LncRNA GAS5 Inhibits Temozolomide Chemoresistance to Glioma Via Inactivating Wnt/β-Catenin Pathway by Interacting with PTBP1

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Research Article

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Abstract

Purpose Temozolomide-based therapeutic resistance has become the crucial cause of chemotherapy failure in glioma treatment. Long non-coding RNA (lncRNA) growth arrest-specific 5 (GAS5) is reported to be downregulated in glioma and to inhibit tumor progression and metastasis. This study aimed to investigate function and potential regulatory mechanism of GAS5 in temozolomide (TMZ) chemoresistance to glioma.

Methods qRT-PCR, western blotting and immunofluorescence were used to measure the levels of GAS5 and proteins. RNA-binding protein immunoprecipitation assay was used to analyze the interaction between GAS5 and PTBP1. Flow cytometry apoptosis assay, CCK-8 assay, colony formation assay and nude mice xenograft experiments were used to detect the effects of GAS5 on TMZ resistance in glioma.

Results Downregulation of GAS5 might predict a poor prognosis in glioma patients. Overexpression of GAS5 improves the sensitivity to TMZ in glioma cells. Mechanistically, GAS5 could interact with polypyrimidine tract binding protein 1 (PTBP1) to downregulate its expression, thereby inactivating the Wnt/β-catenin pathway. Moreover, GAS5 could increase the anti-tumor effect of TMZ *in vivo*.

Conclusion This study indicated that GAS5 contributed to TMZ chemoresistance of glioma through interacting with PTBP1, and then inhibiting Wnt/β-catenin pathway, which provides a novel approach to develop promising therapeutic strategy.

Introduction

Glioma, characterized by early metastasis and high recurrence rate[1], is a devastating malignant intracranial tumor with a 5-year survival rate of approximately 5% and the survival time of about 50% patients is less than 14.6 months[2, 3]. The standard therapies for glioma include surgical resection, radiotherapy and chemotherapy[4]. Temozolomide (TMZ) is a first-line alkylating agent in glioma therapy with effective effect on inhibiting proliferation and facilitating apoptosis of glioma cells[5, 6]. However, the occurrence of intrinsic or acquired chemoresistance causes a limited response to TMZ and the exact mechanism of TMZ resistance to glioma still confuse us[7]. Therefore, it is critical to comprehensively understand the key regulators underlying glioma chemoresistance to TMZ, so as to develop effective therapeutic strategies for dealing with TMZ resistance according to novel intervention targets.

Long noncoding RNAs (lncRNAs), lacking protein coding potential, are families of endogenous cellular RNAs longer than 200 nucleotides in length, which play important molecular and cellular functions via regulating DNA expression and formatting complex with different cellular macromolecules including RNAs and proteins[8, 9]. It has been widely identified that lncRNAs could play a significantly regulative role in multiple human diseases[10, 11], including cancers [12]. Growth-arrest specific transcript 5 (GAS5), a spliced long non-coding RNA belongs to the 5’ terminal oligopyrimidine class, originally identified as a small nucleolar RNA which functions as a tumor suppressor in diversified cancers[13]. Commonly, researches have revealed that GAS5 could prevent carcinogenic progression via inducing cell apoptosis

and inhibiting cell proliferation[14]. Otherwise, exogenously increasing the level of GAS5 can reduce the occurrence of tumor chemo-resistance [18]. A research has demonstrated that GAS5 serves as a tumor suppressor to inhibit glioma proliferation, migration, and invasion via interacting with miR-106b-5p in glioma [19]. However, the role of GAS5 in glioma chemo-resistance to TMZ has rarely been investigated.

Polypyrimidine tract-binding protein 1 (PTBP1, also known as hnRNP I), which is a part of the subfamily of heterogeneous nuclear ribonucleoproteins (hnRNPs)[15], regulates almost all aspects of mRNA metabolism including alternative splicing, polyadenylation, IRES-mediated translation and mRNA stability[16]. Increasing evidences have identified the interactions between lncRNAs and PTBP1 in the tumor chemo-resistance[17, 18]. For instance, linc-ROR confers gemcitabine resistance to pancreatic cancer cells by inducing autophagy and modulating the miR-124/PTBP1/PKM2 axis[19]. Knockdown of lnc00462717 increases the accumulation of doxorubicin (Dox) in glioma via interacting with PTBP1 to inhibit the miR-5p/Occludin signaling pathway[20]. Furthermore, PTBP1, mediated by lncRNA MIR155HG, could upregulate the activation of Wnt/β-catenin signaling pathway in TMZ resistance to glioma has been demonstrated in our previous research [21]. Up to date, the activation of Wnt/β-catenin pathway occurs widely in in glioma chemoresistance to TMZ [22, 23]. However, whether GAS5 could regulate glioma chemoresistance to TMZ through PTBP1, following by regulating Wnt/β-catenin signaling pathway remains to be elucidated.

In current study, based on the Gene Set Enrichment Analysis (GSEA), we have demonstrated the lower expression of GAS5 in glioma patients than in normal controls. GAS5 has highly improved the sensitivity of glioma to TMZ through interacting with PTBP1 to decrease its expression, leading to the inactivation of Wnt/β-catenin signaling pathway. To the best of our knowledge, these results provide a novel guidance for clinical glioma chemo-resistance to TMZ.

Material And Methods

Cell Culture and Reagents

The human glioma cell lines of U251 and A172 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and the Chinese Academy of Sciences Cell Bank (Shanghai, China), respectively. The U251 and A172 cells were cultured in Dulbecco's Modified Eagle's Medium(DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum(FBS, Gemini, California, USA), supplemented with 2 mM glutamine (HyClone, Logan, Utah, USA) and 100 U/ml penicillin–streptomycin (HyClone, Logan, Utah, USA) at 37 ^{o}C with 5% CO_2. The Temozolomide (TMZ) and LiCl were purchased from the Sigma Chemical Co., Ltd. (Sigma, Loui- siana, USA).

