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Original Research

MST1 suppresses viability and promotes apoptosis of glioma cells via upregulating SIRT6 expression

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It has been well established that mammalian sterile 20-like 1 (MST1) functions as a suppressor via regulating cell progression in many tumors. However, the molecular mechanism of MST1 on regulating glioma progression remains unclear. Here, we discovered that MST1 was robustly down-regulated in glioma tissues and cells. Functional analysis showed that over-expression of MST1 downregulated viability and colony formation and promoted apoptosis of glioma cells. Our results also identified that MST1 positively regulated expression of SIRT6 (Sirtuin 6) via transcriptional factor FOXO3a (Forkhead box O3a). Furthermore, the functional role of MST1 in glioma cell viability (or apoptosis) were significantly reversed after knocking down of SIRT6. Our research indicates that MST1 is a potential biomarker for the prognosis and diagnosis of glioma and provides new direction on the molecular mechanism of glioma progression and development.

Keywords

Glioma; mammalian sterile 20-like 1; Sirtuin 6; viability; apoptosis; molecular mechanisms; immunohistochemistry

1. Introduction

Glioma comprises 30% of all brain tumors and central nervous system tumors, and accounting for 80% of all malignant brain tumors (Goodenberger and Jenkins, 2012). Glioma is characterized by uncontrolled proliferation, invasion and angiogenesis (Chang et al., 2017). The average survival time of glioma is generally ranging from 12 to 15 months (Huse et al., 2011; Grossman et al., 2010). A common treatment method is a combination of surgery, radiation therapy, and chemotherapy (Sun et al., 2013; Zach et al., 2009). After use of this method however, the postoperative survival rate have shown to be unsatisfactory due to high risk for metastasis and invasion (Wu et al., 2017; Bohman et al., 2009). Therefore, finding new targets and elucidating the molecular mechanisms of glioma remains necessary.

MST1 (mammalian sterile 20-like 1), is widely expressed as serine/threonine-protein kinase, acts upstream of the stress-induced mitogen-activated protein kinase (MAPK) cascade and plays an important role in a variety of cellular physiological activities (Oh et al., 2009; Dong et al., 2009). Research has shown that MST1 regulates cell cycle and induces cells apoptosis via signaling pathway related to p53 (Yuan et al., 2011). Moreover, MST1 functions as a tumor suppressor in various cancers (Grusche et al., 2010; Huang et al., 2005; Rawat et al., 2013; Xu et al., 2013). Therefore, MST1 is developed as a prognostic biomarker and therapeutic target in human cancer via its regulatory role in cell proliferation and apoptosis (Jia et al., 2003). However, the effects and molecular mechanism of MST1 in the progression of glioma are still unclear.

SIRT6 (Sirtuin 6) is a stress responsive protein deacetylase and functions in various molecular pathways involved in aging, DNA repair, telomere maintenance, glycolysis and inflammation (Frye, 2000). SIRT6 could be used as a prognostic biomarker in hepatocarcinoma (Elhanati et al., 2016). SIRT6 also acts as a tumor suppressor in various cancer cells (Sebastian et al., 2012). For example, the down-regulation of SIRT6 was proved to promote the progression of ovarian cancer (Zhang et al., 2015). Particularly, SIRT6 is mildly expressed in glioma, and over-expression of SIRT6 inhibits PCBP2 (Poly(rC)-binding protein 2) expression and suppresses glioma growth (Chen et al., 2014). In addition, SIRT6 inhibits glioma cell progression via inactivation of JAK2/STAT3 signaling pathway (Feng et al., 2016). These investigations indicated the involvement of SIRT6 in progression of glioma.

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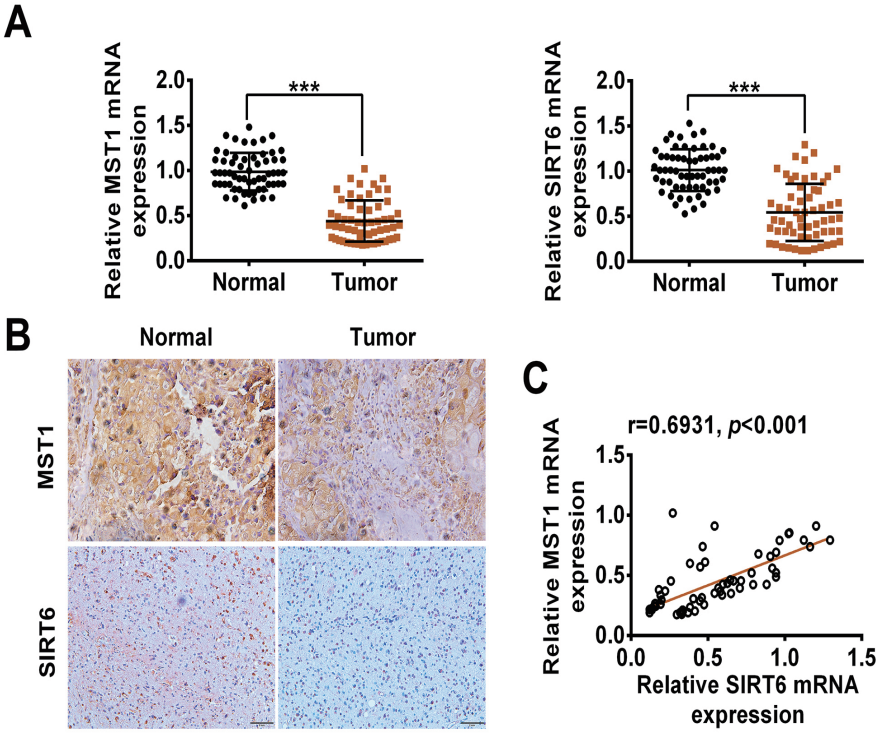

Figure 1. Down-regulation of MST1 in glioma. (A) The mRNA expression of MST1 ($P = 0.00018$) and SIRT6 ($P = 0.00029$) in human glioma tissues (n = 50, Tumor) were lower than that in adjacent normal tissues (n = 50, Normal). *** represents Tumor *vs*. Normal. (B) Immunohistochemistry (IHC) staining showed lower expression of MST1 and SIRT6 in glioma tissues than that in Normal tissues. (C) Positive correlation between MST1 level and SIRT6 expression ($P = 0.00033$).

Previous study indicated that MST1 is implicated in the control of FOXO-dependent neuronal cell death via phosphorylating FOXO3a at Ser-207 (Yuan et al., 2011). Besides, FOXO3a transcriptional factor was shown to positively regulate the expression of SIRT6 (Kim et al., 2010). Based on the above studies, we hypothesized that MST1 might be involved in glioma progression and a close relationship might exist between MST1 and SIRT6. The results of the present study show that MST1 was down-regulated in glioma tissues and cells. Over-expression of MST1 suppressed viability and promoted apoptosis of glioma cells. Finally, we provided preliminary evidence for MST1 positively regulating the expression of SIRT6 by transcriptional factor FOXO3a, and consequently affecting glioma progression.

