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FoxM1 regulates Sirt1 expression in glioma cells

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Abstract. – BACKGROUND AND OBJECTIVES: Glioma accounts for most of primary malignant brain tumors and usually results in poor survival. However, the key signaling networks regulating glioma cell proliferation remain poorly defined. The forkhead box M1 (FoxM1) is a key transcription factor regulating multiple aspects of cell biology. Prior studies have shown that FoxM1 is overexpressed in glioma and plays a critical role in cancer development and progression.

MATERIALS AND METHODS: Western blot and Real-time PCR assays were used to determine the regulation roles of FoxM1 on Sirt1 (Sirtuin1) expression. Small interfering RNAs (siRNAs) were used to silence the expression of FoxM1. Luciferase assays were used to measure binding of FoxM1 to the promoter region of Sirt1. Direct binding of FoxM1 to promoter of Sirt1 was assessed by chromatin immunoprecipitation (CHIP) assays.

RESULTS: We found that FoxM1 positively regulated mRNA expression of Sirt1. FoxM1 overexpression promoted while its knockdown inhibited Sirt1 expression. Besides, we identified a minimal FoxM1 binding site on the promoter region of Sirt1.

CONCLUSIONS: Our results for the first time add a new FoxM1-Sirt1 connection that mediates cell proliferation in glioma.

Key Words:
Glioma, FoxM1, Sirt1, mRNA transcription, Promoter.

Introduction

Glioma accounts for most of primary malignant brain tumors and usually results in poor survival[1]. Aberrant expression and (or) activity of several oncogenes or tumor suppressors plays key roles in the initiation and progression of glioma[2,3].

FoxM1, also named as HFH-11, MPP2, or Trident, belongs to the large family of forkhead transcription factors[4]. Through binding promoter regions for a consensus [TAAACA] sequence, FoxM1 controls the expression of genes required for cell-cycle transition, leading to cell proliferation and invasion[5,6]. In agreement, FoxM1 expression is up-regulated and correlate with poor prognosis and metastasis in several types of human cancers, including breast, gastric, prostate and colon cancers[7-8]. Moreover, FoxM1 is also essential for the proliferation, migration, and angiogenesis in glioma, at least in part, through regulation of Wnt/□-catenin signaling and Anxa1 expression[9,10]. Therefore, better understanding the roles of FoxM1 may help to provide a new therapeutic strategy against glioma[11].

Sirtuin 1 (Sirt1), a NAD+-dependent histone deacetylase, is implicated in multiple biologic processes, through modifying many transcription factors, such as P53, NF-kB/P65, FoxOs[12]. Recent studies suggest that Sirt1 is overexpressed and (or) catalytically activated in tumor cells, to block apoptosis and enhance cell proliferation[13]. Therefore, in the present study, we speculate that whether Sirt1 expression is regulated by FoxM1 in two glioma cell lines.

Materials and Methods

Cell Culture

Glioma cells (SHG-44 and U251) were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum: FBS (Gibco, Shanghai, China).

RNA Extraction and Real-time

Total RNAs were isolated by TRIzol reagent (Invitrogen, Shanghai, China), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, China) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was

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performed using a SYBR Green Premix Ex Taq (Takara, Tokyo, Japan) on Light Cycler 480 (Roche, Basel, Switzerland). Expression of b-actin was determined as an internal control.

Western Blot

Cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 10000× g for 15 min at 4°C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies, followed by HRP-linked secondary antibodies (Cell Signaling, Irvine, CA, USA). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions. Anti-FoxM1, anti-Sirt1, anti-P53 antibodies were purchased from Abcam Company (Cambridge, MA, USA). Protein levels were normalized to GAPDH (Santa Cruz, Santa Cruz, CA, USA).

siRNA and Cell Transfection

Small interfering RNA oligos (siRNA) targeting FoxM1 or GFP were obtained from Dharmacon Company (Lafayette, CO, USA). GFP siRNA was employed as a negative control. Cells were seeded on to 6-well plates then transfected with 50 nM siRNA oligos. All the transient transfections were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols.

