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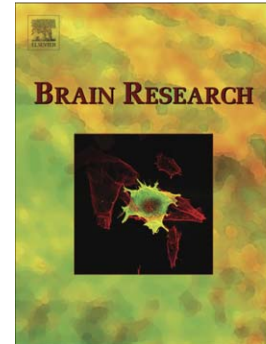

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Expression of Targeting Protein for Xenopus Kinesin-like Protein 2 is Associated with Progression of Human Malignant Astrocytoma

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Abstract

In humans, the targeting Protein for Xenopus Kinesin-like Protein 2 (TPX2) is a cell cycle-associated protein, and altered TPX2 expression has been found in various malignancies. However, the contribution of TPX2 expression to astrocytoma progression is unclear. The aim of this study was to investigate TPX2 expression in human astrocytoma samples and cell lines. TPX2 protein expression was detected in the nucleus of astrocytoma tissues by immunohistochemistry and immunofluorescence staining. Real-time PCR and western blot analysis showed that the expression levels of TPX2 were higher in high-grade astrocytoma tissues and cell lines than that in low-grade astrocytoma tissues and normal cell lines. Immunohistochemical analysis of tumor tissues from 52 patients with astrocytoma showed that TPX2 over-expression was significantly associated with decreased patient survival. In addition, down-regulation of the TPX2 gene by RNA interference inhibited proliferation of U87 cells. TPX2 gene silencing also increased early-stage apoptosis in U87 cells. Western blotting and real-time PCR showed changes in the protein and mRNA expression of Aurora A, Ran, p53, c-Myc and cyclin B1 in U87 cells that had been transfected with pSUPER/TPX2/siRNA. These data suggest that TPX2 expression is associated with the progression of malignant astrocytoma.

Research Highlights

Demonstrated that a gene known as targeting protein for Xenopus kinesin-like protein 2 (TPX2) may serve as an attractive anticancer target for malignat astrocytoma. The high expression of TPX2 expression in malignant astrocytoma tissues and cells may be associated with the progression of malignant astrocytoma. This result may offer a novel approach to controlling malignant astrocytoma.

Classification terms:research report

Section: Disease-Related Neuroscience

Keywords: Astrocytoma; TPX2; RNA interference; apoptosis

Abbreviations: TPX2, targeting protein for Xenopus kinesin-like protein 2; PCR, Polymerase Chain Reaction; PA, pilocytic astrocytoma; DA, diffuse

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astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma; DAPI, 4',6-diamidino-2-phenylindole; RNAi, RNA interference; siRNA, small interfering RNA ; c.p.m., counts per minute

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1. Introduction

Malignant astrocytomas are the most common form of primary brain tumor, of which glioblastoma muliforme (GBM) is the most frequent malignant type (Levin, 1999). Despite improvement in treatment strategies, the prognosis for patients with malignant astrocytoma remains poor (Wechsler-Reya and Scott, 2001). Multiple genetic aberrations have been described in malignant astrocytoma, including amplification, homozygous deletion and the mutation of multiple genes (Ichimura et al., 2000; Riemenschneider et al., 2003; Ohgaki and Kleihues, 2007; Balss et al., 2008; Parsons et al., 2008). Several important pathways involved in the regulation of cell division are disrupted in glioblastoma (Cancer Genome Atlas Research Network, 2008). With an improved understanding of the molecular pathogenesis of malignant astrocytoma, targeted molecular therapies have recently evolved (Chi and Wen, 2007; Ohgaki and Kleihues, 2007; Sathornsumetee et al., 2007). The identification of additional astrocytoma-associated tumor markers is thus important for further research on astrocytoma pathogenesis. The targeting protein for Xenopus kinesin-like protein 2 (TPX2) is a cell cycle-associated human protein (Heidebrecht et al., 1997; Zhang et al., 1999) and its expression is tightly regulated by the cell cycle. The aberrant expression of TPX2 has been found in various malignant tumors (Bonatz et al., 1999; Hufton et al., 1999; Rudolph et al., 1999; Gruss et al., 2002; Krams et al., 2003; Tonon et al., 2005; Shigeishi et al., 2009; Kadara et al., 2009; Warner et al., 2009; Satow et al., 2010). These results suggest that TPX2 plays a role in the oncogenesis of some malignancies. However, the expression patterns of TPX2 in astrocytoma are unclear. In the present study, we investigated the expression of TPX2 in human astrocytoma and related cell lines in order to test whether a correlation existed between TPX2 protein levels and the grade of cell differentiation. In addition, we evaluated the proliferation and apoptosis of cells when the TPX2 gene was inhibited by RNA interference in order to examine the relationships between TPX2 and astrocytoma.

