Drug-resistant human bladder-cancer cells are more sensitive to adenovirus-mediated wild-typep53 gene therapy compared to drug-sensitive cells

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DRUG-RESISTANT HUMAN BLADDER-CANCER CELLS ARE MORE SENSITIVE TO ADENOVIRUS-MEDIATED WILD-TYPE *P53* GENE THERAPY COMPARED TO DRUG-SENSITIVE CELLS

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We investigated the therapeutic potential and molecular mechanism of adenovirus-mediated wt *p53* gene therapy for drug-resistant human bladder cancers. KK47, a human bladder-cancer cell line, along with the drug-resistant sublines KK47/DDP10, KK47/DDP20 (cisplatin-resistant) and KK47/ADM (doxorubicin-resistant) were used for the experiments. All 4 KK47 cell lines had genetically normal *p53* genes. Using an *in vitro* cytotoxicity assay, the drug-resistant cell lines were more sensitive to Ad-CMV-p53 cell killing than the KK47 parental cell line. Ad-CMV-p53 induced higher levels of p53 protein and mRNA in the drug-resistant cell lines than in the parental cell line and, consequently, higher levels of *p21* and *Bax* mRNA, which resulted in higher percentages of G_1 cell-cycle arrest and apoptosis. The higher efficiencies of adenoviral gene transfer in the drug-resistant cell lines were confirmed by X-gal staining after infection with Ad-CMV-β-gal. In conclusion, adenovirus-mediated wt *p53* gene therapy was more effective in the drug-resistant bladder-cancer cell lines than in the drug-sensitive bladder-cancer cell line.

Key words: *adenovirus;* p53; *bladder cancer; gene therapy; drug resistance; gene transfer*

Although patients with advanced bladder cancer can be effectively treated initially with combination chemotherapy, generally including isolation (*i.e.*, M-VAC), about 30% of patients receiving M-VAC do not respond, resulting in disease progression.[1] Furthermore, additional chemotherapy provides little benefit to patients who have relapsed after an initial response. Thus, inherent or acquired drug resistance leads to treatment failure. Although much is known about the molecular mechanisms by which tumor cells acquire drug resistance, the treatment of drug-resistant tumors remains a significant problem.[2]

The *p53* gene encodes a 393–amino acid phosphoprotein and overexpression of wt *p53* could cause apoptosis, cell growth arrest and suppression of uncontrolled cell proliferation.[3] Currently, adenoviral delivery of wt *p53* genes into cancer cells (*i.e.*, prostate cancer,[3,4] lung cancer,[5–7] head-and-neck cancer,[8,9] bladder cancer[10]) is being thoroughly investigated in both laboratory studies and clinical trials. These investigations have demonstrated the efficacy and safety of adenovirus-mediated wt *p53* gene therapy.

Previously, Seth *et al.*[11] reported that drug-resistant human breast-cancer cell sublines, MCF-Adr and MCF-Mito, were more sensitive to killing by Ad-wt *p53* compared to the parental cells (MCF-7) and that the downstream signals of p53 expression might be responsible for the sensitivity of drug-resistant MCF-7 cells to Ad-wt *p53*. Similarly, we discovered that the drug-resistant human bladder-cancer cell sublines KK47/DDP10, KK47/DDP20 and KK47/ADM are more sensitive to Ad-wt *p53*-mediated killing than the parental KK47 cells. However, the results of that investigation suggested that the sensitivities of the KK47 cell lines to Ad-wt *p53* were at least partially due to the differential transduction efficacy of adenoviral gene transfer of the wt *p53* gene.

In the present study, we evaluated the therapeutic potential and molecular mechanism of adenovirus-mediated wt *p53* gene therapy of drug-resistant human bladder cancers. Our results suggest that such therapy is more effective in the drug-resistant bladder cancer cell lines compared to the parental cell line, which may partially be explained by the efficiency of adenoviral gene transfer.

MATERIAL AND METHODS

Cells and cell culture

Human bladder-cancer cell lines KK47, KK47/DDP10, KK47/DDP20 and KK47/ADM were generously provided by Dr. S. Naito (Kyushu University, Fukuoka, Japan).[12,13] KK47, the parent line, was established from human TCC of the bladder. The drug-resistant lines KK47/DDP10, KK47/DDP20 (resistance to cisplatin) and KK47/ADM [resistance to doxorubicin (Adriamycin)] were established by stepwise exposure of KK47 cells to cisplatin or doxorubicin. All cell lines were cultured in DMEM (GIBCO BRL, Grand Island, NY) supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml) and 10% FBS (Sigma, St. Louis, MO). Cells were fed 3 times a week with fresh growth medium.

Mutation analysis of p53 *by PCR-SSCP*

Cell lines were grown to about 80% to 90% confluence and then trypsinized and cell pellets were collected for mutation analysis of the *p53* gene. DNA was extracted from cell pellets as previously described[14] and subjected to PCR-SSCP analysis to search for mutations in exons 5–8 of the *p53* gene. Primer sets for amplification of 4 exons of *p53* were designed with fluorescence Cy-5 (Amersham, Aylesbury, UK) at the 5′ site of primers according to Genebank X54156, with the following primers: exon 5, 5′-TTCCTCTTCCTACAGTACTCC-3′ and 5′-GCCCCAGCTGCTCACCATCGC-3′; exon 6, 5′-CACTGATTGCTCTTAGGTCTG-3′ and 5′-AGTTGCAAACCAGACCTCAGG-3′; exon 7, 5′-CCAAGGCGCACTGGCCTCATC-3′ and 5′-TCAGCGGCAAGCAGAGGCTGG-3′; exon 8, 5′-CCTATCCTGAGTAGTGGTAAT-3′ and 5′-GTCCTGCTTGCTTACCTCGCT-3′. PCR-SSCP analysis was performed according to Orita *et al.*[15] PCR products were diluted 50-fold with 95% formamide and dena-

Abbreviations: CAR, coxsackievirus and adenovirus receptor; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; M-VAC, methotrexate, vinblastine, doxorubicin (Adriamycin) and cisplatin; SSCP, single-strand conformation polymorphism; TCC, transitional-cell carcinoma; TdT, terminal deoxynucleotide transferase; TUNEL, TdT-mediated dUTP nick end labeling; wt, wild type.