Luciferase Reporter, Chromatin Immunoprecipitation Assays

Sirt1 promoter was amplified from the human genomic DNA and inserted into pGL3 vector (Promega, Madison, WI, USA). For the luciferase reporter assays, SHG-44 cells were seeded in 24-well plates and transfected with FoxM1 expression plasmids or empty vector (pCDNA-3.1), using Lipofectamine 2000. Cell lysates were harvested 48 hr after transfection. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, USA). Chromatin immunoprecipitation (ChIP) assays were performed following the manufacturer's instructions using kits from Upstate Biotechnology (Millipore, Billerica, MA, USA). In short, SHG-44 cells in 10cm dishes were fixed with 1% formaldehyde. After lysis, genomic DNA was sheared to fragments at 200-1000 bp using sonications. Chromatin was incubated and precipitated with antibodies against 2 μg FoxM1 antibody (Abcam) or IgG (Abcam) overnight at 4-6°C. The results were determined by quantitative real-time PCR.

Statistical Analysis

Data are expressed as the mean±SEM from at least three separate experiments and analyzed by Student's t-test. Statistical significance is displayed as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

Results

Up-regulation of Sirt1 by FoxM1 Overexpression in Glioma Cells

To determine the relationship between FoxM1 and Sirt1, SHG-44 and U251 cells were transfected with expression plasmids containing FoxM1 or empty vector (EV). As shown in Figure 1A and B, FoxM1 overexpression significantly increased mRNA and protein levels of Sirt1 in SHG-44 cells. Besides, similar results were also observed in U251 cells (Figure 1C-D).

FoxM1 Inhibition by siRNA Oligos Reduced Sirt1 Expression

Then, FoxM1 expression was silenced by small interfering RNA (siRNA) oligos. As a result, knockdown of FoxM1 greatly reduced its endogenous expression, as well as Sirt1 mRNA and protein levels in SHG-44 and U251 cells (Figure 2A-2D). Together, our findings indicate that FoxM1 is an up-stream regulator of Sirt1 in glioma cells.

The Human Sirt1 Gene Promoter is a Transcriptional Target of FoxM1

Next, we speculate that whether FoxM1 could regulate Sirt1 at the transcriptional level. Expression plasmids containing full-length or deletion with DNA binding domain of FoxM1 were transfected into SHG-44 cells[14]. As shown in Figure 3A-3B, FoxM1 deleted with DNA binding domain largely abolished the induction of Sirt1, suggesting that FoxM1 regulates Sirt1 expression through its binding to the promoter region. Therefore, we examined the activities of reporters driven by promoter region in human Sirt1 gene in SHG-44 cells (Figure 3C). Consistent with the regulation of the Sirt1 mRNA and pro-

