·Basic Research·

In vitro and in vivo inhibitory effect of Ad-ING4 gene on proliferation of human prostate cancer PC-3 cells

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[Abstract] Background and Objective: Adenovirus vector has been widely used in tumor gene therapy. ING4 is a member of growth inhibiting factors and a potent anti-tumor gene which could induce apoptosis of many tumor cells. This study was to investigate the inhibitory effects of adenovirus-mediated ING4 (Ad-ING4) gene on the proliferation of human prostate cancer PC-3 cells in vitro and in vivo, and to explore its mechanisms. **Methods:** Ad-ING4 was obtained by virus-amplification technique. After transfection of purified Ad-ING4 into PC-3 cells, the expression of ING4 was detected by reverse transcription-polymerase chain reaction (RT-PCR); the influence of Ad-ING4 transfection on cell proliferation was evaluated using MTT assay. Cell apoptosis was assessed using Hoechst33258 staining and flow cytometry. RT-PCR was performed to detect the mRNA levels of the transcription of apoptosis-related genes such as Bcl-2, Bax, p53, and Caspase-3. Athymic nude mice bearing PC-3 tumors were intratumorally injected with Ad-ING4 (100 μL, 1×10^9 pfu/mL). Tumor growth was recorded. All nude mice were killed at the end of the experiment to observe the growth of xenografts. The expressions of Bcl-2, Bax, Caspase-3, and CD34 proteins in tumor tissues were detected by immunohistochemistry. **Results:** Human ING4 gene was successfully transcribed in PC-3 cells and induced apoptosis by up-regulating p53, Bax, Caspase-3 expression and down-regulating Bcl-2 expression. Inhibition of cell proliferation was significant in PC-3 cells. Tumor growth was significantly inhibited in the Ad-ING4 group as compared with that in the Ad-GFP group and the PBS group ($P<0.05$). The weight inhibitory rate was 37.0% in the Ad-ING4 group. The expressions of Bax and Caspase-3 were up-regulated, and the expressions of Bcl-2 and CD34 were down-regulated in the Ad-GFP group. **Conclusions:** Adenovirus-mediated ING4 gene exhibits anti-tumor ability in human prostate cancer PC-3 cells in vitro and in vivo, and induces apoptosis. This may be related to the up-regulations of p53, Bax, Caspase-3 and down-regulation of Bcl-2.

Key words: ING4 gene, adenovirus vector, PC-3 cells, prostate carcinoma, tumor-suppression

Prostate cancer is a common malignancy involving the male reproductive system, and its incidence is increasing. Because prostate cancer has potent infiltration ability and is prone to distant metastasis in its early phase, most men have advanced disease when diagnosed. Treatments, such as surgery, radiotherapy, chemotherapy, steroids, and immunotherapy, cannot control local T3 lesions, as determined clinically or pathologically. The anatomical accessibility and the insights into the pathogenesis of prostate cancer make gene therapy a promising treatment for prostate cancer.[1]

The gene, ING4, a member of the inhibitor of growth (ING) family, was first identified by Shiseki et al.[2] in 2003. In 2004, the gene was determined to be an important inhibitor of tumor growth.[3] The gene is prone to deletion mutations, down-regulation, or both, and is associated with carcinogenesis and the degree of malignancy.[3–5] Exogenous ING4 gene expression takes its anti-tumor effect through multiple pathways, and it is a potential tumor suppressor gene. We have found that AD-ING4 has substantial tumor suppressor effects in liver cancer, pancreatic cancer, lung cancer, gastric cancer, and osteosarcoma.[6–10]

In the present study, we infected PC-3 human prostate cancer cells with a recombinant adenovirus Ad-ING4 carrying the ING4 gene and observed the in vitro and in vivo effects on growth suppression and apoptosis to investigate molecular mechanisms and to determine the feasibility of using gene therapy to treat prostate cancer.

Materials and Methods

Materials

Ad-GFP and recombinant adenovirus Ad-ING4 were

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constructed in our laboratory; PC-3 human prostate cancer cells were preserved in our laboratory. Adenovirus packaging cells, QBI-293A, were provided by professor Zhong Jiang, Fudan University. MTT was purchased from Sigma Company, and Hoechst staining kits were purchased from Nanjing KeyGen Biotech. Co. Ltd. Cell lysis buffer Trizol was purchased from Invitrogen. Reverse transcription MMLV was from Fermentas, and *Taq* was from TaKaRa Company. Bcl-2, Bax, and Caspase-3 antibody detection kits were purchased from Jing Mei Biotech Co., Ltd (Shanghai). CD34 detecting kits were purchased from Jing Mei Biotech Co., Ltd (Shen Zhen). Up- and down-stream primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Table1). Male BALB/c nude mice aged 4 to 6 weeks were purchased from the Shanghai laboratory animal center of the Chinese Academy of Sciences. Streptavidin peroxidase (SP) super-sensitive kits were purchased from Fuzhou Maixin Biotech. Co. Ltd.

