Technology in Cancer Research & Treatment
2017, Vol. 16(5) 533–545

DOI: 10.1177/1533034616656315
journals.sagepub.com/home/tct
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Overexpression of Inhibitor of Growth 4 Enhances Radiosensitivity in Non-Small Cell Lung Cancer Cell Line SPC-A1

Xuan Pan, MD[1,2], Rui Wang, PHD[3], Haibo Bian, MD[2], Wei De, PHD[4], Ping Zhang, MD[5], Chenchen Wei, MD[2], and Zhaoxia Wang, PHD[2]

Abstract

Inhibitor of growth 4 is a member of the inhibitor of growth family proteins, which is involved in cell apoptosis, migration, invasion, and cell cycle progress. In this study, we investigated the inhibitor of growth 4 level in non-small cell lung cancer tissues and explored the antitumor activity of inhibitor of growth 4 *in vitro* and *in vivo* using non-small cell lung cancer cell line SPC-A1 and its underlying molecular mechanisms. We also explored its role on the radiosensitivity in SPC-A1 cells. The level of inhibitor of growth 4 protein was significantly decreased in 28 cases of non-small cell lung cancer tissues in comparison with corresponding noncancerous lung epithelial tissues. Upregulation of inhibitor of growth 4 by plasmid pcDNA3.1-ING4 delivery could suppress proliferation and increase apoptosis of SPC-A1 cells both *in vitro* and *in vivo*. Additionally, we found that overexpression of inhibitor of growth 4 in SPC-A1 cell line could lead to a higher Bcl-2/Bax ratio, which might be an important factor in the apoptosis regulation. Furthermore, overexpression of inhibitor of growth 4 enhanced the radiosensitivity of SPC-A1 cells to irradiation. Inhibitor of growth 4 upregulation plus radiotherapy induced synergistic tumor suppression in SPC-A1 xenografts implanted in athymic nude mice. Thus, the restoration of inhibitor of growth 4 function might provide a potential strategy for non-small cell lung cancer radiosensitization.

Keywords

ING4, radiosensitivity, NSCLC, apoptosis, gene therapy

Abbreviations

Cox-2, cyclooxygenase; ING4, inhibitor of growth 4; IL, interleukin; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NLS, nuclear localization signal; NSCLC, non-small cell lung cancer; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription polymerase chain reaction; SC, subcutaneously; TUNEL, TdT-mediated dUTP nick-end labeling.

Received: December 17, 2015; Revised: March 17, 2016; Accepted: May 11, 2016.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of all cases.[1,2] Despite early diagnosis and advances in clinical treatment, patients with NSCLC are often diagnosed at an advanced stage and have a poor prognosis. The 5-year survival rate of NSCLC is only 13%, and the median survival is 8 to 11 months.[3] At present, radiotherapy plays an important role in achieving local control of the NSCLC tumor and in the relief of symptoms resulting from metastatic diseases.[4] However, as a curative modality, radiotherapy has been disappointing. Two major issues that limit the effectiveness of radiotherapy of NSCLC are radioresistance of the tumor and radiation-induced toxicity to normal tissues such as the lung and esophagus.[4] Therefore, it is urgent to exploit

[1] Department of Medical Oncology, Nanjing Medical University Affiliated Cancer Hospital of Jiangsu Province, Cancer Institution of Jiangsu Province, Nanjing, China
[2] Department of Oncology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, China
[3] Department of Medical Oncology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China
[4] Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing, China
[5] Department of Pathology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, China

Corresponding Author:
Zhaoxia Wang, PhD, Department of Oncology, The Second Affiliated Hospital of Nanjing Medical University, Jiangjiayuan Road 121#, Nanjing, 210011, China.
Email: zhaoxiawang88@hotmail.com

novel strategies to overcome the radioresistance of human NSCLC.

The inhibitor of growth (ING) gene family is comprised of 5 evolutionarily conserved proteins (ING1-ING5), which have been considered as candidate tumor suppressor genes.[5-8] They are involved in nucleoprotein modification through their roles in histone acetylation.[9] Recent studies have shown that the ING protein enhances p53 activity and regulates various biological activities, including DNA repair, oncogenesis, cell apoptosis, and cell cycle process.[6-8] Inhibitor of growth 4 is characterized by a highly conserved C-terminal plant homeodomain finger motif and a potential bipartite nuclear localization signal (NLS) domain.[6] Inhibitor of growth 4 has emerged as a tumor suppressor gene, because its expression was markedly downregulated in multiple human malignancies[10-15] and the ING4 gene is frequently mutated in various cancer cell lines.[16,17] Additionally, ING4 expression is associated with pathological features and poor prognosis, suggesting that it might serve as a diagnostic and prognostic marker for human malignancies.[10,15,18,19] Shiseki *et al* demonstrated that ING4 overexpression could induce a decrease cell population in S phase and apoptosis enhancement in a p53-dependent manner.[6] Inhibitor of growth 4 could induce G_2/M arrest and enhance chemosensitivity to antitumor agents in human hepatocellular cancer cells.[20,21] In addition, ING4 was reported to suppress nuclear factor κB (NF-κB) signaling and mediate the transcriptional repression of multiple NF-κB–responsive genes, such as cyclooxygenase (Cox-2), hypoxia-inducible factor 1α, matrix metalloproteinase 2 (MMP-2), MMP-9, CD34 (human hematopoietic stem cell membrane glycoprotein), interleukin 6 (IL-6), and IL-8, leading to angiogenesis inhibition in multiple malignancies.[10,19,22-27] Therefore, these findings revealed that ING4 exerts its tumor-suppressive effects through multiple pathways.

Conventional chemotherapy or radiotherapy together with gene therapy referred as combined therapy is a common practice in human cancer treatment, since it could reduce side effects, reverse chemoresistance to cytotoxic drugs, and improve therapeutic outcome. Recently, several studies have reported that ING4 was significantly decreased in human NSCLC tissues compared with corresponding noncancerous lung tissues.[12,15] Moreover, downregulation of nuclear ING4 was associated with the tumor stage and lymph node metastasis in NSCLC.[15] Upregulation of ING4 induces cell growth inhibition and triggers apoptosis in the NSCLC cells via multiple pathways.[12,28-30] Additionally, adenovirus-mediated upregulation of ING4 gene inhibits microvessel formation through downregulating the expression of CD34 in tumor vessels. We also previously reported that restoration of ING4 expression could reverse chemoresistance of docetaxel-resistant NSCLC cells to docetaxel *in vitro* and *in vivo*.[31] To the best of our knowledge, the protective effect of ING4 in combination with radiation therapy on human NSCLCs has not been investigated.

In our previous study, we first detected the expression of ING4 protein in NSCLC tissues by Western blot and immunohistochemistry assays and found that ING4 was markedly downregulated in NSCLC tissues compared with corresponding noncancerous tissues. Then, we constructed a recombinant plasmid pcDNA3.1-ING4 and selected stable transfectant cells to assess the combined effects of ING4 gene plus X-ray against NSCLC cells. We found that overexpression of ING4 could suppress proliferation, increase apoptosis of SPC-A1 cells, and lead to a higher Bcl-2/Bax ratio both *in vitro* and *in vivo*. Inhibitor of growth 4 upregulation plus radiotherapy induced synergistic tumor suppression in SPC-A1 cells. Taken together, ING4 could serve as a potential therapeutic target in radiation therapy of NSCLC.

Methods

Ethics Statement

Ethics approval was given by the ethics committee of Nanjing University. Written informed consent was obtained from patients before sampling. The animal experiments were carried out in accordance with the Nanjing University Guide for the Care and Use of Laboratory Animals formulated by the National Society for Medical Research.