2. Results

2.1 Expression of TPX2 in astrocytoma tissue

Positive TPX2 immunoreactivity was detected in the nuclear compartments of 35 out of 52 astrocytoma tissue samples tested, while no positive TPX2

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staining was detected in the normal brain tissue (Table 1, Fig. 1A). Analysis of the positive-staining data showed that the TPX2 expression level in high-grade astrocytomas was significantly higher than that in low-grade astrocytomas (P =0.045). Similar results were also obtained by real-time PCR (P <0.001) and western blot analysis (P =0.003) (Fig.1B and Fig.1C). In addition, the median survival durations in patients with strongly positive versus moderately positive or negative TPX2 staining (+++ vs. ++/+ -) were 16.385 months and 29.205 months, respectively. Kaplan-Meier survival curves indicated that increased expression of TPX2 was significantly associated with the overall poor survival of astrocytoma patients (P =0.034) (Fig. 1D).

2.2 Expression of TPX2 in U87 and U251 cell lines

The results of western blotting showed that TPX2 protein expression was more significant in U87 and U251 cells than in normal control cells (Fig. 2A). The results of real-time PCR analysis showed that TPX2 mRNA expression increased significantly in U87 and U251 cells compared with control cells (P <0.001 and P <0.001) (Fig. 2B). Immunofluorescent stianing of U87 cells showed that TPX2 protein was localized to the nucleus and associated with the mitotic spindle (Fig. 2C).

2.3 Effects of TPX2 down-regulation on the proliferation of GBM cells

The TPX2 gene was silenced by pSUPER/TPX2/siRNA3, while there was no significant difference in TPX2 expression in U87 cells transfected with pSU-PER/TPX2/siRNA1 or pSUPER/TPX2/siRNA2 compared with the control group and negative control group (Fig. 3A) . To detect cell proliferation, counts per minute (c.m.p.) was counted by an ^{3}H-TdR incorporation test. c.m.p. was decreased in U87 cells where TPX2 expression was inhibited, as compared with that in control cells and negative control cells (P<0.001, P=0.01) (Fig. 3B).

2.4 Effects of TPX2 down-regulation on the apoptosis of GBM cells

Flow cytometry showed that the down-regulation of the TPX2 gene increased the percentage of early-stage apoptosis of U87 cells compared with control

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cells and negative control cells (P<0.001, P<0.001) (Fig. 4A). Hoechst 33258 staining also showed that the number of apoptotic nuclei was increased (Fig. 4B), suggesting that treatment of TPX2 with siRNA activated the apoptotic pathway.

2.5 Expression of Aurora A, Ran, p53, c-Myc, cyclin B1, and cyclin D1 in U87 cells

The results of western blotting showed that the expression levels of Aurora A, Ran, c-Myc, p53 and cyclin B1 underwent changes in U87 cells where TPX2 was silenced as compared with control cells (Fig. 5A). Real-time PCR analysis showed that the levels of Aurora A, Ran and cyclin B1 mRNA expression in U87 cells transfected with pSUPER/TPX2/siRNA3 were significantly decreased compared with the control group (P <0.001, P =0.027 and P <0.001) (Fig. 5B). The levels of p53 and c-Myc mRNA expression were increased compared with control cells (P <0.001 and P =0.002). No significant difference in cyclin D1 protein and mRNA expression was observed in any of the groups.

