FK506-binding protein 5 inhibits proliferation and stimulates apoptosis of glioma cells

Hui Yang¹, Qing-Xiu Zhang², Dong-Sheng Pei³, Feng Xu¹, Yong Li¹, Ru-Tong Yu⁴

¹Department of Neurosurgery, Xuzhou First People’s Hospital, Xuzhou, China
²Department of Neurology, The Second Affiliated Hospital of Xuzhou Medical College, Xuzhou, China
³Jiangsu Key Laboratory of Biological Cancer Therapy, Xuzhou Medical College, Xuzhou, China
⁴Department of Neurosurgery, Affiliated Hospital of Xuzhou Medical College, Xuzhou, China

Submitted: 2 September 2013
Accepted: 16 September 2013

Arch Med Sci 2015; 11, 5: 1074–1080
DOI: 10.5114/aoms.2015.54864
Copyright © 2015 Termedia & Banach

Abstract

Introduction: FK506-binding protein 5 (FKBP5) is reported to act as a scaffolding protein for Akt to promote the dephosphorylation of AKT Ser473 and suppress pancreatic cancer growth. However, other studies have shown that FKBP5 promotes tumor growth and chemoresistance through regulating NF-κB signaling in other cancers. In this study, we attempted to investigate the role and mechanism of action of FKBP5 in the regulation of proliferation and apoptosis of glioma cells.

Material and methods: The glioma U251 cell line was used as the model. Cell proliferation was detected by MTT assay. Cell apoptosis was detected by annexin-V staining. Protein expression was detected by Western blot analysis.

Results: FKBP5 overexpression inhibited the proliferation of U251 cells significantly (p < 0.05), and promoted the apoptosis of U251 cells significantly (p < 0.05). In addition, FKBP5 overexpression inhibited the phosphorylation of Akt at Ser743, decreased the level of Bcl-2, increased the level of Bax, and enhanced the cleavage of caspase-9 and caspase-3 (p < 0.05 compared to control). In contrast, FKBP5 knockdown enhanced the proliferation of U251 cells, increased the phosphorylation of Akt significantly (p < 0.05), increased the expression of Bcl-2 and decreased the expression of Bax, and decreased the cleavage of caspase-9 and caspase-3 significantly (p < 0.05).

Conclusions: FKBP5 plays the role of a tumor suppressor in glioma by inhibiting the activation of Akt and stimulating the intrinsic mitochondrial apoptotic pathway, and could be used as a new target for gene therapy of glioma.

Key words: FK506-binding protein 5, Akt, glioma, apoptosis.

Introduction

Glioma is one of the most common, aggressive types and still poorly treated primary brain tumors. Several molecules have been identified to be implicated in the development of glioma [1]. However, there is still no efficient therapeutic strategy to control the advanced disease. Thus it is important to develop new approaches for glioma therapy.

Serine/threonine kinase AKT (also called protein kinase B) is a critical kinase that is normally activated in a growth-factor-dependent manner, and becomes overactivated to promote tumorigenesis [2]. The struc-
FK506-binding protein 5 inhibits proliferation and stimulates apoptosis of glioma cells

FK506 binding protein 5 (FKBP5) belongs to a family of immunophilins named for their ability to bind the immunosuppressive drug FK506. FKBP5 has peptidyl-prolyl isomerase (PPIase) activity and can produce cis-trans-isomerization of prolyl bonds in different states of a target protein, which is important for protein folding [7]. FKBP5 protein contains three C-terminal TPR domains and two N-terminal FKBP domains (FK1 and FK2) [8]. A recent study indicated that the FKBP5 C-terminal TPR domain binds to the phosphatase PHLPP, while the N-terminal FK domains are responsible for AKT binding and facilitate PHLPP-mediated dephosphorylation of AKT-Ser473, leading to increased cell sensitivity to chemotherapy [9].

Aberrant expression of FKBP5 is associated with cancer and resistance to chemotherapy via promoting NF-κB activation in melanoma and leukemia [10]. However, other evidence suggested that FKBP may function as a tumor suppressor through negatively regulating AKT activation in pancreatic cell lines [11].

In this study, we attempted to investigate the role of FKBP5 in the activation of Akt, and the regulation of the proliferation and apoptosis of glioma cells using both gain and loss of function approaches.

