

MDM2 Does Not Influence p53-mediated Sensitivity to DNA-damaging Drugs¹

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Abstract

MDM2 inhibits transactivation properties of the tumor suppressor protein p53 by binding to and facilitating proteasomal degradation of p53. Because MDM2 targets p53 for degradation, it was anticipated that cells that overexpress MDM2 would not contain functional wild-type p53 (wtp53). However, p53 and MDM2 in cells with damaged DNA can become phosphorylated, and their binding to each other can become inhibited. Thus, p53 remains functional and induces apoptosis of damaged cells. Here we report the results of experiments designed to investigate whether MDM2 amplification and overexpression can inhibit p53-mediated chemosensitivity to DNA-damaging drugs. Two cell lines in which MDM2 is amplified, NB-1691 and Rh18, were transduced with an adenoviral expression vector for p53 (Ad.p53). Although functional wtp53 was detected, no change in chemosensitivity was observed, suggesting that endogenous wtp53 may have been active in the MDM2-amplified cells. The adenoviral vector Ad.MDM2 was used to generate MDM2 expression in a rhabdomyosarcoma cell line, Rh30-CI.27, engineered to express inducible wtp53. When p53 expression was induced, cells became chemosensitive to actinomycin D in the presence or absence of MDM2 expression; this result suggests that MDM2 cannot inhibit p53-mediated chemosensitivity. There was no evidence of a reduced amount of MDM2-p53 binding after drug exposure, but the remaining unbound wtp53 may be functional and capable of potentiating cytotoxicity. In conclusion, MDM2 expression is important in inhibiting p53 function during tumor development but not during the DNA damage-mediated cytotoxic response.

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Introduction

The tumor suppressor gene that encodes p53 is frequently mutated in human tumors (1, 2). Mutant p53 protein can exhibit a dominant-negative function and a gain of an oncogenic phenotype (3). In addition to being mutated, wtp53⁶ can also be inhibited by its binding to other proteins within the cell. For example, the large T antigen and E6 protein expressed by DNA tumor viruses such as SV40 and papilloma virus, respectively, can bind to and inactivate wtp53 (4). MDM2 is a cellular protein that has a similar function. It physically interacts with the transcription activation domain of p53 (5) and inhibits its transactivation function (6, 7). In addition, MDM2 functions as an E3 ubiquitin ligase that targets p53 for degradation by the proteasome (8–10). *Mdm2* knockout mice do not survive past the embryo stage (11, 12), whereas *p53* knockout (13) and *Mdm2* and *p53* double knockout mice do (11, 12). These results indicate that the ability of MDM2 to negatively regulate p53 activity is an essential cellular function.

After DNA damage or oncogene activation, p53 expression is up-regulated, and this increased expression leads to transactivation of several cellular proteins, including MDM2 (14), the cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} (15), and Bax (16). As a consequence, arrest of the cell cycle during G₁, apoptosis, or both processes can be induced (15, 17, 18). MDM2 plays a role in a negative feedback loop because the protein mediates p53 degradation, thereby inhibiting further induced expression of itself and other p53 target genes (19). However, this feedback loop can become modified in cells with IR-induced DNA damage. ATM phosphorylates both p53 and MDM2, thereby inhibiting their binding (20, 21). In addition, MDM2 can become phosphorylated by a DNA-dependent protein kinase that also inhibits the binding of MDM2 to p53 (22).

The ability of wtp53 to induce apoptosis of cells exposed to DNA-damaging agents is of interest to investigators because p53 may affect the tumor's response to chemotherapeutic agents. Many published reports describe an association between wtp53 expression and chemosensitivity to a wide variety of chemotherapeutic agents (23–27), but the results differ considerably, depending on the model system used. Other reports have indicated that wtp53 expression has no influence on the sensitivity of fibrosarcoma (28) and colon carcinoma cells (29), suggesting that the genetic backgrounds of different cell types influence the effect of wtp53 expression on drug sensitivity.

⁶ The abbreviations used are: wtp53, wild-type p53; IR, ionizing radiation; MOI, multiplicity of infection; TFIID, transcription factor IID; TBS-T, 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20; CAT, chloramphenicol acetyltransferase.

MDM2 is amplified, overexpressed, or both in a proportion of tumors (30–34), particularly in soft-tissue sarcomas (35–38). As an alternative mechanism to gene mutation, high-level *MDM2* expression can inhibit wtp53 function during tumorigenesis or tumor progression. However, results of clinical studies investigating the association between *MDM2* expression and prognosis are contradictory. Overexpression of *MDM2* protein in acute lymphoblastic leukemia and soft-tissue sarcomas is associated with an unfavorable prognosis (39, 40), whereas *MDM2* gene amplification indicates a favorable prognosis for patients with non-small cell lung carcinoma and soft-tissue sarcomas (38, 41). These contradictions may be explained if wtp53 is functional in certain tumors after treatment with chemotherapy. Because *MDM2* is usually overexpressed together with wtp53 in tumor cells, this report directly investigates the effect of *MDM2* expression on wtp53-mediated drug-induced cytotoxicity.

Materials and Methods

Cell Lines. Rh30, Rh18, and SJNB-4 were obtained from Dr. Peter Houghton (St. Jude Children's Research Hospital). NB-1691 was obtained from the Pediatric Oncology Group. These cell lines have been characterized previously (42). Rh30 is heterozygous for an R273C mutation in p53, and SJNB-4 is homozygous for a C176F mutation in p53. Rh18 and NB-1691 contain wtp53 genes and an amplified *MDM2* gene and overexpress *MDM2* protein (42). All four cell lines were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Rh30-CI.27 and Rh30-VC3, which were obtained from Dr. Janet Houghton (St. Jude Children's Research Hospital), are clones of Rh30 that have been described previously (27) and are maintained in 200 units/ml hygromycin. Induction of wtp53 expression in Rh30-CI.27 was achieved by the addition of 5 mM dexamethasone.