Cell Transient Transfection

The overexpression plasmid pCDH-MSCV-MCS-EF1-GFP-PU (pMSCV) was generously donated by Dr. Mo at the Cancer Institute of the University of Mississippi Medical Center. As for the construct of pcDNA3.1-lncRNA GAS5 plasmid, the whole sequence of GAS5 was synthetized and subcloned into pcDNA3.1(+)

plasmid (pMSCV-GAS5) by Shanghai Novoprotein Technology Co., Ltd. (Shanghai, China). The small interfering RNA (siRNA) sequences were designed by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of siRNAs were shown in Supplemental Table 1. All transfections were completed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's manuals. The efficiency of transient transfection is detected by quantitative real-time fuorescence polymerase chain reaction (qRT-PCR, Supplemental Fig.S1A).

Cell Stable Transfection

As for the cell stable transfection, U251 cells were firstly transfected with pMSCV and pMSCV-GAS5. Then the transfected cells were seeded in twelve-well culture plates at a confuency of less than 25% to stably select for at least 7 days with puromycin dihydrochloride (Beyotime, Shanghai, China) at the concentration of 2 μg/ml. The stably transfected pMSCV-GAS5 (sMSCV-GAS5) and pMSCV (sMSCV) U251 cells were ultimately harvested. The efficiency of stable transfection was confrmed by qRT-PCR (Supplemental Fig.S1B).

RNA Extraction, Reverse Transcription and qRT-PCR analysis

The total RNA was extracted from cell lines using RNAeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime, Shanghai, China), according to the manufacturer's protocols. Then extracted RNAs were reversed to complementary DNAs(cDNAs) through using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan). Subsequently, the qRT-PCR detection of mRNA was performed using the TB GreenTM Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Kusatsu, Japan) on the CFX ConnectTM Real-Time System (Bio-Rad, Singapore). The special primers were synthesized from TSINGKE Biological Technology Co., Ltd. (Chongqing, China). The relative expression levels of RNAs were normalized to GAPDH and calculated by the method of $2^{-\Delta\Delta Ct}$. The primer sequences were provided in Supplemental Table 2.

Flow Cytometry Apoptosis Assay

According to our pervious study, the half maximal inhibitory concentrations (IC50s) of TMZ in A172 and U251 cells were 1200 μM and 150 μM, respectively[21]. Based on this result, the transfected U251 and A172 cells were treated with TMZ at IC50 doses for another 72h. The cells were then resuspended in binding buffer containing FITC-Annexin V and propidium iodide (PI) for 15min at room temperature. After diluted with binding buffer, stained cells were immediately analyzed by flow cytometry (Beckman, USA) for determination of the apoptosis rate.

Colony Formation Assay

The stable transfected U251 cells (totally 500) were seeded in six-well plate. After treated with TMZ at IC50 doses for 3 days, the U251 cells were preserved in 10% DMEM containing 10% FBS to form colonies. A week later, the cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal

violet for 30min (Sigma, San Francisco, CA, USA). The appeared colonies were captured under microscope and subsequently counted by ImageJ.

Cell Counting Kit-8 (CCK-8) Assay

The cell viability was evaluated through Enhanced Cell Counting Kit-8 assay (CCK-8, Biosharp, Beijing, China). Briefly, equal numbers of transfected cells were seeded into 96-well plates (3000/well) and cultured in 10%DMEM overnight. Next, the cells in plates were treated with dose TMZ concentrations for 72h. After indicated transfection and treatment times, cells were treated with CCK-8 solution(10 μl/well) for 1h at 37 ^{o}C. The optical desity was read at a wavelength of 450nm using Spectrophotometer Multiskan GO (Thermo Fisher, Vantaa, Finland).

Western Blotting

The cells were lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with the protease inhibitor PMSF (Beyotime, Shanghai, China). Subsequently, protein concentration was measured with the BCA Kit (Beyotime, Shanghai, China). Then protein extracts were segregated by 10% sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes ((Millipore, Carrigtwohill, Ireland). After blocking with 5% BSA in Tris-buffered saline with 0.1% Tween 20(TBST) for 1h at 37°C, the membranes were incubated with the primary anti-GAPDH, anti-β-catenin, anti-c-MYC, anti-cyclinD1, anti-PTBP1(all purchased from Proteintech, Rosemont, USA) at 4 °C overnight. Then the membranes were incubated with HRP-conjugated AfniPure goat anti-mouse/ rabbit IgG (H+L) secondary antibody (Proteintech, Rosemont, USA). The immunoreactive bands for target protein were imaged using WesternBright™ ECL reagent (Advansta, Menlo Park, USA) and the band intensities were normalized to those of GAPDH.

Immunofluorescence

For immunofluorescence, the transfected cells were seeded into 12-well plate, which were placed with glass coverslips in advance. After cultured with 10%DMEM overnight, the cells were fixed with 4% paraformaldehyde for 20min and subsequently washed with cold 0.01M PBS for 3 times. Next, the cells were blocked with 5%BSA for 1h at 37 ^{o}C and incubated with primary antibodies against PTBP1/β-catenin (Proteintech, Rosemont, USA) at 4 ^{o}C overnight. Cells were then labeled with the fuorescent secondary antibody (FITC AfniPure goat anti-rabbit IgG (H+L) 1:100 [EarthOx, San Francisco, USA], Rhodamine (TRITC)- conjugated goat anti-rabbit IgG (H +L) 1:100 [Proteintech, Rosemont, USA]). Cell nuclei were counterstained with DAPI for 5min. Images were captured with the microscope using 40x objectives.

