into 60-mm dishes for determination of PE based on colony-forming ability. After 14 days of incubation, dishes were stained, and colonies with >50 cells were counted. Values represent the mean \pm SD of two to four independent experiments for both cell lines. *B*, annexin V assay of U87 cells showing apoptotic cell death after Ad-p53 or dl312 infection and treatment with IR, cisplatin, or BCNU. Cells (2.2×10^5) were plated as monolayers and treated 24 h later with Ad-p53 or dl312. After 48 h, cells were treated with 9 Gy of radiation, $30 \mu\text{M}$ cisplatin, or $40 \mu\text{M}$ BCNU; 96 h after Ad-p53 infection and 48 h after chemotherapy or IR, cells were collected and subjected to annexin V assay. Values represent the mean \pm SE of three independent experiments.

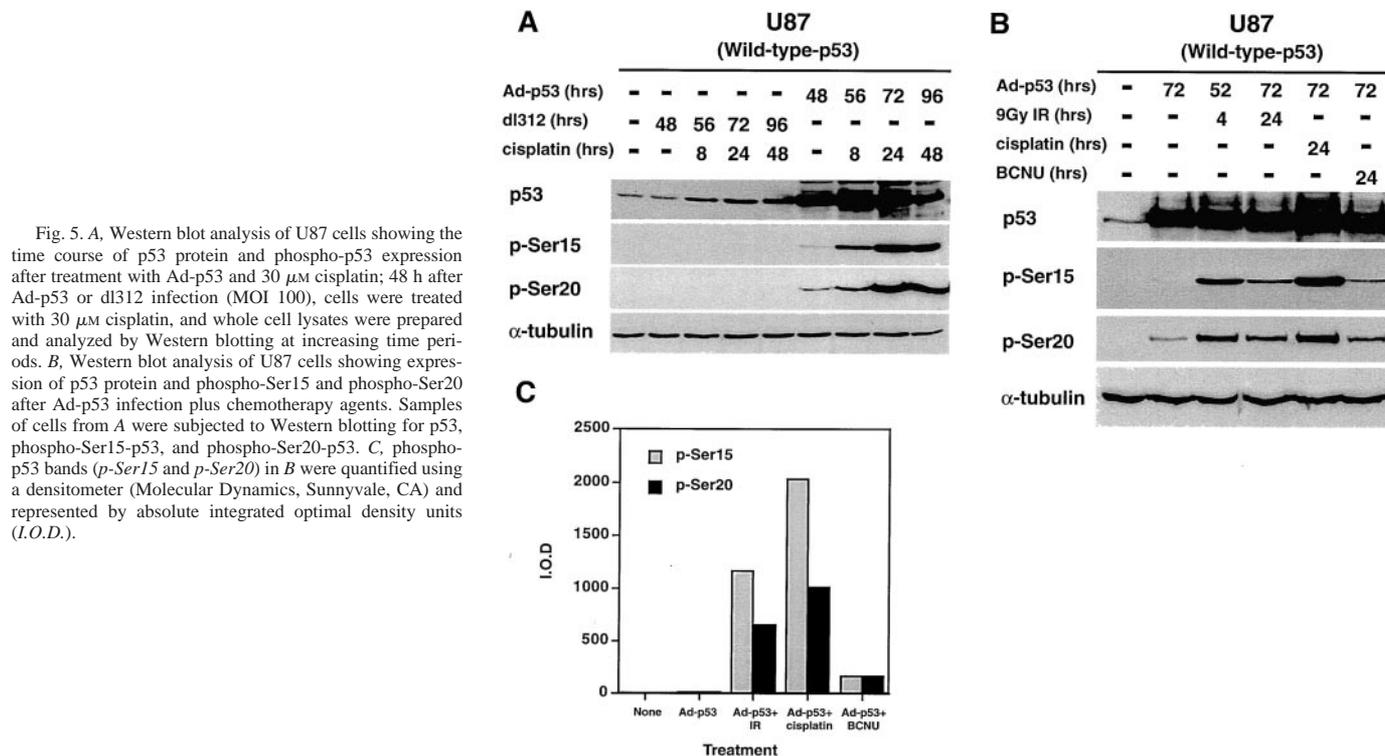


Fig. 5. A, Western blot analysis of U87 cells showing the time course of p53 protein and phospho-p53 expression after treatment with Ad-p53 and 30 μ M cisplatin; 48 h after Ad-p53 or dl312 infection (MOI 100), cells were treated with 30 μ M cisplatin, and whole cell lysates were prepared and analyzed by Western blotting at increasing time periods. B, Western blot analysis of U87 cells showing expression of p53 protein and phospho-Ser15 and phospho-Ser20 after Ad-p53 infection plus chemotherapy agents. Samples of cells from A were subjected to Western blotting for p53, phospho-Ser15-p53, and phospho-Ser20-p53. C, phospho-p53 bands (p-Ser15 and p-Ser20) in B were quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA) and represented by absolute integrated optical density units (I.O.D.).

