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Original Research

TMBIM1 promotes proliferation and attenuates apoptosis in glioblastoma cells by targeting the p38 MAPK signalling pathway

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common and most fatal primary malignant brain tumour in adults. The average survival time of patients after diagnosis is only 12–15 months. And its characteristics of excessive proliferation and apoptosis evasion play a crucial role in the poor prognosis of patients. Therefore, it is worth investigating the molecular mechanism of GBM to find an effective therapeutic target to overcome the dilemma. In the current study, Transmembrane BAX inhibitor motif containing 1 (TMBIM1) was highly expressed in GBM tissues and high TMBIM1 expression in GBM cell lines (U87 and U251) could promote cell proliferation and inhibit cell cycle arrest. In addition, TMBIM1 could significantly attenuate GBM cell apoptosis and decrease the sensitivity of GBM cells to temozolomide (TMZ). In terms of the molecular mechanism, we revealed that TMBIM1 interferes with the p38/MAPK pathway by inhibiting p38 phosphorylation to promote cell proliferation and attenuate cell apoptosis. In vivo experiments showed that the survival time of mice in TMBIM1 knockdown group was significantly prolonged. Our discovery provided an important basis for future intensive molecular mechanism research in GBM and presented a potential target for the treatment of GBM.

Introduction

Glioblastoma, also known as glioblastoma multiforme (GBM), is the most common and most fatal primary malignant brain tumour in adults. The World Health Organization (WHO) classifies this tumour as Grade IV astrocytoma, which represents the highest grade of malignancy, based on its histopathological, molecular, and clinical characteristics [1]. Glioblastoma accounts for approximately 15% of all brain tumours and 50% of gliomas, with an incidence of 1–5 cases per 100,000 people per year [2]. Although patients receive standard therapies such as surgery, TMZ chemotherapy, radiation therapy and rapidly developing immunity therapy, the average survival time of patients after diagnosis is only 12–15 months, with a 5-year survival rate of less than 5% [3]. This short survival time is attributed mainly to the deregulation of many key signalling pathways involving proliferation, apoptosis and temozolomide resistance caused by the highly mutated genome of GBM [4,5]. Therefore, it is worth investigating the molecular mechanism underlying the development and progression of GBM and searching for effective therapeutic targets to overcome the current dilemma.

TMBIM1 (transmembrane BAX inhibitor motif containing 1), a membrane protein localized in late endosomes and lysosomes, inhibits Fas-mediated apoptosis and maintains cellular Ca^{2+} homoeostasis [6,7]. In previous studies, high expression of TMBIM1 is associated with serrated polyposis syndrome, which increases the risk of colon cancer [8]. In addition, TMBIM1 gene variations have been associated with colorectal cancer susceptibility in the Chinese population, and the average expression level of TMBIM1 in colon cancer tumour tissues is significantly higher than that in adjacent normal tissues [9]. Many

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studies have suggested that TMBIM1 and its family members are involved in the regulation of apoptosis in different ways [10–12]. In a word, the molecular mechanism of TMBIM1 and its role in tumour remain to be elucidated. Moreover, the expression pattern and role of TMBIM1 in GBM remains unclear.

P38 mitogen-activated protein kinase is a type of mitogen-activated protein kinases (MAPKs) that is essential for cell stress signalling and plays a crucial role in regulating signals that affect apoptosis, proliferation, cell cycle, and survival [13,14]. And p38 MAPK signalling pathway activation is achieved via a phosphorylation cascade [15]. Previous studies have shown that the phosphorylation state of the p38/MAPK pathway in GBM is decreased, thereby attenuating the apoptosis of GBM cells [16]. Das A et al. found that flavonoids can induce cell apoptosis through activating p38/MAPK pathway in GBM cell lines [17]. P38 is usually considered as a pro-apoptotic inducer, but the activation of p38 MAPK was reported to promote cell invasion and glycolysis in GBM [18,19]. Therefore, it is necessary to continue to explore the regulation of p38 MAPK signalling in GBM.

In our research, we discovered that the expression of TMBIM1 is significantly higher in GBM tissues compared with non-tumour tissues and high TMBIM1 expression is associated with glioma malignancy. TMBIM1 could promote the proliferation of GBM cells and TMBIM1 knockdown could cause cell cycle arrest. Furthermore, TMBIM1 knockdown induced the apoptosis of GBM cells both in vitro and in vivo, and high levels of TMBIM1 decreased the sensitivity of GBM cells to temozolomide (TMZ). In terms of the molecular mechanism, we found that TMBIM1 regulates cell proliferation and apoptosis through the p38/MAPK pathway. This study aimed to provide important data on the effect of TMBIM1 on the development and progression of GBM and to explore whether TMBIM1 can be used as a new therapeutic target for human GBM.

