p38γ overexpression in gliomas and its role in proliferation and apoptosis

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The objective of this study was to confirm the biological role of p38γ in human gliomas. The expression profiles of p38γ and hTERT in human glioma samples were detected by Western Blot and immunohistochemistry. RNA interference was performed in U251 cells by p38γ silencing. Cell proliferation and apoptosis were assayed by CCK-8 and flow cytometric analysis, and then RNA and protein expression levels were measured by real-time RT-PCR and Western Blot, respectively. Telomerase activity assays and Caspase-3, -9 activation assays were also conducted. The results showed p38γ had a positive correlation with the glioma’s malignancy grade and that the treatment of U251 cells with p38γ-siRNA inhibited proliferation and induced apoptosis. Correspondingly, hTERT expression and telomerase activity were down regulated and Caspase-3 and -9 activities were elevated. In conclusion, p38γ may serve as an oncogenic factor promoting the growth and progression of gliomas and may become a useful therapeutic target.
Reduction of p38 expression in U251 cells by siRNA. Quantitative RT-PCR and Western Blotting were performed to determine the effect of RNAi on the expression of p38 in U251 cells. Our results revealed that the p38 mRNA level in p38/siRNA treated cells (1.06 ± 0.05) was significantly down-regulated compared with the level in negative control siRNA-treated cells (13.01 ± 1.62) and blank control U251 cells (14.22 ± 1.14) (P = 0.0013; Figure 3a). Similar results were obtained when detecting the protein level of p38 and hTERT by Western blotting (Figure 3b&c, Supplementary Figure S2). Additionally, the descent of hTERT correlated with p38 silencing (r = 0.667, P = 0.0294).

**Downregulation of p38 by siRNA inhibited cell proliferation in U251 cells.** To investigate cell proliferation, the CCK-8 assay was performed. Compared to the control group, the knockdown of p38 by siRNA reduced U251 cell proliferation to 46.99 ± 2.3% (P = 0.0009; Figure 4a). Furthermore, it was discovered that the telomerase activity (IOD value) in p38-silenced groups (303.3 ± 11.6) was significantly decreased to those of the control groups (P = 0.0010; Figure 4b).

Figure 1 | Up-expression of p38 and hTERT in gliomas. As to p38, the band intensity ratios of IOD were 0.18 ± 0.02 (control), 0.30 ± 0.04 (low-grade glioma), 0.54 ± 0.04 (high-grade glioma). In hTERT, the sequences were 0.05 ± 0.01 (control), 0.14 ± 0.02 (low-grade glioma), 0.25 ± 0.03 (high-grade glioma). Full-length blots are presented in Supplementary Figure S1.

**Reduction of p38 expression in U251 cells by siRNA.** Quantitative RT-PCR and Western Blotting were performed to determine the effect of RNAi on the expression of p38 in U251 cells. Our results revealed that the p38 mRNA level in p38/siRNA treated cells (1.06 ± 0.05) was significantly down-regulated compared with the level in negative control siRNA-treated cells (13.01 ± 1.62) and blank control U251 cells (14.22 ± 1.14) (P = 0.0013; Figure 3a). Similar results were obtained when detecting the protein level of p38 and hTERT by Western blotting (Figure 3b&c, Supplementary Figure S2). Additionally, the descent of hTERT correlated with p38 silencing (r = 0.667, P = 0.0294).

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Figure 2 | Characteristic immunohistochemical staining for p38 and hTERT in patients with different grade gliomas. The “→” point out the positive cells yellowish-brown in cytoplasm (p38) or nucleus (hTERT).
Knockdown of p38 by siRNA in human U251 glioma cells resulted in cell apoptosis. Annexin V staining demonstrated that the amount of apoptotic cells in U251 cells transfected with p38γ siRNA was significantly increased when compared to cells untreated and transfected with control siRNA (Figure 4d, e, and f). The percent of apoptosis increased from 3.20 ± 0.02% in control groups to 11.97 ± 0.41% in p38γ silenced groups (P = 0.0011). This result indicated that siRNA targeting p38γ was able to induce apoptosis in glioma cells.

