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ADENOVIRUS-MEDIATED WILD-TYPE P53 EXPRESSION SUPPRESSES GROWTH OF LUNG ADENO-CARCINOMA CELLS*

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Objective: To study the growth suppression of lung adenocarcinoma cell by the introduction of wild-type P53 gene and explore a gene therapy approach for lung adenocarcinoma. Methods: A replication-deficient adenovirus vector encoding a wild-type P53 was constructed and transfected into the cultured human lung adenocarcinoma cell line GLC-82. The efficiency of gene transfection and expression was detected by immunochemical staining and polymerase chain reaction. The cell growth rate and cell cycle were analysed by cell-counting and flow cytometry. Results: Wild-type P53 gene could be quickly and effectively transfected into the cells by adenovirus vector. Wild-type P53 expression could inhibit GLC-82 cell proliferation and induce apoptosis. Conclusion: The results indicated that recombinant adenovirus expressing wild-type P53 might be useful vector for gene therapy of human lung adenocarcinoma.

Key words: Adenovirus vector, P53 gene, GLC-82 cell, Gene therapy.

Wild-type P53 negatively regulates the growth of tumor cells, and is therefore considered a tumor suppressor gene.[1] Mutations of P53 gene are reported in 50–60% of human cancers.[2] It has now been well documented that replacement of the wild-type P53 gene into P53-deficient tumor cells from widely disparate types of human cancers, such as lung cancer, stomach carcinoma, and bladder carcinoma, could inhibit cellular proliferation and lead to the reversal of tumorigenicity.[3–5] In this study, a replication-deficient adenovirus vector encoding a wild-type P53 was constructed and transfected into the cultured human lung adenocarcinoma cell line GLC-82. We have tested the ability of recombinant adenovirus to suppress tumor growth and explored a gene therapy approach for cancer.

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MATERIALS AND METHODS

Cell Line

Human lung adenocarcinoma cell line GLC-82 was generously provided by Cancer Hospital, Chinese Academy of Medical Sciences, and was maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS).

Construction of Recombinant Wild-type P53 Adenovirus Vector

The human wild-type P53 cDNA fragment encoding the full-length P53 was first inserted into the plasmid vector pRC/CMV (Invitrogen). The P53 minigene cassette from the pRC/CMV plasmid vector, which contains the human cytomegalovirus promotor

(CMV), the P53 cDNA fragment, and the polyadenylation site of the rabbit β-globin gene, was then recovered and transferred into the shuttle plasmid pXCJL-1. The resultant recombinant shuttle plasmid pXCJLP53 was further cotransfected with the PJM17 plasmid into 293 cells using the lipofectin reagent (Life Technologies, Inc). Recombinant viruses were isolated by screening adenovirus plaques from 293 cell monolayers, and further characterized by restriction enzyme digestion mapping. The recombinant adenovirus containing wild-type P53 expression cassette was designated AdCMVP53. High-titer stock of AdCMVP53 virus was prepared by CsC1 ultracentrifugation. AdCMVP53 virus contained 10^{12} viral particles/ml as measured by absorbance at 260 nm (1A260=1×10^{12} viral particles/ml). The replication-deficient recombinant adenovirus expressing the β-gal (AdCMV β-gal), which was used as a control in the experiments, was generated by the same methods.

Determination for Gene Expression Efficiency of Recombinant Adenovirus

The GLC-82 (1×10^5) were seeded on coverslips in six-well culture plates and cultured for 24 h. After infection for 72 h with AdCMV β-gal at various dilutions, the cells were fixed with acetone and formaldenyde. The expression of β-gal in AdCMV β-gal virus-transduced cells was detected by staining the cells with x-gal for 4–12h at room temperature. The efficiency of gene transfection was quantitated by counting the percentage of blue cells.[6]

Analysis of P53 Protein in GLC-82 Cells

Immunochemical staining for detection of P53 protein in cells was performed using the anti-P53 monoclonal antibody (Santa Cruz).