2. Materials and methods

2.1 Patients and sample collection

Glioma tissues and the adjacent normal tissues from 50 patients were collected after surgery in Zhejiang Cancer Hospital. The tissues were embedded in paraffin or immediately frozen and stored at -80 °C for the next experiments. Approval for this study was obtained from Zhejiang Cancer Hospital Ethics Committee. Written informed consents were acquired from all participants. Patient characteristics are shown in Table 1.

2.2 Cell lines and cell culture

Human embryonic brain cells (HEB), human glioma cell lines A-172 (glioblastoma grade II), U-138 MG (glioblastoma grade III) and U-251 MG (glioblastoma grade IV) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM/F-12 media containing 10% fetal bovine serum (GIBCO, Life Technologies, Carlsbad, CA, USA) at an incubator with 37 °C and 5% CO_2.

2.3 Cell transfection and plasmid construction

For knocking down of MST1 or SIRT6, siRNA (siMST1-1: 5'-GCAGGUCAACUUACAGAUATT-3'; siMST1-2: 5'-GACUGAUGGAGCCAAUACUTT-3'; siSIRT6-1: 5'-AAGAATGTGCCAAGTGTAAGA-3'; siSIRT6-2: 5'-CCGGCTCTGCACCGTGGCTAA-3'; siFOXO3a-1: 5'-ATGGCAAGCACAGAGTTGGATGAAGTCCA-3'; siFOXO3a-2: 5'-ATGGCTAGTACCGAACTGGACGAGGTGCA-3') and the negative control (siNC: 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Vigenebio (Jinan, China). U-251 MG cells (4×10^5 per well) were seeded in 12-well plates overnight. The culture medium was then removed, and washed with Phosphate-buffered saline (PBS). The cells were transfected with 40 nM siRNAs by Lipofectamine 2000 (Invitrogen). For the over-expression of MST1 and SIRT6, the primary precursor sequences were PCR and subcloned into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Cells were then transfected with 40 nM pcDNA3.1-MST1 or pcDNA3.1-SIRT6 by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested for further analysis 48 hours after transfection.

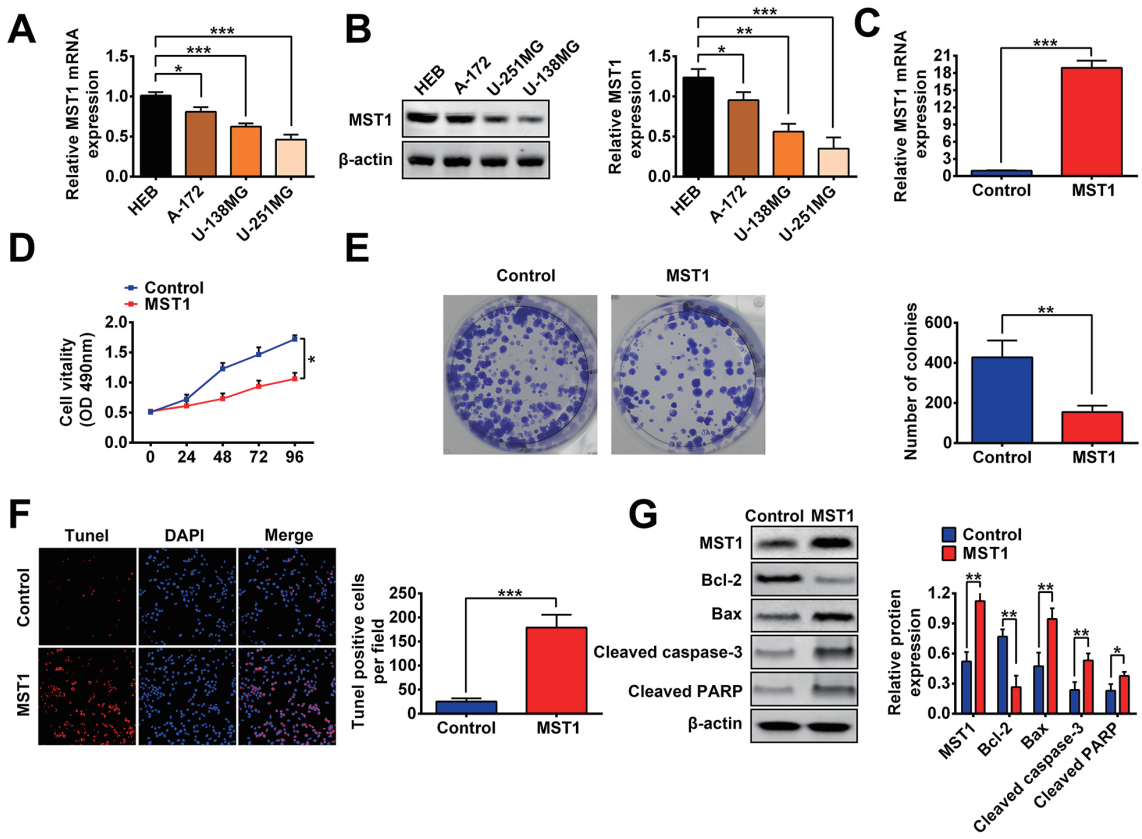

Figure 2. Over-expression of MST1 inhibited cell viability and colony formation and induced cell apoptosis of glioma cells. (A) The mRNA expression of MST1 in glioma cell lines (A-172 ($P = 0.0085$), U-138 MG ($P = 0.0004$) and U-251 MG ($P = 0.0002$)) was lower than that in human embryonic brain cells (HEB). All results are expressed as mean ± SEM of at least 3 independent experiments. $*$, $***$ represents glioma cell lines *vs.* HEB. (B) The protein expression of MST1 in glioma cell lines (A-172 ($P = 0.031$), U-138 MG ($P = 0.0076$) and U-251 MG ($P = 0.00083$)) was lower than human embryonic brain cells (HEB). All results are expressed as mean ± SEM of at least 3 independent experiments. $*$, $**$, $***$ represents glioma cell lines *vs.* HEB. (C) MST1 was increased in U-251 MG cell line after transfecting with pcDNA-MST1 (MST1) ($P = 0.0001$). All results are expressed as mean ± SEM of at least 3 independent experiments. $***$ represents MST1 *vs.* Control. (D) MST1 decreased viability of U-251 MG cells by MTT assay ($P = 0.00067$). All results are expressed as mean ± SEM of at least 3 independent experiments. $***$ represents MST1 *vs.* Control. (E) MST1 suppressed colony formation of U-251 MG cells, which was detected by colony formation assay ($P = 0.0063$). All results are expressed as mean ± SEM of at least 3 independent experiments. $***$ represents MST1 *vs.* Control. (F) MST1 induced apoptosis of U-251 MG cells by TUNEL assay ($P = 0.0006$). All results are expressed as mean ± SEM of at least 3 independent experiments. $***$ represents MST1 *vs.* Control. (G) MST1 decreased protein expression of Bcl-2 ($P = 0.0030$) and increased protein expression of MST1 ($P = 0.0014$), Bax ($P = 0.0090$), cleaved-caspase 3 ($P = 0.0082$) and cleaved PARP ($P = 0.03$) in U-251 MG cells by western blot. All results are expressed as mean ± SEM of at least 3 independent experiments. $*$, $**$ represents MST1 *vs.* Control.