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TABLE I – PRIMERS AND PROBES

β-gal
Forward: 5′-AAACCCTGGCGTTACCCAA-3′
Reverse: 5′-CCTCTTCGCTATTACGCCAGCT-3′
Probe: 5′-TTAATCGCCTTGCAGCACATCCCC-3′

p53
Forward: 5′-TGCGTGTGGAGTATTTGGATG-3′
Reverse: 5′-TGGTACAGTCAGAGCCAACCAG-3′
Probe: 5′-AAACACTTTTCGACATAGTGTGGTGGTGCC-3′

p21
Forward: 5′-AGCAGAGGAAGACCATGTGGAC-3′
Reverse: 5′-TTTCGACCCTGAGAGTCTCCAG-3′
Probe: 5′-TGTCACTGTCTTGTACCCTTGTGCCTCG-3′

Bax
Forward: 5′-AAGCTGAGCGAGTGTCTCAAGC-3′
Reverse: 5′-ACTCGGAAAAAGACCTCTCGG-3′
Probe: 5′-CTGGACAGTAACATGGAGCTGCAGAGGA-3′

CAR
Forward: 5′-CAGAAGCTACATCGGCAGTAATCA-3′
Forward: 5′-CTCTGAGGAGTGCGTTCAAAGTC-3′
Probe: 5′-TCCATGTCTCCTTCCAACATGGAAGGA-3′

GAPDH
Forward: 5′-GAAGGTGAAGGTCGGAGT-3′
Reverse: 5′-GAAGATGGTGATGGGATTTC-3′
Probe: 5′-CAAGCTTCCCGTTCTCAGCC-3′

tured at 80°C for 5 min, followed by rapid cooling on ice. Denatured products were separated on 5% polyacrylamide gels containing 5% glycerol with an automated laser fluorescence DNA sequencer (ALF Express, Amersham) and analyzed to detect aberrant band shifts with the software *Allele Link* (Amersham).

Production of recombinant adenoviruses

Ad-CMV-p53, a classic first-generation, replication-deficient recombinant adenovirus, contains the CMV promoter, wt *p53* cDNA and SV40 polyadenylation signal in an expression cassette that replaces the serotype 5 adenoviral E1 region.[16] The recombinant β-gal control adenovirus (Ad-CMV-β-gal) was constructed in a similar manner. Viral stocks were propagated in 293 cells. Cells were harvested 36 to 40 hr after infection, pelleted, resuspended in PBS and lysed by the 3-time freeze-thaw method. Cell debris was removed and the virus purified by $CeCl_2$ gradient centrifugation. Concentrated virus was dialyzed, aliquotted and stored at −80°C. To screen against the presence of wt replication-competent adenoviruses, a viral protein synthesis assay was performed as previously described;[17] results were confirmed by the absence of plaque formation on A549 cells.[18] A ratio of viral particles (optical density at 260 nm) to plaque-forming units by the plaque assay on 293 cells was used to assess the quality of the preparation.

In vitro *cytotoxicity assay*

Cells were seeded at a density of 500/well in 96-well tissue culture plates on day 0 before viral infection. On day 1, cells were infected by directly adding the adenoviruses at various MOI (Ad-CMV-p53: 5, 25, 50, 75, 100 MOI; Ad-CMV-β-gal: 50, 100 MOI) into each well, which contained 100 μl of medium; and 6 hr after adding the medium with adenoviruses, the medium was exchanged for medium without adenoviruses. At day 7, viable cells were counted by the Alamar blue method, according to the manufacturer's instructions (Alamar, Sacramento, CA). Briefly, 10 μl of Alamar blue were aseptically added to cultures and cultures were returned to the incubator for 3 hr; fluorescence was then measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm by a fluoroscan (Titertek Fluoroscan II; Labsystems, Tokyo, Japan). The survival rate was calculated assuming the rate of uninfected cells to be 100%.

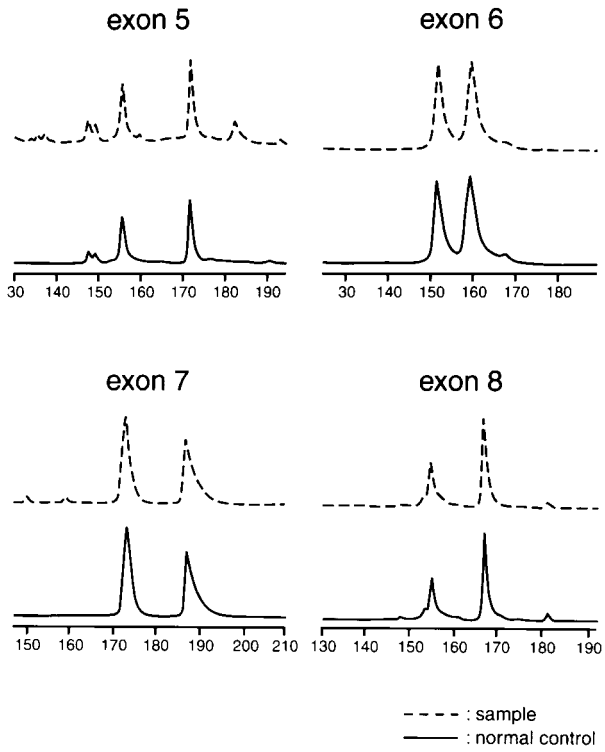

FIGURE 1 – DNA band shifts in all 4 cell lines. No aberrant band shift was detected in exons 5–8 compared to the band of normal control, indicating that there is no mutation in exons 5–8 of the *p53* gene in any of these cell lines.

