Axin induces cell death and reduces cell proliferation in astrocytoma by activating the p53 pathway

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Abstract. Astrocytic tumors are the most common brain tumors with various genetic defects. As a tumor suppressor gene, Axin could control cell death and growth. Axin possesses a separate domain that directly interacts with p53 and regulates the activity of p53 pathway. Our aims were to elucidate the effects of Axin on the progression of astrocytoma. We examined the expression of Axin in 96 cases of astrocytoma using immunohistochemistry. The apoptotic index, proliferactive acitivity and the expression levels of p53 and its downstream genes, p21 and Cyclin D1, were evaluated in the C6 astrocytoma cells with overexpression or silencing of Axin. The results showed the levels of Axin correlated significantly inversely with the grades of astrocytoma (R=-0.286, P<0.05) and correlated negatively with Ki-67 labeling index (R=-0.227, P<0.05). Overexpression of Axin in C6 cells induces cell death, and reduces the cell proliferation, up-regulates the expression of p53. Silencing of Axin reduces p53 expression. The p53 inhibitor, pifithrin-α, was able to counteract the effect of Axin in C6 cells. Our data demonstrate that the expression of Axin is associated negatively with the progression of astrocytoma. In conclusion, Axin induces cell death and reduces cell proliferation, partially by activating the p53 pathway in astrocytoma cells. This knowledge is helpful in understanding the role of Axin in the progression of astrocytoma.

Introduction

Astrocytomas are the most common primary brain tumors and classified according to their histologic characteristics in low-grade astrocytomas and high-grade tumors (including glioblastoma multiforme, GBM). Modern molecular genetic analyses have revealed that the progression of astrocytic tumors results from accumulating inactivation of different tumor suppressor genes and/or amplification of certain oncogenes (1-3).

Axin was originally identified from the characterization of the *Fused* locus (4). Molecular analysis revealed that Axin is a master scaffold protein within the cell (5,6). Axin utilizes different domains for its distinct roles in different signaling pathway. To date, Axin has been implicated in at least four different signaling pathways: the Wnt signaling pathways, the stress-activated protein kinase/Jun N-treminal kinase (SAPK/JNK), transforming growth factor ß (TGF-ß) and p53 signaling pathways (4,5,7-11). Axin acts as a possible mediator for cross-talk in these signaling pathways. Recent data showed that Axin formed a ternary complex with homeodomain-interacting protein kinase-2 (HIPK2) that is bound to p53 (11-13), which is involved in UV-induced p53-dependent apoptosis via phosphorylation of Ser46 of p53 in 293 cells (11). Decreased Axin expression has been reported to correlate with tumor differentiation of oesophageal squamous cell carcinoma (14), whereas high Axin levels may induce cell apoptosis (11,15). We hypothesized that Axin expression may correlate with the progression of astrocytoma. The question of how Axin may be involved in the regulation of progression of astrocytoma cells still need to be addressed. Therefore, in this study we examined the expression levels of Axin in low and high grades astrocytomas and analyzed the relationship between Axin expression and the clinical pathology. Furthermore, we treated the C6 astrocytoma cells with overexpression or silencing of Axin and explored the effect of axin on cellular proliferation and apoptosis and the expression of p53 and its downstream genes, p21 and Cyclin D1 to analyze the mechanisms and molecules of Axin involved in the astrocytoma progression *in vitro*.

Patients and methods

Patients and tumors. We obtained 96 surgically removed astrocytoma tissues from the Xijing Hospital, the affiliated hospital of the Fourth Military Medical University, China. All samples were pathologically confirmed astrocytoma or glioblastoma multiforme (GBM, Grade IV). Patients were

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Table I. Biopsy specimens.

Histopathological diagnosis	Total case no.	Male	Female	Age	
				Median	Range
WHO Grade II	49	30	19	31	3-69
WHO Grade III	31	17	14	45	17-65
WHO Grade IV	16	9	7	53	36-66

2007 WHO Grade II includes pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma and diffuse astrocytoma; WHO Grade III includes anaplastic astrocytoma and WHO Grade IV includes glioblastoma, giant cell glioblastoma and gliosarcoma.

3-69 years of age, and the mean age was 36 years (56 males and 40 females) (Table I). These tumors were classified as Grade II, III and IV according to the classification system of CNS (2007) of World Health Organization (WHO), including 49 cases of Grade II, 31 cases of Grade III, and 16 cases of Grade IV. Because WHO Grade I astrocytoma were biologically and genetically distinct from diffuse astrocytomas and virtually never progress to higher grades, WHO Grade I astrocytoma were not included in our study. Tissue specimens had previously been fixed in 10% buffered formalin and embedded in paraffin for routine histological examination.

