Inhibitor of Growth 4 Induces Growth Suppression and Apoptosis in Glioma U87MG

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Key Words

Apoptosis · Glioma · Inhibitor of growth 4 · Proliferation · Tumor suppressor

Abstract

Objective: Inhibitor of growth (ING) 4 is a member of the ING family proteins. It has been shown to play an important role in cell cycle, transcription and oncogenesis, but the molecular mechanism of ING4 on tumor growth inhibition has not yet been elucidated. The goal of this study is to investigate the inhibitory effects of ING4 on gliomas and its mechanism by transduction of ING4 cDNA into glioma U87MG. **Methods:** The effect and mechanisms of ING4 on proliferation and apoptosis of U87MG were evaluated in vitro by MTT assay, flow-cytometric analysis, TUNEL assay, Western blot analysis and animal experiments. **Results:** The level of ING4 was markedly reduced in glioma tissues, and the extent of reduction correlated with the progression from lower to higher grades of tumors. It was observed that U87MG with exogenous ING4 gene presented with growth suppression, apoptosis enhancement and the deregulation of cell cycle- or apoptosis-regulating proteins. **Conclusion:** ING4 has a potential role on the growth suppression and apoptosis enhancement in gliomas U87MG via the activation of mitochondrial-induced apoptotic pathway and the hindrance of

the cell cycle progression. The low expression and dysfunction of ING4 might be correlated with the tumorigenesis and progression of gliomas.

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Introduction

Inhibitor of growth (ING) gene is a novel tumor suppressor gene family, and the known members are considered candidates of tumor suppressor genes. The gene products are thought to interact with and enhance the function of p53 in gene transcription, apoptosis and DNA repair [1–8], although the exact functions of ING genes have not been elucidated. Six members of the ING gene family, ING1, ING2, ING3, ING4, ING5 and INGx, have been isolated and characterized to date. They all share a highly conserved plant homeodomain finger motif in the C-terminal end and a nuclear localization sequence for ING gene and protein structures [1, 4, 7–10]. ING4 gene has emerged as a strong candidate tumor suppressor gene that functions in cell proliferation, contact inhibition and angiogenesis [11, 12]. It was first identified through a

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computational search of expressed sequence tag clones showing homology to ING1 gene and ING2 gene [7]. ING4 gene maps to human chromosome 12p13–32, including 8 exons and 7 introns, and encodes a 248-amino-acid polypeptide. It is a nuclear factor expressed in normal human tissues, but its expression is markedly reduced in several human malignancies such as brain tumors [11], squamous cell carcinomas [13] and lung adenocarcinoma [14].

Gliomas, which account for 60% of primary brain tumors, are the most common primary tumors of the central nervous system with a lethality approaching 80% within the first year of glioblastoma diagnosis [15]. The incidence is 5–7 per 100,000 person years. Previous studies in gliomas demonstrate that the expression of ING4 is significantly reduced, the level of reduction correlates with the progression from lower to higher grades of tumors, and that it can also inhibit tumor growth and angiogenesis [11]. ING4's precise mode of action has been recognized limitedly. In this research, we cloned the sequence of a cDNA fragment encoding human ING4 and transfected it into human glioma U87MG to study the effect of ING4 on proliferation and apoptosis of U87MG as well as to investigate its inhibitory mechanism on gliomas.

Materials and Methods

Materials and Cell Line

Therm RT-PCR/plat Taq (23191-050), Lipofectamine 2000 (LS11668-027), G-418 (INALCO1758-1811), Trizol and MTT reagent were purchased from Invitrogen (Carlsbad, Calif., USA), In Situ Cell Death Detection Kit (11684817910) from Roche (Nutley, N.J., USA), SABC (goat-IgG)-POD kit from BOSTER (Wuhan, China), goat polyclonal antibody against ING4 (ab3714) from Abcam Inc. (Cambridge, Mass., USA), and the antibodies against Bcl-2 (SC-23960), Bax (SC-70407), Cyt-c (SC-81752), caspase 3 (SC-7148), PARP (SC-56197), Cox-2 (SC-7951), SKP2 (SC-74477), p27 (SC-1641) from Santa Cruz Inc. (Santa Cruz, Calif., USA). The human glioma U87MG was a kind gift from the Department of Immunology at Harbin Medical University. U87MG were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO₂.