Material and methods

Cell culture and transfection

The glioma cell line U251 was purchased from the Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (Sijiqing Biological Co., Hangzhou, China), 50 U/ml penicillin and 0.05 mg/ml streptomycin at 37°C with 5% CO₂.

pCMV6-XL5-FKBP5 vector for the overexpression of FKBP5 was purchased from Origene (Rockville, MD, USA). siRNA duplexes were synthesized by GeneChem (Shanghai, China) with the following sequences: FKBP5 siRNA 5’ UCGGCUUGG-CAGUCUCUCCUAAA 3’; control scramble siRNA 5’ UUUCUCCGAACGUGUCACGU 3’. Transfection of FKBP5 expression vector, FKBP5 siRNA or control siRNA was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

Western blot analysis

Equal amounts of cell lysate were separated on polyacrylamide gels and then electrotransferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). After blocking for 3 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4°C with primary antibodies as follows: mouse monoclonal anti-FKBP5 (Sigma), rabbit polyclonal anti-AKT antibody (sc-8312), rabbit polyclonal p-AKT1/2/3 (Ser 473) (sc-33437), rabbit polyclonal anti-Bcl-2 antibody (sc-783), rabbit polyclonal anti-Bax (sc-493) and rabbit polyclonal anti-actin (sc-10731) (all from Santa Cruz Biotechnology), rabbit polyclonal anti-caspase-3 and anti-caspase-9 (all from Cell Signal Biotechnology). Membranes were then washed and incubated with alkaline phosphatase conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma) in TBST for 2 h, and finally developed using NBT/BCIP color substrate (Promega, Madison, WI, USA). The densities of the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP Upland, CA, USA).

Cell proliferation assay

Cell proliferation was determined using WST-8 dye (Beyotime Inst Biotech, China) according to the manufacturer’s instructions. Briefly, cells were seeded at the density of 5 x 10⁴ cells/well in 96-well plates, grown at 37°C for 24 h, and subsequently were transfected with siRNA or plasmid and then incubated in culture for 24 h, 48 h, 72 h and 96 h, respectively. After 10 μl WST-8 dye was added to each well, cells were incubated at 37°C for 2 h and the absorbance was determined at 450 nm using a microplate reader.

Apoptosis assay

Cells were plated in 24-well plates at 37°C for 24 h, and subsequently were transfected with siRNA or plasmid. Then, after being grown for 24 h the cells were treated with an annexin V-FITC apoptosis detection kit (KeyGEN, Jiangsu, China). Under fluorescence microscopy, six fields were randomly selected from every sample and 100 cells...
were randomly selected from every field. Apoptotic rate = (number of apoptotic cells/100) × 100%.

Statistical analysis
All experiments were performed at least three times. The data were expressed as means ± standard deviations, except as otherwise stated, and analyzed by one-way analysis of variance using SPSS version 13.0 (SPSS, Chicago, USA). Value of \( p < 0.05 \) was considered significant.

Results
FKBP5 inhibits proliferation and promotes apoptosis of glioma cells
First we transfected U251 cells with FKBP5 plasmid or siRNA. Western blot analysis showed that the FKBP5 protein level was increased after transfection of FKBP5 plasmid and decreased after transfection of FKBP5 siRNA (Figures 1 A and B). These results demonstrated that the constructs we used were efficient to overexpress or knockdown FKBP5 in U251 cells.

Next we investigated the effects of FKBP5 overexpression or knockdown on the proliferation of U251 cells. WST-8 assay showed that FKBP5 overexpression inhibited cell proliferation significantly (\( p < 0.05 \), Figure 2 A), while FKBP5 knockdown increased cell proliferation although the difference was not statistically significant (Figure 2 B).

Furthermore, we investigated the effects of FKBP5 overexpression on the apoptosis of U251 cells. The annexin-V staining showed that FKBP5 overexpression promoted apoptosis of U251 cells at an early stage (Figure 2 C). In addition, DAPI staining showed that overexpression promoted apoptosis of U251 cells at a later stage (Figure 2 D). Taken together, these data suggest that FKBP5 inhibits the proliferation and promotes the apoptosis of glioma cells.

FKBP5 inhibits phosphorylation of Akt in glioma cells
To investigate the molecular mechanism by which FKBP5 inhibits the proliferation of U251 cells, we first examined the activation of Akt in these cells, given the previous report that FKBP inhibited pancreatic cancer cell proliferation by negatively regulating Akt activation [11]. Western blot analysis showed that FKBP5 overexpression inhibited the phosphorylation of Akt dramatically (Figure 3 A), while FKBP5 knockdown increased the phosphorylation of Akt (Figure 3 B). These results suggest that FKBP5 may inhibit glioma cell proliferation via inhibiting Akt activity.