RNA Immunoprecipitation (RIP)

According to the manufacturer’s instructions, the EZ-Magna RIP Kit (Millipore, Carrigtwohill, Ireland) was utilized for performing RIP. The mouse monoclonal anti-PTBP1 were obtained from Millipore

(Carrigtwohill, Ireland). Normal mouse anti-IgG (Millipore) were served as the negative control. qRT-PCR was conducted to examined the purified RNA isolated from RIP.

Nude Mice Xenograft Experiments

Male BALB/C nude mice (4–6 weeks old, 18-20 g, SPF, male) were commercially purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) and maintained in an SPF-grade pathogen-free animal laboratory. The xenograft assay was performed as described previously using U251 cells (1×10^8), which stably overexpressed p-MSCV and p-MSCV-GAS5. The mice were randomly divided into two groups (n=6 each group) named s-MSCV and s-GAS5, respectively. About 30 days after xenotransplantation, the mice were injected with TMZ(50 mg/kg/d) dissolved in 5% DMSO PBS solution every 5 days with every five-day interval for two cycles, and the volume of tumors was measured simultaneously. After 45 days, the mice were sacrificed. The tumors were removed to measure the final volumes and weights. The volume was calculated using the formula: volume= (length×width^2)/2. All procedures of animal experiment were approved by the Animal Research Ethics Committee of Chongqing Medical University.

Statistical Analysis

In the whole researches, the quantitative data were presented as mean ± SEM. Differences between two groups were analysed by an unpaired t-test. The log-rank test was utilized to evaluate the statistical correlation of survival rate with GAS5 expression. The experimental data, which was at least three independent replicates were analyzed with Graph Pad Prism Version 5.01 (GraphPad, La Jolla, CA, USA). Statistical significance was expressed as *P < 0.05, **P < 0.01, or ***P < 0.001.

Results

Downregulation of GAS5 is significantly correlated with poor prognosis

Previous studies have demonstrated that GAS5 is involved in the initiation and development of glioma[24]. Moreover, the analysis of microarray dataset from the Gene Expression Omnibus (GEO, accession number: GSE116520, GSE15824 and GSE31095) repository database confirmed that GAS5 expression was reduced in glioma tissues than in normal tissues (Fig. 1a). In addition, we also found that GAS5 expression was negatively correlated with WHO grade of glioma (Fig. 1b). From CGGA and TCGA database, we revealed that glioma patients with low GAS5 expression had the poor survival (Fig. 1c). Thus, these findings indicate that low-level GAS5 could serve as an important factor during glioma progression.

Overexpression of GAS5 inhibits glioma chemoresistance to TMZ

The tumor-suppressive property of GAS5 has been reported in our pervious study[25]. However, the function of GAS5 during glioma chemoresistance to TMZ remains to be unknown. To define whether GAS5 is involved in TMZ resistance in glioma cells, the U251 and A172 cells were firstly transfected with

pMSCV-GAS5 or pMSCV for GAS5 overexpression and si-GAS5 or si-NC for GAS5 knockdown, respectively. The expression of GAS5 after transfection was evaluated by qRT-PCR (Fig. 2a). As expected, CCK-8 assays showed that GAS5 overexpression effectively increased TMZ-induced repression of glioma cell viability. On the contrary, the GAS5 knockdown in glioma cells distinctly led to resistance to TMZ treatment (Fig. 2b). Besides, the apoptosis rate was improved in pMSCV-GAS5 groups compared to that in pMSCV groups with TMZ treatment for 3 days. However, the lower apoptosis rate was observed in GAS5 knockdown glioma cells compared with NC groups (Fig. 2c). Consistently, GAS5 overexpression also potently inhibited the colony formation of glioma cells with or without TMZ treatment (Fig. 2d). Taken together, these data suggested that GAS5 overexpression could promote the sensitivity of glioma cells to TMZ-based chemotherapy.

GAS5 inhibits the chemoresistance of glioma cells to TMZ through interacting with PTBP1

To further identify the molecular mechanism of GAS5-mediated glioma chemoresistance to TMZ, a bioinformatic analysis of Starbase v2.0 (http://starbase.sysu.edu.cn/starbase2/)along with the RNA-protein interaction prediction were used to predict the GAS5-interacting proteins. Based on the results, the PTBP1 was identified as the most likely candidate (Fig. 3a). Subsequent RIP assay validated that GAS5 and PTBP1 could be specifically enriched in the complex precipitated by PTBP1 antibody relative to non-specific immunoglobulin G (IgG) control (Fig. 3b), suggesting that GAS5 could directly or indirectly interact with PTBP1 in glioma cells. Further findings confirmed that the protein level of PTBP1 was downregulated in GAS5 over-expressing U251 cells, while this change could be reversed in GAS5-konckdown U251 cells (Fig. 3c).

Numerous reports have revealed that PTBP1 serves as a proto-oncogene involving in various types of cancers, especially in chemoresistance of glioma[26, 27]. Moreover, PTBP1 expression correlates with poor prognosis of glioma patients[28]. According to our pervious study, PTBP1 is involved in the TMZ resistance of glioma cells[21]. Consistently, as determined by CCK-8 and flow cytometry, si-GAS5-induced promotion of glioma chemoresistance to TMZ was markedly recovered after co-transfecting with GAS5 and PTBP1 siRNA (Fig. 3d-e). Collectively, these results demonstrated that GAS5 increased the sensitivity of glioma cells to TMZ through interacting with PTBP1.

GAS5 inhibits temozolomide chemoresistance to glioma via inactivating Wnt/β-catenin pathway by interacting with PTBP1

As previously demonstrated, GAS5 could inactivate Wnt/β-catenin signaling pathway in breast cancer and colorectal cancer, respectively[29, 30]. However, the role between GAS5 and Wnt/β-catenin pathway in glioma is still unknown. Based on these results above, we further explored the expression level of target proteins (β-catenin, cyclin D1, and c-Myc) of Wnt/β-catenin pathway in GAS5-altered glioma cells. Notably, compared with the control groups, the expression level of β-catenin, cyclin D1 were distinctly reduced in U251 cells with GAS5 overexpression. Moreover, a significant increase of target proteins related to the Wnt/β-Catenin pathway could be detected in the GAS5 knockdown groups in

comparison with the control groups (Fig. 4a). Collectively, these results indicated that GAS5 modulates Wnt/β-catenin pathway activity in U251 cells.