Adenoviral Vectors. Ad.p53 (Av1p53), containing the wtp53 cDNA, was obtained from Genetic Therapy Inc. (Novartis Co., Gaithersburg, MD; Ref. 43). Ad.VC, a control vector, was obtained from Dr. Janet Houghton. Ad.MDM2 was generated by the homologous recombination of pAVS6a.MDM2 (obtained from Dr. John Schuetz, St. Jude Children's Research Hospital) with *Add/327* genomic DNA after cotransfection into 293 cells (American Type Culture Collection, Manassas, VA; Ref. 44). Plaques were allowed to form in an agarose overlay of the cells for 14 days. Individual plaques were isolated, and the virus was allowed to replicate in another monolayer of 293 cells. The presence of human *MDM2* cDNA was confirmed by PCR. After two rounds of plaque purification, the vector was amplified in 293 cells grown in twenty-five 162-cm² flasks and purified by cesium chloride ultracentrifugation (44). The adenoviral vectors were all titered by serial dilution on 2×10^6 293 cells in 1 ml of media. Plaques were counted after 14 days, and the purified virus was stored in aliquots at –80°C. DNA was isolated from the virus, and the absence of replication-competent virus was confirmed by the inability of PCR to detect the adenoviral E1a gene (45). Cells were transduced with viral vectors

at a specific MOI or number of plaque-forming units per cell, as described in the text.

Chemotherapeutic Agents. The cytotoxic agents used in this study were doxorubicin (Cetus Corp., Emeryville, CA), actinomycin D (Merck and Co., Inc., West Point, PA), and vincristine (Eli Lilly, Indianapolis, IN).

Growth Curves and Cytotoxicity Assays. Cells (5×10^4) were plated into 6-well plates (Corning Costar, Cambridge, MA) and allowed to attach overnight. The following day, the cells in three wells were counted to determine the plating efficiency. Cells were then transduced with adenoviral vectors. After 24 h, the virus was removed, and fresh medium was added. To determine the cell doubling time, triplicate wells were counted at least every other day until the cells reached the stationary phase. For the cytotoxicity experiments, the virus-containing medium was replaced with medium containing increasing concentrations of chemotherapeutic drugs. Control cells that were not transduced were treated in the same manner. Cells were exposed to drugs for three cell doublings, after which time the total number of cells in each well was counted. The mean \pm SDs of the cell number were calculated, and the results were presented relative to the growth of non-drug-treated cells. IC₅₀ values were the concentrations of drug that reduced the cell number by 50%.

Antibodies. The horseradish peroxidase-conjugated anti-p53 monoclonal antibody DO-1-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a concentration of 1:200. Polyclonal antibodies against *MDM2* (N20; Santa Cruz Biotechnology), TFIID (Santa Cruz Biotechnology), and p21 (C19; Santa Cruz Biotechnology) and a monoclonal antibody against tubulin (Sigma, St. Louis, MO) were used at concentrations of 1:200, 1:200, 1:500, and 1:1000, respectively. Antibodies were detected by using horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse Ig (Amersham, Arlington Heights, IL) at dilutions of 1:1000. The anti-tubulin and anti-TFIID antibodies were used to confirm that equal amounts of protein had been loaded onto the gel.

Western Blot Analysis. Extracts of soluble cellular protein were prepared by resuspending cell pellets in extract buffer [50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 1 mM EDTA, 0.5 mM DTT, and 0.1% NP40] containing freshly added protease inhibitors (10 μ g/ml each of antipain, aprotinin, and leupeptin; 1 mM sodium orthovanadate; and 2 mM phenylmethylsulfonyl fluoride). Cells were then frozen on dry ice for 5 min and then heated at 37°C for 1 min. This freeze-thaw cycle was repeated twice. After centrifugation at 14,000 rpm (4°C, 10 min), the supernatants were retained, and the pellets were discarded. Protein concentrations of all extracts were determined by the Bradford method (Ref. 46; Bio-Rad Protein Assay kit; Bio-Rad Laboratories, Hercules, CA).

Cell extracts (50 μ g) were subjected to electrophoresis in precast 4–20% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA), and proteins were electroblotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in BLOTTO (5% nonfat dried milk in TBS-T) for 1 h at room temperature to block potential sites of nonspecific protein binding. After this initial incubation period, the membranes were incubated with a

primary antibody diluted in BLOTTO (0.5 $\mu\text{g}/\text{ml}$) for another hour. After three washes in TBS-T, the membranes were incubated with the appropriate secondary antibody for another hour. The final three washes with TBS-T consisted of two washes for 15 min and one wash for 30 min. Finally, enhanced chemiluminescence (Amersham) detection reagents were added as described by the manufacturer. Proteins were visualized by exposure to X-ray film (Kodak BioMax-MR; Eastman Kodak Co., Rochester, NY) for various time intervals, which were dependent on protein abundance.

Immunoprecipitation. Immunoprecipitation of MDM2 was carried out by incubating agarose-conjugated antibody SMP14 (Santa Cruz Biotechnology) and cell extract in lysis buffer [50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, and protease inhibitors as described for Western analysis] for 1 h at room temperature. The immunoprecipitates were then washed three times in 0.5 ml of wash buffer [5% sucrose, 5 mM Tris (pH 7.4), 5 mM EDTA, 0.5 M NaCl, 1% NP40, and protease inhibitors]. Loading buffer was added, and the samples were boiled for 10 min before they underwent SDS-PAGE and Western blot analysis as described above. Data obtained were the same in the presence and absence of 100 nM okadaic acid.