FKBP5 effect on expression of Bax and Bcl-2
To investigate the molecular mechanism by which FKBP5 promotes the apoptosis of U251 cells, we examined the effects of FKBP5 on the levels of Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-9 in U251 cells. Western blot analysis showed that FKBP5 overexpression decreased the protein ex-
FK506-binding protein 5 inhibits proliferation and stimulates apoptosis of glioma cells

Expression of Bcl-2 and increased the protein level of Bax, while FKBP5 knockdown increased the protein expression of Bcl-2 and decreased the protein level of Bax (Figure 4 A). These results suggest that FKBP5 may promote the apoptosis of glioma U251 cells via downregulation of expression of the anti-apoptosis protein Bcl-2 and upregulation of expression of the pro-apoptosis protein Bax.

In addition, we detected the levels of cleaved caspase-3 and caspase-9, which are important executers of apoptosis. We found that FKBP5 overexpression increased the levels of cleaved caspase-3 and caspase-9, while FKBP5 knockdown decreased the levels of cleaved caspase-3 and caspase-9 (Figure 4 B). These data indicate that FKBP5 enhances the cleavage of caspase-9 and caspase-3, thus promoting the apoptosis of glioma U251 cells.

Discussion

Recent studies have shown that FKBP5 is overexpressed in several types of cancer and sustains cancer cell growth, malignancy, and resistance to therapy through promoting NF-κB activation [12]. However, FKBP ligands are potent anticancer agents through regulating protein-protein interaction, in addition to their immunosuppressant activity [11]. In the present study we aimed to investigate the function and mechanism of action of
Figure 3. FKBP5 inhibits phosphorylation of Akt in glioma U251 cell line. A – Western blot analysis showed that protein level of p-Akt was decreased remarkably in U251 cells transfected with FKBP5 expression plasmid. B – Western blot analysis showed that protein level of p-Akt was increased remarkably in U251 cells transfected with FKBP5 siRNA.

*Value of p < 0.05 compared to transfection control. Total Akt was used as loading control.

Figure 4. FKBP5 modulates expression of Bax and Bcl-2 and cleavage of caspase-9 and caspase-3 in glioma U251 cell line. A – Western blot analysis showed that protein level of Bax was increased and that of Bcl-2 was decreased remarkably in U251 cells transfected with FKBP5 expression plasmid, while protein level of Bax was decreased and that of Bcl-2 was increased remarkably in U251 cells transfected with FKBP5 siRNA. B – Western blot analysis showed that levels of cleaved caspase-9 and caspase-3 were increased remarkably in U251 cells transfected with FKBP5 expression plasmid, but decreased in U251 cells transfected with FKBP5 siRNA.

*Value of p < 0.05 compared to transfection control. β-actin was used as loading control.
FKBPs in glioma by using the U251 cell line. Our results showed that FKBPs inhibited the proliferation and promoted the apoptosis of U251 cells and these were associated with the inhibition of Akt activation, the upregulation of pro-apoptotic factor Bax, the downregulation of anti-apoptotic factor Bcl-2, and the increased cleavage of caspase-9 and caspase-3. These results suggest that FKBPs functions as a potential tumor suppressor in glioma.

AKT is an important pro-survival signaling pathway, and misregulation of the pathway can disrupt the balance between cell survival and death, affecting cancer development and therapy. Akt activation is associated with poor prognosis and resistance to chemotherapeutics in a variety of cancers [13]. The most important upstream activator of AKT is PI3K, while lipid phosphatases such as PTEN, PHLPP and PP2A act to inactivate Akt by regulating the dephosphorylation of Akt. It is well documented that two highly conserved residues, T308 within the activation loop of the kinase domain and S473 in a hydrophobic motif just C-terminal to the kinase domain, are essential for the phosphorylation of AKT. Phosphorylation on T308 is necessary for AKT kinase activity, whereas S473 phosphorylation produce a marked effect on the strength and duration of AKT signaling [14]. In this study, we found that overexpression of FKBPs could inhibit S473 phosphorylation of Akt markedly. These results imply that FKBPs acts as a scaffold protein to anchor protein phosphatases such as PTEN to promote the inactivation of Akt and suppress the proliferation of glioma cells and are consistent with a recent study showing that ectopic expression of PTEN suppressed the growth of U251 glioma cells [15].

