Late resistance to adenoviral *p53*-mediated apoptosis caused by decreased expression of Coxsackie-adenovirus receptors in human lung cancer cells

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(Received January 21, 2004/Revised March 8, 2004/Accepted March 8, 2004)

Adenovirus-mediated wild-type *p53* gene transfer induces apoptosis in a variety of human cancer cells. Although clinical trials have demonstrated that a replication-deficient recombinant adenovirus expressing the wild-type *p53* gene (Ad-*p53*) is effective in suppressing growth of non-small cell lung cancer (NSCLC), we often experienced late resistance to this treatment. To elucidate the mechanism of late resistance to Ad-*p53* in human lung cancer cells, we generated 5 different resistant variants from p53-susceptible H1299 NSCLC cells by repeated infections with Ad-*p53*. We first examined the transduction efficiency of adenoviral vector by Ad-*LacZ* transduction followed by X-gal staining in parental and 5 resistant H1299 cell lines. Their sensitivity to viral infection decreased in correlation with the magnitude of resistance, and Ad-*p53*-mediated tumor suppression could be restored by dose escalation of Ad-*p53* in the resistant variants. The expression of Coxsackie and adenovirus receptor (CAR) and αV integrins, which are cellular receptors for attachment and internalization of the virus, respectively, was next investigated in these cell lines. Flow cytometry revealed that αVβ3 and αVβ5 integrin expression was consistent, while p53-resistant cell lines showed that diminished CAR expression correlated with the magnitude of the resistance. Our results demonstrated that decreased CAR expression could be one of the mechanisms of late resistance to Ad-*p53*, which may have a significant impact on the outcome of adenovirus-based cancer gene therapy. (Cancer Sci 2004; 95: 459–463)

It is well known that the *p53* gene is frequently altered in a variety of human cancers, and its product plays a very important role in tumor suppression. Adenovirus-mediated wild-type *p53* gene replacement is an attractive therapeutic strategy for various human cancers and clinical trials of this new therapy are currently being conducted in many countries.[1)] One of the obstacles to this treatment, however, seems to be late resistance. Adenoviral entry into target cells is a multi-step process that includes the initial attachment of the virus capsid via high-affinity interaction of the viral fiber protein with a cell-surface molecule called the Coxsackie-adenovirus receptor (CAR),[2–5)] low-affinity interactions of the penton-base protein in the capsid with αvβ3 and/or αvβ5 integrins[6–8)]; and internalization of the virus capsid into the endosomes.[4)] Recent reports have indicated that there is another interaction with heparan sulfate glycosaminoglycans.[9)] CAR is a 46-kDa transmembrane glycoprotein that functions as a high-affinity receptor for both subgroup C adenoviruses (adenovirus type 2 and 5) and the Coxsackie B viruses,[10, 11)] and is expressed in a wide range of human cell types. Therefore, cellular sensitivity to adenovirus-based gene therapy seems to be considerably affected by expression of CAR and/or αv integrins in target cells.

A recent study has reported that late resistance to Ad-*p53* in bladder carcinoma cells was caused by different expression of genes involved in cell cycle regulation or apoptosis.[12, 13)] In addition, another group has reported a reduced transduction efficiency of adenoviral vector due to decreased expression of αV integrins in human glioma cells.[14)] We have treated nine patients with advanced non-small cell lung cancer (NSCLC) by intratumoral injection of Ad-*p53* as a phase I trial in Japan. The first patient responded well and suppression of tumor growth occurred in this patient for 9 to 10 months after the commencement of treatment. The tumor, however, started to regrow even with repeated Ad-*p53* injections. The present study was designed to elucidate the mechanism of the late resistance to *p53* gene therapy in NSCLC patients. For this purpose, we generated H1299 human NSCLC cells refractory to Ad-*p53* treatment by means of five repeated infections. We found that the transduction efficiency of adenoviral vector and CAR expression markedly diminished as the cells were repeatedly infected with Ad-*p53*. Our results emphasize the importance of CAR expression in target cells for adenovirus-mediated gene therapy.

Materials and Methods

Cells and culture conditions. The human NSCLC cell lines, which contain homozygously deleted *p53*, were propagated in monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 m*M* HEPES, 100 units/ml penicillin and 100 mg/ml streptomycin. The H1299-R1, H1299-R2, H1299-R3, H1299-R4 and H1299-R5 cell lines were generated from H1299 by selection for resistance to Ad-*p53*. They were maintained and propagated in the same medium used for H1299. The transformed embryonic kidney cell line 293 was grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/liter), as well as 10% fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The 293 cells were used for the production of adenoviral vectors.