Thus, the presence of alanine 15 instead of serine 15 prevented phosphorylation of the p53 protein at this site.

To determine whether this inhibition of phosphorylation was able to attenuate the p53-mediated induction of apoptosis after cisplatin, we measured the percentage of apoptosis using annexin V-PE in cells treated with p-p53-(ser15)-EGFP or p-p53-(ala15)-EGFP with or without cisplatin. Because the transfection frequency was approximately 30%, only EGFP-positive cells were counted to reduce the background associated with untransfected cells. As expected, treatment with p-p53-(ser15)-EGFP significantly enhanced the frequency of cisplatin-mediated apoptosis, as shown in Fig. 6B. This sensitization was partially blocked by p-p53-(ala15)-EGFP. Thus, although the inhibition of phosphorylation at serine 15 reduced the amount of apoptosis, it was not sufficient to completely abrogate apoptotic activity.

DISCUSSION

A number of reports have analyzed the role of phosphorylation in regulating p53 activity under physiological or near physiological conditions (22, 23, 26, 35). The investigations presented here address the role of phosphorylation in regulating p53 when it is present at supraphysiological levels, such as those that occur with gene therapy. Our data demonstrate that phosphorylation of exogenous p53 correlates with apoptosis induction during adenovirus-mediated p53 gene delivery to human glioma cell lines. Phosphorylation of p53 therefore appears to be an important mechanism underlying the different responses of mutant p53 and wild-type p53 gliomas to Ad-p53, and moreover, these data suggest that phosphorylation plays a critical regulatory role in the radio- and chemosensitizing actions of Ad-p53.