2.4 qRT-PCR

RNAs from tissues or cells were extracted by Trizol reagent (Invitrogen). The RNAs were then reverse transcribed into cDNAs using miScript Reverse Transcription kit (Qiagen, Hilden, Germany). cDNAs (10 ng) were amplified by using SYBR1 Premix Ex Taq^{TM} II (Takara, Shiga, Japan) under the following conditions: Denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s in a total 40 cycles. U6 was used as the internal reference. Three technological replicates were used to ensure the reliability of the analysis. For relative quantification, the fold change was determined with $2^{-\triangle\triangle Ct}$ method, The primer sequences were shown:
MST1, 5'-ACAAATCCTCCTCCCACATTCCG-3' (forward) and 5'-CACTCCTGACAAATGGGTGCTG-3' (reverse);
SIRT6, 5'-CCAAGTTCGACACCACCTTT-3' (forward) and 5'- CGGACGTACTGCGTCTTACA-3' (reverse);
FOXO3a, 5'-AGTGGATGGTGCGCTGTGT-3' (forward) and 5'-CTGTGCAGGGACAGGTTGT -3' (reverse);
U6, 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse);
GAPDH, 5'-TGTTCGTCATGGGTGTGAAC-3' (forward) and 5'-ATGGCATGGACTGTGGTCAT -3' (reverse).

2.5 Immunohistochemistry

The extracted glioma tumor specimens and the adjacent normal tissues were cut into slices of 5 μm. The slices were embedded in paraffin, and then dewaxed in xylene and rehydrated in ethanol. The sections were then blocked for endogenous peroxidase by incubating in 3% H_2O_2 and then washed in PBS containing 0.05

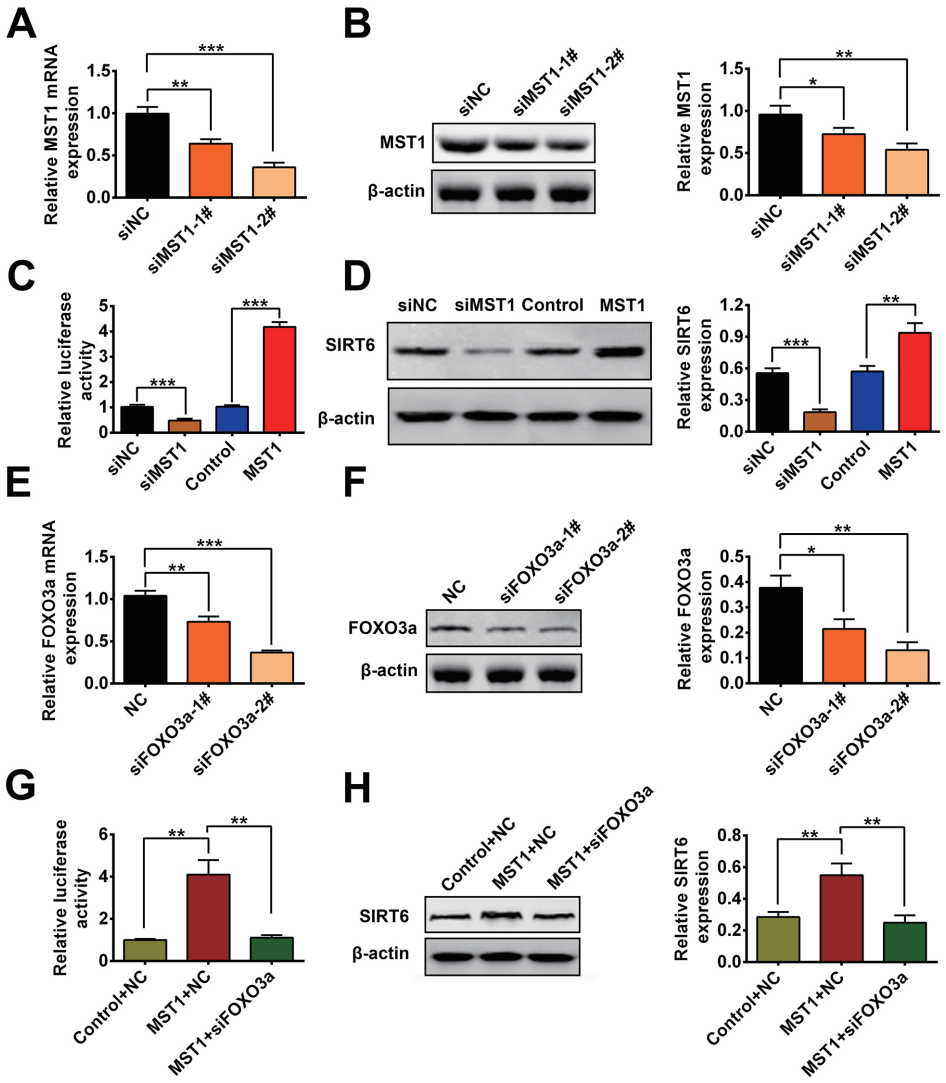

Figure 3. MST1 positively regulated SIRT6 expression via FOXO3a (A) MST1 mRNA expression was decreased in U-251 MG cells that transfected with siMST1-1# ($P = 0.0030$) or siMST1-2# ($P = 0.00054$) as compared to the control group. All results are expressed as mean ± SEM of at least 3 independent experiments. **, *** represents siMST1 *vs.* siNC. (B) MST1 protein expression was decreased in U-251 MG cells that transfected with siMST1-1# ($P = 0.0378$) or siMST1-2# ($P = 0.0065$) as compared to the control group. All results are expressed as mean ± SEM of at least 3 independent experiments. *, ** represents siMST1 *vs.* siNC. (C) siMST1 decreased the luciferase activity of SIRT6 ($P = 0.00036$) promoter while MST1 over-expression increased the activity ($P = 0.00019$) by luciferase reporter assay. All results are expressed as mean ± SEM of at least 3 independent experiments. *** represents siMST1 vs. siNC or MST1 *vs.* Control. (D) siMST1 decreased the protein expression of SIRT6 ($P = 0.0003$) while MST1 over-expression increased the protein expression of SIRT6 ($P = 0.004$) in U-251 MG cells by western blot. All results are expressed as mean ± SEM of at least 3 independent experiments. **, *** represents siMST1 *vs.* siNC or MST1 *vs.* Control. (E) FOXO3a mRNA expression was decreased in U-251 MG cells that transfected with siFOXO3a-1# ($P = 0.0035$) or siFOXO3a-2 ($P = 0.00078$). All results are expressed as mean ± SEM of at least 3 independent experiments. **, *** represents siFOXO3a *vs.* siNC. (F) FOXO3a protein expression was decreased in U-251 MG cells that transfected with siFOXO3a-1# ($P = 0.01$) or siFOXO3a-2as compared to the control group($P = 0.0017$). All results are expressed as mean ± SEM of at least 3 independent experiments. *, ** represents siFOXO3a *vs.* siNC. (G) The increased luciferase activity induced by MST1 over-expression ($P = 0.0015$) was decreased after transfecting an addition of siFOXO3a ($P = 0.0018$) by luciferase reporter assay. All results are expressed as mean ± SEM of at least 3 independent experiments. ** represents MST1 *vs.* Control or MST1 + siFOXO3a. (H) The increased SIRT6 protein expression in U-251 MG cells induced by MST1 over-expression ($P = 0.0046$) was decreased after transfecting an addition of siFOXO3a ($P = 0.0040$) by western blot. All results are expressed as mean ± SEM of at least 3 independent experiments. ** represents MST1 *vs.* Control or MST1 + siFOXO3a.