Recombinant adenoviruses. The recombinant adenoviral vector expressing human wild-type *p53* cDNA (Ad-*p53*) was constructed as described previously.[15–18)] Ad-*LacZ* is E1 and E3-deleted, and expresses the reporter *LacZ* gene under the control of the cytomegalovirus-promotor region. Recombinant viruses were purified by ultracentrifugation in cesium chloride step gradients, and their titer was determined by plaque-forming assay in the 293 cells.

X-gal staining. The transduction efficiency of adenoviral vector was assessed by X-gal staining. Cells were seeded in 6-well plates (2×10^5 cells/well) and infected with Ad-*LacZ* at indicated multiplicities of infection (MOIs). X-gal staining was per-

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formed 24 h after infection according to the protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO).

Western blot analysis. H1299 and H1299-R5 cells infected with Ad-*p53* were collected 20 h after infection, lysed in lysis buffer (10 m*M* Tris-HCl [pH 7.5], 400 m*M* NaCl, 1 m*M* DTT, 5 m*M* NaF, 1 m*M* EDTA, 0.5% Na_3VO_4, 10% glycerol, 0.5% NP-40, 0.1 m*M* PMSF, 1 mg/ml leupeptin and 1 mg/ml aprotinin) for 30 min on ice, and centrifuged at 15,000 rpm for 30 min. Protein concentration was measured by means of the Bradford assay. Equal amounts of protein containing sample buffer (62.5 m*M* Tris-HCl [pH 6.8], 2% SDS, 10% glycerol and 5% β-mercaptoethanol) were boiled for 5 min and electrophoresed under reducing conditions on 12.5% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to Hybond-polyvinylidene difluoride (PVDF) transfer membranes (Amersham, Arlington Heights, IL), and incubated with primary antibody against *p53* (Ab-2; Oncogene Research Products, San Diego, CA) or rabbit anti-human actin monoclonal antibody (Sigma-Aldrich), followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amersham) was used to detect secondary probes.

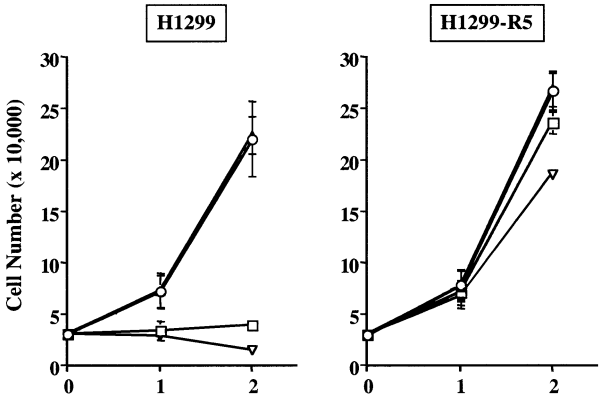

Fig. 1. Effect of Ad-*p53* on the growth of parental H1299 and p53-resistant H1299-R5 human NSCLC cells. H1299 and H1299-R5 cells (3×10^4 cells) were plated in each well of triplicate 24-well plates and infected with control Ad-*LacZ* or Ad-*p53* at an MOI of 30 or 50 for 24 h. Cell viability was determined daily by trypan blue staining. Mock-infected cell growth was used as a control. Data represent the mean±SD of triplicate determinations. ○ Mock, △ Ad-*LacZ* 50 MOI, □ Ad-*p53* 30 MOI, ▽ Ad-*p53* 50 MOI.

Flow cytometric analysis. The expressions of CAR, αvβ3 and αvβ5 integrins were evaluated by flow cytometric analysis using FACScalibur (Becton Dickinson, Mountain View, CA). Cells were washed twice with phosphate-buffered saline (PBS) containing 2% FCS, and for antibody incubation, 2×10^5 cells were incubated with RmcB for anti-CAR (Upstate Biolab, Lake Placid, NY), LM609 for anti-αvβ3 or P1F6 for αvβ5 (Chemicon International, Temecula, CA) for 30 min on ice. An isotype-matched normal mouse IgG1 conjugated to FITC (Serotec, Oxford, UK) was used as a negative control. The cells were then washed twice with PBS containing 2% FCS, and incubated with FITC-labeled rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA) for 30 min on ice. The cells were again washed twice with PBS containing 2% FCS, and resuspended in 1 ml of PBS containing 2% FCS. They were analyzed immediately by flow cytometry.

