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FKBP51 acts as a biomarker of early metastasis and is related to carmustine sensitivity in human glioma cells

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Abstract. – OBJECTIVE: Given that FK506 binding protein 51 (FKBP51) is upregulated in multiple cancers, we designed the present study to characterize its role as well as underlying regulatory mechanisms in glioma in the presence and absence of the chemotherapeutic carmustine (BCNU).

MATERIALS AND METHODS: Through lentiviral overexpression and shRNA knockdown of FKBP51, we examined the effects on BT325 glioma cell proliferation, migration and invasion using quantitative reverse transcription PCR (qRT-PCR), CCK-8 assay, flow cytometry, and transwell assay.

RESULTS: The upregulation of FKBP51 resulted in significantly decreased BT325 cell proliferation and cell viability, cell cycle arrest, reduced BCNU chemosensitivity and AKT pathway inactivation. However, FKBP51-overexpressed BT325 cells showed enhanced migration and invasion, which was supported by corresponding increase in phosphorylated IKKα (p-IKKα), MMP-2, and MMP-9 levels, as well as increased NF-κB p65 nuclear translocation. By contrast, FKBP51-suppressed BT325 cells showed excessive proliferation and BCNU resistance due to increased p-AKT activation and attenuated migration and invasion.

CONCLUSIONS: We demonstrated that the effects of FKBP51 on BT325 glioma cell proliferation, migration, invasion and BCNU chemosensitization are modulated *via* the AKT and NF-κB pathways. Furthermore, our findings suggest the potential of FKBP51 as a prognostic glioma biomarker and an indicator of patient response to chemotherapy.

Key Words:
FK-506 binding protein 51, Proliferation, Carmustine, Migration, Invasion, Glioma.

Abbreviations

FK506 binding protein 51 (FKBP51); peptidylprolyl isomerase (PPIase); nuclear factor-κB (NF-κB); carmustine (BCNU); Roswell Park Memorial Institute (RPMI); green fluorescent protein (GFP); polyvinylidene fluoride (PVDF); propidium iodide (PI); cyclin-dependent kinase inhibitors (CDKIs); matrix metalloproteinases (MMPs); serine/threonine protein kinase (AKT), inhibitor of nuclear factor kappa (IκB); IκB kinase (IKK); glycogen synthase kinase-3 beta (GSK-3β).

Introduction

Despite being the most common primary brain tumor in adults[1], the etiology and pathogenesis of malignant (high-grade) glioma remain unclear. An epidemiological study showed that the incidence of glioma in men is approximately 1.5-fold to 2-fold higher than that in women regardless of etiology, suggesting a gender dependency[2-4]. The androgen receptor (AR) has been found to play a role in glioma tumorigenesis[5,6]. The standard treatment for glioma includes surgery, radiation and chemotherapy[7]. In general, the median survival time of glioma patients is less than 1.5 years from the point of diagnosis. The poor disease prognosis is due to the anatomical position, which poses difficulty in surgical resection, and invasiveness of the tumor, which promotes chemoresistance[7,8]. Thus, elucidation of the mechanisms underlying glioma proliferation, invasion and chemoresistance as well as prognostic biomarkers would provide useful insights and plug the gaps in the field.

FK506 binding protein 51 (FKBP51) is a 51-kDa protein that belongs to a family of immunophilins. It consists of a C-terminal TPR three-tandem repeat domain that mediates protein-protein interactions, and two N-terminal FK506 binding domains (FK1 and FK2). Functions in FK-506binding and peptidylprolyl isomerase (PPIase) activity are controlled by the FK1 domain, while the FK2 domain determines protein structure[9-12].

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FKBP51 is ubiquitous in many human tissues and is stimulated by steroid hormones, such as progesterone, glucocorticoid and androgen, via their nuclear receptors[13]. In turn, FKBP51 regulates steroid hormone receptor activity[14,15]. FKBP51 is differentially expressed in different cancer tissues of various malignancy grades and has been implicated in tumor progression and chemotherapy response[16-20]. Both AR and FKBP51 are highly expressed in glioma tissue[5,21]. Examination of FKBP51 expression in 223 brain glioma patients and 23 normal brain tissues from patients with brain injury in our previous study revealed FKBP51 upregulation in glioma tissue and found that elevated FKBP51 expression was positively correlated with glioma pathology grade (China Modern medicine 23:12-14, 2016). FKBP51 negatively regulates all 3 isoforms of the serine/threonine protein kinase AKT (AKT 1, AKT 2, and AKT3) via protein-protein interactions[22]. Aberrant AKT activation is well known to play a pivotal role in the development, progression, and chemoresistance of a variety of tumors, including gliomas[23-26]. Furthermore, FKBP51 can reportedly regulate both canonical and noncanonical nuclear factor-κB (NF-κB) activation[27,28], which controls tumor migration and invasion[29,30].

In the present study, we characterized the role of FKBP51 in glioma cell proliferation, migration, and invasion, and elucidated the underlying molecular mechanism that regulates carmustine [1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU)] chemosensitivity.

Materials and Methods

Reagents

BCNU, TNF-α, AKT inhibitor (A6730) and NF-κB inhibitor (BAY11-7082) were purchased from Sigma-Aldrich Trading Co., Ltd., Shanghai, China. The primary antibodies for FKBP51 (sc-271547) and GAPDH (sc-47724) were purchased from Santa Cruz Biotechnology, Shanghai, China. Antibodies to S176S180-p-IKKα (ab17943), IKKα (ab109749), IκB-α (ab32518), NF-κB p65 (ab32536), S9-p-GSK3β (ab75814), GSK3β (ab32391), P21 (ab109520), P27 (ab32034), p-FOXO1A (S256; ab 31339), FOXO1A (ab52857), BAX (ab32503), BCL-2(ab32124), α-tubulin (ab52866), MMP-2 (ab92536) and MMP-9 (ab76003) were purchased from Abcam, Cambridge, MA, USA. The primary antibodies for Ser473-p-AKT (4060S), AKT (4685S) and histone H3 (9715) were purchased from Cell Signaling Technology, Inc., Danvers, MA, USA. NE-PER nuclear and cytoplasmic extraction kit (78833) was purchased from Thermo Fisher Scientific, Rockford, IL, USA.

Cell Line and Culture

Human glioma cell line BT325 (Chinese Academy of Science, Shanghai, China) was cultured in Roswell Park Memorial Istitute-1640 (RPMI-1640) basic complete medium (Gibco® Thermo Fisher Scientific, Suzhou, China) supplemented with 15% fetal bovine serum (Gibco, Suzhou, China) and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator.

