TMEFF2 Promoter Hypermethylation Is an Unfavorable Prognostic Marker in Gliomas

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Abstract

Background: Transmembrane protein with EGF-like and two follistatin-like domains 2 (TMEFF2) is a transmembrane protein in the tomoregulin family. Little research has been performed to determine if TMEFF2 methylation can be a prognostic marker in adult diffuse gliomas.

Methods: In this study, we investigated TMEFF2 expression in surgical tissue samples of gliomas. In addition, we conducted bisulfite amplicon sequencing (BSAS) and methylation-specific PCR (MSP) to evaluate TMEFF2 methylation in glioblastoma (GBM) cells. Subsequently, we investigated the biological function of TMEFF2 in GBM cells. Moreover, we explored the prognostic significance of TMEFF2 in gliomas through analyzing a cohort dataset from TCGA.

Results: Immunohistochemistry analysis of 75 paired glioma tumor and peritumoral tissues proved that glioma tumor tissue expressed TMEFF2 at levels that were lower than those of peritumoral tissues ($P<0.001$). The methylation level of the TMEFF2 promoter was higher in glioblastoma cells than it was in SVG p12 cells ($P<0.001$). Inhibition of methylation reduced TMEFF2 methylation and improved its expression in LN229 and T98G cells ($P<0.05$). Knockdown of TMEFF2 expression significantly promoted the proliferation of U87MG cells and primary GBM cells ($P<0.05$). TMEFF2 methylation is negatively associated with IDH1, ATRX and TP53 mutations, and the subtype of gliomas harboring combined IDH1/ATRX/TP53 mutations was linked to low levels of TMEFF2 methylation. Survival analysis confirmed that low levels of TMEFF2 methylation are associated with a good prognosis in glioma patients.

Conclusions: Our results suggest that TMEFF2 DNA methylation might be associated with glioma tumor progression and could serve as a valuable prognostic marker of adult diffuse gliomas.

Introduction

Diffuse gliomas account for approximately 80% of malignant tumors originating in the central nervous system (CNS) in adults. Despite the development of standard treatments and other emerging treatments for adult diffuse gliomas, the outcomes of glioma patients remain relatively poor[1, 2]. Inter- and intratumoral heterogeneity may contribute to the different outcomes in glioma patients[3, 4]. Based on these current issues, the updated 2016 World Health Organization (WHO) classification of tumors of the CNS emphasized the role of molecular markers, such as IDH1/2, EGFR, TP53, and MGMT, in the diagnosis and prediction of prognosis of adult diffuse gliomas[2].

The isocitrate dehydrogenase (IDH) gene is mutated in > 70% of diffuse lower-grade gliomas and in some glioblastomas[5, 6]. Mutant IDH protein produces oncometabolite D-2-hydroxyglutarate (2HG), affecting epigenetic regulation, especially DNA methylation, of the genome of glioma cells[7, 8]. Despite its role in tumor initiation, mutant IDH is a hallmark of favorable prognosis in glioma patients[9–11]. However, the outcomes for glioma patients with IDH mutations are also remarkably different[12]. Research on new

markers would help us understand the molecular events during adult diffuse glioma progression and provide better guidance for patient prognosis and treatment.

TMEFF2 (transmembrane protein with EGF-like and two follistatin-like domains 2, also known as HPP1 or TPEF) encodes a transmembrane protein of the tomoregulin family[13]. It has been shown that TMEFF2 is downregulated by promoter hypermethylation in several neoplastic diseases, such as colon cancer, esophageal cancer, gastric cancer, and prostate cancer[14–18]. Interestingly, it has been demonstrated that TMEFF2 can act as a promotor as well as suppressor during tumor progression, depending on its alternative splicing and ectodomain shedding[19–22]. Although it was reported that the level of TMEFF2 promoter methylation varies in different adult glioblastoma subtypes in the database from The Cancer Genome Atlas (TCGA)[23], the expression and promoter methylation in glioma cells remain unconfirmed. Moreover, little is known about the correlation between TMEFF2 and the prognosis of adult diffuse gliomas.

In this study, we attempted to investigate the expression and promoter methylation of TMEFF2 in primary glioma tissue samples and in vitro cultured glioblastoma cells. We conducted TCGA database mining to evaluate the role of TMEFF2 promoter methylation as a potential prognostic marker in adult diffuse gliomas.

Materials And Methods

Patients and tissue samples

Patients enrolled in this study were independently diagnosed with primary glioma by two pathologists in a double-blinded manner according to the criteria of the 2016 WHO classification. They had undergone routine surgery at the Department of Neurosurgery of Nanfang Hospital (Guangzhou City, Guangdong Province, China) between 2013 and 2019 without radiotherapy or chemotherapy prior to surgery. Brain tissues beyond MRI indicated peritumor edema area were collected as peritumor tissues. Temporal tissue samples from 3 epilepsy patients undergoing surgical treatment were used as normal brain tissues. This study was permitted by the Ethics Committee of Southern Medical University, and informed consent was obtained from each of the enrolled patients.

Cell lines and culture

Human glioma cell lines U87MG, T98G, LN229, and SVG p12 were purchased from American Type Culture Collection (ATCC: Rockville, MD, USA). The primary human glioblastoma cell line GBM1 was derived and cultured from a patient pathologically diagnosed with GBM (WHO IV) who underwent routine surgery at the Department of Neurosurgery of Nanfang Hospital (Guangzhou City, Guangdong Province, China)[24]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM glucose 4.5 g/L; Biological Industries) with 10% fetal bovine serum (FBS; Biological Industries) at 37 °C in a humidified incubator with 5% CO_2.

Immunohistochemistry (IHC)

Seventy-five paired human glioma tumor and peritumor tissues were used for immunohistochemistry experiments to study altered TMEFF2 protein expression using the two-step plus poly-horseradish peroxidase (HRP) method. Briefly, 4 μm sections were mounted on amino propyl ethoxysilane (APES) slides. The slides were deparaffinized, rehydrated, immersed in 10 mM sodium citrate buffer (pH 6.0), pretreated in a microwave oven for 20 min, and then rinsed for 15 min with phosphate-buffered saline (PBS). Endogenous peroxidase was quenched by incubation of the sections in 0.3% hydrogen peroxide for 30 min at room temperature. Nonspecific binding was blocked by incubation with nonimmune serum (1% bovine serum albumin for 15 min at room temperature). The sections were incubated overnight with two polyclonal antibodies against TMEFF2 (rabbit anti-TMEFF2, ab50002, Abcam, Cambridge, UK; and rabbit anti-TMEFF2, 11928-1-AP, Proteintech, Rosemont, IL, USA) at a dilution of 1:1000. The next day, the slides were stained with a two-step plus Poly-HRP Anti-Rabbit IgG Detection System (PV-6001; ZSGB-Bio, Beijing, China) to detect TMEFF2. After visualization of the reaction with 3,3'-diaminobenzidine, the slides were counterstained with hematoxylin and mounted with synthetic medium. Gastric cancer tissue was used as a positive control, and PBS replaced the anti-TMEFF2 primary antibody to provide a negative control condition.