3. Discussion

In the present study, we present both clinical and experimental evidence that TPX2 expression affects the progression of human malignant astrocytoma. The mitotic spindle apparatus segregates and redistributes chromosomes during mitosis (Sharp et al., 2000) and is composed of a highly dynamic microtubule skeleton that is associated with a variety of proteins, including kinesin-like and microtubule-associated proteins (Desai and Mitchison, 1997). As a cell microtubule and cycle-associated human protein (Heidebrecht et al., 1997), TPX2 has non-redundant functions essential for chromatin-induced microtubule assembly (Gruss et al., 2001) and spindle formation (Sharp et al., 2000) during mitosis. TPX2 strictly colocalizes with spindle microtubules in the M-phase (Trieselmann et al., 2003). Previous studies have shown that TPX2 is over-expressed in some carcinomas (Bonatz et al., 1999; Hufton et al., 1999; Rudolph et al., 1999; Gruss et al., 2002; Krams et al., 2003; Tonon et al., 2005; Shigeishi et al., 2009; Kadara et al., 2009; Warner et al., 2009; Satow et al., 2010). Down-regulation of TPX2 in a variety of human tumor cell lines results in apoptotic cell death and gross cell cycle aberrations (Morgan-Lappe et al., 2007). This re-

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sult suggests that TPX2 plays a role in promoting a highly malignant behavior in human cancers. However, its role and molecular mechanisms in astrocytoma are unclear. In the present study, we found both clinical and experimental evidence altered in TPX2 expression in human astrocytoma. Our data demonstrate that there is a correlation between TPX2 expression levels and astrocytoma differentiation grade. Furthermore, we compared the survival time of astrocytoma patients with different TPX2 expression levels. The results suggest that the TPX2 expression status may be a valuable metric for predicting the survival of patients with malignant astrocytoma. We also found that transfection with TPX2/siRNA resulted in the suppression of proliferation and the induction of early-stage apoptosis in U87 cells. Our study also suggests that down-regulation of TPX2 may decrease the proliferation and induce the apoptosis of malignant astrocytoma cells. Finally, we analyzed TPX2 expression to see whether it was correlated with the cell cycle- and tumor-associated proteins. Previous studies have shown that TPX2 promotes microtubule assembly around chromosomes by targeting Aurora kinase A (AURKA) to spindle microtubules (Kufer et al., 2002). AURKA is a key regulator of genome stability and plays an important role in centrosome maturation and mitotic spindle assembly. TPX2 directly binds to the kinase catalytic domain of Aurora A (Eyers and Maller, 2004). TPX2-AURKA interaction leads to the activation of AURKA, and in turn the phosphorylation of TPX2 by this kinase (Eyers and Maller, 2004). And TPX2 and AURKA integrate with each other for spindle assembly (De Luca et al., 2006).Previous studies have shown that the disruption of Aurora A/TPX2 interaction results in defective spindles that generate chromosomal abnormalities (Bibby et al., 2009). It has been proposed that TPX2 is a target of RanGTP in the spindle assembly pathway (Ems-McClung et al., 2004). Ran is a small GTPase that belongs to the RAS superfamily and plays a critical role in mediating the effects of several mitotic factors including TPX2 (Gruss and Vernos, 2004). The interaction between TPX2 and AURKA was stimulated by RanGTP (Tsai et al., 2003). TPX2 is bound to the nuclear import factors importin α and β during M-phase. This complex is dissociated by RanGTP and once released TPX2 promotes microtubule assembly (Gruss et al., 2001). This function of TPX2 may be related to the apoptosis of tumor cells.