Lentivirus Infection

BT325 cells (1×10^5) were seeded in 24-well plates and cultured overnight in antibiotic-free RPMI 1640 medium supplemented with 15% FBS till approximately 60% confluency. The cells were then infected with GFP-labeled FKBP51 lentivirus (Shanghai Genetimes Technology, Inc., Shanghai, China) for 8 h at a multiplicity of infection (MOI) of 20 in the presence of 5 μg/ml polybrene to improve infection efficiency. Transfection with an empty lentiviral vector was used as the negative control. The GFP-positive lentiviral-infected cells were visualized using a fluorescence microscope (Molecular Devices ImageXpress® Micro Confocal System, San Jose, CA, USA) 72 h after infection and 3 visual fields at ×200 magnification were used for counting.

FKBP51 shRNA Plasmid Transfection

BT325 cells (1×10^5) were seeded in 24-well plates with antibiotic-free RPMI-1640 medium supplemented with 15% FBS. The cells were cultured overnight until 60-80% confluency. BT325 cells were then transfected with 0.2 μg of shRNA plasmid (Santa Cruz Biotechnology Inc., Shanghai, China) and negative control plasmid and 3 μl of transfection reagent (Santa Cruz Biotechnology Inc., Shanghai, China) for 8 h according to the manufacturer's instructions. The shRNA plasmid used consists of a pool of 3 different shRNA plasmids (sc-35380-SHA, sc-35380-SHB and sc-35380-SHC). Sequences for the 3 shRNA plasmids are as follows: sc-35380-SHA, 5'-GATCCGATGGAATATGGTTTATCATTCAAGAGATGATAAACCATATTCCATCTTTTT-3'; sc-35380-SHB, 5'-GATCCCAAGGGTGACTTTGAGAAATTCAAGAGATTTCTCAAAGTCACCCTTGTTTTT-3'; sc-35380-SHC, 5'-GATC-

CGAAGGGGTCACTAATGAAATTCAAGAGATTTCATTAGTGACCCCTTCTTTTT-3'). The culture medium was refreshed after 8 h of infection and cultured for 2 days before 2 μg/ml puromycin (Sigma-Aldrich Trading Co., Ltd., Shanghai, China) selection for 3-5 days.

Cell Proliferation Assay

Transfected BT325 cells (5.0×10^3/well) were plated in 96-well plates and CCK-8 assays were performed daily for 3 consecutive days. The optical density (OD) of each well was measured at 450 nm using a Bio-Tek microplate reader (Bio-Tek Instruments, Thermo Fisher Scientific, Winooski, VT, USA). Triplicate wells were used for each time point.

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using a Mini BEST Universal RNA Extraction Kit (TaKaRa, Otsu, Shiga, Japan). The extracted RNA (1 μg) was reverse transcribed into cDNA using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) and random hexamer primers in a final volume of 20 μl. Next, qRT-PCR was performed using a Roche LightCycler 480 and SYBR green (TaKaRa Bio Inc, Dalian, China) to quantify the FKBP51 mRNA levels under the following conditions: 10 min at 95°C; 45 cycles of 95°C for 10 sec, 60°C for 10 sec; and 72°C for 10 sec. GAPDH was used as a control to compare gene expression. The specific qRT-PCR primer pairs used were as follows: FKBP51: 5'-AAA AGG CCA AGG AGC ACA AC-3' (sense), 5'-TTG AGG AGG GGC CGA GTT C-3' (antisense); GAPDH: 5'-GGT ATC GTG GAA GGA CTC-3' (sense), 5'-GTA GAG GCA GGG ATG ATG-3' (antisense). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression[31].

Western Blotting

Cellular protein was extracted using a cell protein extraction kit (Thermo Fisher Scientific, Waltham, MA, USA), and protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted proteins (20 μg per lane) were separated using 10% SDS-PAGE, and then, transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies include p-GSK3β and GSK3β at 1:5000 dilution; IκB-α at (1:2000 dilution, and the other primary antibodies at1:1000 dilution). Next, the PVDF membranes were washed 3 times with TBST before incubation with goat anti-rabbit horseradish peroxidase-labeled secondary antibody (1:5000 dilution; Z2301, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. After washing 3 times with TBST, the immunoreactive bands were visualized using Immobilon™ Western HRP Substrate Peroxide Solution (WBKLS00100, Millipore, Billerica, MA, USA). GAPDH, α-tubulin and H3 proteins were used as internal controls for total protein, plasma protein and nucleoprotein, respectively. Each experiment was performed in triplicate. Image-Pro Plus (version 6.0.0.260, Media Cybernetics, Inc. Rockwille, MD, USA) was used to quantify the protein bands.

Cell Cycle Analysis

A total of 1×10^6 transfected BT325 cells were collected and washed with PBS before fixing in 70% ice-cold ethanol. After incubation for at least 3 h at -20°C, the cells were washed again and incubated with 200 μl of propidium iodide (PI)-containing Muse™ Cell Cycle Reagent (Millipore, Billerica, MA, USA. Cat # MCH100106) for 30 min at room temperature in the dark. Flow cytometry analysis was performed to determine the percentages of cells in the G0/G1, S and G2/M phases using a Muse Cell Analyzer (Millipore, Billerica, MA, USA). Each experiment was performed in triplicate and data were expressed as mean ± standard deviation (SD).

Transwell Invasion Assay

Matrigel-coated or uncoated 24-well transwell inserts with membranes (8 μm pore size; BD Biosciences, San Jose, CA, USA) were used for migration and invasion assays. Next, 1×10^5 and 2×10^5 cells in serum-free medium were added to the upper transwell migration and invasion chambers, respectively. Medium containing 15% FBS was added to the lower chambers as a chemoattractant. Matrigel and the cells remaining in the upper chamber were removed by cotton swabs after 24 h incubation in a humidified incubator with 5% CO_2 at 37°C. The migrated/invaded cells observed in the lower chamber were fixed in 95% ethanol for 15 min, then, stained with hematoxylin for 10 min, and differentiated with 1% hydrochloride alcohol for 5 s. Ten high-power fields were randomly chosen for counting of migrated/invaded cells under a light microscope. The average number of each field was calculated.

Statistical Analysis

Statistical analyses were conducted using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). Data with normalized distribution were expressed as mean ± SD. Comparisons among different treatment groups were performed using One-way analysis of variance (ANOVA) after checking the homogeneity of variance, and comparisons between any two groups following one-way ANOVA were performed using Student-Newman-Keuls (SNK) test. Values of $p < 0.05$ were considered statistically significant.

Results

FKBP51-Overexpressed and FKBP51-Suppressed BT325 Cell Lines Were Successfully Established

FKBP51 expression levels vary in glioma cell lines and tumors harvested from patients[21]. Since FKBP51 is moderately expressed in BT325 cell line, we selected it for our experiments in the present study. Using EGFP-labeled BT325 cells, we found that FKBP51 lentiviral infection efficiency was approximately 80% (Figure 1A). Furthermore, qPCR (Figure 1B) and Western blot analyses (Figure 1C) revealed successful FKBP51 shRNA knockdown and lentiviral-induced FKBP51 overexpression.