Immunohistochemistry

The expression of ING4 in various grades of glioma tissues was detected by immunohistochemical technique. One hundred and twenty cases of formalin-fixed and paraffin-embedded glioma samples (January 2005 to November 2007) were obtained from the Department of Pathology at the Affiliated Tumor Hospital of Harbin Medical University. The samples were divided into 2 groups according to the tumor differentiation grades: 60 cases of low-grade gliomas (astrocytoma) and 60 cases of high-grade gliomas (glioblastoma). The brain tissues from cerebrovascular

malformation were selected as positive control, the primary antibody replaced with PBS as negative control. The study was conducted according to the Declaration of Helsinki principles and approved by the Medical Ethics Committee of the Affiliated Tumor Hospital of Harbin Medical University. All patients gave informed consent.

Four-micrometer sections were analyzed following the manufacturer's protocol of the SABC (goat-IgG)-POD kit. A semi-quantitative scoring system was used to evaluate the immunostain result of ING4. Immunoreactivity was assessed by 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: non-staining as 0; shallow brown as 1; brown as 2; deep brown as 3). The final score obtained was the multiplication of the proportion of positive cells and strength of staining: 0–2 scores as (–), 3–5 as (+), 6–9 as (++), >9 as (+++).

RT-PCR

The transcription level of ING4 mRNA was detected by RT-PCR in various grades of glioma tissues conserved in liquid nitrogen. Total RNAs were isolated with Trizol reagent from 15 cases of normal brain tissues (cerebrovascular malformation), 15 cases of low-grade glioma tissues (astrocytoma) and 15 cases of high-grade glioma tissues (glioblastoma). RT-PCR was performed according to the manufacturer's protocol of a first-strand synthesis kit and a PCR kit as well as β -actin as a control. The primer sequences for the human ING4 gene were as follows: forward primer p1: 5'-TTG AAT TCA TGG CTG CTG GGA TGT ATT TGG GAAC-3'; reverse primer p2: 5'-CCT CTA GAT CAG ATA CAT CCA CAC CTT TTA GCG-3'. The primer sequences for β -actin were as follows: forward primer p1: 5'-ACCACAGTCCATGC-CATCAC-3'; reverse primer p2: 5'-TCCACCACCCTGTTGCT-GTA-3'.

Plasmid Construction and Cell Transfection

The recombinant plasmid pcDNA3.1-ING4 was constructed with reference to our previous results [14]. The recombinant pcDNA3.1-ING4 and the empty vector pcDNA3.1 were transfected into U87MG with lipofectamine 2000 reagent. The stable transfectant clones were obtained by selecting the clones in the media containing G418 (600 μ g/ml) for 2 weeks and were identified by indirect immunofluorescence (IF) staining and Western blot analysis.

Indirect IF Staining

The slides with seeded cells were washed with PBS, and then fixed in 4% paraformaldehyde. Fixed cells were permeabilized with 0.5% Triton X-100 and blocked with 1% BSA. For staining, the cells were incubated with primary antibody against ING4 (dilution 1:400) and mouse anti-goat secondary antibody conjugated with fluorescent materials, and the results were documented under a fluorescence microscope.

Western Blot Analysis

Seventy micrograms of total protein extracted from U87MG clones with pcDNA3.1-ING4 was loaded to electrophoresis, and then transferred onto a regular nitrocellulose membrane. The regular nitrocellulose membrane was incubated in primary antibodies, including antibodies for ING4 (dilution 1:500) as well as Bcl-2, Bax, Cyt-c, caspase 3, PARP, Cox-2, Skp2 and p27 (dilution

1:200). The antigen-antibody reaction was visualized using the enhanced chemiluminescence method. The protein expression levels were quantitated with the Quantity One software (Bio-Rad, Hercules, Calif., USA). U87MG clones with pcDNA3.1 served as control.

MTT Assay

To observe the cell propagation, U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1 were seeded in 96-well plates at a density of 1×10^3 cells/well and cultured for 24, 48, 72 and 96 h. Cell viability was measured by the MTT method. The optical density (OD) value was determined on the 490-nm wavelength. The culture medium without cells was used as blank control.