CAT Reporter Gene Assays. Transcriptional activity of p53 was evaluated in cell lines that had been transiently transfected with pCOSX1CAT (19), a plasmid in which the CAT reporter gene is regulated by the p53-responsive *MDM2* promoter sequence, or with p1634CAT, the parental vector containing no promoter sequences. Transfections were carried out in triplicate with LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) under conditions described by the manufacturer. After 48 h, cells were harvested, and whole cell extracts were assayed for CAT activity by a two-phase liquid scintillation counting assay using [^3H]acetyl-CoA (Amersham) as a substrate (47). CAT activity was calculated as cpm [^3H]acetylchloramphenicol/h/mg protein. The results for extracts of cells transfected with the control p1634CAT were subtracted from the results for the other extracts.

Results

Characterization of Endogenous p53 Function. Two rhabdomyosarcoma (Rh30 and Rh18) and two neuroblastoma (SJNB-4 and NB-1691) cell lines were chosen for this study. One of each pair of cell lines had either an *MDM2* gene amplification (Rh18 and NB-1691) or a mutant p53 gene [Rh30 and SJNB-4 (control cell lines)]. These genotypes suggested that the function of p53 in all four lines was attenuated. We tested this hypothesis by evaluating the p53 response in these cells after their exposure to IR. Four h after cells received an IC_{80} dose of IR, the cells were harvested and evaluated by Western blot analysis. No induction of the p53 target gene *p21* was observed in Rh30, SJNB-4, or NB-1691 cells treated with IR (Fig. 1). In contrast, expression of both p53 and p21 was increased in Rh18 cells, a result indicating that despite *MDM2* overexpression, wtp53 transactivated at least one of its target genes, *p21*.

The Effect of Exogenous p53 on Chemosensitivity. To generate transcriptionally active wtp53, we used an adenovirus containing a gene for wtp53 (Ad.p53) to transduce the

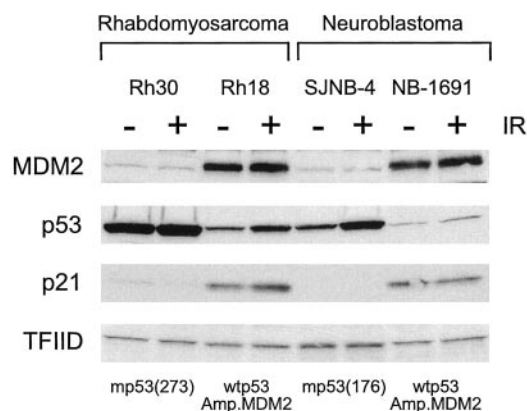


Fig. 1. Western blot analysis of cell extracts before and 4 h after exposure to an IC_{80} dose of IR. For Rh30, the dose was 7.25 Gy; for Rh18, the dose was 6.25 Gy; for SJNB-4, the dose was 6.40 Gy; and for NB-1691, the dose was 7.8 Gy. TFIIID was used as a loading control.

two cell lines that have an amplified *MDM2* gene (Rh18 and NB-1691) and the two control cell lines that express mutant p53 (Rh30 and SJNB-4). The amount of Ad.p53 for each cell line was carefully chosen such that expression of functional wtp53 was achieved while cell proliferation was maintained. The MOI of virus used to transduce Rh30, Rh18, and NB-1691 was 1; that used to transduce SJNB-4 was 2. We measured the transcriptional activity of wtp53 in transduced cells by using CAT reporter assays (Fig. 2). In Rh30, SJNB-4, and NB-1691 cells, the relative increase in CAT activity after transduction with the p53 vector was 4–5-fold. No significant increase in CAT activity was observed in similarly transduced Rh18 cells, but the results of the CAT assays showed that these cells demonstrated high-level endogenous wtp53 activity. The chosen MOIs resulted in expression of wtp53 that was sufficient to induce p21 expression in all four cell lines (data not shown).

Rh30, Rh18, NB-1691, and SJNB-4 cells were transduced with Ad.p53, and after 48 h, the transduced cells were exposed to increasing concentrations of doxorubicin. Expression of wtp53 sensitized the two mutant p53-expressing cell lines, Rh30 and SJNB-4, to the cytotoxic effects of doxorubicin (Fig. 3). In contrast, no p53-mediated chemosensitization was observed in the two lines in which *MDM2* was amplified.

Rh18 and NB-1691 cells that had been transduced with Ad.p53 were also exposed to increasing concentrations of actinomycin D and vincristine. In this experiment, Rh30 cells served as a control. Table 1 contains the IC_{50} values generated from dose-response curves for the three cell lines exposed to actinomycin D or vincristine. Rh30 cells that expressed exogenous wtp53 were chemosensitized to actinomycin D, whereas Rh18 and NB-1691 cells that expressed the same protein were not. As we expected, expression of wtp53 was unable to potentiate the cytotoxicity of vincristine in any of the Ad.p53-transduced cell lines (Table 1).

The Effect of MDM2 Expression on wtp53-mediated Chemosensitivity. To directly evaluate the effect of *MDM2* expression on wtp53-mediated chemosensitivity, exoge-