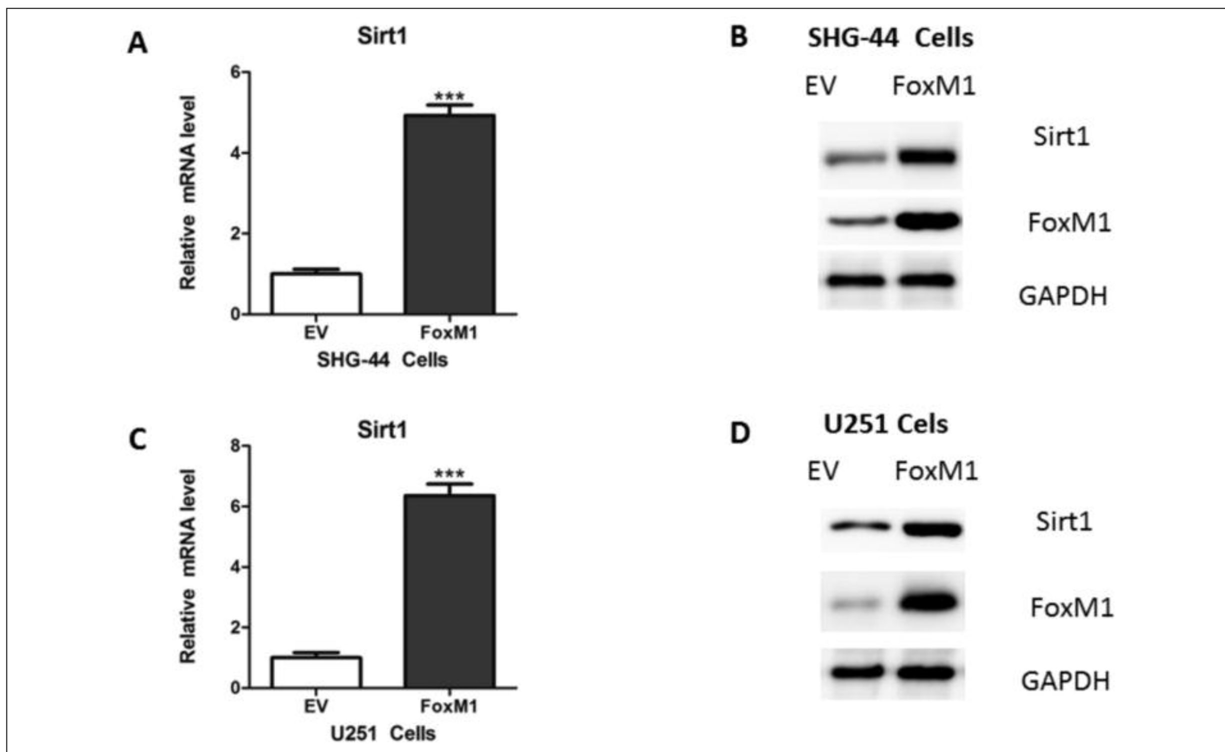

Figure 1. Up-regulation of Sirt1 by FoxM1 overexpression. ***A-D,*** mRNA and protein levels of Sirt1 were analyzed by Real-time PCR and western blot in SHG-44 ***(A, B)*** and U251 ***(C, D)*** cells transfected with expression plasmids containing empty vector (EV) or FoxM1. ***$p < 0.001$.

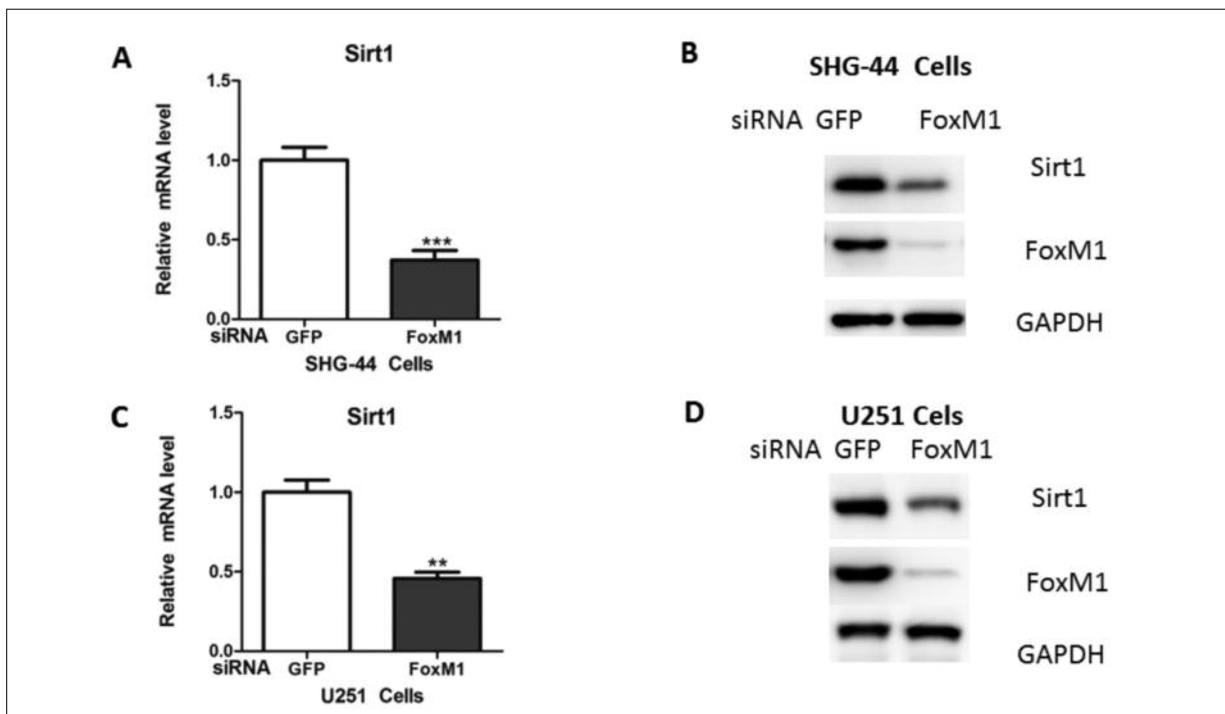

Figure 2. Down-regulation of Sirt1 by knockdown of FoxM1. ***A-D,*** mRNA and protein levels of Sirt1 were analyzed by Real-time PCR and western blot in SHG-44 ***(A, B)*** and U251 ***(C, D)*** cells transfected with siRNA oligos targeting FoxM1 or GFP. **$p < 0.01$, ***$p < 0.001$.