RNA isolation and qRT-PCR

Twenty-three paired human glioma tumor and peritumor tissues were used in qRT-PCR experiments to detect TMEFF2 mRNA expression. RNA isolation and qRT-PCR were carried out as previously described[25]. Total RNA was isolated from twenty-three paired glioma tumor and peritumor tissues as well as LN229, T98G, U87MG, and GBM1 cells using TRIzol (Invitrogen), and RNA samples (600 ng per sample) were used to generate cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Cat# RR047A) according to the manufacturers' instructions. The obtained cDNA samples were used as templates for qPCR amplifications using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Cat# RR820A). GAPDH was used as a corresponding internal control. All mRNA levels were quantified by the 2-ΔΔCT method. Each reaction was performed in triplicate. The primer sequences of TMEFF2 were as follows: forward 5'-GCTGCTTTCCCTACCTCCTT-3'; reverse 5'-AGCCACACACAGGCACATAG-3'. The primer sequences of GAPDH were as follows: forward 5'-TGACTTCAACAGCGACACCCA-3'; reverse 5'-CACCCTGTTGCTGTAGCCAAA-3'. The primer sequences of DNMT1 were as follows: forward 5'-GATCTCCTACAACGGGGAGC-3'; reverse 5'- AGCCACCAATGCACTCATGT-3'.

DNA bisulfite conversion and methylation analysis

A DNA extraction kit (Takara, Cat# 9765) was used to isolate DNA from GBM and SVG p12 cells according to the manufacturer's instructions. Sodium bisulfite conversion of 600 ng of extracted DNA was performed using a DNA Bisulfite Conversion Kit (Tiangen, Cat# DP215-02). Bisulfite conversion was followed by bisulfite amplicon sequencing (BSAS) or methylation-specific PCR (MSP). Bisulfite amplicon sequencing (BSAS) was performed within CpG sites of the promoter region of TMEFF2. The four primers that were used to amplify TMEFF2 are shown in Table S3. Methylation data were then analyzed, and the average methylation levels at all sites were calculated using MethylKIT software (Access Date 2019/09/26). The methylation level of each CpG site is defined as the ratio of the number of methylated

reads to the combination of methylated and unmethylated reads (values between 0 and 1). For methylation-specific PCR, an EpiScope MSP Kit (Takara, Cat# RR100A) was used, and qRT-PCR was performed. The primers specific for TMEFF2 in MSP are shown in Table S3. For MSP of clinical glioma samples, tumor tissues from forty-three patients who had undergone whole exome sequencing in the clinic, which determined the mutation status of the IDH1, ATRX and TP53 genes, were used. Temporal tissue samples from 3 epilepsy patients undergoing surgical treatment were used as normal controls.

5-Aza-2-deoxycytidine decitabine (DAC) treatment

DAC was purchased from Selleck Chemical Co. (Selleck, Cat# S1200) and was dissolved in dimethyl sulfoxide (DMSO) (Sigma, Cat# D2650). The stock solution was diluted with PBS to an original concentration of 10 mM and stored at -20℃. Further working solutions were added to the cell culture medium immediately before use. Appropriate DMSO controls were implemented. Cells were treated with DAC at a concentration of 5 μM for 96 h, after which total RNA or DNA was extracted from the cells.

Transient knockdown of DNMT1 and TMEFF2 in GBM cells

Cells were transfected with chemosynthetized siRNAs purchased from Kidan Biotechnology Co. (Guangzhou, China) using Lipofectamine 2000 reagent (Invitrogen, Cat# 11668) for 8 h according to the manufacturer's protocol. The sequences of the siRNAs are shown in Table S3.

Cell proliferation assay

Cell proliferation was measured by Cell Counting Kit-8 (CCK8) assays and EdU assays.

For CCK8 assays, cells were seeded in 96-well plates at a density of 2000 cells/well and then were incubated for 12 h to allow cell attachment. Then, 10 μL of CCK-8 solution (Bimake, Cat# B34304) was added to each well on days 1, 2, 3, 4, 5 and 6, which was followed by another 2 h incubation. The optical density was then measured at 450 nm using a microplate reader.

For EdU assays, cells were plated at a density of 20000/dish in confocal dishes. After 24 h of incubation, cells were treated with EdU reagent (RiboBio, Cat# C10310-1) for 2 h according to the manufacturer's instructions and then were fixed with 4% paraformaldehyde. One hundred microliters of 1X Apollo®567 staining reaction solution and Hoechst 33342 (RiboBio, Cat# C10310-1) was added to each dish and then was incubated for 30 min at room temperature on a decolorization shaker. Cells were then visualized using a BX63 automatic intelligent fluorescence microscope (Olympus, Tokyo, Japan).

Database and data analysis

Validation cohort data was collected from the GBM and LGG projects of The Cancer Genome Atlas (TCGA). DNA Methylation (Illumina 450K), Mutations and Clinical Data sets were downloaded from the cBioPortal database[26], and RNA HiSeq V2 RSEM data were downloaded from the GDC Data Portal[27]. RNA HiSeq V2 RSEM data were transformed from FPKM into TPM data before merging into the matrix.

Statistical and survival analysis

R software version 3.5.0 was used to assess the relationship between the TMEFF2 methylation level and mRNA expression through the use of Spearman's correlation analysis. Kruskal-Wallis tests were used to analyze TMEFF2 methylation or mRNA expression in different cells as well as in different tumor grades. The Wilcox test was used to analyze the correlation between TMEFF2 methylation or mRNA expression and gene mutations. Kaplan-Meier analysis was used to assess the association of TMEFF2 methylation or TMEFF2 mRNA expression with patient prognosis. A scanning model found that β value = 0.1 was the best cutoff for TMEFF2 methylation and that TPM = 6.07 was optimal for assessing TMEFF2 mRNA expression in Kaplan-Meier analysis of the TCGA cohort. Differences between survival rates were analyzed using a log-rank test, and survival curves were plotted using R software. Differences in TMEFF2 methylation and expression in different treatment groups were analyzed using Student's t test, and CCK8 results were analyzed using variance analysis of two-factor repeated measures. Statistical analyses were carried out using SPSS statistical software (version 20.0, SPSS, Inc., Chicago, IL, USA), and the significance level was assigned at $P < 0.05$.