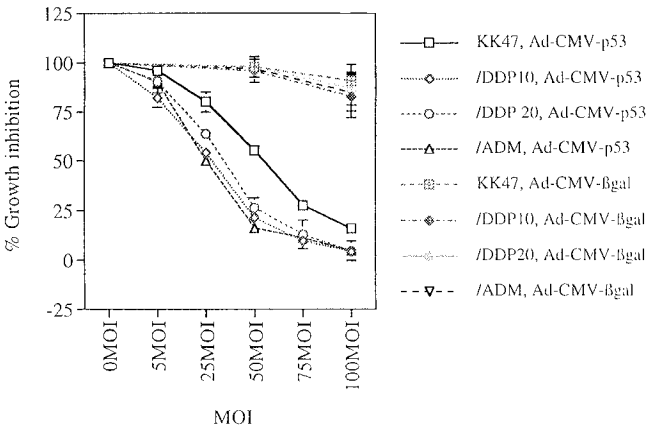

FIGURE 2 – *In vitro* cytotoxicity of Ad-CMV-β-gal and Ad-CMV-p53 infections in KK47 cell lines. While no cytotoxicity was observed with Ad-CMV-β-gal exposure, significant Ad-CMV-p53 cytotoxicity was observed at 25 to 100 MOIs in all of the cell lines. Drug-resistant cell lines (KK47/DDP10, KK47/DDP20 and KK47/ADM) have significantly higher sensitivities to Ad-CMV-p53 cell killing compared to the parental KK47 cell line. Each point represents triplicate averages, with SD bars shown when the SD was >5%.

Real-time quantitative RT-PCR of mRNA expression of the β-gal, p53, p21, Bax *and* CAR *genes*

*Extraction of total RNA from cells infected with Ad-CMV-β-gal or Ad-CMV-p53.*Cells were infected with adenoviruses by 6 hr cultivation in medium containing various MOI (0, 5, 25, 50, 75 and 100) of Ad-CMV-β-gal or Ad-CMV-p53. At 3 days postinfection, cells were trypsinized and cell pellets collected by centrifugation at

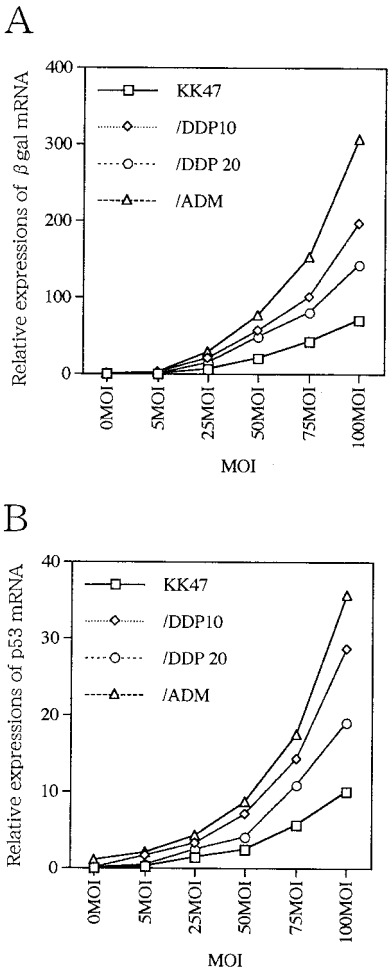

FIGURE 3 – Relative mRNA levels of *β-gal (a)* and *p53 (b)* by quantitative RT-PCR of cells exposed to various MOI (5, 25, 50, 75, 100) of Ad-CMV-β-gal *(a)* and to various MOI (0, 5, 25, 50, 75, 100) of Ad-CMV-p53 *(b)*. Ad-CMV-β-gal and Ad-CMV-p53 mediate higher levels of *β-gal* and *p53* mRNA expression in drug-resistant cell lines than in the KK47 parental cell line at every MOI. Values represent relative levels of mRNA normalized to the KK47 parental cell line at 5 MOI (for *β-gal* mRNA expression) or the KK47/ADM cell line at 0 MOI (for *p53* mRNA expression) with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA.

1,000*g* for 5 min. Isogen (Nippon Gene, Tokyo, Japan) was used to extract total RNA from the cells.

Quantitative RT-PCR using the TaqMan fluorogenic detection system. Quantitative RT-PCR using the TaqMan fluorogenic detection system (Perkin-Elmer Applied Biosystems; Foster City, CA) was performed according to previously described methods.[19,20] Briefly, primers and the TaqMan probe for β-gal, p53, p21, Bax and CAR were designed using the primer design software *Primer Express* (Perkin-Elmer Applied Biosystems). Table I shows the sequences for the TaqMan probes and primers. Primers and the TaqMan probe for GAPDH (TaqMan GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems. RNA for GAPDH was used as an endogenous control. Fifty microliters of reaction mixture were used, containing 10 ng of extracted total RNA; 1 × TaqMan buffer A; 5.5 mmol/l $MgCl_2$; 300 μmol/l dATP, dGTP and dCTP; 600 μmol/l dUTP; 0.2 μmol/l forward and reverse primers; 0.1 μmol/l TaqMan probe; 1.25 units of AmpliTaq Gold; 12.5 units of MuLV reverse transcriptase; and 20 units of RNase inhibitor (Perkin-Elmer Applied Biosystems). The conditions of 1-step RT-PCR were as follows: 30 min at 48°C (stage 1, RT), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation) and then 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3, PCR). The assay used an instrument capable of measuring fluorescence in real time (ABI prism 7700 Sequence Detector, Perkin-Elmer Applied Biosystems). Data were analyzed using the software *Sequence Detector* (Perkin-Elmer Applied Biosystems).

X-Gal staining after infection with Ad-CMV-β-gal

After infection with Ad-CMV-β-gal at 50 MOI for 6 hr, cells were grown in 2-well chamber slides to about 70–90% confluence for 3 days and the medium was discarded and fixed with 0.05% glutaraldehyde. After discarding the fixative solution, cells were rinsed thoroughly 3 times in PBS at room temperature. An X-gal solution mixture [0.5 ml; 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6.3H_2O$, 1 mM $MgSO_4$ and 1 mg/ml X-gal (Sigma)] was added to cover cells. Cells were then incubated overnight at 37°C and counterstained with hematoxylin. Positive cells expressing β-gal were stained blue and the percentage of blue cells was calculated.