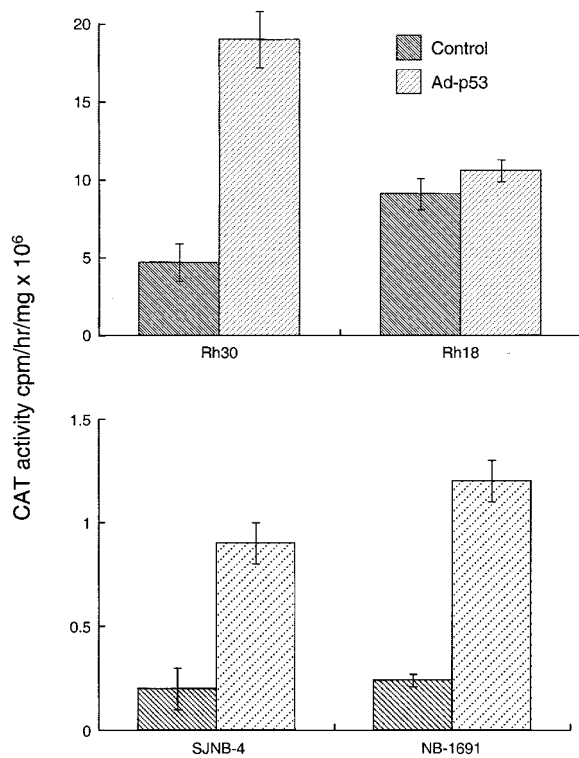


Fig. 2. CAT reporter assays for Rh30, Rh18, SJNB-4, and NB-1691 cells. Before or 24 h after transduction with Ad.p53, each cell line was transiently transfected with a plasmid in which the CAT gene was linked to a p53-responsive promoter. CAT activity was defined as cpm/h from which the CAT activity of cells transfected with a promoterless CAT construct was subtracted. Each value was then divided by the protein concentration to normalize for the number of cells in each experiment. Values represent the means \pm SDs of at least three independent transfections.

nous MDM2 protein was expressed in Rh30-CI.27 and a control cell line, Rh30-VC3. In Rh30-CI.27 cells, expression of wtp53 was controlled by a dexamethasone-inducible promoter (27). Induction of wtp53 in these cells results in sensitization to chemotherapeutic agents *in vitro* (27).

Exogenous expression of MDM2 was achieved by using Ad.MDM2, an adenoviral vector containing the human *MDM2* cDNA. A dose-dependent increase in MDM2 expression was observed in Rh30-CI.27 cells transduced with Ad.MDM2 at MOIs from 0 to 100 (Fig. 4A). A MOI of 25 was chosen for subsequent experiments because it was the lowest amount of virus that could generate significant MDM2 expression (Fig. 4A). This amount of virus generated equivalent levels of MDM2 protein compared with the amount expressed in the two *MDM2* gene-amplified cell lines, Rh18 and NB-1691 (Fig. 4B). The adenoviral vectors were used to transduce Rh30-CI.27 and Rh30-VC3 cells that had been treated with 5 μ M dexamethasone. The induction of p21 protein indicated that transcriptionally active wtp53 was induced in Rh30-CI.27 cells in the presence of high levels of MDM2 (Fig. 5A). Twenty-four h after the induction of wtp53 expression and exposure to the viral vector encoding MDM2, we added increasing concentrations of actinomycin D to the cells. Consistent with published data was our finding that

Rh30-CI.27 cells expressing wtp53 were more sensitive than Rh30-VC3 cells to the cytotoxic effects of actinomycin D (Ref. 27; Fig. 5B). Exogenous expression of MDM2 in Ad.MDM2-transduced cells did not influence the chemosensitivity of either Rh30-CI.27 or Rh30-VC3 cells (Fig. 5B).

Immunoprecipitation of MDM2 and p53 Complexes.

Loss of MDM2-p53 binding may have released functional wtp53 in the *MDM2* gene-amplified cells. To investigate whether MDM2-p53 binding was affected in the doxorubicin-treated cells, we performed immunoprecipitation analyses. In Rh18 cells, a large proportion of wtp53 was bound to MDM2, and the amount of wtp53-MDM2 complexes appeared to increase after exposure to doxorubicin (Fig. 6). In NB-1691 cells, a slight reduction in the amount of MDM2-p53 complex appeared to occur 24 h after treatment with doxorubicin. Subsequent immunoprecipitations were carried out to remove all of the MDM2-bound p53 from the extract. Four consecutive immunoprecipitations were required to remove the large amount of MDM2-p53 complexes in Rh18 cells, whereas only two were required to remove all of the MDM2-bound p53 from the NB-1691 extract. However, the presence of detectable levels of unbound p53 in the supernatant after the last immunoprecipitation demonstrated that not all of the endogenous wtp53 was bound to MDM2 in NB-1691 and Rh18 cells (Fig. 6). In Rh18 cells, the slight increase in the amount of MDM2-bound p53 after drug exposure was consistent with the reduced amount of p53 detected in the supernatant.

Similar immunoprecipitations were carried out with cell lysates from Rh18 and NB-1691 cells treated with a higher 500 nM concentration of doxorubicin. In additional experiments, the cells were exposed to IR at doses of 2 Gy, IC₈₀ (6.25 and 7.8 Gy for Rh18 and NB-1691, respectively), and 10 Gy. Radiation was used as a control because it had been previously shown to promote loss of MDM2-p53 binding in other cell types. Cells were harvested at 30 min, 4 h, and 24 h after doxorubicin or IR exposure. After treatment with either doxorubicin or IR, a significant loss of MDM2-p53 binding in Rh18 and NB-1691 cells could not be detected (data not shown). However, in control ML1 myeloid leukemia cells, no MDM2-p53 binding could be detected 4 h after exposure to 10 Gy of IR (data not shown). This control demonstrated that our assay was capable of detecting loss of binding in a previously characterized model system.

Discussion

In cells containing DNA damage, a normal p53-mediated response results in the induction of expression of p53 and p53-responsive genes, such as *p21* (15). We evaluated the p53 response in four tumor cell lines and found that three of them, Rh30, SJNB-4, and NB-1691, expressed no functional wtp53 (Fig. 1). Rh30 and SJNB-4 expressed high levels of mutant p53, and, consequently, no induction of p21 was observed in these cell lines. Rh18 and NB-1691 overexpress MDM2; although there was no induction of p53 or p21 in NB-1691, induced expression of both genes was observed in Rh18. It has been demonstrated previously that p53 and MDM2 can become phosphorylated in response to IR and that this phosphorylation inhibits their binding and activates

Fig. 3. Doxorubicin growth inhibition assays for Rh30, SJNB-4, Rh18, and NB-1691 cells. ■, cells that were not pretreated with adenovirus; ●, cells transduced with Ad.VC; ▲, cells transduced with Ad.p53. Cells were transduced 24 h before drug treatment. Each point represents the mean \pm SD of cells in triplicate wells in a representative experiment.

