

INHIBITION of GSK-3 β SUPPRESSES GLIOMA CELLS PROLIFERATION by DOWN-REGULATING MUTANT p53

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Abstract : Glycogen synthase kinase-3 β (GSK-3 β) has been identified as a therapeutic target for many diseases and is associated with tumor suppressor or pro-cancer mechanisms in many tumors. Mutant p53 plays a carcinogenic role in most tumors. Glioma is an intracranial tumor with high incidence, poor therapeutic effect and easy recurrence. The present research aims to investigate the regulation and effects of GSK-3 β on mutant p53 and its biological role in glioma development. In addition, the effect of GSK-3 β inhibitor 1-AKP interfering with GSK-3 β on cell proliferation was studied in glioma cells. And the effects of lentivirus-mediated RNA interference of p53 on glioma cell proliferation. GSK-3 β can positively regulate p53 and inhibition of GSK-3 β could down-regulate the expression of mutant p53 thus inhibiting the proliferation of glioma cells. To sum up, GSK-3 β inactivation regulates cell proliferation by inhibiting the expression of mutant p53.

Key words — Glioma, GSK-3 β , 1-AKP, Mutant p53, Cell proliferation

I. INTRODUCTION

Glioma is the most frequent primary malignant tumor in the nervous system, accounting for 40-50% of the incidence of all primary intracranial tumors^[1]. High-grade glioma has rapid proliferation and strong invasiveness^[2], neither surgery nor chemoradiotherapy can completely remove tumor tissue without harming normal tissue^[3]. Glioma has a short survival period and poor prognosis, especially glioblastoma (GBM). The 2016 WHO Classification of Tumors of the Central Nervous System replacing traditional histology-based glioma diagnostics with an integrated histological and molecular classification system with the requisite diagnostic biomarkers include IDH1/2 mutations, 1p/19q codeletion, H3F3A mutations and C11orf95-RELA fusions and so on^[4]. Although molecular markers are helpful to the clinical diagnosis and prognosis, the improvement of treatment still faces severe challenges. Therefore, for glioma, it is necessary to further explore the molecular mechanism of its carcinogenesis and find a more effective treatment plan for glioma.

Glycogen synthase kinase-3 β (GSK-3 β), which acts on a variety of signaling proteins and transcription factors, has been identified as a therapeutic target for a variety of diseases. Emodin enhanced the neuroprotective effect through AMPK/GSK-3 β pathway, and has therapeutic effects on neurodegenerative diseases^[5]. The GR/ROR signaling pathway mediated by GSK-3 was involved in the autophagy of hippocampal neurons induced by white LED light^[6]. Inhibition of GSK-3 β prevented induction of Puma, thereby apoptosis upon growth factor withdrawal^[7]. However, role of GSK-3 β in tumors is a matter of debate. Numerous reports have confirmed that GSK-3 β plays opposite roles in different tumor types through different signaling pathways. Studies had shown that activation

of GSK-3 β negatively regulates the Wnt/ β -catenin signaling pathway, thereby inhibiting tumor proliferation^[8]. GSK-3 β effectively regulated STAT3 transcriptional pathways to control the progression and growth of pancreatic cancer^[9]. Conversely, inhibition of GSK-3 induced cell cycle arrest at the G1 phase in MLL leukaemia^[10]. Furthermore, the overexpression of GSK-3 β in human colon cancer tissues could activate Akt, leading to nuclear accumulation of β -catenin, suggesting that GSK-3 β may also be involved in the tumorigenesis of colon cancer^[11]. In fact, different researchers had different opinions on the role of GSK-3 β in glioma. Some reports had confirmed that GSK-3 β promoted the malignant progression of glioma, GSK-3 β mediated Tip60S86 phosphorylation inhibits the apoptosis induction of p53^[12], and the inhibition of GSK-3 β could weaken the migration and invasion ability of glioma cells^[13]. Conversely, it had also been shown that activation of GSK-3 β in glioma could negatively regulate the Wnt/ β -catenin signaling pathway, so that inhibited tumor proliferation^[14]. Therefore, the mechanism of GSK-3 β in the malignant progression of glioma needs to be further explored.

p53 signaling pathway is considered as an indispensable molecular pathway for the regulation of glioma^[15]. The WHO revised diagnosis of glioma in 2016 clearly showed that p53 mutation plays an important role in the molecular diagnosis of high-grade glioma^[4]. Overexpression of p53 mutant in glioma cells showed characteristics of glioma stem cell, and the cells overcame apoptosis and proliferation barriers to acquire carcinogenicity^[16]. And GSK-3 β regulation p53 had been widely recognized^[8]. GSK-3 β inhibitors were used to regulate the direct target gene TREM2 of p53 and inhibit its expression in innate immune system^[17]. And regulatory role of GSK-3 β was demonstrated by large reductions of p53-induced increases in the levels of MDM2, p21 and Bax when GSK-3 β was inhibited^[18].