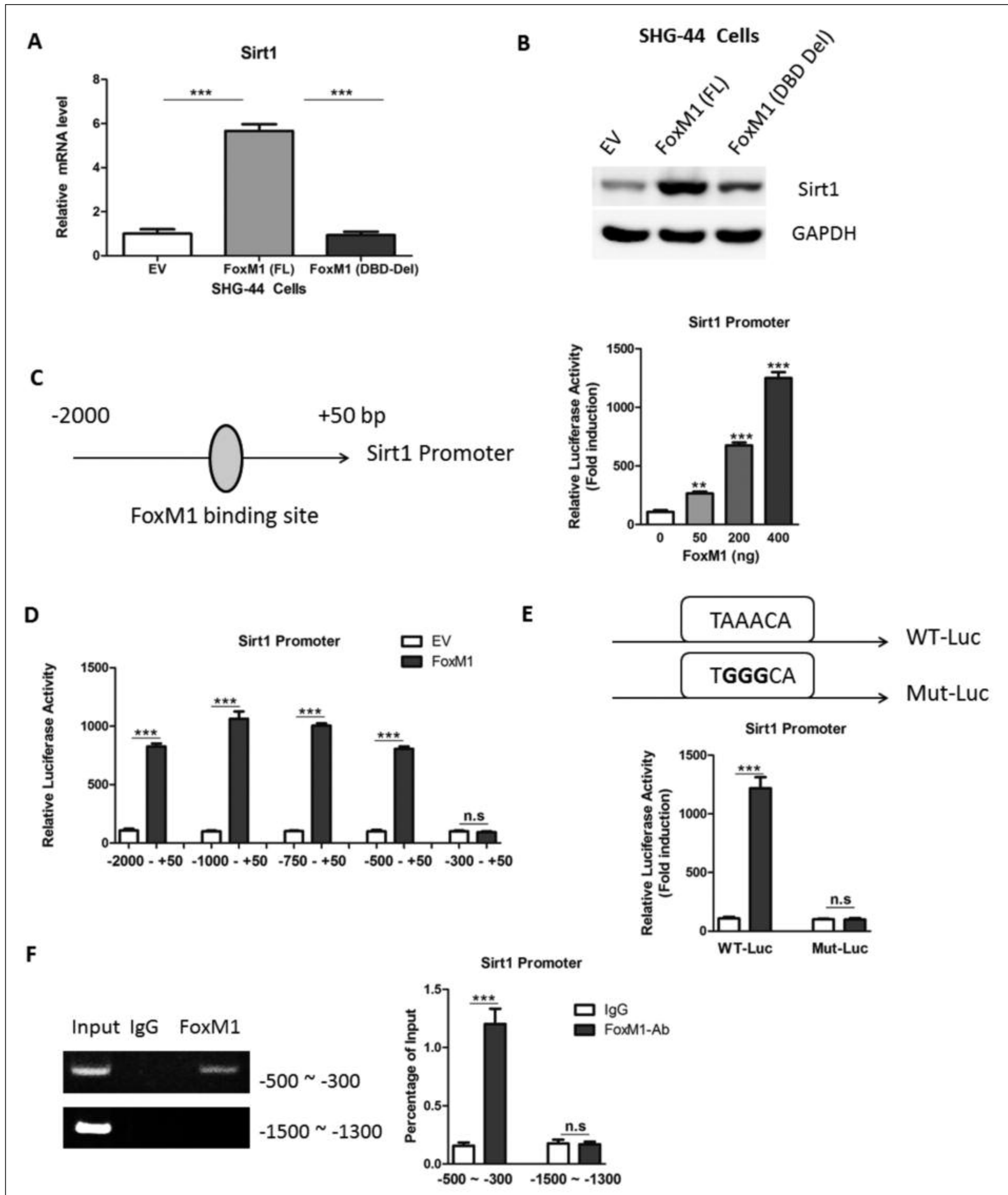

Figure 3. The human Sirt1 gene promoter is transcriptional targets of FoxM1. ***A-B,*** mRNA and protein levels of Sirt1 were analyzed by Real-time PCR ***(A)*** and western blot ***(B)*** in SHG-44 cells transfected with expression plasmids containing empty vector (EV), FoxM1 with full-length (FL), or DNA binding domain deletion (DBD-Del). $^{***}p < 0.001$. ***C,*** Human Sirt1 promoter construction and analysis. The promoter region from -2000 to +50 bp was cloned and co-transfected with FoxM1 expression plasmids in SHG-44 cells. 48 hr after transfection, cells were harvested and luciferase was determined. The transcription start site was set as +1 bp. ***D,*** The Sirt1 promoter analysis by series of deletion. ***E,*** Activation of wild-type (WT) and mutant (Mut) Sirt1 promoter by FoxM1 overexpression. The point-mutation was shown in bold. ***F,*** The recruitment of FoxM1 onto Sirt1 promoter was shown by chromatin immunoprecipitation assays and quantified by real-time PCR. The promoter region from -1500 to -1300 bp was employed as a negative control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

tein expression, FoxM1 activated Sirt1 promoter activity in a dose dependent manner (Figure 3C). Through serials deletion of this promoter, we defined a FoxM1-responsive region (487 to 482 bp upstream of the transcriptional start site) that contained a consensus binding site for FoxM1 (5'-TAAACA-3') (Figure 