Patient and Tissue Samples

Non-small cell lung cancer and adjacent noncancerous tissue samples were obtained at the time of surgery from 28 patients with NSCLC at the Jinling Hospital (China) from 2008 to 2009. Tumor and adjacent noncancerous tissue samples were rapidly frozen in liquid nitrogen after resection and stored at −80°C.

Cell Culture

Human NSCLC cell line SPC-A1 was obtained from the Department of Medical Oncology, Jinling Hospital. SPC-A1 was cultured in Dulbecco-modified Eagle medium (GIBCO, USA) supplemented with 10% fetal bovine serum at 37°C in humidified atmosphere with 5% CO_2.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 3-μm-thick sections. The sections were incubated with primary goat monoclonal ING4 antibody (Santa Cruz Biotechnology, Santa Cruz, California), followed by incubation with a secondary antibody (horseradish peroxidase conjugated to goat antigoat immunoglobulin). The adjacent noncancerous lung tissues were selected as a positive control. Negative control consisted of substituting phosphate-buffered saline (PBS) solution for the primary antibody.

The immunostain result of ING4 was evaluated by a semiquantitative scoring system. The percentage of positive cells (score: 1 for <10%; 2 for 11%-50%; 3 for 51%-75%; 4 for >75%) and strength of staining (score: no staining as 0; shallow brown as 1; brown as 2; deep brown as 3) were used to assess the immunoreactivity. The immunohistochemistry score was recorded from the 2 areas: 0 to 2 scores as (−), 3 to 5 as (+), 6 to 9 as (++), and >9 as (+++).

Conventional Reverse Transcription Polymerase Chain Reaction Analysis

Total cellular RNA was extracted from cells and was reverse transcribed using reverse transcription polymerase chain reaction (RT-PCR) kits (Applied Biosystems, USA). The *XhoI*/*EcoRI* site was inserted into the primer as follows: the forward primer: 5′-CGCTCGAGATGGCTGCGGGGATGTATTTG-3′ (letters underline: *XhoI*); the reverse primer 5′-CGGAATTCCTATTTCTTCTTCCGTTC-3′ (letters underline: *EcoRI*); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, and the forward primer, 5′-GGAGTCAACGGATTTGGTCG-3′ and the reverse primer, 5′-CATCGCCCCACTTGATTTTG-3′ were used. The PCR conditions included denaturation (94°C for 30 seconds), annealing (55°C for 30 seconds; 58°C for GAPDH), and extension (72°C for 30 seconds). The amplified products were separated by 1% agarose gel electrophoresis stained for ethidium bromide and visualized by UV light.

Construction and Transfection of Recombinant Plasmid pcDNA3.1-ING4

The ING4 complementary DNA fragment (GenBank NM_016162) was subcloned into pcDNA3.1(−) by XhoI and EcoRI restriction enzymes to produce pcDNA3.1-ING4 vector. The recombinant plasmid was amplified in *Escherichia coli* JM109 and was identified by PCR and DNA sequencing. The empty pcDNA3.1(−) was used as a negative control.

The vectors were transfected into human NSCLC cells SPC-A1 with Lipofectamine 2000 reagent using standard transfection procedures. Then, the colonies were selected to produce stable transfectants by adding G418 (600 mg/mL) for 2 weeks. G418-resistant colonies were further verified by RT-PCR and Western blot analysis.

MTT (3-[4,5-dimethylthazol-2-yl]-2,5-diphenyltetrazolium bromide) Assay

The control or transfected SPC-A1 cells were seeded in 96-well plates at a density of 2000 cells/well (200 μL). After culture for 0, 6, 12, 24, 48, and 96 hours, cell viability was measured by the MTT procedure. Twenty microliters of MTT (5 mg/mL) was added to each well, and the plates were then continuously incubated for 4 hours. Reactions were terminated with 200 μL Dimethyl Sulphoxide (DMSO) for 5 minutes. The optical density value was measured at 490 nm wavelength by an ELx800 Automated Microplate Reader (Biotech, USA).

Cell Survival Assay

For the radiosensitivity assay, the cells were X-ray irradiated 24 hours after seeding at different doses of 0, 2, 4, 6, 8, and 10 Gy and at an average dose rate of 100 cGy/min using the X-ray irradiator (ELEKTA Precise Treatment System, Sweden). Immediately following irradiation, the cells were incubated at 37°C with 5% CO_2. After incubation for 4 days, an MTT assay was performed to evaluate the effect of ING4 overexpression on the cell survival after X-ray irradiation. The surviving fraction at 2 Gy (SF_{2Gy}) was calculated. D_{37} was defined as the dose that required for 37% surviving fraction.

Colony Formation Assay

Human SPC-A1 cells, transfected with pcDNA3.1(−) or pcDNA-3.1-ING4 plasmid, were seeded onto 6-well plates at 5.0×10^3 cells/well and incubated at 37°C in 5% CO_2 environment. After incubation for 14 days, the colonies were fixed with methanol, stained with crystal violet, and counted manually. Colony-forming efficiency was calculated as colonies/plated cells × 100%.

Western Blot Analysis

Cells and tissue proteins were extracted in the extraction buffer, and protein concentration was determined by protein assay (Bio-Rad, Canada). The protein lysates were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene fluoride membrane. Blotting was performed for ING4, Bcl-2, Bax, and caspase-3 with ING4, Bcl-2, Bax, and active caspase-3 primary antibody (Santa Cruz, California), respectively. β-Actin (Sigma, USA) was used as an internal control.

Flow Cytometric Analysis

Cells were harvested and suspended in cold PBS, counting 5×10^5 to 1×10^6 cell/mL. Apoptosis analysis was performed using annexin-V-FITC/PI (fluorescein isothiocyanate/Propidium Iodide) double staining according to the manufacturer's instructions, and apoptotic cells were analyzed using flow cytometry (FACS Calibur; Becton Dickinson, USA).

TdT-Mediated dUTP Nick-End Labeling Assay

Transfected SPC-A1 cells were plated on the slides. Apoptotic cells were detected using the *in situ* cell death detection kit (Roche, Swiss) according to the manufacturer's instructions. Photos of the slides were taken using an Olympus (Olympus, Japan) fluorescence microscopy. For *in vivo* assay, the xenograft tissues were cut into 5-μm-thick sections. The tissue sections were assessed by hematoxylin and eosin staining, immunohistochemical staining, and TdT-mediated dUTP nick-end labeling (TUNEL) assay, respectively.

In Vivo Studies

Animal experiments were performed in accordance with the institutional guidelines set forth by the ethics board. The female BALB/c athymic nude mouse (5-6 weeks of age) were purchased from the Experimental Animal Centre of Nanjing Medical University and maintained under pathogen-free conditions (n = 5/group). A total of approximately 5.0×10^6 cells (SPC-A1/pcDNA3.1-ING4 and SPC-A1/pcD NA3.1) were injected