Immunohistochemistry. Immunohistochemistry was carried out as previously described (16). Briefly, the deparaffinized and rehydrated slides were blocked with with 20 ml/l fetal calf serum for 30 min to reduce non-specific binding. Then the anti-Axin antibody (1:200; Santa Cruz, USA) and anti-Ki-67 antibody (1:200; NeoMarkers, USA) were applied to the sections and incubated at 4˚C overnight. The breast cancers were used the positive control of Axin and Ki-67. Negative controls were obtained by replacing the primary antibody with non-immune mouse or rabbit serum. The sections were subsequently incubated with the second antibody (Dako, Denmark) at 37˚C for 40 min, and stained with DAB-H_2O_2 for 5-10 min and counterstained with hematoxylin. The slides were independently evaluated by two observers. Specimens containing ≥5% stained tumor cells were considered positive and <5% were considered negative.

For the immuno-fluorescent analysis, cells grew on the glass cover slides to 60% confluency. Some cells were treated with pifithrin-α (30 μM/ml) or Axin siRNA for 24 h, and fixed with 4% paraformaldehyde. The TRITC or FITC labeled secondary antibodies were used, and the nuclei stained with Hoechst 33258 (Sigma, USA) for 10 min. The slides were examined by fluorescence E-1000 microscopy (Nikon, Japan).

Cell culture and transfection. C6 astrocytoma cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) at 37˚C, 5% CO_2. The cells were transfeced with pCMV-HA and pCMV-HA-Axin (gift from Dr Sheng-Cai Lin) using Lipofectamine 2000 (Invitrogen, USA). To investigate the roles of p53 in the cells over-expressing Axin, the transfected C6 astrocytoma cells were treated with the specific p53 inhibitor pifithrin-α (PFT-α; Sigma) for 24 h. To determine the roles of Axin in the apoptosis and cell proliferation, siRNA specific to Axin (Genepharma, USA) were used to knockdown the endogenous Axin as previously described (17). To establish the C6 astrocytoma cells stably expressing Axin gene, cells were separately transfected with the plasmid pIRES2-EGFP (C6-EGFP) or pIRES2-EGFP-Axin (C6-Axin). The G418-resistant clones were grown in a media containing G418 until the cells stably expressing Axin gene appeared (18).

Growth curve assay, flow cytometric analysis and colony formation analysis. For the growth curve assay (MTT methods), C6 cells were plated in a 96-well plate at a density of 600 cells per well, and incubated in a humidified 5% CO_2 at 37˚C. At 1, 3, 5, and 7 days, to cells were added methyl thiazolyl tetrazolium (MTT) 20 μl per well. After 4-h incubation, the supernatants were removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well and swirled for 10 min to solubilize crystals, and the absorbance at 490 nm was measured.

For analysis of cell cycles, ~1x10^6 cells were washed twice with PBS and pelleted at 1,000 x g for 5 min. Cells were resuspended in ice-cold 75% ethanol and stained with propidium iodide (PI) followed by analysis of flow cytometry.

One hundred cells were evenly seeded in a 6-well plate in each group and cultured 2-3 weeks in a humidified atmosphere, with 5% CO_2, at 37˚C. The colonies were fixed and stained with Giemsa, and counted under a microscope.

Apoptosis analysis. TUNEL method was used in apoptosis analysis with the apoptosis detection kit (Boster, China) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde for 30 min. After washing with PBS, the sections were digested with proteinase K for 3 min. Sections were labeled with TdT and DIG-d-UTP for 2 h at 37˚C, and blocked with the blocking reagents at room temperature for 30 min. The anti-Dig-antibody were applied to the cells and incubated at 37˚C for 30 min. After washing with PBS, sections were incubated with SABC (Boster) at 37˚C for 60 min, and visualized with DAB, and counter-stained with hematoxylin. The apoptotic cells in five different fields were counted and the apoptotic index was calculated as the ratio of apoptotic cells for total cells.

Table II. Primer sequences and real-time RT-PCR conditions.