FKBP51 Inhibits Cell Cycle Progression and Proliferation of BT325 Cells

CCK-8 assays showed decreased BT325 cell proliferation when FKBP51 was overexpressed but increased BT325 cell proliferation when FKBP51 was downregulated (Figure 2A). Given that cell proliferation is regulated by the distribution of cell cycle phases[32], we examined the relative proportion of cells in their respective cell cycle phases for both FKBP1-overexpressed and suppressed BT325 cells. Flow cytometry analysis revealed increased proportion of cells in the G0/G1 phase and decreased S-G2/M phase cells in FKBP1-overexpressed cells. By contrast, a higher percentage of cells in the G2/M phase but decreased percentage of the G0/G1 phase cells was observed upon FKBP51 shRNA knockdown (Figure 2B). These findings showed that FKBP51 overexpression can induce cell cycle arrest at the G0/G1 phase while FKBP51 knockdown promotes S-G2/M phase cell cycle progression.

FKBP51 Reduces AKT Pathway Activation

Given that AKT is well known in regulating cell proliferation and G1/S cell cycle transition in many cell types[33], we next determined its role in regulating FKBP51-mediated inhibition of BT325 cell proliferation, Western blot analyses showed that Ser473-phospho-AKT expression was decreased in FKBP51-overexpressed BT325 cells but increased in FKBP51-suppressed BT325 cells (Figures 3D-F).

Next, we verified the involvement of the AKT pathway using an AKT inhibitor. We treated negative control and shRNA FKBP51-transfected BT325 cells with different concentrations of the AKT inhibitor (0, 5, 10 and 20 μM) and examined the effects on cell proliferation and cell cycle distribution using CCK-8 assay and cell cycle analysis. In the presence of 10 μM AKT inhibitor, cell proliferation was decreased in a dose-dependent manner (Figures 3A and B), and cells were arrested at the S and G2/M phases in both groups (Figure 3C). These results indicated that AKT inhibition induced G1/S cell cycle arrest, thereby decreasing BT325 cell proliferation.

We next examined the expression levels of downstream substrates of the AKT pathway, GSK3β, FOXO-1, BCL-2, P21 and P27, which regulate cell cycle progression, cell proliferation and apoptosis. In FKBP51-overexpressed BT325 cells, reduced AKT, Ser9-phospho-GSK3β (p-GSK3β), Ser256-phospho-FOXO-1 (p-FOXO-1) and BCL-2 levels but increased P21 and P27 levels, were observed. The reverse was observed in FKBP51-shRNA knockdown BT325 cells (Figures 3D-F). These results indicate that FKBP51 inhibits AKT Ser473 phosphorylation, leading to GSK3β and FOXO-1 accumulation in the nucleus, and activating gene transcription of the cyclin-dependent kinase inhibitors (CDKIs) P21 and P27, thereby inducing cell cycle arrest and decreasing cell proliferation. This is consistent with reduced antiapoptotic BCL-2 expression (Figures 3D-F).

FKBP51 Sensitizes BT325 Cells to BCNU Treatment by Inhibiting AKT

Next, we examined the role of FKBP51 in modulating BT325 cell response to BCNU treatment. CCK-8 assays revealed decreased cell viability of FKBP51-overexpressed cells but increased cell viability of FKBP51-repressed cells upon BCNU treatment. These findings suggested that FKBP51 sensitized BT325 cells to BCNU treatment (Figure 4A). Given the established role of the AKT

