Molecular Medicine

Identification of *ING4* (Inhibitor of Growth 4) as a Modulator of Docetaxel Sensitivity in Human Lung Adenocarcinoma

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Resistance to docetaxel (DTX) usually occurs in patients with lung adenocarcinoma. To better elucidate the underlying molecular mechanisms involved in resistance to DTX-based chemotherapy, we established a DTX-resistant lung adenocarcinoma cell line (SPC-A1/DTX). By gene array analysis, the expression of *ING4* was found to be significantly downregulated in SPC-A1/DTX cells. Additionally, the decreased expression of the *ING4* gene was induced upon DTX treatment of SPC-A1 cells. Overexpression of *ING4* reverses DTX or paclitaxel resistance of DTX-resistant lung adenocarcinoma cells (SPC-A1/DTX or A549/Taxol) by inducing apoptosis enhancement and G_2/M arrest, and small interfering RNA–mediated *ING4* knockdown renders DTX-sensitive lung adenocarcinoma cells more resistant to DTX or paclitaxel. Also, overexpression of *ING4* could enhance the *in vivo* sensitivity of SPC-A1/DTX cells to DTX. The phenotypical changes of SPC-A1/DTX cells induced by overexpression of *ING4* might be associated with the decreased ratio of Bcl-2/Bax, which resulted in the activation of caspase-3. The level of *ING4* expression in tumors of nonresponding patients was significantly lower than that in those of responders, suggesting that the expression of *ING4* was positively correlated with tumor response to DTX. Our results provide the first evidence that *ING4* might be essential for DTX resistance in lung adenocarcinoma. Thus, *ING4* will be a potential molecular target for overcoming resistance to DTX-based chemotherapies in lung adenocarcinoma.

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INTRODUCTION

Non–small cell lung cancer (NSCLC) has been one of the most lethal malignancies around the world. Adenocarcinoma of the lung is the most common type of lung cancer and accounts for 30% to 35% of primary lung tumors (1). Currently, systemic chemotherapy is still an important treatment option for patients with advanced lung adenocarcinoma (2). Docetaxel (DTX) has become an integral part of several commonly used chemotherapy regimens in NSCLC (3). However, the development of intrinsic or acquired resistance to DTX remains the greatest obstacle to the successful treatment of patients with lung adenocarcinoma. Thus, elucidating the mechanisms by which DTX resistance arises in lung adenocarcinoma remains a critical issue for overcoming and predicting DTX resistance in NSCLC.

Recently, a wealth of novel insights into molecular targets and mechanisms of cancer chemosensitivity and resistance have yielded much progress in genomic and proteomic studies (4). DTX (Taxotere™) is a novel microtubule-stabilizing agent that has been synthesized from a precursor extracted from a renewable natural source, the needles of the European yew, Taxus baccata (5). This agent can enhance microtubule assembly and inhibit the depolymerization of tubulin. In the clinical setting, DTX has been used for adjuvant therapy after resection of localized NSCLC and in combination with radiation for locally advanced NSCLC and treatment of patients with advanced NSCLC (6,7). However, the therapeutic results in some patients with advanced NSCLC have been unsatisfying, as in cases of intrinsic or acquired chemoresistance. The dysregulation of oncogenes such as Bcl-2 family members or tumor suppressors such as *PUMA* (p53 upregulated modulator of apoptosis) has been found to be associated with DTX resistance of tumor cells (8–10). However, the molecular mechanisms of DTX resistance are very complicated and require further elucidation.

Previously, we successfully established a DTX-resistant lung adenocarcinoma cell line (SPC-A1/DTX) from a DTX-nonresistant lung adenocarcinoma cell

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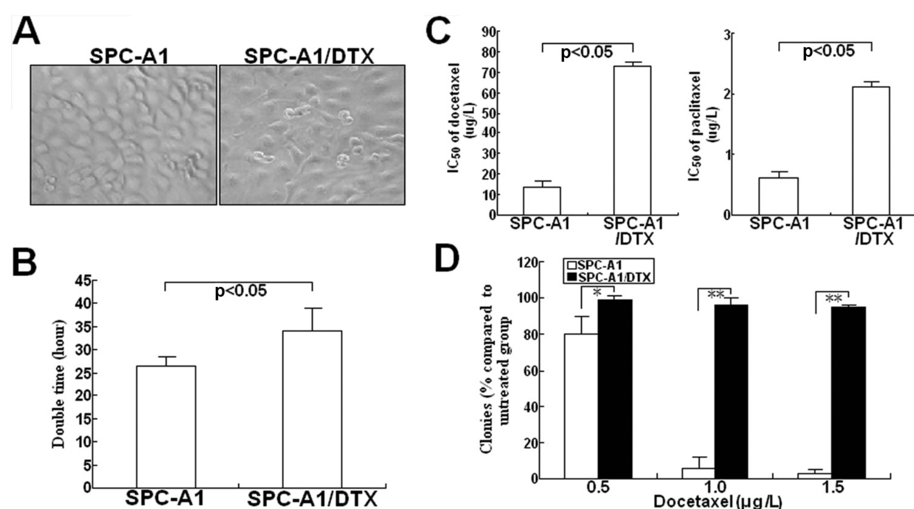

Figure 1. DTX-resistant cells (SPC-A1/DTX) were derived from lung adenocarcinoma cell lines (SPC-A1). (A) Morphologies of SPC-A1 and SPC-A1/DTX cells. Cells were grown to 80% confluency and then photographed under 40× magnification. (B) The doubling time of SPC-A1 and SPC-A1/DTX cell lines was 34.0 h and 26.5 h, respectively. (C) The IC_{50} values of DTX or paclitaxel in SPC-A1 and SPC-A1/DTX cells were determined. (D) The colony formation of SPC-A1 and SPC-A1/DTX cells treated with various concentrations of DTX (0.5, 1.0 and 1.5 μg/L). Results represent the average of three independent experiments (mean ± SD). *$p < 0.05$ and **$p < 0.01$ compared with parental SPC-A1.

line (SPC-A1). To gain further insight into the mechanisms of DTX resistance and explore novel potential therapeutic targets for reversing the DTX resistance of lung adenocarcinoma, we performed a microarray analysis on lung adenocarcinoma cells using the Affymetrix U133A microarray, which showed that a total of 2332 genes that were differentially expressed between the SPC-A1 and SPC-A1/DTX cell lines. Among these genes, *ING4* (inhibitor of growth 4) was found to be significantly downregulated in the SPC-A1/DTX cell line in comparison with the parental SPC-A1 cell line. *ING4*, a novel member of the inhibitor of growth (ING) gene family, has attracted much attention as a tumor suppressor because of its ability to suppress tumor growth, angiogenesis and invasion (11). In our previous studies, we also showed that downregulation of *ING4* was associated with poor prognosis of patients with lung adenocarcinoma (data not published). However, there have been no reports about the association of *ING4* expression with DTX sensitivity of lung adenocarcinoma.

In the present study, we attempted to investigate the roles of *ING4* in docetaxel-induced drug resistance and its possible molecular mechanisms. Herein, we report our finding that restoration of *ING4* expression could reverse the resistance of NSCLC cells to DTX both *in vitro* and *in vivo* by inducing apoptosis enhancement and cell cycle G_2/M arrest. Also, the expression of *ING4* in advanced lung adenocarcinoma might be positively correlated with the response of patients to DTX. Taken together, our results indicate that *ING4* might be a key regulator of DTX resistance in lung adenocarcinoma cells and has the potential of being a therapeutic target for chemosenstization of lung adenocarcinoma.

MATERIALS AND METHODS

Cell Lines and Chemotherapeutic Reagents

The human lung adenocarcinoma cell lines (SPC-A1 or A549) and taxol-resistant human lung adenocarcinoma cell line (A549/Taxol) were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The DTX-resistant lung adenocarcinoma cell line (SPC-A1/DTX) was established and preserved in our lab. The DTX-resistant SPC-A1 cell line was selected by continuous exposure to increasing concentrations of DTX. DTX was added into exponentially growing cultures of SPC-A1 cells at a concentration of 0.008 μg/L and allowed to remain in the culture until cell growth resumed. The cultures were then split and treated again with progressively higher concentrations of DTX. Over the course of selection, the DTX concentration was increased to 5.0 μg/L. The resulting subline was designated as SPC-A1/DTX cells (SPC-A1/DTX). The taxol-resistant A549 cell line (A549/Taxol) was preserved in a 0.2-μg/mL final concentration of taxol according to the manufacturer's instruction. All cell lines were cultured in RPMI 1640 (GIBCO-BRL, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in humidified air at 37°C with 5% CO_2. DTX and paclitaxel and pancaspase inhibitor (Z-VAD-FMK) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of DTX and paclitaxel (1.0 μg/mL) were prepared with dimethyl sulfoxide and diluted with phosphate-buffered saline (PBS) to the required concentrations before each experiment.