Examination of Wild-type P53 cDNA by PCR

Genomic DNA was prepared from infected and noninfected GLC-82 cells by using standard proteinase K digestion and phenol extraction conditions. Oligonucleotide primers (sense: 5'CTA ACC GCG GTC CCT TCC CAG AAA ACC TAC3'; antisense: 5'TAC AGT CAG AGC CAA CCT CAG GCG3') were synthesized. A PCR was performed using 100ng of genomic DNA for 30 cycles of denaturation (95ºC, 1 min), annealing (50ºC, 30 sec), and polymerization (72ºC, 1 min). PCR products were analyzed by ethidium bromide staining on a 1.5% agarose gel.

Effect of wild-type P53 gene on the growth of GLC-82 Cells

GLC-82 cells (0.6×10^5 cells/well) were plated in 24-well plates and allowed to adhere 24 h at 37ºC. The cells were then infected with either AdCMVP53 or AdCMV β-gal for an additional 24, 48, 72, 96, and 120 h of incubation at 37ºC. The cell numbers were counted each day for 5 days.

Flow Cytometric Analysis

Single cell suspensions collected at each time point were fixed with 70% ethanol. After incubation in 50 μg/ml RNase for 30 min at 37ºC, the cells were suspended in 50 μg/ml propidium iodide for 30 min at 4ºC and then subjected to flow cytometry.[7]

RESULTS

AdCMV β-gal Infection *in Vitro*

To determine the efficiency of adenovirus infection, GLC-82 cells were infected with AdCMV β-gal at different concentration from 0 to 10^9 pfu/ml. After infection, the cells were stained for β-galactosidase activity. The result showed that infection of GLC-82 with AdCMV β-gal at the concentration of 10^8 pfu/ml for 4 h leads to the transduction of 100% of GLC-82.

Expression of P53 in AdCMVP53-infected Cells

The expression of P53 in AdCMVP53-infected and uninfected GLC-82 was confirmed by immunochemical staining. As expected, AdCMVP53-infected GLC-82 displayed high levels of expression of P53 (Figure 1), while P53 expression was not found in uninfected GLC-82, suggesting that wild-type P53 gene can be introduced and express efficiently in cultured GLC-82 cells.

Detection of Wild-type P53 cDNA

Successful gene transfer into cultured GLC-82 was analysed by PCR using oligometric primers specific

for human P53 cDNA. As shown by agarose gel electrophoresis (Figure 2), P53 cDNA present in cells infected with AdCMVP53. In contrast, a product of PCR was not obtained in uninfected and AdCMV β-gal-infected GLC-82.

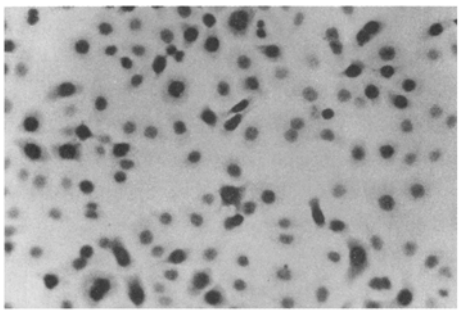

Fig. 1. Immunochemical staining for P53 protein expression in AdCMVP53-infected GLC-82

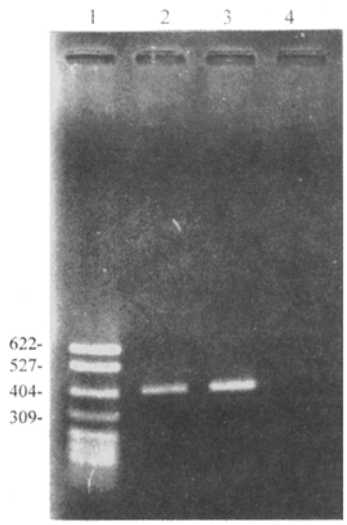

Fig. 2. Detection of PCR products of P53 cDNA by 1.5% agarose gel electrophoresis

Lane 1: PBR322/MspI marker

Lane 2: positive control (pRC/CMVP53)

Lane 3: PCR products of infected cells

Lane 4: PCR products of uninfected cells

Growth Suppression of GLC-82 Cells by the Introduction of Wild-type P53 Gene

To test further the activity of the P53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of GLC-82. As shown in Figure 3, the uninfected and AdCMV β-gal-infected cells proliferated rapidly during the first 96 h. In contrast, infection with AdCMVP53 resulted in a 94.1% reduction in GLC-82 growth at 96 h. The result revealed that introduction of wild-type P53 gene via a recombinant adenovirus vector suppresses growth of GLC-82 cells.

