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Introduction of wild-type *p53* gene downregulates the expression of H-*ras* gene and suppresses the growth of bladder cancer cells

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Abstract Retroviral vectors were used to introduce the wild-type *p53* gene into human bladder cancer cell lines BIU-87 and EJ, which express endogenous *wt-p53* gene and have a mutation in H-*ras* gene. The expression of the exogenous *wt-p53* gene in cells suppresses the growth of the bladder cancer cells in standard culture and in soft agar and blocks the cell cycle progression in G1. The BIU-87 and EJ cells developed tumors with average volumes of 6.53 cm^3 and 6.61 cm^3 in nude mice in 9 weeks after inoculation, while the cells transduced with *wt-p53* gene failed to form tumors. The expression of H-*ras* gene in bladder cancer cells was reduced at mRNA level. These results suggest that the overexpression of the *wt-p53* gene suppresses the expression of mutant H-*ras* gene and inhibits the tumor cell growth in vivo and in vitro.

Key words Bladder cancer · Cell line · Anti-oncogene · Gene therapy · Suppression

Mutations in the *p53* gene have been associated with a wide range of human tumors. Loss of *wt-p53* function plays a role in the initiation and progression of human malignant tumors, including human bladder cancer in particular [1, 10, 12, 14]. Studies have shown that the incidence of *p53* gene mutations appears to be much higher in invasive-type and high-grade bladder cancers than in superficial and low-grade ones [6, 7]. Patients with bladder cancers containing mutant *p53* have a significantly poorer prognosis. Introduction of *wt-p53* gene into various cancer cell lines containing *p53* gene alterations, such as lung cancers, gastric cancers, colon cancers and osteosarcoma, suppresses their malignant proliferation and tumorigenicity. More than 50% of human cancers have no alteration in the *p53* gene. It is not definitely known whether the expression of exogenous *wt-p53* suppresses the growth of tumor cells, which express endogenous *wt-p53*.

In this study, we introduced cDNA coding for *wt-p53* into human bladder cancer cells, which express endogenous *wt-p53* and have mutations in H-*ras* gene. We analyzed the growth and tumorigenicity of the bladder cancer cells transfected with *wt-p53* gene, and also determined whether the overexpression of *wt-p53* gene in tumor cells regulates the expression of mutant H-*ras* gene.

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Materials and methods

Cells and culture condition

Human bladder cancer cell line BIU-87 was established at the Institute of Urology, Beijing Medical University, and cell line EJ was kindly supplied by Dr. Diener at Alberta University. Both cell lines have a point mutation in H-*ras* gene. Cells were routinely propagated in monolayer culture in RPMI 1640 (Sigma, St. Louis, Mo., USA) containing 10% newborn calf serum at 37 °C. The amphotropic packaging cell line PA317 was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS).

Retroviral vector design and infection of tumor cells

A 1.8-kilobase long *wt-p53* cDNA segment was obtained from the plasmid pc53-SN3 by digestion with restriction enzyme *Bam*HI. The segment was amplified by polymerase chain reaction (PCR) to develop a 1.2-kilobase *Eco*RI and *Sal*I fragment and then inserted into the restriction site *Eco*RI/*Sal*I present upstream from the initiation codon of the neo^R gene of the modified N2 retroviral vector pDOR-neo^R, to generate vector construct pDOR-p53 (Fig. 1). The construct pDOR-p53 and the vector pDOR-neo^R were introduced into the virus packaging cell line PA317 without or with lipofectin,