Flow Cytometry

To analyze the cell cycle, U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1 were harvested, counting 5×10^5 to 1×10^6 cells/ml, were fixed with 70% ethanol and then re-suspended in PBS buffer containing 20 μ g/ml propidium iodide (PI) and 50 μ g/ml RNase A. The DNA contents were measured as the PI signal to determine cell cycle profiles on flow cytometry (FCM). Furthermore, U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1, at a density of 5×10^5 to 1×10^6 cells/ml, were fixed with FITC-labeled annexin V. Apoptotic cells were quantitatively determined by PI staining on FCM.

TUNEL Assay

The slides with seeded cells were washed with PBS, and then the TUNEL assay was done to identify apoptotic cells following the manufacturer's instruction. The results were evaluated by apoptotic index (AI), the percentage of positive cells by counting 1,000 cells under 10 random high visual fields. All above experiments were performed in triplicate at minimum.

Animals

Forty BALB/C (nu/nu) male nude mice (16–20 g; 4–6 weeks of age) were purchased from the Animal Institute at the Chinese Academy of Medical Sciences. Animals were fed in pathogen-free environment with constant temperature and humidity, allowed standard rat chow and water ad libitum, and maintained on a 12-hour light-dark schedule. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of Harbin Medical University.

Tumorigenicity in Nude Mice

Forty nude mice were randomly divided into 4 groups, with 10 mice per group. Exponential U87MG/pcDNA3.1-ING4, at a density of 1×10^7 cells/ml, was subcutaneously inoculated (0.2 ml) into the abdomen of nude mice. U87MG/pcDNA3.1 and U87MG were used as the control groups and physiological saline as the blank group. The mice were weighed and the tumor size was measured once a week. After 4 weeks, the animals were sacrificed by transection of spinal cord and the xenografts were removed. Tumor volume and weight were measured. Tumor volume was calculated as: $V = ab^2/2$, where a is the longest diameter and b is the diameter perpendicular to a. The xenografts were performed by routine HE and immunohistochemical staining with specific polyclonal antibody against ING4 (dilution 1:400) as well as Bcl-2, Bax, Cyt-c, caspase 3, PARP, Cox-2, Skp2 and p27 (dilution 1:200). At the same time, the transcription level of ING4 mRNA was also

detected by RT-PCR in the xenografts of U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1.

Statistical Analysis

Data was expressed as values $\pm$ SD. Wilcoxon rank sum test, Kruskal-Wallis H test, paired t test, one-way ANOVA and q test were used for statistical analysis. $p < 0.05$ was considered statistically significant.

Results

The Expression of ING4 in Various Grades of Gliomas

The level of ING4 in 120 cases of various-grade gliomas was detected by using immunohistochemical technique with specific antibodies against ING4 (dilution 1:400). The results are shown in figure 1 and table 1. ING4 had obvious low expression in gliomas compared with normal brain tissues, and the level was lower in high-grade glioma tissues (glioblastoma; grade IV) than low-grade glioma tissues (astrocytoma; grade II). In glioblastoma tissues, the expression of ING4 was mostly lost. A statistically significant difference was obtained between glioblastoma and astrocytoma ($n = 60$; $U = 4.5 > 2.35$, $p < 0.01$). The expression of ING4 gene in various grades of glioma tissues was also examined by RT-PCR analysis. The results are shown in figure 2 and table 2. The level of ING4 mRNA in glioma tissues was obviously decreased compared with normal brain tissues, and the expression level in high-grade than in low-grade glioma tissues. A statistical difference was observed between 3 groups ($n = 15$; $p < 0.01$). Moreover, the survival period of patients with various-grade gliomas was also analyzed. In table 3, the different survival period of patients with glioblastoma or astrocytoma was observed accompanying different expression levels of ING4 in their corresponding tissues. The results indicate that patients with higher expression level of ING4 had a longer survival period.