Methods and materials

Bioinformatics analysis

TMBIM1 expression profiles in various human cancers were analysed using The Cancer Genome Atlas (TCGA) database. The Gliovis portal (http://gliovis.bioinfo. cnio.es/) [20] was used to clarify the expression and prognostic role of TMBIM1 in gliomas. The data for the expression of TMBIM1 and survival analysis were from the TCGA-GBM, TCGA-GBMLGG, CGGA, Gravendeel and Rembrandt datasets, which were downloaded from Gliovis. In these datasets, LGG includes WHO grade II and III. The relationship between TMBIM1 expression and the cell cycle was analysed by gene set enrichment analysis (GSEA).

Human GBM and control brain tissues

All GBM tissue specimens (collected from surgical resection) and control brain tissues (collected from patients with traumatic brain injury during emergency surgeries) used in this study was obtained from the Department of Neurosurgery, Renmin Hospital of Wuhan University, China. All GBM specimens had a confirmed pathological diagnosis by pathologists at Renmin Hospital of Wuhan University. Procurement and rational use of specimens in this study required written patient informed consent and was approved by the Institutional Ethics Committee of the Faculty of Medicine, Renmin Hospital Affiliated to Wuhan University (approval number: 2012LKSZ (010) H).

Antibodies and reagents

The antibodies included the following: anti-TMBIM1 (CSB-PA003955, Cusabio, China), anti-p38 (#4511, Cell signalling Technology, USA), anti-p-p38 (#9212, Cell signalling Technology), anti-Bcl2 (12,789–1-AP, Proteintech, China), anti-Caspase 3 (#19,677–1-AP, Proteintech, China), anti-BAD (10,435–1-AP, Proteintech, China), anti-BAX (#60,267–1-Ig, Proteintech, China), anti-CyclinB1 (ab32053, Abcam, UK), anti-Cleaved-Caspase 3 (#19,677–1-AP, Proteintech, China), anti-GAPDH (#GB11002, Servicebio, China), and anti-β-actin (GB11001, Servicebio, China). The p38 MAPK inhibitor BIRB796 was obtained from MCE (HY-10,320, USA).

Cell culture

Human GBM cell lines (U87 and U251) were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in high-glucose DMEM (Genom, Hangzhou, China) supplemented with 10% foetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Biosharp, Anhui, China) and the incubation temperature was 37 °C with 5% CO_2. BIRB796 was used at 1 μM.

Stable cell lines establishment

We purchased the knockdown lentivirus vector of TMBIM1(rLV-shRNA-TMBIM1) from Huamengbio (Wuhan,China) and verified all lentiviral vectors by DNA sequencing. Cells were infected by lentivirus according to the manufacturer's instruction. Twenty-four hours after infection, the virus-infected cells were cultured in the medium containing puromycin (2 ug/ml) for selection. The surviving cells were used in the subsequent experiments. The knockdown efficiency of TMBIM1 was verified by Western blot. The TMBIM1 shRNA sequence was as follows: shTMBIM1-F:5'-GATCCGGAGAGAGCGGTGAGTGATAGCTCGAGCTATCACTCACCGCTCTCTCCTTTTTTG-3′, shTMBIM1-R: 5'-AATTCAAAAAAGGAGAGAGCGGTGAGTGATAGCTCGAGCTATCACTCACCGCTCTCTCCG-3′.

Cell viability assay

A CCK-8 cell counting kit (CCK-8) was used to detect the proliferation-promoting activity of TMBIM1. Following the manufacturer's instructions (Topscience, Shanghai, China), the transfected U87 and U251 cells were inoculated in 96-well plates, seeded with 5000 cells in each well, and cultured in a 37 °C constant temperature cell incubator. At 0 h, 24 h, 48 h, 72 h and 96 h, 10 microlitres of the CCK-8 reagent was added to each well of the corresponding group and incubated for 2 h in the incubator. Finally, the OD value was measured with a microplate reader at 450 nm. The experiments were conducted in triplicate.