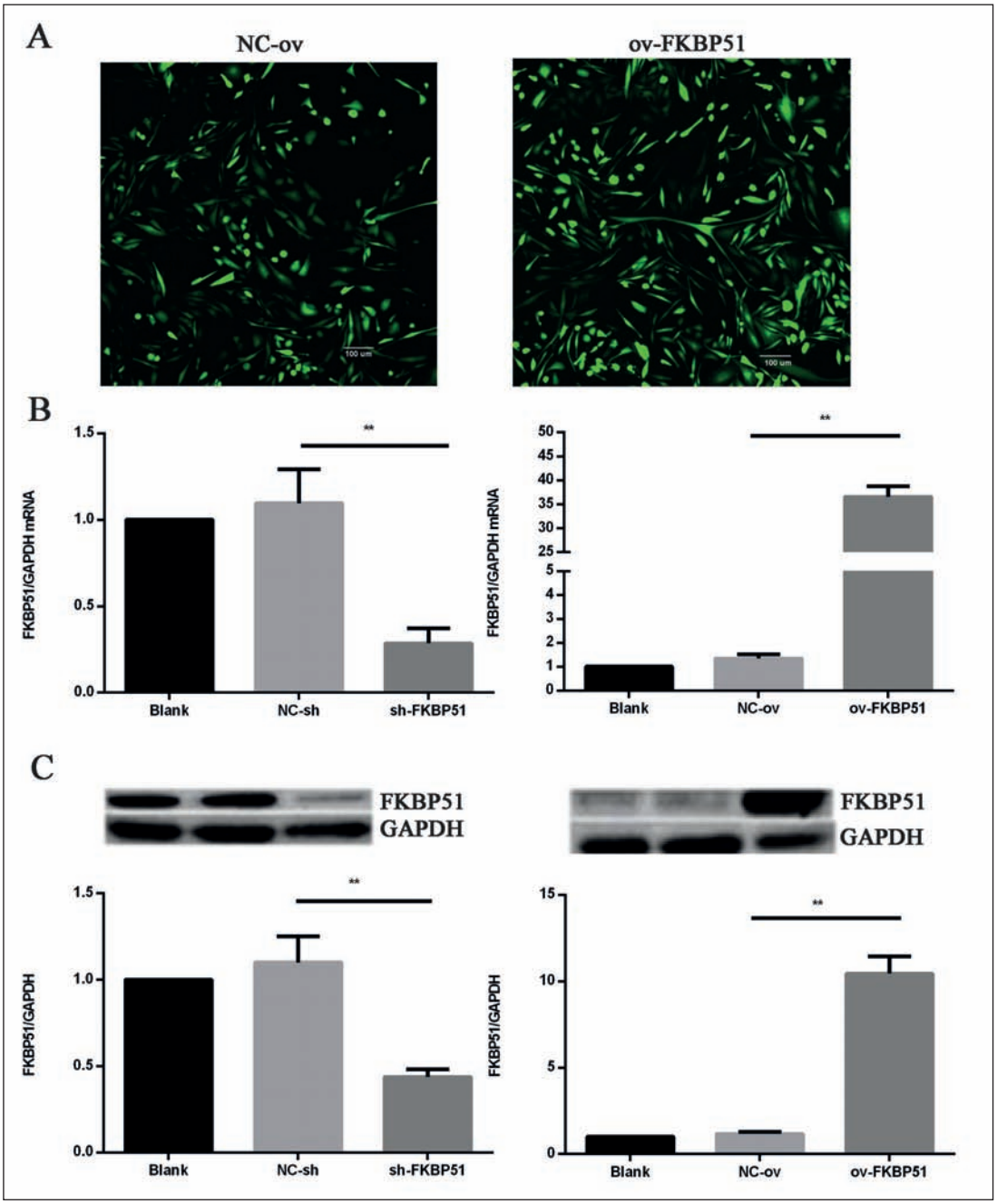

Figure 1. FKBP51 expression levels in FKBP51 lentiviral-overexpressed and shRNA knockdown BT325 cells. **A**, Overlapping bright field and EGFP fluorescence images at ×100 magnification. **B-C**, FKBP51 mRNA and protein levels in FKBP5 lentiviral-overexpressed and shRNA knockdown BT325 cells. BLANK: BT325 cells, NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression. GAPDH was used as a reference control. Data of 3 independent experiments are shown as the mean ± SD; $^{**}p<0.01$.

pathway in regulating chemoresistance, we investigated its involvement in mediating the response of BT325 cells to BCNU treatment. We treated negative control and FKBP51 shRNA knockdown BT325 cells with different concentrations of an AKT inhibitor (A6730) for 48 h and assessed the cell viability using CCK-8 assays. Cell viability gradually decreased in a dose-dependent manner in both groups (Figure 4B). Next, we treated FKBP51 shRNA knockdown BT325 cells with three concentrations of BCNU (2, 5 and 10 μM) for 72 h and monitored the effect on the AKT pathway.

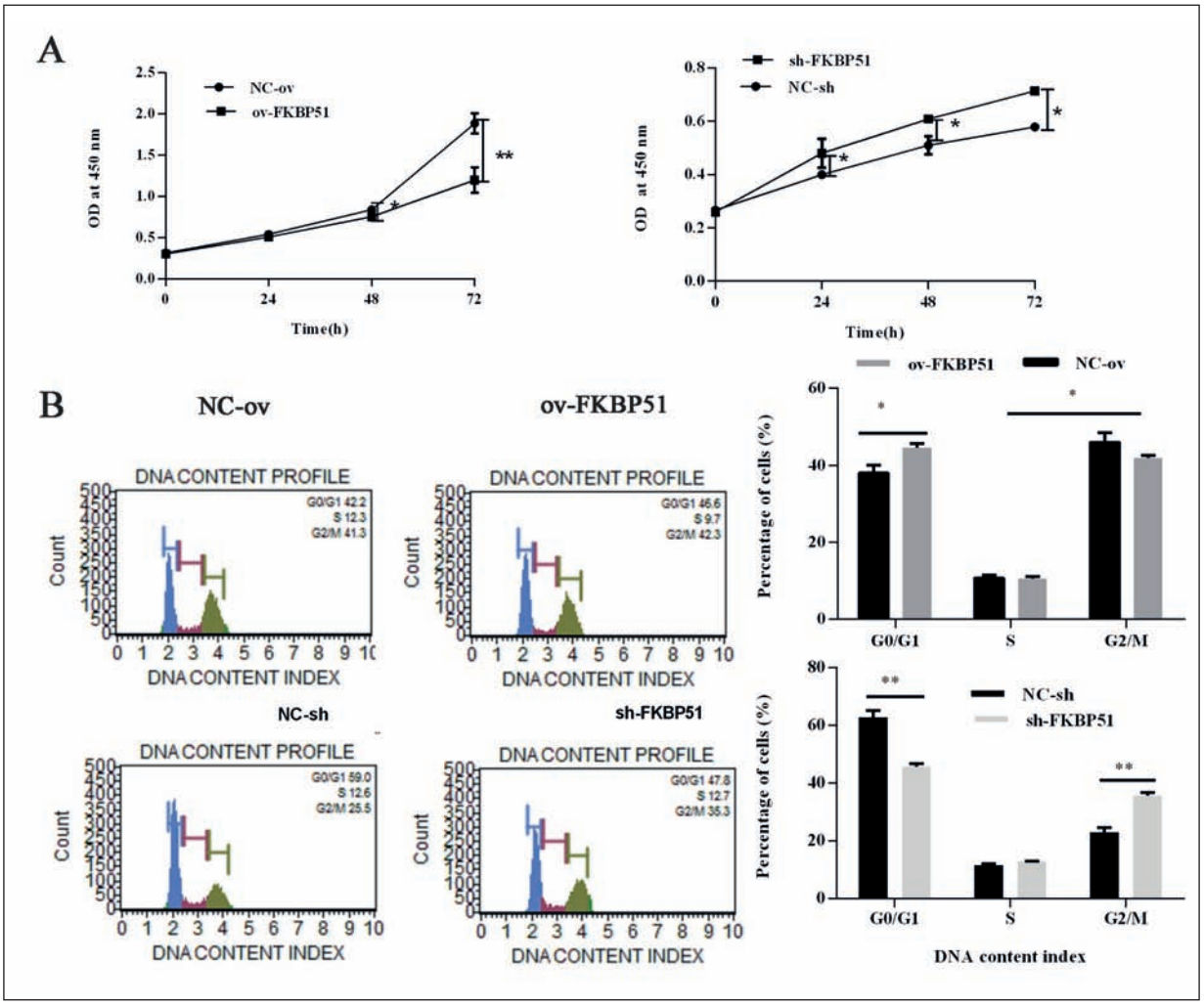

Figure 2. FKBP51 inhibits BT325 cell cycle progression and cell proliferation. **A**, CCK-8 analysis of FKBP51-overexpressed and shRNA knockdown BT325 cells. The absorbance (OD) at 450 nm in each group was measured to assess cell proliferation. **B**, Flow cytometry profiles showing cell cycle phase distribution in FKBP51-overexpressed and shRNA knockdown BT325 cells. Each experiment was performed in triplicate. NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression. Data of 3 independent experiments are shown as the mean ± SD; *$p<0.05$, **$p<0.01$.

Decreased Ser473-phospho-AKT (p-AKT) and p-GSK3β levels but increased P21 and P27 levels were observed. Antiapoptotic BCL-2 protein level started to decrease at 10 μM BCNU (Figure 4C). These results suggest that AKT signaling modulates the response of BT325 cells to BCNU.

FKBP51 Promotes BT325 Cell Migration and Invasion

Following the confirmation of the association between FKBP51 and BT325 cell proliferation, we next proceeded to determine the role of FKBP51 in BT325 cell migration and invasion. Transwell assays with and without Matrigel demonstrated positive correlation between FKBP51 expression and BT325 cell migration and invasion (Figure 5).