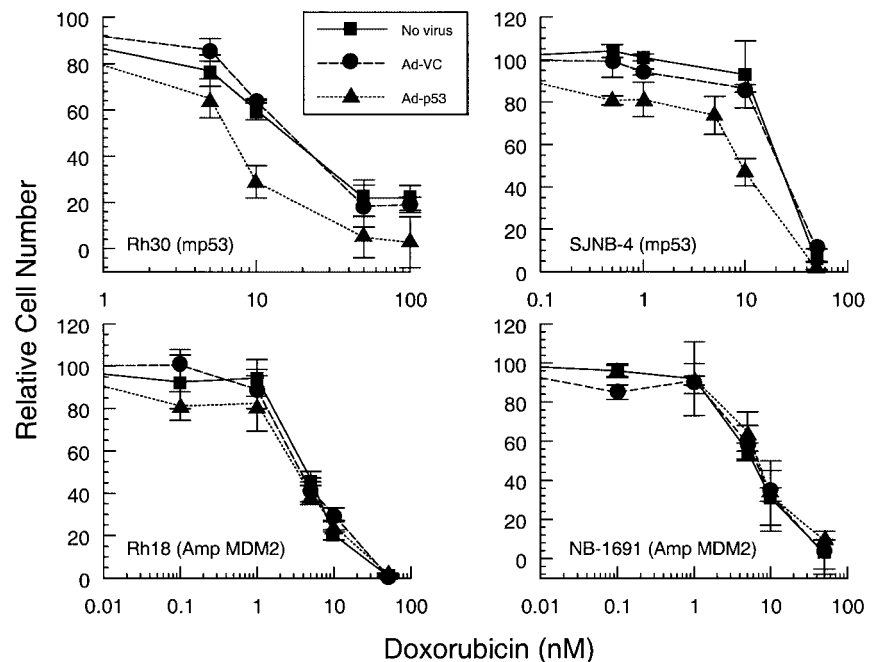


Table 1 IC₅₀ values of actinomycin D and vincristine in representative experiments in which cells were treated with drug after adenoviral transduction

Data are representative of at least two independent experiments.

	Cell line	No virus	Ad.VC	Ad.p53
Actinomycin (nM)	Rh30	0.48	0.47	0.24
	Rh18	0.04	0.03	0.05
	NB-1691	0.95	1.00	0.85
Vincristine (nM)	Rh30	0.5	0.4	0.4
	Rh18	0.7	0.8	0.6
	NB-1691	3.2	2.2	3.0

p53 expression (20–22). However, at an IC₈₀ dose of IR, only the p53 in Rh18 cells became active. The high level of endogenous wtp53 expression and transcriptional activity in Rh18 cells could account for these functional differences (Figs. 2 and 6).

Expression of wtp53 mediated by an adenoviral vector (Ad.p53) enhanced p53-mediated promoter activity in Rh30, SJNB-4, and NB-1691 cells, but not in Rh18 cells (Fig. 2). Rh18 demonstrated high levels of endogenous p53 activity that did not increase significantly after transduction with Ad.p53. These results demonstrate either that endogenous wtp53 is active in the presence of high-level MDM2 expression or that the transfection procedure itself induced a stress response that up-regulated wtp53 activity. The first of these two possibilities is more likely because the MDM2 expressed in Rh18 cells has been demonstrated to increase the half-life of p53 protein, suggesting that the E3 ubiquitin ligase function of MDM2 may not be functional in these cells (48). Nevertheless, after transduction with Ad.p53 but before drug exposure, all four cell lines expressed significant levels of transcriptionally active wtp53.

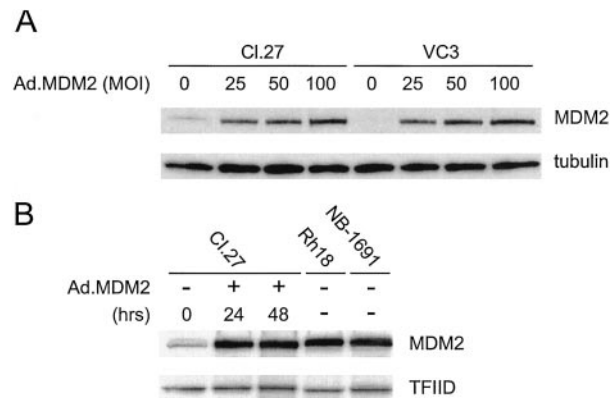


Fig. 4. A, Western blot analysis of Rh30-Cl.27 (Cl.27) and Rh30-VC3 (VC3) cells 24 h after transduction with Ad.MDM2 at increasing MOIs. The amount of tubulin was evaluated to determine whether similar amounts of protein were loaded in each well. B, MDM2 Western blot analysis of Rh30-Cl.27 (Cl.27) cells transduced with Ad.MDM2 (MOI of 25) for 24 and 48 h compared with MDM2 expression in Rh18 and NB-1691 cells. TFIID was used as a loading control.

