

Reversal of 5-fluorouracil resistance by adenovirus-mediated transfer of wild-type p53 gene in multidrug-resistant human colon carcinoma LoVo/5-FU cells

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Abstract

AIM: To observe the reversal effects of wide-type p53 gene on multi-drug resistance to 5-FU (LOVO/5-FU).

METHODS: After treatment with Ad-p53, LOVO/5-FU sensitivity to 5-FU was investigated using tetrazolium dye assay. Multidrug resistance gene-1 (MDR1) gene expression was assayed by semi-quantitative reverse transcription-polymerase chain reaction and the expression of p53 protein was examined by Western blotting.

RESULTS: The reversal activity after treatment with wide-type p53 gene was increased up to 4.982 fold at 48 h. The expression of MDR1 gene decreased significantly after treatment with wide-type p53 gene, and the expression of p53 protein lasted for about 5 d, with a peak at 48 h, and began to decrease at 72 h.

CONCLUSION: Wide-type p53 gene has a remarkable reversal activity for the high expression of MDR1 gene in colorectal cancers. The reversal effects seem to be in a time dependent manner. It might have good prospects in clinical application.

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INTRODUCTION

Resistance to cytotoxic agents is the major cause of failure of medical treatment of human cancer and it is known that tumor cells can become resistant to anticancer drugs by a variety of different mechanisms^[1]. MDR (multidrug-resistance) is a form

of drug resistance characterized by decreased cellular sensitivity to a broad range of chemotherapeutic agents and due significantly to the over expression of MDR1 mRNA and its product P-gp^[2,3]. As an integral membrane protein, the M_r 170 000 P-gp is thought to be an energy-dependent membrane pump involved in the excretion of toxins in normal cells^[4]. Elevated expression of P-gp in malignant cells results in increased efflux and therefore reduced intracellular accumulation of cytotoxic agents, such as 5-FU, anthracyclines, Vinca alkaloids, and epipodophyllotoxins, but is not cross-resistant to alkylating agents, antimetabolites, and cisplatin. This decrease is considered as the basic mechanism of the MDR phenomenon^[4-7]. While a number of pharmacological agents have been shown to partially reverse MDR *in vitro*^[8], there remains a need to identify more potent, more specific, and less toxic chemosensitizers for clinical use.

Inactivity of p53 gene by missense mutation or deletion is the most common genetic alteration in human cancers^[9]. The loss of p53 function has been reported to enhance cellular resistance to a variety of chemotherapeutic agents^[10-13]. Our preliminary experiments^[14] also showed that there was a strong relationship between the MDR1 gene expression and mutated p53 gene in colorectal cancers.

Thus, in light of the fact that MDR1 is over-expressed in colorectal cancer that often lacks a functional p53^[14], we ask whether p53 plays a role in the control of MDR1 gene. Therefore, we sought to determine whether the introduction of the wild - type p53 gene in LOVO/5-FU cells by an adenoviral vector could increase the sensitivity of cells to a DNA cross-linking agent 5-FU *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell culture

LoVo cells were derived from a human colon adenocarcinoma. 5-FU-resistant LoVo sub-line (LoVo/5-FU) was obtained from the LoVo cell line by selection with 5-FU^[15], and maintained in medium containing 5 ng/mL 5-FU as described previously. LoVo/5-FU cells were kindly provided by Dr. Xue-Qing Yao. Monolayer cultures of LoVo and LoVo/5-FU were maintained in RPMI 1640 medium supplemented with 150 g/L heat-inactivated fetal calf serum (Sigma Chemical Co), 10 mL/L of a 200 mmol/L glutamine solution, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 g/L vitamin solution for minimum essential Eagle's medium purchased from GIBCO-BRL (Gaithersburg, MD). Cell lines grown in the presence of agents were passed in drug-free medium 2 to 3 times prior to use. All cells were grown at 37 °C in a humidified atmosphere of 50 mL/L CO₂ and 950 mL/L air.

Adenovirus vectors

Construction and identification of Ad-p53 and Ad-LacZ were finished and kindly provide by Dr. Xin-Hua Yin. Briefly, recombinant p53 adenovirus, Ad5CMV-p53 containing cytomegalovirus promoter, wild-type p53 cDNA, and SV40

early lopyadenylation signal in a minigene cassette was inserted into the E1-deletion region of modified Ad5. p53 shuttle vector and recombinant plasmid pJM 17 were cotransfected into 293 cells (Ad-transformed human embryonic kidney cells) by a liposome-mediated technique. The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. Control Ad-LacZ virus was generated in a similar manner. Viral titers were determined by plaque forming activity in 293 cells. Concentrated virus was dialyzed, aliquoted, and stored at -80 °C. Cells were harvested 36-40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by double cesium chloride gradient ultracentrifugation.