M EDTA followed by 4% paraformaldehyde. Tissues were incubated in 4% dry milk and 0.3% goat serum in PBS solution for 20 minutes to block non-specific binding. Then the sections were incubated overnight at room temperature with anti-MST1 or SIRT6 (6 μg/mL; Santa Cruz, CA, USA) antibody in the presence of 10% rabbit serum. After washing, sections were incubated for another

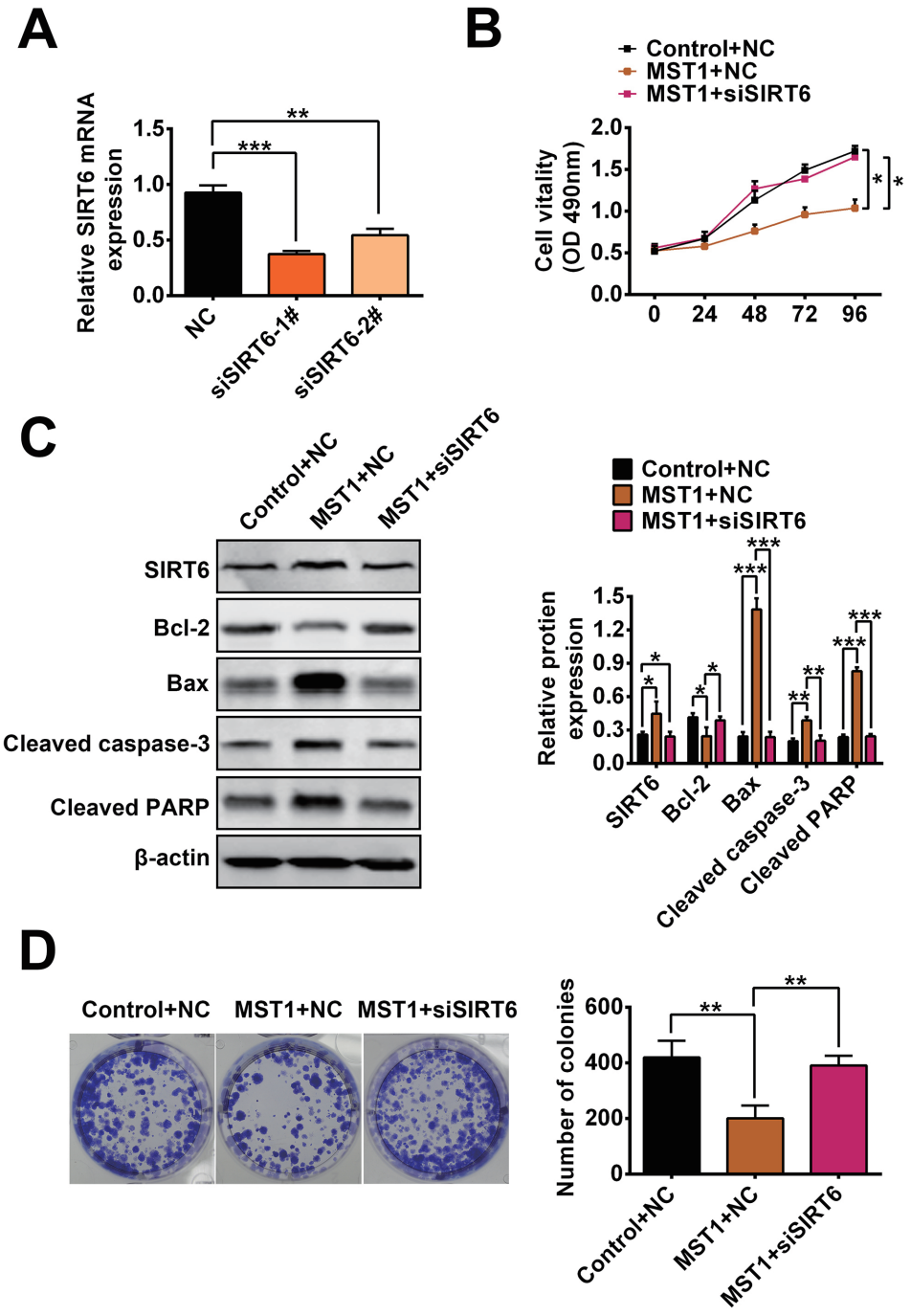

Figure 4. MST1 inhibited cell viability and induced cell apoptosis of glioma cells through regulating SIRT6 expression (A)SIRT6 mRNA expression was decreased in U-251 MG cells transfected with siSIRT6-1# ($P = 0.0002$) or siSIRT6-2# ($P = 0.0017$). All results are expressed as mean ± SEM of at least 3 independent experiments. $**$, $***$ represents siSIRT6 *vs.* siNC. (B) The decreased viability of U-251 MG cells induced by MST1 over-expression ($P = 0.00059$) was increased after transfecting with siSIRT6 ($P = 0.00047$) by MTT assay. All results are expressed as mean ± SEM of at least 3 independent experiments. $*$ represents MST1 *vs.* Control or MST1 + siSIRT6. (C) MST1 decreased expression of Bcl-2 ($P = 0.028$) and increased expression of SIRT6 ($P = 0.046$), Bax ($P = 0.00063$), cleaved-caspase 3 ($P = 0.0013$) and cleaved PARP ($P = 0.00057$) protein expression of U-251 MG cells by western blot. Co-transfection of MST1 over-expression and siSIRT6 increased expression of Bcl-2 ($P = 0.046$) and decreased expression of SIRT6 ($P = 0.040$), Bax ($P = 0.00038$), cleaved-caspase 3 ($P = 0.0059$) and cleaved PARP ($P = 0.00046$) protein expression. All results are expressed as mean ± SEM of at least 3 independent experiments. $*$, $**$, $***$ represents MST1 *vs.* Control or MST1 + siSIRT6. (D) The decreased number of colonies of U-251 MG cells induced by MST1 over-expression ($P = 0.0075$) was increased after transfecting with siSIRT6 ($P = 0.0048$) ($P = 0.0082$) by colony formation assay. All results are expressed as mean ± SEM of at least 3 independent experiments. $**$ represents MST1 *vs.* Control or MST1 + siSIRT6.