Table 1 Primers for polymerase chain reaction (PCR)

Gene	Sequences
ING4	Sense: 5′-GCGTCGACATGGATGATGGGATGTATTTGGAAC-3′;
	antisense: 5′-GCAAGCTTCTAGTGGTGGTGGTGGTGGTGTTTC-3′
p53	Sense: 5′-CCTCCTCAGCATCTTATCCG-3′;
	antisense: 5′-CACAAACACGCACCTCAAA-3′
Bcl-2	Sense: 5′-TGTGGCCTTTCTTTGAGTTCG-3′;
	antisense: 5′-CTACCCAGCCTCCGTTATCC-3′
Bax	Sense: 5′-GGATGCGTCCACCAAGAA-3′;
	antisense: 5′-GCACTCCCGCCACAAAGA-3′
Caspase-3	Sense: 5′-ACCTCAGAGAGACATTCAC-3′;
	antisense: 5′-CCCCACTCCCAGTCATTCCTTT-3′
β-actin	Sense: 5′-TGTTTGAGACCTTCAACACCC-3′;
	antisense: 5′-AGGAAGGCTGGAAGAGTGC-3′

Methods

Amplifying the Ad-ING4 virus Amplification of Ad-ING4 virus was accomplished with routine methods.[11]

Determining the potency of the Ad-ING4 virus The potency of Ad-ING4 virus was determined with routine methods.[11]

Morphology of PC-3 human prostate cancer cells infected with Ad-ING4 PC-3 cells in their logarithmic growth phase were divided into three groups: an experimental group [Ad-ING4 group with 100 MOI (1×10^9 pfu/mL)], a negative control group [Ad-GFP group with 100 MOI (1×10^9 pfu/mL)], and a cell control group (PBS group). All groups were cultured at 37℃ with 5% CO_2 for 72 h and then observed under fluorescent microscopy.

Exogenous ING4 gene expression induced by adenovirus in PC-3 human prostate cancer cells PC-3 cells in their logarithmic growth phase were divided into three groups according to the method described above in section 1.2.3 and infected for 72 h, followed by centrifugation to harvest cells. Then, 1 mL of the cell lysis buffer Trizol was added to each group. Total RNA was extracted according to routine methods in our laboratory.[10] cDNA was obtained by reverse transcription according to the manufacturer's instructions, and RT-PCR identification with ING4 primers was performed according to routine methods of our laboratory.[9]

Determining the impact of Ad-ING4 on PC-3 cell growth with the MTT method PC-3 cells in their logarithmic growth phase were digested by trypsin and re-suspended with complete medium to make a single cell suspension that was then adjusted for its density after counting. Cells were seeded to 96-well plates with 1×10^4 cells in each well. After the cells attached to the wall, they were divided into three groups as described.

There were three dual-wells in each group. Cells were cultured in an incubator with 5% CO_2 at 37℃. MTT 10 μL (5 mg/mL), was added to each well on days 0, 1, 2, 3, 4, and 5, the wells were then incubated at 37℃ for 4–6 h, and stopping solution [10% SDS+1% HCl(1 mol/L)], 100 μL/well was added. The solution was dissolved at 37℃ and examined for absorbance value (A) at 570 nm with an enzyme-labeling instrument. The growth curve was drawn with the absorbance value on the vertical axis and time on the horizontal axis. Growth inhibition rate was calculated by following formula:

growth inhibition = (1–A value of experimental group/A value of negative control group) ×100%.

Detecting the impact of recombinant adenovirus on PC-3 apoptosis by Annexin-V-PE/7-AAD double-staining flow cytometry PC-3 cells in their logarithmic growth phase that had been infected with a recombinant adenovirus were divided into three groups: an experimental group [Ad-ING4 group with 100 MOI (1×10^9 pfu/mL)], a negative control group [Ad-GFP group with 100 MOI (1×10^9 pfu/mL)], and a cell control group (PBS group). Groups were cultured at 37℃ with 5% CO_2 for 72 h, followed by digestion with trypsin free of EDTA, cell harvesting, and two PBS washings. The cell concentration was adjusted to 1×10^6 to 1×10^7/mL by 1×buffer. The 100-μL cell suspension was mixed on ice with 10 μL Annexin-V-PE in an EP tube, which was then kept in the dark for 15 min. The solution was combined with 380 μL 1× buffer and 10 μL 7-AAD, then kept in the dark and examined with flow cytometry within 1 h.

Effects of Ad-ING4 on the morphological changes of the nuclei of PC-3 cells Groupings and specific procedures were the same as described. Suspended cells were harvested after incubation at 37℃ and 5% CO_2 for 72 h, washed with PBS two or three times, fixed with 4% paraformaldehyde, stained with Hoechst 33258 for 30 min, covered with glycerol, then observed under laser scanning confocal microscopy for apoptotic bodies in cells.