3D). Besides, point-mutation of this site completely abolished the effect of FoxM1 on the Sirt1 transcriptional activity (Figure 3E). Furthermore, our chromatin immunoprecipitation (ChIP) assays also revealed that FoxM1 could bind to the promoter region of Sirt1 gene in SHG-44 cells (Figure 3F).

FoxM1 Regulates P53 Signaling in Glioma Cells

As mentioned before, Sirt11 has been suggested to inhibit p53-dependent functions through deacetylating the P53 protein[12]. Therefore, we sought to determine whether FoxM1 overexpression could affect P53 signaling. As shown in Figure 4A-4B, FoxM1 overexpression led to a reduction of P53 acetylation in SHG-44 and U251 cells, without changing total amount of P53 protein. Consistently, FoxM1 deficiency enhanced the acetylation status of P53 protein (Figure 4C-4D), suggesting that FoxM1 could regulate P53 signaling by altering its aceylation.

Discussion

In the current study, for the first time, we demonstrate that FoxM1 regulates Sirt1 expression in two human glioma cells. FoxM1 overexpression promotes while its deficiency reduces mRNA and protein levels of Sirt1. At the molecular level, we further identify a functional FoxM1 binding site in the promoter region of human Sirt1 gene. Therefore, our results indicate that FoxM1 could be a positive regulator of Sirt1 gene in glioma. In addition, it would be interesting to further investigate whether FoxM1 could regulate Sirt1 in other types of human cancer cells.