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To sum up, to explore the regulatory role and mechanism of GSK-3 β and p53 in glioma will provide more effective reference for the treatment of glioma.

II. MATERIALS AND METHODS

A. Cell culture and inhibitor treatment

The glioma cell lines U251 and U-118MG were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in an atmosphere containing 5% CO₂ at 37 °C. For GSK-3 β inhibitor treatment plated cells were pretreated with 10 μ M inhibitors for 48-72 hours and then cell morphologic changes and biologic behaviors were observed.

B. CCK-8 assay

Cells were seeded into a 96-well plate at a density of 3000 cells per well and after incubation cell proliferation was detected using Cell Counting Kit-8 (CCK-8, Selleck, USA) 10 μ l of CCK-8 reagent was added to each well, and the cells were cultured for another 1h. At the end of the incubation, the optical density at 450nm was analyzed with a microplate reader (BioTek, USA).

C. Colony formation assay

Total of 700 cells were placed in a well of a 6-well plate and maintained in media containing 10% FBS, the medium was replaced every 3 days. After 12 days, the cells were fixed with 4% paraformaldehyde for 30 minutes and then stained with 0.1% crystal violet (Beyotime, China) for 20 minutes, visible colonies were counted manually, and each group included triplicate wells.

D. Western blotting

Cells were completely lysed using RIPA Lysis Buffer containing PMSF on the ice for 30 minutes and supernatants were acquired by centrifugation at 12,000g for 30 minutes at 4°C. Cell lysates were quantified for protein content using the BCA method. Then, 20 μ g of whole protein were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to PVDF membranes (Millipore, USA). After closing with 5% skim milk powder in Tris-buffered saline containing 0.2% Tween-20 (TBST), and incubated with primary antibodies for p53 (1:1000 dilution), p-GSK-3 β ^{Ser9} (1:1000 dilution) and GAPDH (1:1000 dilution, Cell Signaling Technology, USA) at 4°C overnight. After washing with TBST, the membranes were then incubated with HRP-conjugated secondary antibodies (Millipore, USA) for 1h, immunoreactive bands were then visualized by the ECL chemiluminescence system (Thermo Fisher Scientific Inc., MA, USA). Densities of the immunoreactive bands were determined by ImageJ (National Institutes of Health, USA).

E. Viral infections and generation of stable cell lines

For generation of lentiviral vectors encoding shRNA targeting p53, corresponding shRNA oligos were cloned into the PLKO.1-TRC vector pre-cleaved by Age I and EcoR I, which was used to co-transfected HEK-293T cells with psPAX₂ (Addgene, 12260) and pMD2.G (Addgene, 12259) packaging plasmids using CaCl₂. Lentiviral particles were collected after 48 h and concentrated by ultracentrifugation at 19,400g for 2.5h. U251 and U-118MG glioma cells were transduced with shp53, HEK-293T cells were transfected with shNC empty vector as negative control. After 48 h, medium containing puromycin was used to

select stable clones. The clones with stable knockdown of p53 were identified and verified using Western blotting.

F. Statistical analysis

Data analyses and construction of statistical charts were performed using the GraphPad Prism 6.0 software package (GraphPad Software, CA, USA). The results are presented as the mean value (\pm SD). All data were analyzed using ANOVA or t-test, which were employed to establish whether there was any difference between the control and experimental data. $p < 0.05$ was considered to be significantly different.