Results

TMEFF2 is hypermethylated, and its expression is reduced in gliomas

After evaluation of immunostained paired tumor and peritumoral specimens from 75 glioma patients and normal brain specimens from 5 epilepsy patients, we observed a relatively low level of TMEFF2 protein expression in 70.67% (53/75) of all tumor specimens. TMEFF2 expression in tumor specimens was lower than it was in peritumoral specimens ($P < 0.001$) (Fig. 1a, 1b). The immunohistochemistry staining results were consistent when using another rabbit anti-TMEFF2 antibody (data not shown). RT-PCR of 23 paired glioma samples showed that TMEFF2 mRNA expression levels were lower in tumor tissues than they were in peritumor tissues ($P < 0.01$) (Fig. 1c). To identify changes in methylation of the TMEFF2 promoter in glioma, we performed bisulfite amplicon sequencing (BSAS) in 1 patient-derived primary glioblastoma cell line (GBM1) and 3 glioblastoma cell lines (T98G, LN229, and U87MG), and we also analyzed a normal astroglial cell line (SVG p12). Using four pairs of different primers, we amplified the DNA sequences of four CpG islands in the TMEFF2 promoter and detected the methylation level of all CpG sites involved in these islands to generate a landscape of TMEFF2 promoter methylation in GBM and SVG p12 cells. All glioblastoma cells showed higher average TMEFF2 promoter methylation levels than SVG p12 cells ($P < 0.001$) (Fig. 1d, 1e). To further interrogate the methylation sites of TMEFF2 in glioma, we identified 49 differentially methylated cytosines (DMCs) in a total of 73 CpG sites in the TMEFF2 promoter that were hypermethylated in glioblastoma cells relative to SVG p12 cells (Table S1, Fig. 1f and G, Fig S1a-f).

TMEFF2 expression is negatively correlated with its promoter methylation level in glioblastoma cells

Methylation-specific PCR (MSP) verified TMEFF2 promoter hypermethylation in glioblastoma cells (Fig. 2a). Conversely, RT-PCR demonstrated that TMEFF2 mRNA expression was lower in glioblastoma cells than it was in the other cells tested (Fig. 2b). To confirm the correlation between promoter methylation and expression of TMEFF2 in gliomas, we queried a dataset from TCGA and found a significant negative correlation (Pearson's $r = -0.38$, $p < 0.001$) between the TMEFF2 promoter methylation level and mRNA expression in glioma (Fig. 2c). Treatment with a demethylating agent, decitabine (DAC), decreased TMEFF2 promoter methylation to 41.48% of untreated levels ($P < 0.001$) in LN229 cells and 39.85% of untreated levels ($P < 0.001$) in T98G cells (Fig. 2d). Furthermore, treatment with DAC caused a 2.97-fold ($P < 0.001$) and 3.03-fold ($P < 0.05$) increase in TMEFF2 mRNA in LN229 and T98G cells, respectively (Fig. 2e). Furthermore, we used siRNAs to knock down the expression of the human DNA methyltransferase DNMT1 in LN229 and T98G cells. The efficiency of DNMT1 interference in both cell lines was verified by RT-PCR (Fig. 2f). Knocking down DNMT1 inhibited TMEFF2 promoter methylation to 60.51% ($P < 0.01$) and 44.65% ($P < 0.001$) and upregulated TMEFF2 mRNA expression by 3.677 ($P < 0.001$) and 4.76 ($P < 0.05$) fold, in LN229 and T98G cell lines, respectively (Fig. 2g and Fig. 2h).

TMEFF2 might participate in regulating the proliferation of glioblastoma cells

To verify the biological function of the TMEFF2 protein in glioblastoma cells, we examined the effects of TMEFF2 knockdown by two distinct siRNAs on the glioblastoma cell line U87MG and the primary glioblastoma cell line GBM1. Compared with a nontargeting control siRNA (siNC), treatment with either TMEFF2 siRNA significantly reduced TMEFF2 expression in both cell lines (Fig. 3a). Knockdown of TMEFF2 in both U87MG and GBM1 glioblastoma cells significantly increased the ratio of EdU + cells, as shown by EdU tests (Fig. 3b and c), and knockdown promoted cell proliferation, as shown by CCK8 tests (Fig. 3d and e). Thus, TMEFF2 might act as an inhibitor of the proliferation of adult diffuse glioma.

TMEFF2 promoter methylation might be negatively correlated with the IDH1+/ATRX+/TP53 + subtype of glioma

To further explore the correlation between TMEFF2 methylation and adult diffuse glioma, we combined the Brain Low Grade Glioma and Glioblastoma Multiforme datasets from TCGA and generated a pan-glioma cohort with 1122 primary glioma samples (Table S2). In our cohort, TMEFF2 promoter methylation was found to increase with glioma tumor grade (Fig. 4a); inversely, its mRNA expression declined from low- to high-grade glioma (Fig. 4b). Since adult diffuse gliomas are highly heterogeneous, we hypothesized that TMEFF2 promoter methylation was correlated with one of glioma subtypes. To test this hypothesis, we filtered gene mutations associated with TMEFF2 promoter methylation and found that TMEFF2 promoter methylation was negatively correlated with IDH1 mutation, ATRX mutation and TP53 mutation (Fig. 4c). In addition, in either IDH1 or ATRX or TP53 mutant samples, TMEFF2 was hypomethylated, and its mRNA expression was upregulated (Fig. 4d, 4e). Next, we divided the samples in our cohort into the IDH1, ATRX and TP53 combined mutant group (IDH1+/ATRX+/TP53+) and the -

combined mutant group (-com, IDH1 wild-type and IDH1 mutant but ATRX or TP53 wild-type). Compared to the -com group IDH1+/ATRX+/TP53 + gliomas showed lower TMEFF2 promoter methylation ($P < 0.001$) and higher TMEFF2 mRNA expression ($P < 0.001$) (Fig. 4f, 4 g). Furthermore, we verified these results in clinical glioma tissues. IDH1 mutant glioma tissues (n = 23) harbored lower TMEFF2 methylation than IDH1 wild-type glioma tissues (n = 20) ($P < 0.05$) (Fig. 4h). IDH1+/ATRX+/TP53 + glioma tissues (n = 6) showed lower TMEFF2 methylation than non-combined mutant glioma tissues (n = 37) ($P < 0.05$) (Fig. 4i).