Amplicon	Primer (5'-3')	Product size (bp)	Tm
ß-actin	P1-5'-GGAGATTACTGCCCTGGCTCCTA-3'	150	64.9
	P2-5'-GACTCATCGTACTCCTGCTTGCTG-3'		64.6
Axin	P1-5'-CAGTAGCGTGGATGGAAT-3'	113	50.6
	P2-5'-AAGTGCGAGGAATGTGAG-3'		50.5
Cyclin D1	P1-5'-AGGAGCAGAAGTGCGAAGA-3'	196	55.5
	P2-5'-GGGCGGATAGAGTTGTCAGT-3'		56.5
P21	P1-5'-CAAAGTATGCCGTCGTCTGT-3'	110	56.0
	P2-5'-CAAAGTATGCCGTCGTCTGT-3'		56.2
P53	P1-5'-GCGTTGCTCTGATGGTGA-3'	232	55.1
	P2-5'-CAGCGTGATGATGGTAAGGA-3'		56.6

Real-time RT-PCR analysis. Total cellular RNA was extracted with TRIzol Reagent (Invitrogen). Total cellular RNA was reverse transcribed with RNA reverse transcriptal PCR Kit (Takara, Japan), according to the manufacturer's protocol. The levels of mRNA were analyzed by real-time PCR performed with the Bio-Rad iQ4 Multicolor Real-time iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The primers for Axin, p53, p21 and Cyclin D1 are listed in Table II. Amplification products were verified by agarose gel electro-phoresis and melting curves. The levels of mRNA were normalized internally to ß-actin expression, accounting for differences in primer efficiencies (19).

Western immunoblot analysis. C6 cells were collected at 1,000 x g for 3 min and washed with PBS. The cells pellet was resuspended in lysis buffer (0.1% SDS, 62.5 mM Tris-HCl, pH 8.5 10% glycerol, 1 mM phenylmethylsulfony1 fluoride (PMSF), 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 5 μg/ml aprotinin) and sonicated for 10 sec, followed by centrifugation at 12,000 x g for 10 min. Protein concentrations were determined with the protein quantity kit (Bio-Rad Laboratories). An equal amount of protein (20 μg) was separated by SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were blocked with 5% skim-milk in TBST at room temperature for 1 h. The antibodies of Axin (A-0481 Sigma), p53 (Santa Cruz), p21 and Cyclin D1 (ABZOOM, USA) were used to detect the protein levels in total cell lysates. The intensity of ß-actin immunostaining was used as internal loading control.

Statistical analysis. Analyses were carried out using SPSS11.5 software. The Pearson or Spearman's correlation test was used to access the relationship of the expression levels of Axin with the clinicopathologic alterations of astrocytoma. The Mann-Whitney U test and the Kruskal-Wallis test were used to analyze the results of real-time RT-PCR, flow cytometry, MTT, and apoptosis. P-values <0.05 were considered statistically significant.

Table III. The expression of Axin in 96 cases of astrocytoma.

	Axin expression (-)	(+)	P-value	
Grades				
II	26	23		
III	23	8	R=-0.286*	P<0.05
IV	14	2		
Sex				
Female	22	18	R=-0.189	P>0.05
Male	41	15		
Age				
≤40	34	17	R=0.023	P>0.05
>40	29	16		
Ki-67 labeling index				
≤5%	33	25	R=-0.227*	P<0.05
>5%	30	8		
Location				
Frontoparietal	22	8		
Cerebral ventricle	5	8		
Temproparietal	13	7	R=0.008	P>0.05
Cerebellum	12	3		
Others	11	7		

Results

Axin expression correlated with reduced cellular activity in astrocytoma. The immunohistochemistry was employed to investigate the expression of Axin and Ki-67 in the astrocytoma (Fig. 1). As shown in Table III, 33 cases in the