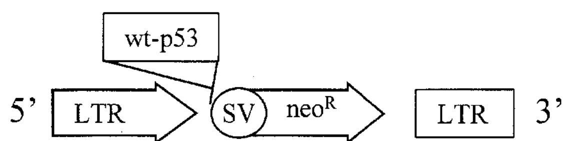

Fig. 1 Construction of the retroviral vector pDOR-p53

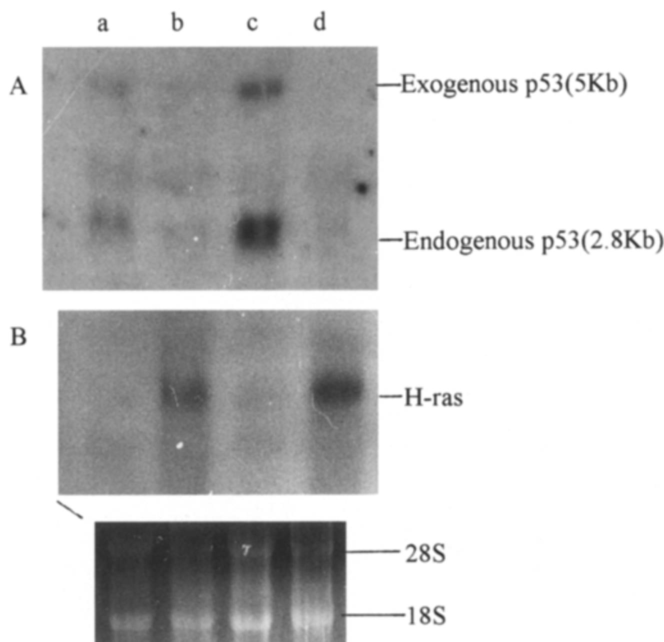

Fig. 2A, B Northern hybridization. The total RNA was isolated and electrophoresed as described in "Materials and methods." The A blot was hybridized with *p53* probe, the B blot with H-*ras* probe. *a* BIU87-pDOR-p53, *b* BIU-87, *c* EJ-pDOR-p53, *d* EJ

respectively. Clones were isolated by G418 selection and expanded to cell lines. The cell free supernatants were tested for the presence of virus using NIH3T3 cells. Supernatants of the cell lines secreting high titer of virus ($1 \times 10^{5 \sim 6}$ CFU/ml) were used to infect the BIU-87 and EJ cells. For transfection, 5×10^5 tumor cells were seeded into 10-cm culture dishes 24 h before the transfection. The viral supernatant containing Polybrene 8 μg/ml was then added to the cells. After 12 h, the viral supernatant was removed and the cells were grown in RPMI 1640 medium with 20% FCS for an additional 3 days to allow for integration and expression of the transfected DNA. Geneticin-resistance clones were selected in the medium containing an optimal concentration of G418 (400 μg/ml for the EJ cell line, 800 μg/ml for the BIU-87 cell line) for 14–16 days.

Northern blot analysis

Total RNA was extracted by a guanidinium isothiocyanale method [4]. Thirty micrograms per lane of RNA was electrophoresed in 1% agarose/2.2 M formaldehyde gel and transferred to nylon membranes (Bio-Rad). Fifty to 100 ng *p53* cDNA probe or H-*ras* cDNA probe was labeled with [α-^{32}P]dCTP (Amersham Co.) by random oligo-primer extension as described [8]. The nylon membranes were UV cross-linked, hybridized, washed and then exposed to Kodak XAR-5 X-ray film at -70 °C for 3–10 days.

Cell growth curve and growth in soft agar

The cells were seeded into 24-well plates with 3×10^5 cells in each well and the cells from the replicate well were counted every day for 6 days. For the studies in soft agar, cells were seeded at 1000 cells/dish into 30-mm dishes with 3% LMP agarose containing 10% FCS. The clones were counted after 14 days and the results were calculated as the average of three dishes per cell line.

Flow cytometric (FCM) analysis

The cells were harvested, washed and incubated in the medium containing RNase 200 μ/ml at 37 °C for 30 min, and then adjusted to 1×10^6 cells/ml in phosphate-buffered saline (PBS) and stained with propidium iodide. The cells were analyzed by FACScan (Becton Dickinson, Mountain View, Calif., USA).

Tumorigenicity test

The cells were harvested, washed and 5×10^6 cells injected s.c. in 0.3 ml PBS into the right flank of BALB/c nude mice. Tumors were measured every week for 9 weeks and excised at various times for further analysis.