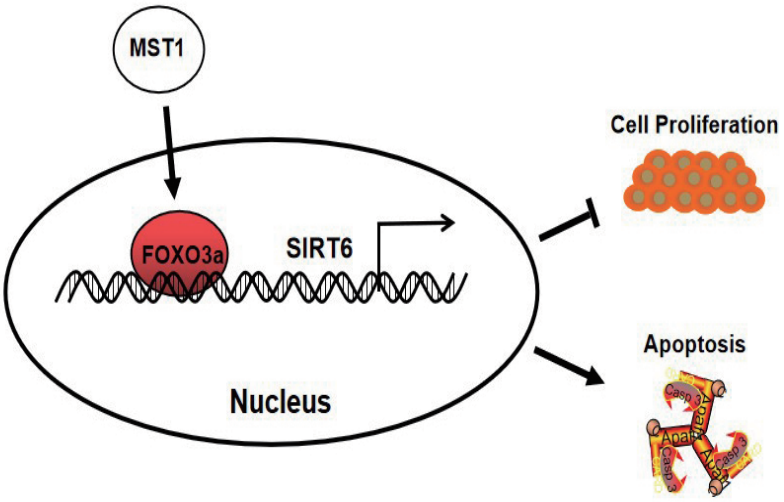

Figure 5. Summary diagram to illustrate the hypothesized pathway and effects of MST1 on glioma progression. MST1 could promote the phosphorylation of FOXO3a, and destruct the binding of FOXO3a to 14-3-3 protein in the cytoplasm, thus dissociating FOXO3a and promoting translocation into nucleus. Moreover, FOXO3a binds with promoter of SIRT6, and positively regulates the expression of SIRT6, thus leading to the inhibition of cell proliferation and promotion of cell apoptosis.

2 h with horseradish peroxidase (HRP) goat anti-rabbit IgG secondary antibody. Slides were counterstained with hematoxylin to stain cell nuclei in order to identify cells. Slides were then dehydrated and examined under a light microscope (Olympus, Tokyo, Japan).

2.6 Western blot

Sample proteins were extracted from tissues or cells using RIPA buffer (KeyGen, Nanjin, China). Protein concentration was determined via BCA protein assay kit (Boster, China). Protein lysates (30 μg) were loaded onto 10% SDS-PAGE, and then transferred to PVDF membrane. The membrane was blocked in PBS-T with 5% BSA for 2 hours. PVDF membranes were then probed with rabbit anti-MST1 antibody (80 kDa, 1 : 1500, Abcam, Cambridge, MA, USA), Bcl-2 (26 kDa) or Bax (21 kDa) (1 : 1000, Abcam), Cleaved caspase-3 (17 kDa) or Cleaved Poly(ADP-Ribose)-Polymerase (PARP; 25 kDa) (1 : 2000, Abcam), SIRT6 (37 kDa) or FOXO3a (72 kDa) (1:2500, Abcam), β-actin (1 : 3000, Abcam) overnight at 4 °C. The PVDF membrane was washed with Tris Buffered Saline with Tween 20 (TBST, 10 min $\times$ 3 times) and labeled with horseradish peroxidase (HRP)-conjugated secondary antibodies (1 : 5000; Abcam) for 1 hour. Immunoreactivities were detected by enhanced chemiluminescence (KeyGen, Nanjin, China), and the band intensity was quantified by ImageJ software. The relative protein levels were calculated based on β-actin protein as a loading control.

2.7 MTT assay

U-251 MG cells were cultured in 96-well plates at concentration of 1×10^4 per well for 24 hours and then were treated with Anlotinib for another 24 hours. The 3-(4, 5-dimethylthiazal-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay (Solarbio, Beijing, China) was utilized to estimate cell viability. The results were calculated by the means of spectrophotometric plate reader (BioTek, VT, USA) at 490 nm. All the results were tested at least by three independent experiments.

2.8 Colony formation assay

Following heat to 56 °C, the DMEM (4.1 mL) and agar (4%, 0.9 mL) were then cooled for precipitation. The 1 $\times$ 104 cells/mL single-cell suspension was made via dilution of 1 $\times$ 106 U-251 MG cells per mL in DMEM with 0.36% agar. The colonies were stained with 0.04% crystal violet-2% ethanol in PBS and then incubated at 37 °C for 3 weeks. The stained colonies were photographed under a light microscope (Olympus).

2.9 Cell apoptosis assay

U-251 MG cells were harvested at concentration of 1×10^6 per well, and then fixed with 4% paraformaldehyde at 27 °C for 1 hour. Permeated with 0.1% Triton-X 100 and 0.1% sodium citrate for 2 minutes, the cells were then added by terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) solution (In Situ Cell Death Detection Kit; Roche, IN, USA) and incubated at dark in 37 °C for 1 hour. 0.5 μg/mL 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) was utilized to stain the nuclei. The fluorescence was also analyzed by fluorescent microscopy (Olympus, Tokyo, Japan).

2.10 Luciferase reporter assay

The promoter sequence of SIRT6 mRNA was cloned into pGL-luciferase plasmid (Promega, Madison, Wisconsin, USA) to construct luciferase reporter plasmid. U-251 MG cells were seeded at concentration of 3×10^4 per well for triplicates in 24-well plates 24 hours before transfection, and co-transfected with siSIRT6, siFOXO3a or siNC. Forty-eight hours later, the cells were then harvested and lysed, and the luciferase activities were performed with the Lucifer Reporter Assay System (Promega).

Table 1. Correlation of the expression of MST1 and SIRT6 with clinicopathological features.

Characteristics	N	Relative MST1 expression		χ^2 value	*P* value	N	Relative MST1 expression		χ^2 value	*P* value
		High (n=24)	Low (n=36)				High (n=26)	Low (n=34)		
Age				0.286	0.593				0.045	0.832
45	25	9	16			27	10	14		
≥ 45	35	15	20			33	16	20		
Gender				0.100	0.752				0.025	0.875
female	31	13	18			33	14	19		
male	29	11	18			27	12	15		
Tumor size				4.922	0.027*				7.186	0.007**
<5 cm	32	17	15			31	19	13		
≥ 5 cm	28	7	21			29	7	21		
Peritumoral Brain Edema				1.358	0.244				0.667	0.414
≥ 1 cm	27	13	14			25	15	16		
< 1 cm	33	11	22			35	11	18		
WHO stage				10.223	0.017*				15.934	0.001*
I	16	9	5			18	11	4		
II	17	9	7			15	10	6		
III	12	3	11			11	2	10		
IV	15	3	13			16	3	14		

*P values < 0.05 was considered statistically significant.

2.11 Statistical analysis

All data are expressed as mean ± SEM of at least 3 independent experiments. All statistical analyses were using GraphPad Prism software (GraphPad Prism Software Inc., San Diego, USA). Statistical analyses were performed with one-way analysis of variance (ANOVA). $P < 0.05$ was considered as significant.

3. Results

3.1 MST1 and SIRT6 were down-regulated in glioma tissues

We firstly determine the expression level of MST1 in glioma tissues. As shown in Fig. 1A, the mRNA expression level of MST1 in 50 glioma tissues was lower than that in the adjacent normal tissues. The mRNA expression level of SIRT6 in glioma tissues was also lower than that in the adjacent normal tissues (Fig. 1A). Consistently, the downregulation of MST1 and SIRT6 in glioma tissues were also confirmed by immunohistochemical staining, demonstrating lower protein expression of MST1 and SIRT6 in glioma tissues than those in adjacent normal tissues (Fig. 1B). The correlation analysis showed a positive relationship between MST1 level and SIRT6 expression (Fig. 1C), suggesting the potential relationship with glioma.