Results

Generation of H1299 cells resistant to Ad-*p53*. We have previously demonstrated that Ad-*p53* infection at an MOI of 50 resulted in exogenous *p53* gene expression, leading to induction of apoptosis as early as 24 h after infection in H1299 human NSCLC cells.[17] In order to establish H1299 cells resistant to Ad-*p53*, the parental H1299 cell line was repeatedly infected with Ad-*p53* at an MOI of 100. H1299 cells (1×10^6) were grown stably in T75 flasks and then exposed to Ad-*p53* at an

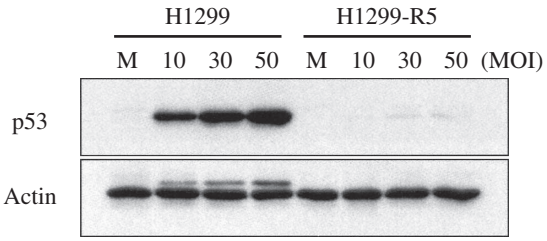

Fig. 2. Western blot analysis for detection of p53 protein expression in adenovirally transduced H1299 and H1299-R5 cells. Cells were treated with Ad-*p53* at an MOI of 10, 30 or 50 and then collected 20 h after infection. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane, probed with anti-p53 antibody, and then visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and re-probing with anti-actin antiserum.

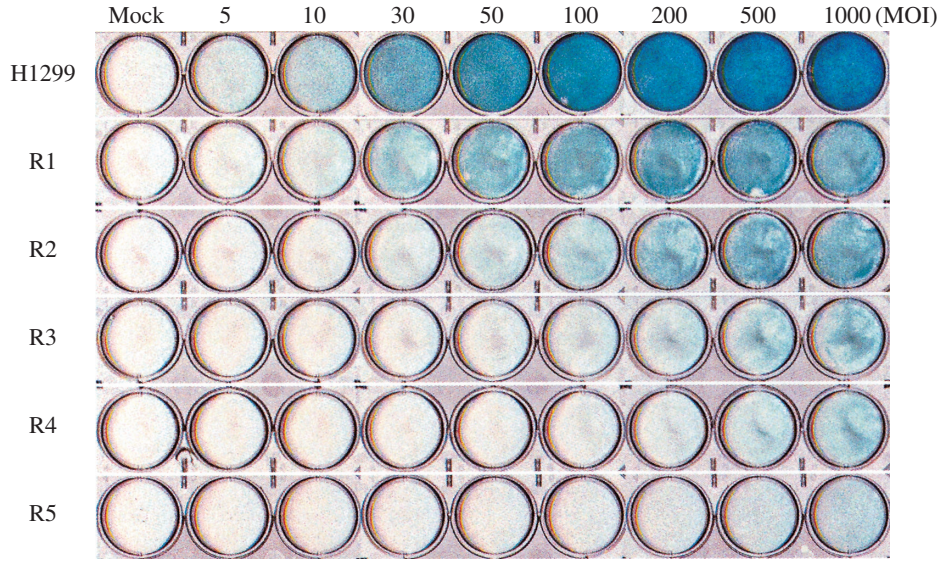

Fig. 3. Transduction efficiency of the adenoviral vector in p53-resistant H1299 variants. Parental H1299 cells and p53-resistant variants (R1 to R5) (2×10^5 cells/well) were plated onto 6-well culture plates, infected with Ad-*LacZ* at the indicated MOI, and stained with X-gal 24 h after infection.

MOI of 100. Twenty-four hours after infection, the medium containing Ad-*p53* was replaced with fresh complete medium without Ad-*p53*. Four days later, when most cells had undergone apoptosis, the medium containing dead, floating cells was removed and then replaced with the fresh medium every 3 to 4 days. Recovered viable H1299 cells were collected 18 days after the first Ad-*p53* infection. These cells were secondly exposed to Ad-*p53* at an MOI of 100 for 24 h, processed by the same method as described for the first infection, and then collected 13 days after the second Ad-*p53* infection. These cells were thirdly infected with Ad-*p53* at an MOI of 100 for 24 h, and then collected 7 days later. The number of viable H1299 cells increased twice within 7 days. Recovered cells were treated with two more rounds of Ad-*p53* infection at an MOI of 100. These 5 cell lines resistant to Ad-*p53* were designated H1299-R1 through H1299-R5. In fact, most H1299-R5 cells survived after infection with Ad-*p53* at an MOI of less than 50, although the growth rate of H1299-R5 cells was similar to that of the parental H1299 cells (Fig. 1).