TMEFF2 promoter hypomethylation might be an indicator of better OS in patients with IDH1 + glioma

To determine the correlation between TMEFF2 promoter methylation and glioma patients' overall survival (OS), we initially performed Kaplan-Meier survival curves with a log-rank test based on the best separation model. The results showed that patients whose primary tumors showed low methylation of TMEFF2 obtained longer survival and better prognosis compared with those patients whose primary tumors showed high methylation of TMEFF2 (HR = 0.35, $P < 0.001$; Fig. 5a). Additionally, survival analysis of TMEFF2 expression in primary gliomas implicated high expression of TMEFF2 in better overall survival than low expression (HR = 0.21, $P < 0.001$; Fig. 5b).

To further understand the prognostic value of TMEFF2 promoter methylation, we performed pairwise comparisons of the 4 cohorts grouped by the various combinations of IDH1 mutation (MUT/WT) and TMEFF2 promoter methylation (Low/High). We observed that samples with IDH1.MUT-TMEFF2_Methylation.Low showed the best outcome with the lowest HR of OS (HR = 0.09) (Fig. 5c). In comparing IDH1.MUT-TMEFF2_Methylation.Low with IDH1.MUT-TMEFF2_Methylation.High alongside IDH1.WT-TMEFF2_Methylation.Low and IDH1.WT-TMEFF2_Methylation.High, we found that among 427 IDH1 mutant samples, patients with low TMEFF2 promoter methylation (n = 400/427) had better outcomes than patients with high TMEFF2 promoter methylation (n = 27/427) with an OS of 105.12 months versus 61.96 months ($P = 0.038$).

Discussion

TMEFF2 is a transmembrane protein in the tomoregulin family that has been demonstrated to be hypermethylated and underexpressed in various tumor types. It has been reported that in gastric cancer, TMEFF2 deregulation may play an important role in the progression of gastric carcinogenesis[21]. However, it was also proposed that in prostate cancer, the extracellular domain of TMEFF2 would shed from the cell membrane and promote cell proliferation by combining the ErbB1 receptor[22]. In the current study, we assessed the expression and promoter methylation of TMEFF2 and explored its clinical implication in adult diffuse glioma.

TMEFF2 has been reported to be highly expressed in human brain tissues[17]. In our current study, we confirmed that in comparison to healthy tissue, TMEFF2 expression is significantly decreased in glioma tumor tissues and GBM cells. In addition, from the data of TCGA GBM and LGG cohorts, we ascertained

that TMEFF2 expression is progressively downregulated during the progression from Grade II to Grade IV glioma. Our data are also consistent with previous reports that TMEFF2 expression is downregulated and negatively correlated with tumor histologic grade in gastric cancer and coral cancer[18, 28].

High TMEFF2 expression levels have been reported to be associated with both growth-promoting and growth-suppressing functions in various studies in multiple cancers. In most studies, TMEFF2 has been found to suppress growth[19–21], while Nazim Ali and Vera Knauper proposed that shedding of the soluble TMEFF2 ectodomain would induce proliferation by inducing ERK1/2 phosphorylation in an ErbB1-dependent manner in prostate cancer cells[22]. In our study, cell biological function assays showed increased proliferation in TMEFF2 knockdown GBM cells, indicating that TMEFF2 acted as an inhibitor of proliferation of GBM cells. These data suggest that TMEFF2 may play important roles in suppressing the growth of adult diffuse glioma. Downregulation of TMEFF2 may be related to glioma tumor progression.

We performed bisulfite amplicon sequencing (BSAS) in GBM cells compared with SVG p12 cells and provided the first identification of hypermethylated CG sites in the TMEFF2 promoter of GBM cells. Our MSP work also illustrated TMEFF2 hypermethylation in GBM cells. Moreover, we confirmed the negative correlation between TMEFF2 methylation and mRNA expression. Taken together, low TMEFF2 expression in gliomas may be due to the methylation regulation of its promoter. It was demonstrated that histone deacetylases as well as c-Myc, STAT1 and STAT3 may contribute independently to the transcriptional suppression of TMEFF2 in colon cancer, prostate cancer and gastric cancer[29–32]. However, more detailed studies need to be undertaken to better understand the regulatory mechanism of promoter methylation and transcription of TMEFF2 in gliomas.

However, we failed to clearly clarify the expression of TMEFF2 in glioma tumor tissues or cells by Western blot (WB) using commercial antibodies (data not shown). We noticed that O-methylguanine-DNA methyltransferase (MGMT) is an important biomarker for the chemosensitivity of gliomas, and methylation detection (not IHC) is more precise for the clinical testing of MGMT in glioma patients[1, 33, 34]. Andreas Herbst et al. and Su Man Lee et al. detected methylated free-circulating DNA (ctDNA) for TMEFF2 in the blood of metastatic colorectal cancers and non-small cell lung cancer, respectively[35, 36]. It has also been reported that sequencing ctDNA in cerebrospinal fluid (CSF) can provide a landscape of the tumor genome for glioma patients and is associated with disease outcome[37]. In this study, we successfully and stably detected TMEFF2 DNA methylation levels in GBM cells and in glioma patients' tumor tissues. Thus, methylated TMEFF2 DNA could also be a used as a detectable indicator in glioma patients' tumor specimens or cerebrospinal fluid in the future.

Mounting evidence has confirmed that mutation of IDH1 is a hallmark of favorable patient outcomes in adult diffuse gliomas. In our previous research, we demonstrated that mutation of IDH1 occurs frequently and predicts favorable prognosis in Chinese glioma patients[38]. Mutant IDH1 in gliomas causes broad epigenetic alterations, including DNA hypermethylation, and results in a subtype of glioma with a CpG island methylator phenotype (G-CIMP)[39, 40]. In our study, we observed a small subset (27/427) of IDH1 mutant gliomas with a high degree of TMEFF2 methylation that showed a poor prognosis compared to

the large subset (400/427), which had less TMEFF2 methylation. Due to tumor heterogeneity between glioma patients, TMEFF2 methylation may be a biomarker of poor prognosis in IDH1 mutant glioma patients. Genome-wide methylation of G-CIMP gliomas shifts substantially in tumor recurrence[41]. Our data have not shown whether TMEFF2 methylation is associated with malignant tumor transformation during tumor recurrence. More studies could be performed to clarify the role of TMEFF2 methylation in tumor recurrence.