3.2 Over-expression of MST1 inhibited cell viability and colony formation and induced cell apoptosis of glioma cells

MST1 expression profiles were also detected in three independent glioma cell lines (A-172, U-138 MG and U-251 MG) and human embryonic brain cell line (HEB). The results showed that the mRNA (Fig. 2A) and protein (Fig. 2B) expression of MST1 in glioma cell lines were significantly down-regulated as compared with that in HEB cells. Of which, U-138 MG cell line demonstrated a 50% reduction in both mRNA and protein expression of MST1, and A-172 demonstrated a 20% reduction in both mRNA and protein expression of MST1 (Fig. 2A,B). Particularly, U-251 MG cell line performing about 50% reduction in mRNA expression and more than 50% reduction in protein expression than that in HEB cells, respectively (Fig. 2A,B), which was selected for the following functional analysis. U-251 MG cell line with stable over-expression of MST1 was established, and the expression of MST1 was higher about ninefold in U-251 MG cells transfected with MST1 over-expression than that in the control cells (Fig. 2C). MTT assay revealed that MST1 over-expression significantly decreased viability of U-251 MG cells (Fig. 2D). MST1 over-expression also suppressed colony formation of U-251 MG cells (Fig. 2E), as shown by the decreased number of colonies in cells with MST1 over-expression compared to control cells. Moreover, TUNEL staining analysis showed that MST1 over-expression also induced apoptosis in cells with MST1 over-expression as compared to the control cells (Fig. 2F). Besides that, the expression of anti-apoptotic protein Bcl-2 was decreased and the expression of pro-apoptotic proteins Bax, cleaved-caspase 3 and cleaved PARP were increased in cells with MST1 over-expression as compared to the control cells (Fig. 2G). These results revealed that MST1 over-expression inhibited cell viability and colony formation and induced cell apoptosis of glioma cells.

3.3 MST1 positively regulated SIRT6 expression via FOXO3a

The underlying mechanism was then explored. MST1 was knocked down in U-251 MG cell line by siRNAs (siMST1-1# and siMST1-2#), and the transfection efficiency was confirmed by the down-regulated MST1 mRNA (Fig. 3A) and protein expression (Fig. 3B). Of which, siMST1-2# performing about 60% reduction in mRNA expression and 50% reduction in protein expression (Fig. 3A,B), which was selected for the subsequent analy-

sis. The luciferase reporter assay demonstrated that siMST1 decreased the luciferase activity of SIRT6 promoter while MST1 over-expression increased the activity as compared to the corresponding controls (Fig. 3C). Besides, siMST1 performed about 60% reduction, and MST1 over-expression performed 50% induction of SIRT6 protein expression as compared to the corresponding controls (Fig. 3D). These investigations suggested that MST1 positively regulated SIRT6 expression.

FOXO3a transcriptional factor was shown to positively regulate the expression of SIRT6 (Kim et al., 2010). In our study, U-251 MG cell was transfected with siRNAs (siFOXO3a-1# and siFOXO3a-2#) , showing 40% and 60% reduction in both mRNA (Fig. 3E) and protein (Fig. 3F) expression as compared to the corresponding controls, respectively. Of which, siFOXO3a-2# performing less mRNA and protein expression of FOXO3a (Fig. 3E, F), and was selected for the following assays. Indeed, luciferase reporter assay revealed the increased luciferase activity induced by MST1 over-expression was decreased after co-transfection of siFOXO3a and MST1 over-expression (Fig. 3G). Moreover, the increased SIRT6 protein expression induced by MST1 over-expression was also decreased by co-transfection of siFOXO3a and MST1 over-expression (Fig. 3H). Overall our results indicate that MST1 positively regulated SIRT6 expression via FOXO3a.

3.4 MST1 inhibited cell viability and induced cell apoptosis of glioma cells through regulating SIRT6 expression

Based on the results, it raises the possibility that SIRT6 may be involved in the effect of MST1 on glioma progression. We thus used SIRT6 specific siRNAs (siSIRT6-1# and siSIRT6-2#) to knock down SIRT6 expression, as shown in Fig. 4A. Of which, siSIRT6-1# demonstrated 70% reduction and siSIRT6-2# demonstrated 50% reduction of SIRT6 mRNA expression as compared to the control (Fig. 4A), and siSIRT6-1# was selected for the following experiments. Firstly, MTT assay showed that the cell viability inhibited by MST1 was promoted by addition of siSIRT6 (Fig. 4B). Secondly, the decreased expression level Bcl-2 or the increased expression levels of Bax, cleaved-caspase 3 and cleaved PARP by MST1 over-expression were increased or decreased by addition of siSIRT6, respectively (Fig. 4C). The colony formation assay showed that the inhibitory activity of MST1 on colony formation of glioma cells was induced by addition of siSIRT6 (Fig. 4D). These results demonstrated that the regulatory effects of MST1 on glioma cell viability and colony formation was at least partially through suppression of SIRT6. As shown in Figure 5, MST1 could promote the phosphorylation of FOXO3a, and destruct the binding of FOXO3a to 14-3-3 protein in the cytoplasm, thus dissociating FOXO3a and promoting translocation into nucleus. Moreover, FOXO3a binds with promoter of SIRT6, and positively regulates the expression of SIRT6, thus leading to the inhibition of cell proliferation and promotion of cell apoptosis.

4. Discussion

It has been well established that the development of glioma is closely related to abnormal cell proliferation (Tate and Aghi, 2009). Many proteins or other molecules related to the inhibition of glioma cell proliferation have been identified in previous studies (Yang et al., 2014; Wang et al., 2014). For example, transmembrane protein 140 (TMEM140) (Li et al., 2015) or centrosomal protein of 55 (CEP55) (Wang et al., 2016) have been proved to promote viability of glioma cells, and silencing of TMEM140 or CEP55 could suppress the viability, migration, and invasion of glioma cells. Moreover, over-expression of miR-136 played a tumor-suppressive role in inducting glioma cell apoptosis (Yang et al., 2012). Our current study investigated the effects of MST1 on glioma cell viability and apoptosis and revealed the underlying molecular mechanisms.

Here, we found that MST1 was down-regulated in human glioma tissues. Previous studies, in turn, showed that MST1 was either highly or lowly expressed in different glioma tissues (Chao et al., 2015). This may be due to the different cell types, grades and locations of tumor tissues. Moreover, MST1 was also down-regulated in different glioma cell lines. It seems that MST1 was differently expressed in various grades of cell lines, since U-251 MG with glioblastoma grade IV demonstrated the lowest expression of MST1, and A-172 with glioblastoma grade II demonstrated the highest expression of MST1. Previous study has shown that TGF-β promotes glioma malignancy through repression of MST1 (Guo et al., 2017), and NDRG2 (N-myc downstream regulated gene 2) gene methylation was shown to be glioma malignancy-dependent (Skiriute et al., 2014). We speculate that the expression of MST1 was also dependent on glioma malignancy, for the higher grade of glioma cells, the lower expression of MST1.