FKBP51 Facilitates MMP-2 and MMP-9 Expression

Given the role of matrix metalloproteinases (MMPs) as key mediators of basement membrane degradation, angiogenesis and tumor invasion in glioma[34], we examined the expression of the key MMPs, MMP-2 and MMP-9 in BT325 cells. Increased and decreased MMP-2 and MMP-9 expression levels were observed in FKBP51-overexpressed and FKBP51-suppressed BT325 cells respectively (Figures 6A-B). This suggests that MMP-2 and

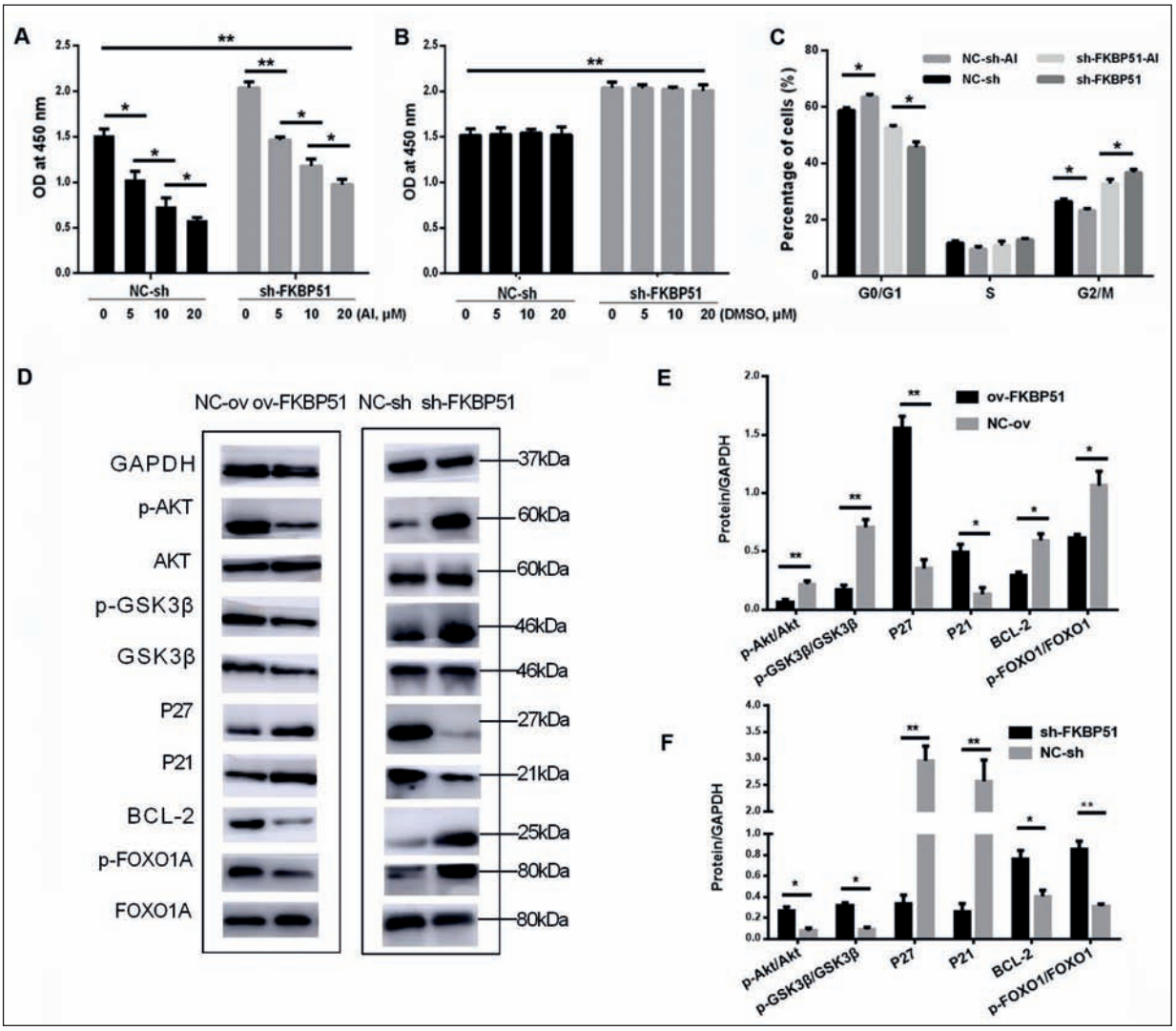

Figure 3. FKBP51 decreases BT325 cell proliferation by inhibiting AKT signaling. **A-B**, CCK-8 cell viability profiles of the negative control and FKBP51 shRNA-transfected BT325 cells after 24 h treatment with (**A**) A6730 (AKT inhibitor) at four 0, 5, 10 and 20 μM, or (**B**) a vehicle control (DMSO). **C,** Flow cytometry profile showing the distribution of cell cycle phases in A6730-treated (10 μM) control- and FKBP51 shRNA-transfected BT325 cells. **D,** Western blot showing the expression of AKT and its downstream targets P21, P27, BCL-2, FOXO1 and GSK3β in FKBP51-overexpressed and shRNA knockdown BT325 cells. **E-F**, Profiles showing the relative protein expression levels of AKT and its downstream targets. Data of three independent experiments are shown as the mean ± SD. NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression. AI: AKT inhibitor, DMSO: dimethyl sulfoxide; *$p<0.05$, **$p<0.01$.

MMP-9 may be involved in FKBP51-induced BT325 cell migration and invasion (Figures 6A-B).

FKBP51 Promotes MMP-2 and MMP-9 Expression by Activating the NF-κB Pathway

Since the NF-κB signaling pathway has been shown to regulate MMP-2 and MMP-9 expression in various cancers[35], we examined the relationship between FKBP51 and the NF-κB pathway. In FKBP51-overexpressed BT325 cells, expression of inhibitor of nuclear factor kappa (IκB) was decreased while NF-κB p65 and phosphorylated IκB kinase (IKK) α were increased in both the cytoplasm and the nucleus. In contrast, increased IκBα expression but significantly reduced p-IKKα and NF-κB p65 expression, as well as p65 nuclear translocations were observed in FKBP51 shRNA-transfected BT325 cells compared with that in negative control cells (Figures 6A, C and D).