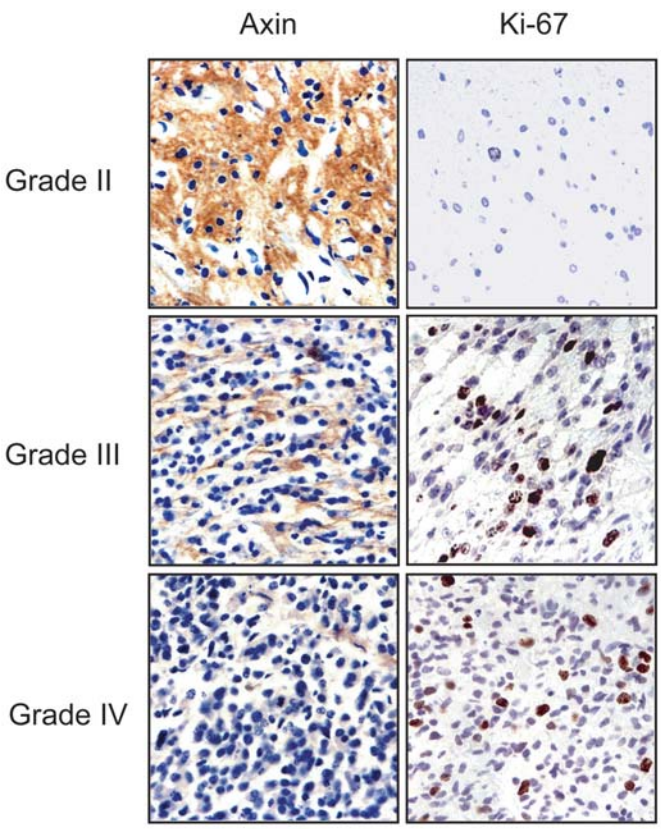

Figure 1. Immunohistochemical staining of Axin and Ki-67 in astrocytoma. The localization of Axin was preserved in both cytoplasma and nuclei, and the expression level of Axin was lower in high-grade astrocytoma compared to low-grade astrocytoma (left panel); Ki-67 was expressed in nuclei, and the expression of Ki-67 was lower in low-grade astrocytoma compared to the high-grade astrocytoma (right panel). The expression of Axin was negatively correlated with the grade of astrocytoma (R=-0.286;*P<0.05).

96 samples of astrocytoma showed Axin positive expression (34.4%). 23 cases of 49 Grade II samples (46.9%), 8 of 31 Grade III samples (25.8%), 2 of 16 Grade IV samples (12.5%) showed Axin positive staining. The Spearman's correlation test revealed that Axin positive expression was significantly inversely correlated with the grades of astrocytoma (R=-0.286; P<0.05).

The correlative analysis between Axin expression and the clinicopathologic characteristics of patients with astrocytoma is summarized in Table III. Axin immunoactivity was correlated negatively with Ki-67 labeling index (R=-0.227, P<0.05). However, the expression level of Axin did not differ significantly by age (P>0.05), sex (P>0.05), and location (P>0.05).

Axin induces apoptosis, and inhibits proliferative activity of astrocytoma cells. To determine the role of Axin in the astrocytoma, C6 cells were transfected with pCMV5 vector containing Axin. The expression of Axin was detected using immunoblotting, and the apoptotic cells were detected by TUNEL staining. As shown in the Fig. 2A and B, apoptotic index of cells with overexpressed Axin significantly increased compared to the pCMV5 group and the blank control (P<0.01). The cells with Axin overexpression were shrunken and the nuclei were condensed (Fig. 2A and C). These data indicated