Microarray Analysis

Total RNA from the lung adenocarcinoma cell line (SPC-A1) or the corresponding DTX-resistant lung adenocarcinoma cell line (SPC-A1/DTX) was isolated by use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometry methods. Complementary DNA (cDNA) microarray analysis was performed with reagents and according to protocols provided by Affymetrix (Santa Clara, CA, USA). Briefly, we prepared double-stranded

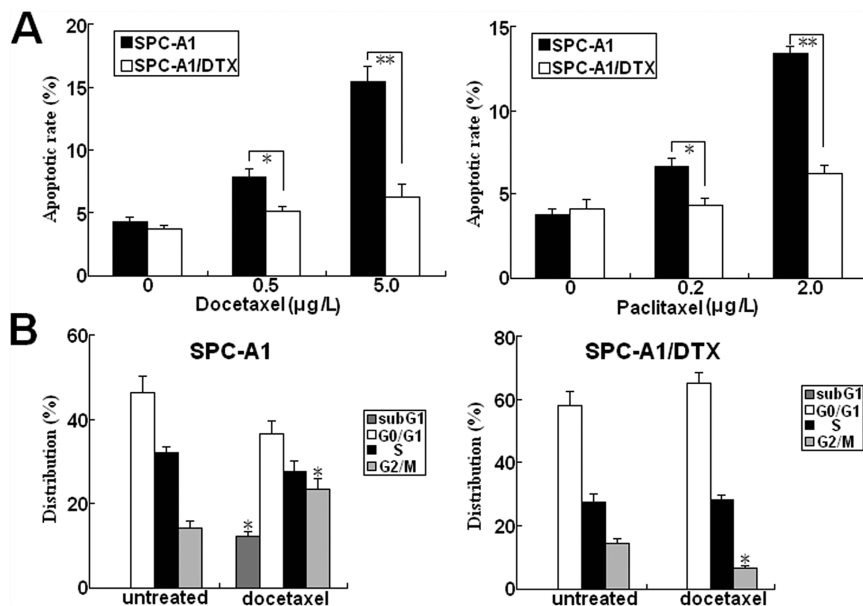

Figure 2. DTX or paclitaxel resistance is correlated with decreased apoptosis and changes in cell-cycle distribution on exposure to drugs. (A) Flow cytometric analysis of apoptosis in parental SPC-A1 and DTX-resistant SPC-A1/DTX cell in the presence of the indicated concentrations of DTX (0, 0.5, 5.0 μg/L) or paclitaxel (0, 0.2, 2.0 μg/L). (B) Flow cytometric analysis of cell cycle in parental SPC-A1 and DTX-resistant SPC-A1/DTX cell without or with DTX treatment (1.0 μg/L). Results represent the average of three independent experiments (mean ± SD). *$p < 0.05$ and **$p < 0.01$ compared with parental SPC-A1.

cDNA using the One-Cycle cDNA synthesis kit. The GeneChip IVT labeling kit was then used to synthesize biotin-labeled cRNA, which was then fragmented prior to hybridization. The labeled, fragmented cRNA samples were then hybridized to Affymetrix U133A microarrays, and the array was washed, stained and scanned with the Affymetrix GeneChip Scanner 3000. The acquired image was analyzed by the Affymetrix GeneChip operating software version 1.0. Differential cDNA expression was determined with a two-sided t test on a single cDNA basis. Differentially detected signals were generally accepted as true when the ratio of the p value was < 0.05 and were then selected for cluster analysis.

Real-Time Reverse Transcription–Polymerase Chain Reaction Assay

Total cellular RNA was prepared using TRIzol reagent (Invitrogen) and reversely transcribed according to the manufacturer's instruction. Real-time polymerase chain reaction (PCR) products were detected with SYBR Green I dye by using a Light Cycler instrument (Roche, Basel, Switzerland). The *GAPDH* gene was amplified as an internal control. Relative quantitation was done by using the ΔCt (threshold cycle) method by taking the difference (ΔCt) between the Ct of GAPDH and ΔCt of each transcript and computing $2^{-\Delta Ct}$. We obtained the sequences of primers for *ING4* by referring to Tzouvelekis *et al.* (12). The sequences of primers for the *ING4* gene were as follows: sense 5′-AGCTT GCCAT GCAGA CCT-5′; reverse 5′-GCGCA CGAGC TTTAA CTT-3′. Ct values were normalized to the reference gene *GAPDH*. The sequences of primers for the *GAPDH* gene (sense 5′-GAAGG TGAAG GTCGG ATGC-3′; reverse 5′-GAAGA TGGTG ATGGG ATTTC-3′) were designed by using the Primer Premier 5.0 software package.

Drug Sensitivity Assay

Single-cell suspensions were prepared and dispersed in 96-well plates. After incubation for 72 h with the DTX compounds (Sigma), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma) solution (0.5 mg/mL) was added. Following incubation for 4 h, 100 μL of extraction buffer was added to each well. After an overnight incubation, absorbance at 490 nm was measured with a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Construction of Plasmid Vectors

To ectopically express *ING4*, the *ING4* coding region (Genbank NM_016162) was subcloned into plasma control DNA (pcDNA)3.1(+) (Invitrogen) by use of a PCR method with the following primers: sense, 5′-GGGCT AGCAT GGCTG CGGGG ATGTA TTTG-3′; reverse, 5′-CCCTT AAGGA TAAAG AAGAA GGCAA GAAC-3′. The pSilencer4.1-CMVneo vector was obtained from Ambion (Austin, TX, USA), and DNA template oligonucleotides corresponding to the *ING4* gene and a negative control oligonucleotide having no homology with human beings or mice were designed and synthesized as follows: *shING4*, sense: 5′-GATCC GAGGC TGATC TCAAG GAGAA ATTCA AGAGA TTTCT CCTTG AGATC AGCCT CAGA-3′; negative control short hairpin RNA (shRNA), sense: 5′-AAGCT GAAGT ACAAC CTTCT TCAAG AGAGA AGGTT GTACT TCAGC TTAG-3′. All of the above sequences were inserted into the *Bgl*II and *Hin*d III enzyme sites of the pSilencer4.1-CMVneo vector, respectively. The recombinant plasmids were named pcDNA/ING4, pcDNA/control, pSil/shING4 and pSil/shcontrol, respectively.

Transfection and Stable Selection

The parental SPC-A1 or A549 and resistant SPC-A1/DTX or A549/Taxol cells were seeded into 6-well plates at 2.0 × 10^4 cells/well, respectively, and cultured overnight to 80% confluence prior to transfection. We performed transfection

Table 1. Differentially expressed genes with a >15.0-fold change in the DTX-resistant SPC-A1 cell line (SPC-A1/DTX) compared with the parental SPC-A1 cell line.

cDNA expression profiles					
Upregulated in SPC-A1/DTX			Downregulated in SPC-A1/DTX		
Gene symbol	Fold change[a]	P[b]	Gene symbol	Fold change[a]	P[b]
PDE1A	99.7	0.003	*SERPINB5*	60.9	< 0.001
SLC7A7	83.9	0.012	*LUM*	57.3	0.007
ALDH2	49.9	0.005	*ING4*	39.4	0.001
BIRC7	41.1	0.004	*CYP1A1*	33.8	0.003
TESCALCIN	37.5	0.021	*RASIP1*	24.5	0.006
MUC13	37.5	0.006	*RNASEK*	22.1	0.011
NOTCH1	34.5	< 0.001	*TMPRSS11F*	20.4	<0.001
CHST13	34.3	0.009	*GALR2*	18.7	0.006
STMN3	31.8	<0.001	*KRT15*	18.5	<0.001
KLHDC9	30.7	0.014	*RUNX3*	17.8	0.009
UBE2L6	30.5	0.001	*$ABCG_2$*	17.1	0.001
OLFM1	29.7	0.001	*$ABCC_3$*	16.5	0.006
FNDC5	22.8	0.003			
PTGDS	21.9	0.005			
CTSH	21.0	<0.001			
TCAM1P	20.7	0.018			
NAP1L5	18.5	0.007			
FAM18A	18.5	0.004			
KIR2DL1	17.5	0.002			

[a]Fold change values were generated from the median expression of the miRNAs in the groups compared.
[b]Student *t* test *p* values.