p53 expression after Ad-*p53* infection. In order to confirm functional *p53* gene transduction, we conducted western blot analysis using antibody against human p53 for parental H1299 cells and p53-resistant H1299-R5 cells. As shown in Fig. 2, western blotting indicated that readily detectable p53 protein expression was achieved in parental H1299 cells 20 h after Ad-*p53* infection at an MOI of 10, but not in H1299-R5 cells infected with less than 50 MOI of Ad-*p53*. These results suggest that the adenovirus-mediated *p53* gene transfer is highly attenuated in H1299-R5 cells.

Transduction efficiency of adenoviral vector in p53-resistant H1299-R5 cells. We next examined the transduction efficiency of the adenoviral vector in Ad-*p53*-resistant cells by means of X-gal staining. H1299, H1299-R1, H1299-R2, H1299-R3, H1299-R4 and H1299-R5 cells (2×10^5 cells/well) were plated onto 6-well culture plates, infected with Ad-*LacZ* at an indicated MOI, and stained with X-gal 24 h after infection. As shown in Fig. 3, the percentage of blue-stained LacZ-positive cells gradually decreased as the number of Ad-*p53* infections increased. These results implied that repeated infections with Ad-*p53* reduced the transduction efficiency of adenoviral vector in a frequency-of-treatment-dependent manner. To overcome the low transduction efficiency, we used very high doses of Ad-*p53* to infect H1299-R5 cells. Western blot analysis demonstrated detectable expression of p53 protein in H1299-R5 cells after Ad-*p53* infection at more than 500 MOI (Fig. 4A). Most H1299-R5 cells could be killed by Ad-*p53* infection at an MOI of 5000 within 48 h (Fig. 4B). In contrast, 5000 MOI of Ad-*LacZ* had no apparent effect on the viability of H1299-R5 cells, suggesting that there is no toxicity of adenovirus infection alone. Thus, the function of downstream mediators of p53 may be intact in H1299-R5 cells and Ad-*p53* resistance is directly associated with the reduced transduction efficiency of the adenoviral vectors.

Expression of CAR and integrins on Ad-*p53*-resistant H1299 variants. Considerable evidence has accumulated that the transduction efficiency of adenoviruses relies on the expression of cell-surface molecules involved in adenoviral entry, such as CAR, αvβ3 and αvβ5 integrins. In an attempt to understand the mechanism of the low transduction efficiency of adenoviral vectors in Ad-*p53*-resistant H1299 cells, we compared the cell

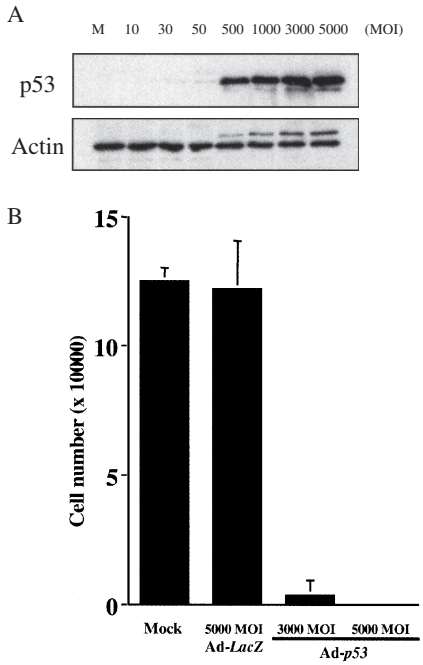

Fig. 4. A: Western blot analysis of p53 protein in H1299 and H1299-R5 cells transduced with high doses of Ad-*p53*. Total cell extracts were prepared from cells mock-infected or infected with Ad-*p53* at indicated MOIs at 24 h after infection, and then analyzed on western blots using antibodies against human p53 or actin. B: Cytotoxic effects of high doses of Ad-*p53* on H1299-R5 cells. H1299-R5 cells were cultured as monolayers in triplicate in 6-well culture plates, infected with 5000 MOI of Ad-*LacZ* or Ad-*p53* at indicated MOIs, and assessed for cell viability 48 h after infection. Mock-infected cells were used as a control. Data represent the mean±SD of triplicate experiments.