Consist with our pervious study, the lncRNA MIR155HG have been confirmed to promote TMZ resistance of glioma by regulating the Wnt/β-catenin pathway via interact with PTBP1[21]. Based on this perspective, we reasonably hypothesized that PTBP1 could be involved in role of GAS5 in regulating the sensitivity of TMZ to glioma through Wnt/β-catenin pathway. To further illuminate the potential mechanism of TMZ resistance to glioma, subsequent western blot assays confirmed that the level of β-catenin and its target genes (c-Myc and Cyclin D1) induced by the repression of GAS5 could be distinctly reversed when co-transfecting with both GAS5 and PTBP1 siRNA (Fig. 4b). Based on the results that the promotion of TMZ sensitivity caused by PTBP1 knockdown could be weakened by Lithium (LiCl, an activator of the Wnt/β-catenin pathway[31]), we concluded that GAS5 could inhibit TMZ chemoresistance to glioma via inactivating Wnt/β-catenin pathway by interacting with PTBP1.

GAS5 enhances TMZ sensitivity to glioma *in vivo*

To further examine the underlying effects of GAS5 on the TMZ-resistant phenotype *in vivo*, cells with stably overexpression of pMSCV-GAS5 or pMSCV were subcutaneously injected into each nude mouse to generate tumor xenograft, followed by the administration with TMZ treatment. As shown in Fig. 5a and 5b, GAS5 overexpression dramatically decreased the tumor volume and weight when compared with the sMSCV group. Furthermore, the tumor growth has been more strongly inhibited by simultaneous GAS5 overexpression together with TMZ treatment, suggesting the vital role of GAS5 on TMZ sensitivity *in vivo.* All of these results revealed that overexpression of GAS5 could enhance TMZ sensitivity *in vivo*, which provides a potential therapeutic target to overcome TMZ resistance to glioma.

Discussion

Glioma is one of the most importance malignancies world accompanied by a high rate of morbidity and mortality[3, 32]. TMZ, the main chemotherapy utilized for glioma, is an alkylating pro-drug which methylates DNA at O^6 position of guanine. During DNA replication, the maintenance of this leads to genomic instability and eventually cell death[33]. Even though TMZ is the frontline chemotherapeutic agent, it exhibits a minimal increase in the overall median survival due to the acquisition of chemo-resistant phenotype that develops during treatment[33]. Preventing TMZ resistance is still a challenge in glioma chemotherapy[34]. Recently, lncRNAs have come into limelight with carcinoma research. Emerging evidence demonstrated that aberrant lncRNA expression is strongly implicated in the drug resistance of glioma[35]. However, the molecular mechanism for regulation of GAS5 in TMZ chemoresistance to glioma have not been well elucidated. Intriguingly, our previous findings substantiated that the expression of GAS5 was distinctly downregulated in glioma tissues compared with the normal samples[25]. In this study, we found that the knockdown of GAS5 remarkedly enhanced TMZ resistance to glioma, which manifests as decreased cell viability, clonal number and promotion of apoptosis. Conversely, overexpression of GAS5 could attenuate the TMZ resistance to glioma. Moreover,

by the subcutaneous injection of nude mice with stably transfected U251 cells and then treated with TMZ, we found that GAS5 upregulation could restrain the growth of TMZ-treated tumors. Therefore, these findings highlight the importance of GAS5 dysfunction in the development of drug resistance. However, given that glioma is an intracranial tumor, the different microenvironment between the in situ model and subcutaneous xenograft model may still exist some limitations during the *in vivo* experiments.

We next explored the molecular underlying GAS5-mediated inhibition in TMZ resistance to glioma. As one of the most critical participants in the regulation of intracellular RNA biological function, RNA-binding proteins have been involved in the progression and chemoresistance of glioma[27]. In our study, through RIP assay and correlation analysis of online website. We confirmed that PTBP1 has been identified as the most potential target to interact with GAS5. Additionally, former studies have indicated that high expression of PTBP1 predicts the poor prognosis[37, 38]. Knockdown of PTBP1 notably enhances the anti-tumor effect of TMZ to glioma cells[39]. Currently, we found that the decrease of TMZ resistance created by GAS5 knockdown could be partially reversed when co-transfected with both GAS5 and PTBP1 siRNA in glioma cells, which further demonstrated that GAS5 facilitates TMZ sensitivity of glioma cells through interacting with PTBP1 to downregulate its expression. Intriguingly, we found that GAS5 could regulate PTBP1 expression both in transcriptional and post-transcriptional level. It has been confirmed that the abundance of mRNA has a direct relationship to guide the synthesis of proteins, with the mRNA abundance being transcriptional intensity and degradation rate[40, 41]. We speculate that the overexpression of GAS5 may restrain the transcriptional level of PTBP1 mRNA, thereby reducing the post-transcriptional level of PTBP1 protein. Furthermore, the following experiments should be conducted in depth.