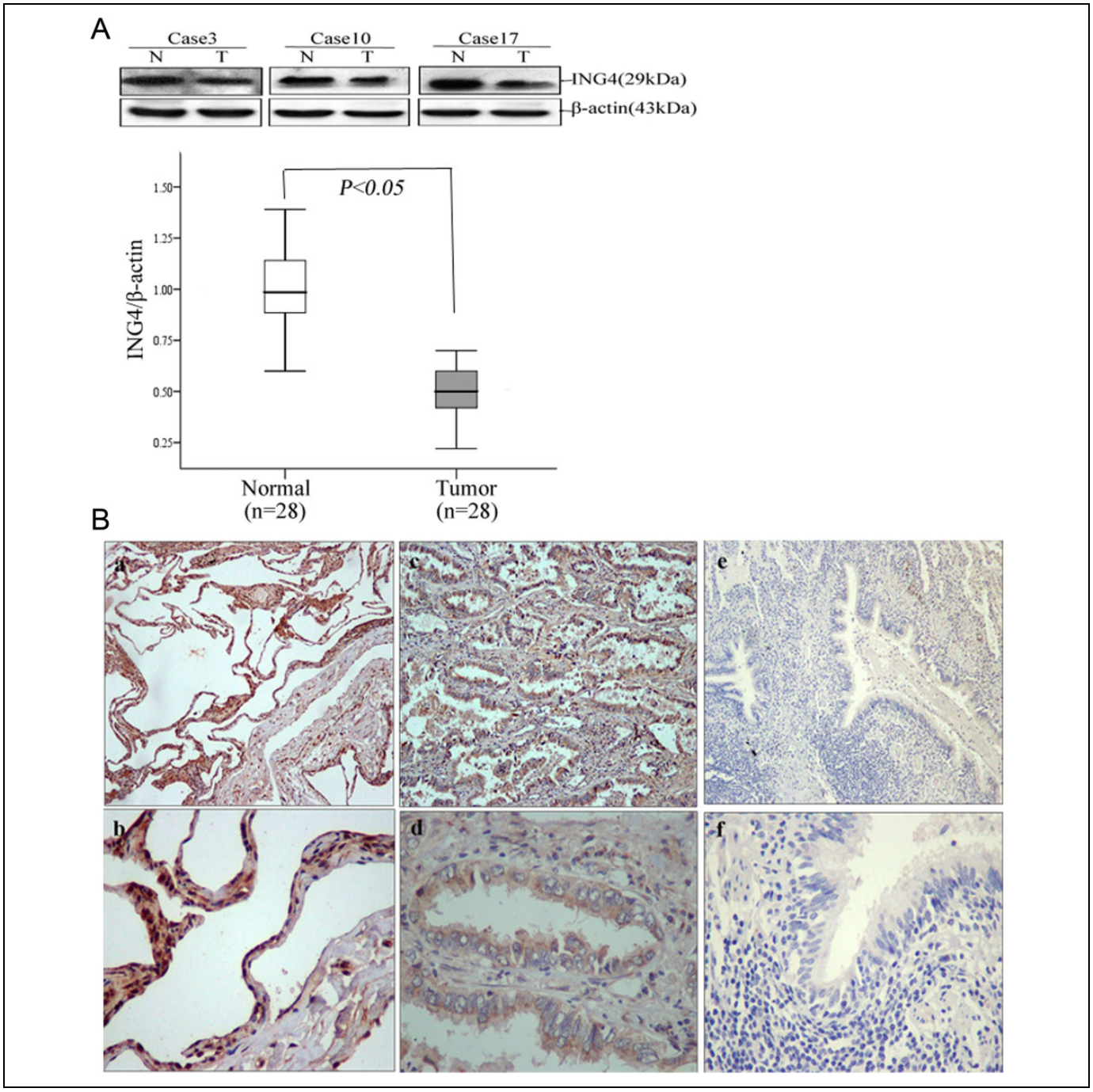

Figure 1. Expression of ING4 in human NSCLC cancers. A. Relative expression levels of ING4 protein were detected in 28 cases of NSCLC tissue samples and corresponding non-tumor lung tissues by immunoblotting. β-actin protein was used as an internal control. N, non-tumor tissues; T, NSCLC tissues. The data is representive of 3 independent experiments. B. Immunohistochemistry analysis of the expression of the ING4 protein in NSCLC tissues. Positive expression of ING4 was determined as brown staining of the nucleus and cytoplasm. Immunostaining of ING4 was strongly positive in corresponding non-tumor lung tissues (a, b), and was positive in the cytoplasm but not the nucleus in lung adenocarcinomas (c, d) or almost negative in squamous cell carcinomas (e, f). Original magnification, ×100.

subcutaneously (SC) into the posterior flank of nude mouse. Tumor growth was examined weekly for at least 4 weeks. After 28 days, the mice were killed and autopsies performed. Tumor volumes were calculated using the formula: $V\,(\text{in mm}^3) = A \times B^2/2$, where A is the largest diameter and B is the perpendicular diameter. For the radiosensitivity assay, 2 weeks after tumor cell implantation, SPC-A1 SC xenografted tumor-bearing mice were irradiated 10 Gy totally using an X-ray irradiator. Growth delay was calculated 15 days after X-ray irradiation. The primary tumor tissues were used to perform immunostaining

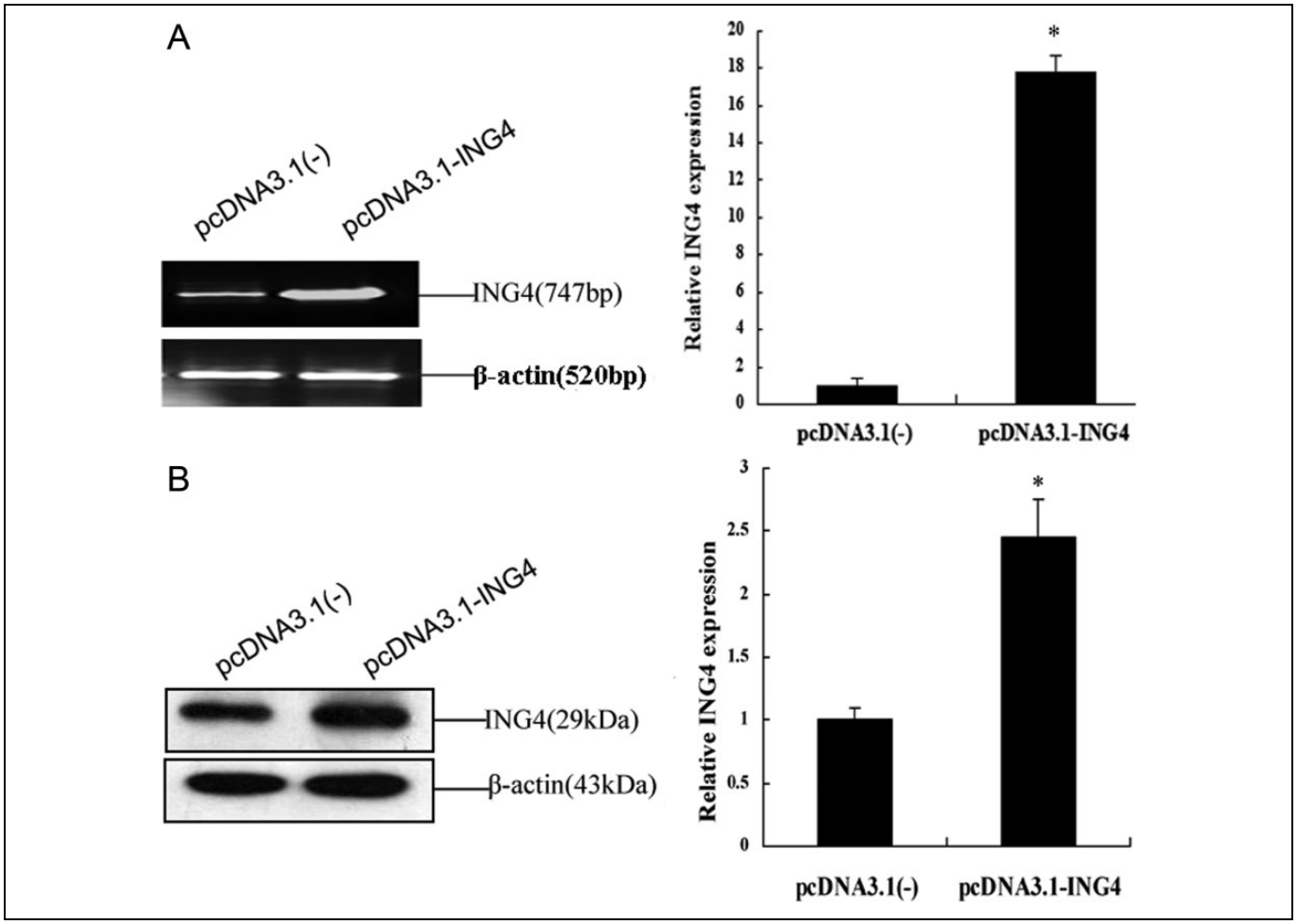

Figure 2. RT-PCR and western blot analysis of ING4 expression in SPC-A1/pcDNA3.1-ING4 compared with SPC-A1/pcDNA3.1. A. The epression of ING4 mRNA was upregulated in SPC-A1/pcDNA3.1-ING4 by RT-PCR and β-actin was used as an internal control. B. ING4 protein expression was upregulated in SPCA1/pcDNA3.1-ING4 by Western blotting and β-actin was used as an internal control.

analysis of proliferating cell nuclear antigen (PCNA), Bcl-2, and Bax protein.