Analysis of Cells Apoptosis by Flow cytometry

The apoptotic cells were determined by flow cytometry. As shown in Figure 4, 48 h or 72 h after infection, overexpression of wild-type P53 induced apoptosis of cells.

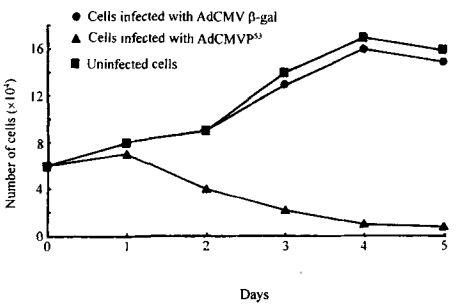

Fig. 3. Effect of P53 gene on the growth of GLC-82 cells

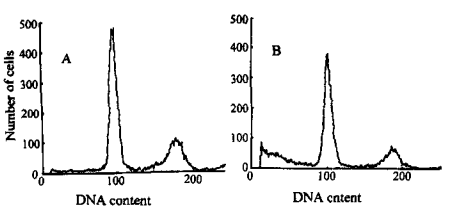

Fig. 4. Analysis of cells apoptosis by flow cytometry

A: uninfected GLC-82

B: AdCMVP53-infected GLC-82

DISCUSSION

P53 gene, a DNA of 20 kb which transcribes a full-length mRNA of 2.8 kb and encodes a nuclear protein containing 393 amino acids, was located in short arm of chromosome 17. Recent studies have shown that

wild-type P53 functions a tumor suppressor. However, mutated P53 gene is considered an oncogene. Mutation of P3 gene is the most common genetic alteration in human cancers. Loss of wild-type P53 function is associated with the uncontrolled growth of many types of human cancers. In its proposed role as a "guardian of the genome", the P53 gene product acts as a transcriptional activator of other genes which inhibit cell cycle progression from G1 to S phase in normal cells.[8] Wild-type P53 protein has been implicated as a molecule of genomic stability to prevent mutation or deletion of functional genes because of its involvement in DNA damage-induced G1 cell cycle arrest, apoptosis, and gene amplification.[9] Its levels rise and accumulate in response to DNA damage, leading to G1 arrest and repair, terminal differentiation. If overexpression of wild-type P53 or too much DNA damage, cell apoptosis is induced by P53.

Of note, introduction of wild-type P53 gene into P53-deficient tumor cells could suppress growth and result in the reversal of tumorigenicity, indicating the potential application of wild-type P53 in cancer gene therapy. In this study, we constructed replication-deficient adenovirus vectors encoding either a wild-type P53, AdCMVP53, or a β-galactosidase, AdCMV β-gal. The lung adenocarcinoma cell line GLC-82 was infected with AdCMV β-gal and expressed very-high levels of β-gal after 4 h, suggesting the high transfer efficiency of adenovirus vector. Furthermore, the expression of wild-type P53 in AdCMVP53-infected and uninfected GLC-82 confirmed by immunochemical staining and PCR. High levels of P53 protein and P53 cDNA were observed 24 h after infection in AdCMPVP53-infected GLC-82, while no P53 cDNA and P53 protein were detected in uninfected cells. Importantly, most AdCMVP53-infected cells were arrested in G0 or G1 phase of the cell cycle 48 h after infection as assayed by flow cytometry. As expected, apoptosic cells were found in AdCMVP53-infected cells 72 h after infection. Taken together, these results revealed that wild-type P53 mediated by adenovirus vector could suppress growth of tumor cells by regulation of cell cycle progression and induction of apoptosis, and might be used in the therapy of lung adenocarcinoma.

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