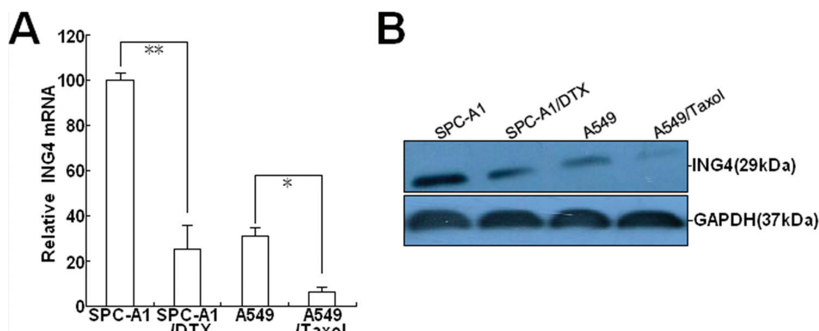

Figure 3. *ING4* was significantly downregulated in SPC-A1/DTX or A549/Taxol cell line. (A) Real-time quantitative RT-PCR analysis of *ING4* mRNA expression in SPC-A1 or SPC-A1/DTX and A549 or A549/Taxol cells. *$p < 0.05$ compared with SPC-A1 or A549 cell line. Results represent the average of three independent experiments (mean ± SD). (B) Western blot analysis of *ING4* protein expression in SPC-A1 or SPC-A1/DTX and A549 or A549/Taxol cells. GAPDH was used as an internal control.

using LipofectAMINE Plus (Grand Island, NY, USA) with standard transfection procedures. At 48 h posttransfection, 600 μg/mL G418 (Sigma) was added to select stable transfectants, and individual clones were isolated and maintained in a medium containing G418 (200 μg/mL). The stably transfected cells were named SPC-A1/DTX/ING4, SPC-A1/DTX/control, SPC-A1/shING4, SPC-A1/shcontrol, A549/Taxol/ING4, A549/Taxol/control, A549/shING4 and A549/shcontrol, respectively.

Western Blot Assay

We analyzed the levels of ING4, Bcl-2, Bax, procaspase-3, cleaved caspase-3 and GAPDH protein using standard Western blot procedures described previously (13) with rabbit anti-human ING4, Bcl-2, Bax, procaspase-3, cleaved caspase-3 and GAPDH primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively.

Flow Cytometric Analysis of Apoptosis

An annexin V-fluorescein isothiocyanate apoptosis detection kit (Oncogene Research Products, Boston, MA, USA) was used to detect apoptosis according to the manufacturer's instructions.

Flow Cytometric Analysis of Cell Cycle

Cells were harvested at the 70% confluent stage and fixed in 70% ethanol at –20°C. After being washed with PBS, the cells were treated with PBS containing RNase A (100 mg/mL) at 37°C for 30 min. After centrifugation, the cells were resuspended in PBS containing propidium iodide (50 μg/mL) and stained at room temperature for 30 min. DNA content was evaluated by use of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson).

Assay of Caspase-3 Activity

Caspase-3 activity was determined by use of the colorimetric CaspACE Assay System (Promega Corp., Madison, WI, USA) following the manufacturer's instructions, as previously reported (14). Each determination was performed in triplicate.

Immunohistochemistry

Paraffin-embedded, formalin-fixed tissues were immunostained for *ING4* and proliferating cell nuclear antigen (PCNA) proteins (Santa Cruz Biotechnology) by use of standard immunohistochemistry procedures described previously (15). Positive tumor cytoplasm results were

scored separately as follows: 0 = less than 5% of immunostained cells; 1 = 5–30% of positive cells; 2 = 30–60% of positive cells; and 3 = greater than 60% of positive cells.

In Vivo Chemosensitivity Assay

Animal studies were performed according to institutional guidelines. Approximately 5.0×10^6 SPC-A1/DTX/control or SPC-A1/DTX/ING4 cells were suspended in 100 μL PBS and injected subcutaneously into the right side of the posterior flank of female BALB/c athymic nude mice (Department of Comparative Medicine, Jinling Hospital, Nanjing, China) at 5 to 6 wks of age. Tumor growth was examined every other day with a vernier caliper. Tumor volumes were calculated by using the equation: $V = A \times B^2/2$ (mm^3), wherein A is the largest diameter, and B is the perpendicular diameter. When the average tumor size reached about 50 mm^3, DTX was given through intraperitoneal injection with a concentration of 1.0 mg/kg, 1 dose every other day with 3 doses totally. After 2 wks, all mice were killed, and necropsies were performed. The primary tumors were excised, paraffin embedded and formalin fixed. Then we performed hematoxylin and eosin staining and immunostaining analysis for PCNA protein expression and analyzed the apoptosis with a TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) apoptosis detection kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions.

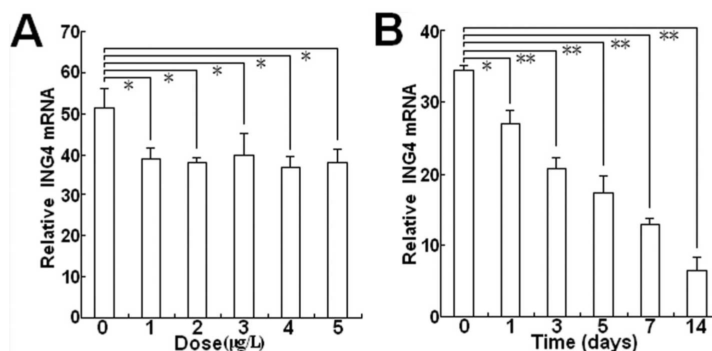

Figure 4. Induction of the decreased *ING4* expression by DTX in parental SPC-A1 cells. (A) SPC-A1 cells were cultured in the presence of various concentrations of DTX (0, 1, 2, 3, 4 or 5 μg/L) for 24 h. Real-time quantitative RT-PCR assay was performed to detect the expression of *ING4* mRNA. $*p < 0.05$ compared with 0 μg/L. (B) SPC-A1 cells were cultured in the presence of DTX (1.0 μg/L) for various time (0, 1, 3, 5, 7 or 14 d). Real-time quantitative RT-PCR was performed to detect the expression of *ING4* mRNA. Results represent the average of three independent experiments (mean ± SD). $*p < 0.05$ and $**p < 0.01$ compared with 0 d.

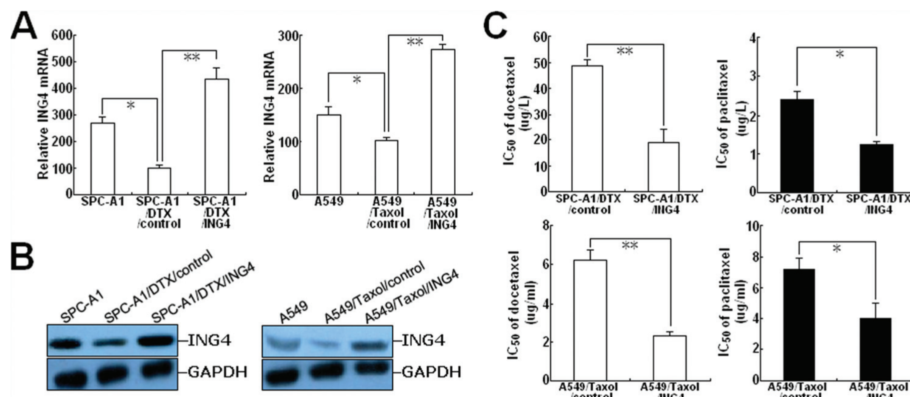

Figure 5. Effect of *ING4* overexpression on the *in vitro* sensitivity of SPC-A1/DTX or A549/Taxol cells to DTX or paclitaxel. (A) Real-time quantitative RT-PCR analysis of *ING4* mRNA expression in the stably transfected SPC-A1/DTX/ING4 (or SPC-A1/DTX/control or parental SPC-A1) and A549/Taxol/ING4 cells (or A549/Taxol or parental A549). (B) Western blot analysis of ING4 protein expression in the stably transfected SPC-A1/DTX/ING4 (or SPC-A1/DTX/control or parental SPC-A1) and A549/Taxol/ING4 cells (or A549/Taxol or parental A549). (C) MTT analysis of the IC_{50} values of DTX or paclitaxel in SPC-A1/DTX/ING4 (or SPC-A1/DTX/control) and A549/Taxol/ING4 (or A549/Taxol/control) cells. Results represent the average of three independent experiments (mean ± SD). $*p < 0.05$ and $**p < 0.01$ compared with SPC-A1/DTX/control or A549/Taxol/control cell line.