Transient transfections

Adherent cells were plated in the wells of a 12-well plate. After 24 h, Ad-LacZ was transfected into the cells in different diluting densities. Forty-eight transfection, the cells were washed twice with PBS and fixed for 2 h with 40 g/L formaldehyde solution at 4 °C, then washed twice again with PBS and stained with 1 g/L X-gal solution for about 8 h at 37 °C. Observed under the electron microscope, the cells with blue staining were positive cells with LacZ expression, and calculated to determine the transfection efficiency.

Drug sensitivity testing

Chemosensitizers in the various cell lines were determined by using the tetrazolium-based colorimetric assay (MTT [*i.e.*, 3-(4,5-dimethyl-2-thiazoly)-2-m-5-diphenyl-2H-tetrazolium bromide] assay). Cells (LoVo/5-FU/Ad-p53 cells, LoVo/5-FU cells, LoVo cells;) were seeded in a 96-well microtiter plate at a density of 1×10^5 cells/well and each well contained 200 μ L medium. Cells were exposed 24 h after plating to various concentrations of 5-FU for 4 h. After treatment, medium was removed and replaced with 200 μ L of drug-free medium. Plates were incubated for further 72 h. At this time, 15 μ L of MTT (4 mg/mL) solution was added to each well. After a 4-h incubation at 37 °C, the cellular supernatant was gently aspirated, and insoluble formazan crystals were dissolved by adding 180 μ L 1 000 g/L DMSO (dimethyl sulfoxide) to each well, then the plates were shaken for about 10 min. The absorbance of each well was determined by a spectrophotometry at the wavelength of 570 nm with a microculture plate reader. Inhibition of cell growth was determined as a percentage of absorbance of vehicle-treated control cultures. IC₅₀ was the concentration of drugs that reduced staining (A₅₇₀) to 50% of vehicle-treated controls.

The effect of chemosensitizers on drug resistance was studied by exposing cells to a range of concentrations of cytotoxic drugs in the absence or presence of concentrations of chemosensitizers. Dose-response curves were corrected for the inhibition of cell growth caused by chemosensitizers alone, and the "fold reversal" of MDR for 5-FU plus chemosensitizer combination was calculated as follows:

$$\text{Fold reversal} = \frac{\text{IC}_{50} \text{ drug alone}}{\text{IC}_{50} \text{ drug +chemosensitizer}}$$

Western blot analysis of wild-type p53 expression

Cells infected with the indicated adenovirus and MOI were grown to about 80% confluence in 10-cm plates. After washed with PBS, cells were scraped from the plate, washed once with ice-cold PBS containing 1 mmol/L phenylmethylsulfonyl fluoride, and centrifuged at 1 000 r/min for 4 min. The cell pellet was resuspended in ice-cold radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, and 1 g/L SDS, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin and 1 μ g/mL pepstatin A)

and sonicated. After centrifugation at 12 000 g at 4 °C for 20 min, the resulting supernatant was harvested as the total cell lysate. The protein was aliquoted and stored at -70 °C until use. Protein samples were electrophoresed through a 125 g/L polyacrylamide gel containing 1 g/L SDS and the gel was run at 30 mA for 3 h. Separated proteins were transferred onto nitrocellulose paper by electroblotting. After blocked with 50 mL/L blocking reagent, the nitrocellulose membrane was probed with a monoclonal antibody against human wild-type p53 from Oncogene Science. Detection was accomplished using ECL western blotting detection reagents from Amersham Pharmacia Corp.