III. RESULTS

A. Positive correlation between expressions of GSK-3 β and p53

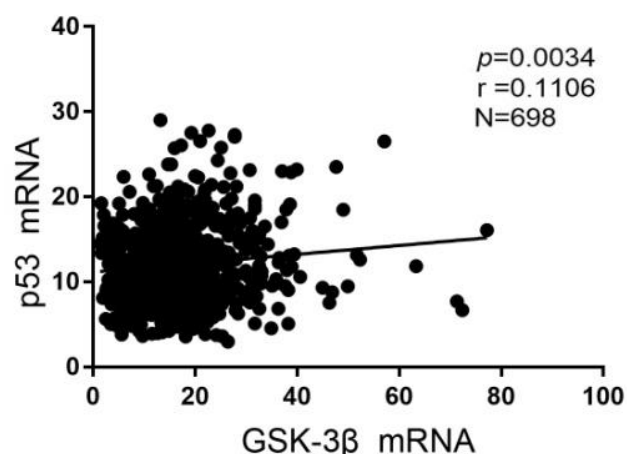


Fig.1. Linear correlation of GSK-3 β and p53 genes in glioma

All gene expression data of 698 glioma patients were downloaded from TCGA. The mRNA expression of GSK-3 β and p53 in glioma samples was analyzed by linear regression. The results showed that with the increase of GSK-3 β mRNA expression, the mRNA expression of p53 also increased ($p = 0.0034$, $r = 0.1106$). Although the difference was statistically significant, the correlation coefficient was low, and the results were for reference only (Fig. 1). These results suggest that there may be a positive correlation between the expression of GSK-3 β and mutant p53 in glioma. Then we selected U251 and U-118MG glioma cells with p53 hot-spot mutation sites for the following experiments to investigate the relationship between GSK-3 β and mutant p53 in glioma cells.

B. Inhibition of GSK-3 β restrain the proliferation of glioma cells

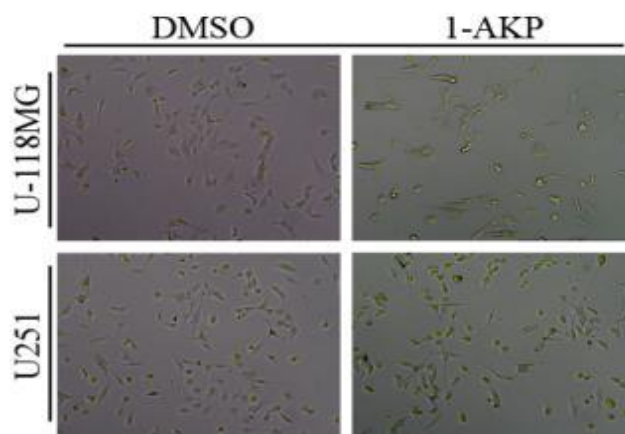


Fig.2 Comparison of cell morphology before and after GSK-3 β inhibitor administration

In this study, we treated cells with GSK-3β's inhibitor 1-AKP for 3 days, we observed some changes in the morphology of glioma cells. When GSK was inhibited, the cells showed a more obvious fusiform and the pseudopod was more slender(Fig. 2). Moreover, cell growth rates were significantly slower in the inhibitor group than in the DMSO group. This phenomenon suggests that inhibition of GSK-3β may affect cell growth in glioma cells.

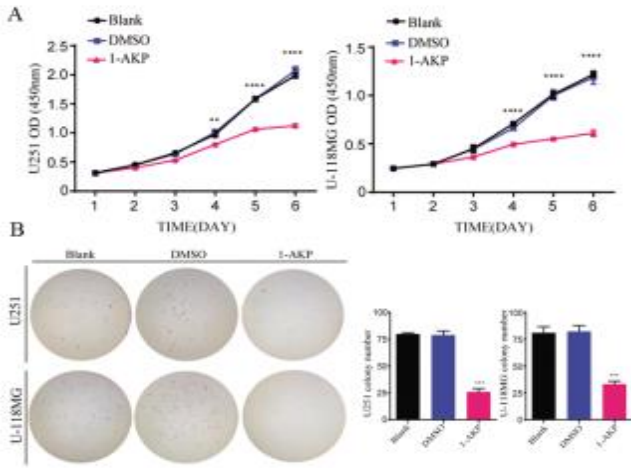


Fig.3. The proliferation after inhibiting GSK-3β A. CCK-8 assays were performed at different time points to detect the effect of 1-AKP on the growth of glioma cells. B. Colony-forming assays were performed on the same cell lines; the histograms illustrate the number of colonies. Data are presented as the mean ± SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Immediately, we investigated the effect of inhibiting GSK-3β in glioma cell proliferation using CCK-8 assays. The results showed that the cell proliferation of U251 and U-118MG cells was decreased after inhibition of GSK-3β with 1-AKP. In addition, the effect of GSK-3β inhibition was also investigated with colony formation assays. Similarly, the results revealed that the number of colonies decreased significantly following GSK-3β inhibition(Fig. 3). The above results suggested that inhibiting GSK-3β inhibited the proliferative ability of glioma cells.