Expression of ING4 in U87MG Transfectants

After the pcDNA3.1-ING4 and pcDNA3.1 were transfected into U87MG cells, the expression of ING4 was evaluated by indirect IF staining and Western blot analysis. By indirect IF staining, it displayed weak stain for ING4 in the nucleus of U87MG/pcDNA3.1, but strong stain and aggregation in the nucleus of U87MG/pcDNA3.1-ING4 (fig. 3). Western blot analysis revealed that the level of ING4 was significantly increased in U87MG/pcDNA3.1-ING4 compared with U87MG and U87MG/pcDNA3.1 (fig. 4).

Fig. 1. The expression level of ING4 by immunohistochemical technique in high- and low-grade glioma tissues: glioblastoma (a $\times 100$; b $\times 400$); astrocytoma (c $\times 100$; d $\times 400$); normal brain tissues (e $\times 100$; f $\times 400$). ING4 showed significantly low expres-

sion in gliomas compared with normal brain tissues. The level was lower in glioblastoma than astrocytoma, with statistically significant difference ($U = 4.57$; $p < 0.01$). The expression of ING4 was mostly lost in glioblastoma tissues.

Table 1. Comparison with the expression of ING4 in various grades of glioma tissues

Staining intensity	Astrocytoma	Glioblastoma	U value	p
–	7	49		
+	27	10		
++	23	1		
+++	3	0		
Total	60	60	4.57	<0.01

Statistical analysis was performed using two-sample U test of Wilcoxon rank sum test ($U = 4.57$, $p < 0.01$), suggesting that the expression of ING4 was statistically significantly different between low- and high-grade glioma tissues.

Table 2. The density value analysis of band brightness for ING4/ β -actin ($n = 15$) in various grades of glioma tissues

Groups	Cases	Ratio for ING4/ β -actin
Normal brain tissues	15	1.479 ± 0.247
Astrocytoma	15	0.632 ± 0.057
Glioblastoma	15	0.177 ± 0.032

The level of ING4 in normal brain tissues was significantly higher than in glioma tissues ($t = 12.971$, 20.249 , $p < 0.01$), and the level of ING4 in low-grade glioma tissues was significantly higher than in high-grade glioma tissues ($t = 26.765$, $p < 0.01$).

Fig. 2. The transcription level of ING4 mRNA by RT-PCR in various grades of glioma tissues: glioblastoma (lane a); astrocytoma (lane b); normal brain tissues (lane c).

Fig. 3. ING4 expression was determined by indirect IF staining: strong stain and aggregation of the nucleus in U87MG/pcDNA3.1-ING4 (a) and weak stain in U87MG/pcDNA3.1 (c). b, d The nucleus of U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1, respectively, counterstained with Hoechst.

Color version available online

ING4 Regulates Negative Proliferation of U87MG and Enhances Apoptosis *in vitro*

To observe the effects of ING4 on U87MG growth, the cell viability of U87MG clones with or without exogenous ING4 gene was measured at 4 time courses by the MTT method. The result is shown in figure 5. The OD value of U87MG/pcDNA3.1-ING4 cultured for 24, 48, 72 and 96 h was 0.253 ± 0.035 , 0.371 ± 0.038 , 0.488 ± 0.035 and 0.694 ± 0.056 , respectively, and 0.345 ± 0.038 , 0.486 ± 0.024 , 0.712 ± 0.042 and 1.022 ± 0.073 , respectively, in U87MG/pcDNA3.1. There was a statistical difference at 24, 48, 72 and 96 h for U87MG/pcDNA3.1-

ING4 and U87MG/pcDNA3.1 ($t = 3.670, 5.792, 7.411$ and 10.755 , respectively; $p = 0.035, 0.010, 0.005$ and 0.002 , respectively). The OD value of U87MG/pcDNA3.1-ING4 was lower than that of U87MG/pcDNA3.1 at 4 time courses, and the difference was more conspicuous with longer culture time. The result revealed that the growth speed of U87MG was significantly slower after transfected exogenous ING4 gene, suggesting that the proliferation of U87MG with exogenous ING4 overexpression was suppressed.

To explore the intrinsic mechanism of ING4 on cell growth, cell cycle analysis was performed in U87MG

Fig. 4. ING4 expression was determined by Western blot analysis: the level of ING4 was significantly higher in U87MG/pcDNA3.1-ING4 (lane a) than U87MG/pcDNA3.1 (lane b) and U87MG (lane c).