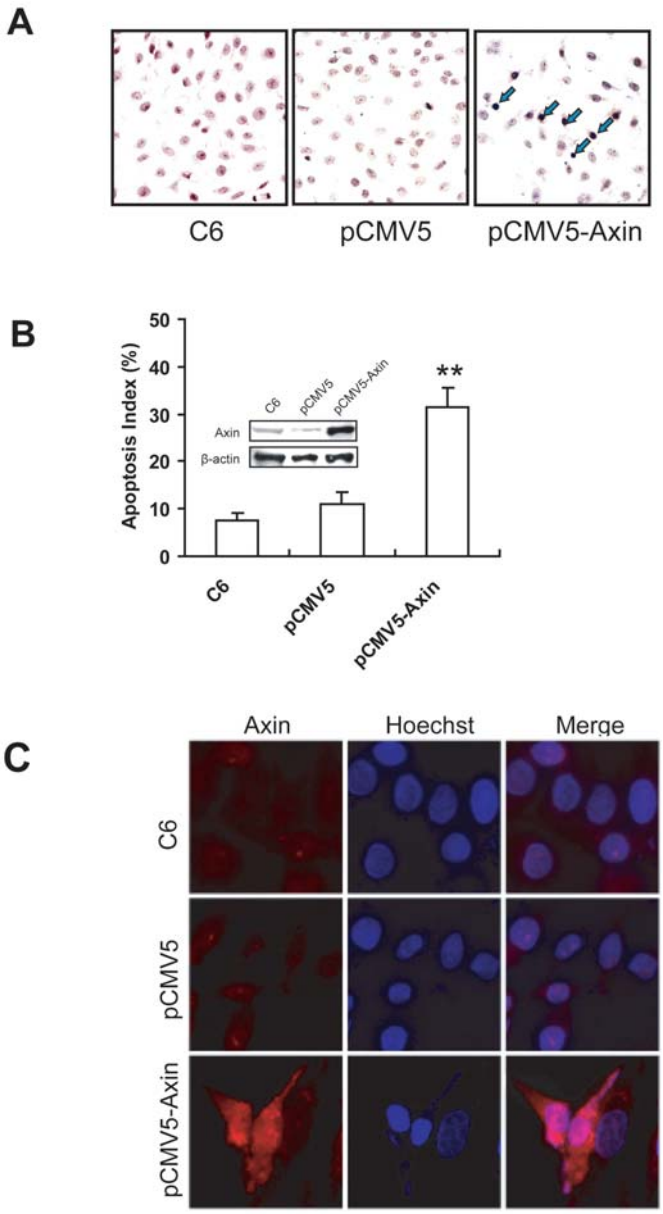

Figure 2. Axin induced apoptosis in C6 astrocytoma cells. After transfection for 48 h, the expression of Axin was detected using immunoblotting, and the apoptosis were examined using TUNEL staining and Hoechst 33258. (A) Apoptosis was detected by TUNEL staining. After transfection with Axin, the nuclei of apoptotic C6 cells became condensed and dark. (B) The apoptosis index of C6 transfected with Axin (33.2±4.2%) was significantly increased compared to C6-pCMV5 (10.79±2.6%) and C6 cells (7.8±1.6%). (C), The nuclei (blue) of cells with overexpression of axin (red) are condensed. n=3; **P<0.01.

that overexpression of Axin induced cell death in the astrocytoma cell line.

To determine the involvement of Axin in the astrocytoma cell growth, C6 astrocytoma cells were transfected with pIRES2-EGFP-Axin, which could express both Axin and EGFP separately. The cells stably expressing Axin were established by G418 selection. The siRNA specific for Axin was introduced to knockdown Axin expression. The expression levels of Axin was detected using immunoblotting, and the cell cycles were examined by flow cytometry analysis (Fig. 3A). The results indicated that overexpression of Axin

