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Original article

TGF-β-mediated repression of MST1 by DNMT1 promotes glioma malignancy

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ABSTRACT

Human gliomas are related to high rates of morbidity and mortality. TGF-β promotes the growth of glioma cells, and correlate with the degree of malignancy of human gliomas. However, the molecular mechanisms involved in the malignant function of TGF-β are not fully elucidated. Here, we showed that TGF-β induced the downregulation of MST1 expression in U87 and U251 glioma cells. Treatment of glioma cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-AzadC) prevented the loss of MST1 expression. Addition of 5-AzadC also reduced the TGF-β-stimulated proliferation, migration and invasiveness of glioma cells. Furthermore, Knockdown of DNMT1 upregulated MST1 expression in gliomas cells. In addition, the inhibition of DNMT1 blocked TGF-β-induced proliferation, migration and invasiveness in glioma cells. These results suggest that TGF-β promotes glioma malignancy through DNMT1-mediated loss of MST1 expression.

1. Introduction

Gliomas are the most common primary malignant intracranial tumors in adults. The World Health Organization (WHO) classified glioma as grades I–IV on the basis of histological features [1]. Glioblastoma multiforme (WHO grade IV) is the most aggressive malignant type of glioma, despite recent advances in surgery, adjuvant radio chemotherapies for glioma, the prognosis for patients with glioblastoma multiforme is still grim, the median survival of patients with glioblastoma is about 14.6 months after standard treatment [2]. Therefore, searching novel and effective therapeutic targets is an urgent need for the treatment of glioma.

MST1 (Mammalian Sterile 20 Like Kinase 1) is forms part of the 'Hippo' signaling pathway and were cloned in 1995 by Chernoff's group [3]. The catalytic domain of MST1 is situated in the N-terminal region while a non-catalytic tail at the C-terminal region contains two caspase cleavage sites, two nuclear export sites (NES) and dimerisation domains [4,5]. MST1 plays a key role in regulating gene expression, cell proliferation and apoptosis, organ size, and tumorigenesis [6,7]. Additionally, loss of cytoplasmic MST1 expression has been reported as a marker of tumor progression, suggesting a tumor suppressor role for MST1 in human colorectal cancer [8]. Kim et al. found that MST1 expression was significantly reduced in various lymphoma/leukemia patient samples, which implied that the loss of MST1 could promote tumorigenesis in hematopoietic lineages [9]. Recently, loss of MST1 expression due to DNA methylation was reported in soft tissue sarcoma [10].

DNA methylation mediated by the actions of DNA methyltransferases (DNMTs) is a fundamental epigenetic mark that plays a prominent role in gene expression regulation and control of differentiation [11]. In mammals, DNA methylation is sustained by DNMTs via the transfer of a methyl group to the 5-carbon in the cytosine of a CpG dinucleotide. Accumulating evidence has demonstrated that DNA hypermethylation of promoter regions leads to tumor suppressor genes silencing [12,13]. DNA methyltransferase 1(DNMT1), located on chromosome 19p13.2, is the

Abbreviations: WHO, World Health Organization; MST1, Mammalian Sterile 20 Like Kinase 1; NES, nuclear export sites; DNMTs, DNA methyltransferases.

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main methyltransferase enzyme responsible for maintaining the methylation pattern, overexpression of DNMT1 has been found in various types of malignant tumors, including glioma [14,15]. TGF-β plays an important role in cancer development and cell migration. Previous studies demonstrated that TGF-β was highly active in high-grade glioma and has prognostic values in glioma [16]. TGF-β has also been shown to increase expression of DNMT1 and global DNA methylation in lung fibroblasts [17]. The actions of TGF-β on DNA methylation machinery are context specific, and the mechanisms by which TGF-β affects expression of MST1 in glioma are not yet known.

In this paper, we demonstrate that TGF-β induced the reduction of MST1 in glioma cells; however the inhibition of DNMT1 by 5-AzadC or knockdown reversed the reduction of MST1 by TGF-β stimulation. In addition, the inhibition of DNMT1 by 5-AzadC or knockdown inhibits glioma cell proliferation, migration and invasion mediated by TGF-β stimulation. Our data uncover the new mechanism of epigenetic repression MST1 involved in TGF-β-mediated glioma malignancy.

2. Materials and methods

2.1. Cell culture

Human glioma cell lines (U87 and U251) were obtained from Chinese Academy of Medical Sciences (Shanghai, China). U87 and U251 glioma cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HYCLONE, USA). All cells were supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotics (100 U/ml penicillin and 100 mg/l streptomycin), and incubated in a humidified 5% CO_2 atmosphere at 37 °C.