Gliomas with mutant IDH and 1p/19q noncodeletion mostly harbor loss-of-function mutations in ATRX and gain of new function mutations in TP53[2]. In low-grade gliomas, this subtype is mainly composed of astrocytoma and anaplastic astrocytoma and displays a favorable prognosis. In glioblastomas, mutated IDH is present in only < 10% of all cases, but in those cases there is a better prognosis[10, 42]. The molecular profiles of glioblastoma with mutated IDH are similar to those of astrocytoma with mutated IDH, including frequent ATRX and TP53 mutations and MGMT hypermethylation. In our study, through TCGA database analysis and clinical specimen verification, we found that TMEFF2 promoter methylation is negatively correlated with mutations in IDH1, ATRX and TP53. The IDH1, ATRX and TP53 combined mutant (IDH1+/ATRX+/TP53+) samples in our glioma cohort presented lower levels of TMEFF2 promoter methylation and higher levels of TMEFF2 expression than other samples. Thus, low TMEFF2 methylation may be a new detectable molecular marker used to identify IDH1+/ATRX+/TP53 + gliomas.

Conclusion

In this study, we determined the promoter methylation and expression of TMEFF2 in glioma and highlighted the clinical significance of TMEFF2 methylation in glioma. The TMEFF2 methylation level may serve as a prognostic marker for adult diffuse gliomas. Low TMEFF2 methylation may be a new molecular marker used to identify IDH1+/ATRX+/TP53 + gliomas. Further molecular, cellular, and animal model studies should be performed to achieve a comprehensive understanding of the mechanism of TMEFF2 in carcinogenesis and tumor progression in adult diffuse gliomas.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of Southern Medical University and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

SD, YX, TP and HW conducted most of the bench work, assembled the results, and wrote the manuscript. JL performed clinical specimens embedding and IHC. YF performed total RNA extraction and qRT-PCR. JG and XF performed MSP detection. YQ performed transient knockdown and cell proliferation assays. All authors read and approved the final manuscript.

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Figures

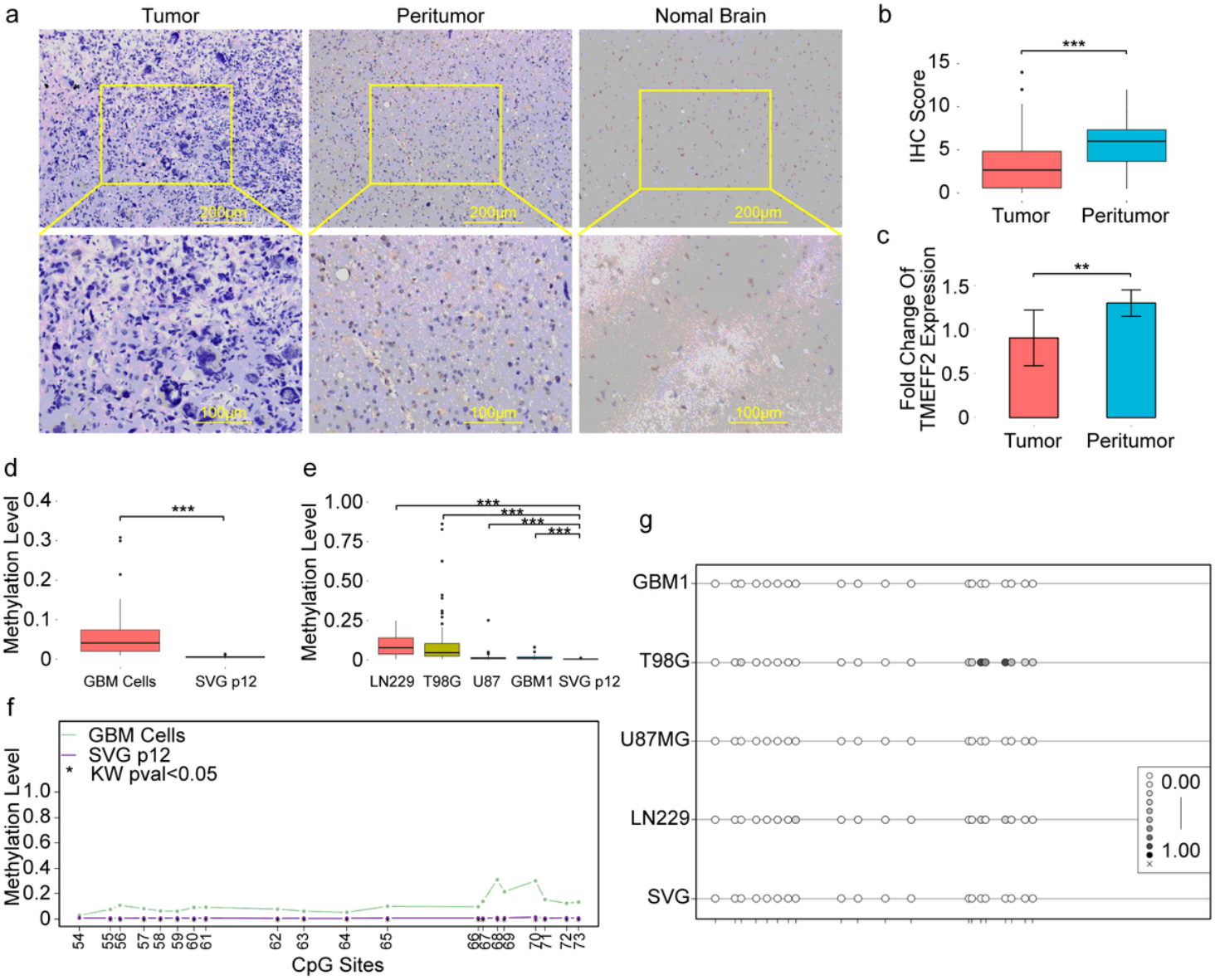

Figure 1

TMEFF2 expression and methylation in glioma tissues and glioblastoma cells a Representative images of TMEFF2 immunohistochemical staining of paired tumor/peritumor tissues from one glioma patient and tissue from one epilepsy patient. b Boxplot of the TMEFF2 immunohistochemistry staining score in tumor and peritumor tissues (n = 75). c Bar gragh of the RT-PCR of TMEFF2 expression in paired tumor/peritumor glioma patient tissues (n = 23). Error bars represent the SD of tissues. d Boxplot of the total methylation levels of BSAS in GBM cells and SVG p12 cells. e Boxplot of the total methylation levels of BSAS in each GBM cell and SVG p12 cell sample. f Line plot of CpG sites of the fourth CpG island of the TMEFF2 promoter in GBM cells and SVG p12 cells. g Methylation plot of the fourth CpG island of the TMEFF2 promoter in GBM cells and SVG p12 cells. CpG sites are presented at each point on each line. **P<0.01, and ***P<0.001.