Changes in apoptosis-related gene transcription detected by semi-quantitative RT-PCR PC-3 cells in their logarithmic growth phase were grouped as described, infected with virus for 72 h, and concentrated by centrifugation. Then, 1 mL of cell lysis buffer Trizol was added to each group. Total RNA was extracted according to routine methods in our laboratory.[10] cDNA was obtained by reverse transcription according to the manufacturer's instructions. Amplification with PCR was performed with the cDNA as the template and Bcl-2, Bax, p53, Caspase 3, and β-actin up-and down-stream primers (Table 1). The conditions for PCR were 94℃ for 5 min, 94℃ for 30 s, 56℃ for 30 s, 72℃ for

1 min for 30 cycles, then followed by extension at 72℃ for 10 min. PCR products were subjected to 1.0% agarose gel electrophoresis. The results were observed with a gel imaging system and analyzed with BandScan software.

An animal model of PC-3 human prostate cancer cells PC-3 prostate cancer cells were cultured in RPMI-1640 complete medium containing 10% calf serum and incubated with 5% CO_2 at 37℃. PC-3 cells in their logarithmic growth phase were digested with trypsin, washed by PBS twice, centrifuged at 1 500 r/min for 5 min and re-suspended with PBS to make a cell suspension. Then, cell density was adjusted to 2×10^7/mL. The right anterior axilla of 15 nude mice were inoculated with 100-μL of cell suspensions (2×10^6 cells). These mice were fed in a specific pathogen free environment. The experiment began when the tumors grew to 5 mm in diameter.

Experimental groups and anti-tumor treatment The 15 nude mice were divided into 3 groups of 5 mice each. All tumors in the experimental, negative control, and cell control groups were injected with 100 μL of Ad-ING4 (1×10^9 pfu/mL), Ad-GFP (1×10^9 pfu/mL), or PBS, respectively. The injections were performed every other day for 5 cycles.

Tumor volume, weight, and tumor suppression rate The short and long axes of the tumor were measured at every injection, and tumor volume was calculated as: $V=ab^2\times0.5$ (where a is the long axis and b is the short axis). Changes in tumor volume were graphed. The mice were killed 1 week after the complete of treatment. The tumor was removed and weighted to calculate the tumor suppression rate of the treatment group: tumor suppression rate = (mean tumor weight of the control group minus mean tumor weight of the treatment group)/mean tumor weight of the control group×100%. Tumor specimens were fixed overnight with 10% neutral formaldehyde, embedded in paraffin for hematoxylin and eosin (HE) and immunohistochemical staining.

Determining apoptosis after Ad-ING4 treatment HE staining of tumor specimens was performed routinely. Tumor cell apoptosis was indicated by: tumor cell shrinkage, cytoplasm condensation, nuclear shrinkage, chromatin condensation with coagulation blocks, aggregation of chromatin along the inner side of the nuclear membrane, or the presence of apoptotic bodies formed by nuclear lysis.

Molecular mechanisms of the in vivo anti-tumor effect of Ad-ING4 on nude mice Bcl-2, Bax, Caspase-3, and CD34 expressions in the paraffin sections were detected with the streptavidin peroxidase immunohistochemistry method in a preliminary investigation of potential molecular mechanisms. Positive cells were defined as those with scattered or diffuse brown-yellow granularity in the cytoplasm or nucleus. Each section was observed under × 400 light microscopy, and counts of 10 visual fields was used in statistical analysis.

Statistical methods The SPSS11.0 software was used for data processing. Data are expressed as means and standard deviations (SD) and analyzed with One-Way ANOVA. Alpha was set at 0.05.

Results

Morphological changes of PC-3 cells after Ad-ING4 virus infection

PC-3 cells were infected by Ad-ING4 and Ad-GFP (1×10^9 pfu/mL) with doses of 100 MOI for 72 h, and their morphology was observed under fluorescence and light microscopy. PC-3 cells in the Ad-GFP and PBS groups had attached to the well walls, were growing, and had intact structures, whereas those of the Ad-ING4 group showed remarkable abnormalities: they had became round and adrift, and the number of cells attached to the walls of the well was substantially lower than that in the control groups. Cells in both the Ad-ING4 experimental group and Ad-GFP negative control group exhibited a strong green fluorescence under fluorescence microscopy (Fig. 1).

Identifying the expression of the exogenous ING4 gene in PC-3 cells

PC-3 cells were infected with 100 MOI Ad-ING4 and Ad-GFP and total RNA was extracted. ING4 gene expression in PC-3 cells was detected with RT-PCR. The Ad-ING4 experimental group showed positive bands of ING4 and β-actin, whereas Ad-GFP and PBS groups showed only a β-actin band (Fig. 2).

Detecting the inhibitory effects of Ad-ING4 on PC-3 cell with MTT

PC-3 cells were infected with 100 MOI Ad-GFP and Ad-ING4, then cell growth was detected with MTT for the next 4 days (Fig. 3). Ad-ING4 significantly inhibited the proliferation of PC-3 cells with a inhibition rate of 58% on day 4 ($P<0.05$). The results indicate that ING4 gene transcription induced by adenovirus significantly inhibited the proliferation of PC-3 cells, while the effect of Ad-GFP was unremarkable, which was not significantly different from that of PBS ($P>0.05$).