Patients and Tissue Samples

A total of 18 lung adenocarcinoma tissues were collected from patients with advanced lung adenocarcinoma who received chemotherapy at the Department of Medical Oncology, Jinling Hospital, between March 2005 and September 2006. Patients met all of the following criteria: suffering from primary lung adenocarcinoma; a histological diagnosis of lung adenocarcinoma with at least one measurable lesion; a clinical stage of IIIB–IV; first-line chemotherapy either with DTX 75 mg/m^2 and cisplatin 100 mg/m^2 or DTX 75 mg/m^2 and carboplatin AUC (area under the curve) 6 mg/mL/min administered every 3 wks for a maximum of 5 cycles; and availability of sufficient tumor tissue in paraffin blocks for assessment by immunohistochemistry. Tumor response was examined by computed tomography and evaluated according to the Response Evaluation Criteria in Solid tumors (RECIST) as a complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD), as described previously (16). Written permission to use human tumor tissues was obtained from the patients.

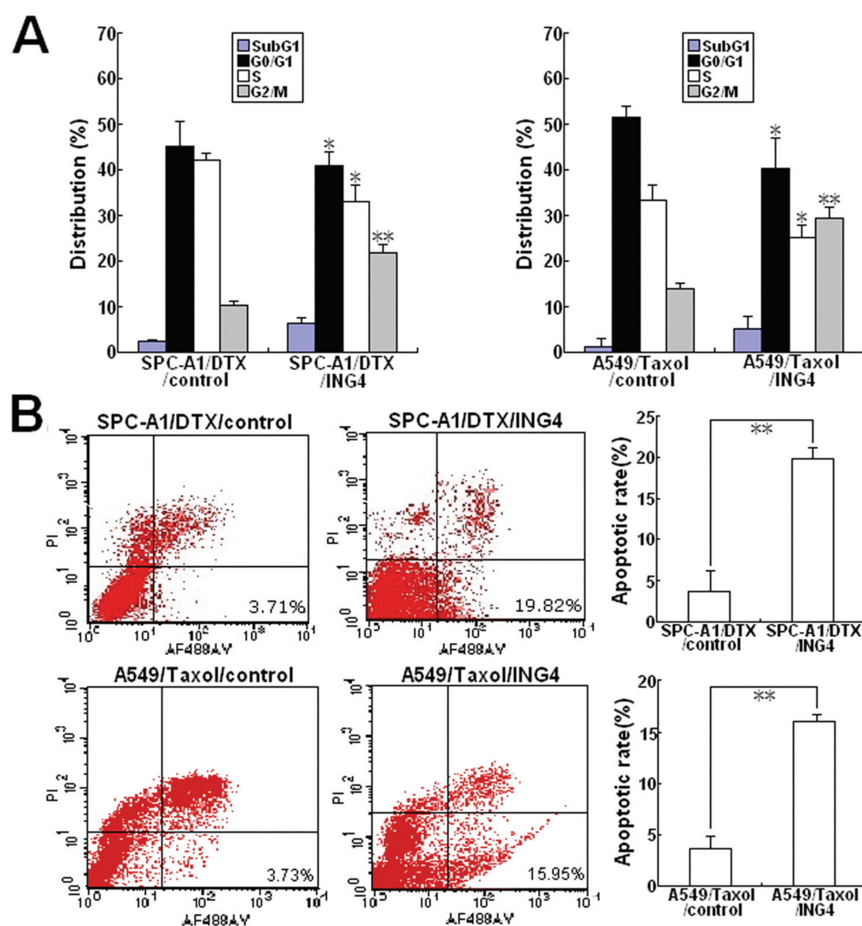

Figure 6. Effect of *ING4* overexpression on apoptosis and cell cycle of SPC-A1/DTX or A549/Taxol cells. (A) Flow cytometric analysis of cell cycle in SPC-A1/DTX/ING4 (or SCP-A1/DTX/control) and A549/Taxol/ING4 (or A549/Taxol/control) cells. (B) Flow cytometric analysis of apoptosis in SPC-A1/DTX/ING4 (or SPC-A1/DTX/control) and A549/Taxol/ING4 (or A549/Taxol/control) cells. Results represent the average of three independent experiments (mean ± SD). $*p < 0.05$ and $**p < 0.01$ compared with SPC-A1/DTX/control or A549/Taxol/control cell line.

Statistical Analysis

Experimental data were expressed as the mean ± SD of at least three independent assays. Statistical analyses were carried out using one-way ANOVA and Student t test to evaluate the continuous variables. Progression-free survival (PFS) was assessed from the first day of chemotherapy administration to the date of objective disease progression. The probability of survival was plotted by the Kaplan-Meier method and compared by the log-rank test. Differences between groups were considered significant at $p < 0.05$. All statistical analyses were performed using the SPSS 13.0 statistical software.

All supplementary materials are available online at www.molmed.org.

RESULTS

Analysis of Growth or Cytotoxicity of DTX in DTX-Nonresistant or -Resistant Lung Adenocarcinoma Cells

The DTX-resistant cell line (SPC-A1/DTX), was developed from the DTX-nonresistant cell line (SPC-A1). As observed by optical microscopy (Figure 1A), the morphology of the SPC-A1 and SPC-A1/DTX cells was significantly different and the SPC-A1/DTX cells appeared as large swellings or spindle- or rhombus-shaped cell forms. The doubling time of the SPC-A1/DTX cell line (34.0 h) was significantly longer than that of the SPC-A1 cell line (26.5 h) ($p < 0.05$; Figure 1B). With the relative resistance (as a resistance factor) calculated via the ratio of the half maximal inhibitory concentration (IC_{50})-resistant variant/IC_{50} of the parental cell line, the SPCA-1/DTX cell line was 5.37-fold resistant to DTX (13.58 μg/L) and was 3.46-fold resistant to paclitaxel (0.61 μg/L) (Figure 1C), suggesting that SPC-A1/DTX cell line acquired resistance to cross-resistant to paclitaxel. Colony formation assays also showed significant DTX resistance in the SPC-A1/DTX compared with the SPC-A1 cell line (Figure 1D). Therefore, the sensitivity of DTX was shown to be significantly different between the SPC-A1 and SPC-A1/DTX cell lines, a finding that might provide us better cell models to investigate the molecular mechanisms of DTX resistance in lung adenocarcinoma.

DTX Resistance of Lung Adenocarcinoma Cells was Correlated with Loss of the G_2/M Checkpoint in the Cell Cycle

To investigate whether resistance to DTX was correlated with drug-induced apoptosis and/or cell-cycle distribution, we performed a flow cytometry assay to detect the changes in apoptosis and cell cycles. In the SPC-A1/DTX cell line, there was a significant decrease in apoptosis on exposure to various doses of DTX in comparison with the parental SPC-A1 cell line (Figure 2A). Likewise, following treatment with various doses of paclitaxel, a significant reduction in the extent of cell death was also seen by comparison of parental SPC-A1 and SPC-A1/DTX cells. Next, the perturbation in cell cycle following exposure to DTX (1.0 μg/L) was analyzed. After 24-h exposure to DTX in parent SPC-A1 cells, the percentage of G_2/M-phase cells was significantly increased ($p < 0.05$), and an apoptosis

peak (sub-G1) could be seen before the G_1 phase. However, in DTX-resistant SPC-A1/DTX cells, there were no obvious changes in the sub-G1 population. Furthermore, the percentage of G_2/M-phase cells was obviously decreased in resistant SPC-A1/DTX cells relative to parental SPC-A1 cells ($p < 0.05$; Figure 2B). Therefore, it was concluded that loss of the G_2/M cell-cycle checkpoint function and abrogation of apoptosis might be involved in the acquired DTX resistance of lung adenocarcinoma cells.