Our study also revealed that MST1 over-expression inhibited viability and colony formation of U-251 MG cells, in line with the previous study, demonstrating the anti-proliferation effect of MST1 on glioma progression (Guo et al., 2017). Moreover, MST1 over-expression also promoted apoptosis by decreasing Bcl-2 expression and increasing the pro-apoptotic proteins Bax, cleaved caspase-3 and cleaved PARP. Generally, cell apoptosis is well-known modulated by apoptotic and anti-apoptotic proteins , Bcl-2 superfamily (Faiao-Flores et al., 2013). The relative amounts of active anti-apoptotic protein (Bcl-2 (Lazou et al., 2006)) and pro-apoptotic protein (Bax (Mayer and Oberbauer, 2003)) are involved in the regulation of cell apoptosis (Rybnikova et al., 2006). Consistently, previous study results have shown that hMOB3 (Human Mps One Binder 3) inhibited MST1 cleavage-based apoptosis process to promote tumor growth in glioblastoma multiform (Tang et al., 2014).

Although recent study showed that through regulation of AKT/mTOR signaling pathway, MST1 promoted cell proliferation and growth, while inhibited the cell apoptosis of glioma (Chao et al., 2015), the direct downstream target of MSTI in glioma cells remains unclear. In the present study, we identified a new potential target, for MST1 in glioma cells: SIRT6. We found that over-expression of MST1 could increase the luciferase activity of the SIRT6 promoter, suggesting the relationship between MST1 and SIRT6. Moreover, functional assays showed that the inhibitive ability of MST1 on glioma cell viability, and the enhancing ability of MST1 on glioma cell apoptosis, was reversed by SIRT6 knockdown. Finally, our results indicated that MST1 suppresses viability and promotes apoptosis of glioma cells via upregulating SIRT6 expression.

Consistently, previous studies have shown that MST1 could promote the phosphorylation of FOXO3a, and destruct the binding of FOXO3a to 14-3-3 protein in the cytoplasm, thus dissociating

FOXO3a and promoting translocation into nucleus (Lehtinen et al., 2006; Yu et al., 2013). Moreover, FOXO3a binds with a promoter of SIRT6, and positively regulates the expression of SIRT6 (Kim et al., 2010). This is the first evidence demonstrating the regulatory role of MST1 on glioma cell viability and apoptosis, and showing the relationship between MST1, FOXO3a and SIRT6. However, due to the complicated pathogenesis of glioma malignancy, molecular mechanisms of MST1 on the progression of glioma need to be further investigated.

In summary, the present study demonstrated that overexpression of MST suppressed glioma cell viability and promoted cell apoptosis through positively regulating SIRT6 via FOXO3a, thus acted as a tumor suppressor in glioma. This finding illuminated the relationship between MST1/ SIRT6 regulatory axis and indicates that MST1 is a potential biomarker for the prognosis and diagnosis of glioma, and provides new light on the molecular mechanism of glioma progression and development.

Abbreviations

MST1: mammalian sterile 20-like 1; SIRT6: Sirtuin 6; FOXO3a: Forkhead box O3a; PCBP2: Poly(rC)-binding protein 2.

Ethics approval and consent to participate

The animal use protocol has been approved by Zhejiang Cancer Hospital Approval No. IACUC DB-16-029.

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Conflict of interest

The authors declare no competing interests.

Authors' Contributions

DPZ designed the research study. CS and BW performed the research. XQ provided help and advice on the ELISA experiments.

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References

Bohman, L. E., Gallardo, J., Hankinson, T. C., Waziri, A. E., Mandigo, C. E., McKhann, G. M., Sisti, M. B., Canoll, P. and Bruce, J. N. (2009) The survival impact of postoperative infection in patients with glioblastoma multiforme. *Neurosurgery* **64**, 828-834.

Chang, N., Ahn, S. H., Kong, D. S., Lee, H. W. and Nam, D. H. (2017) The role of STAT3 in glioblastoma progression through dual influences on tumor cells and the immune microenvironment. *Molecular and Cellular Endocrinology* **451**, 53-65.

Chao, Y., Wang, Y., Liu, X., Ma, P., Shi, Y., Gao, J., Shi, Q., Hu, J., Yu, R. and Zhou, X. (2015) Mst1 regulates glioma cell proliferation via the AKT/mTOR signaling pathway. *Journal of Neuro-oncology* **121**, 279-288.

Chen, X., Hao, B., Liu, Y., Dai, D., Han, G., Li, Y., Wu, X., Zhou, X., Yue, Z., Wang, L., Cao, Y. and Liu, J. (2014) The histone deacetylase SIRT6 suppresses the expression of the RNA-binding protein PCBP2 in glioma. *Biochemical and Biophysical Research Communications* **446**, 364-369.

Dong, Y., Du, X., Ye, J., Han, M., Xu, T., Zhuang, Y. and Tao, W. (2009) A cell-intrinsic role for Mst1 in regulating thymocyte egress. *Journal of Immunology* **183**, 3865-3872.

Elhanati, S., Ben-Hamo, R., Kanfi, Y., Varvak, A., Glazz, R., Lerrer, B., Efroni, S. and Cohen, H. Y. (2016) Reciprocal regulation between SIRT6 and miR-122 controls liver metabolism and predicts hepatocarcinoma prognosis. *Cell Reports* **14**, 234-242.

Faiao-Flores, F., Coelho, P. R., Toledo Arruda-Neto, J. D., Maria-Engler, S. S., Tiago, M., Capelozzi, V. L., Giorgi, R. R. and Maria, D. A. (2013) Apoptosis through Bcl-2/Bax and cleaved caspase up-regulation in melanoma treated by boron neutron capture therapy. *PloS One* **8**, e59639.

Feng, J., Yan, P. F., Zhao, H. Y., Zhang, F. C., Zhao, W. H. and Feng, M. (2016) SIRT6 suppresses glioma cell growth via induction of apoptosis, inhibition of oxidative stress and suppression of JAK2/STAT3 signaling pathway activation. *Oncology Reports* **35**, 1395-1402.

Frye, R. A. (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochemical and Biophysical Research Communications* **273**, 793-798.

Goodenberger, M. L. and Jenkins, R. B. (2012) Genetics of adult glioma. *Cancer Genetics* **205**, 613-621.

Grossman, S. A., Ye, X., Piantadosi, S., Desideri, S., Nabors, L. B., Rosenfeld, M., Fisher, J. and Consortium, N. C. (2010) Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. *Clinical Cancer Research* **16**, 2443-2449.

Grusche, F. A., Richardson, H. E. and Harvey, K. F. (2010) Upstream regulation of the hippo size control pathway. *Current Biology* **20**, R574-R582.

Guo, Z., Li, G., Bian, E., Ma, C. C., Wan, J. and Zhao, B. (2017) TGF-beta-mediated repression of MST1 by DNMT1 promotes glioma malignancy. *Biomedicine & Pharmacotherapy* **94**, 774-780.