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Some studies have found that p53 and c-Myc participate in promoting the TPX2 function. As apoptosis-associated proteins, p53 and c-Myc have the ability to induce the apoptosis of tumor cells. P53 is able to inhibit AURKA activity (Furukawa et al., 2006) and this inhibitory effect can be suppressed by the prior binding of AURKA to TPX2 (Eyers and Maller, 2004). The activation of AURKA by TPX2 is required for the full accumulation and phosphorylation of p53 (Pascreau et al., 2009). Aurora-A kinase regulates telomerase activity through c-Myc in tumor cells (Yang et al., 2004). During the apoptotic-execution phase, cells undergo dramatic structural rearrangements that require a functioning yet remodeled cytoskeleton (Moss and Lane, 2006). Therefore, there is a close association between microtubules and nuclear fragments in apoptotic cells (Moss et al., 2006). The release of nuclear RanGTP is required for apoptotic microtubule assembly (Moss et al., 2009). As a cell cycle-associated protein, cyclin B is involved in the generation of Ran-GTP in mitotic chromosomes (Li and Zheng, 2004). Our study also confirmed that these proteins interact with TPX2. These data suggest that TPX2 might affect the process of astrocytoma through regulating the function of cell cycle- or/and apoptosis-associated proteins. Nevertheless, further research on the function of TPX2 is needed. Accordingly, TPX2 might be an attractive anticancer target because of its ability to regulate the cell cycle and microtubule dynamics, which are important mechanisms of tumor formation.

In summary, our study demonstrated a correlation between TPX2 expression levels and astrocytoma differentiation grade. High TPX2 expression was detected in malignant astrocytoma, and increased TPX2 expression was associated with poor overall survival in astrocytoma patients. The inhibition of TPX2 reduced the proliferation of GBM cells and induced the apoptosis of GBM cells. TPX2 expression was associated with the expression of a cell cycle-associated gene (cyclin B1) and apoptotic genes (p53 and c-Myc) in malignant astrocytoma, which may be one of the mechanisms through which TPX2 regulates highly malignant tumor behaviors. These findings suggest that TPX2 may play important roles in the progression of malignant astrocytoma. Furthermore, targeting microtubule and cell cycle-associated proteins including TPX2 may represent a novel approach to controlling malignant as-

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trocytoma progression. Further investigation on the direct function of these proteins in the pathogenesis of cancer is needed.

4. Experimental Procedures

4.1 Patients and tissue samples

Samples were obtained from 52 patients with newly diagnosed primary astrocytoma (5 PA, WHO grade I; 16 DA, WHO grade II; 13 AA, WHO grade III; 18 GBM, WHO IV), who had not received therapy before sample collection. Samples were collected between 2004 and 2006 in Changzheng Hospital, the Second Military Medical University (Shanghai, China). The research protocol was approved by the Ethics Committee of Second Military Medical University. All samples were confirmed by pathological examination according to WHO criteria (Kleihues et al., 2002). Normal brain tissue samples were obtained from five patients who underwent decompressive craniectomy. The tumors were calssified into low-grade (WHO grade I and II) and high-grade (WHO grade III and IV) groups for statistical analysis.

4.2 Cell lines

Human GBM cell lines U87 and U251 were purchased from ATCC and grown and maintained in DMEM containing 10% fetal bovine serum (Hyclone, UT, USA) with 2% agarose-coated slides (NUNC, Denmark).Human astrocytes-cerebellar (HAc) astroglial cells were used as controls.

4.3 Immunohistochemical analysis

Paraffin-embedded sections were immunohistochemically stained with a polyclonal antibody against human TPX2 (ab71816; Abcam, UK). The nuclei were then counterstained with hematoxylin. Staining was classified by a three-tiered system according to the percentage of positive cells and staining intensity as previously described (Gong et al., 2005): negative (0/+), moderately positive (++), or strongly positive (+++). Scoring of staining intensity was conducted in a blinded manner to prevent bias resulting from the knowledge of the clinical data.

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4.4 Patient survival analysis

Of the 52 patients whose tissue samples were investigated for TPX2 expression by immunohistochemical analysis, overall survival time of all patients was measured from the date of diagnosis to the date of death. All patients had complete 3-year follow-up information.

4.5 Immunofluorescence staining

U87 cells were incubated with the anti-TPX2 polyclonal antibody. The cells were subsequently incubated with mouse antibody against γ-tubulin (Sigma, USA). The nuclei were finally counterstained with DAPI. Fluorescence microscopy was performed using a confocal microscope system.