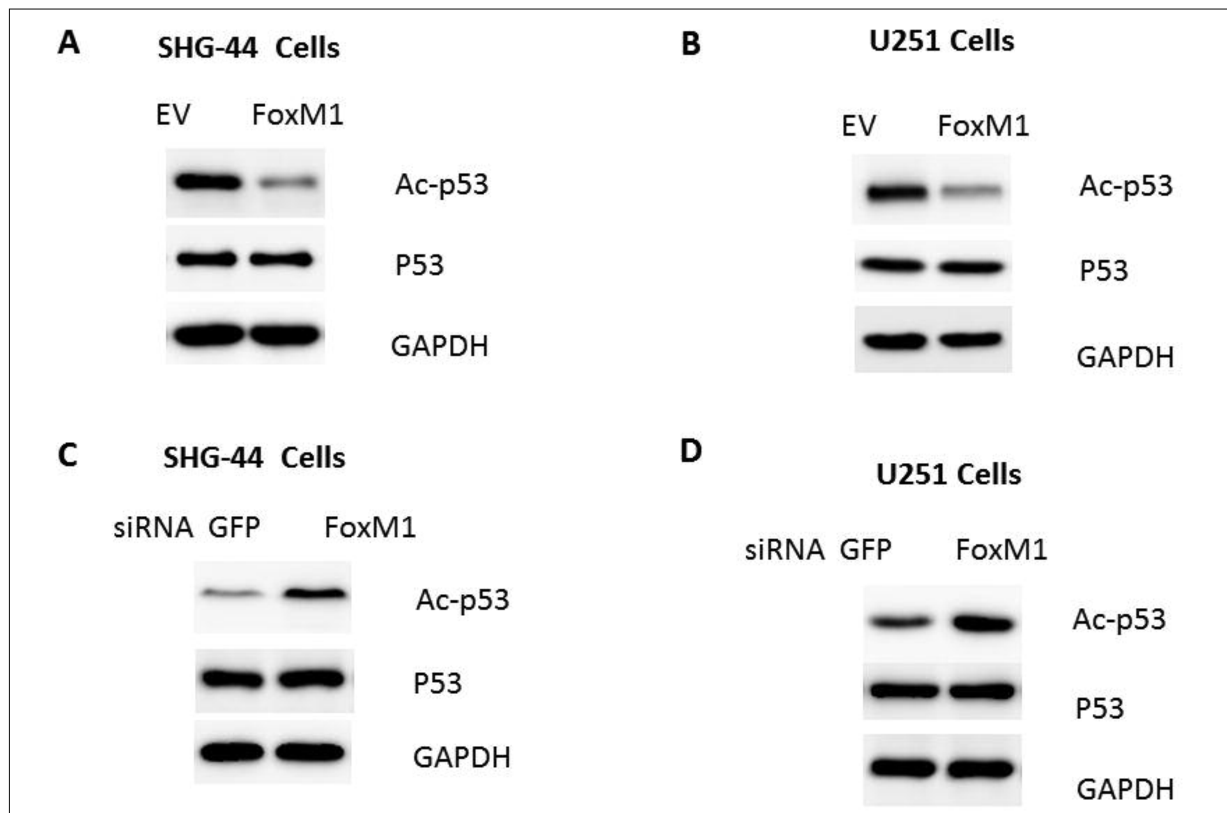

Figure 4. FoxM1 regulates P53 signaling in Glioma cells. *(A-B)* Protein levels of acetylated P53 were analyzed by western blot in SHG-44 *(A)* and U251 *(B)* cells transfected with expression plasmids containing empty vector (EV) or FoxM1. *(C-D)* Protein levels of acetylated P53 were analyzed by western blot in SHG-44 *(C)* and U251 *(D)* cells transfected with with siRNA oligos targeting FoxM1 or GFP.

Sirt1 is initially considered as a tumor suppressor, as evidenced its gene knockout mice[15]. Sirt1 null mice show impaired DNA damage response, genomic instability and tumorigenesis, while its activation Sirt1 protects against mutant BRCA1-induced breast cancer[15]. Besides, Sirt1 inhibits intestinal tumorigenesis in mouse model of colon cancer through regulation of Wnt/b-catenin signaling[16]. However, subsequent studies find that Sirt1 could promote proliferation of breast cancer and hepatocellular carcinoma, in which expression of Sirt1 is significantly higher in tumor tissues, compared with adjacent non-cancerous tissues[17,18]. Besides, recent studies suggest that Sirt1 could be a promoter factor on tumorigenesis of glioma through PTEN/PI3K/AKT signaling pathway[19]. Sirt1 may act as a G1-phase promoter and a survival factor in glioma cells[20]. Besides, knockdown of SirT1 expression enhances radiosensitivity and radiation-induced apoptosis in glioma CD133-positive cells[21]. Moreover, Sirt1 activity is also required for glioma stem cell proliferation[22]. Consistent with these reports, our data add a novel mechanism for the aberrant expression of Sirt1 in glioma.

In addition, we find that FoxM1 could regulate P53 signaling in glioma cells, as shown by its acetylation status. Interestingly, previous reports demonstrate that P53 could modulate FoxM1 transcription through an E2F-binding site located within the proximal promoter region in breast cancer cells[23]. Therefore, the FoxM1-P53 regulatory axis might form a feedback in cancer cells, although its roles and mechanisms are still needed to be further explored.

Conclusions

A new insight is provided into how FoxM1 could affect the Sirt1 expression. These findings might have implications for understanding the molecular machanisms and developing therapeutics for human glioma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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