Table 3. The expression level of ING4 in various grades of glioma tissues and the survival period of patients

Survival period	Astrocytoma				Glioblastoma			
	-	+	++	+++	-	+	++	+++
<6 months	1	1	0	0	28	2	0	0
7-12 months	5	12	2	0	21	7	1	0
13-24 months	1	7	8	1	0	1	0	0
>24 months	0	7	13	2				

Statistical analysis was performed using multiple-samples H test of Kruskal-Wallis test. It showed $H = 16.35$, $C = 0.896$, $H_c = 18.25$, $v = 3$ and $p < 0.05$ for low-grade gliomas (astrocytoma), and $H = 99.32$, $C = 0.762$, $H_c = 130.3$, $v = 3$ and $p < 0.05$ for high-grade gliomas (glioblastoma). This suggested in both low- and high-grade gliomas that the survival period of patients was prolonged with higher expression level of ING4.

clones with or without exogenous ING4 gene by FCM. As shown in figure 6, significantly increased percentages of cells in G1 phase were observed in U87MG/pcDNA3.1-ING4 (55.4%) compared with U87MG/pcDNA3.1 (38.6%). Moreover, decreased percentages of cells in S phase were also observed in U87MG/pcDNA3.1-ING4 (28.4%) compared with U87MG/pcDNA3.1 (53.5%). These data indicate that ING4 could negatively regulate the cell growth of U87MG and induce G1/S arrest of cell cycle.

To demonstrate the effect of ING4 on apoptosis, we further analyzed the apoptosis of U87MG clones with or without exogenous ING4 gene by FCM and TUNEL as-

Fig. 5. The cell viability of transfected U87MG detected by MTT method after cultured for 24, 48, 72 and 96 h: the OD value of U87MG/pcDNA3.1-ING4 was obviously lower than that of U87MG/pcDNA3.1.

say. By annexin V FITC analysis on FCM, a significantly increased percentage of apoptotic cells was observed in U87MG/pcDNA3.1-ING4 (fig. 7). The total apoptotic rate was $13.78 \pm 1.37\%$ in U87MG/pcDNA3.1-ING4 and $3.23 \pm 0.50\%$ in U87MG/pcDNA3.1. There was a statistically significant difference between the 2 groups ($t = 8.53 > 2.896$, $p < 0.01$). The percentage of apoptotic cells in early stage was higher in U87MG/pcDNA3.1-ING4 ($2.19 \pm 0.33\%$) compared with that in U87MG/pcDNA3.1 ($0.99 \pm 0.25\%$), with a statistical difference ($t = 6.32 > 2.896$, $p < 0.01$). TUNEL assay showed that more apoptotic cells were found in U87MG/pcDNA3.1-ING4 ($AI 7.10 \pm 2.38\%$) compared with U87MG/pcDNA3.1 ($AI 0.55 \pm 0.61\%$; fig. 8), with a statistical significance ($t = 8.260$, $p < 0.05$). These results suggest that ING4 could enhance apoptosis of U87MG in vitro.

The Inhibitory Effect of ING4 on the Growth of Nude Mice Xenografts in vivo

Nude mice models with gliomas were established by subcutaneously inoculating U87MG/pcDNA3.1-ING4, while the control tumors were established by subcutaneously inoculating U87MG and U87MG/pcDNA3.1. Two weeks later, the tumor nodules were touched, and the xenografts were removed 4 weeks later. The size of U87MG/pcDNA3.1-ING4 xenografts was significantly smaller than the control tumors (fig. 9; table 4), and a statistically significant difference was observed in the weight and volume of U87MG/pcDNA3.1-ING4 xenografts and the control tumors, suggesting that tumor growth was significantly repressed by ING4. Furthermore, positive ex-

Fig. 6. Cell cycle analysis of transfected U87MG by FCM. Increased cell percentages in G1 phase and decreased cell percentages in S phase were found in U87MG/pcDNA3.1-ING4 compared with U87MG/pcDNA3.1.