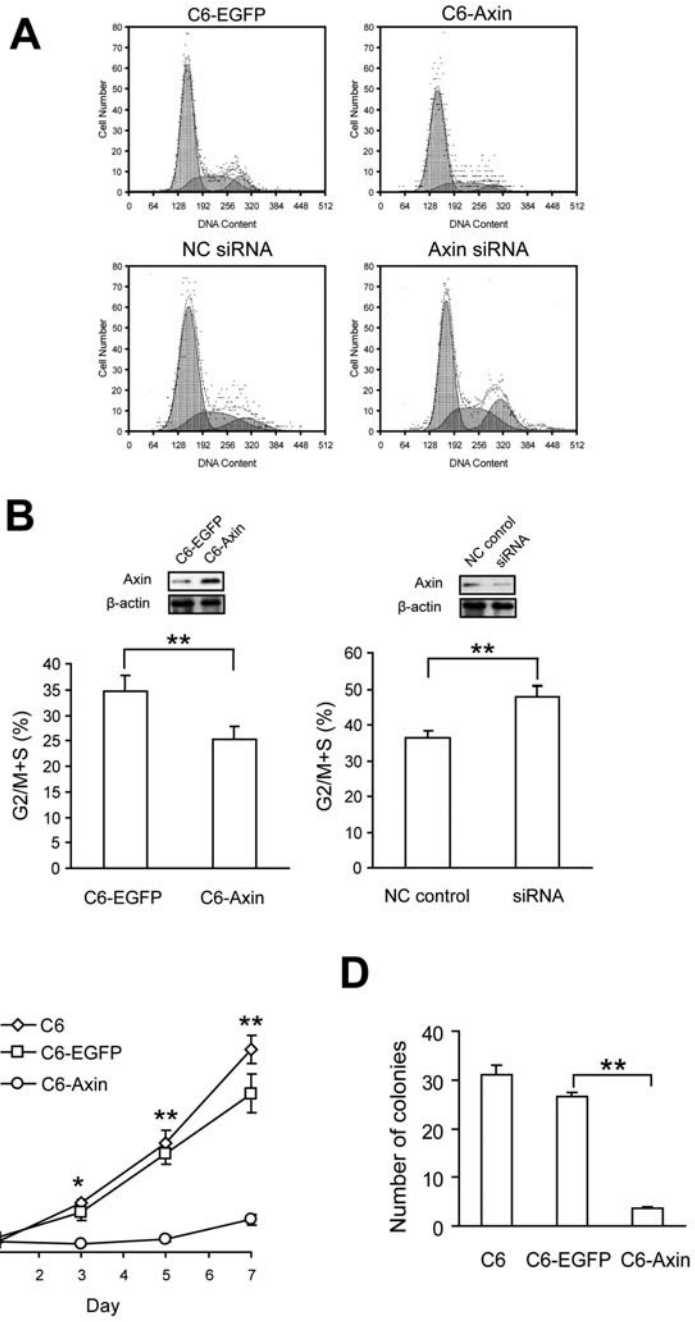

Figure 3. Axin reduces cell proliferative activity in C6 cells. (A) The cell cycle was analyzed by flow cytometry according to DNA content. (B) The percentage of G2/M + S-phase cells was lower in C6 cells transfected with pIRES-EGFP-Axin (C6-Axin) compared to C6 cells transfected with pIRES2-EGFP (C6-EGFP). Furthermore, the percentage of G2/M + S-phase cells in C6 cells transfected with Axin siRNA was higher compared to the C6 cells with negative control siRNA (NC siRNA). The expression levels of Axin are shown in the upper panel. (C) The MTT assay indicated that the growth rate of C6-Axin group was significantly lower than C6-EGFP and C6 groups. (D) The colony-forming ability was markedly reduced in C6-Axin group compared with the C6-EGFP group (3.5±0.5 clones/100 cells versus 31±2 clones/100 cells). n=3; *P<0.05; **P<0.01.

could reduce the distribution of G2/M + S-phase cells (25.2% versus 34.6%, P<0.01), and led to G1-phase cell cycle arrested (Fig. 3B). Furthermore, knockdown of Axin in C6 cells could increase the distribution of G2/M + S-phase cells (48.0% versus 36.3%, P<0.01).

In order to investigate the proliferation inhibitory effect of Axin on astrocytoma cells, MTT assay and colony-forming ability experiments were used. MTT assay results showed that the level of proliferation in C6 cells overexpressing Axin was significantly lower than that of C6 cells or C6 cells transfected with pIRES2-EGFP (P<0.05 at days 3, 5 and 7) (Fig. 3C). Similarly, the colony-forming ability markedly reduced in the cells overexpressing Axin compared with the C6 cell transfected with EGFP (3.5±0.5 clones/100 cells versus 31±2 clones/100 cells, P<0.01) (Fig. 3D). These results suggested that Axin could inhibit the proliferative activity of astrocytoma cells.

Axin activates the p53 pathway in C6 astrocytoma cells. We next assessed the effect of Axin on the expression of p53 and its downstream genes, p21 and Cyclin D1, which are known to regulate apoptosis and cell cycle distribution (20-22).

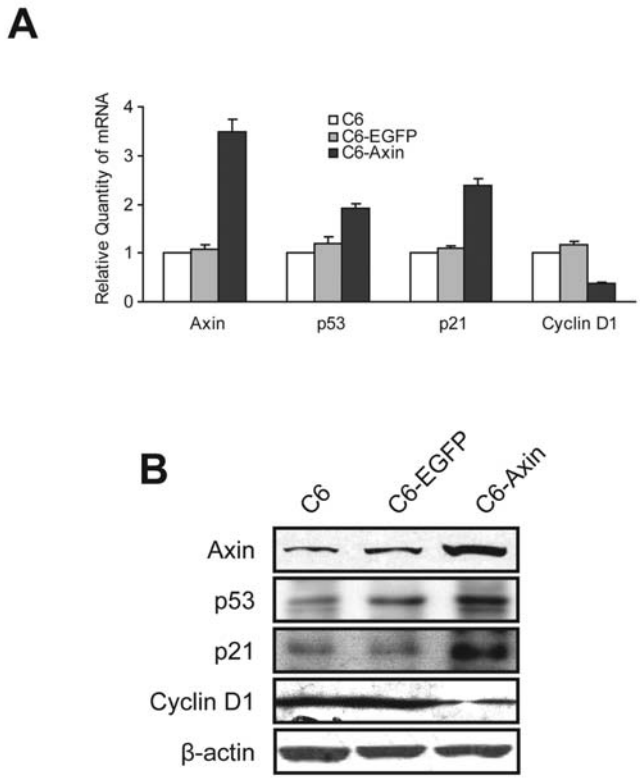

Figure 4. Protein and mRNA levels of Axin, p53, p21, and Cyclin D1 in C6 cells transfected with Axin. (A) The mRNA levels were measured by semi-quantitative real-time PCR. The relative expressions of Axin, p53, and p21 mRNA were enhanced, and Cyclin D1 was reduced in C6 cells transfected with pIRES2-EGFP-Axin (C6-Axin) compared to the C6 cells transfected with pIRES2-EGFP (C6-EGFP). (B) The protein expression of Axin, p53, and p21 enhanced, and Cyclin D1 protein was reduced in C6-Axin cells compared to C6-EGFP cells.