4.6 Real-time PCR

RNA extraction and RT-PCR analysis of the expression of TPX2 mRNA was performed as follows. Cells were collected and total RNA was extracted with a spin column (Qiagen, Germany) following the manufacturer's instructions. The first strand cDNA was synthesized using a cDNA synthesis kit (Promega, WI, USA). The gene expression levels were analyzed by quantitative real-time PCR conducted on an ABI 7500 Real-Time PCR system (Applied Biosystems, CA, USA). The primers used in the experiments are shown in Table 2. After an initial incubation for 2 min at 50℃, cDNA was dena tured at 95℃ for 10 min, followed by 40 cycles of PCR (95℃, 15s, 60℃, 60s) . All results were obtained from at least three independent experiments. Using β-actin as an internal control, the mRNA levels of all genes were normalized.

4.7 Western blot

Tissue section lysates or proteins were prepared from specimens and cell lines. Anti-TPX2 and β-actin antibody (Sigma, CA, USA) were incubated for 1 h, and the secondary antibody was incubated for 30 min at room temperature in PBS-T containing 2% BSA. Signals were detected by the ECL Plus Western Blotting Detection System according to the manufacturer's specifications (Amersham, UK).

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4.8 RNA interference

siRNA was designed by a siRNA Target Finder program (Ambion, TX, USA). The oligonucleotides that encoded the chosen siRNA sequences were designed for insertion into the pSUPER plasmid (OligoEngine, WA, USA) following the published protocols (Brummelkamp et al., 2002) . The final sequence-specific siRNA expression vectors were respectively named TPX2/siRNA1, TPX2/siRNA2, TPX2/siRNA3, and the negative control was abbreviated as pSUPER-neo. Untransfected cells were used as controls. Stable transfection of pSUPER/TPX2 siRNA was performed in U87 cells using the Lipofectamine 2000 (Invitrigen, Gibco, USA) following the guidelines provided by the manufacturer. The levels of knock down of TPX2 mRNA and protein were determined by western blotting and real-time PCR analysis.

4.9 Evaluation of proliferation of GBM cells by ^{3}H-TdR incorporation test

Cells were incubated in 96-well microtiter plates. Then, 1μCi of ^{3}H-TdR (GE, CT, USA) was added to each well and thoroughly mixed for 14 h before harvest. [^{3}H]Thymidine incorporation was measured as c.p.m. as detected by a MicroBeta beta counter (PerkinElmer, RI, USA).

4.10 Assessment of U87 cell apoptosis for flow cytometry and Hoechst staining

Preparation of single U87 cells was performed as follows. Apoptosis was induced in a 1×10^6 cells/ml suspension by the addition of 1 mg/ml staurosporine. Cells were Incubated in a 37 ℃, 5% CO_2 incubator for 1 h. 500 μl non-induced cell suspension was added to a second plastic 12×75 mm test tube, followed by addition of 5 μl annexin V FITC conjugate and 10 μl propidium iodide solution. The fluorescence of the cells was determined immediately (BD Biosciences, NJ, USA). At the indicated times during treatment, cells were fixed on the dishes with methanol and stained for 10 min with Hoechst 33258 (Beyotime, Jiangsu, China) at 0.5pg/ml. The percentage of cells containing apoptotic nuclei was determined by fluorescence microscopy.

4.11 Statistical Analysis

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Statistical analysis was performed using SPSS software package. Results were presented as mean values (± SD). One-way ANOVA analysis or the Kruskal–Wallis test was used to compare the data between the groups. Survival was assessed by a Kaplan-Meier analysis with a log-rank test for determining statistical significance. A value of $P<0.05$ was considered significant.