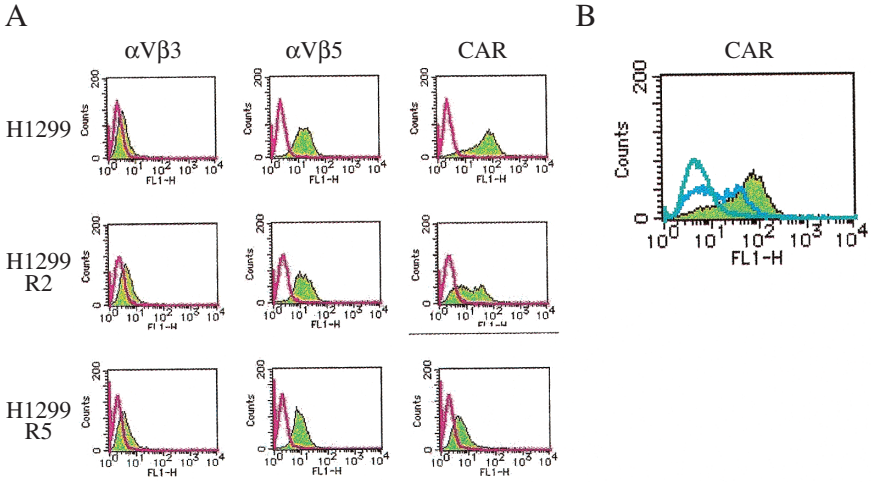

Fig. 5. Expression of CAR and integrins on parental H1299 and p53-resistant H1299 variants. A: Flow cytometric analysis of integrin αvβ3, integrin αvβ5 and CAR. Parental H1299, moderately p53-resistant H1299-R2, and highly p53-resistant H1299-R5 cells were incubated with the mAbs RmcB (CAR), LM609 (αvβ3) or P1F6 (αvβ5), followed by detection with FITC-labeled secondary antibody. An isotype-matched normal mouse IgG1 conjugated to FITC was used as a control in all experiments (pink line). B: Merged images of CAR expression. Parental H1299 (green), H1299-R2 (blue) and H1299-R5 (light blue) cells were measured for CAR expression with indirect flow cytometry.

surface expression of CAR and αv integrins by flow cytometry. CAR was abundantly expressed in parental H1299 cells. However, CAR expression levels were slightly diminished in H1299-R2 cells, but were lower in H1299-R5 cells. In contrast, parental H1299, H1299-R2 and H1299-R5 cells showed a similar expression pattern of αvβ3 and αvβ5 integrins (Fig. 5A). These results indicate that the reduced transduction efficiency of adenoviral vector in Ad-*p53*-resistant cells might be caused by decreased expression of CAR, but not αv integrins.

Discussion

The major finding of the present study was that the late resistance to adenoviral *p53*-mediated apoptosis in human NSCLC cells is associated with diminished transduction efficiency of the adenoviral vector. Recent reports have demonstrated that bladder carcinoma cells resistant to Ad-*p53* expressed the same amounts of p53 protein after Ad-*p53* infection; however, the expression of some genes, such as PIGs, varied.[12, 13] The p53 protein induces cell cycle arrest or apoptosis through its function as a sequence-specific transcriptional activator. It regulates the expression of a number of target genes including *p21*$^{WAF1/CIP1}$,[19, 20] *p53R2*,[21] *MDM2*,[22] *Noxa*[23] and *p53AIP1*.[24] Post-transcriptional modification activates p53 protein and may select its target promoter sequences.[25] As the differential regulation of p53 target genes might decide the fate of the cells, the findings by Maxwell *et al*.[12, 13] that the late resistance is due to varied expression of genes involved in *p53*-mediated apoptosis seems reasonable. However, one of the mechanisms hypothesized in their reports to explain the selection of resistant variants is debatable. Although they suggested that p53 exerted an effect at the molecular level in parental cells, it is unlikely that adenovirus-mediated transient gene expression affects the genome of tumor cells in such a short time period.