Determination of MDR1 mRNA expression by RT-PCR

Expression of the MDR1 gene was determined by measuring the mRNA level from total RNA by SUPERSRIPT™ first-strand synthesis system (GIBCO BRL, Gaithersburg, MD), using MDR1-specific primer pairs. Briefly, total RNA was extracted from established lines with TRIzol reagent (Life Technologies, Inc.). Equal amounts of RNA, as determined by absorbance at 260 nm, were denatured and size was fractionated by electrophoresis through a 12 g/L agarose gel containing formaldehyde and 2 μ g/dL ethidium bromide (Figure 1). Equal amounts (1 μ g) of total RNA from each tissue were treated with RNase-free DNase I to remove any contaminated DNA. DNase-treated RNA was then used to synthesize single-strand cDNAs by AMV reverse transcriptase in the presence of random primers, followed by PCR amplification. The primers used for PCR reaction were: MDR1-1, GTA CCC ATC ATT GCA ATA GC and MDR1-2, CAA ACT TCT GCT CCT GAG TC. These two MDR1 primers bracketed the COOH-terminal region of the MDR1 cDNA and gave a PCR product of 147 bp. PCR amplification was performed by pre-denaturing at 95 °C for 3 min, and then running 30 cycles in the following conditions: denaturation at 94 °C for 40 s, annealing for 40 s at 55 °C, and extension at 72 °C for 50 s. As internal control, RT-PCR for β -actin was carried out using 5' CGT GGG CCG CCC TAG GCA CCA 3' (forward primer) and 5' TTG GCC TTA GGG TTC AGG GGG 3' (reverse primer) in the same conditions as described for MDR1, and showed a PCR product of 243 bp. The PCR products were electrophoresed in a 20 g/L agarose gel with 0.5 μ g/mL ethidium bromide and visualized under UV light. Quantitation of relative band volume counts was performed using Molecular analyst for windows software. To estimate MDR1 expression levels, a MDR1 index was designated as a band volume count ratio of MDR1 to constitutively expressed β -actin, because β -actin mRNA was expressed constitutively in cells. The higher the MDR1 index, the higher the expression level of MDR1 mRNA in cells.

RESULTS

Sensitivity of LoVo and LoVo/5-FU to 5-fluorouracil

The sensitivity of LoVo and LoVo/5-FU cells to 5-fluorouracil was evaluated using the MTT assay as shown in Table 1. Previous studies utilizing MTT assays have shown that the LoVo/5-FU cell line is 8.988-fold more resistant to 5-fluorouracil than parental LoVo cells. We found the two cell lines showed differences in sensitivity to 5-fluorouracil, too. The concentration inducing 50% inhibition of cell proliferation for 5-Fluorouracil was 0.82 μ g/mL for the parental line and 7.37 μ g/mL for the LoVo/5-FU cells.

Effect of wtp53 gene on cell growth in LoVo/5-FU cells

Figure 1 showed the effect of wild-type p53 on the LoVo/5-FU cells. It suggested that the dose of Ad-p53 for experiment didn't affect the growth rate of LoVo/5-FU cells during the six days after transfection.

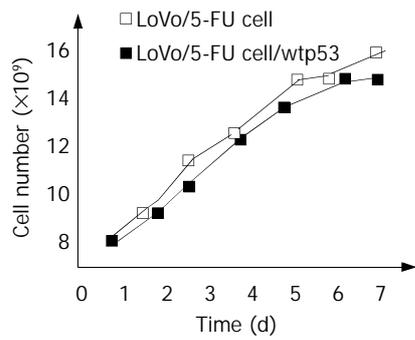


Figure 1 Effect of wild-type p53 on the growth rate of LoVo/5-FU cells.

Effect of wild-type p53 on LoVo/5-FU cells sensitivity to 5-fluorouracil

The effect of wild-type p53 on 5-fluorouracil sensitivity was examined using the MTT assay. A dose of 8.17×10^{10} pfu/mL Ad-p53 was chosen as the dose to modulate 5-fluorouracil sensitivity. For this study, cells were treated with wild-type p53 for 48 h, 5-fluorouracil was then added to the final concentration of 0.25 to 15 $\mu\text{g/mL}$ for 3 h, and then the medium was removed and replaced with a fresh medium without drugs and the incubation was continued. Cell growth was determined after an addition for 72 h. Treatment with wild-type p53 appeared to decrease the resistance of LoVo/5-FU cells to 5-fluorouracil (Table 1).

Table 1 Effect of wild-type p53 on LoVo/5-FU sensitivity to 5-fluorouracil

Cell lines	IC ₅₀ ($\mu\text{g/mL}$)	Resistant time	Reverse time
LoVo cells	0.82		
LoVo/5-FU cells	7.37	8.988	
LoVo/5-FU cells+wild-type p53	1.48 ^b	1.804	4.982

^b $P < 0.01$ compared with LoVo/5-FU cell control group.

Expression of wild-type p53 protein in cell lines

The X-gal staining suggested that the dilution of 1:20 for Ad-p53 could make about more than 80% cells infected successfully. LoVo/5-FU was transduced *in vitro* with the human wild-type p53 cDNA by exposing to Ad-p53 (1:20). To make sure that the constructed p53 expression vector, Ad-p53, efficiently expressed functional wild-type p53, we determined its protein expression and transactivating function. Western blot analysis showed a higher level of wild-type p53 protein expression as early as 48 h after infection with Ad-p53, and which lasted for 5 d, then decreased significantly on the sixth day. But no wild-type p53 was detected in parental (uninfected) cells or control cells infected with Ad-LacZ (data not shown), suggesting that the transfer and expression of p53 by Ad-p53 were highly efficient (Figure 2).