Fig. 7. The apoptotic cells were detected by annexin V FITC analysis on FCM. The sum of UR (upper right) and LR (lower right) represents the percentage of apoptotic cells, UR represents apoptotic cells in early stage. The percentage of apoptotic cells in U87MG/pcDNA3.1-ING4 (15.01%) was obviously higher than that in U87MG/pcDNA3.1 (3.12%). The percentage of apoptotic cells in early stage was significantly high in U87MG/pcDNA3.1-ING4 (2.42%) compared with that in U87MG/pcDNA3.1 (0.95%).

Fig. 8. The apoptosis of transfected U87MG was determined by TUNEL assay: more apoptotic cells were observed in U87MG/pcDNA3.1-ING4 (a) compared with U87MG/pcDNA3.1 (b).

Fig. 9. The size of nude mice xenografts by subcutaneous inoculation with U87MG, U87MG/pcDNA3.1 and U87MG/pcDNA3.1-ING4, respectively. The size of xenografts in U87MG/pcDNA3.1-ING4 group was significantly smaller than those in the control tumors.

Fig. 10. The expression of ING4 in nude mice xenografts by immunohistochemical technique: U87MG/pcDNA3.1-ING4 (a), U87MG/pcDNA3.1 (b) and HE staining (c).

pression of ING4 was found in xenografts of U87MG/pcDNA3.1-ING4, and mostly no staining was observed in the control tumor by immunohistochemical technique (fig. 10). By RT-PCR analysis, the level of ING4 gene was also found to be obviously higher in xenografts of U87MG/pcDNA3.1-ING4 than in control tumors (fig. 11). As shown in table 5, there was a significant difference between the 2 groups ($n = 10$; $t = 38.82$, $p < 0.01$).

The Expression of Proteins Involved in Proliferation and Apoptosis in U87MG Clones with or without Exogenous ING4 Gene

To explore what molecular mechanisms could be involved, we analyzed the levels of several proteins involved in cell proliferation and apoptosis, including p27, SKP2, Bcl-2, Bax, Cyt-c, caspase 3, PARP and Cox-2, in U87MG clones with or without exogenous ING4 gene by Western

Table 4. The suppression effects of ING4 on the growth of nude mice xenografts (n = 5)

Groups	Weight of nude mice g	Weight of xenografts g	Volume of xenografts mm ³
U87MG/pcDNA3.1-ING4	22.98 ± 0.28	0.99 ± 0.12	679.28 ± 12.36
U87MG/pcDNA3.1	25.70 ± 0.55	1.28 ± 0.12	804.59 ± 12.74
U87MG	26.92 ± 0.12	1.36 ± 0.14	840.36 ± 18.96

Statistically significant difference was observed in volume and weight of xenografts of U87MG/pcDNA3.1-ING4 and the control tumors. In the weight of xenografts: $q = 5.11$, $p < 0.01$ for U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1; $q = 6.51$, $p < 0.01$ for U87MG/pcDNA3.1-ING4 and U87MG; $q = 1.41$, $p > 0.05$ for

U87MG/pcDNA3.1 and U87MG. In the volume of xenografts: $q = 18.69$, $p < 0.01$ for U87MG/pcDNA3.1-ING4 and U87MG/pcDNA3.1; $q = 24.02$, $p < 0.01$ for U87MG/pcDNA3.1-ING4 and U87MG.

Fig. 11. The level of ING4 in nude mice xenografts by RT-PCR analysis: U87MG/pcDNA3.1 (lane a) and U87MG/pcDNA3.1-ING4 (lane b).

blot analysis. The results showed that the level of p27, Bax, Cyt-c and PARP (31k Da) was upregulated; however, the level of SKP2, Bcl-2, caspase 3, PARP (116k Da) and Cox-2 was downregulated in U87MG/pcDNA3.1-ING4 compared with U87MG/pcDNA3.1 (fig. 12). The level of p27, SKP2, Bcl-2, Bax, Cyt-c, caspase 3, PARP and Cox-2 was also analyzed in nude mice xenografts by immunohistochemical technique. The positive reaction of Bcl-2, Bax and Cyt-c was located in plasma, that of Cox-2 in cell membrane, that of p27, SKP2 and PARP in nucleus, and that of caspase 3 in nucleus and plasma. Higher expression of p27, Bax and Cyt-c as well as lower expression of SKP2, Bcl-2, caspase 3, PARP and Cox-2 were observed in xenografts of U87MG/pcDNA3.1-ING4 compared with those of U87MG/pcDNA3.1 (fig. 13).