Detecting apoptosis with Annexin-V-PE/7-AAD double-staining flow cytometry

PC-3 cells infected with 100 MOI Ad-GFP and Ad-ING4 for 72 h were subject to Annexin-V-PE/7-AAD double-staining flow cytometry to detect apoptosis. Ad-ING4 significantly induced PC-3 apoptosis, at a rate of about 19% (Fig. 4), which was significantly higher than those of Ad-GFP and PBS groups ($P<0.05$).

Impact of Ad-ING4 on the morphological changes in PC-3 cells

Laser scanning confocal microscopy of PC-3 cells after treatment with Ad-ING4 revealed the dense staining, condensation, and even breakage of the nucleus that is characteristic of apoptosis, whereas the nuclei of the Ad-GFP and PBS groups were normal and showed no evidence of apoptosis (Fig. 5).

Detecting the apoptosis-related genes Bcl-2, Bax, p53, and Caspase-3 in Ad-ING4-infected PC-3 cells

PC-3 cells were infected with 100 MOI Ad-GFP and Ad-ING4 for 72 h, collected for total RNA extraction, and subjected to RT-PCR to semi-quantitatively detect mRNA transcriptions of

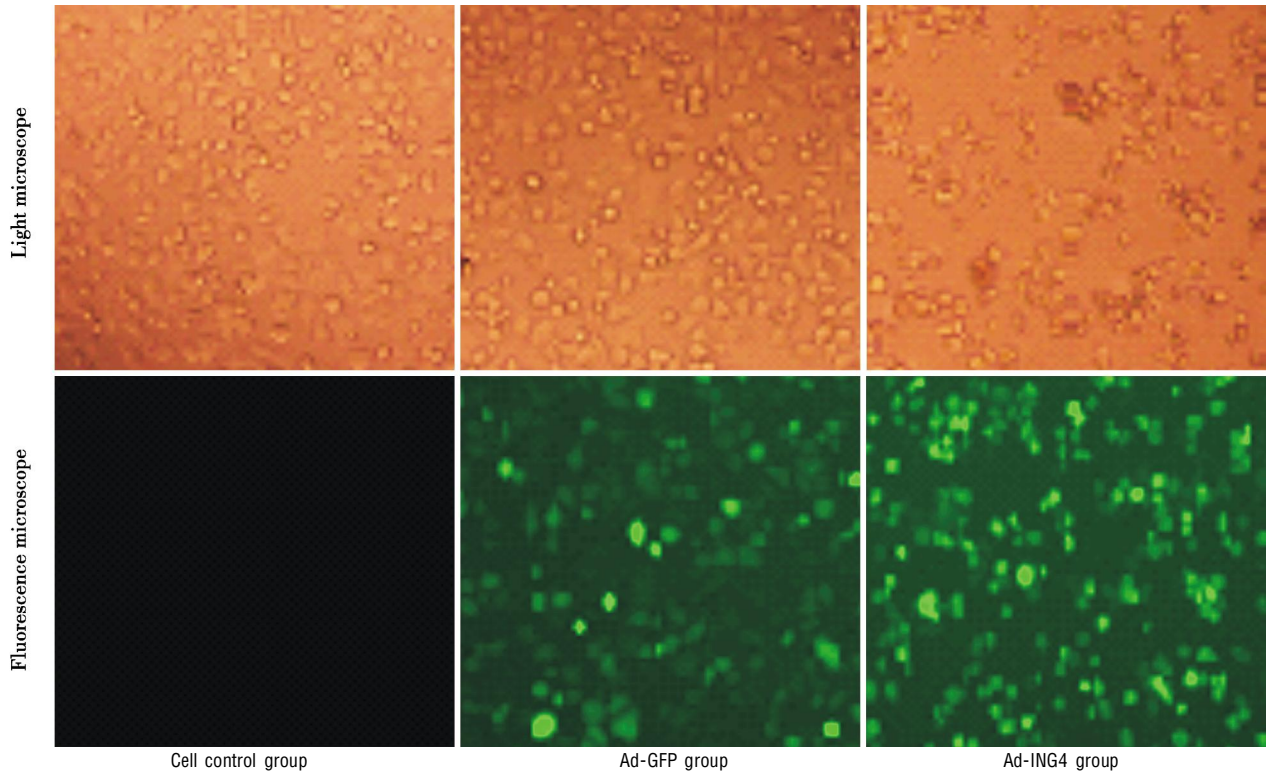

Figure 1 Morphologic changes of PC-3 cells after adenovirus transfection (×100)

Many PC-3 cells with green fluorescence are presented in Ad-GFP group and Ad-ING4 group. No green fluorescence is found in cell control group.

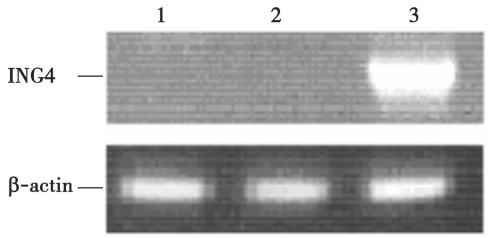

Figure 2 Identification of ING4 in PC-3 cells by reverse transcription-PCR (RT-PCR)

Lane 1, PBS group; lane 2, Ad-GFP group; lane 3, Ad-ING4 group.