On the other hand, Yamamoto *et al*.[14] indicated that the late resistance to Ad-*p53* is caused by reduced transduction efficiency in glioma cells, which is consistent with our data; the precise mechanism, however, was different from ours. They showed that decreased expression of αv integrins on the cell surface is involved in reduced transduction efficiency. You *et al*.[26] have recently demonstrated that the integrins αvβ3 and αvβ5 are not always necessary for adenovirus transduction, while the expression of CAR is essential for and positively correlated with its efficiency. In addition, Mizuguchi *et al*.[27] reported that the CAR-binding-ablated adenoviral vector mediates about 1% of gene transfer activity compared with conventional adenoviral vectors, whereas αv integrin-binding-ablated adenoviral vector maintained at least 76% of the gene transfer activity. These observations indicate that CAR expression might be more important than expression of αv integrins with respect to the entry of adenoviral vectors. In fact, we demonstrated here that the reduction of transduction efficiency with Ad-*p53* is caused by low CAR expression in the course of repeated Ad-*p53* infections. These results suggest that the most important receptor for adenovirus entry might be dependent on the cell type or other conditions.

Previous studies compared the sensitivity of parental cells to Ad-*p53*-mediated apoptosis with merely that of the final resistant variants generated by repeated Ad-*p53* infections, making it difficult to clearly define the process of resistance acquisition. We used not only the final resistant variant, but also intermediates to allow us to determine how the tumor cells acquire resistance to Ad-*p53*. Most parental H1299 cells expressed high levels of CAR, whereas the moderately resistant variant H1299-R2 displayed a two-peak pattern, suggesting a heterogeneous CAR expression (Fig. 5B). Moreover, CAR expression was quite homogeneous in the highly resistant variant H1299-R5, with the majority of the cells exhibiting low levels of CAR expression (Fig. 5B). These results indicate that the Ad-*p53*-resistant population becomes gradually dominant during repeated Ad-*p53* infections. Although the differential expression of other adenoviral receptors such as heparan sulfate, as well as other families of integrins, could not be ruled out, down-regulation of CAR expression seems to be one of the mechanisms of the resistance. We have experienced a late resistance to Ad-*p53* treatment during a clinical trial in patients with advanced NSCLC. Experiments to examine differential expression of adenovirus receptors as well as p53 target genes in biopsy samples obtained before and after Ad-*p53* injections are under way in our laboratories.

The level of CAR expression may be one of the most important determinants of the efficacy of gene therapy using adenoviral vectors.[28] Moreover, Okegawa *et al*.[29] reported that increased expression of CAR protein inhibited tumor growth of prostate cancer cell lines; in our experimental model, however, down-regulation of CAR expression affected neither the *in vitro* growth rate (Fig. 1) nor the morphology (data not shown). When CAR expression is reduced after repeated Ad-*p53* infections, a strategy to overcome the resistance is required. Dose escalation of Ad-*p53* is likely to be effective (Fig. 4); however, it appears not to be practical in clinical trials because of the associated toxicity. One possible strategy is re-targeting of the adenoviral vector to alternative cellular receptors in order to circumvent CAR deficiency. Recently, it has been reported that fiber-modified vectors containing RGD peptide and/or polylysine could mediate CAR-independent cell entry and overcome the low expression of CAR.[30, 31] In fact, the tumor-selective replication-competent oncolytic adenovirus, TRAD,[32] could not efficiently kill H1299-R5 cells, whereas TRAD-RGD, which contains a RGD motif in the fiber knob, exhibited a profound cytotoxicity against H1299-R5 cells (Taki, M., Fujiwara, T., Mizuguchi, H., unpublished data). Another study has demonstrated that an adenovirus vector containing adenovirus type 5/ adenovirus type 35 chimeric fiber protein exhibits altered and expanded tropism, because adenovirus type 35 recognizes unknown receptors other than CAR.[33] Modification of the adenoviral tropism would be of great benefit to overcome the resistance caused by reduced transduction efficiency.

In conclusion, we have shown that the late resistance to Ad-*p53* in human NSCLC cells is associated with reduced CAR expression. The importance of CAR expression in adenovirus-mediated gene therapy is clear. Our results provide a rationale for developing new methods to overcome resistance to adenovirus-mediated *p53* gene therapy, as well as other adenoviral therapies for human cancers, and should be of clinical utility.

We are grateful to Drs. Takeshi Kawashima, Tatsuo Umeoka and Masahiko Nishizaki for helpful discussions. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by grants from the Ministry of Health, Labour and Welfare, Japan (Health Sciences Research Grants [Research on Human Genome and Gene Therapy]).

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