Statistical Analysis

General statistical and survival analysis was performed by SPSS 10.0 program. Experimental data were expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by Student *t* test or analysis of variance. All tests performed were 2 sided. $P < .05$ was considered statistically significant.

Results

ING4 Is Significantly Downregulated in Human NSCLC Tissues

Firstly, the expression of ING4 protein in 28 cases of matched NSCLC and noncancerous lung tissue samples was detected by Western blot assays. As shown in Figure 1A, the average expression level of ING4 protein in NSCLC tissues was significantly lower than that in the corresponding nontumor lung tissues ($P < .05$). We confirmed these findings by immunostaining analysis of ING4 expression in NSCLC tissues. The ING4 protein was mainly located in the nucleus of normal lung epithelial or tumor cells (Figure 1B). Of note, we observed a significant reduction in the expression of ING4 in NSCLC tissues compared with normal tissues, while most tumor cells in the NSCLC samples showed little to no nuclear staining.

Interestingly, we found some tumors with ING4 expression in the cytoplasm but lack expression in the nucleus, which needs to be further investigated. These results were consistent with a recent report by Wang *et al*,[15] suggesting that downregulation of ING4 might play an important role in the initiation and progression of NSCLC.

The Expression of ING4 Was Significantly Upregulated in SPC-A1 Transfected by pcDNA3.1-ING4

After pcDNA3.1-ING4 or pcDNA3.1 was transfected into the SPC-A1 cells, ING4 expression in the transfected SPC-A1 cells

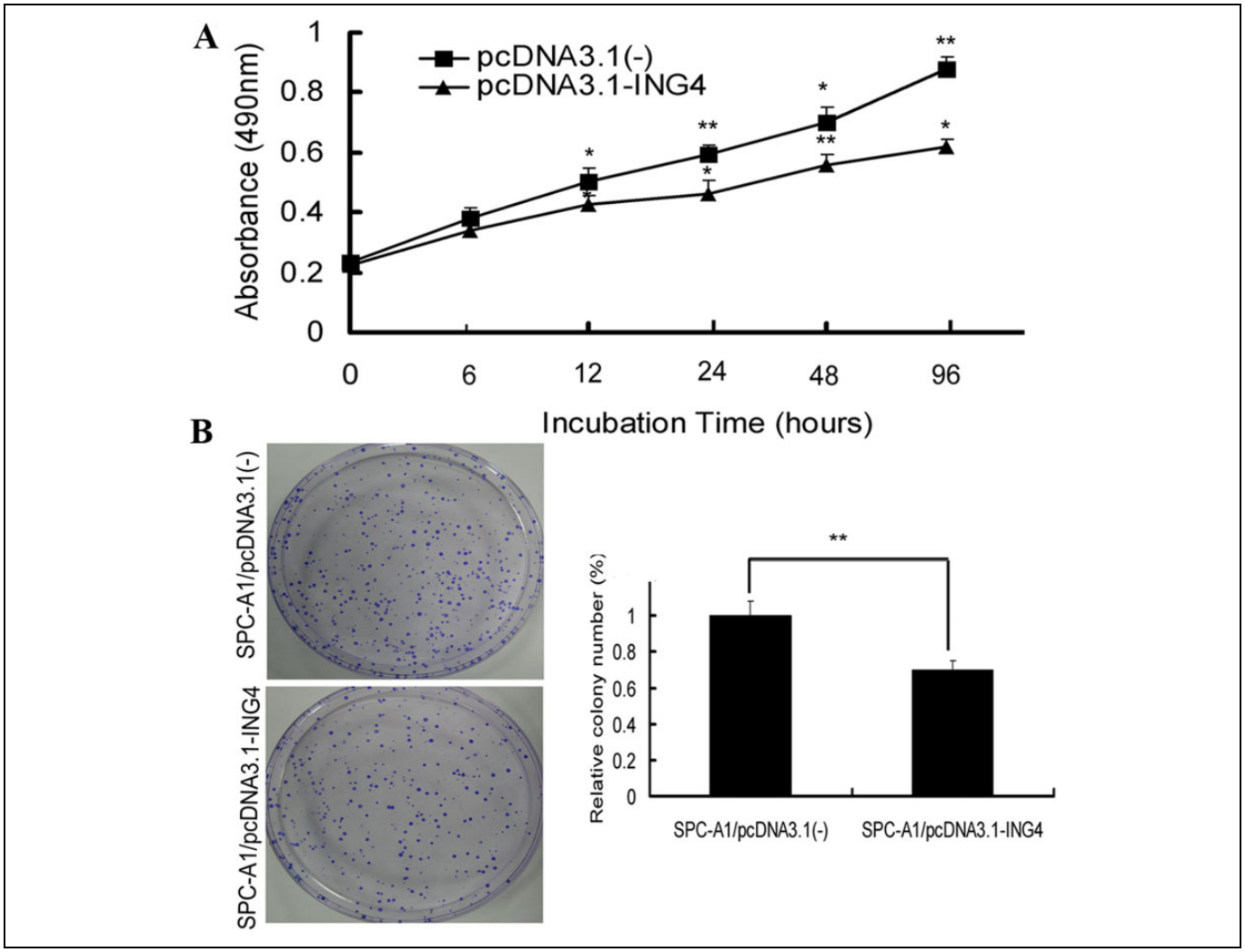

Figure 3. Ectopic ING4 expression inhibits *in vitro* growth of NSCLC cells. SPC-A1 cells were transfected with pcDNA3.1(-) or pcDNA3.1-ING4, respectively. A. At 24 h after transfection, the MTT assay was performed to determine the proliferation of SPC-A1 cells. B. Representative results of colony formation of SPC-A1 stably transfected with pcDNA3.1(-) or pcDNA3.1-ING4. Data represents the mean ± s.d from three independent experiments at a *, $P < 0.05$ and **, $P < 0.01$.

was detected by RT-PCR and Western blot analysis. Reverse transcription PCR and Western blot analysis revealed that the expression of ING4 at messenger RNA and protein level was obviously increased in SPC-A1/pcDNA3.1-ING4 compared with SPC-A1/pcDNA3.1 (Figure 2A and B). The above results confirmed the expression of the recombinant plasmids in transfected SPC-A1 with pcDNA3.1-ING4, indicating that the exogenous ING4 gene was successfully transfected into SPC-A1 cells.