Having assessed the mechanism of GAS5 in the regulation of chemoresistance to TMZ, we further investigated that potential signal pathway involved in this mechanism. Our previous study has reported that lncRNA MIR155HG could promote TMZ chemoresistance to glioma via activating the Wnt/βcatenin pathway[21]. Additionally, we have also demonstrated that lncRNA ST7-AS1 modulates Wnt/βcatenin pathway by directly binding to PTBP1[28]. In this study, our data also showed that GAS5 could play a vital role in regulating the Wnt/β-catenin pathway. Besides, GAS5 knockdown decreased the expression of β-catenin and its downstream targets c-Myc and cyclinD1. Activation of Wnt/βcatenin pathway caused by GAS5 knockdown could be obviously reversed through the co-transfection with si-GAS5 and si-PTBP1. Additionally, our findings have confirmed that activation of Wnt/β-catenin pathway caused by LiCl treatment could increase the chemoresistance to TMZ compared with PTBP1 knockdown group[21], which demonstrated PTBP1 could regulate TMZ resistance through Wnt/β-catenin pathway. Collectively, these results suggest that GAS5 reduces TMZ chemoresistance to glioma cells by suppressing PTBP1 via the inhibition of Wnt/β-catenin pathway.

It has been regarded that Wnt/β-catenin pathway, also known as the canonical Wnt pathway, is perhaps the best characterized during the development of glioma[23]. The central feature of the canonical Wnt pathway is the stabilization of cytosolic β-catenin followed by translocation to the nucleus[22]. Moreover, previous studies have reported that PTBP1 proteins are localized in the nucleus, while phosphorylated

PTBP1 is cytoplasmic[42]. Thus, we speculated that the downregulation of GAS5 could further decrease the phosphorylated level of PTBP1, and therefore restrains the translocation of β-catenin from cytosol to nucleus, ultimately inactivates the Wnt/β-catenin pathway during glioma procession. Nevertheless, we cannot exclude the possibility that GAS5-mediated inhibition of glioma development could partly proceed by β-catenin independent or “non-canonical” Wnt signaling pathway.

In conclusion, our present study indicated that low expression of GAS5 is correlated with poor prognosis in glioma patients. Furthermore, Overexpression of GAS5 could reduce TMZ chemoresistance through interacting with PTBP1 to downregulate it expression in glioma cells, thereby inactivating Wnt/β-catenin pathway. Accordingly, our present data strongly support GAS5 as a promising target for overcoming glioma chemoresistance.

Declarations

Author contributions

Xin He, Jie Sheng and Yingxi Chen finished the most part of the experiments and wrote this manuscript. Xuan Long and Wei Yu assisted to finish some part of these experiments. Qian Liu has designed the experiments and modified the manuscript.

Data Availability Statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest All authors declare no conflicts of interest.