Results

Expression of *wt-p53* gene and H-*ras* gene in bladder cancer cells

The retroviral vectors pDOR-p53 and the control vectors pDOR-neo^R were introduced into the bladder cancer cell lines BIU-87 and EJ, respectively. The G418-resistant clones were selected and expanded to cell lines for further analysis. Northern hybridization with *wt-p53* probe showed the successful gene insertion and transcription (Fig. 2A), and H-*ras* probe showed the suppressed expression of H-ras mRNA in the BIU87-pDOR-p53 and EJ-pDOR-p53 cells (Fig. 2B), in comparison with their parental cell lines.

Inhibition of tumor cell growth in vitro

To study whether the expression of exogenous *wt-p53* in cells would affect the growth of the bladder cancer cells in vitro, cell proliferation was measured under standard culture conditions and in soft agar. As shown in Fig. 3, the growth of the BIU87-pDOR-p53 and EJ-pDOR-p53 cell lines in standard culture and in soft agar was significantly suppressed, in comparison with their parental cells.

Cell cycle analysis by FCM

The distribution of cells within the cell cycle can be determined by their DNA content through measurement of fluorescence emission after propidium iodide

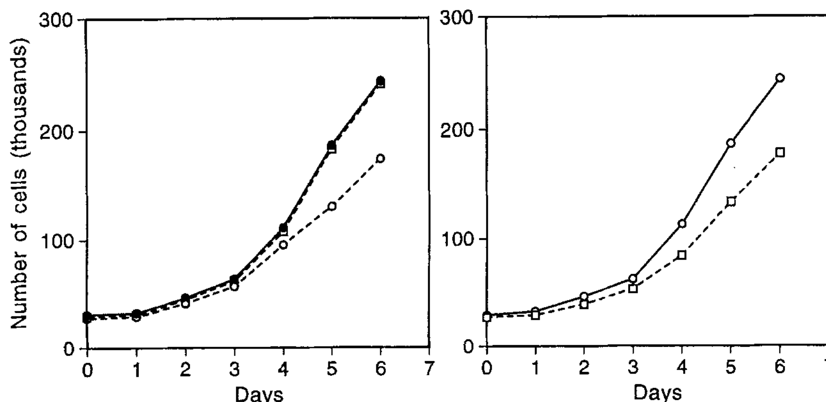

Fig. 3 Cell growth curve. The growth of BIU87-pDOR-p53 and EJ-pDOR-p53 was significantly suppressed ($P < 0.01$). *Left*: -●- EJ; --□-- EJ-pDOR-neo, -○- EJ-pDOR-p53; *right*: -○- BIU87; --□-- BIU-pDOR-p53

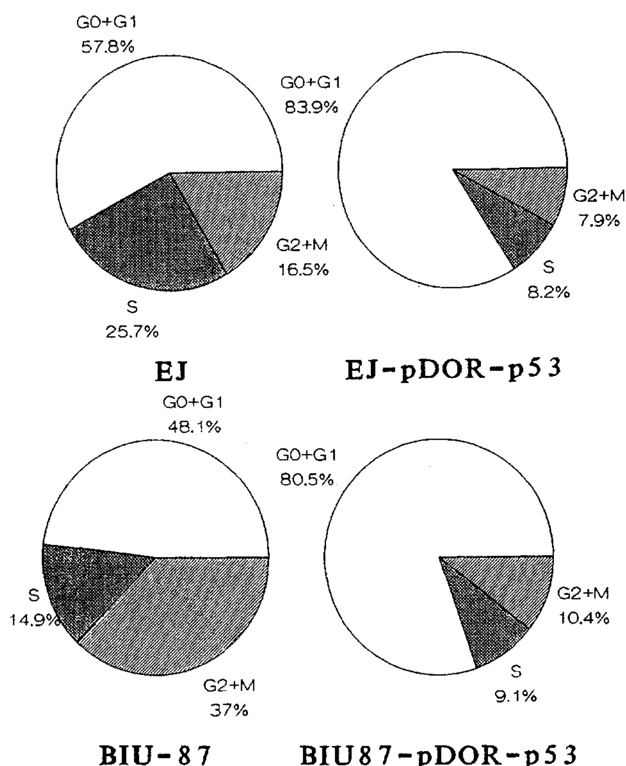

Fig. 4 Cell cycle analysis. The proportion of BIU87-pDOR-p53 cells and EJ-pDOR-p53 cells in G0/G1 phases is increased, while that in S phase and G2/M phases is significantly decreased, in comparison with BIU87 and EJ cells