Overexpression of ING4 Could Suppress In Vitro Growth of NSCLC Cells

To test whether ING4 may function as a tumor suppressor, MTT assay was performed to analyze the effect of ING4 upregulation on the *in vitro* growth of NSCLC. As shown in Figure 3A, SPC-A1/pcDNA3.1-ING4 cells grew more slowly than SPC-A1/pcDNA3.1 cells. To further testify the antiproliferative effect of ING4 on the growth of NSCLC cells, colony formation assay was performed. Compared with SPC-A1/pcDNA3.1 cells, the colony numbers of SPC-A1/pcDNA3.1-ING4 cells decreased by about 30% ($P < .01$; Figure 3B). Thus, the results of colony formation assay were consistent with those of MTT assay and further indicated that overexpression of ING4 could inhibit *in vitro* proliferation capacity of NSCLC cells.

Overexpression of ING4 Triggered Apoptosis in NSCLC Cells

To determine whether apoptosis was at the source of the growth inhibition, we further analyzed the apoptosis of SPC-A1 cells with or without exogenous *ING4* gene by flow cytometric analysis. Annexin V FITC/PI staining of SPC-A1/

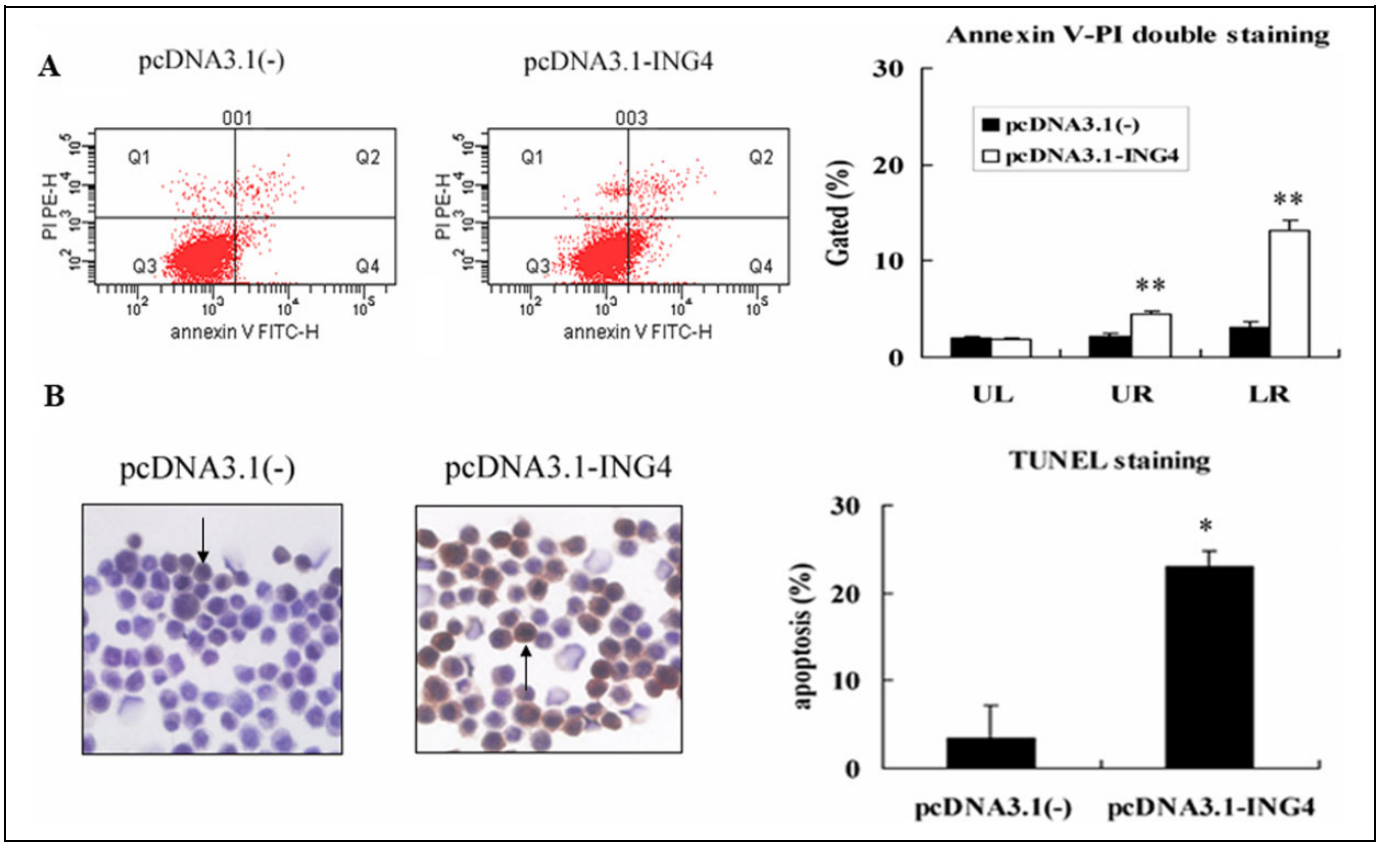

Figure 4. Ectopic ING4 expression enhances apoptosis in NSCLC cells. After transfection with pcDNA3.1(-) or pcDNA3.1-ING4, SPC-A1 cells were collected for apoptosis analysis. A. The apoptotic rates of cells were detected by flow cytometry. B. TUNEL staining showed apoptosis of NSCLC cells. Arrows indicate apoptotic cells. The results were reproducible in three independent experiments. *, P <0.05 and **, $P < 0.01$.

pcDNA3.1-ING4 cells was analyzed by flow cytometry, and a significant increase in the level of apoptotic cells was observed (Figure 4A). In the SPC-A1/pcDNA3.1-ING4 cells, the rates of early (lower right quadrant) and late (upper right quadrant) apoptosis were 13.24% ± 1.26% and 4.52% ± 0.34%, respectively. In contrast, the rates were 3.17% ± 0.38% and 2.10% ± 0.54%, respectively, in empty vector transfection ($P < .01$). We confirmed these results by performing a TUNEL assay where we noted a ~6-fold increase in levels of apoptotic cells in ING4-transfected SPC-A1 cells (23.13% ± 3.62%) compared with pcDNA3.1(−) (3.52% ± 1.09%; $P < .05$; Figure 4B). Taken together, these results suggest that ectopic expression of ING4 can trigger apoptosis in NSCLC cells.

Effect of ING4 on the In Vitro Sensitivity of NSCLC Cells to Irradiation

The aberrant activation of survival pathways and inactivation of apoptosis pathways in tumor cells can enhance resistance to anticancer treatment. To explore the role of ING4 in cell survival and response to radiotherapy, SPC-A1 cells were transfected with the pcDNA3.1-ING4, and their radiosensitivity to X-ray irradiation was assessed by MTT and flow cytometry assays. We first compared the survival fraction of the cells with escalating doses of irradiation (0-10 Gy). As shown in Figure 5A, there was a significant difference in survival fraction between SPC-A1/pcDNA3.1-ING4 and SPC-A1/pcDNA3.1 cells. SPC-A1/pcDNA3.1-ING4 cells were significantly more sensitive to the cytotoxic effects of X-ray irradiation as shown by a reduction in cell survival rate at 4.0, 6.0, 8.0, and 10.0 Gy ($P < .05$; Figure 4A). The D_{37} value of SPC-A1/pcDNA3.1-ING4 cells was 5.5 Gy, whereas in control cells, it was 8.7 Gy (Table 1).

Next, we measured the apoptosis in irradiated SPC-A1 cells by flow cytometry. Compared with irradiation alone, transfection of pcDNA3.1-ING4 enhanced the radiosensitivity of SPC-A1 cells. Twenty-four hours after 6.0 Gy single-dose irradiation, the apoptotic rate of SPC-A1/pcDNA3.1-ING4 was nearly 3 times higher (28.02% ± 1.84%) relative to empty vector control (10.16% ± 0.68%; $P < .05$; Figure 5B). While at 48 hours, the apoptotic rate was almost identical (Figure 5C). In conclusion, our data revealed that upregulation of ING4 could increase the *in vitro* sensitivity of SPC-A1 cells to X-ray irradiation via the induction of apoptosis.