To further explore the role of p38γ in the apoptotic-signaling pathway, we examined the activities of caspase-3 and caspase-9 (Figure 4c). A significant enhancement of the two indexes was observed in U251 glioma cells treated with p38γ siRNA. The activity of caspase-3 was 103.2 ± 1.74 in the treatment group compared with 43.98 ± 0.64 (BC) and 46.49 ± 0.94 (NC) in the control groups (P = 0.0016). For caspase-9, the sequences were 42.21 ± 1.67 (p38γ/ siRNA), 24.28 ± 0.69 (BC), and 26.24 ± 0.89 (NC) (P = 0.0018).

Discussion

Important causes of tumor related deaths from the central nervous system are gliomas characterized by an unlimited proliferation and progressive local invasion. Unfortunately, the underlying molecular mechanisms that result in astrocytomagenesis, local invasion, and recurrence remain unclear and are a major obstruction in finding novel therapeutic strategies.

Many researches have shown that p38 MAPKs participated in tumorigenesis. p38 was also involved in the cytotoxicity of troglitazone (TGZ) in renal cell carcinoma (RCC) cell lines, while its activation was obligate in tumor cell apoptosis induced by drugs. It was found that a lack of p38γ abrogates the radiosensitizing effect of 5-Fluorouracil (5-FU) in colorectal HCT116 cell lines. As to human gliomas, researches indicated that the tumor occurrence was closely related to the MKK3/p38 pathway activation, and inhibition of p38 by LY479754 greatly sensitized arrested glioma cells to cytotoxic therapies. Studies also detected that p38 activation...
was one of the major causes for the increased chemosensitivity to CDDP on glioma cells. Moreover, p38 inhibition was found to strongly reduce invasion of U251 glioblastoma cells in an inflammatory microenvironment.

However, all of these researches have evident limitations. Most of the time, the inhibition of p38 was accomplished through inhibitors aimed specially at p38α/β, such as SB202190. Few reports addressed the p38γ and p38δ isoforms. Recent studies indicated that the Ras oncogene positively regulated the expression of p38γ, which increases Ras-dependent growth or inhibits stress induced cell-death independent of phosphorylation. This role that p38 played may be achieved through the regulation of ERK (extracellular signal-regulated kinase) expression or banding with PTP1H (protein-tyrosine phosphatase H1). Furthermore, p38γ overexpression led to a marked cell cycle arrest in the G2/M phase. All of these suggests p38γ could be involved in the tumor process. Therefore, p38γ was regarded as a potential drug target in recent experiments. It has been found that a depletion of p38γ suppressed Ras transformation in rat intestinal epithelial cells. Knockdown of p38γ expression in mouse breast cancer cell lines 4T1 resulted in an obvious decrease in cell proliferation and colony formation in vitro and a dramatic retardation of tumorigenesis in vivo. In addition, down-regulation of p38γ initiated the activation of AKT signaling. The effect of targeting p38γ could be promoted by inhibition of this feedforward loop with various P38/AKT signaling inhibitors. Nonetheless, it was not known how p38γ might play a role in glioma tumorigenesis.

In this study, we first examined the expression of p38γ in gliomas of different degrees by Western Blot and immunohistochemistry. The data showed that p38γ was positively correlated with the glioma’s malignancy grade. Previous research has indicated that hTERT depletes p38 expression and telomerase activity both declined. With the histological data above, we can deduce that hTERT may represent an indicator of progression and poor prognosis. Our result of hTERT expression corresponds with this characterization. Moreover, there was cooperativity in the expression of p38γ and hTERT, which was also shown in sarcomas.

p38γ silencing experiments showed that p38γ was involved in the cell proliferation of glioma cells. Along with the downregulation of p38γ by siRNA, the hTERT expression and telomerase activity both declined. Combined with the histological data above, we can deduce that hTERT may be a downstream target of p38γ that participates in cell suppression in glioma. It is not known how the p38γ in the cytoplasm is taken into nucleus and regulates hTERT expression. Up to now, available data didn’t reveal the mechanism in detail. However, the latest research has revealed that a lack of either p38γ or p38δ in K-Ras-transformed fibroblasts increased cell migration and MMP-2 secretion, and a lack of p38γ led to increased cell proliferation as well as tumorigenesis. Additionally, the p38α/β inhibitor SB203580 was found to have no effect on abrogating the inhibitory effect of TNFα on hTERT in myeloid cells. It was confirmed that p38γ phosphorylation decreases p38γ protein expression via c-Jun-dependent ubiquitin-proteasome pathways, while its inhibition increases cellular p38γ concentrations, indicating an active role of p38δ phosphorylation in negatively regulating p38γ protein expression. Therefore, these conflicting results suggest that the p38 MAPK expression distribution of each subtype and their interactions should be included in future research.