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Figures

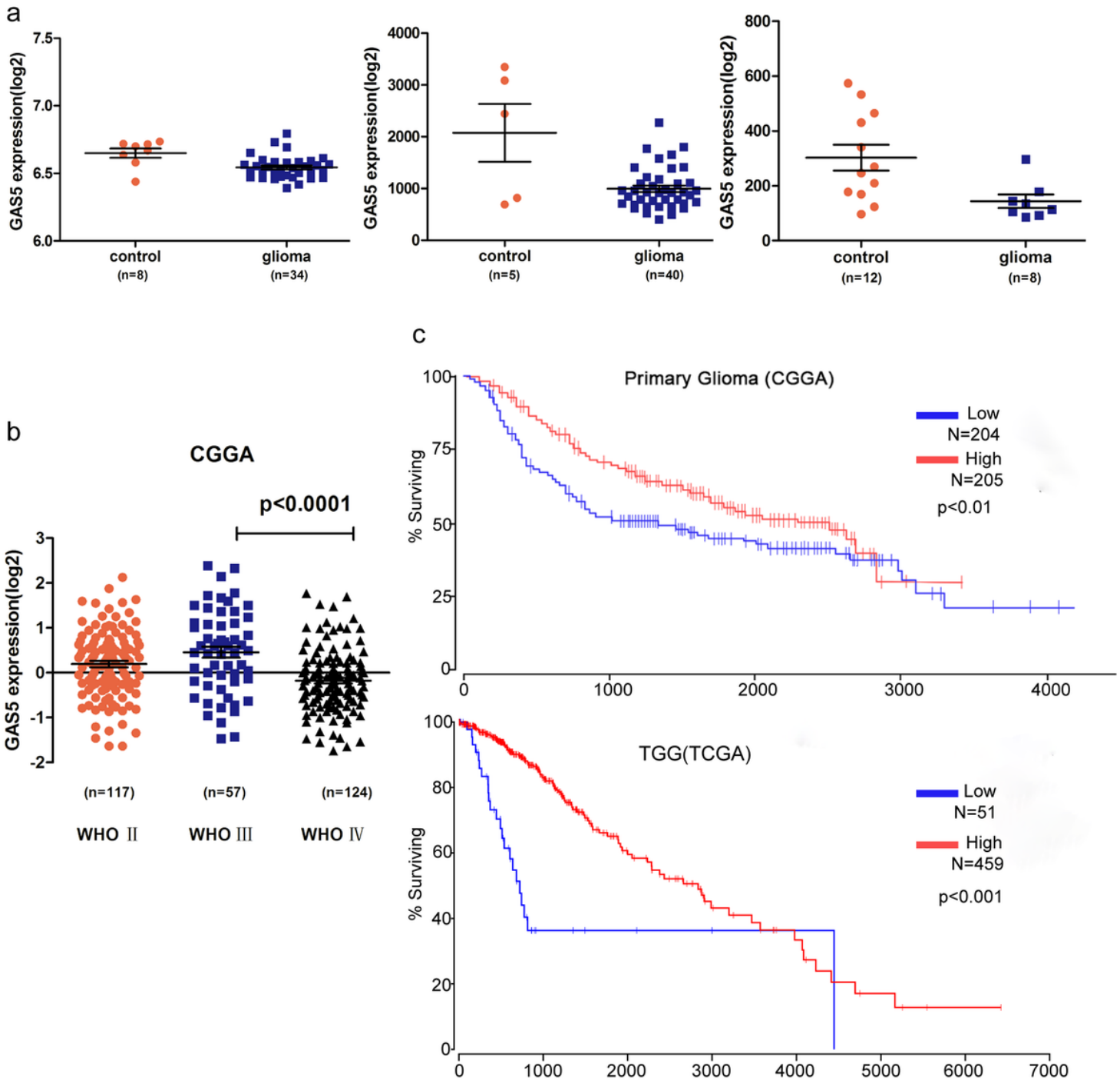

Figure 1

Downregulation of LncRNA GAS5 in glioma cells is relevant to poor prognosis. a Levels of GAS5 were analyzed in normal brain and glioma samples in the GSE116520 (ncontrol=8, nglioma=34), GSE15824 (ncontrol=5, nglioma=40) and GSE31095 (ncontrol=12 nglioma=8) datasets. b Levels of GAS5 were analyzed in glioma samples within different clinical grades in CGGA (nWHO II=117, nWHO III=57, nWHO IV=124). c CGGA (nlow =204, nhigh=205) database and TCGA (nlow =51, nhigh=459) database. The unpaired t-test and log-rank test were performed in clinical data analysis. Data are shown as the mean±SEM

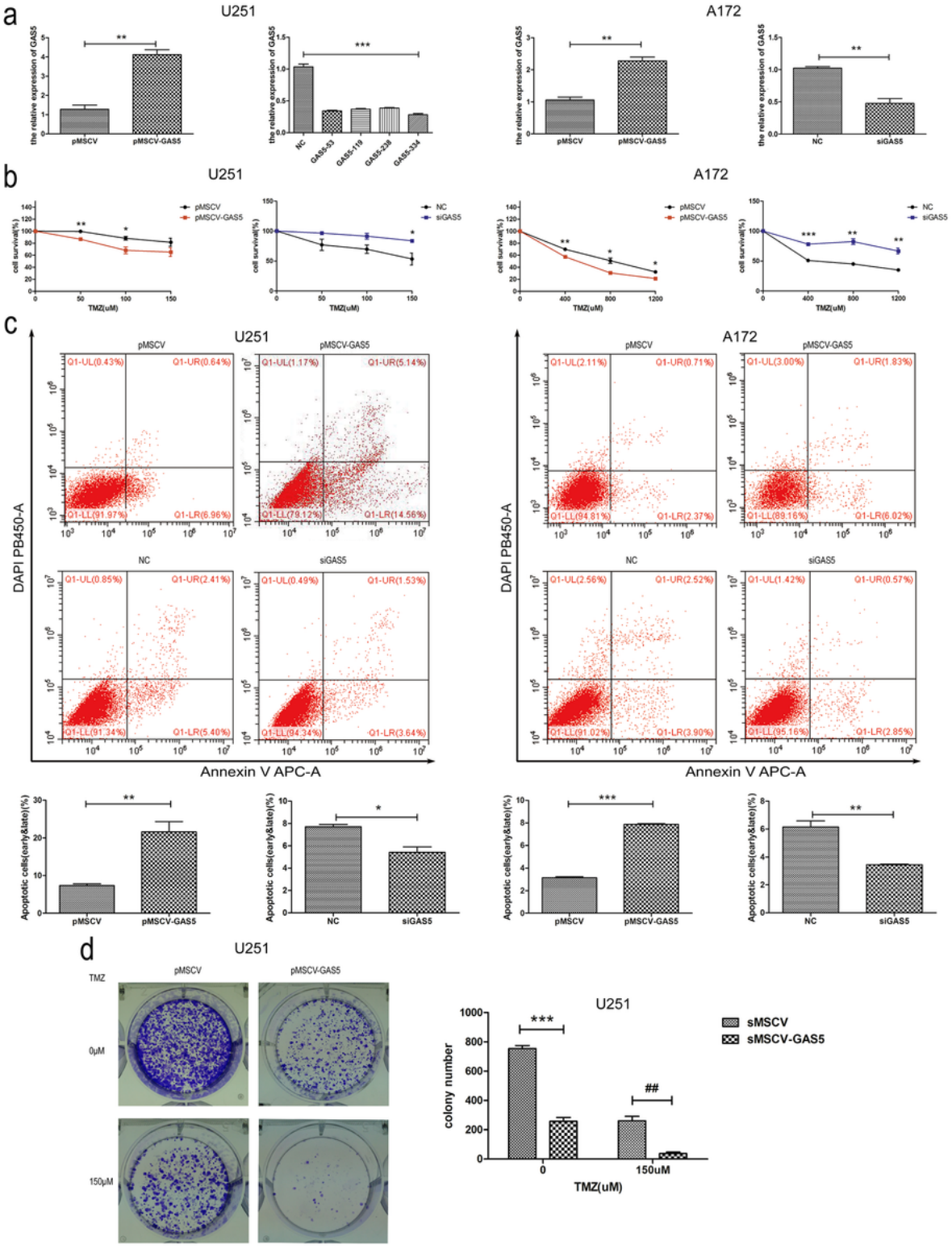

Figure 2

Downregulation of GAS5 in glioma cells is positively correlated with TMZ resistanc. a U251 and A172 cells with altered expression of GAS5 were treated with a dose of TMZ for 72 h, GAS5 expression was detected by qRT-PCR. b U251 and A172 cells with altered expression of GAS5 were treated with a dose of TMZ for 72 h, cell viability was tested by the CCK-8 assay. c GAS5-altered U251 and A172 cells were exposed to 150 μM TMZ and 1200 μM TMZ, respectively, for 72 h and the apoptosis rate was determined

by fow cytometry. d Colony formation assays were performed with sMSCV U251 cells and sMSCV-GAS5 U251 cells after 150 μM TMZ treatment for 72 h. The log-rank test and unpaired t-test were performed. Data are shown as the mean±SEM of at least three replicates (*P<0.05, **P<0.01, ***P<0.001, ##P<0.01)