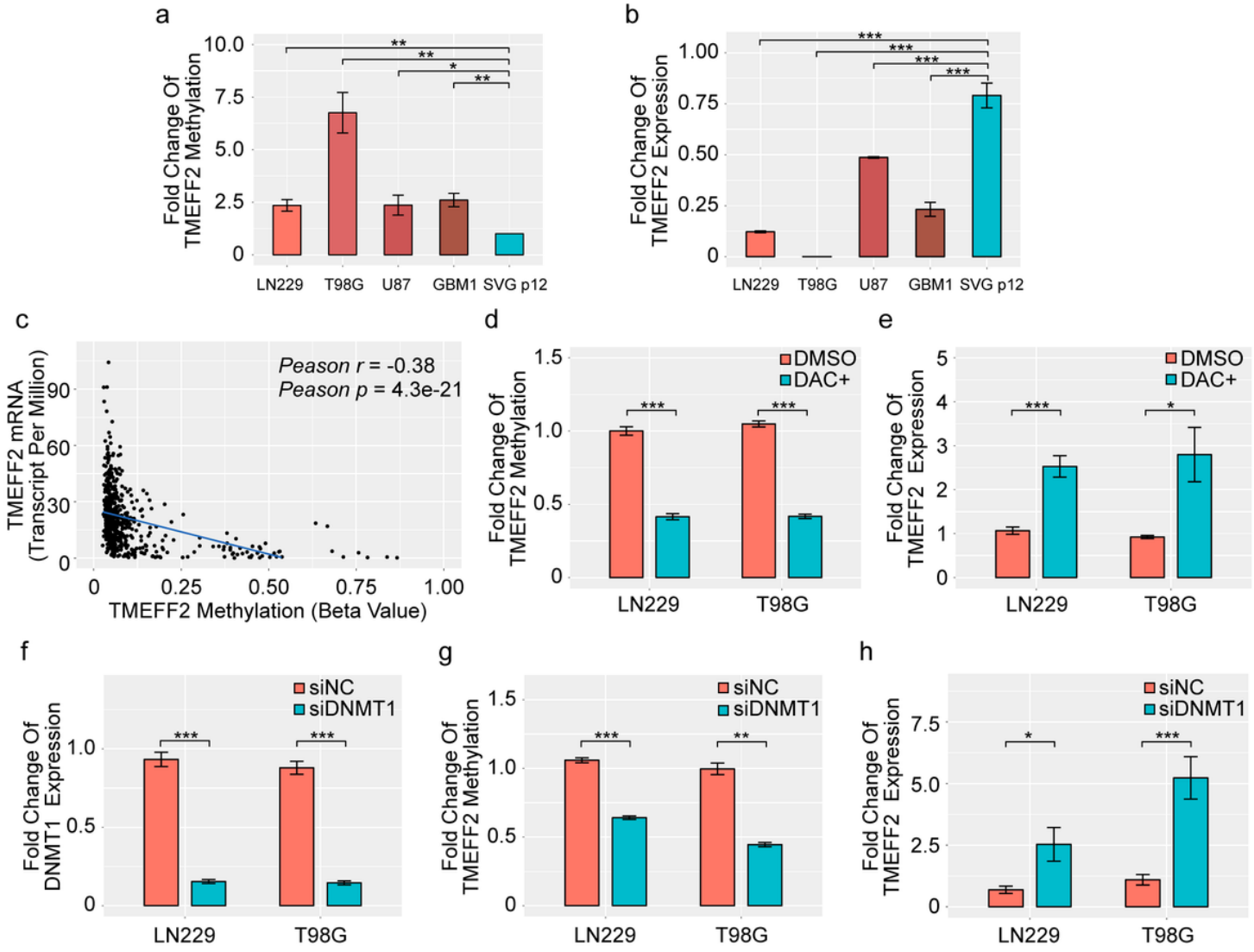

Figure 2

Inhibition of methylation upregulates TMEFF2 mRNA expression in glioblastoma cells a Bar graghBar gragh of the MSP analysis of GBM cells and SVG p12 cells. Error bars represent the SD of repeats of each cell. b Bar gragh of the RT-PCR analysis of GBM cells and SVG p12 cells. Error bars represent the SD of repeats of each cell. c The correlation between TMEFF2 methylation and mRNA expression in primary gliomas (n = 565). d Bar gragh of the MSP analysis of TMEFF2 in LN229 and T98G cells following treatment with DAC (5 μM) for 96 h. Error bars represent the SD of repeats of each cell. e Bar gragh of the RT-PCR analysis of TMEFF2 in LN229 and T98G cells following treatment with DAC (5 μM) for 96 h. Error bars represent the SD of repeats of each cell. f Bar gragh of the RT-PCR analysis of DNMT1 in LN229 and T98G cells treated with DNMT1 siRNA. Error bars represent the SD of repeats of each cell. g Bar gragh of the MSP analysis of TMEFF2 in LN229 and T98G cells following treatment with DNMT1 siRNA. Error bars represent the SD of repeats of each cell. h Bar gragh of the RT-PCR analysis of TMEFF2 in LN229 and T98G cells treated with DNMT1 siRNA. Error bars represent the SD of repeats of each cell. *P<0.05, **P<0.01, and ***P<0.001.