Our research also revealed that siRNA targeting p38γ was able to induce apoptosis in glioma cells and reduce expression levels of Caspase-3/9. Recently, p38γ was thought to induce cell apoptosis according to regulation of the cell cycle. One study presented that p38γ deletion sensitizes cells to ultraviolet ray (UV) exposure, accompanied by prolonged S phase cell cycle arrest and an increased rate of apoptosis. However, other tests performed in breast cancer cells indicated that p38γ overexpression resulted in cell cycle arrest in the G2/M phase, loss of p38 could induce pleiotropic mitotic defects, and the majority of p38-depleted cells die at mitotic arrest or soon after abnormal exit from M-phase. This remains to be researched further.

In summary, our results indicated that p38γ is likely to be an oncogenic factor promoting growth and progression in gliomas. Meanwhile, p38γ induced tumorigenesis may act towards regulating the expression of hTERT. Therefore, p38γ may be a potential therapeutic target in glioma.

Methods

Patient samples. Human surgical biopsy samples taken from 71 patients with glioma were collected at the time of primary resection in the Neurosurgery Department of the Xiangya Hospital of Central South University from June to October in 2011. None of the patients had received chemotherapy or radiation before surgery. Five specimens (2 traumatic brain injury were used as nonneoplastic controls. All specimens were assessed by a pathologist according to the WHO Classification of Tumors of the Central Nervous System (4th edition, 2007), which were divided into low-grade glioma and high-grade glioma. Informed consents were obtained from the involved patients. This study was approved by the Ethics Committee of the Xiangya Hospital of Central South University.

Cell culture and transfection. The human U251 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FBS), penicillin (300 IU/ml), and streptomycin (50 mg/ml). p38γ siRNA (primers: 5'-GGGAAGCGGUUACUUACAATT-3' (sense), 5'-UUUGAAGUACACGCGUUGCATT-3' (antisense), and negative control siRNA were purchased from Shanghai GenePharma Co., Ltd. Before the transfection procedure, U251 cells were seeded (2 × 10⁵ cells/well) on six-well plates and grown to 70% confluence. Lipofectamine 2000 (Invitrogen) was utilized for transfection according to the manufacturer’s instructions. After incubation for 20 min at room temperature, the mixture of lipofectamine 2000 reagent and respective siRNA were diluted with culture medium and added to each well. Forty-eight hours after transfection, cells were harvested for quantitative real-time PCR and Western Blot analysis.

Quantitative PCR. Real-time PCR was done using SYBR Green PCR Master Mix (ABI, 4309153) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal reference control. Primer sequences for p38γ were 5'-CCGACACATGATGAAATGGAA-3' (forward) and 5'-TCAGGGAGAATTGAGTGGT-3' (reverse), while for GAPDH they were 5'-CAATGACCCCTATTGACC-3' (forward) and 5'-GACAAGCCTCGGTTCAG-3' (reverse). Primers were obtained from Sangon Biotech (Shanghai, China).

Cell viability assay. The cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay (Shanghai Beyotime Biotechnology Ltd., #C0038) with process steps from the kit’s instructions. The optical density (OD) at 450 nm was recorded on a Microplate Reader (Bio-Rad, Hercules, CA, USA). The relative cell proliferation rate (% of control) was expressed as the percentage of (ODtest – ODblank)/ODcontrol – ODblank, where ODtest is the optical density of the cells given siRNA, ODcontrol is the control sample, and ODblank is of the wells without U251 cells. Each experiment was performed three times.