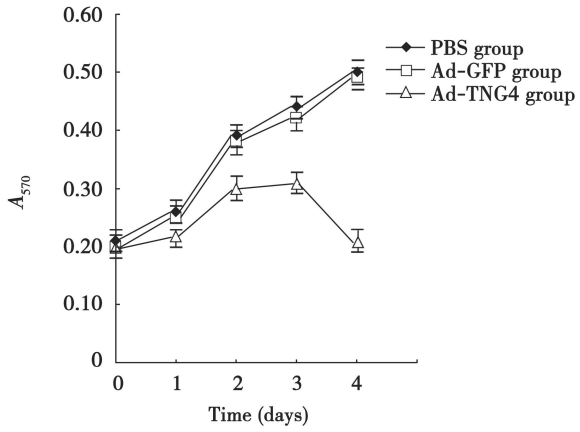

Figure 3 Effects of Ad-ING4 on proliferation of PC-3 cells

Bcl-2, Bax, p53, and Caspase-3. Results were further analyzed with the BandScan grey scale software. Compared with PBS and Ad-GFP control groups, PC-3 cells treated with Ad-ING4 showed up-regulated Bax, Caspase-3, and p53 genes (P<0.05), whereas the Bcl-2 gene was down-regulated (P<0.05) (Fig. 6).

In vivo tumor suppressive effect of Ad-ING4 on tumor load in nude mice

All 15 nude mice were successfully inoculated as indicated by the presence of subcutaneous tumors 5 days later. The tumors grew continuously thereafter. The diameter of the tumor reached 5 to 7 mm at 14 days. During this 2-week treatment period, tumors in the PBS and Ad-GFP control groups grew continuously and their volume markedly increased, whereas tumors in Ad-ING4 experimental group grew more slowly (P< 0.05) (Fig. 7).

Tumors in the Ad-ING4 experimental group had smaller volumes than those in the in negative and cell controls (P<0.05) (Table 2). Mean volumes did not differ significantly between PBS and Ad-GFP groups. The tumor growth inhibition rate was as high as 37%. No deaths or adverse reactions occurred during treatment.

Pathologic findings of tissue sections

Tumor specimens stained with HE showed that in PBS and Ad-GFP groups, tumor cells grew well with irregular shape, and pathologic mitosis. The Ad-ING4 experimental group had regions of massive necrosis that were uniformly stained pink, without