C. Inhibition of GSK-3β activity reduced the expression of p53

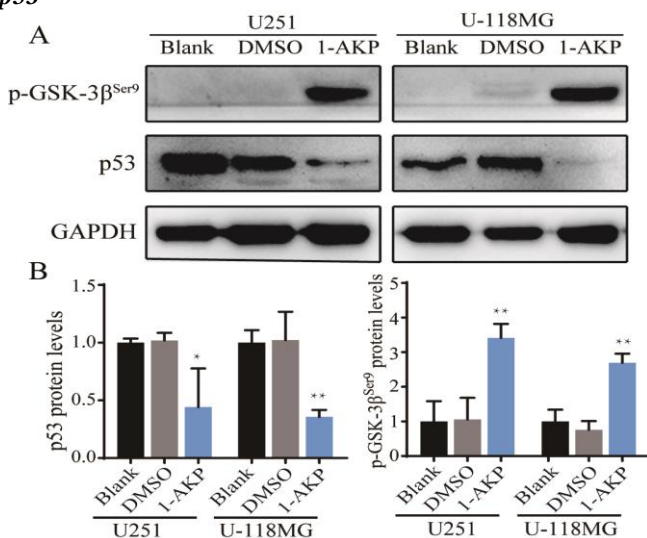


Fig.4. GSK-3β inactivation inhibited the expression of p53. A. Western blot analysis of p-GSK-3β^{Ser9} and p53 protein expressions in U251 and U-118MG glioma cells after inhibition of GSK-3β. B. histograms illustrate the number of colonies. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Previous studies had shown that GSK-3β modulates p53 activity, which regulates the expression of many genes^[8]. GSK-3β was regulated by multiple mechanisms, the best characterized being its self-inhibition following phosphorylation of its N-terminal serine-9^[19]. Subsequently, changes in the protein expressions of p-GSK-3β^{Ser9} and p53 were detected after using 1-AKP in glioma cells. It was found that the expression of p53 decreased after GSK-3β was inactivated(Fig. 4). These results suggest that the regulation of proliferation capacity in glioma cells by GSK-3β may be partially realized through the p53 pathway.

D. Construction of lentivirus interference vector targeting p53

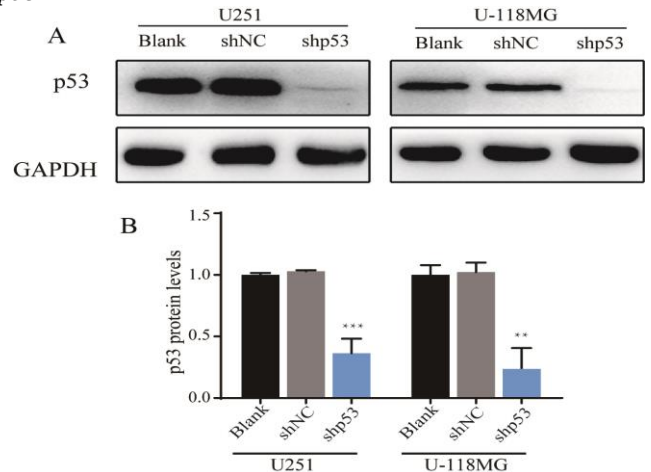


Fig.5. The expression of p53 after shp53 A. Western blot analysis of p53 protein expression in U251 and U-118MG glioma cells after knockdown of p53. B. histograms illustrate the number of colonies. Data are presented as the mean ± SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

Transfection of cells with lentiviral vector shp53 to determine whether GSK-3β regulates glioma cell function by mutant p53. We detected the expression of p53 protein levels to confirm knockdown efficiency. The results showed that the protein levels of p53 were significantly downregulated in shp53 transfected cells (shp53 group) compared with shNC transfected cells (shNC group) (Fig. 5).