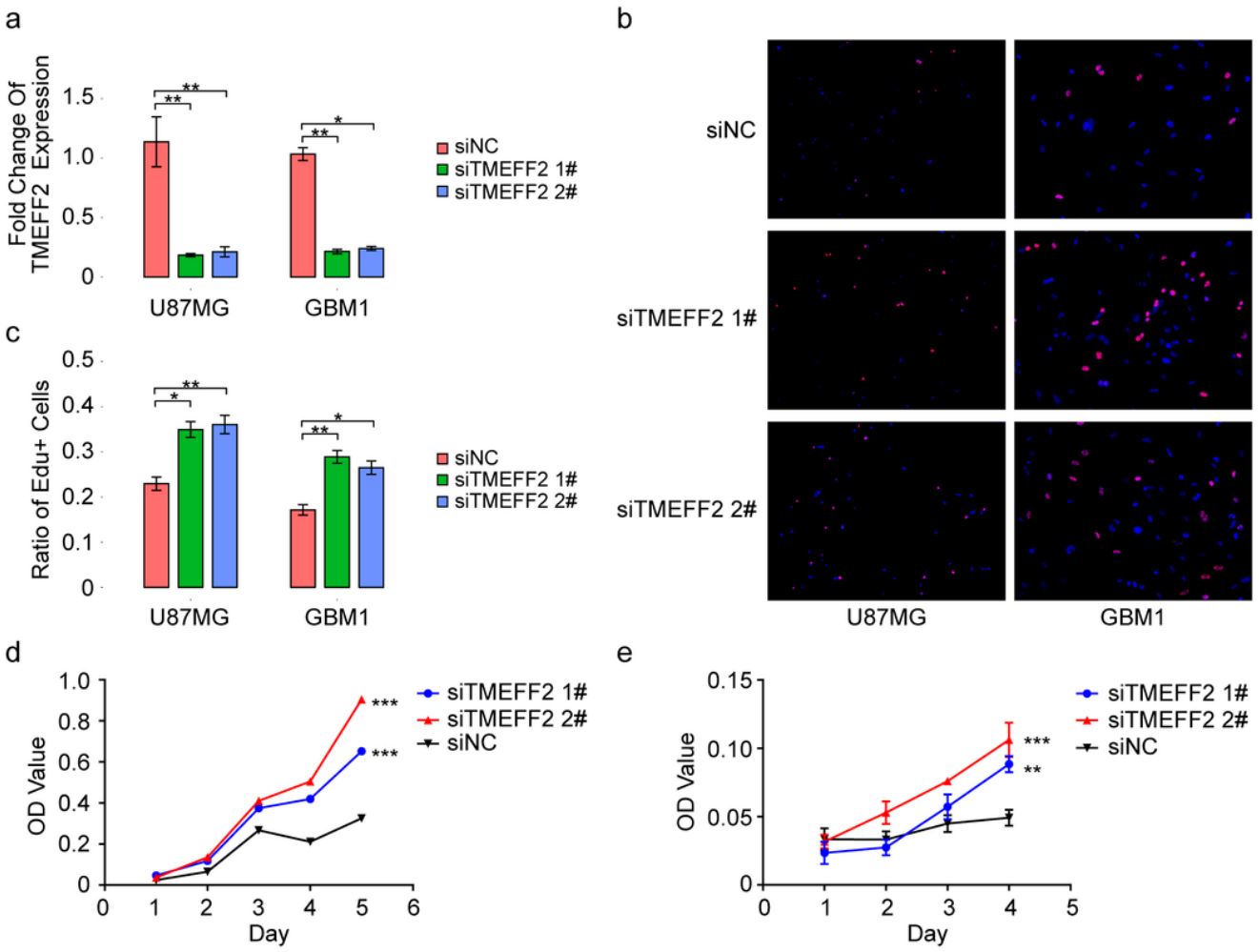

Figure 3

Knockdown of TMEFF2 in U87 and GBM1 cells enhanced GBM cell proliferation a Bar gragh of the RT-PCR of TMEFF2 mRNA expression in U87MG and GBM1 cells. Error bars represent the SD of repeats of each cell. b and c EdU assay showing different cell proliferation rates in siTMEFF2- and siNC-treated U87MG and GBM1 cells. Error bars represent the SD of repeats of each cell. d and e CCK8 assay showing different cell proliferation rates in siTMEFF2- and siNC-treated U87MG and GBM1 cells, respectively. *P<0.05, **P<0.01, and ***P<0.001.