Huang, J., Wu, S., Barrera, J., Matthews, K. and Pan, D. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie. *The Drosophila Homolog of YAP Cell* **122**, 421-434.

Huse, J. T., Phillips, H. S. and Brennan, C. W. (2011) Molecular subclassification of diffuse glioma: seeing order in the chaos. *Glia* **59**, 1190-1199.

Jia, J., Zhang, W., Wang, B., Trinko, R. and Jiang, J. (2003) The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes & Development* **17**, 2514-2519.

Kim, H. S., Xiao, C., Wang, R. H., Lahusen, T., Xu, X., Vassilopoulos, A., Vazquez-Ortiz, G., Jeong, W. I., Park, O., Ki, S. H., Gao, B. and Deng, C. X. (2010) Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metabolism* **12**, 224-236.

Li, B., Huang, M. Z., Wang, X. Q., Tao, B. B., Zhong, J., Wang, X. H., Zhang, W. C. and Li, S. T. (2015) TMEM140 is associated with the prognosis of glioma by promoting cell viability and invasion. *Journal of Hematology & Oncology* **8**, 1-11.

Lazou, A., Iliodromitis, E. K., Cieslak, D., Voskarides, K., Mousikos, S., Bofilis, E. and Kremastinos, D. T. (2006) Ischemic but not mechanical preconditioning attenuates ischemia/reperfusion induced myocardial apoptosis in anaesthetized rabbits: the role of Bcl-2 family proteins and ERK1/2. *Apoptosis* **11**, 2195-2204.

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villen, J., Becker, E. B., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T. K. and Bonni, A. (2006) A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* **125**, 987-1001.

Mayer, B. and Oberbauer, R. (2003) Mitochondrial regulation of apoptosis. *News in Physiological Sciences* **18**, 89-94.

Oh, S., Lee, D., Kim, T., Kim, T. S., Oh, H. J., Hwang, C. Y., Kong, Y. Y., Kwon, K. S. and Lim, D. S. (2009) Crucial role for Mst1 and Mst2 kinases in early embryonic development of the mouse. *Molecular and Cellular Biology* **29**, 6309-6320.

Rawat, S. J., Creasy, C. L., Peterson, J. R. and Chernoff, J. (2013) The tumor suppressor Mst1 promotes changes in the cellular redox state by phosphorylation and inactivation of peroxiredoxin-1 protein. *The Journal of Biological Chemistry* **288**, 8762-8771.

Rybnikova, E., Sitnik, N., Gluschenko, T., Tjulkova, E. and Samoilov, M. O. (2006) The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobaric hypoxia in rats. *Brain Research* **1089**, 195-202.

Sebastian, C., Zwaans, B. M., Silberman, D. M., Gymrek, M., Goren, A., Zhong, L., Ram, O., Truelove, J., Guimaraes, A. R., Toiber, D., Cosentino, C., Greenson, J. K., MacDonald, A. I., McGlynn, L., Maxwell, F., Edwards, J., Giacosa, S., Guccione, E., Weissleder, R., Bernstein, B. E., Regev, A., Shiels, P. G., Lombard, D. B. and Mostoslavsky, R. (2012) The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. *Cell* **151**, 1185-1199.

Skiriute, D., Steponaitis, G., Vaitkiene, P., Mikuciunas, M., Skauminas, K., Tamasauskas, A. and Kazlauskas, A. (2014) Glioma malignancy-dependent NDRG2 gene methylation and downregulation correlates with poor patient outcome. *Journal of Cancer* **5**, 446-456.

Sun, J., Guo, M., Pang, H., Qi, J., Zhang, J. and Ge, Y. (2013) Treatment of malignant glioma using hyperthermia. *Neural Regeneration Research* **8**, 2775-2782.

Tang, F., Zhang, L., Xue, G., Hynx, D., Wang, Y., Cron, P. D., Hundsrucker, C., Hergovich, A., Frank, S., Hemmings, B. A. and Schmitz-Rohmer, D. (2014) hMOB3 modulates MST1 apoptotic signaling and supports tumor growth in glioblastoma multiforme. *Cancer Research* **74**, 3779-3789.

Tate, M. C. and Aghi, M. K. (2009) Biology of angiogenesis and invasion in glioma. *Neurotherapeutics: the Journal of the American Society for Experimental NeuroTherapeutics* **6**, 447-457.

Wang, G., Liu, M., Wang, H., Yu, S., Jiang, Z., Sun, J., Han, K., Shen, J., Zhu, M., Lin, Z., Jiang, C. and Guo, M. (2016) Centrosomal protein of 55 regulates glucose metabolism, proliferation and apoptosis of glioma cells via the Akt/mTOR signaling pathway. *Journal of Cancer* **7**, 1431-1440.

Wang, J. B., Dong, D. F., Gao, K. and Wang, M. D. (2014) Mechanisms underlying the biological changes induced by isocitrate dehydrogenase-1 mutation in glioma cells. *Oncology Letters* **7**, 651-657.

Wu, W., Zhong, D., Zhao, Z., Wang, W., Li, J. and Zhang, W. (2017) Postoperative extracranial metastasis from glioblastoma: a case report and review of the literature. *World Journal of Surgical Oncology* **15**, 1-5.

Xu, C. M., Liu, W. W., Liu, C. J., Wen, C., Lu, H. F. and Wan, F. S. (2013) Mst1 overexpression inhibited the growth of human non-small cell lung cancer in vitro and in vivo. *Cancer Gene Therapy* **20**, 453-460.

Yang, J. T., Li, Z. L., Wu, J. Y., Lu, F. J. and Chen, C. H. (2014) An oxidative stress mechanism of shikonin in human glioma cells. *PloS One* **9**, e94180.

Yang, Y., Wu, J., Guan, H., Cai, J., Fang, L., Li, J. and Li, M. (2012) MiR-136 promotes apoptosis of glioma cells by targeting AEG-1 and Bcl-2. *FEBS Letters* **586**, 3608-3612.

Yu, T., Ji, J. and Guo, Y. L. (2013) MST1 activation by curcumin mediates JNK activation, Foxo3a nuclear translocation and apoptosis in melanoma cells. *Biochemical and Biophysical Research Communications* **441**, 53-58.

Yuan, F., Xie, Q., Wu, J., Bai, Y., Mao, B., Dong, Y., Bi, W., Ji, G., Tao, W., Wang, Y., Yuan, Z. (2011) MST1 promotes apoptosis through regulating Sirt1-dependent p53 deacetylation. *The Journal of Biological Chemistry* **286**, 6940-6945.

Zach, L., Stall, B., Ning, H., Ondos, J., Arora, B., Uma, S., Miller, R. W., Citrin, D. and Camphausen, K. (2009) A dosimetric comparison of four treatment planning methods for high grade glioma. *Radiation Oncology* **4**, 1-7.

Zhang, G., Liu, Z., Qin, S. and Li, K. (2015) Decreased expression of SIRT6 promotes tumor cell growth correlates closely with poor prognosis of ovarian cancer. *European Journal of Gynaecological Oncology* **36**, 629-632.