Our data demonstrate that there is specificity to the particular site of

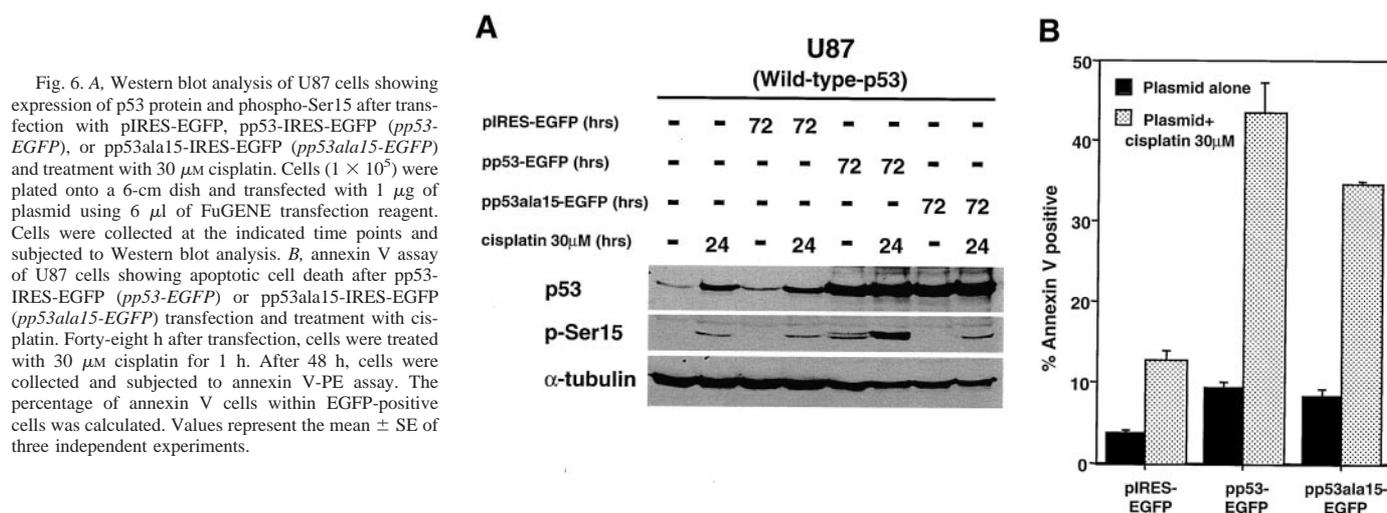


Fig. 6. A, Western blot analysis of U87 cells showing expression of p53 protein and phospho-Ser15 after transfection with pIRES-EGFP, pp53-IRES-EGFP (pp53-EGFP), or pp53ala15-IRES-EGFP (pp53ala15-EGFP) and treatment with 30 μ M cisplatin. Cells (1×10^5) were plated onto a 6-cm dish and transfected with 1 μ g of plasmid using 6 μ l of FuGENE transfection reagent. Cells were collected at the indicated time points and subjected to Western blot analysis. B, annexin V assay of U87 cells showing apoptotic cell death after pp53-IRES-EGFP (pp53-EGFP) or pp53ala15-IRES-EGFP (pp53ala15-EGFP) transfection and treatment with cisplatin. Forty-eight h after transfection, cells were treated with 30 μ M cisplatin for 1 h. After 48 h, cells were collected and subjected to annexin V-PE assay. The percentage of annexin V cells within EGFP-positive cells was calculated. Values represent the mean \pm SE of three independent experiments.

phosphorylation (*i.e.*, phosphorylation of serine 15 and serine 20 but not serine 392) correlated with apoptosis induction in mutant *p53* and wild-type *p53* gliomas. The relative importance of serines 15 and 20 compared with serine 392 in mediating apoptosis was particularly evident when Ad-p53 was combined with IR or DNA-damaging chemotherapeutic agents. Our findings are consistent with other reports on the role of phosphorylation of these sites under more physiological conditions. Although Aschcroft *et al.* (36) suggested that phosphorylation of most NH₂-terminal serines, including serines 15 and 20, was not critical to the function of p53 protein, Unger *et al.* (25) demonstrated that conversion of serines 15 and 20 to alanine with subsequent loss of phosphorylation at these sites significantly reduced p53-mediated apoptotic activity. Moreover, serine 15 has been shown to be a primary site of phosphorylation in normal cellular response to DNA damage from IR. Indeed, the gene product of the *ATM* gene, which is critical for radiation sensitivity, has been shown to specifically phosphorylate p53 at serine 15 in response to IR (37, 38). Likewise, Chehab *et al.* (24) have provided genetic and biochemical evidence that serine 20 phosphorylation is critical for p53 function after IR. Phosphorylation of p53 at both serines 15 and 20 has been shown to occur under physiological conditions after treatment with cisplatin (39). In contrast to these NH₂-terminal sites, to our knowledge there has been no association reported between phosphorylation of the COOH-terminal serine 392 and apoptosis induction. Although phosphorylation of serine 392 by casein kinase II has been shown to stimulate DNA binding (35) and potentiate tetramerization, the biological consequence of this modification has not been fully elucidated. Because serine 392 was phosphorylated in wild-type *p53* cells that did not undergo apoptosis after treatment with Ad-p53, our data suggest that this event is not critical to apoptosis induction. Moreover, our finding that serine 392 phosphorylation did not correlate with increases in apoptosis that occur during Ad-p53-mediated radiosensitization is consistent with the findings of Kapoor and Lozano (40), who showed that UV irradiation, but not γ -irradiation, induces phosphorylation at this site. Thus, despite the supraphysiological levels of p53 that occur after Ad-*p53* gene therapy, the activation of this exogenous p53 appears to be regulated in a manner similar to more physiological conditions, and the biological result of specific sites of phosphorylation is similar to that seen under physiological conditions. This suggests that the response of the cell to Ad-p53 depends on the context of the cell, that is, the array of p53-regulatory proteins and p53 sites that these proteins phosphorylate.