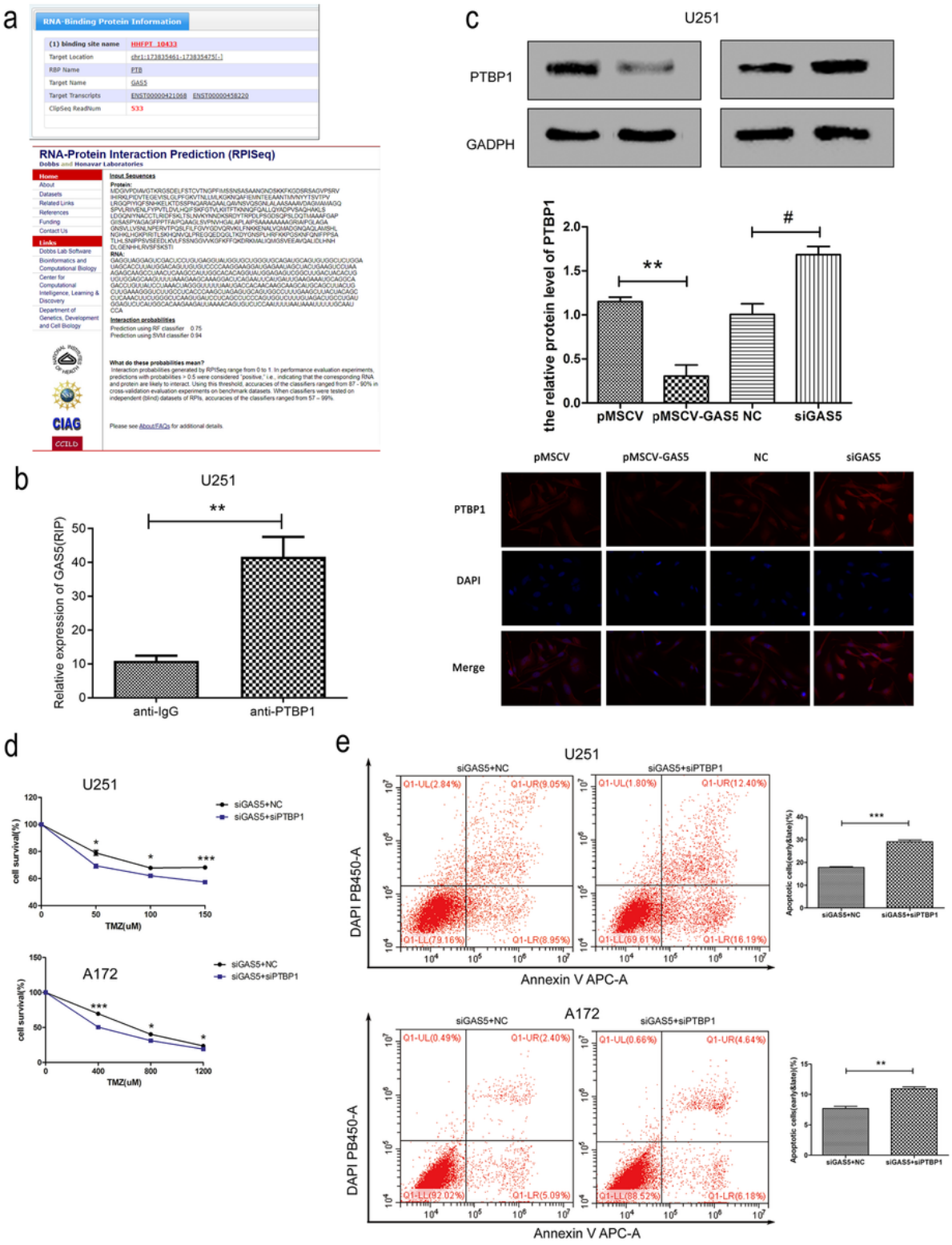

Figure 3

GAS5 inhibits the TMZ resistance of glioma by regulating PTBP1. a Relation - ship between GAS5 and PTBP1 predicted by Star-base v2.0 and RPIseq. b The expression of GAS5 using anti-PTBP1 antibody

and anti-IgG antibody was determined by the RIP assay. c The protein expression of PTBP1 in MIR155HG-altered U251 cells was determined by western blotting and immunofluorescence; scale bars =50 μm. d Cell viability of cells co-transfected with siGAS5 and siPTBP1 was detected by the CCK-8 assay. e The apoptosis rate of cells co-transfected with siGAS5 and siPTBP1 was determined by fow cytometry. All results represent the means ±SEM in triplicate and were analyzed by unpaired t-tests (N.S. non-sense, *P <0.05, **P<0.01, ***P<0.001,#P<0.05)