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Table and Figure legends

Table 1 TPX2 expression in 52 patients with astrocytoma

Grade	n	TPX2 positive index				
		-	+	++	+++	Positive rate
I	5	2	1	2	0	40%
II	16	3	4	6	3	56.25%
III	13	2	2	5	4	69.23%
IV	18	0	3	9	6	83.33%

Note: Number of the samples detected by immunohistochemical staining.
- or +, negative; ++, moderate positive; +++, strong positive

Table 2 The primers for real-time PCR

Gene	Forward primer	Reverse primer
TPX2	TTTGGTATCAGACCCGAAGC	TCCCCATGTTTGTCAGTGAA
Aurora A	CGGCGGCGGAAGATG	ACTTAGTCCGTTTTTGCAGCTTCT
Ran	CGGGACCATGGACAGCTTT	GCCACGAAGCGGTCGAT
p53	GCCCCCAGGGAGCACTAA	TCTCCTCCACCGCTTCTTGT
c-Myc	TTTGCGGTGGGCAGAAA	GCGGTCCCTACTCCAAGGA
Cyclin B1	CCCTGCTGCAACCTCCAA	TTTGTTACCAATGTCCCCAAGAG
Cyclin D1	AGACCTTCGTTGCCTCTTGTG	CCATGGAGGGCGGATTG
β-Actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA

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Figure 1

Expression of TPX2 in normal human brain and astrocytoma tissues. TPX2 immunohistochemical staining on the tissues of tumor and normal brain (Original magnification 200×, inset magnification 400×) (A). Protein expression of TPX2 in astrocytoma tissues detected by western blotting (B). TPX2 mRNA expression by real-time PCR (C). Correlation between TPX2 expression and survival of the patients with astrocytoma (D). *p<0.001 versus control; **p<0.001 versus low-grade.

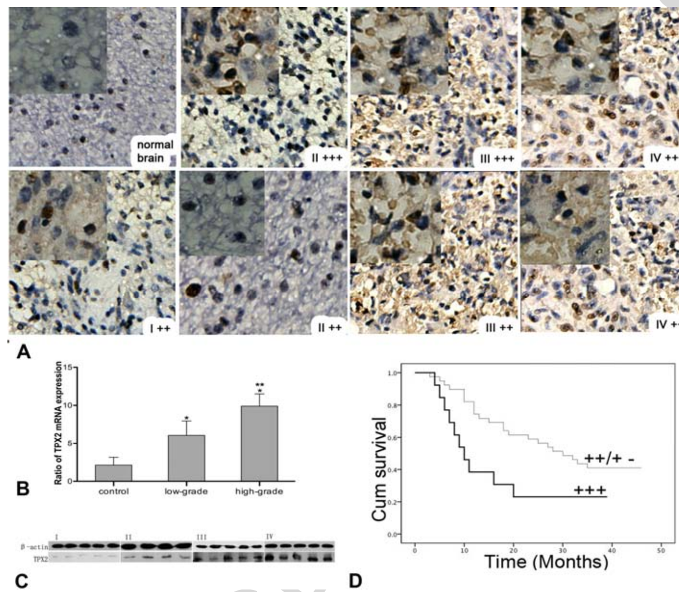

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Figure 2

Expression of TPX2 in U87 and U251 GBM cell lines. Protein expression of TPX2 by western blotting (A). TPX2 mRNA expression by real-time PCR analysis (B). Subcellular localization of TPX2 in U87 cells identified by immunofluorescent staining (C). TPX2 protein was visualized (green), centrosomes were visualized with antibody against γ-tubulin and the rhodamine-conjugated secondary an antibody (red). The nucleus was labeled with DAPI (blue). (Magnification, 1,000×).*p<0.001 versus control, **p>0.05 versus U87 cells.

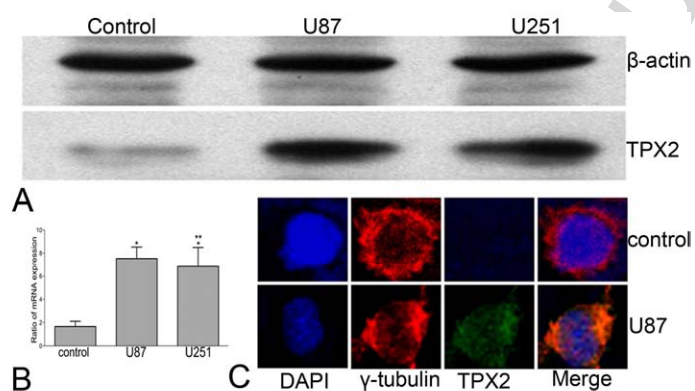

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Figure 3

Down-regulation of TPX2 expression alters GBM cell proliferation. TPX2 gene was silenced by transfection with TPX2/siRNA3 (A). c.m.p. was lower in U87 cells with transfected TPX2/siRNA3 compared with control cells (B). *p<0.001 versus control cells,**>0.05 versus control cells.