E. Knockdown of p53 inhibited the proliferation of glioma cells

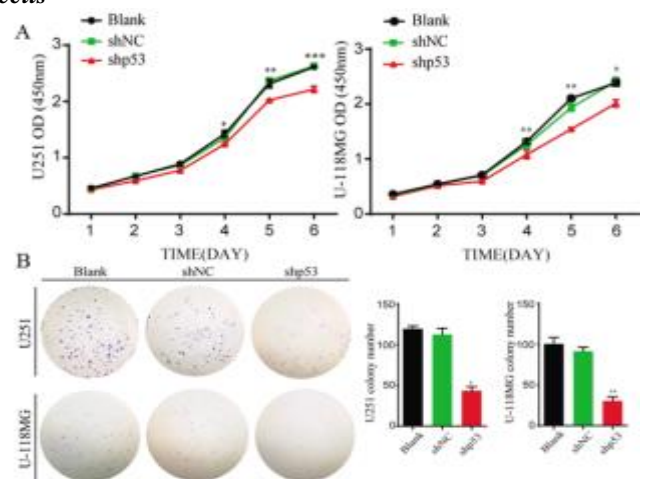


Fig.6. The proliferation after knockdown of p53 A. CCK-8 assays were performed at different time points to detect the effect of knockdown of p53 on the growth of glioma cells. B. Colony-forming assays were performed on the same cell lines; the histograms illustrate the number of colonies. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We also used the CCK-8 assay to determine the effect of p53 knockdown on the proliferation of both glioma cells. The results showed that the proliferation of U251 and U-118MG cells was inhibited after knocking down p53 compared to the shNC group. The results of plate cloning against U251 and U-118MG cells showed that the ability of cell clone formation after p53 inhibition was significantly reduced, and the number of cell clones in the shp53 group was significantly reduced compared with the shNC group (Fig. 6). The above results showed that the proliferation ability of glioma cells decreased after knockdown of p53. It could be understood that GSK-3 β 's regulation of glioma cell proliferation in previous experiments was indeed achieved through the p53 pathway.

IV. DISCUSSION

In the present study, we determined that GSK-3 β and p53 may have a positive correlation in glioma. Decreased cell proliferation was detected in glioma cells after GSK-3 β inactivation, in addition, we showed that GSK-3 β inactivation regulates cell proliferation by inhibiting the expression of mutant p53.

We verified the relationship between GSK-3 β and mutation p53 in glioma through the data of TCGA database, and found that GSK-3 β and mutation p53 showed a positive correlation at mRNA level and protein level, this result can only be used as a reference due to the low determination coefficient. The carcinogenic mechanism of some genes in glioma is different, which makes the formulation of the treatment plan for glioma more complicated. Among them, the role of GSK-3 β in glioma was given contrary opinions by different studies^[14,20,21]. A previous study reported that GSK-3 β phosphorylation of RNPC1 promotes the translation of p53 mRNA and promotes p53 mRNA translation^[22]. Targeting GSK-3 β /p53 pathways may improve therapy and overcome therapeutic resistance in colorectal cancer^[23]. We selected the glioma cell lines U251 and U-118MG with high expression of p53 R273H/R213Q mutation site respectively for the following work.

GSK-3 β inhibitor 1-AKP inhibits GSK-3 β activity led to increased b-cell proliferation and insulin secretion^[24]. We detected proliferation of glioma cells and found that the growth ability and colony-forming ability were decreased when GSK-3 β was inhibited. At the same time, the decrease of expression of p53 occurred with the inhibition of GSK-3 β . This was consistent with a recent study that GSK-3 β phosphorylation of RNPC1 promotes the translation of p53 mRNA and promotes p53 mRNA translation^[22]. Targeting GSK-3 β /p53 pathways may improve therapy and overcome therapeutic resistance in colorectal cancer^[23]. Based on this study and the previous studies, we propose that inhibition of GSK-3 β reduced cell proliferation in glioma cells, and it may be achieved by GSK-3 β 's regulation of p53.

The tumor suppressor gene p53 is lost or mutated in about half of all tumors^[25]. Mutant p53 not only result in loss of transcriptional activity, but also acquire new carcinogenic functions^[26]. We knockdown p53 to confirm the central role of mutant p53 in regulation of GSK-3 β to regulating cell proliferation. Then we found that depletion of p53 could directly cause the decrease of the growth ability and colony-forming ability. This finding was similar to a previous study which reported that p53 mutation can up-regulate cell cycle genes, and the loss of wild-type p53-mediated normal cell cycle checkpoints

leads to abnormal DNA synthesis and increased cell proliferation^[27].

In conclusion, our study showed that GSK-3 β inactivation regulates cell proliferation by inhibiting the expression of mutant p53. Thus, we propose that GSK-3 β plays an essential role in glioma progression, and it may be used as a potential prognostic marker and therapy target for glioma.

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