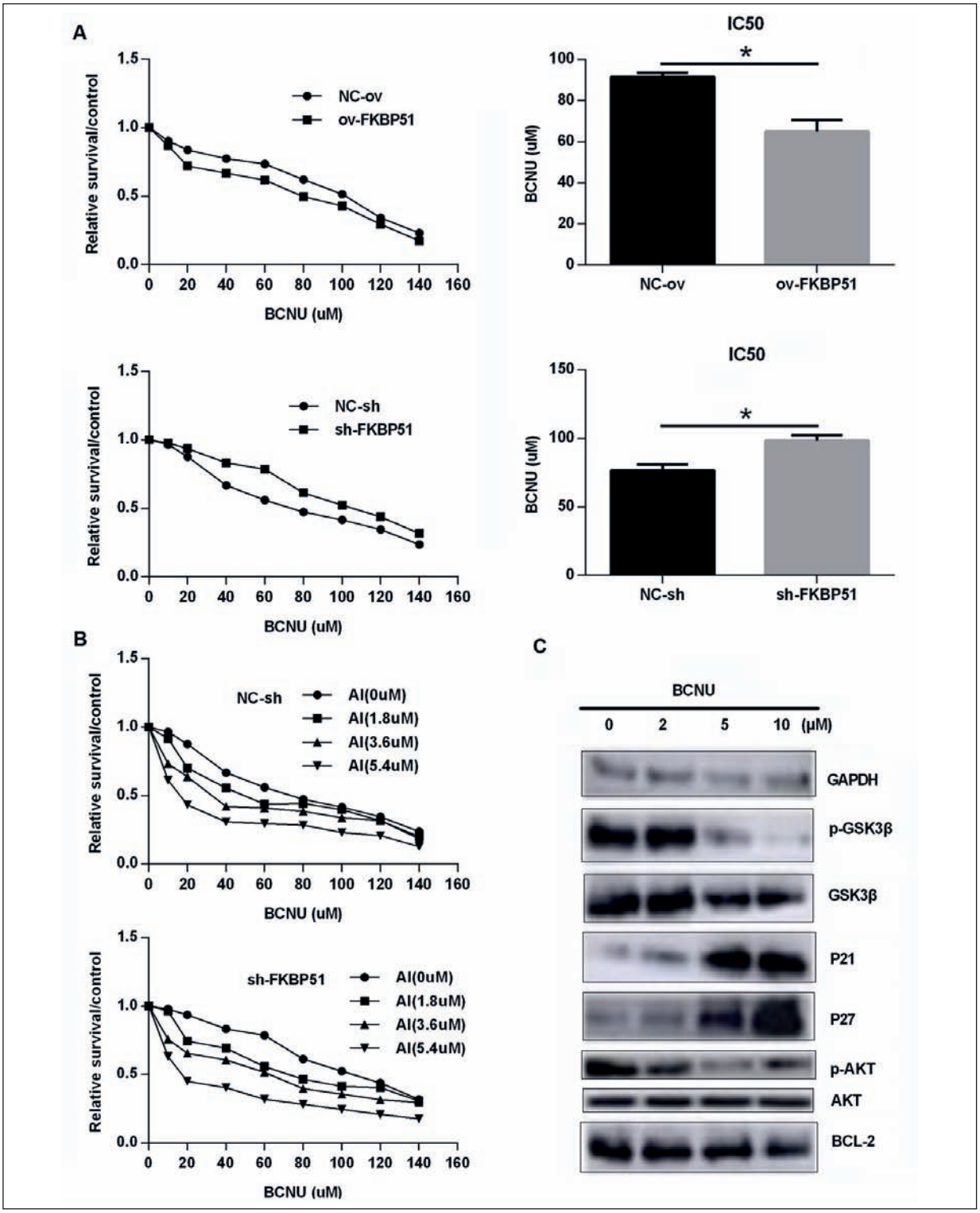

Figure 4. FKBP51 sensitizes BT325 to BCNU via AKT inhibition. **A**, CCK-8 cell viability and IC_{50} profiles of FKBP51-overexpressed and shRNA knockdown BT325 cells after 48 h treatment with BCNU at 20-140 μM doses. **B**, CCK-8 cell viability profiles of negative control and FKBP51 shRNA knockdown BT325 cells after 48 h treatment with AKT inhibitor (1.8, 3.6 and 5.4 μM) and BCNU (20-140 μM). **C**, Western blot analysis of AKT and its downstream targets GSK3β, P21, P27 and BCL-2 in FKBP51 shRNA knockdown BT325 cells after 48 h treatment of BCNU at 2, 5 and 10 μM. All cells were treated with LPS (1 μg/ml) for 15 min before protein extraction. NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression; *p<0.05.