Although our main goal was to examine the mechanisms underlying the therapeutic application of p53, our results provide some insight into the physiological regulation of p53. In this regard, there is significant evidence suggesting that under physiological conditions, the level of p53 in a cell is important for regulating the cellular decision toward either cell cycle arrest or apoptosis (41–43). For example, Chen *et al.* (41), using an inducible p53 cell line, demonstrated that low levels of p53 resulted in cell cycle arrest, whereas higher levels induced apoptosis. Moreover, because phosphorylation of serine 15 results in dissociation of p53 from its negative regulator mdm-2, it has been suggested that the primary effect of phosphorylation is to increase p53 levels (22). However, our results suggest that activation of p53 via phosphorylation is at least as important as the level of p53 for inducing apoptosis. Specifically, although mutant *p53* cells were sensitive to Ad-p53-mediated apoptosis, and wild-type *p53* cells were resistant, both cell types contained extremely high levels of exogenous p53 protein after Ad-p53 delivery. However, only the sensitive mutant cells contained phosphorylated p53. Moreover, we have shown previously that a 10-fold decrease in the amount of Ad-p53 delivered to the mutant *p53* cells did not result in a decrease in apoptosis (18). Likewise, when Ad-p53 was combined with IR or

chemotherapy, increases in p53 levels were barely detectable relative to the high level of p53 present after Ad-p53 alone. However, the pool of phosphorylated p53 increased dramatically after IR and chemotherapy. Thus, phosphorylation of p53 correlates better with apoptosis induction than does the level of p53.

Our results suggest that significant differences exist in the upstream mechanisms that control phosphorylation of p53 in mutant *p53* compared with wild-type *p53* cell lines. Phosphorylation of exogenous p53 in wild-type *p53* cells occurs only in response to DNA-damaging signals, as would be expected. However, in mutant *p53* cells, exogenous p53 delivered by adenovirus was constitutively phosphorylated, and the endogenous mutant p53 was also phosphorylated in unstimulated cells (Fig. 1). These data suggest that the signaling pathway that leads to p53 phosphorylation is dysregulated in these mutant *p53* cells. Because phosphorylation of p53 is controlled by a variety of kinases, including ATM, ATR, DNA-dependent PK, cyclin-dependent kinases, casein kinase I and II, and the checkpoint kinases 1 and 2 (37, 38, 44, 45), the dysregulation of p53 phosphorylation in mutant *p53* tumor cells may be mediated by overexpression of one or more of these kinases. Alternatively, underexpression of p53-regulatory phosphatases may also be postulated (28). In addition, it has been reported recently that p53 is capable of down-regulating the PK CK2 (46), suggesting that wild-type p53 may negatively modulate its own regulatory kinases. Thus, the aberrant regulation of phosphorylation in mutant *p53* cells may be a direct consequence of p53 inactivation in these cells. Alternatively, because p53 “guards the genome” from DNA damage (47), its inactivation within mutant *p53* cells may result in a progressive accumulation of DNA damage that may constitutively activate p53 signaling pathways.