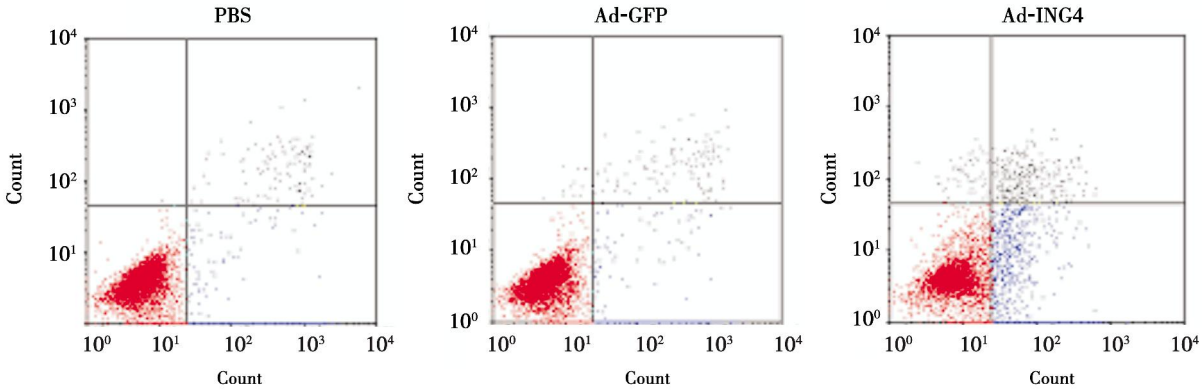

Figure 4 Apoptosis rate of PC-3 cells detected by flow cytometry

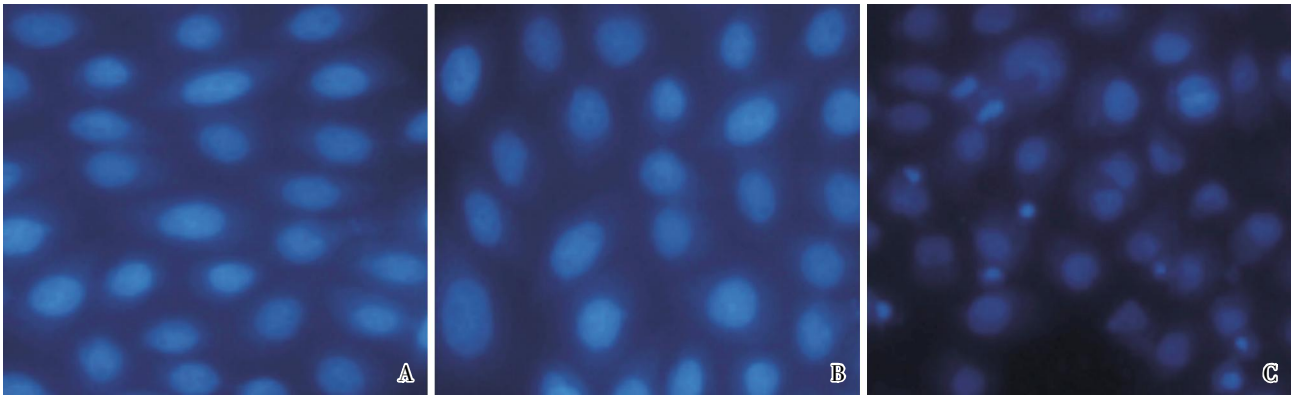

Figure 5 Apoptosis of PC-3 cells 72 h after transfection of Ad-ING4 (Hoechst 33258×400)

Apoptotic cells were observed in Ad-ING4 group (C), but less were found in Ad-GFP group (B) and PBS group (A).

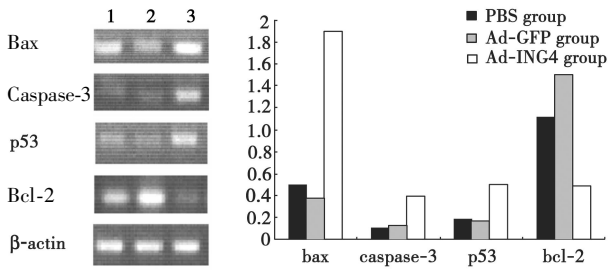

Figure 6 The analysis of mRNA expression of Bcl-2, Bax, p53, Caspase-3 in PC-3 cells by RT-PCR

1, PBS group; 2, Ad-GFP group; 3, Ad-ING4 group.

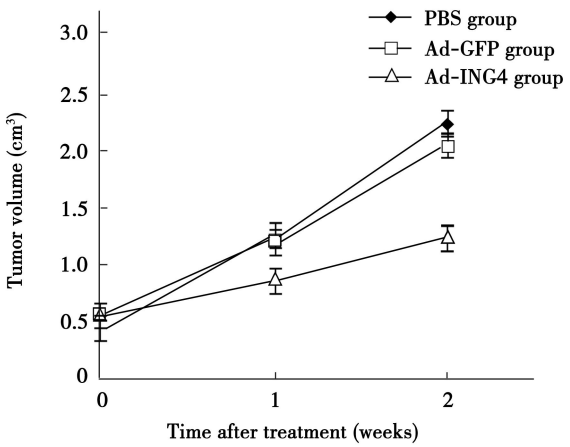

Figure 7 Growth curves of tumor cells in nude mice after treatment

cellular structures, and the cells were fragmented (Fig. 8).

Changes in Bcl-2, Bax, Caspase-3, and CD34 protein expression in PC-3 xenografts after Ad-ING4 transfection

Paraffin-embedded sections were stained with the SP immunohistochemical method (Fig. 9). The Bax and Caspase-3 genes in the Ad-ING4 experimental group were up-regulated and showed large amounts of brown-yellow granules in the stained sections (especially the Caspase-3, $P<0.05$). The Bcl-2 and angiogenesis-marked gene CD34 were down-regulated ($P<0.05$), especially the Bcl-2 gene, which had the greatest down-regulation. The gene expressions in the negative and cell control groups did not differ significantly (Table 3).

Table 2 Comparison of the volume, weight and inhibition rate of tumors in nude mice 34 days after transplantation

Group	Tumor volume(cm^3)	Tumor weight(mg)	Inhibition rate(%)
PBS	2.25±0.41	3044.8±816.4	0
Ad-GFP	2.05±0.44	2678.6±332.1	12.02
Ad-ING4	1.24±0.81[a]	1926.1±961.4[a]	36.74

[a]$P<0.05$, vs. Ad-GFP group and PBS group.

Table 3 Expressions of Bax, Caspase-3, Bcl-2, and CD34 in nude mice

Group	Expression ($\bar{x}\pm s$, $\times 10^4$)			
	Bax	Caspase-3	Bcl-2	CD34
PBS	6.21±4.33	5.83±5.01	40.84±8.53	58.44±7.76
Ad-GFP	6.41±5.44	5.06±3.32	33.79±9.02	58.19±5.92
Ad-ING4	15.72±8.74[a]	13.79±6.48[a]	18.56±2.99[a]	23.83±6.89[a]

[a]$P<0.05$, vs. Ad-GFP group and PBS group.