Table 5. The density value analysis of band brightness for ING4/ β -actin (n = 10)

Xenografts	Cases	Ratio for ING4/ β -actin	t	p
U87MG/pcDNA3.1-ING4	10	0.899 ± 0.051	38.82	<0.01
U87MG/pcDNA3.1	10	0.173 ± 0.031		

The level of ING4 gene in xenografts of U87MG/pcDNA3.1-ING4 was significantly higher than in xenografts of U87MG/pcDNA3.1, with a statistically significant difference ($t = 38.82$, $p < 0.01$).

Discussion

Tumor suppressor genes are very important in resisting tumorigenesis and preventing tumor progression. They are often inactivated in cancer cells, which are considered as one of the primary mechanisms of tumorigenesis and progression. ING4, a novel candidate tumor suppressor gene from the ING family, was implicated in a variety of processes including oncogenesis, apoptosis, DNA repair and cell cycle control [16]. Previous study demonstrated that the expression of ING4 is significantly reduced in many human malignant tumors, including gliomas, and the level of reduction correlates with the progression from lower to higher grades of tumors [11–14]. In present experiments, we also detected the expression of ING4 in various grades of gliomas. By immunohistochemical technique and RT-PCR analysis, the level of ING4 was all found significantly reduced in glioma tissues compared with normal brain tissues, and its level in high-grade glioma (glioblastoma) was significantly lower than that in low-grade glioma (astrocytoma). The results were in ac-

Fig. 12. The expression of proteins involved in cell cycle and apoptosis including p27, SKP2, Bcl-2, Bax, Cyt-c, caspase 3, PARP and Cox-2 by Western blot analysis: U87MG/pcDNA3.1-ING4 (lane a) and U87MG/pcDNA3.1 (lane b).

cordance with previous research [11], which implies the low expression and dysfunction of ING4 might be correlated with the tumorigenesis and progression of gliomas. We found the expression of ING4 in high-grade glioma (glioblastoma) was mostly loss. Loss of heterozygosity of chromosome 12p13.3 for the ING4 locus is infrequent in gliomas, so we presumed the loss of ING4 in glioblastoma might be correlated with the mutation of ING4 gene, such as deletion of 465C, and cause expression suppression of wild ING4 protein [12]. We then found the expression of ING4 was associated with the prognosis of patients by analyzing the correlation between the survival period of patients and the expression of ING4. The patients with higher ING4 level had a longer survival period.

The action mechanism of ING4 remains largely unclear. The overexpression of ING4 could induce a decreased cell population in S phase of cell cycle and apoptosis in a p53-dependent manner with increasing p21 and Bax expression [7, 13, 17]. To further confirm the correlation of ING4 abnormality and gliomas, we transfected a cDNA fragment coding ING4 into U87MG to establish some stable U87MG clones with constitutive overexpression of ING4.

We first examined the effects of ING4 overexpression on the proliferation of U87MG. MTT assay indicated that the growth of U87MG clones with exogenous ING4 gene was inhibited with obviously decreased cell viability compared with the controls. By FCM, the ratio of G1 cells was

significantly increased and the ratio of S cells was decreased in U87MG clones with exogenous ING4 gene compared with U87MG clones with empty plasmid, suggesting that ING4 could inhibit cell cycle progression by inducing G1/S arrest of cell cycle. These results indicate that the overexpression of ING4 could play an inhibition role on U87MG proliferation in vitro. To further strengthen the evidences, the tumorigenicity of U87MG clones with exogenous ING4 gene was observed in nude mice. As expected, its tumorigenicity was significantly decreased, and the volume and weight of xenografts were significantly smaller than the control tumors, with statistical significance. These results suggest that the overexpression of ING4 could negatively regulate glioma U87MG growth in vitro and in vivo. To further analyze the effect of ING4 on U87MG growth, we also examined the apoptosis of transfected U87MG clones. By both FCM and TUNEL assay, we found ING4 significantly induced apoptosis and more apoptotic cells were found in U87MG clones with exogenous ING4 gene. These data suggested that ING4 might play a promoting role on apoptosis of U87MG and might indirectly inhibit cell growth by enhancing apoptosis.