Colony formation assay

First, we established stable TMBIM1 knockdown and negative control GBM cells (U87 and U251), added approximately 500 cells to each 6-well culture plate, and cultured them with 2 ml DMEM containing 1% penicillin/streptomycin and 10% FBS. At approximately 2 weeks, obvious colony formation was observed. At this time, the cells were fixed with 4% paraformaldehyde (Biosharp, Anhui, China) for half an hour and then stained with 0.5% crystal violet. After taking a photo of each well with a camera, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to count the cell colonies.

EdU-DNA synthesis assay

A Cell-Light EdU Apollo567 In Vitro Kit (C10310–1, RiboBio, Guangzhou, China) was used to detect the proliferating cells. The cells were inoculated on sterilized slides placed in 6-well plates and incubated for 24 h. According to the manufacturer's instructions, 1 ml of 50 μM EDU medium was added to each well and incubated for 2 h, and then 1 ml of 4% paraformaldehyde was added to each well for 30 min. After decolorization and membrane rupture, 1 ml of 1X Apollo staining reaction solution was added and incubated for 30 min and then the cells

were counterstained with Hoechst 33,342 under dark conditions for 30 min. Immediately after staining, the fluorescence of Hoechst 33,342 and EdU was observed under a fluorescence microscope (Olympus BX51, Japan). ImageJ software was used to count the cells, and the percentage of EdU-positive cells was calculated using the number of EdU-positive cells and the total number of cells stained by Hoechst.

Cell cycle assay

Flow cytometry and Cell Cycle staining kits (Multisciences, Hangzhou, China) were utilized to analyse the cell cycle. Referring to the manufacturer's protocol, the cells were digested into a single cell suspension with 0.25% trypsin (without EDTA), and approximately 6×10^5 cells were collected by centrifugation at 1000 RPM. After washing twice with PBS, 1 ml DNA staining solution and 10 μl permeabilization solution were added. Immediately after staining at room temperature and avoiding light for 30 min, a FACS Calibur Flow Cytometer (BD Biosciences, USA) was used for detection. The data were quantified using Modfit LT 5.0 software.

Apoptosis assay

A commercial Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China) was used to detect the apoptosis rate of the GBM cells. According to the manufacturer's instructions, the cells were collected by centrifugation after digestion with trypsin without EDTA. After washing twice with PBS, the cells were suspended in 400 μl Annexin V binding solution, and then 5 μl Annexin V-FITC was added for 15 min and 10 μl PI was added for 5 min in the dark. Immediately after staining, a CytoFLEX flow cytometer (Beckman Coulter) was used for detection. In the four quadrants, the cells in the lower left quadrant where Annexin V-FITC and PI were negative were live cells, the cells in the upper right quadrant where both Annexin V-FITC and PI were positive were late apoptotic or dead cells, and the cells in the lower right quadrant where Annexin V-FITC were positive and PI were negative were early apoptotic cells. The apoptosis rate was calculated by the sum of apoptosis in the right upper and lower quadrants for further statistical analysis.

TUNEL assay

We measured the fragmentation of DNA, which is a feature of apoptotic cells, by using a TUNEL kit (Roche Diagnostics, Mannheim, Germany). The TUNEL assay was completed according to the manufacturer's instructions, and images were taken using a fluorescence microscope (Olympus, Japan). The TUNEL-positive cell count was determined using ImageJ software.

Western blot analysis

The cells were lysed with RIPA buffer (Beyotime, Shanghai, China) containing PMSF (Beyotime) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) on ice for 30 min. After lysis, the protein concentration was determined by the BCA method, the samples were mixed with loading buffer (Beyotime) and heated on a 100 °C thermostat for 5–10 min. The processed protein was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Germany). Then, the PVDF membrane was blocked with 5% skim milk for 1 h. The membrane was placed in the diluted primary antibody solution, incubated overnight in a shaker at 4 °C Celsius, and then incubated with the secondary antibody (Proteintech, Wuhan, China) for one hour. The imaging was performed by using a ChemiDoc Touch (Bio-Rad, USA). Finally, ImageJ software was used to quantify the band intensity. The relative protein level was normalized to GAPDH or β-actin.

Immunofluorescence staining assays

The cells were fixed with 4% paraformaldehyde for 30 min, ruptured with 0.5% Triton X-100 for 10 min, and blocked with 1% BSA for 30 min. Then, the cells were incubated with the diluted primary antibodies (including anti-BCL2 and anti-cleaved caspase 3) overnight at 4 °C. The next day, the cells were incubated with Alexa Fluor-labelled secondary antibodies (Antgene, China) for 1 h under dark conditions. The nuclei were stained with DAPI (Antgene, China) in the dark for 5 min. An Olympus BX51 microscope (Olympus, Japan) was used to capture the images.