Microarray Analysis of Differentially Expressed Genes Associated with DTX Resistance of Lung Adenocarcinoma Cells

To investigate the molecular mechanisms of DTX resistance in lung adenocarcinoma cells, we performed a microarray analysis on lung adenocarcinoma cells using the Affymetrix U133A microarray. The microarray data showed that a total of 2332 genes were differentially expressed between the SPC-A1 and SPC-A1/DTX cell lines (Supplementary Figure S1A; $p < 0.05$). By the fold-change analysis, we found that 338 or 31 of the 29,187 flagged cDNAs in SPC-A1/DTX cells showed at least a 4.0- or 15.0-fold change in expression level compared with parental SPC-A1 cells. Compared with SPC-A1 cells, 12 genes were significantly downregulated in SPC-A1/DTX cells, whereas the other 19 genes were significantly upregulated in SPC-A1/DTX cells (Table 1). Then, a real-time quantitative reverse transcription (RT)-PCR assay was employed to validate the top three downregulated and top three upregulated genes (*PDE1A*, *SLC7A7*, *ALDH2*, *SERPINB5*, *LUM*, *ING4*) between the two cell lines. The expression of the 6 genes showed concordance with the microarray data (Supplementary Figure S1B).

The Expression of *ING4* Gene is Downregulated in SPC-A1/DTX and A549/Taxol Cells

Our gene expression data showed that *ING4* was one of the top three

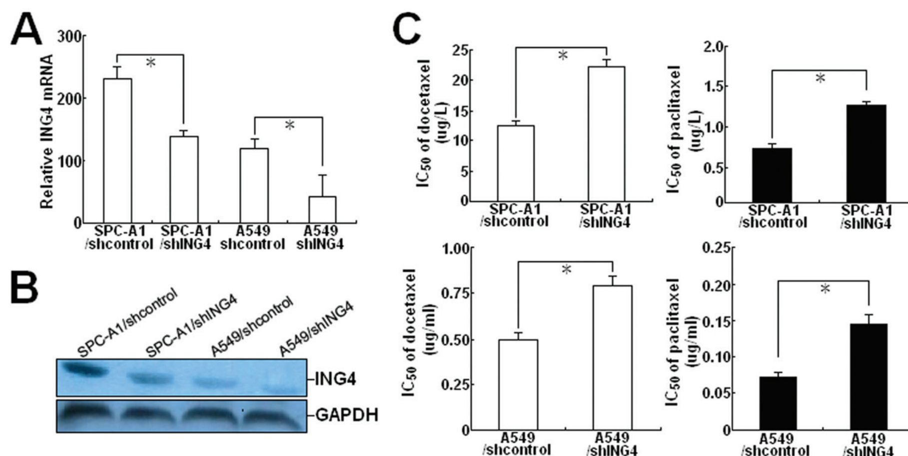

Figure 7. Effect of *ING4* downregulation on the *in vitro* sensitivity of SPC-A1 or A549 cells to DTX or paclitaxel. (A) Real-time quantitative RT-PCR analysis of *ING4* mRNA expression in the stably transfected SPC-A1/shING4 (or SPC-A1/shcontrol) and A549/shING4 (or A549/shcontrol) cells. (B) Western blot analysis of ING4 protein expression in the stably transfected SPC-A1/shING4 (or SPC-A1/shcontrol) and A549/shING4 (or A549/shcontrol) cells. (C) MTT analysis of the IC_{50} values of DTX or paclitaxel in SPC-A1/shING4 (or SPC-A1/shcontrol) and A549/shING4 (or A549/shcontrol) cells. Results represent the average of three independent experiments (mean ± SD). *$p < 0.05$ and **$p < 0.01$ compared with SPC-A1/shcontrol or A549/shcontrol cell line.

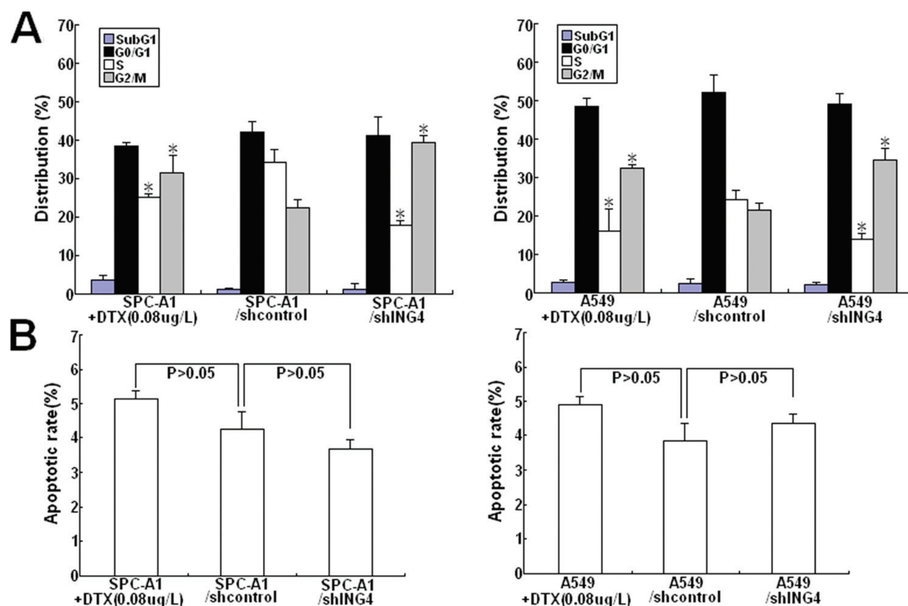

Figure 8. Effect of *ING4* downregulation on apoptosis and cell cycle of SPC-A1 or A549 cells. (A) Flow cytometric analysis of cell cycle in SPC-A1/shcontrol or A549/shcontrol, SPC-A1/shING4 or A549/shING4 and parental SPC-A1 or A549 cells combined with DTX treatment. *$p < 0.05$ compared with SPC-A1/shcontrol or A549/shcontrol cell line. (B) Flow cytometric analysis of apoptosis in SPC-A1/shcontrol or A549/shcontrol, SPC-A1/shING4 or A549/shING4 and parental SPC-A1 or A549 cells combined with DTX treatment. Results represent the average of three independent experiments (mean ± SD).

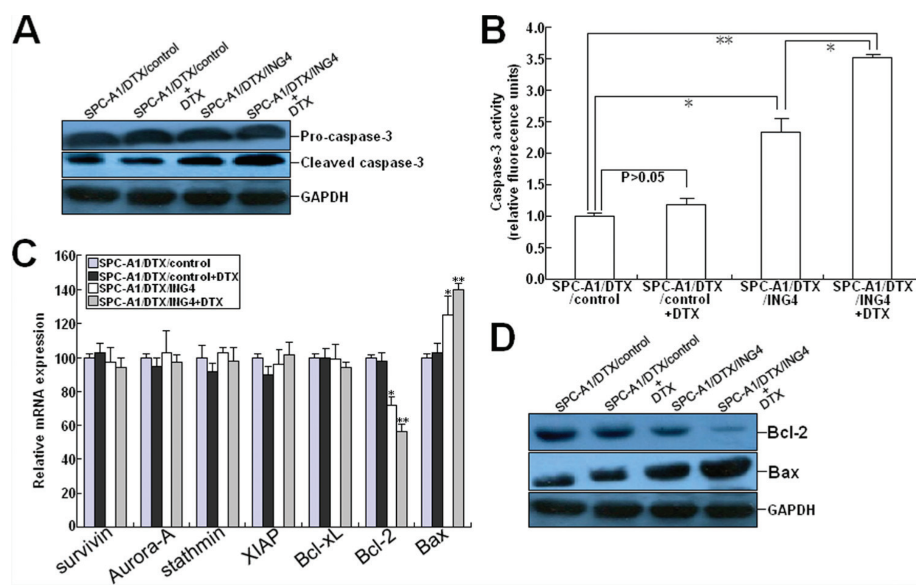

Figure 9. Effects of *ING4* overexpression on the survival pathway in SPC-A1/DTX cells. (A) Western blot detection of both procaspase-3 and cleaved caspase-3 proteins expression in stably transfected SPC-A1/DTX/control and SPC-A1/DTX/ING4 cells. Equal loading was confirmed by showing equal GAPDH levels. (B) Detection of caspase-3 activity of SPC-A1/DTX/control and SPC-A1/DTX/ING4 cells. *$p < 0.05$ and **$p < 0.01$ compared with SPC-A1/DTX/control or SPC-A1/DTX/ING4 cell line. (C) Real-time quantitative RT-PCR analysis of survivin, Aurora-A, stathmin, XIAP, Bcl-xL, Bcl-2 and Bax mRNA expression. *$p < 0.05$ and **$p < 0.01$ compared with SPC-A1/DTX/control or SPC-A1/DTX/ING4 cell line. (E) Western blot analysis of Bcl-2 and Bax protein expression. GAPDH was used as an internal control. Results represent the average of three independent experiments (mean ± SD).