To find out what molecular mechanism could be involved, we then analyzed the effect of ING4 on the expression of proliferation-regulating proteins p27 and SKP2 in U87MG. Unregulated levels of p27 and downregulated levels of SKP2 were observed significantly in U87MG clones with exogenous ING4 gene compared with the

Fig. 13. The expression of p27, SKP2, Bcl-2, Bax, Cyt-c, caspase 3, PARP and Cox-2 in nude mice xenografts by immunohistochemical technique: xenografts in the U87MG/pcDNA3.1-ING4 and the U87MG/pcDNA3.1 group.

control. p27, an important cdk inhibitor, can prevent cell cycle progression before the late G1 restriction point [18]. SKP2 can specifically ubiquitinate and degrade p27, and induce cell proliferation through S phase from G1 phase. SKP2-dependent degradation of p27 is essential for cell cycle progression. Our results revealed that ING4 might inhibit cell proliferation by preventing cell development at G1 phase by upregulation of p27 and downregulation of Skp2. These results were in accordance with cell cycle analysis of transfected U87MG by FCM, in which ING4 induced G1/S arrest of cell cycle. Simultaneously, we also analyzed the expression of apoptosis-regulating proteins

Bcl-2, Bax, Cyt-c, caspase 3, PARP and Cox-2 in U87MG clones with or without exogenous ING4 gene. Western blot analysis revealed that Bax, Cyt-c and PARP (31 kDa) were significantly elevated, while Bcl-2, caspase 3, PARP (116 kDa) and Cox-2 were significantly decreased in U87MG clones with exogenous ING4 gene, suggesting that ING4 could induce U87MG apoptosis via activating cascade reaction of mitochondrial-induced apoptotic pathway. ING4 might upregulate the expression of Bax and downregulate the expression of Bcl-2, which promotes the release of Cyt-c from mitochondrial and subsequent activation of caspase 3 due to cleavage. Then PARP

(116 kDa), one of the main cleavage targets of caspase 3, could be cleaved by activated caspase 3 into 2 fragments: a 85- and a 31-kDa cleavage product. The cleaved PARP loses the ability of repairing the DNA and keeping the genome intact, and the cells started apoptosis inevitably. Cox-2, a central enzyme in the prostaglandin biosynthetic pathway, plays a key role in early stages of carcinogenesis by promoting tumor cell proliferation, resistance to apoptosis, angiogenesis, tumor cell invasion and setting up of the metastatic process [19]. Cox-2 could inhibit apoptosis via upregulation of Bcl-2 [20]. It was found in our research that Cox-2 was significantly decreased in U87MG clones with exogenous ING4 gene, suggesting that ING4 might enhance apoptosis by downregulating the synthesis of Cox-2 and subsequent downregulation of Bcl-2. To further confirm the effect of ING4 on the above proteins, we detected the immunohistochemical expression of these proteins in xenografts of U87MG clones with or without exogenous ING4 gene. The results were similar to the above results by Western blot analysis. High expression of p27, Bax and Cyt-c as well as low expression of SKP2, Bcl-2, caspase 3, PARP and Cox-2 were observed in xenografts of U87MG clones with exogenous ING4 gene compared with the control tumors. Based on the above

results, ING4 might induce the activation of mitochondrial-induced apoptotic pathway and the interference of the cell cycle progression through its regulation on the expression of several proteins involved in cell cycle progression and apoptosis.

In summary, our research suggests that ING4 could negatively regulate cell proliferation and enhance apoptosis of glioma U87MG via the activation of mitochondrial-induced apoptotic pathway and the G1/S arrest of the cell cycle progression. The low expression and dysfunction of ING4 that occurred in gliomas might be implicated in the tumorigenesis and progression of gliomas and affect the prognosis of patients with low- or high-grade gliomas.

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