staining. There is a doubling of the DNA content in the progression from G1 phase to G2/M phase, when DNA synthesis occurs during S phase. The BIU87-pDOR-p53, EJ-pDOR-p53 cell lines and their parental cell lines were measured by FCM for cell cycle analysis. As shown in Fig. 4, the proportions of BIU87-pDOR-p53 and EJ-pDOR-p53 cells in G0/G1 phase were significantly increased, while the proportions of that in S phase and G2/M phase were reduced. This suggests that overexpression of *wt-p53* blocks progression through the cell cycle in G1 prior to S phase.

Suppression of tumor formation in BALB/c nude mice

Five $\times 10^6$ cells were injected into BALB/c nude mice. The BIU87-pDOR-p53 and EJ-pDOR-p53 cells failed to develop a tumor up to 9 weeks. The control cells BIU-87, EJ, and EJ-pDOR-neo^R progressively formed tumors; at 9 weeks, tumor volumes averaged 6.53 cm^3, 6.61 cm^3 and 6.10 cm^3, respectively. The histopathology of the tumors growing in nude mice corresponded to that in BIU-87 and EJ cells.

Discussion

A number of studies have shown frequent alterations in *p53* gene in bladder cancer. Investigations to the introduction of the *wt-p53* gene into various tumor cells, such as human lung cancer, gastric cancer, colon carcinoma and leukemia cells containing altered *p53* gene, have shown it to suppress their growth and tumorigenicity [2, 11, 13, 15, 16]. These studies give us an understanding of the function of the *p53* gene and suggest the future use of human tumor therapy with the *wt-p53* gene.

In this study, we introduced the *wt-p53* gene into human bladder cancer cell lines BIU-87 and EJ, which have no alteration in *p53* gene determined by PCR-sequencing and have a point mutation in the H-*ras* gene. The expression of exogenous *wt-p53* in cells suppressed their growth in vitro. The BIU87-pDOR-p53 and EJ-pDOR-p53 cells lost the ability to form clones in semi-solid medium and showed complete inhibition of their tumorigenicity in nude mice. Thus far, most of the experiments with transfection of *wt-p53* gene were performed on tumor cells containing mutation or inactivation in *p53* gene. The effect of exogenous *wt-p53* in bladder cancer cells, which express endogenous *wt-p53*, remained unclear. Diller et al. [5] transfected the *wt-p53* gene into nine osteosarcoma cell lines (eight with altered *p53* gene and one with normal expression of *p53* gene). Their study revealed a similar growth sup-

pression upon the nine cell lines. The present study suggests that increasing the expressive dosage of *wt-p53* gene in bladder cancer cells reduces the ability of the proliferation of tumor cells and blocks cell cycle progression in G1.

We next examined whether the introduction of *wt-p53* gene into human bladder cancer cells affects the expression of H-*ras* gene. Both the BIU-87 and EJ cell lines contain a point mutation in the H-*ras* gene and express a high level of H-*ras* mRNA. It is known that the mutant H-*ras* gene increases genetic instability and affects cell differentiation [3,9]. The BIU87-pDOR-p53 and EJ-pDOR-p53 cells express a lower level of H-*ras* mRNA than their parental BIU-87 and EJ cells. This result indicates that the overexpression of *wt-p53* gene in cells suppresses the expression of mutant H-*ras* gene at transcriptional level.

In conclusion, to increase the *wt-p53* dosage in cells appears to increase its growth-suppressive activity in bladder cancer cells. The overexpression of *wt-p53* gene directly blocks the cell cycle progression in G1 phase, downregulates the expression of mutant H-*ras* gene and suppresses the growth of bladder cancer cells. These results suggest that the effects of *wt-p53* occur not only in cells with inactivation of *p53* gene, but also in cells expressing *wt-p53* gene and containing certain other oncogene lesions.

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