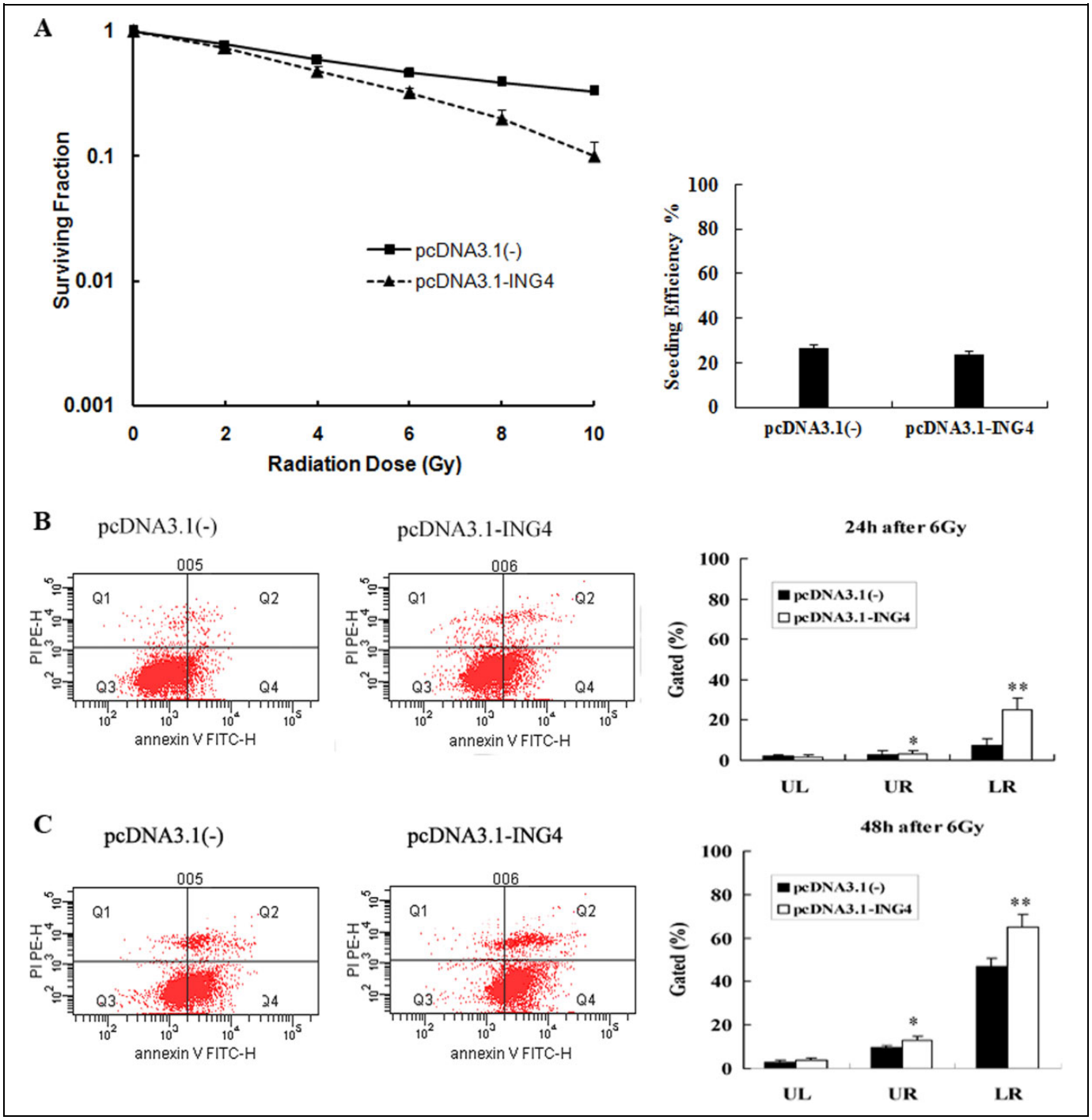

Figure 5. Ectopic ING4 expression enhances radiosensitivity of NSCLC cells. A. Radiation cell survival curves of SPC-A1 cells after pcDNA3.1(-) and pcDNA3.1-ING4 transfection. The results were reproducible in three independent experiments. At 24 h B and 48h C after radiation, SPC-A1/pcDNA3.1(-) and SPC-A1/pcDNA3.1-ING4 cells were collected for apoptosis analysis. The apoptotic rates of cells were detected by flow cytometry. All experiments were repeated in triplicate with similar results. *, P <0.05 and **, P < 0.01.

Effect of ING4 on the In Vivo Sensitivity of NSCLC Cells to Irradiation

As overexpression of ING4 could increase the radiosensitivity of SPC-A1 cells *in vitro*, we evaluated the effect of combining ectopic ING4 expression and localized radiation therapy *in vivo*. SPC-A1 cells transfected with pcDNA3.1-ING4 or pcDNA3.1(−) vector were injected into female nude mice, respectively. Nude mice with established tumor xenografts were treated with localized X-ray irradiation. Interestingly, we found that tumors derived from SPC-A1 cells/pcDNA3.1-ING4 grew substantially slowly, compared to the control mice

Table 1. The Survival Curve Parameters of SPC-A1 Cells After Radiation.

	SF_{2Gy}	D_{37}[a]	*P* Value
pcDNA3.1(−)	0.79	8.7	
pcDNA3.1-ING4	0.74	5.5	.01

[a]D_{37} called the "mean lethal dose," is the dose on the straight-line portion of the survival curve to decrease the survival to 37%.

during the entire tumor growth period. After 4-week inoculation, average volume of tumors derived from SPC-A1/pcDNA3.1-ING4 cells was 2 times (810 ± 36 mm^3) smaller than those inoculated with empty vector (SPC-A1/pcDNA3.1[−]; 1630 ± 51 mm^3; $P < .05$; Figure 6A). The apoptotic rate of tumors measured by TUNEL assay in SPC-A1/pcDNA3.1-ING4 (20.83% ± 2.24%) cells was approximately 6-fold than that of tumors in control cells (3.50% ± 1.51%; $P < .01$; Figure 6B).

To investigate whether the upregulation of ING4 could potentiate the inhibition of tumor growth by irradiation *in vivo*, tumor-bearing mice were irradiated with 10 Gy of X-ray. The results of the tumor growth curve showed that tumor growth was retarded by radiation alone (Figure 6A). Moreover, the therapeutic effect of ING4 overexpression was more significant than that of radiation alone. Furthermore, the radiation effect on tumor xenografts was enhanced by combined ING4 overexpression. Exposure to 10 Gy irradiation treatment resulted in a decrease in the volume of the xenografts to 615 ± 43 vs 942 ± 18 mm^3 ($P < .05$, Figure 6A). These results suggest that ING4 was significantly associated with *in vivo* proliferation and apoptosis capacity of NSCLC cells, and restoration of ING4 function reversed the radioresistance of NSCLC cells to X-ray irradiation.

Effects of ING4 on the Survival Pathways of NSCLC Cells

To further explore the mechanisms underlying the ING4-induced growth suppression and proapoptotic function in SPC-A1 cells, we analyzed the expression of apoptosis-related proteins by immunoblotting. Activation of caspase-3 was found in SPC-A1/pcDNA3.1-ING4 cells compared with SPC-A1/pcDNA3.1(−) cells, suggesting that the ectopic expression of ING4 might induce apoptosis in NSCLC cells in a caspase-3–dependent manner (Figure 7A). We also measured the expression of the antiapoptotic protein, Bcl-2, and found that levels of Bcl-2 decreased with overexpression of ING4 (Figure 7A). Immunostaining analysis revealed that the positive rate of PCNA in SPC-A1/pcDNA3.1-ING4–positive tumors significantly decreased relative to controls (Figure 7B), suggesting that ING4 downregulates proliferation *in vivo*. We also assessed the levels of Bcl-2 and Bax in nude mice xenografts by immunohistochemistry. We detected Bcl-2 and Bax at the plasma membrane and in the plasma (Figure 7B). Higher expression of Bax and lower expression of Bcl-2 were observed in xenografts of SPC-A1/pcDNA3.1-ING4 compared with those of SPC-A1/pcDNA3.1(−).