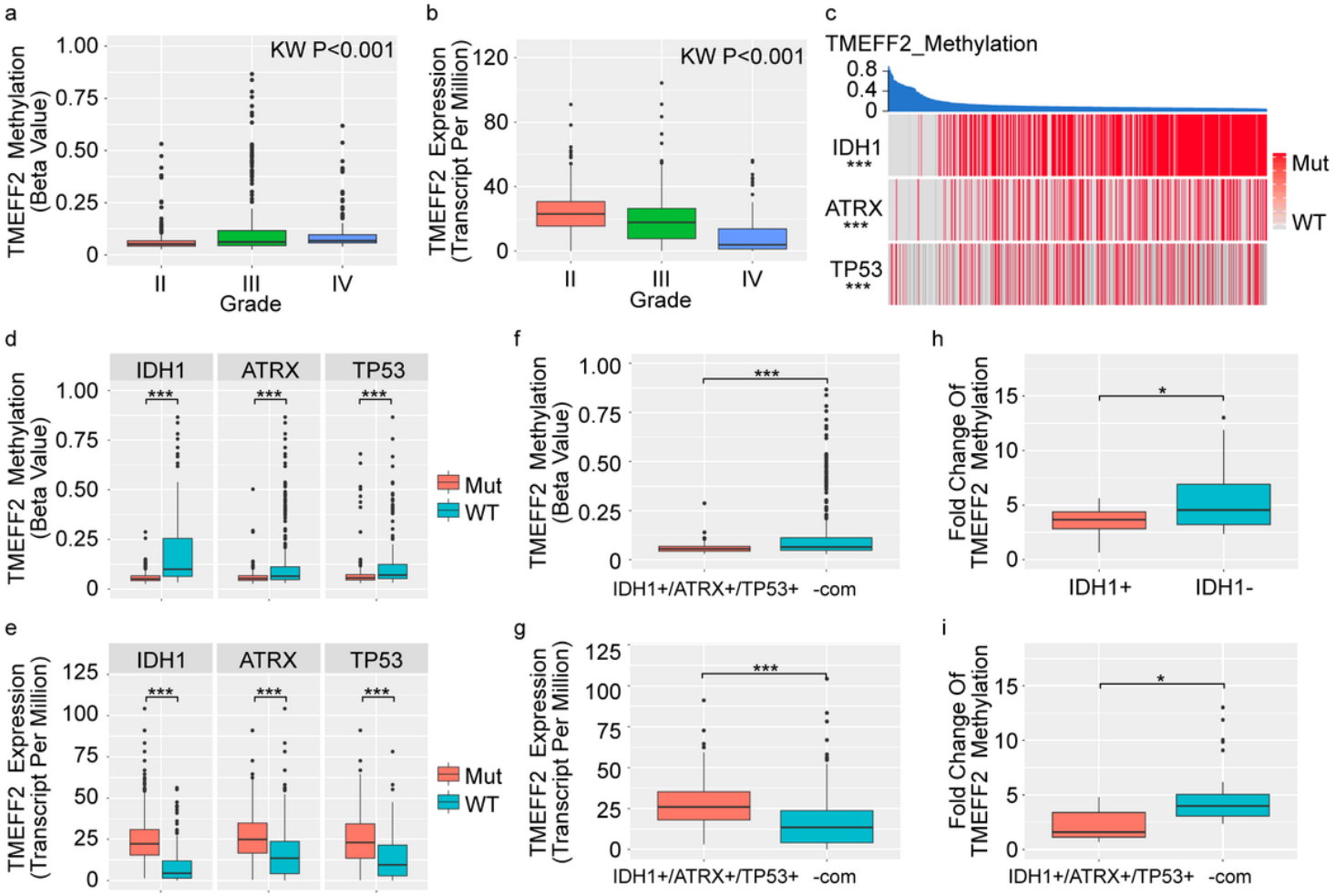

Figure 4

TMEFF2 methylation and mRNA expression levels in the TCGA dataset a Boxplot of the TMEFF2 methylation level in different grades of primary gliomas (n = 1122). Kruskal-Wallis P<0.001. b Boxplot of the TMEFF2 mRNA expression in different grades of primary gliomas (n = 1122). Kruskal-Wallis P<0.001. c Negative association of TMEFF2 methylation with IDH1, ATRX, and TP53 mutations in primary gliomas. d Boxplot of the TMEFF2 methylation level in IDH1, ATRX and TP53 mutant/wild-type primary gliomas. e Boxplot of the TMEFF2 mRNA expression levels in IDH1, ATRX and TP53 mutant/wild-type primary gliomas. f Boxplot for TMEFF2 methylation level in IDH1/ATRX/TP53 combined mutant and non-combined mutant primary gliomas (n = 573). g Boxplot of the TMEFF2 mRNA expression in IDH1/ATRX/TP53 combined mutant and non-combined mutant primary gliomas (n = 588). h Boxplot for TMEFF2 methylation level in IDH1 mutant and IDH1 wild-type primary glioma patients' tumor tissues. i Boxplot for TMEFF2 methylation level in IDH1/ATRX/TP53 combined mutant and non-combined mutant primary glioma patients' tumor tissues. *P<0.05, and ***P<0.001.

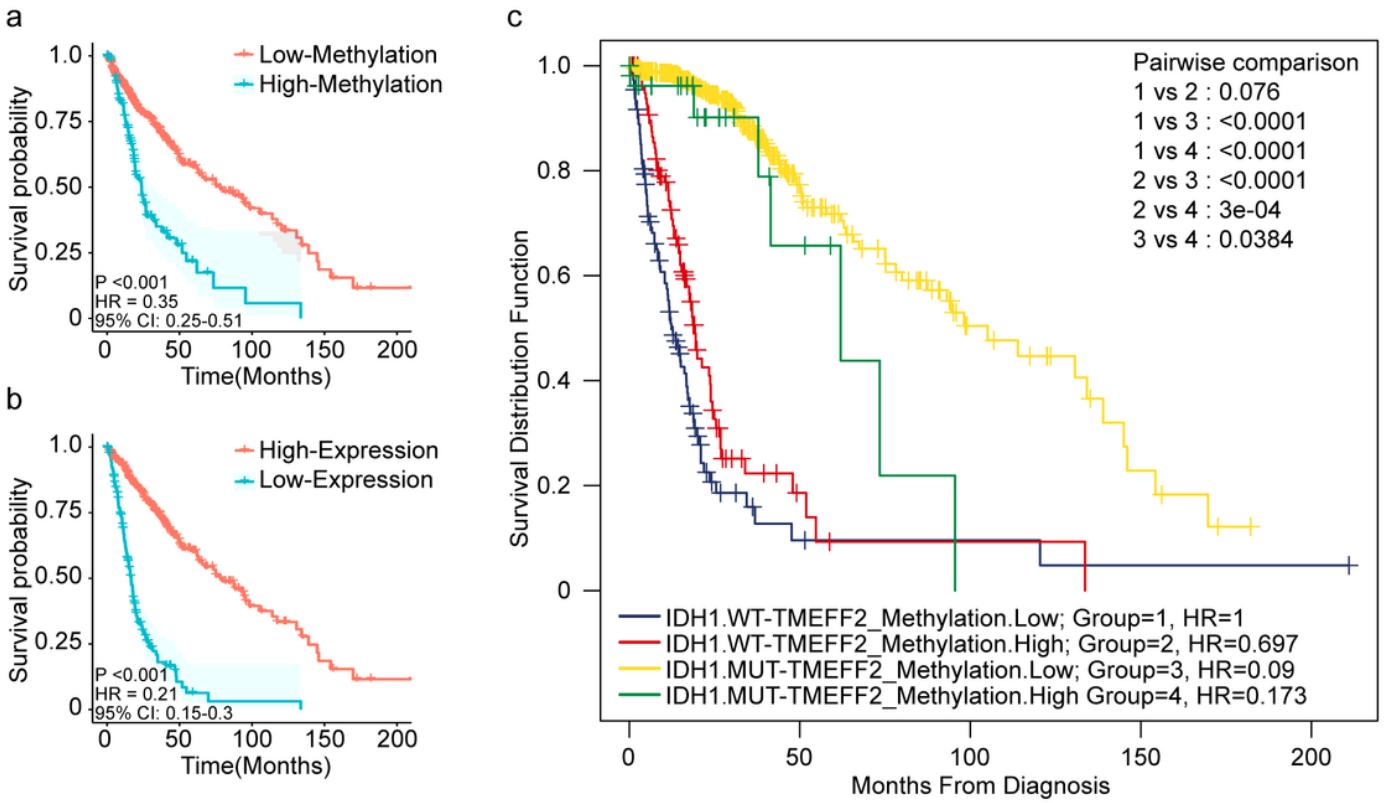

Figure 5

Prognostic value of TMEFF2 methylation in primary gliomas in TCGA dataset a Kaplan-Meier curve for OS (month) in patients with low levels of TMEFF2 methylation (n = 519) versus high levels of TMEFF2 methylation (n = 137) in primary glioma. b Kaplan-Meier curve for OS (month) in patients with low levels of TMEFF2 mRNA expression (n = 164) versus high levels of mRNA expression (n = 501) in primary glioma. c OS (month) for four subgroups of primary glioma patients stratified by combinations of both factors: patients with mutant IDH1 and low levels of TMEFF2 methylation (IDH1.MUT-TMEFF2_Methylation.Low), patients with mutant IDH1 and high levels of TMEFF2 methylation (IDH1.MUT-TMEFF2_Methylation.High), patients with wild-type IDH1 and low levels of TMEFF2 methylation (IDH1.WT-TMEFF2_Methylation.Low), and patients with wild-type IDH1 and high levels of TMEFF2 methylation (IDH1.WT-TMEFF2_Methylation.High).

Supplementary Files

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