Three drugs were used in the cytotoxicity assays: doxorubicin and actinomycin D, both of which are DNA-damaging topoisomerase II inhibitors, and vincristine, which does not damage DNA but inhibits the mitotic spindle. Expression of wtp53 sensitized the two mutant p53-expressing cell lines, Rh30 and SJNB-4, to doxorubicin (Fig. 3). There was no significant difference between the dose-response curves for Ad.VC-transduced cells and cells that had not been transduced. This result demonstrated that viral transduction alone was not responsible for the observed effects. Ad.p53 did not potentiate the cytotoxicity of doxorubicin in cells containing an amplified MDM2 gene. Similar results were obtained when

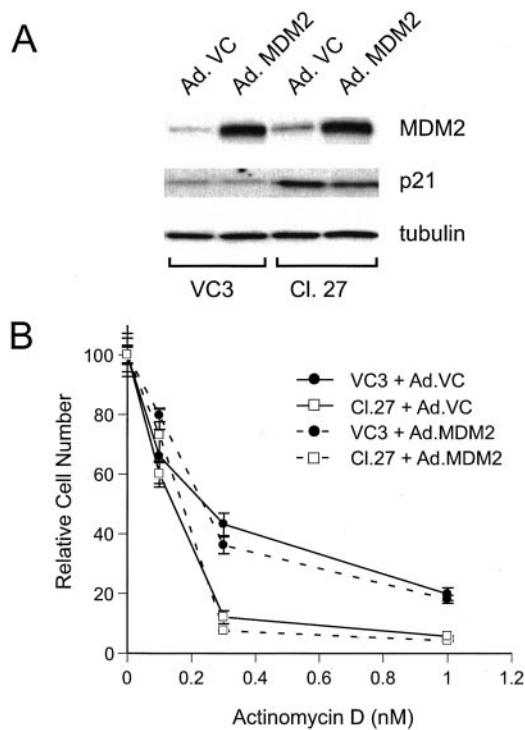


Fig. 5. A, Western blot analysis of Rh30-Cl.27 (Cl.27) and Rh30-VC3 (VC3) cells 24 h after transduction with either Ad.VC or Ad.MDM2 (MOI of 25). Dexamethasone was added to both cell lines at the time of transduction to induce wtp53 expression in the Rh30-Cl.27 cells. Rh30 cells expressed high levels of mutant p53 protein; therefore, an elevation in wtp53 could not be detected (data not shown). Transcriptionally active wtp53 was expressed in response to the addition of dexamethasone, as demonstrated by the elevated level of p21. The amount of tubulin was evaluated to determine whether similar amounts of protein were loaded in each well. B, evaluation of the chemosensitivity of transduced Rh30-Cl.27 and Rh30-VC3 cells after treatment with dexamethasone and actinomycin D. Cells were exposed to dexamethasone and either Ad.MDM2 or Ad.VC 24 h before their exposure to actinomycin D. This pretreatment allowed expression of wtp53 and MDM2 before the drug was added. Data for Rh30-VC3 are shown as closed circles, whereas data for Rh30-Cl.27 data are shown as open squares. The solid lines represent cells transduced with Ad.VC, and the dashed lines represent cells transduced with Ad.MDM2. Data points are the mean \pm SD of values of cells in triplicate wells in a representative experiment.

the cells were exposed to actinomycin D (Table 1). As expected, wtp53 did not potentiate vincristine cytotoxicity in any of the cell lines (Table 1). These results demonstrate that exogenous wtp53 expression can sensitize these mutant p53-expressing cells to the cytotoxic effects of the DNA-damaging agents doxorubicin and actinomycin D. In contrast, wtp53 expression had no effect on the chemosensitivity of cells that overexpressed MDM2.

The lack of p53-mediated sensitivity of the cell lines in which MDM2 was amplified suggests two potential hypotheses. First, MDM2 may inhibit p53-mediated chemosensitivity by a novel mechanism, even though wtp53 is transcriptionally active. Second, endogenous wtp53 may already be active before exposure to DNA-damaging agents or may become activated after exposure to DNA-damaging agents. If so, it may not be possible for exogenous wtp53 expression to further enhance the chemosensitivity of the cells.

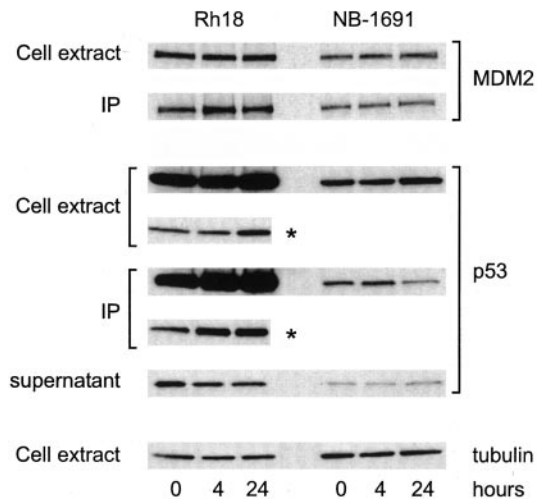


Fig. 6. Western blot analysis of Rh18 and NB-1691 cell extracts 0, 4, and 24 h after treatment with 10 and 20 nM doxorubicin, respectively, both were the IC₈₀ concentrations for the two cell lines. The top panel shows MDM2 present in cell extracts and in an initial immunoprecipitate obtained by using an agarose-conjugated anti-MDM2 antibody. The middle panel shows the same Western blot after incubation with an anti-p53 antibody. The cell extract panels show the total amount of p53 present in the sample, and the immunoprecipitation (IP) panels reveal the proportion of p53 bound to MDM2. The short panels of bands labeled with an asterisk show the results of the same Western blot above them, but the film was exposed for a shorter time. Consecutive rounds of MDM2 immunoprecipitation were carried out to remove all of the p53 associated with MDM2, as described in the text. The bands labeled supernatant show p53 present after four and two consecutive rounds of immunoprecipitation for the Rh18 and NB-1691 cell extracts, respectively. Therefore, p53 observed in the supernatant was not bound to MDM2. The bottom panel shows the amount of tubulin, which was evaluated to determine whether similar amounts of cell extract were loaded in each well.

To directly evaluate whether MDM2 expression could inhibit wtp53-mediated chemosensitization, we transduced Rh30-Cl.27 cells with Ad.MDM2. High-level expression of MDM2 did not inhibit the transactivation potential of wtp53, whose expression was induced by the addition of dexamethasone (Fig. 5A). As expected, Rh30-Cl.27 cells were sensitized to actinomycin D upon induction of wtp53 expression (27), but the sensitivity of these cells to the drug did not change when they were transduced with Ad.MDM2 (Fig. 5B). Therefore, MDM2 overexpression was unable to inhibit p53-mediated chemosensitization of the cells.