Discussion

Inhibitor of growth family members play important roles in tumorigenesis, cell apoptosis, and angiogenesis. Inhibitor of growth 4 was first identified in 2003 by searching for homology with ING1, the founding member of ING family proteins.[6] Inhibitor of growth 4 was found to be significantly downregulated in lung carcinoma,[12] breast cancer,[14] head and neck squamous cell carcinomas,[11] colorectal cancer,[13] and astrocytoma,[19] corroborating our findings in NSCLC. In this study, we showed that ING4 expression was markedly decreased in NSCLC tissues compared with corresponding nontumor lung tissues. Moreover, most tumor cells in the NSCLC samples showed little to no nuclear staining, indicating that ING4 gene was associated with initiation and progression of lung cancer. Taken together, ING4 gene could be served as a diagnostic marker for human malignancies. As the size of tissue samples in this study was limited, further investigation in a larger patient cohort is necessary to confirm its clinical significance in NSCLC. Evidence also shows that the ING4 gene is frequently mutated in various cancer cell lines.[17] The N214D mutation dramatically dampened the ability of ING4 to inhibit proliferation, anchorage-independent growth, or cell migration or to sensitize to cell death. The N214D mutation also correlates with reduced protein stability of ING4 due to increased proteasome-mediated degradation.[17] Recently, Li and his colleagues reported that ING4 was induced by breast cancer melanoma suppressor 1–inhibited melanoma angiogenesis.[23] However, the molecular mechanism for ING4 downregulation in human cancers is currently unclear and needs to be further elucidated.

Inhibitor of growth 4 is known to bind *p53* via its NLS region and enhance the transcriptional activity of *p53*.[6,32] Recent studies found that transient ING4 expression, which is dependent on Myc and Phosphatase and Tensin Homolog (PTEN), is absolutely required for the prostate epithelial differentiation. And ING4 is lost in the majority of human prostate cancers.[33] As a candidate tumor suppressor gene, ING4 may affect tumor inhibition via multiple pathways. Overexpression of ING4 enhanced the chemosensitivity of cells to the anticancer agents, CDDP, doxorubicin, and etoposide in hepatocellular carcinoma cells.[20,21] Meanwhile, Xie *et al* showed that adenovirus-mediated ING4 plus cisplatin (CDDP) has an *in vitro*- and *in vivo*-enhanced antitumor effect associated with the cooperative regulation of extrinsic and intrinsic apoptotic pathways in hepatocarcinoma cells SMMC-7721.[21] Our previous studies have found a decreasing level of ING4 expression occurred in docetaxel-resistant lung adenocarcinoma tissues. And overexpression of ING4 enhanced the sensitivity of SPC-A1 cells to docetaxel.[31,34] In the present study, we found that overexpression of ING4 could significantly affect the growth of NSCLC cells both *in vitro* and *in vivo*, and the SPC-A1 cell growth inhibition by

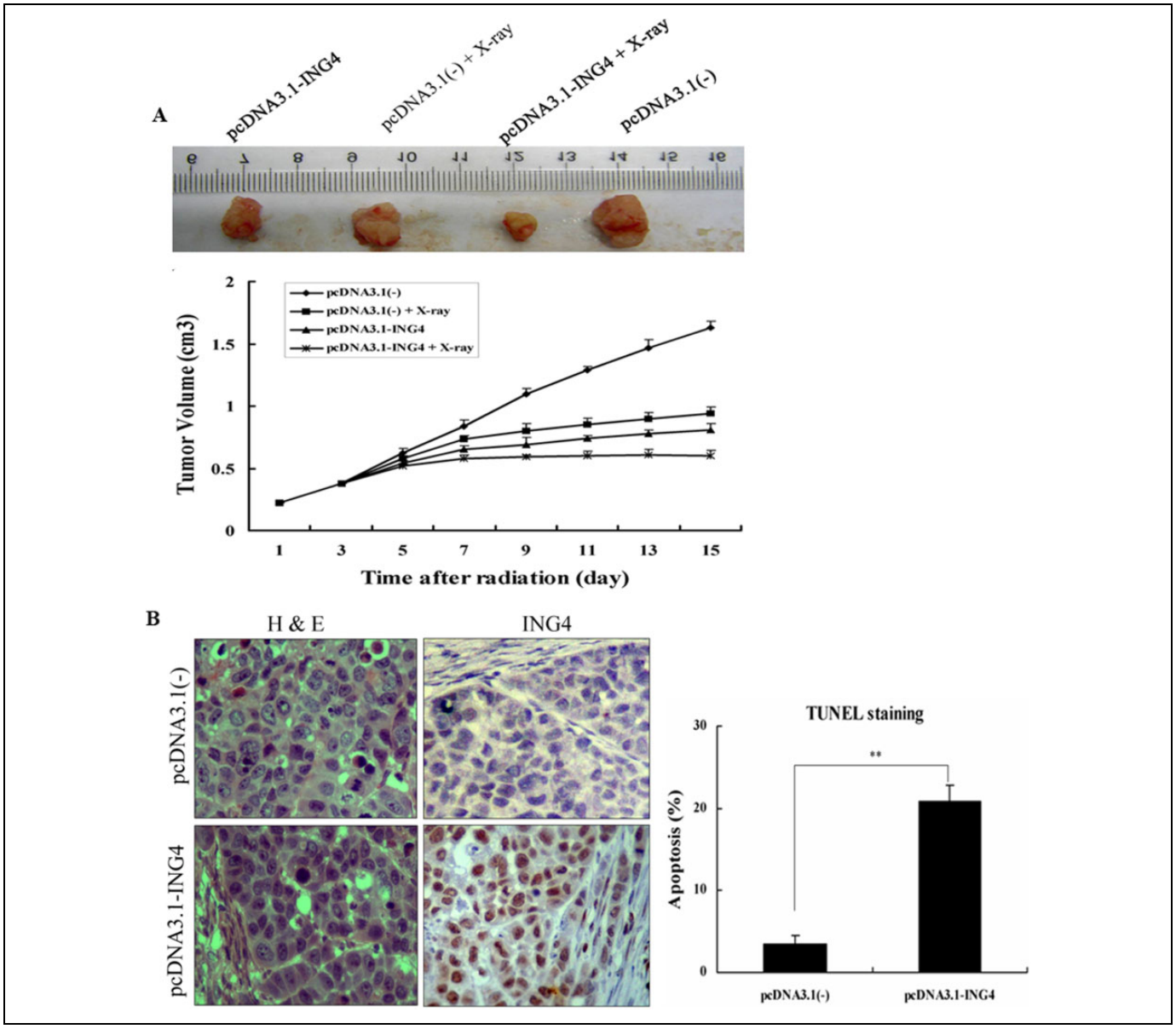

Figure 6. Ectopic ING4 expression induces radiosensitivity *in vivo*. A. Tumor growth curves measured after injection of SPC-A1 cells stably transfected with pcDNA3.1(-) or pcDNA3.1-ING4. The tumor volume was calculated every 2 days with or without X-ray. B. Left: The expression of ING4 in xenografts derived from SPC-A1/pcDNA3.1-ING4 was significantly overexpressed compared with SPC-A1/pcDNA3.1(-). Right: TUNEL staining was performed to determine the apoptosis of NSCLC xenografts derived from SPC-A1 cells. All experiments were repeated in triplicate with similar results. *, $P < 0.05$ and **, $P < 0.01$.

ectopic ING4 expression might be associated with caspase-3–dependent apoptosis enhancement. In addition, our findings showed that overexpression of ING4 in NSCLC cells led to a reversal of the Bcl-2/Bax ratio, which finally induced activation of caspase-3.