Western immunoblotting

After infection with Ad-CMV-p53 at 50 MOI for 6 hr, cells were grown in a 100 mm dish to about 70–90% confluence for 3 days and the cell lysate was made by adding 20% SDS containing 1 mM PMSF. The lysate was sonicated for 30 sec on ice, followed by centrifugation for 5 min at 4°C. Twenty micrograms of each protein were loaded in reduced conditions on a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. After blocking the membrane with PBS containing 5% powdered milk, the membrane was incubated with anti-p53 (DO-1) antibody (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr, followed by incubation with anti-mouse IgG. After extensive washing, proteins were visualized with an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

Flow-cytometric analysis

To analyze the effects of Ad-CMV-p53 treatment on the cell cycle, cells were infected with Ad-CMV-p53 by 6 hr cultivation in medium containing 50 MOI Ad-CMV-p53. At 4 days postinfection, cells were harvested and fixed on ice for 30 min in PBS (pH 7.4) containing 2% formaldehyde. After several washes, cells were centrifuged and resuspended in 3 ml of 80% ice-cold ethanol for postfixation and stored at –20°C. Before flow cytometry, cells were washed and incubated for 15 min in phosphate citric acid buffer (20% Triton X, 5 mg/ml RNase A in PBS) and then resuspended in 50 mg/ml of propidium iodide. Cells were incubated for at least 15 min at room temperature in the dark and the DNA content of preparations was analyzed by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ). A 488 nm laser was run at 15 mW and fluorescence was passed through a 585/42 nm bandpass filter. At least 20,000 events were acquired using *CELL Quest* software (Becton Dickinson). Each experiment was performed at least twice.

Detection of apoptotic cells by TUNEL assay

After infection with Ad-CMV-p53 at 50 MOI for 6 hr, cells were grown in 2-well chambers for 4 days, then used for the histopathologic apoptotic cell analysis. Apoptosis was assessed by morphologic criteria and by immunohistochemistry *in situ* using the ApopTag assay (Oncor, Gaithersburg, MD). This method detects nucleosome-sized DNA fragments by tailing the 3′-OH ends of fragments with digoxigenin-nucleotide using TdT.[21] DNA fragments were then tailed with digoxigenin-nucleotide by TdT and incubated with an antidigoxigenin antibody conjugated with FITC. Fluorescent signals (green) in the ApopTag assay and counterstaining with propidium iodide were detected using a confocal laser microscope (MRC 1024; Bio-Rad, Hemel Hempstead, UK). The percentage of cells exhibiting TUNEL-positive nuclei was

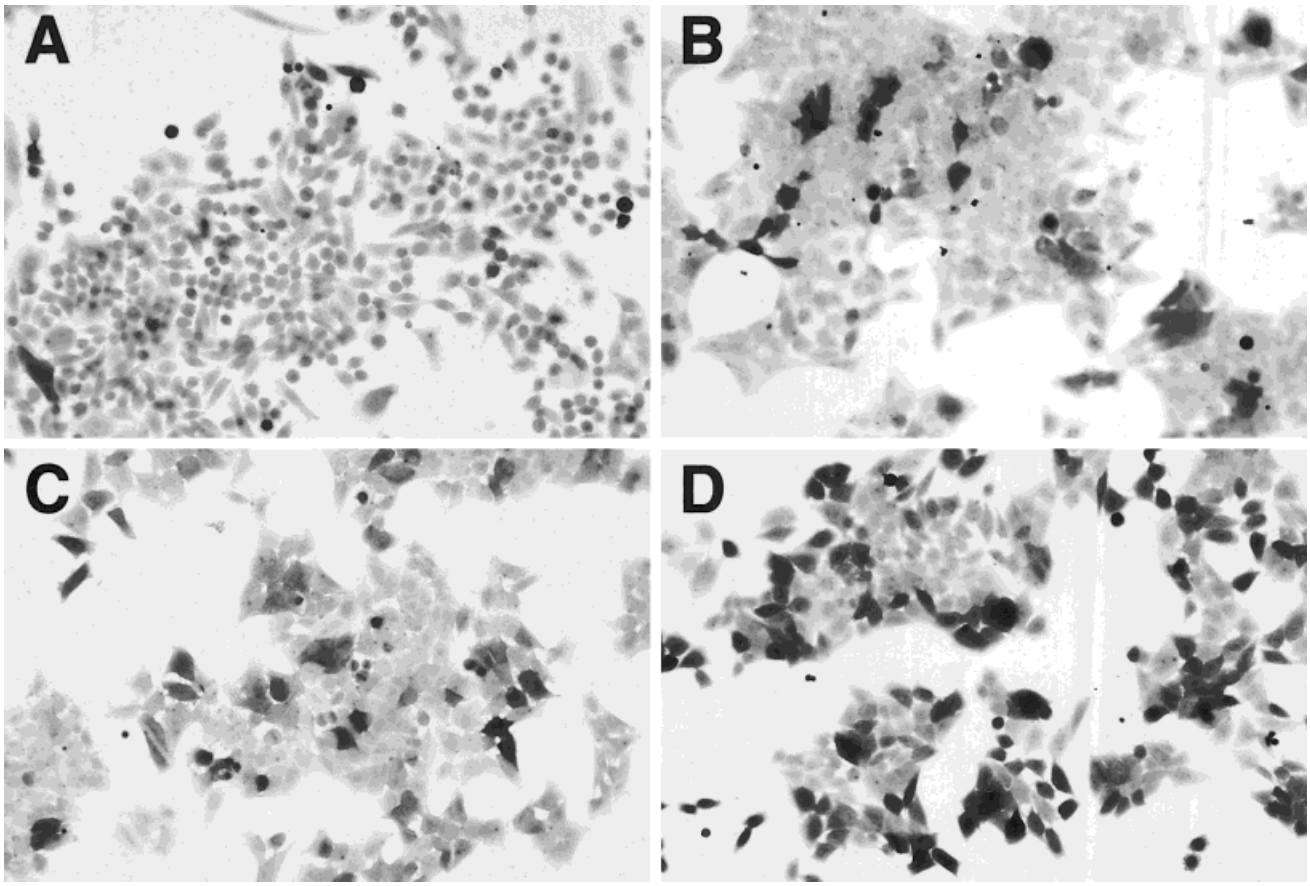

FIGURE 4 – X-gal staining of KK47 cell lines after exposure to 50 MOI of Ad-CMV-β-gal. Blue, cells exposed to 50 MOI of adenovirus, demonstrating excellent adenoviral transduction efficiency. The percentages of blue cells from drug-resistant cell lines were significantly higher than those of the KK47 cell line [% positive cells: *(a)* KK47 5.2 ± 3%, *(b)* KK47/DDP10 33.4 ± 6%, *(c)* KK47/DDP20 26.1 ± 7%, *(d)* KK47ADM 41.5 ± 3%].