2.2. TGF-β and 5-aza-2′-deoxycytidine treatment

U87 and U251 glioma cells were placed overnight in culture dishes and serum-starved with 0.5% FCS for 24 h before adding 5 ng/ml TGF-β (Peprotech, USA). 5 μM 5-AzadC (Sigma-Aldrich, St. Louis, MO) was added and refreshed every 24 h for 48 h [18].The medium containing PBS only was regarded as a control.

2.3. Construction of DNMT1 shRNA lentivirus vector and cell infection

The following oligonucleotide was synthesized. The negative control shRNA was 5′-UUCUCCGAACGUGUCACGUTT-3′. DNMT1 shRNA was 5′- GGGACUGUGUCUCUGUUAUTT-3′. The stem-loop-stem oligos (short-hairpin RNAs, shRNAs) were synthesized, annealed, and ligated into the Nhe I/Pac I-linearized pFH1UGW vector. The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing. The generated plasmids were named as pFH1UGW-shDNMT1 or -shCon. Recombinant lentiviral vectors and packaging vectors were then transfected into 293T cells. Supernatants containing lentivirus expressing DNMT1 shRNA or control shRNA were harvested 48 h after transfection. Then, the lentiviruses were purified using ultracentrifugation, and the titer of lentiviruses was determined. U87 and U251 cells were infected with the lentivirus constructs at multiplicity of infection (MOI) = 10 and mock-infected cells were used as negative controls.

2.4. Cell proliferation assay

Cell Counting Kit-8 (CCK8, sigma, USA) was used to determine the cell's proliferation ability. U87 and U251 glioma cells were seeded into 96-well plates at the density of 3000 cells/well. Roughly 20 μl of CCK8 regent was added to each well, and incubated at 37 °C for 2 h. Absorbance was measured at a wavelength of 490 nm by using a ST-360 micro-plate reader (KHB, Shanghai, China). Three replicate wells were set up in each group.

2.5. Real-time PCR analysis

Total RNA was extracted from glioma cells using TRIzol reagent (Invitrogen). Real-time quantitative PCR analysis was performed using SYBR Green Master Mix Kit on Thermo Fisher connect Real-Time PCR platform. In brief, each PCR reaction mixture containing 10 μl of 2 × SYBR Green Master Mix, 1 μl of sense and antisense primers (5 μmol/μl) and 1 μl of cDNA (10 ng) was run for 40 cycles with 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 volume of 20 μl. For relative quantification, $2^{-\ddot{A}\ddot{A}CT}$ was calculated and used as an indication of the relative expression levels, which were calculated by subtracting CT values of the control gene from the CT values of MST1 and DNMT1. Real time-PCR was carried out under a standard protocol using the following primers: DNMT1 (forward: 5′-CGGCTTCAG-CACCTCATTTG-3′; reverse: 5′-AGGTCGAGTCGG

AATTGCTC-3′), MST1 (forward: 5′-ACAAATCCTCCTCCCA-CATTCCG-3′;

reverse: 5′-CACTCCTGACAAATGGGTGCTG-3′). GAPDH was applied as an internal control. The primer sequences of GAPDH were 5′-AGCAAGAGCACAAGAGGAAG-3′ and 5′-GGTTGAGCACAGGG-TACTTT-3′.

2.6. Western blotting

U251 and U87 glioma cells were lysed with RIPA lysis buffer (Beyotime, China). Whole extracts were prepared, and protein concentrations were determined using the BCA protein assay kit (Boster, China). Whole-cell extracts (30 μg) were then fractionated by electrophoresis through an 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at a 120 V for 2 h before transfer onto a PVDF membrane (MilliporeCorp, Billerica, MA, U.S.A.). After blocking against non-specific protein binding, nitrocellulose blots were incubated for 1 h with primary antibodies diluted in TBS/Tween20 (0.075% Tween20) containing 3% Marvel. Anti-DNMT1 and anti-MST1 were diluted 1:400. Following incubation with the primary antibody, blots were washed three times in TBS/Tween-20 before incubation for 1 h with mouse anti-rabbit horseradish peroxidase conjugated antibody at a 1:10,000 dilution in TBS/Tween-20 containing 5% milk. After extensive washing in TBS/Tween-20, the blots were rinsed with distilled water and proteins were detected using the enhanced chemiluminescence system. Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).

2.7. In vitro migration and invasion assays

U251 and U87 glioma cells (1×10^5) were placed on the top side of polycarbonate Transwell filters (without Matrigel for Transwell assay) or plated on the top side of polycarbonate Transwell filter coated with Matrigel (for Transwell matrix penetration assay) in the upper chamber of the QCM™ 24-Well Cell Invasion Assay (Cell Biolabs, INC, USA). For migration assays, cells were suspended in medium without serum, and medium without serum was used in the lower chamber. For the invasion assay, cells were suspended in medium without serum, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated at 37 °C for 24 h (migration assay) or (invasion assay). The non-migratory or non-invasive cells in the top chambers were removed with cotton swabs. The