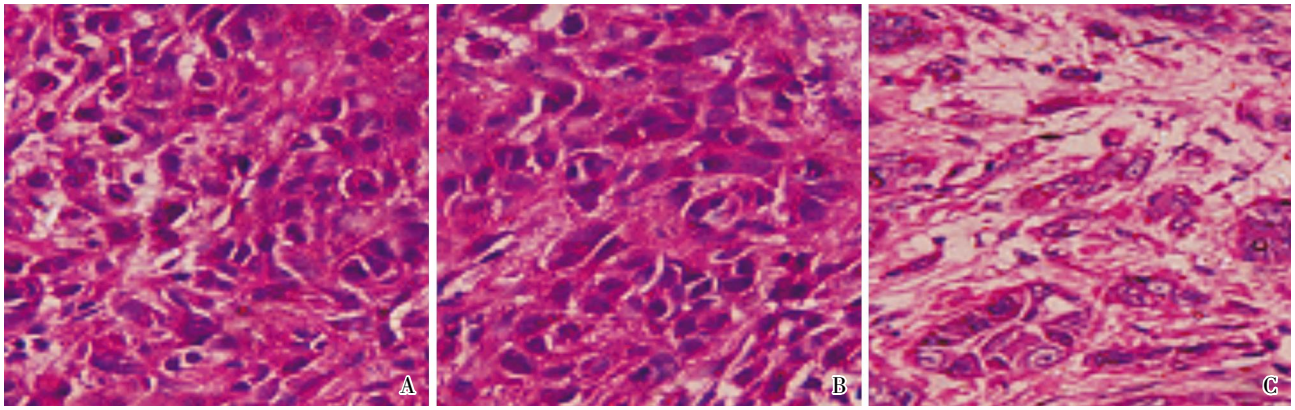

Figure 8 Morphology of the xenograft tumors in nude mice (HE ×100)

A lot of necrotic cells are presented in the tumor tissues in nude mice with transfection of Ad-ING4 (C), while tumor cells grow well in the mice with transfection of Ad-GFP (B)or PBS (A).

Discussion

The Inhibitor-of-Growth (ING) family of genes includes the potential tumor-suppressor genes, including ING1, ING2, ING3, ING4, and ING5. These genes are involved in regulating the cell cycle, apoptosis, and DNA repair. ING4 can enhance p53 transcription by increasing its acetylation, which in turn can shorten the S phase of RKO colon cancer cells and enhance the G_2/M phase arrest of $HepG_2$ liver cancer cells, inducing tumor cell apoptosis.[12] Moreover, ING can interact with the P65 (RelA) subunit of the nuclear factor kappa B (NF-κB) to inhibit its transcription and down-regulate the genes downstream from NF-κB, including IL-8 and Cox-2, thereby inhibiting the growth of glioma and associated angiogenesis. ING can also enhance the sensitivity of liver cancer cells to chemical agents that can damage DNA (such as Etoposide and Adriamycin),[13] restore the contact inhibition among tumor cells,[14] and inhibit HIF activation by combining with hypoxia inducible factor (HIF) α_1, thereby specifically inhibiting angiogenesis and tumor growth.[13]

ING4 can suppress tumor cell movement by interacting with liprin α_1 protein,[15,16] and it can inhibit tumor cell infiltration and metastasis.[17] These actions all indicate that ING4 creates its anti-tumor effects through multiple pathways and can induce tumor cell apoptosis, which makes it a novel tumor suppressor gene. Ad-ING4 inhibits liver cancer, pancreatic cancer, lung cancer, gastric cancer, and osteosarcoma.[6-10] The effect of an ING4 recombinant adenovirus vector on gene therapy for prostate cancer has not been reported.

Therefore, we infected PC-3 cells in vitro with adenovirus to investigate the effect of Ad-ING4 in inducing PC-3 cell apoptosis and to identify the associated mechanisms. RT-PCR showed that the ING4 gene was successfully transfected into PC-3 cells. Flow cytometry and MTT indicated that adenovirus-induced ING4 gene expression significantly inhibited PC-3 cell proliferation and enhanced apoptosis. The apoptosis rate was 19.5%, which was significantly higher than that in the negative and cell control groups. The cells were then stained with Hoechest 33258 and observed under laser scanning confocal microscopy. These results further confirmed that ING4 induced cell apoptosis and nuclear morphological changes in PC-3 cells, whereas there were no apoptotic nuclear morphological changes in Ad-GFP and PBS groups.

We then investigated the molecular mechanisms of the anti-tumor effects of ING4 genes by RT-PCT. The results showed that after ING4 transfection, apoptosis-promoting gene bax transcription was significantly up-regulated, and anti-apoptosis gene Bcl-2 was down-regulated, increasing the Bax/Bc1-2 ratio. Meanwhile, the transcription of the tumor suppressor gene p53 was increased, thereby activating Caspase-3 and subsequently cleaved caspase-3, indicating that ING4 enhanced p53 transcription, activated caspase-3, and induced tumor cell apoptosis by a p53-dependent pathway.

We also treated tumors in nude mice with Ad-ING4 in multiple drug administrations. Although the transplanted tumor size was not reduced, its growth rate was significantly decreased.