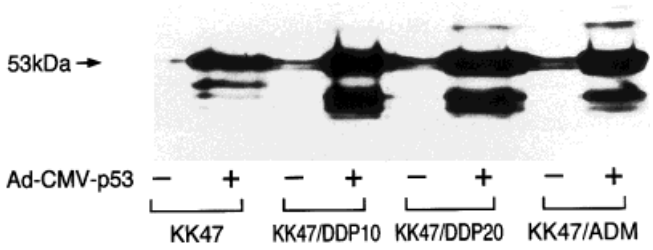

FIGURE 5 – Representative Western blots of p53 protein in KK47 cell lines. Each lane contains 100 μg protein. Arrows at left correspond to 53 kDa immunoreactive protein. Compared to KK47, a clearly higher p53-immunoreactive density was observed in drug-resistant cell lines. Lower bands correspond to unphosphorylated p53 protein.

analyzed quantitatively as an apoptotic index. The total apoptotic index was the average of 5 individual values.

Statistical analysis

Student's *t*-test was employed for statistical analysis and $p < 0.005$ was considered statistically significant.

RESULTS

p53 *exons 5–8*

In all 4 cell lines, no aberrant band shift was detected in exons 5–8 (Fig. 1), indicating that there is no mutation in exons 5–8 of *p53* in any of the 4 cell lines and that their *p53* genes are functionally normal. Thus, the rationale of adenoviral wt *p53* gene therapy for these human bladder-cancer cells is based on overexpression, but not replacement, of wt *p53.*

Sensitivity to adenoviral wt p53 *gene therapy*

While Ad-CMV-β-gal induced no cell growth inhibition in the cell lines, Ad-CMV-p53 induced significant cell growth inhibition in all 4 cell lines at 25 to 100 MOI (Fig. 2). The drug-resistant cell lines (KK47/DDP10, /DDP20, /ADM) had significantly higher sensitivity to Ad-CMV-p53 treatment compared to the parental KK47 cell line.

Expression of β-gal and p53 *mRNA*

Figure 3*a* shows the mean relative quantification of *β-gal* mRNA expression found in the cells at various MOI (5, 25, 50, 75, 100) of Ad-CMV-β-gal infection in triplicate determinations of each sample. To normalize for differences in the amount of total RNA, GAPDH was selected as an endogenous RNA control. The relative quantification was calculated by dividing the value of KK47 infected by 5 MOI of Ad-CMV-β-gal. *β-Gal* mRNA expression in the Ad-CMV-β-gal–infected drug-resistant cell lines was higher than that in the Ad-CMV-β-gal–infected parental cell line at every adenoviral MOI, indicating that the CMV promoter-based adenoviral vector could efficiently mediate gene transduction in drug-resistant cells compared to the drug-sensitive parental cells.

Consistent with the *β-gal* gene transduction, every MOI of Ad-CMV-p53 transduced higher *p53* gene expression in drug-

resistant cells compared to parental cells (Fig. 3*b*). Figure 3*b* shows the mean relative quantification of *p53* mRNA expression at various MOI (0, 5, 25, 50, 75, 100) of Ad-CMV-p53, calculated by dividing the value of noninfected KK47/ADM cells. In noninfected cells, expression of *p53* mRNA was detected only in KK47/ADM cells but not in the other noninfected cells.

Figure 3 shows that the higher adenoviral gene-transfer efficiencies were seen in the drug-resistant cells at every MOI compared to drug-sensitive cells and that Ad-CMV-p53 vector transferred higher levels of *p53* to the drug-resistant cells at every MOI.

Expression of β-gal protein

Consistent with the real-time quantitative PCR data, Ad-CMV-β-gal (50 MOI) induced higher expression of β-gal protein in the drug-resistant cell lines compared to the KK47 cell line (Fig. 4). The percentages of positive cells were as follows: KK47, 5.2 ± 3%; KK47/DDP10, 33.4 ± 6%; KK47/DDP20, 26.1 ± 7%; KK47/ADM, 41.5 ± 3%.

Expression of p53 *protein*

Consistent with *p53* mRNA expression, expressions of p53 protein in drug-resistant cells was higher than in KK47 cells (Fig. 5), indicating that higher levels of *p53* gene transfer could induce higher levels of p53 protein.

Expression of p53, p21 *and* Bax *mRNA*

Figure 6*a* shows the mean relative quantification of *p53* mRNA expression, calculated by dividing the value of noninfected KK47/ADM cells. In noninfected cells, expression of *p53* mRNA was detected only in KK47/ADM cells but not in the other cell types.

Figure 6*b,c* shows the mean relative quantifications of *p21* and *Bax* mRNA expression. Consistent with *p53* mRNA expression, Ad-CMV-p53 induced higher *p21* mRNA expression in drug-resistant cells compared to parental cells.