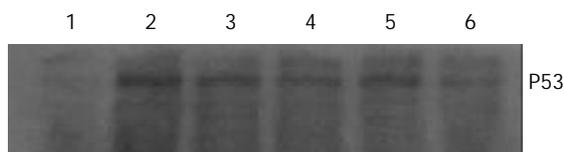


Figure 2 Western blot analysis of wild-type p53 in LoVo/5-FU cell line and LoVo/5-FU cells transiently transfected with p53 expression vector (Ad-p53). Lane 1: LoVo/5-FU cell line. Lanes 2, 3, 4, 5, 6: LoVo/5-FU cells transiently transfected with Ad-p53 for 2, 3, 4, 5, 6 d accordingly. Levels of wild-

type p53 protein (lanes 2-6) were determined by Western-blot analysis with an antibody which could detect wild-type forms of p53 protein.

Expression of wild-type p53 suppressed endogenous MDR1 gene expression

We asked whether wild-type p53 expression could indeed inhibit endogenous MDR1 gene expression in LoVo/5-FU cells. Successful generation of cell lines constantly expressing wild-type p53 was tested in human LoVo/5-FU cells transfected with Ad-p53 as above, and these cells constantly maintained wild-type p53 for about 6 d and provided a valuable reagent for studying drug sensitivity. As seen in Figure 2, vector-transfected LoVo/5-FU cells expressing wild-type p53, had a lower MDR1 mRNA expression than LoVo/5-FU cells without wild-type p53 expression, as determined by RT-PCR. There was a decrease in MDR1 transcripts compared with the empty vector-transfected cell line, suggesting that constitutive expression of p53 could inhibit endogenous MDR1 transcription in LoVo/5-FU cells, and this suppression showed a time-dependent mode (Figure 3, 4 and Tables 2, 3).

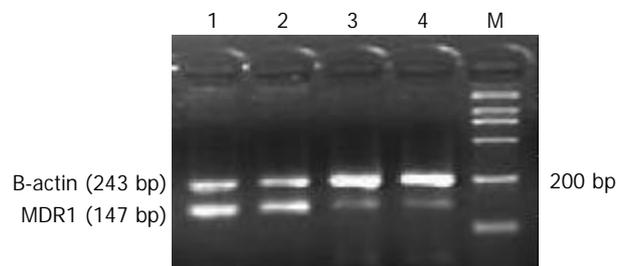


Figure 3 Results of RT-PCR for expression of MDR1 mRNAs in p53-null and wild-type p53 expressing LoVo/5-FU cells. M: marker; Lanes 1 and 2: results of untransfected LoVo/5-FU cells; Lanes 3 and 4: results of transfected LoVo/5-FU cells.

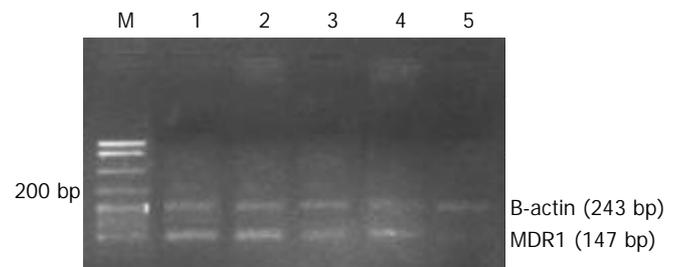


Figure 4 Results of RT-PCR for expression of MDR1 mRNAs of transfected LoVo/5-FU cells at different time. M: marker; Lanes 1, 2, 3, 4, 5: the cells transfected with Ad-p53 for 2, 3, 4, 5, 6 d accordingly.

Table 2 MDR1 expression in different cell lines

	Lane 1	Lane 2	Lane 3	Lane 4
MDR1/ β -actin	1.26	1.19	0.42	0.33

Table 3 MDR1 expression of LoVo/5-FU cells on different day after dealing with Ad-p53

Time/d	2	3	4	5	6
MDR1/ β -actin	1.22	1.24	1.06	0.86	0.42

DISCUSSION

Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology; patients with colorectal

cancer are generally unresponsive to chemotherapy. The goal of our current cancer research was to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interactions between the gene products and chemotherapeutic drugs.