downregulated genes in the resistant cell line. To confirm this finding, we performed real-time quantitative RT-PCR and Western blot assays to detect the expression of *ING4* in DTX-resistant SPC-A1/DTX and taxol-resistant A549/Taxol cell lines. As shown in Figure 3A, the relative level of *ING4* mRNA in the SPC-A1/DTX cell line was significantly lower than that in the parental SPC-A1 cell line ($p < 0.01$), and the A549/Taxol cell line showed significantly lower mRNA expression of *ING4* than the parental A549 cell line. Meanwhile, the expression of *ING4* protein was also significantly downregulated in the SPC-A1/DTX cell line, and the A549/Taxol cell line showed lower protein expression of *ING4* than the parental A549 cell line (Figure 3B). Thus, downregulation of *ING4* might be involved in the formation of DTX resistance in lung adenocarcinoma.

Decreased *ING4* mRNA expression Is Induced in the SPC-A1 Cell Line with DTX Treatment

To further determine whether *ING4* downregulation was involved in DTX-induced resistance in SPC-A1 cells, we investigated whether various concentrations of DTX or prolonged DTX treatment (1.0 μg/L) could induce the decreased expression of *ING4* mRNA. Upon treatment with various concentrations of DTX (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/L) for 24 h, the relative level of *ING4* mRNA expression was significantly decreased, but did not decreased sequentially with the concentration of exposure (Figure 4A). Upon treatment with prolonged DTX (1.0 μg/L) for 0, 1, 3, 5, 7 and 14 d, the relative level of *ING4* mRNA expression decreased sequentially with time of exposure (Figure 4B). Therefore, a decreasing level of *ING4* expression occurred in lung adenocarcinoma cells in response to DTX.

Overexpression of *ING4* Leads to the Increased Chemosensitivity of SPC-A1/DTX or A549/Taxol Cells to DTX

To investigate whether *ING4* expression affects the sensitivity of lung adenocarcinoma cells to DTX, a pcDNA/ING4 vector expressing *ING4* was stably transfected into SPC-A1/DTX and A549/Taxol cells. Compared with parental SPC-A1 or SPC-A1/DTX/control cells, the level of *ING4* mRNA or protein expression in SPC-A1/DTX/ING4 cells was significantly increased (Figures 5A, B). Likewise, the level of *ING4* mRNA or protein expression in A549/Taxol/ING4 cells was also significantly increased compared with A549 or A548/Taxol/control cells (Figures 5A, B). Then, the IC_{50} value of DTX or paclitaxel in SPC-A1/DTX or A549/Taxol cells was determined using an MTT assay. Compared with SPC-A1/DTX/control cells, the IC_{50} value of DTX or paclitaxel in SPC-A1/DTX/ING4 was significantly decreased by 58.6% or 36.8%, respectively (Figure 5C). Compared with A549/Taxol cells, the IC_{50} value of DTX or paclitaxel in A549/Taxol/ING4 cells was significantly decreased by 64.3% or 45.6%, respectively (Figure 5C). Next, we analyzed cell-cycle distribution in SPC-A1/DTX/ING4 or A549/Taxol/ING4 cells. Compared with the SPC-A1/DTX/control or A549/Taxol cell line, the SPC-A1/DTX/ING4 or A549/Taxol/ING4 cell line triggered an accumulation of cells at the G_2/M stage, whereas the numbers of cells in S-phase and G_0/G_1 phase accordingly decreased (Figure 6A). Then, we examined the changes in apoptosis. Compared with SPC-A1/DTX/control cells, the apoptotic rate of SPC-A1/DTX/ING4 cells was significantly enhanced by approximately 16.11% ($p <$ 0.05; Figure 6B). These results strongly suggest that overexpression of *ING4* could reverse the resistance of SPC-A1/DTX cells to DTX or paclitaxel by induc-

ing a G_2/M-phase arrest and apoptosis enhancement.

Downregulation of *ING4* Leads to the Decreased Chemosensitivity of SPC-A1 or A549 Cells to DTX

To extrapolate the finding that overexpression of *ING4* could reverse the chemoresistance of SPC-A1/DTX or A549/Taxol cells, we employed RNA interference to downregulate *ING4* expression and analyze the changes of chemosensitivity in sensitive SPC-A1 or A549 cells. Compared with the SPC-A1/shcontrol or A549/shcontrol cells, the level of *ING4* mRNA and protein expression in SPC-A1/shING4 or A549/shING4 cells was significantly decreased (Figures 7A, B). Then, we determined the IC_{50} value of DTX or paclitaxel using an MTT assay. Compared with SPC-A1/shcontrol cells, the IC_{50} value of DTX or paclitaxel in SPC-A1/shING4 cells was significantly increased by 39.6% or 73.7%, respectively ($p < 0.05$; Figure 7C). Additionally, compared with A549/shcontrol cells, the IC_{50} value of DTX or paclitaxel in A549/shING4 cells was significantly increased by 52.2% or 68.5%, respectively ($p < 0.05$; Figure 7C). Then, we analyzed the changes of cell-cycle distribution (Figure 8A). Compared with SPC-A1/shcontrol or A549/shcontrol cells, the numbers of SPC-A1/shING4 or A549/shING4 cells in G_2/M-phase accordingly decreased and the numbers of SPC-A1/shING4 or A549/shING4 cells in S-phase significantly increased ($p < 0.05$). However, the numbers of SPC-A1/shING4 or A549/shING4 cells in G_0/G_1-phase showed no difference ($p > 0.05$). In addition, it was also shown that the numbers of DTX (0.08 μg/L)-treated SPC-A1 or A549 cells in G_2/M-phase accordingly decreased and the numbers of DTX (0.08 μg/L)-treated SPC-A1 or A549 cells in S-phase significantly increased. In other previous research, it was shown that *ING4* could impede the progression of the cell cycle by regulating the expression of cell cycle regulators (such as p27, cyclinD1 and SKP2),