Expression of CAR *mRNA*

Figure 7 shows the mean relative quantification of *CAR* mRNA expression. There were no significant difference in *CAR* mRNA

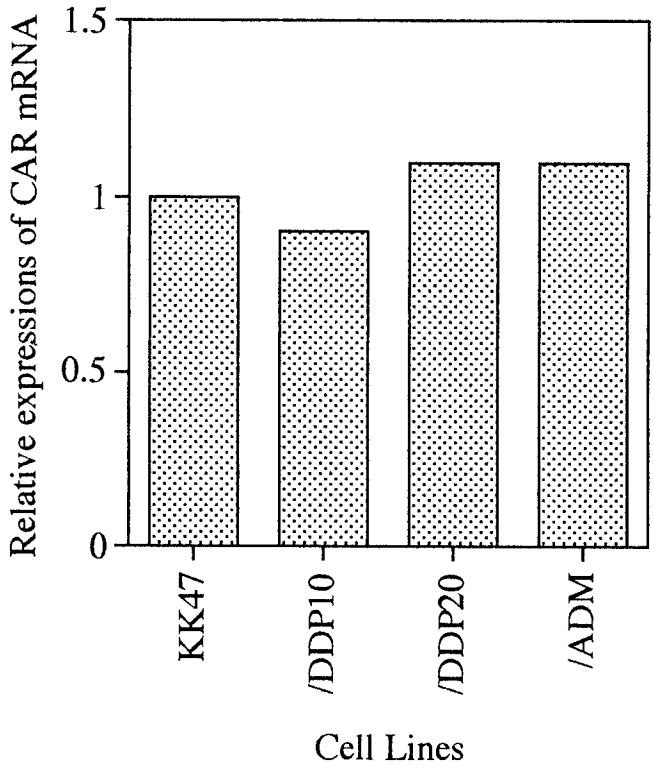

FIGURE 7 – Relative mRNA levels of *CAR* by quantitative RT-PCR of the cell lines. No significant difference in *CAR* mRNA expression was observed among the KK47 cell lines. Values represent relative levels of mRNA normalized to the KK47 parental cell line with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA.

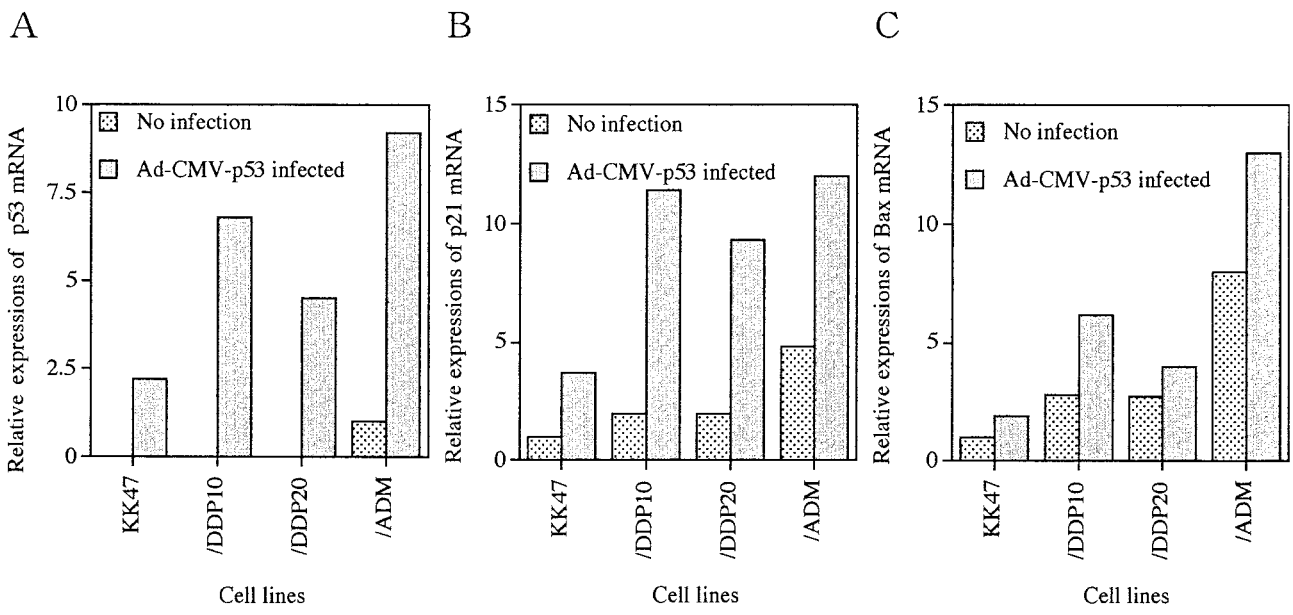

FIGURE 6 – Relative mRNA levels of *p53 (a), p21 (b)* and *Bax (c)* by quantitative RT-PCR of KK47 cell lines with and without exposure to 50 MOI of Ad-CMV-p53. *(a)* Ad-CMV-p53 exposure transduces higher *p53* mRNA expression in drug-resistant cell lines compared to the parental KK47 cell line. Values represent relative levels of mRNA normalized to the noninfected KK47/ADM cell line with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. *(b,c)* Cells exposed to Ad-CMV-p53 have increased mRNA levels of *p21* and *Bax.* After Ad-CMV-p53 exposure, expression of *p21* and *Bax* mRNA was higher in drug-resistant cell lines than in the parental cell line. Values represent relative levels of mRNA normalized to the noninfected KK47 cell line with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA.