Resistance to cytotoxic agents is the major cause of failure of medical treatment of human cancer and it is known that tumor cells could become resistant to anticancer drugs by a variety of mechanisms^[1]. In numerous *in vitro* models, this resistance has been shown to be mediated by MDR1 gene, whose product P-gp is thought to be an energy-dependent membrane pump involved in the excretion of toxins in normal cells. Elevated expression of P-gp in malignant cells could result in increased efflux and therefore reduced intracellular accumulation of cytotoxic agents. This decrease has been considered as the basic mechanism of the MDR phenomenon^[2-7].

LoVo/5-FU cells were selected from the LoVo parental cell line through culture in the presence of 5-fluorouracil and were resistant to 5-FU which was toxic to LoVo parental cells. LoVo/5-FU cells had cross-resistance to the drugs comprising the classical MDR phenotype^[15]. In this study, we found the resistance subline overexpressed MDR1 mRNA and no wild-type p53 protein was expressed.

An adenovirus system has potential advantages for gene delivery *in vivo*, such as easy production of high titer virus, high infection efficiency, and infectivity for many types of cells^[16-18]. The stability and duration of the expression of introduced gene are still controversial. For chemo-gene therapy, the expression levels and high infectivity may be more significant than the duration of expression because drugs can kill infected cells within several days. In our model, the expression of wild-type p53 gene was driven independently by the cytomegalovirus promoter contained in an Ad-p53 vector. The expression of wild-type p53 protein by Ad-p53 peaked after 48 h and decreased to a low level by the sixth day. This suggests that a transiently high level of wild-type p53 expression is sufficient to initiate the cytotoxic program in cancer cells.

We found that pretreatment of LoVo/5-FU cells with Ad-p53 was capable of restoring cell sensitivity to 5-fluorouracil. In the parental cell line, however, Ad-p53 did not potentiate 5-fluorouracil toxicity. The functional reversal of MDR by Ad-p53 in LoVo/5-FU cells appeared to be mediated by changes of the MDR1 gene expression.

Several agents have been described to affect MDR1 gene expression or the MDR phenotype in human or rodent cells, such as the Ca²⁺ channel blocker, verapamil, calmodulin inhibitor, trifluoperazine, which have been shown to reverse the MDR phenotype *in vitro* through direct competition with drugs for P-gp binding in the absence of changes in P-gp expression. Treatment with these agents could result in increased intracellular concentrations of cytotoxic drugs, which are thus a final common pathway in the reversal of the multidrug-resistant phenotype. They are presently used in clinical trials to patients with drug refractory tumors. However, they could induce significant cardiotoxicity, thus which limiting their clinical usefulness^[19,20]. The Ad-p53 concentrations reported here are active *in vivo* in the absence of significant toxicity.

Wild-type p53, a M_r 53 000 nuclear phosphoprotein involved in the control of cell growth and apoptosis is the most commonly altered gene in human tumors^[21]. Its role as a tumor suppressor has been well documented as its inactivation was strongly correlated with human cancer^[21-23]. p53 protein had different domains for DNA binding, transactivation, and tetramerization functions^[24,25]. After binding to consensus for DNA sequences, p53 could positively regulate the expression of downstream effector genes, including Gadd45, p21(WAF1/CIP1)^[26], and mouse muscle creatin kinase^[27]. In addition,

wild-type p53 could also negatively regulate a variety of genes that lack a p53 consensus binding site, including DNA topoisomerase II^[28], MDR1^[29-32], c-fos^[33], and other viral and cellular promoters. It has been suggested that transcriptional repression by p53 could result from its direct interactions with transcription factors, such as TATA-binding protein^[34,35], Sp1^[36,37] and CCAAT-binding factor^[28]. Taken together, these observations strongly imply that p53 acts directly with the transcription machinery to modulate MDR1 transcription.

Many tumors have no functional p53, and others express high levels of MDR1. Whether there is a direct connection has remained to be determined^[38]. However, our results suggested that restoration of wild-type p53 in tumor cells could overcome the uncontrolled up-regulation of MDR1 gene expression, and could add to the beneficial effect of wild-type p53 gene therapy that would restore cell growth inhibition and apoptosis pathway to facilitate drug-induced cell killing.

A variety of treatment protocols, including surgery, chemotherapy, and radiotherapy, have been tried for human colorectal cancer, but the long-term survival rate remains unsatisfactory. The combination therapy we present here might be effective as an adjuvant treatment to prevent local recurrence following primary tumor resection or as a treatment that could be given by intralesional injections in drug-resistant primary, metastatic, or locally recurrent colorectal cancer. Protocols are being developed to explore these clinical applications.

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