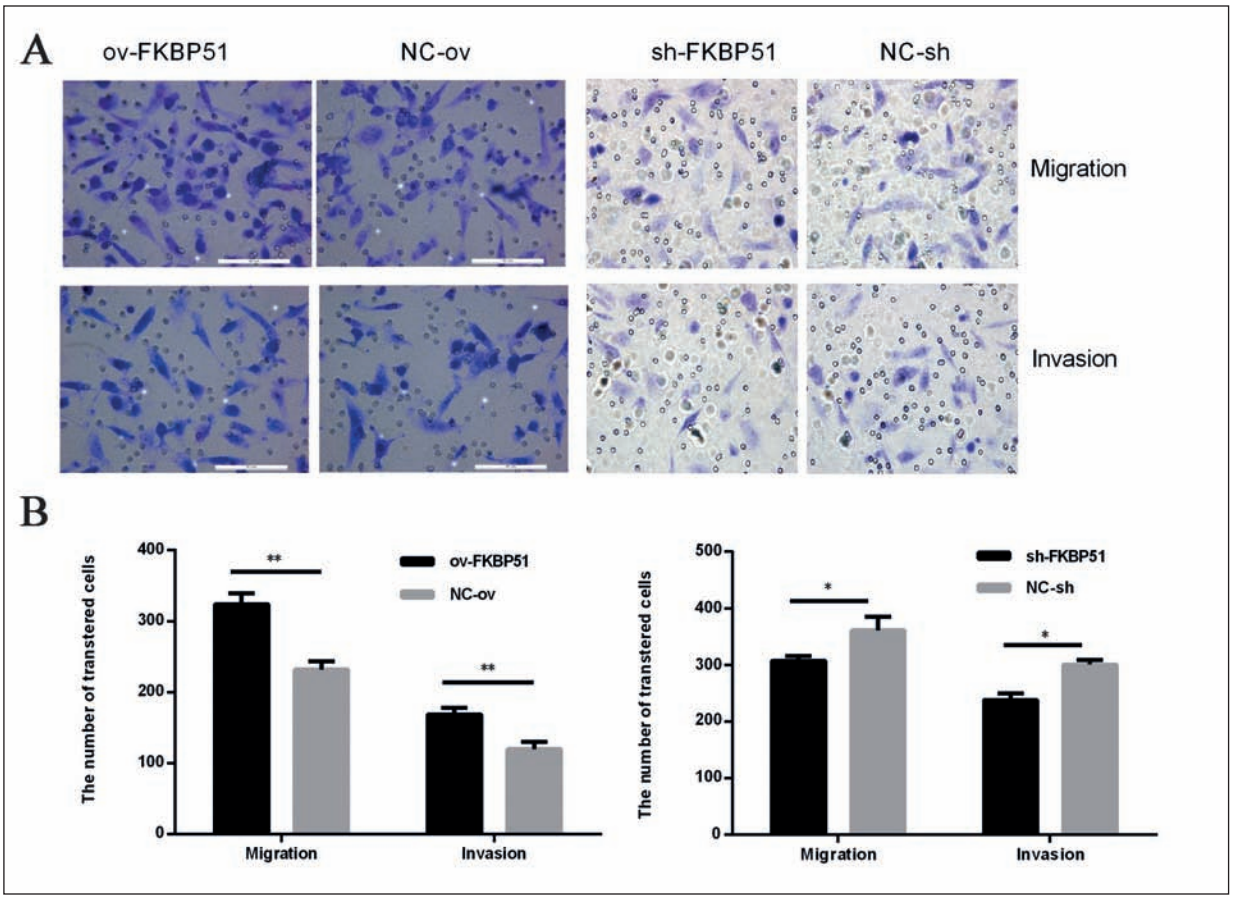

Figure 5. FKBP51 promotes BT325 cell migration and invasion. **A**, Images at ×400 magnification of FKBP51-overexpressed and shRNA knockdown BT325 cells from Transwell assay in the presence and absence of Matrigel. The number of migrated cells in each group was used to assess migration and invasion ability (upper: migration, lower: invasion; white dots in 5A are artifacts and have no practical relevance). **B**, Profiles showing the relative migration and invasion abilities of FKBP51-overexpressed and FKBP51 shRNA-knockdown BT325 cells. NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression. The data of three independent experiments are expressed as the mean ± SD; * $p<0.05$, ** $p<0.01$.

Western blot analysis revealed significantly decreased MMP-2 and MMP-9 expression in negative control and FKBP51-overexpressed BT325 cells that have been treated with an NF-κB inhibitor (Figure 7). These results suggest that FKBP51-mediated activation of classical NF-κB signaling promotes MMP-2 and MMP-9 production.

Discussion

In the current study, we investigated the effects of FKBP51 on BT325 glioma cell proliferation, migration and invasion, as well as the response to BCNU treatment. We found that FKBP51 overexpression inhibited BT325 cell proliferation by inducing G0/G1 cell cycle arrest, and its knockdown accelerated cell cycle progression and promoted cell proliferation. Our findings are however inconsistent with those of Jiang et al[36], as they found that cell proliferation was suppressed in FKBP51 siRNA knockdown A172 cells and enhanced in FKBP51-overexpressing U87 cells, indicating possible glioma cell line-specific differences in growth-regulating pathways or interference of FKBP51 expression at the basal level in glioma cells *in vitro*. Thus, further research is necessary to clarify these inconsistencies.

We next examined the mechanism underlying the association between FKBP51 expression and BT325 cell proliferation. Specifically, we analyzed the expression of the CDKIs P21 and P27, which inhibits cyclin D1 CDK4/6 complex formation, thereby promoting cell cycle arrest at G0/G1 and S phases[37]. FKBP51 overexpression significantly increased P21 and P27 expression levels,

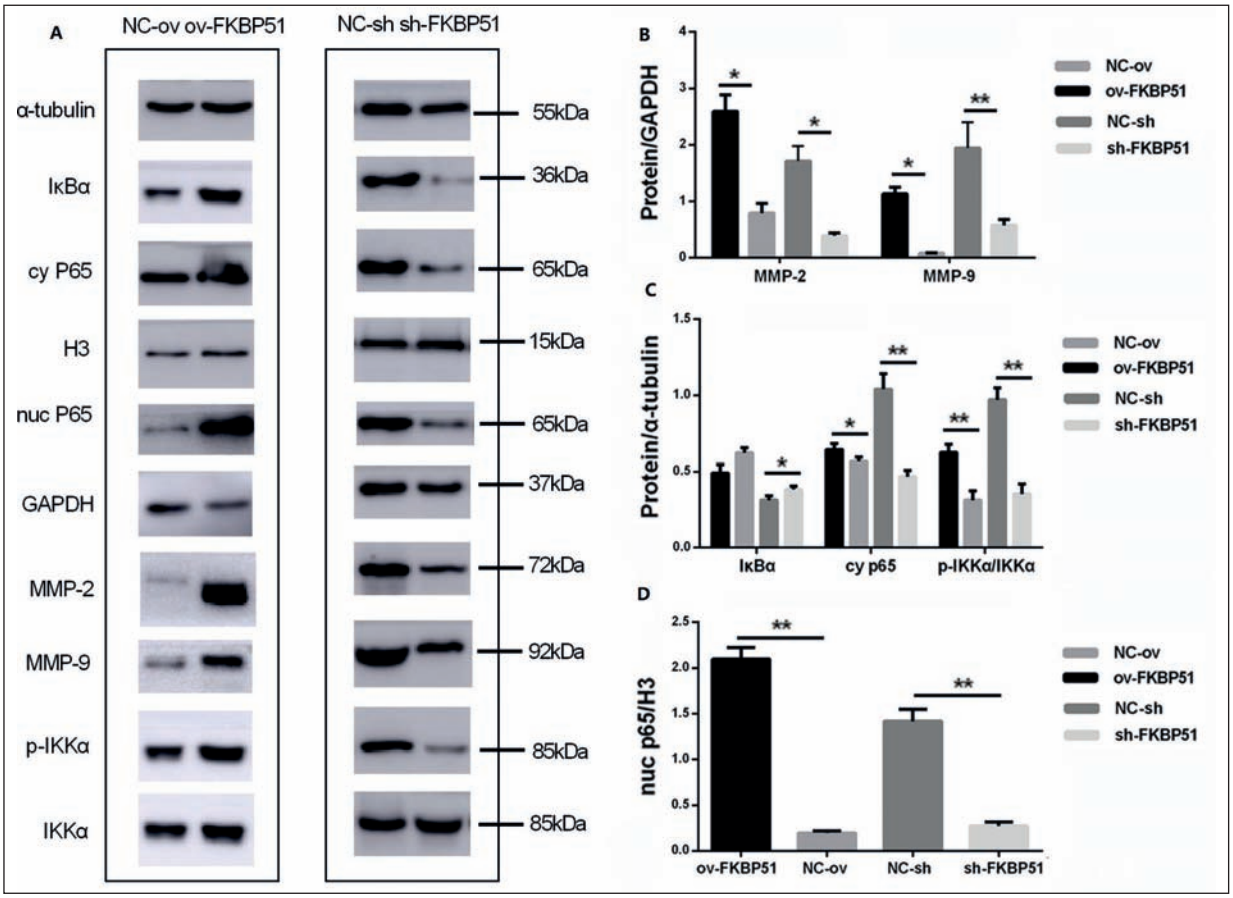

Figure 6. FKBP51-induced NF-κB activation increases MMP-2 and MMP-9 expression. ***A***, Western blot showing the expression levels of MMP-2 and MMP-9 (total protein); IκBα, p-IKKα and IKKα (cytoplasmic protein); and NF-κB p65 (cytoplasmic and nuclear protein). All cells were treated with TNF-α (20 ng/ml) for 15 min before protein extraction. The experiment was repeated 3 times. ***B***, The relative expression levels of MMP-2 and MMP-9 proteins (total protein). Profiles showing the relative expression levels of (***C***) NF-κB p65, IκBα and phosphorylated IKKα in the cytoplasm and of (***D***) NF-κB p65 in the nucleus in FKBP51-overexpressed and shRNA knockdown BT325 cells. Data are shown as the mean ± SD. NC-sh: negative control for sh-FKBP51, sh-FKBP51: shRNA-FKBP51, NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression; *$p<0.05$, **$p<0.01$.