During tumor development, overexpression of MDM2, such as that resulting from MDM2 gene amplification, serves to inhibit wtp53 function. However, the binding of MDM2 and p53 can be inhibited in cells that have been exposed to IR (20–22). We hypothesized that if MDM2 and p53 fail to bind in cells that have an amplified MDM2 gene and have been treated with doxorubicin, wtp53-mediated drug sensitivity could result. The proportion of endogenous wtp53 bound to MDM2 before and after drug exposure in the cell lines was determined. In NB-1691 cells, there was a slight decrease in the amount of p53-MDM2 complex 24 h after exposure to the drug (Fig. 6). However, the opposite result was observed in Rh18 cells: an increased amount of p53-MDM2 complex was observed after treatment with doxorubicin. This increase in the amount of p53 in complex with MDM2 coincided with a

slight induction of p53 expression and with a reduction of p53 protein that remained in the supernatant after four consecutive immunoprecipitations. However, because wtp53 was detected in the immunoprecipitation supernatants of both Rh18 and NB-1691 after MDM2 had been removed, this p53 that was not bound to MDM2 probably contributed to the chemosensitive phenotype observed in the cell lines containing the amplified *MDM2* gene (Figs. 3 and 6). The contradictory clinical results concerning the prognosis of patients in whose tumors MDM2 expression is elevated may be explained in part by variations in the amount of wtp53 in complex with MDM2 in the tumors. In addition, it is possible that different therapeutic agents induce specific phosphorylation patterns that may differentially affect the binding of p53 and MDM2. The synergistic effects of Ad.p53 and chemosensitivity reported previously by Horio *et al.* (49) in cells with endogenous wtp53 probably reflected insufficient endogenous p53 expression to elicit a biological response.

In summary, we demonstrated that exogenous expression of wtp53 in cell lines containing mutant p53 can induce chemosensitivity to DNA-damaging agents in a cell type-specific manner. The cytotoxicity of DNA-damaging drugs was not potentiated by the exogenous expression of transcriptionally active wtp53 in two cell lines that possessed an amplified *MDM2* gene and active endogenous wtp53. Exogenous overexpression of MDM2 in Rh30-CI.27 cells, which are known to display wtp53-mediated chemosensitivity, did not affect this phenotype, a result confirming that MDM2 expression does not influence p53-mediated chemosensitivity. In conclusion, *MDM2* amplification may be important for the inhibition of p53 activity during tumor development, but MDM2 overexpression does not influence the chemosensitivity phenotype mediated by wtp53.

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References

- Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumor suppressor gene. *Nature (Lond.)*, 351: 453–455, 1991.
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. *Science (Wash. DC)*, 253: 49–53, 1991.
- Zambetti, G. P., and Levine, A. J. A comparison of the biological activities of wild-type and mutant p53. *FASEB J.*, 7: 855–865, 1993.
- Mietz, J. A., Unger, T., Huibregtse, J. M., and Howley, P. M. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6-oncoprotein. *EMBO J.*, 11: 5013–5020, 1992.
- Brown, D. R., Deb, S., Munoz, R. M., Subler, M. A., and Deb, S. P. The tumor suppressor p53 and the oncoprotein simian virus 40 T antigen bind to overlapping domains on the MDM2 protein. *Mol. Cell. Biol.*, 13: 6849–6857, 1993.
- Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The MDM2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, 69: 1237–1245, 1992.
- Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature (Lond.)*, 362: 857–860, 1993.
- Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature (Lond.)*, 387: 296–299, 1997.
- Kubbutat, M. H., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature (Lond.)*, 387: 299–303, 1997.
- Honda, R., Tanaka, H., and Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.*, 420: 25–27, 1997.
- Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature (Lond.)*, 378: 203–206, 1995.
- Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature (Lond.)*, 378: 206–208, 1995.
- Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.*, 4: 1–7, 1994.
- Barak, Y., Juven, T., Hoffner, R., and Oren, M. MDM2 expression is induced by wild-type p53 activity. *EMBO J.*, 12: 461–468, 1993.
- El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W., and Vogelstein, B. WAF1/CIP1 is induced upon p53-mediated growth arrest and apoptosis. *Cancer Res.*, 54: 1169–1174, 1994.
- Miyashita, T., and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. *Cell*, 80: 293–299, 1995.
- El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. P., Parsons, R., Trent, J. M., Liu, D., Merrer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75: 817–825, 1993.
- Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G₁ arrest. *Cell*, 76: 1013–1023, 1994.
- Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.*, 7: 1126–1132, 1993.
- Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, 91: 325–334, 1997.
- Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y., and Shkedy, D. Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proc. Natl. Acad. Sci. USA*, 96: 14973–14977, 1999.
- Mayo, L. D., Turchi, J. J., and Berberich, S. J. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res.*, 57: 5013–5016, 1997.
- Lowe, S. W., Ruley, H. E., Jacks, T., and Houseman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer drugs. *Cell*, 74: 957–967, 1993.
- Fan, S., El-Deiry, W. S., Bae, I. J. F., Jondel, D., Blatia, K., Fornace, A. J., Magrath, I., Kohn, K. W., and O'Connor, P. M. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res.*, 54: 5824–5830, 1994.
- Yang, B., Eshleman, J. R., Berger, N. A., and Markowitz, S. D. Wild-type p53 protein potentiates cytotoxicity of therapeutic agents in human colon cancer cells. *Clin. Cancer Res.*, 2: 1649–1657, 1996.
- Kohn, K. W., Jackman, J., and O'Connor, P. M. Cell cycle control and cancer chemotherapy. *J. Cell. Biochem.*, 54: 440–452, 1994.
- Gibson, A. A., Harwood, F. G., Tillman, D. M., and Houghton, J. A. Selective sensitization to DNA damaging agents in a human rhabdomyosarcoma cell line with inducible wild-type p53 overexpression. *Clin. Cancer Res.*, 4: 145–152, 1998.
- Labrecque, S., and Matlashewski, G. J. Viability of wild-type p53-containing and p53 deficient tumor cells following anticancer treatment: the use of human papilloma virus E6 to target p53. *Oncogene*, 11: 387–392, 1995.