Telomerase activity assay. Activity of telomerase was determined with TRAP-silver (TRAP-eze Telomerase Assay Kit, Biozzi Midwest Genetic Science and Technology Ltd., #NK155LM). Briefly, 2 μl telomerase extraction was added to 50 μl of a solution containing 5 μl 10× TRAP buffer, 1 μl dNTPs, 1 μl Taq-DNA polymerase, 1 μl TS primer, 2 μl telomerase extraction, 39 μl DEPC H₂O, and 1 μl CX primer. Then, the reaction mixture was subjected to 30 cycles of PCR amplification (94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s) for 5 min). PCR products (9 μl) were electrophoresed in 1 μl 10% loading buffer on 10% nondenaturing polyacrylamide gel (PAG) at 220 V for 120 min. A silver staining positive result was the appearance of a ladder with a 6 bp increment. According to the Gel imaging analysis system (Bioshine GelX 1650), telomerase activity was shown by relative absorbance (integrated optical density, IOD).

Detection of apoptosis. The apoptosis was investigated using the Annexin V-FITC & PI Apoptosis Detection Kit (ADL, A0001a). All operations were performed in accordance with the instructions of the kit. Briefly, 5 μl annexin V-FITC and 10 μl PI were used per sample. The apoptosis of the U251 Cells (%) was analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer and Cell Quest software.

Caspase-3, 9 activation assay. The activity of caspase-3 was detected by cleavage of chromogenic caspase-3 substrates Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide). Protein was extracted using ice-cold cell lysis buffer and total protein (1–3 mg/ml) was added to the reaction buffer containing 10 μl Ac-DEVD-pNA (2 mg/ml) and then incubated 60–120 min at 37 °C. The pNA cleaved by caspase-3 precursor can be quantified using a spectrometer at 405 nm. A similar process was performed in the caspase-9 activity assay, but the substrates changed to Ac- LEHD-pNA (acetyl-Leu-Glu-His-Asp p-nitroanilide).

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Western blot analysis. The procedures below were implemented to both tissue samples and U251 cells. The total proteins were prepared using the Total Protein Extraction Kit (ProMab, USA) and assayed quantitatively using the Bradford Protein Assay Kit (Beyotime, China). After conventional electrophoresis with 12% SDS-PAGE, separated proteins were transported onto a NC membrane (Pierce, Rockford, USA). Subsequently, the membrane was incubated with primary antibody against p38 (SANTA, USA, 1: 400) or hTERT (Epitomics, USA, 1: 1000) overnight at 4°C. After washing, the membrane was incubated with each corresponding secondary antibody before visualization by chemiluminescence. Mouse monoclonal GAPDH (ProMab, USA, 1:1000) was used as the primary Ab for control. The densities of Western blot bands were detected using the software Gel Pro 4.0 with presentation of IOD (integrated optical density). The band intensity ratio of p38 or hTERT to GAPDH (p38/GAPDH, hTERT/GAPDH) from the same electrophoresis run was analyzed.

Immunohistochemistry and criterion. Immunohistochemistry (IHC) staining of 3 mm sections of glioma samples was performed with the HRP-Polymer anti-Mouse/ Rabbit IHC Kit (Maxzin, Bio, Fuzhou, China) in standard procedures. The primary antibodies were mouse monoclonal p38 antibody (Origene, Rockville, MD, USA, 1:150) and rabbit monoclonal antibody against human telomerase reverse transcriptase (hTERT) (Abcam, Cambridge, MA, USA, 1:400). The positive cells were yellowish-brown in the cytoplasm (p38) or nucleus (hTERT) while unstained in negative cells.

Statistical analysis. SPSS 17.0 statistical software was used for the statistical analysis. Data was expressed as Mean ± SEM. One-way analysis of variance (ANOVA) and the Student–Newman–Keuls tests were used to analyze the significance of differences between study groups. Data was considered statistically significant at p < 0.05.

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Author contributions
K.Y. performed most of the experiments, analyzed the data, and wrote the manuscript. Z.L. and J.L. collected clinical samples and analyzed clinical data. X.C., C.L. and Y.Z. performed and analyzed cellular experiments. X.C. assisted with figures and experimental design. Y.L. designed the experiments and wrote the manuscript.

Additional information
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