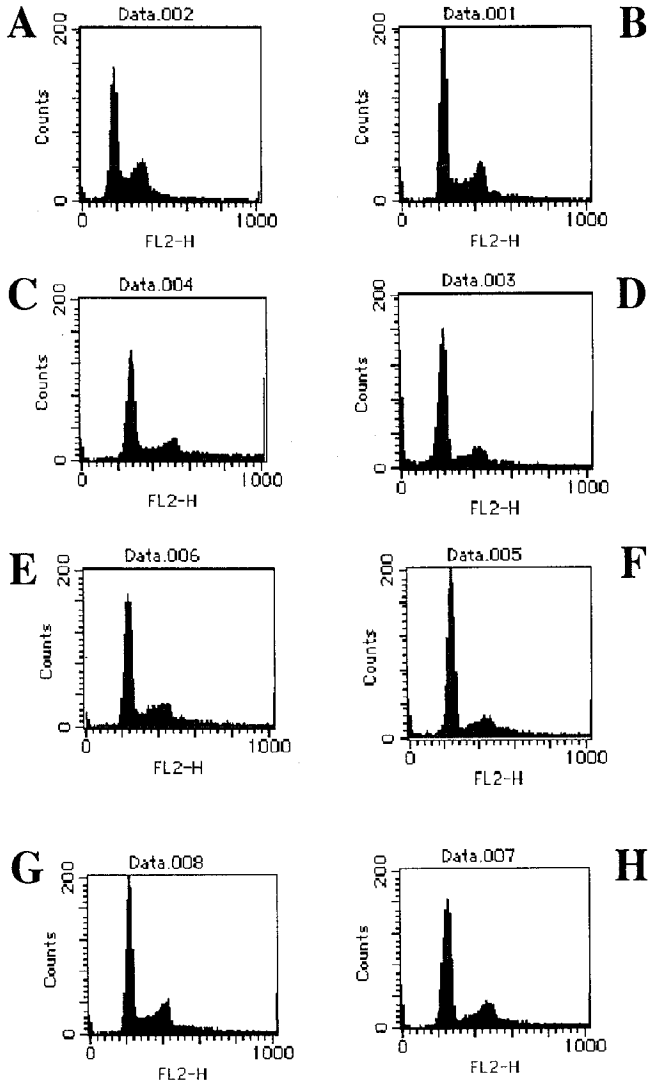

FIGURE 8 – Representative DNA histograms of KK47 cell lines, which were stained with propidium iodide, using FACScan. *(a)* Control KK47 cells. *(b)* KK47 cells after Ad-CMV-p53 infection. *(c)* Control KK47/DDP10 cells. *(d)* KK47/DDP10 cells after Ad-CMV-p53 infection. *(e)* Control KK47/DDP20 cells. *(f)* KK47/DDP20 cells after Ad-CMV-p53 infection. *(g)* Control KK47/ADM cells. *(h)* KK47/ADM cells after Ad-CMV-p53 infection.

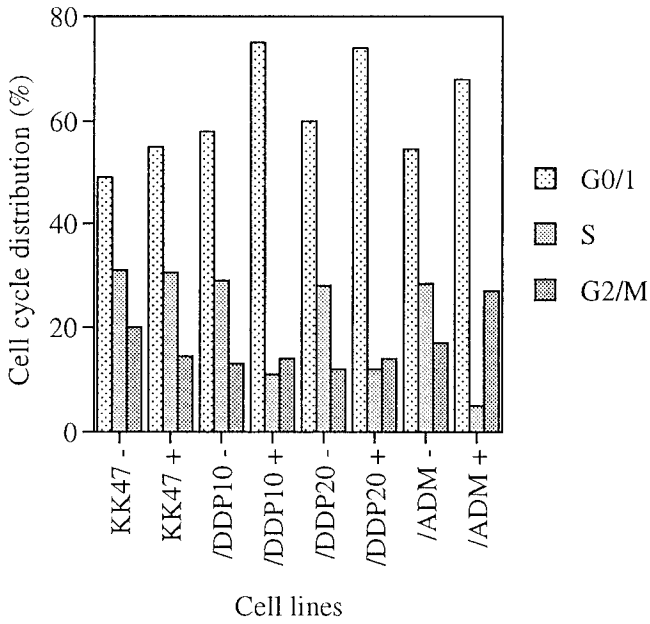

FIGURE 9 – Effect of Ad-CMV-p53 (50 MOI) infection on cell-cycle distribution (–, no infected control cells; +, Ad-CMV–infected cells). G_1 arrest was observed in all 4 cell lines after Ad-CMV-p53 infection. After Ad-CMV-p53 infection, the ratio of G_1 phase was significantly higher in the drug-resistant cell lines than in the KK47 cell line.

expression between drug-resistant cell lines and the parental cell line, suggesting that the differences in gene-transfer efficiency between KK47 and the drug-resistant sublines were independent of CAR expression.

G_1 cell-cycle arrest

The effect on cell-cycle distribution was examined by flow cytometry 4 days after infection (Fig. 8). G_1 cell-cycle arrest was observed after Ad-CMV-p53 (50 MOI) infection in all 4 cell lines, the G_0/G_1 proportions (infected %/noninfected %) being KK47, 55/49 (1.12); KK47/DDP10, 75/58 (1.29); KK47/DDP20, 74/60 (1.23); KK47/ADM, 68/54.6 (1.25) (Fig. 9). In this assay, greater G_1 cell-cycle arrest was observed in the drug-resistant cell lines than in the KK47 cell line.

Apoptosis

To determine whether the cells underwent apoptosis after Ad-CMV-p53 infection, the TUNEL assay was used. Apoptosis was rarely observed in noninfected control cell lines (apoptotic index 0% to 0.8 %). However, after Ad-CMV-p53 infection, apoptosis (apoptotic indices KK47 9.5 ± 4%, KK47/DDP10 47.8 ± 11%, KK47/DDP20 39.8 ± 7%, KK47/ADM 55.3 ± 9%) was observed in the cell lines (Fig. 10). The TUNEL assay also suggested that Ad-CMV-p53 could induce greater apoptosis in the drug-resistant cell lines compared to the parental cell line.

DISCUSSION

Mutations of the *p53* gene are found in 40–60% of tumors in patients with TCC.[22] Preclinical study has suggested that adenovirus-mediated transfer of wt *p53* is growth-inhibitory to TCC cells with mutated *p53* as well as with wt *p53*.[23] Thus, TCC patients with or without mutations of *p53* are being enrolled in a phase I clinical trial of Ad-CMV-p53 gene therapy.[24] Adenovirus-mediated overexpression of wt *p53* induced apoptosis or cell growth arrest in several tumors,[3,11,25] and the efficacy of Ad-CMV-p53 gene therapy correlated with that of adenoviral gene transfer.[25]

We employed a human bladder-cancer cell line that has genetically normal *p53* gene and drug-resistant sublines that also have genetically normal *p53* to investigate adenoviral wt *p53* gene therapy and we demonstrate that Ad-CMV-p53 could mediate higher expression of *p53* in the drug-resistant cell lines than in the drug-sensitive parental line and, consequently, induced higher expression of the downstream effector *Bax* and *p21* genes, which induced cells to undergo apoptosis and G_1 cell-cycle arrest. The TUNEL assay is a valuable method to detect apoptosis.[26] Our TUNEL assay data indicated that Ad-CMV-p53 induced greater apoptosis in the drug-resistant cell lines compared to the parental cell line, consistent with the *in vitro* cytotoxicity of Ad-CMV-p53.