Immunoblotting and real-time PCR were using to evaluate the protein and mRNA level of p53, p21 and Cyclin D1. As shown in the Fig. 4A, the mRNA level of p53 in C6 cells with overexpressed Axin markedly increased compared with the C6 cells transfected with pIRES2-EGFP ($P<0.05$). Moreover, the mRNA level of p21 was significantly increased ($P<0.05$), and the Cyclin D1 mRNA decreased ($P<0.05$) compared with the control groups. Similarly, the immunoblotting analysis revealed that Axin induced the expression of p53 and p21 in C6 cells, and reduced the expression of Cyclin D1 (Fig. 4B). These results suggested that Axin could activate the expression of p53, and then upregulate the p21 expression and downregulate the Cyclin D1.

To further investigate the role of p53 in the Axin-induced apoptosis and inhibition of cell growth, pifithrin-α, which is a reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription, was used to treat the cells. The protein levels of Axin, p53 and its downstream genes were evaluated by immunoblot analysis. We found that the C6 cells with endogenous or overexpressed Axin treated with p53 inhibitor resulted in significantly reduction of p53 and p21, and induction of Cyclin D1 compared with C6 cells with overexpressed Axin (Fig. 5A and B), suggesting the p53 inhibitor could counteract the effect of Axin.

Furthermore, the effect of p53 inhibitor on the apoptosis induced by Axin was investigated using TUNEL staining.

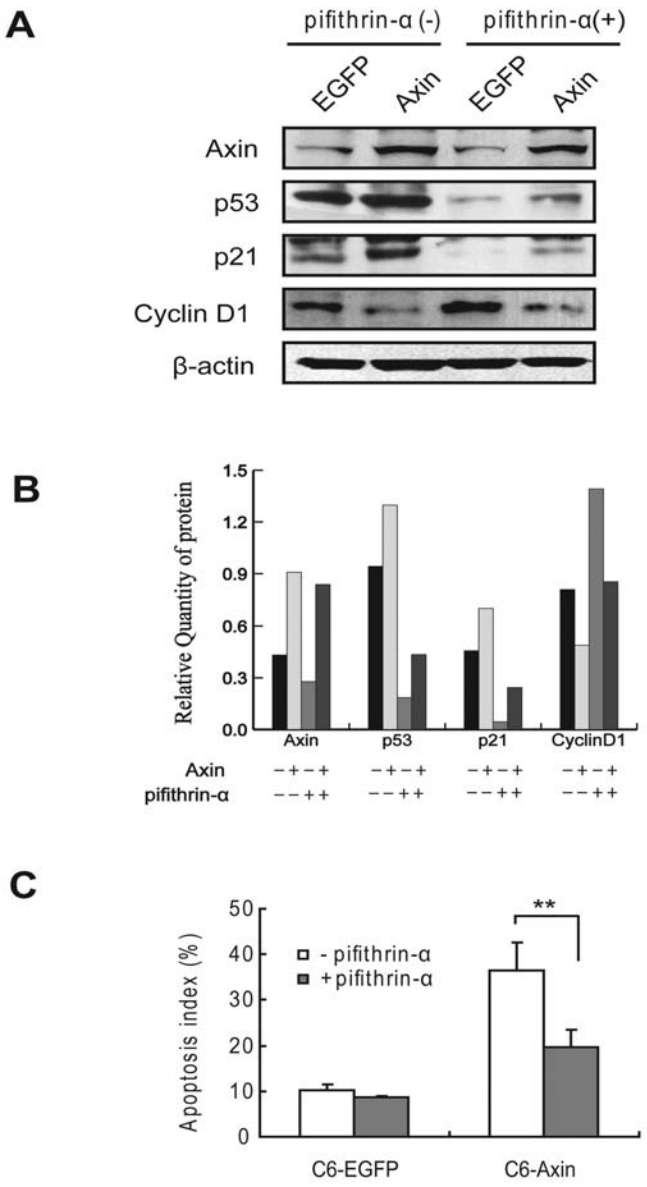

Figure 5. The p53 pathway plays an important role in Axin induced cell death. (A) Expression of Axin, p53, p21, and Cyclin D1 were examined by immunobloting in C6 astrocytoma cells with or without overexpressed Axin. Cells treated with pifithrin-α, a p53 inhibitor, resulted in significant reduction of p53 and p21, and induction of Cyclin D1 compared with non-treated cells. (B) The relative quantity of Axin, p53, p21 and Cyclin D1 protein was analyzed by Quantity One software. (C) The effect of pifithrin-α on the apoptosis induced by Axin was investigated. The apoptotic index of C6 with overexpressed Axin (C6-Axin) treated with pifithrin-α was 19.5%, and was decreased nearly 50% compared with the non-treated group. n=3; $^{**}P<0.01$.