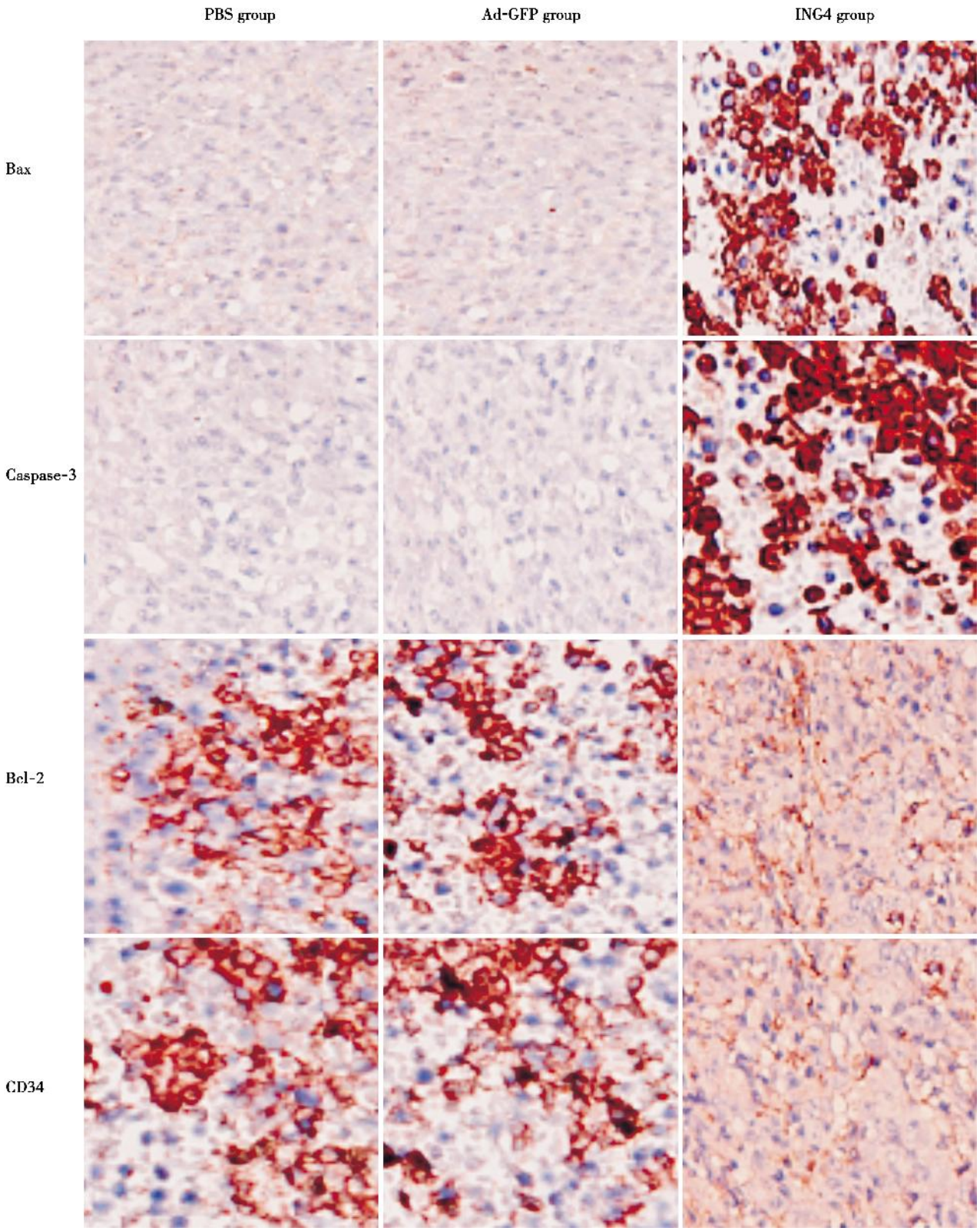

Figure 9 Expression of Bax, Caspase-3, Bcl-2, and CD34 in transplanted human prostate cancer tumors (SP×400)

Bax (in brown) is expressed in cytoplasm. Caspase-3 (in brown) is expressed in cytoplasm and around nuclei.

Bcl-2 (in brown) is expressed in cytoplasm.

CD34 (in brown) is expressed in cell membrance.

Meanwhile, the mice grew normally without gross toxicities. These results indicate that ING4 can specifically inhibit the growth of the transplanted tumor. Pathologic examination and the degree of apoptosis detected in the tumor in situ revealed massive regions of necrosis in mice receiving Ad-ING4, whereas tumor cells remained intact in the negative and cell control groups, with more cells in the mitotic phase and smaller necrotic regions.

The apoptotic index of Ad-ING4 group was significantly higher than those in the Ad-GFP and PBS control groups. We attribute the anti-tumor effect of Ad-ING4 to up-regulation of the Bax gene and down-regulation of Bcl-2 gene, which in turn activates the Caspase-3 pathway to induce apoptosis. Meanwhile, the inhibition of angiogenesis by CD34 down-regulation is also important. These results are consistent with those of in vitro studies.

This study not only provides experimental evidence supporting the possible use of gene therapy for treating prostate cancer with Ad-ING4 but also suggests an approach for studying the mechanisms of the anti-tumor effect of ING4, as well as for creating gene-modified tumor vaccine.

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