Although in wild-type *p53* gliomas phosphorylation of *exogenous* p53 occurs in response to DNA-damaging signals, our results demonstrate that *endogenous* p53 is not phosphorylated in wild-type p53 cells (U87 and D54) in response to these signals (Fig. 3). This lack of phosphorylation of endogenous p53 correlates with the low levels of apoptosis observed in these cells after treatment with IR, cisplatin, or BCNU (see Fig. 4B and Ref. 18) and supports the notion that p53 phosphorylation is associated with apoptosis induction. In fact, the inability to phosphorylate endogenous p53 and the resistance to apoptosis may be partly responsible for the dismal clinical outcome associated with conventional cytotoxic treatments of gliomas. More importantly, the inability of endogenous wild-type p53 to be phosphorylated after exposure to DNA-damaging agents suggests either that the DNA-damaging signal is itself dysregulated in these cells or that the endogenous p53 is not available to receive the signal. Because *exogenous* p53 is phosphorylated after treatment with DNA-damaging agents, the signaling pathway that leads to p53 phosphorylation appears to be functional in these tumor cells. Thus, our data suggest that endogenous p53 is not capable of receiving the DNA-damaging signal. In this regard, it is known that wild-type p53 cells typically have high levels of mdm-2 (48), the primary negative regulator of p53 (22, 26). High levels of mdm-2 may prevent the DNA-damaging signal from phosphorylating p53. Thus, the resistance to apoptosis of wild-type p53 gliomas after conventional therapy may result at least in part from inhibition of endogenous p53 phosphorylation, and the efficacy of conventional treatments may be potentiated by augmenting the pool of p53 that can be phosphorylated. As we have demonstrated, this increase in p53 can be accomplished by infection with Ad-p53. Alternatively, inactivation of mdm-2 may be another approach to achieve the same goal.

Although our studies demonstrate important roles of serines 15 and 20 in apoptosis induction, they also provide insight into the complexity of p53 regulation. In particular, site-specific mutagenesis of serine 15 to alanine attenuated but did not completely abrogate the enhance-

ment of apoptosis that occurred when exogenous p53 was combined with cisplatin (Fig. 6). Thus, although phosphorylation of serine 15 is important for apoptosis, it is not essential. Apoptosis may depend equally on phosphorylation of serine 20 or may require the sequential phosphorylation of both serines (serines 15 and 20) rather than the phosphorylation of one site (28). Alternatively, in addition to serines 15 and 20, other sites may also be important in apoptosis induction. In this regard, Oda *et al.* (27) have provided evidence that phosphorylation of serine 46 is specifically associated with increased expression of p53-regulated apoptosis-inducing protein 1 (p53AIP1), an apoptosis-specific protein that induces cell death by dissipating mitochondrial membrane potentials. Thus, although our studies demonstrate the importance of phosphorylation of p53 for apoptosis induction, detailed investigations into the relative contribution of each phosphorylation site should be the focus of further investigation.

Our studies have implications for the clinical application of p53 gene therapy. Because phosphorylation of p53, rather than just the level of the protein, appears to be critical to apoptosis induction, improvements in p53 gene therapy may be achieved by rational manipulation (*i.e.*, activation) of the regulators of p53 function. Defining these regulators and understanding the stimuli that activate them may provide insight into ways of promoting apoptosis after treatment with Ad-p53. This will be especially important for tumor cells that contain wild-type p53 alleles and are resistant to Ad-p53 treatment by itself. Because most gliomas, particularly the very aggressive glioblastomas multiforme (WHO grade IV), contain mixed populations of mutant and wild-type p53 cells, activating exogenous p53 by combining Ad-p53 with other agents will be necessary for this gene therapy approach to be effective against all gliomas. Therefore, analyzing phosphorylation of apoptosis-specific sites within p53 may provide a mechanism for rapidly screening and stratifying the potential effectiveness of agents that are synergistic with Ad-p53 and that can be combined with Ad-p53 in the clinical setting. In addition, p53 phosphorylation may be used as a surrogate marker for p53-mediated apoptosis and may provide a method for analyzing p53-induced apoptosis in human glioma tissue specimens obtained directly from the patient. Enhancing phosphorylation of p53 may thereby be used to improve the clinical efficacy of p53 gene therapy and as a diagnostic marker of effective therapy.

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