Cumulative evidence suggests that the dysregulation of anti-apoptotic and pro-apoptotic Bcl-2 family members is one of the defining features of cancer cells and significantly contributes to the resistance to chemotherapy and radiotherapy [2, 16, 17]. The pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2 are two important members of this family that exhibit opposite effects on regulation of the intrinsic mitochondrial apoptotic pathway [18–20]. Once this pathway is activated, it leads to downstream activation of components of the caspase cascade, such as caspase-9 and caspase-3, which are cleaved and act as executers of the apoptotic process. To understand the molecular mechanism by which FKBPs regulates the apoptotic pathway in U251 cells, we decided to examine the anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, and caspase-3 and -9. Our results showed that FKBPs overexpression reduced the expression of Bcl-2 and increased the expression of Bax, and facilitated the cleavage/activation of caspase-3 and -9. In contrast, FKBPs knockdown increased the expression of Bcl-2 and decreased the expression of Bax, and inhibited the cleavage/activation of caspase-3 and -9. Taken together, these data indicate that FKBPs activates the intrinsic mitochondrial apoptotic pathway to promote apoptosis of U251 cells.

In conclusion, this study demonstrates that FKBPs inhibits the activation of Akt and stimulates the intrinsic mitochondrial apoptotic pathway in glioma U251 cells, which may contribute to the inhibition of cell proliferation and the promotion of apoptosis. These data suggest that FKBPs plays the role of a tumor suppressor in glioma and could be used as a new target for gene therapy of glioma.

Acknowledgments
Hui Yang and Qing-Xiu Zhang — these authors contributed equally to this work.

Conflict of interest
The authors declare no conflict of interest.

References
1. Zhao WJ, Zhang W, Li GL, Cui Y, Shi ZE, Yuan F. Differential expression of MMP-9 and AQP4 in human glioma samples. Folia Neuropathol 2012; 50: 176-86.
2. Engelman JA, Luo J Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet 2006; 7: 606-19.
3. Franke TF, PI3K/Akt: getting it right matters. Oncogene 2008; 27: 6473-88.
4. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell 2007; 12: 9-22.
5. Padmanaban S, Mukhopadhyay A, Narasimhan SD, Tesz G, Czech MP, Tissenbaum HA. A PP2A regulatory subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. Cell 2009; 136: 939-51.
6. Pan Ji, Chang QS, Wang X, et al. Activation of Akt/GSK-3beta and Akt/Bcl-2 signaling pathways in nickel-transformed BEAS-2B cells. Int J Oncol 2011; 39: 1285-94.
7. Fruman DA, Burakoff SJ, Bierer BE. Immunophilins in protein folding and immunosuppression. FASEB J 1994; 8: 391-400.
8. Sinars CR, Cheung-Flynn J, Rimerman RA, et al. Structure of the large FK506-binding protein FKBPs, an Hsp90-binding protein and a component of steroid receptor complexes. Proc Natl Acad Sci USA 2003; 100: 868-73.
9. Pei H, Li L, Fridley BL, et al. FKBPs affect cancer cell response to chemotherapy by negatively regulating Akt. Cancer Cell 2009; 16: 259-66.
10. Romano SA, Sorrentino A, Di Pace AL, et al. The emerging role of large immunophilin FK506 binding protein 51 in cancer. Curr Med Chem 2011; 18: 5424-9.
11. Hou J, Wang L. FKBPs as a selection biomarker for gemcitabine and Akt inhibitors in treatment of pancreatic cancer. PloS One 2012; 7: e36252.
12. Romano SM, Mallardo M, Romano MF. FKBPs and the NF-kappaB regulatory pathway in cancer. Curr Opin Pharmacol 2011; 11: 288-93.
13. West KA, Castillo SS, Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. Drug Resist Update 2002; 5: 234-48.
14. Yang JP, Cron P, Good VM, et al. Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. Nat Struct Biol 2002; 9: 940-4.
15. Han L, Zhang AL, Xu P, et al. Combination gene therapy with PTEN and EGFR siRNA suppresses U251 malignant glioma cell growth in vitro and in vivo. Med Oncol 2010; 27: 843-52.
16. Ye F, Zhong B, Dan G, et al. Therapeutic anti-tumor effect of exogenous apoptin driven by human survivin gene promoter in a lentiviral construct. Arch Med Sci 2013; 9: 561-8.
17. Mielczarek-Palacz A, Kondera-Anasz Z, Sikora J. Higher serum levels of tumour necrosis factor and its soluble receptors are associated with ovarian tumours. Arch Med Sci 2012; 8: 848-53.
18. Ulukaya E, Acilan C, Yilmaz Y. Apoptosis: why and how does it occur in biology? Cell Biochem Funct 2011; 29: 468-80.
19. Liang QL, Wang BR, Li ZY, Chen GQ, Zhou Y. Effect of TSLC1 gene on growth and apoptosis in human esophageal carcinoma Eca109 cells. Arch Med Sci 2012; 8: 987-92.
20. Liu QL, Liang QL, Li ZY, Zhou Y, Ou WT, Huang ZG. Function and expression of prolyl hydroxylase 3 in cancers. Arch Med Sci 2013; 9: 589-93.