Functional genomics screening utilizing mutant mouse embryonic stem cells identified that ING4 was implicated in radiation response.[35] Zhao *et al* found that the combination of ING4 gene and ^{25}I radiation therapy had a synergistic effect in pancreatic cancer cell growth suppression and apoptosis induction.[36] Until recently, very few studies have been performed to explore the molecular mechanisms of ING4 in modulating the radiosensitivity to X-ray irradiation in NSCLC. In this study, our functional results confirmed that exogenous ING4 significantly increased the radiosensitivity of SPC-A1 cells to X-ray irradiation *in vitro* and *in vivo*. Therefore, these data suggest that appropriate combination of X-ray application with ING4 might be a potential approach to NSCLC therapy. For higher dose, X-ray would produce potentially serious toxic effects such as radiation esophagitis and lung fibrosis, and combination of X-ray with ING4 upregulation for the treatment of NSCLC would contribute to lower dose X-ray exposure and

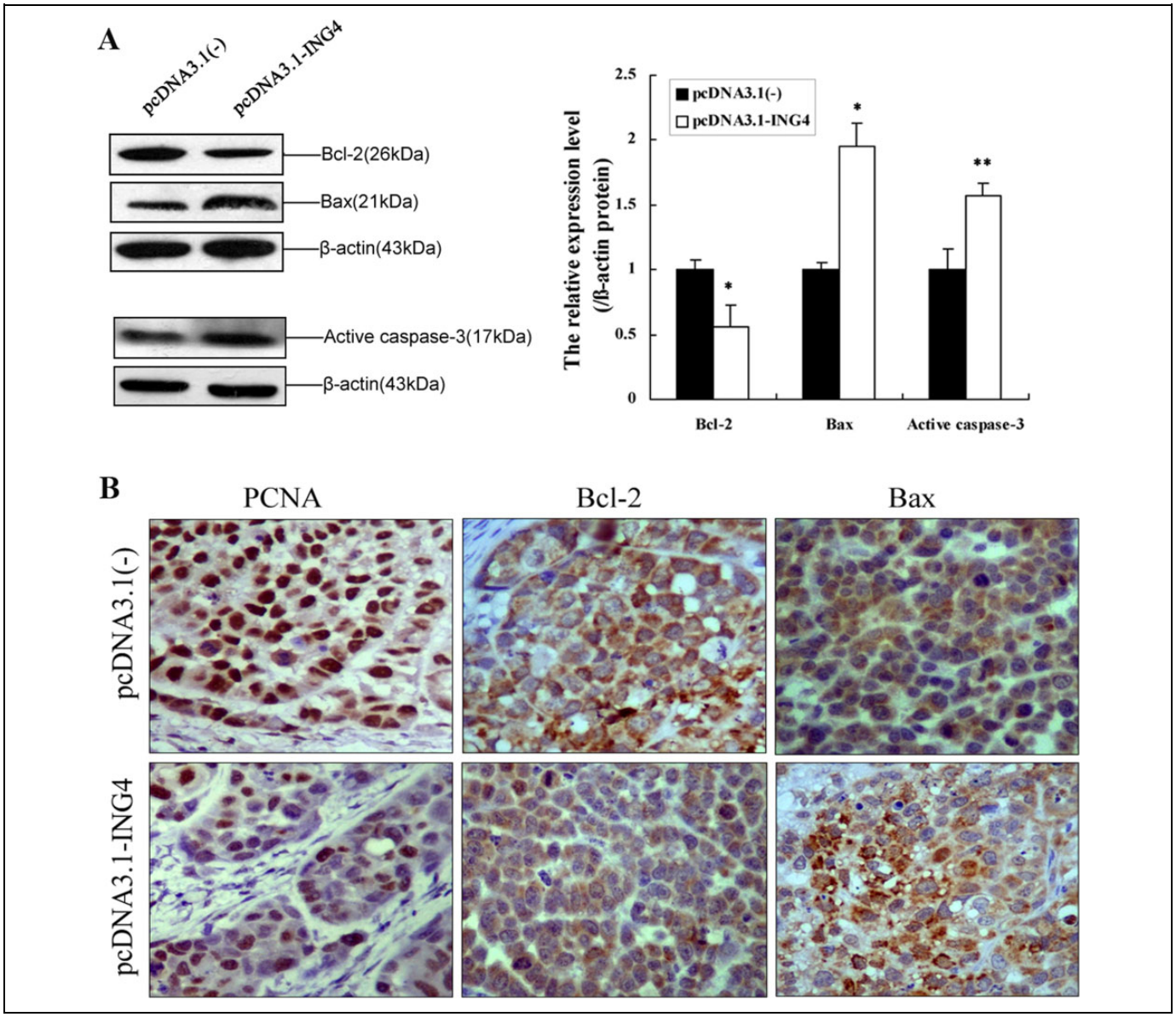

Figure 7. Expression analysis of Bcl-2, Bax, activated caspase-3 and PCNA after pcDNA3.1-ING4 transfection. A. Western blotting analysis for determining expression of Bcl-2, Bax, activated casepase-3 in SPC-A1 cells after pcDNA3.1-ING4 transfection *in vitro*. β-actin protein was used as an internal control. B. Immunostaining was used to detect PCNA, Bcl-2 and Bax-2 in the xenografts of nude mice *in vivo*.

result in a reduction of X-ray toxic side effects. However, whether the upregulation of ING4 in human SPC-A1 cells enhanced radiosensitivity through mitochondrial apoptosis pathway needs to be further elucidated.

Many experiments investigated that ING4 also plays a critical role in tumor invasion and migration. Garkavtsev *et al* firstly reported that ING4 could inhibit brain tumor growth and angiogenesis by associating with p65 (RelA) subunit of NF-κB.[10] Inhibitor of growth 4 and NF-κB interact, but the interaction doesn't prevent NF-κB activation or DNA-binding activity. Inhibitor of growth 4 could attenuate NF-κB–mediated activities to reduce the expression of NF-κB–regulated genes, Cox-2 and MMP-9.[10,23] Li and his colleagues reported inhibited melanoma angiogenesis by suppressing NF-κB activity and IL-6 expression.[23] Therefore, we propose that the absence of proper ING4 activities may enable already activated NF-κB to perpetually induce the expression of genes that contribute to tumorigenesis.

Conclusion

In summary, the current study revealed that frequent down-regulation of ING4 is observed in NSCLC and shown that ING4 functions as a tumor suppressor gene in NSCLC. Our experimental data may provide a new insight that combination of ING4 upregulation and X-ray irradiation might be a potential strategy for the treatment of human NSCLC in future.

Authors' Note

Xuan Pan, Rui Wang, and Zhaoxia Wang performed the research, analyzed the data, and drafted the manuscript. Haibo Bian performed the apoptosis assays and the statistical analysis. Wei De participated in the design of the study. Ping Zhang performed the immunostaining assays. All authors approved the final version of the manuscript. Xuan Pan and Rui Wang contributed equally to this work and should be regarded as joint first authors.

Acknowledgments

Thanks to every one of the Department of Biochemistry and Molecular Biology, Nanjing Medical University for their sincere help.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grants from the National Natural Science Foundation of China (No. 81472198, 81272601, 81502678).

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