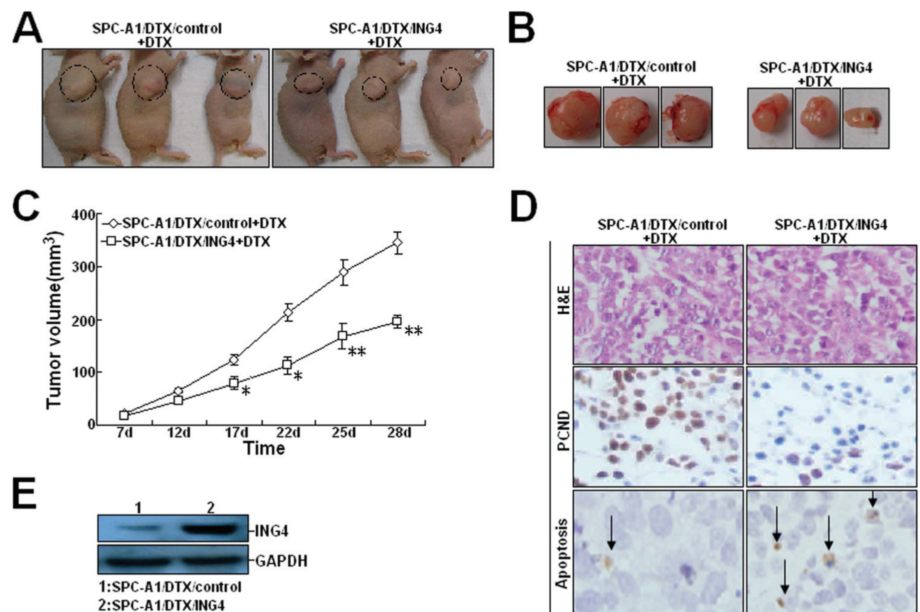

Figure 10. Effect of *ING4* expression on the *in vivo* sensitivity of SPC-A1/DTX cells to DTX. (A) After 28 d, SPC-A1/DTX/ING4 cells with DTX treatment produced smaller tumors than SPC-A1/DTX/control cells with DTX treatment. (B) Representative picture of tumors from SPC-A1/DTX/control or SPC-A1/DTX/ING4 group with DTX treatment. (C) The growth curve of tumor volumes. Each data point presents the mean ± SD of 6 mice. *$p < 0.05$ and **$p < 0.01$ compared with SPC-A1/shcontrol combined with DTX treatment. (D) Tumors developed from SPC-A1/DTX/ING4 cells with DTX treatment showed a lower level of PCNA antigen and increased level of apoptosis than tumors developed from SPC-A1/DTX/control cells combined with DTX treatment. Upper: H&E staining; Intermediate: immunostaining. Lower: TUNEL staining. (E). After 28 d, Western blot analysis of ING4 protein expression in tumors formed from SPC-A1/DTX/control or SPC-A1/DTX/ING4 cells. GAPDH was used as an internal control. Results represent the average of three independent experiments (mean ± SD).

which led to cell arrest in G_2/M phase (17). Next, flow cytometry was used to detect apoptosis. Compared with SPC-A1/shcontrol (or A549/shcontrol) cells, DTX-treated SPC-A1 (or A549) and SPC-A1/shING4 (or A549/shING4) cells showed no difference in apoptosis ($p > 0.05$; Figure 8B). Thus, the increased resistance of SPC-A1 or A549 cells to DTX or paclitaxel induced by *ING4* downregulation was associated with a decreased number of cells in G_2/M-phase and an increased number of cells in S-phase.

Effects of *ING4* Expression on the Survival Pathway of SPC-A1/DTX Cells

To investigate further insight into the mechanism of apoptosis enhancement induced by overexpression of *ING4*, we first determined the involvement of caspase-3. Compared with SPC-A1/DTX/control cells combined with DTX treatment (5.0 μg/L), the activation of caspase-3 protein was observed in SPC-A1/DTX/ING4 cells combined with DTX treatment (Figure 9A). Meanwhile, compared with SPC-A1/DTX/control cells combined with DTX treatment (5.0 μg/L), the activity of caspase-3 activity was significantly increased by approximately 251.3% in SPC-A1/DTX/ING4 cells combined with DTX treatment ($p < 0.01$; Figure 9B). Then, we used a quantitative RT-PCR assay to detect the mRNA expression levels of some apoptosis-related genes, including *survivin*, *Aurora-A*, *stathmin*, *XIAP*, *Bcl-2*, *Bcl-xL* and *Bax*, which has been reported to be associated with the sensitivity of tumor

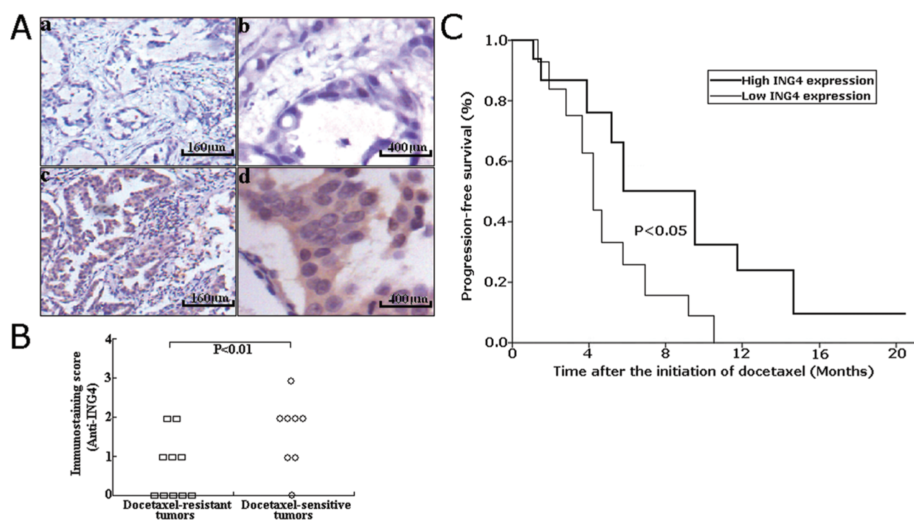

Figure 11. *ING4* expression was downregulated in DTX nonresponding tumors from lung adenocarcinoma patients. (A) Immunostaining of ING4 protein in lung adenocarcinoma tissues. Positive ING4 protein staining was mainly located in the nucleus of tumor cells. a, negative staining (20×); b, negative staining (100×); c, positive staining (20×); d, positive staining (100×). (B) Scores of ING4 immunostaining in lung adenocarcinoma tissues. Tumor tissues were obtained from 8 responding and 10 nonresponding patients. (C) Statistical analysis of PFS of lung adenocarcinoma patients according to the level of ING4 protein expression in tumor tissues. The *p* value was determined with the log-rank test. Scale bars: 160 μm (a, c); 400 μm (b, d).

cells to taxanes. As shown in Figure 9C, SPC-A1/DTX/ING4 cells combined with DTX treatment showed a significant reduction in the level of Bcl-2 mRNA expression and a significant increase in the level of Bax mRNA expression (Figure 9C). Western blot assay confirmed the decreased expression of Bcl-2 protein and the increased expression of Bax protein (Figure 9D). Therefore, the decreased expression of Bcl-2 and increased expression of Bax in the SPC-A1/DTX/ING4 cells leads to the downregulated Bcl-2/Bax ratio, which finally induces the activation of caspase-3.

Overexpression of *ING4* Increases *In Vivo* Chemosensitivity of SPC-A1/DTX Cells to DTX

The SPC-A1/DTX xenograft model in nude mice was employed to explore the possible effect of ectopic *ING4* expression on *in vivo* sensitivity of SPC-A1/DTX cells to DTX. At 28 d after inoculation, all the mice developed tumors at the end of the experiment (Figure 10A), and the tumor volume was measured. Following the treatment with DTX, the average volume of tumors formed from SPC-A1/DTX/ING4 cells (195.4 mm^3) was significantly smaller than that of tumors formed from SPC-A1/DTX/control cells (344.8 mm^3), and therefore, ectopic expression of the *ING4* gene led to an approximately 43.3% suppression of tumor growth ($p < 0.001$; Figures 10B, C). Following the treatment with DTX, immunostaining showed that PCNA-positive cells were significantly decreased in tumors formed from SPC-A1/DTX/ING4 cells compared with that in tumors formed from SPC-A1/DTX/control cells (Figure 10D and Supplementary Table S1; $p < 0.05$). Also, following treatment with DTX, TUNEL-staining assay showed that the rate of apoptotic tumor cells was significantly increased in tumors formed from SPC-A1/DTX/ING4 cells compared with that in tumors formed from SPC-A1/DTX/control cells (Figure 10D). From these data, we concluded that overexpression of *ING4* could increase the *in vivo* chemosensitivity of SPC-A1/DTX cells to DTX.