Adenoviruses have been widely used in gene therapy trials for several reasons. They can be grown to a very high titer and easily concentrated to reach 10^{13} to 10^{14} virus particles per milliliter and they are very effective for gene transfer with high transduction efficiency in a variety of cell types.[27] The efficiency of adenoviral gene transfer is critical for adenoviral vector-based gene therapy.

Adenoviral entry into target cells is the rate-limiting step of gene delivery. The initial binding of adenovirus to the cell surface is a

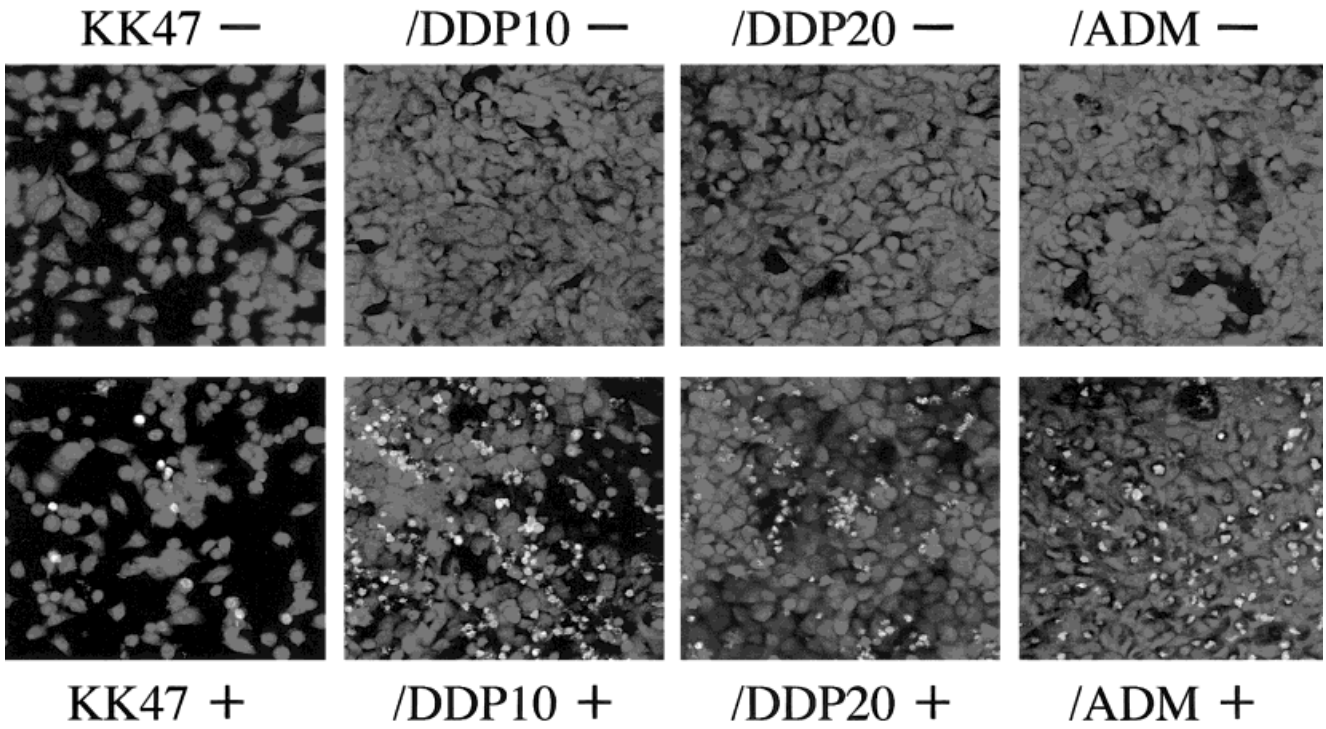

FIGURE 10 – Apoptotic cell detection by TUNEL assay was performed using a confocal laser microscope (–, no infected control cells; +, Ad-CMV-p53–infected cells). Green fluorescent signals represent apoptotic cells. Red signals show nuclei counterstained with propidium iodide. After Ad-CMV-p53 (50 MOI) infection, the ratios of green cells were significantly higher in the drug-resistant cell lines than in the KK47 cell line.

receptor-mediated process.[28,29] The fiber protein is responsible for the initial attachment of the virus to the cellular receptor. A cDNA clone (CAR) encoding a 46 kDa protein has been identified as the receptor for group C adenovirus (*e.g.*, adenovirus types 2 and 5).[30] The efficiency of adenoviral gene transfer to several tumors, including bladder cancer, correlates with the expression of CAR.[25,30,31] In our study, however, no significant differences of CAR expression were observed between the drug-resistant cell lines and the parental cell line, suggesting that the differences in gene transfer efficiency between the drug-resistant cell lines and the parental cell line might be independent of CAR expression.

Ad-CMV-β-gal induced higher *β-gal* gene expression in the drug-resistant cell lines compared to the parental cell line, indicating greater efficiency of adenovirus-mediated gene transfer in the drug-resistant cell lines compared to the parental line. The mechanism of the high efficiency of adenoviral gene transfer in the drug-resistant cells is unclear. However, it could be that DNA damage caused by exposure to an anticancer drug (cisplatin or doxorubicin) increases gene transfer by adenoviral vector. DNA damage by γ-irradiation, UV irradiation or anticancer drugs may induce unscheduled DNA synthesis, causing unexpected viral DNA replication, increased viral binding or transport or decreased viral DNA degradation. These molecular mechanisms are currently under investigation.[33,34]

Adenovirus-mediated wt *p53* gene therapy was more effective in our drug-resistant bladder-cancer cell lines compared to the parental cell line because of the efficiency of adenoviral gene transfer, which was independent of CAR expression.

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