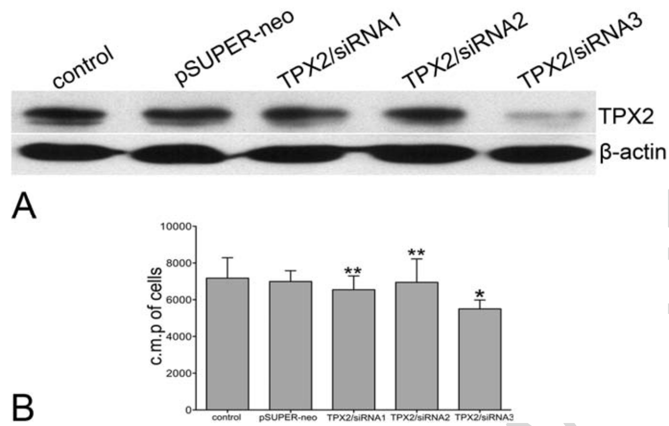

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Figure 4

Apoptosis of U87 cells for flow cytometry and Hoechst staining. TPX2-silenced U87 cells show much greater rates of early apoptosis (A). The number of apoptotic nuclei (small with condensed chromatin) increased in TPX2-silenced U87 cells (B).

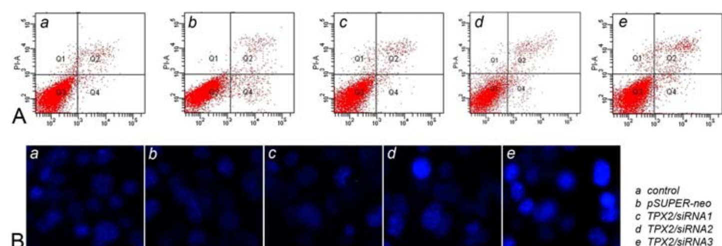

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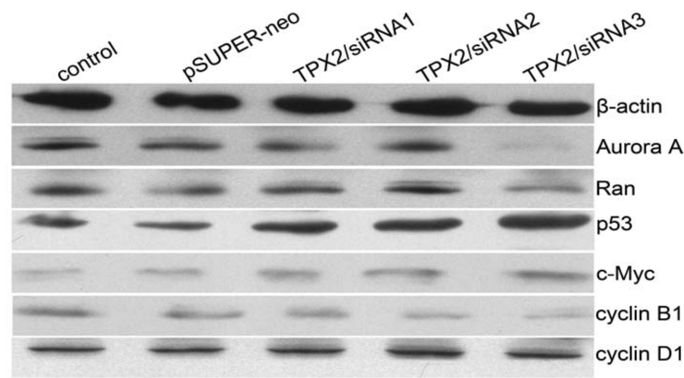
Figure 5

Expression of Aurora A, Ran, p53, c-Myc, cyclin B1 and cyclin D1 in U87 cells. Aurora A, Ran, p53, c-Myc and cyclin B1 expression by western blotting (A) and real-time PCR analysis (B) were significantly changed in transfected U87 cells when compared with control cells. *p<0.001 versus control cells. #p<0.05 versus control cells. **p>0.05 versus control cells.



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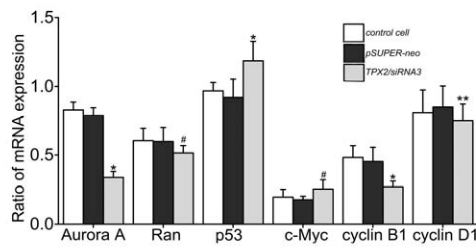

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