29. Slichenmeyer, W. J., Nelson, W. G., Slebos, R. J., and Kastan, M. B. Loss of a p53-associated G₁ checkpoint does not decrease cell survival following DNA damage. *Cancer Res.*, 53: 4164–4168, 1993.
30. Reifemberger, G., Liu, L., Ichimura, K., Schmidt, E. E., and Collins, V. P. Amplification and overexpression of the *MDM2* gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res.*, 53: 2736–2739, 1993.
31. Ladanyi, M., Cha, C., Lewis, R., Jhanwar, S. C., Huvos, A. G., and Healey, J. H. *MDM2* gene amplification in metastatic osteosarcoma. *Cancer Res.*, 53: 16–18, 1993.
32. Corvi, R., Savelyeva, L., Breit, S., Wenzel, A., Handgretinger, R., Barak, J., Oren, M., Amler, L., and Schwab, M. Non-syntenic amplification of *MDM2* and *MYCN* in human neuroblastoma. *Oncogene*, 10: 1081–1086, 1995.
33. Ladanyi, M., Lewis, R., Jhanwar, S. C., Gerald, W., Huvos, A. G., and Healey, J. H. *MDM2* and *CDK4* gene amplification in Ewing's sarcoma. *J. Pathol.*, 175: 211–217, 1995.
34. McCann, A. H., Kirley, A., Carney, D. N., Corbally, N., Magee, H. M., Keating, G., and Dervan, P. A. Amplification of the *MDM2* gene in human breast cancer and its association with *MDM2* and p53 protein status. *Br. J. Cancer*, 71: 981–985, 1995.
35. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature (Lond.)*, 358: 80–83, 1992.
36. Leach, F. S., Tokino, T., Meltzer, P., Burrell, M., Oliner, J. D., Smith, S., Hill, D. E., Sidransky, D., Kinzler, K. W., and Vogelstein, B. p53 mutation and *MDM2* amplification in human soft tissue sarcomas. *Cancer Res.*, 53: 2231–2234, 1993.
37. Cordon-Cardo, C., Latres, E., Drobnyak, M., Oliva, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chen, J., Brennan, M. F., and Levine, A. J. Molecular abnormalities of *mdm2* and *p53* genes in adult soft tissue sarcomas. *Cancer Res.*, 54: 794–799, 1994.
38. Bartel, F., Meye, A., Wurl, P., Kappler, M., Bache, M., Lautenschlager, C., Grunbaum, U., Schmidt, H., and Taubert, H. Amplification of the *MDM2* gene, but not expression of splice variants of *MDM2* mRNA, is associated with prognosis in soft tissue sarcoma. *Int. J. Cancer*, 95: 168–175, 2001.
39. Gustafsson, B., Stal, O., and Gustafsson, B. Overexpression of *MDM2* in acute childhood lymphoblastic leukemia. *Pediatr. Hematol. Oncol.*, 15: 519–526, 1998.
40. Wurl, P., Meye, A., Berger, D., Lautenschlager, C., Bache, M., Holzhausen, H. J., Schmidt, H., Dralle, H., Rath, F. W., and Taubert, H. Significance of retinoblastoma and *mdm2* gene expression as prognostic markers for soft-tissue sarcoma. *Langenbeck's Arch. Surg.*, 383: 99–103, 1998.
41. Higashiyama, M., Doi, O., Kodama, K., Yokouchi, H., Kasugai, T., Ishiguro, S., Takami, K., Nakayama, T., and Nishisho, I. *MDM2* gene amplification and expression in non-small-cell lung cancer: immunohistochemical expression of its protein is a favourable prognostic marker in patients without p53 protein accumulation. *Br. J. Cancer*, 75: 1302–1308, 1997.
42. McPake, C. R., Tillman, D. M., Poquette, C. A., George, E. O., Houghton, J. A., and Harris, L. C. Bax is an important determinant of chemosensitivity in pediatric tumor cell lines independent of Bcl-2 expression and p53 status. *Oncol. Res.*, 10: 235–244, 1998.
43. Pirolo, K. F., Hao, Z., Rait, A., Jang, Y.-J., Fee, W. E., Jr., Ryan, P., Chiang, Y., and Chang, E. H. p53 mediated sensitization of squamous cell carcinoma of the head and neck to radiotherapy. *Oncogene*, 14: 1735–1746, 1997.
44. Graham, F. L., and Prevec, L. Methods for constructing adenoviral vectors. *Mol. Biotechnol.*, 3: 207–220, 1995.
45. Zhang, W.-W., Koch, P. E., and Roth, J. A. Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *BioTechniques*, 18: 444–447, 1995.
46. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248–254, 1976.
47. Neumann, J. R., Morency, C. A., and Russian, K. O. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *BioTechniques*, 5: 444–447, 1987.
48. Keleti, J., Quezado, M. M., Abaza, M. M., Raffeld, M., and Tsokos, M. The *MDM2* oncoprotein is overexpressed in rhabdomyosarcoma cell lines and stabilizes wild-type p53 protein. *Am. J. Pathol.*, 149: 143–151, 1996.
49. Horio, Y., Hasegawa, Y., Sekido, Y., Takahashi, M., Roth, J. A., and Shimokata, K. Synergistic effects of adenovirus expressing wild-type p53 on chemosensitivity of non-small cell lung cancer cells. *Cancer Gene Ther.*, 7: 537–544, 2000.