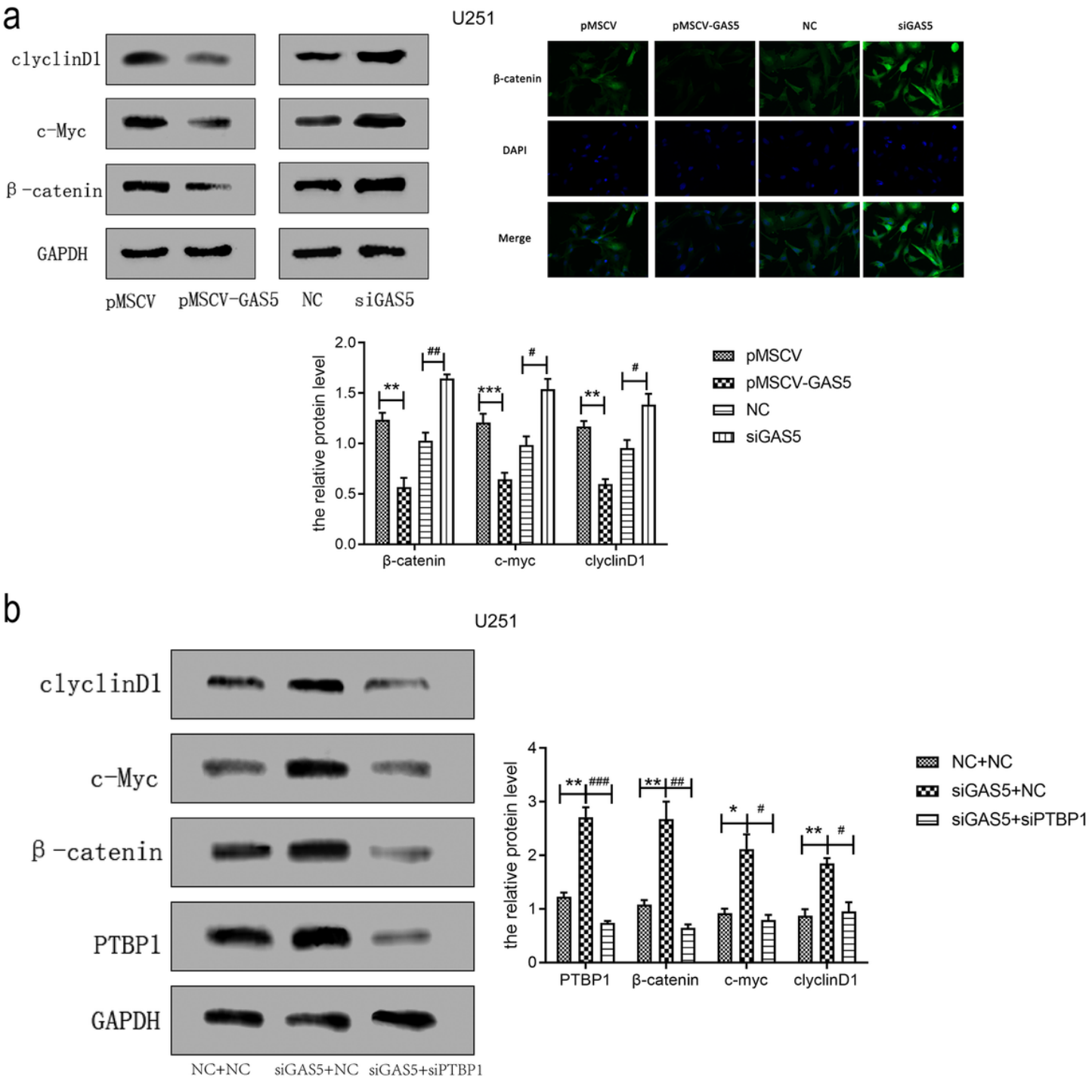

Figure 4

The relative protein expression of the Wnt/β-catenin pathway infuenced by GAS5 and PTBP1. a The protein expression of the Wnt/β-catenin pathway infuenced by GAS5 was determined by western blotting and immunofluorescence, scale bars=50 μm. b The protein expression of the Wnt/β-catenin pathway and PTBP1 infuenced by siGAS5 and siPTBP1 was determined by western blotting. Experiments were independently repeated in triplicate, and data are shown as the mean±SEM analyzed by unpaired t-tests (*P <0.05, **P<0.01, ***P<0.001,#P<0.05, ##P<0.01, ###P<0.001)

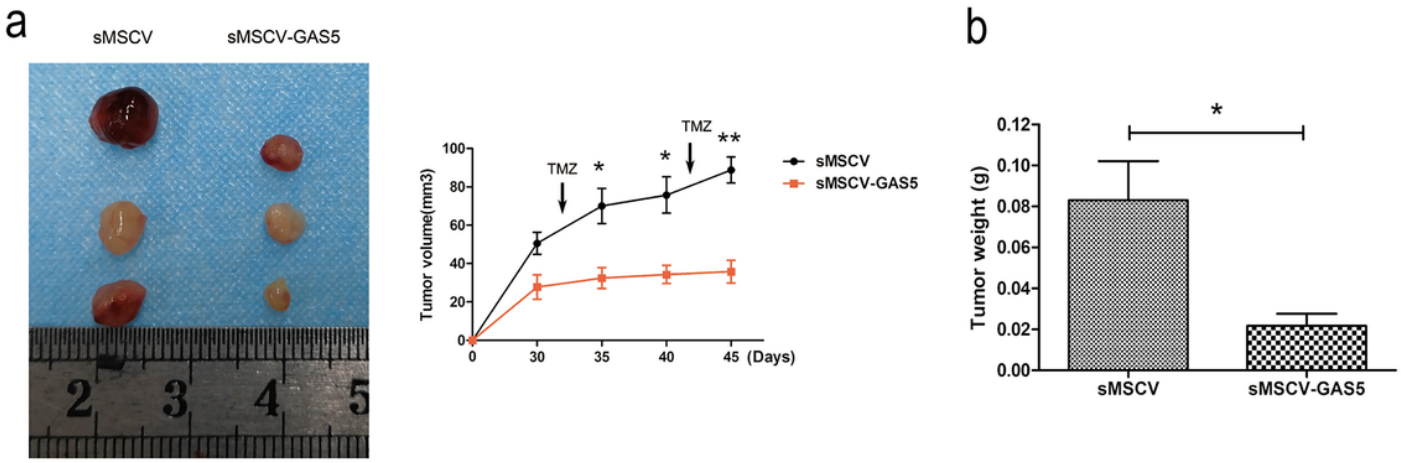

Figure 5

Stable overexpression of GAS5 increases the sensitivity of glioma to TMZ in vivo. a The tumors of nude mice were photographed and the volumes of tumors were measured at 30, 35, 40 and 45 days. b Tumors were weighed when they were harvested. The unpaired t-test was applied. The results are shown as the mean±SEM in triplicate (*P<0.05)