The Expression of *ING4* Is Downregulated in DTX-Resistant Lung Adenocarcinoma Tissues

To investigate the association between *ING4* expression in lung adenocarcinoma tissues and the clinical response to DTX-based regimens, semiquantitative immunohistochemistry on tumor biopsy specimens from 18 eligible patients with advanced lung adenocarcinoma treated with DTX combined with platinum agents was performed. As shown in Figure 11A, the staining of *ING4* protein was mainly located in the cytoplasm of tumor cells. In 6 of the 18 cases, the ING4 protein was negatively expressed in the cytoplasm of tumor cells (a, b: tumor cells with ING4 expression <5%). In 5 of the 18 cases, the ING4 protein was weakly expressed (tumor cells with ING4 expression <30%). In 6 of the 18 cases, the ING4 protein was moderately expressed (c, d: tumor cells with *ING4* expression 30–60%). In one case, the ING4 protein was strongly expressed (tumor cells with ING4 expression >60%). The clinicopathological factors of patients are shown in Supplementary Table.S2. Tumors were divided into two groups: responding (CR + PR) and nonresponding (SD + PD). Compared with that of responding tumors, the immunostaining scores were significantly lower in nonresponding tumors ($p < 0.01$; Figure 11B). Then, ROC (receiver operating characteristic) curve analysis was performed to establish the optimal cutoff value for the histochemical score (HSCORE) of the *ING4* expression level, which yielded a value of 166.8 (data not shown). Patients with a low level of *ING4* expression (HSCORE < 166.8) had a significantly shorter progression-free survival (PFS) than did those with a high level of *ING4* expression (HSCORE ≥ 166.8) ($p < 0.001$; Figure 11C). Therefore, the expression of *ING4* in advanced lung adenocarcinoma might be positively correlated with the response of patients to DTX.

DISCUSSION

Multiple factors associated with sensitivity of tumor cells to taxanes (paclitaxel and DTX) have been reported, including the *MDR* gene, alteration in tubulin dynamics, and differences in β-tubulin isotype expression (18,19). In particular, overexpression of β-tubulin III could induce paclitaxel or DTX resistance in association with reduced effects on microtubule dynamic instability, which suggests that upregulation of a neuronal tubulin isotype is a key contributor to taxane sensitivity in human cancers (20). Additionally, aberrant expression of some oncogenes could affect sensitivity of tumor cells to DTX (21–23). The Aurora-A gene is essential for the proper arrangement of centrosomes and microtubules, and overexpression of Aurora-A has been reported to induce increased resistance to taxanes via a decrease in spindle checkpoint activity *in vitro* (24). Other reports showed that suppression of Aurora-A expression could enhance chemosensitivity to DTX in human cancers, including esophageal squamous cell carcinoma, breast cancer and prostate cancer (25–27). Stathmin, also designated Op18, is a highly conserved ubiquitous cytoplasmic protein that has recently been shown to destabilize microtubules (28). Mutant p53 breast cancers exhibiting high levels of stathmin may be resistant to antimicrotubule agents and siRNA-mediated stathmin downregulation could enhance taxane chemosensitivity of tumor cells, including prostate cancer and osteosarcoma (29,30). Recently, epigenetic events, including DNA methylation and post-transcriptional regulation (microRNAs), were found to be involved in the control of gene expression, which is known to play an important role in cancer and chemotherapy drug resistance (31,32). Previously, we have identified microRNA expression profiles in DTX-resistant human NSCLC cells (SPC-A1), which provide a better understanding of mechanisms involved in drug sensitivity or resistance of NSCLC (33).

To further investigate the mechanisms of DTX resistance and provide theoretical support for drug-resistant reversal induced by DTX, a DTX-resistant variant of the human lung adenocarcinoma cell line SPC-A1 (SPC-A1/DTX) was previously established. The incipient concentration of DTX was 0.008 μg/L, and the SPC-A1/DTX cell line stably grew into 5.0 μg/L DTX. An MTT assay showed that the index of drug resistance of SPC-A1/DTX was 13.20 to DTX. The establishment of a DTX-resistant lung adenocarcinoma cell model provided the foundation for further research on its chemoresistant mechanisms. In the present study, we focused specifically on improving the sensitivity to DTX and the selection of a therapeutic program for each individual patient, to identify novel genes which define the cDNA expression profiles of the parental SPC-A1 cell line in comparison with its DTX-resistant subclone SPC-A1/DTX using a cDNA microarray. Then, we attempted to provide strong molecular, biochemical and biological evidence that downregulation of the *ING4* gene played an important role in SPC-A1 cell progression to DTX resistance. *ING4*, a novel member of the ING family, which comprises six members characterized by a highly conserved C-terminal plant home domain (PHD)-like zinc-finger domain, has been reported to be involved in a variety of processes including oncogenesis, angiogenesis and DNA repair (34). However, whether downregulation of *ING4* is involved in tumor chemoresistance is still unknown. To date, there have been only three reported investigations about the association of *ING4* expression with chemosensitivity of tumor cells. Zhang *et al.* showed that exogenous *ING4* could significantly increase cell death from exposure to some DNA-damaging agents, such as topside and doxorubicin, which indicates that *ING4* could enhance chemosensitivity to certain DNA-damaging agents in HepG2 cells (35). Li, *et al.* reported that *ING4* could enhance the sensitivity of A549 cells to both radiotherapy and chemotherapy (5-fluorouracil) (36). And *ING4* plus CDDP (cisplatin) was closely associated with the cooperative regulation of extrinsic and intrinsic apoptotic pathways and synergistic inhibition of tumor angiogenesis (37).

In the present study we aimed firstly to explore the roles of *ING4* in DTX resistance of lung adenocarcinoma cells. We showed that the *ING4* gene was significantly downregulated in DTX-resistant SPC-A1 or taxol-resistant A549 cell lines when compared with nonresistant SPC-A1 or A549 cells at both transcriptional and translational levels. Ectopic expression of *ING4* could significantly decrease the IC_{50} value of SPC-A1/DTX or A549/Taxol cell lines, whereas siRNA-mediated *ING4* downregulation could increase the IC_{50} value of DTX-sensitive SPC-A1 or A549 cells. Overexpression of *ING4* could inhibit *in vivo* growth of SPC-A1/DTX cells combined with DTX treatment. Moreover, we also found that a decreasing level of *ING4* expression occurred in lung adenocarcinoma cells that responded to DTX. We then further elucidated the possible mechanisms of chemosensitivity enhancement induced by overexpression of *ING4*. Our results showed that overexpression of *ING4* could significantly induce apoptosis enhancement and G_2/M-phase arrest in SPCA-1/DTX cells, and the apoptosis enhancement might be associated with activation of caspase-3 induced by the decreased ratio of Bcl-2/Bax. Next, we analyzed the effects of *ING4* expression on the expression of some apoptosis-related genes such as *survivin*, *XIAP*, *Bcl-xL*, *Bcl-2*, *Bax*, *Aurora-A* and *stathmin*, which have been reported to affect the sensitivity of tumor cells to paclitaxel or DTX (38). We found that upregulation of *ING4* could decrease *Bcl-2* expression and increase *Bax* expression but had no effect on other genes at both transcriptional and translational levels. The decrease in the mitochondrial Bcl-2/Bax ratio is associated with caspase-3–dependent apoptosis, which is consistent with the findings of other researchers. Although Li and his colleagues showed that *ING4* might play an inhibitory role in A549 cell prolifera-

tion and tumor growth in lung adenocarcinoma by means of inactivation of Wnt-1/β-catenin signaling (36), whether *ING4* regulates the sensitivity of lung adenocarcinoma cells to DTX by Wnt-1/β-catenin signaling remains unclear. Also, whether *ING4* regulates expression of Bcl-2 family proteins and mitochondria apoptosis pathway by other molecular regulatory pathways such as PI_3K/Act and NF-dB signaling pathways is still unclear and is under investigation by other members of our research group. Given that the expression of *ING4* in advanced lung adenocarcinoma might be positively correlated with the response of patients to DTX, it is also conceivable that examining the level of *ING4* in tumor tissues might provide important information regarding the sensitivity of lung adenocarcinoma to DTX treatment.

CONCLUSION

Taken together, our results indicate that *ING4* is a modulator of DTX sensitivity in lung adenocarcinoma cells and that upregulation of *ING4* will be a potential strategy for overcoming DTX resistance in human adenocarcinoma. Of course, this study has several limitations. Firstly, because the size of the tissue sample in the present study is small, further investigation of a larger patient population will be necessary to confirm the association of *ING4* expression with therapeutic responses of patients with DTX treatment. Secondly, in the present study, we selected only two DTX-resistant lung adenocarcinoma cell lines, further research on other DTX-resistant lung adenocarcinoma cells will be helpful to strengthen the significance of our study.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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