The apoptotic index of C6-Axin treated with p53 inhibitor was 19.5%, and nearly 2-fold lower compared to the non-treated group (Fig. 5C), suggesting the p53 inhibitor could antagonize the cell death-inducing effect of Axin. Collectively, these results demonstrate that the p53 pathway plays an importrant role the apoptosis induced by Axin in astrocytoma.

Discussion

Axin was identified in 1997 by Zeng *et al* (4). Deregulation of Axin has been found in different forms of cancer. Nakajima

et al (14) found that Axin expression correlated inversely with depth of invasion, lymph node metastasis, and lymphatic invasion and suggested that reduced Axin expression correlated with tumor progression of esophageal squamous cell carcinoma. Zhou and Gao (23) also concluded that reduced expression of Axin might play a pivotal role in the carcinogenesis and metastasis of oral squamous cell carcinoma. Xu *et al* (24) showed that reduced expression of Axin is common in lung cancer. In the present study, we confirmed that Axin expression was negatively correlated with the grades of astrocytoma. These results indicate that Axin plays an important role in the progression of astrocytoma, and the expression level of Axin may provide useful information for estimating malignancy of astrocytoma.

Ki-67 is a useful marker of proliferative information (25). We detected the expression of Ki-67 in astrocytoma. The result showed that high-grade astrocytoma exhibited higher Ki-67 immunoreactivity compared to low-grade tumors, this result concurs with previous investigations (26-28). The result indicated that Axin expression was correlated negatively with Ki-67 immunoactivity.

To further investigate the role of Axin in astrocytoma, we introduced Axin into C6 astrocytoma cells and examined the changes of apoptosis and cell growth. The results showed that overexpression of Axin induce cell death, inhibit cell growth, and reduce the colony formation ability in C6 astrocytoma cells. Axin has been suggested to be a tumor suppressor for many reasons: Axin plays a central role in the control of the oncogenic protein ß-catenin in Wnt signaling pathway (29); Satoh *et al* (30) and Hsu *et al* (31) also found that introduction of wild-type Axin into the cancer cells could lead to cell death; high levels of programmed cell death occurred in the thymus and spleen of transgenic animals overexpressing Axin (31). Moreover, Axin stimulates p53, facilitating p53-mediated transcriptional activity and apoptosis (11). Our study provided further evidence for Axin as a tumor suppressor.

We also investigated the effect of Axin on the expression of p53 and its downstream genes p21 and CylinD1, which are known to regulate apoptosis and cell cycle distribution (20-22). Our results revealed that Axin induced the mRNA and protein levels of p53 and p21, reduced the level of Cyclin D1, while Axin silencing reduced p53 and p21 expression. These effects were abolished by the p53 inhibitor pifithrin-α. The present result suggest that Axin induces cell apoptosis and reduces the distribution of G2/M + S-phase cells by the p53 pathway. To achieve the role of p53 in tumor suppression, activated p53 seems to trigger two separable, albeit not mutually exclusive pathways, one to induce p21 for cell cycle arrest (32) and the other to induce proapoptotic factors such as Bax and Puma (33-35). Rui *et al* (11) showed that Axin and HIPK2 may preferentially trigger the apoptosis pathway but not the G1 cell cycle arrest pathway. However, our present study showed that Axin induced p53 and p21 for cell cycle arrest in astrocytoma. Additional studies will be necessary to elucidate the Axin-induced p53 pathway for apoptosis in astrocytoma cells.

In conclusion, we report for the first time that the expression level of Axin was correlated with the progression of astrocytoma, as well as Ki-67 labeling index. Axin induced cell death and reduced cell proliferative activity. Moreover, we found Axin could induce the expression of p53 and its targeting gene of p21, and downregulated the expression of Cyclin D1. Interestingly, the specific p53 inhibitor, pifithrin-α, counteracted the cell-death-inducing and cell proliferation effect. Our data suggest that Axin induced apoptosis and inhibited cell proliferation in astrocytoma partially by activating the p53 pathway. Our finding will expand the avenues to understand the functions of Axin and p53 as both a tumor suppressor and a developmental regulator. In particular, induction of Axin overexpression has the potential to be developed into a future treatment for astrocytoma patients.

Acknowledgements

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