while FKBP51 knockdown strongly abrogated their expression. Thus, our results suggest the involvement of P21 and P27 in FKBP51-induced cell cycle arrest and growth inhibition. AKT pathway activation has been reported to promote cell proliferation and inhibit apoptosis[38-41]. AKT activation inhibits P21 and P27 and inactivates GSK-3β, leading to cyclin D1 degradation, G1 progression and cell proliferation[42,43]. Therefore, we determined the involvement of AKT in FKBP51-mediated cell cycle arrest. Indeed, we confirmed the association between FKBP51 and the AKT pathway and their interplay in cell cycle progression and cell proliferation. Furthermore, we showed that FKBP51 overexpression enhanced the susceptibility of BT325 cells to BCNU, the standard adjuvant chemotherapeutic for glioma that can easily pass through the blood-brain barrier[44]. In addition, we confirmed the involvement of AKT signaling in regulating FKBP51-induced chemosensitivity in BT325 cells. Thus, FKBP51 suppression can lead to excessive AKT signaling thereby promoting cycle progression, inhibiting apoptosis, and giving rise to resistance to BCNU.

Tumor cell invasion is a primary cause of poor prognosis in patients with glioma. In this study, we demonstrated the positive correlation between FKBP51 expression and BT325 cell migration and invasion. The invasiveness of glioma cells is partially regulated by cell interactions with the extracellular matrix (ECM). MMPs are known to play a pivotal role in ECM degradation[47]. In particular, MMP-2 and MMP-9 have been implicated in tumor metastasis[45]. Since the molecular regulation

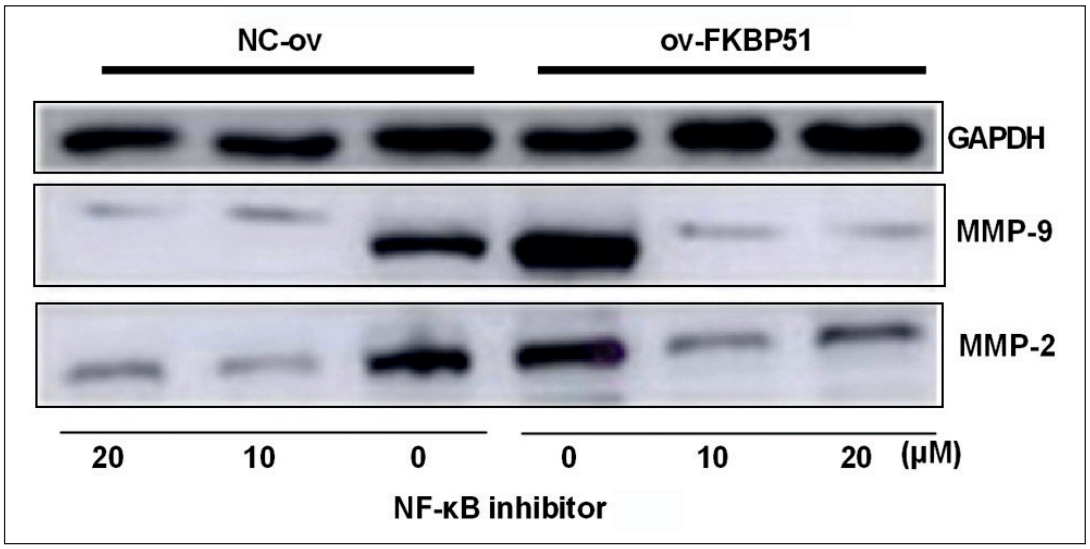

Figure 7. NF-κB inhibition suppresses MMP-2 and MMP-9 expression in BT325 cells. Western blot showing the protein expression of MMP-2 and MMP-9 in negative control and FKBP51-overexpressed BT325 cells after 24 h treatment with an NF-κB inhibitor (BAY 11-7082) at 0, 10 and 20 μM. NC-ov: negative control for ov-FKBP51, ov-FKBP51: FKBP51 overexpression.

of MMP-2 and MMP-9 in glioma cells has not been fully elucidated, we first investigated their association with FKBP51 expression, and then, determined the underlying molecular mechanism regulating glioma migration and invasiveness. We found that MMP-2 and MMP-9 expression levels were not only positively correlated with FKBP51 expression in BT325 cells, but also associated with glioma metastasis. Since NF-κB could reportedly regulate MMP proteolytic enzymes and promote tumor invasion[34,46,47] we examined its crosstalk with FKBP51 in mediating BT325 cell migration and invasion. Consistent with Jiang et al[36], our findings support FKBP51-mediated NF-κB activation in regulating MMP-2 and MMP-9 expression in BT325 cells. Furthermore, our observations support the reported role of FKBP51 as an important cofactor in the isomerization of the IKK catalytic subunit[28]. The dose-dependent reduction in MMP-2 and MMP-9 expression levels using the NF-κB inhibitor, BAY 11-7082, which inhibits TNF-α-induced IκB-α phosphorylation, further confirm the roles of FKBP51 and the classical NF-κB pathway in BT325 cell migration and invasion *via* transcriptional regulation of the MMP-2 and MMP-9 genes. Interestingly, FKBP51-induced NF-κB activation acts *via* the upstream IKKs instead of the AKT pathway. Nonetheless, given that cross-regulation between AKT pathway and other major signaling pathways such as NF-κB is a common phenomenon[48], we believe that these signaling pathways work in synergy to modulate glioma phenotypes.

Conclusions

We characterized the effect of FKBP51 in BT325 glioma cell proliferation, cell cycle progression, migration, invasion and response to BCNU treatment. In addition, we elucidated the involvement of the AKT and NF-κB pathways in regulating glioma phenotypes. Our findings propose a potential role for FKBP51 as a prognostic biomarker for glioma malignancy and an indicator of chemotherapy response.

Conflict of Interests

The authors declare that they have no financial or commercial conflict of interest.

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