Immunohistochemistry and HE staining

The brain tissues were embedded in paraffin after being immobilized in formalin and cut into slices. Antigen retrieval was performed in 10 mM sodium citrate (pH, 6.0) after deparaffinizing and hydrating the tissues. The sections were incubated with 3% H_2O_2 for 10 min and blocked with serum for 1 h. Then, the tissues were incubated with the primary antibodies (anti-Bad, anti-BAX, anti-cleaved-Caspase3, anti-Bcl-2, anti-p-p38, anti-cyclinB1, and anti-TMBIM1) at 4 °C overnight and then they were incubated with the secondary antibody at room temperature for 1 h. Finally, the tissues were stained with DAB (Servicebio), followed by haematoxylin counterstaining. HE staining was performed according to standard procedures. An Olympus BX51 microscope (Olympus) WERE was used to visualize the images. A semiquantitative score was applied to describe the intensity of IHC staining (0 = negative, 1 = low positive, 2 = positive, 3 = high positive).

Intracranial xenograft model

First, we purchased 14 6-week-old BALB/c nude mice and randomly divided the mice into two groups ($n = 7$). Then, stable TMBIM1 knockdown and negative control U87 cells (4×10^5 cells suspended in 4 μL of PBS) were injected into the right striatum of the nude mice in each group. We regularly observed the state of the mice and euthanized them when they showed severe neurological symptoms and/or obvious weight loss (> 20% of their body weight). Then, we removed the brains of the mice, fixed them in 4% paraformaldehyde, embedded them in paraffin and sectioned them for haematoxylin and eosin (H&E) staining and immunohistochemistry. ImageJ software was used to measure tumour areas from H&E stained sections and extrapolate the volumes. The Committee of Animal Care and Use of Renmin Hospital of Wuhan University approved all experiments with animals in this study.

Statistical analysis

All experimental results are presented as the mean ± standard deviation (SD) and were repeated at least three times. GraphPad Prism 8 was used for the statistical analysis. Two groups of means were compared using unpaired t-tests. One-way analysis of variance was used for the comparisons amongst the different groups. When the analysis of variance was significant, post hoc testing of differences between the groups was performed using the Student-Newman-Keuls test. Survival differences were detected using the log-rank test and Kaplan–Meier analysis. Pearson's test was used to analyse the correlation between TMBIM1 and the other genes. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ were regarded as significant.

Results

TMBIM1 is upregulated in human GBM

First, the public TCGA database was used to analyse the expression profiles of TMBIM1 in various human cancers. We found that TMBIM1 expression in GBM was relatively high among 17 categories of human

cancer (Fig. 1A). Next, we examined 5 GBM tissues and 3 nontumour tissues from patients by western blot. We found that the TMBIM1 protein level in GBM tissues was significantly higher than that in nontumour tissues (Fig. 1B and C). Further analysis through the TCGA, CGGA, Rembrandt and Gravendeel datasets proved that the expression of TMBIM1 in GBM was significantly higher than that in low-grade gliomas (LGG) (Fig. 1D). In addition, TMBIM1 was highly expressed in IDH wild-type patients (Supplementary Fig. S1). Taken together, these results indicate that TMBIM1 is upregulated in human glioblastomas and high TMBIM1 expression is significantly associated with glioma malignancy.

TMBIM1 promotes cell proliferation in human GBM cells

The above results indicate that TMBIM1 is upregulated in GBM and is a potential oncogene. Therefore, we further explored the role of TMBIM1 in GBM. Functional studies were carried out in U87 and U251 GBM cell lines. We established stable TMBIM1 knockdown GBM cell lines including U87 and U251 (shTMBIM1) and corresponding negative control (shNC). Their knockdown efficacies were checked by western blot (Fig. 4G). CCK-8 was used to detect the viability of U87 and U251 cells, and the results showed that the TMBIM1 knockdown group showed lower cell viability in both U87 and U251 cells (Fig. 2A and B). Colony formation assay showed that in the two GBM cell lines, the knockdown group had significantly fewer and smaller colonies than the control group (Fig. 2C and D). In addition, the results of the EdU-DNA synthesis assay demonstrated that in the U87 and U251 cell lines, the number of EdU-positive cells in the knockdown group was significantly less than that in the control group (Fig. 2B, E and F). These results show that TMBIM1 can promote the proliferation of GBM cells.

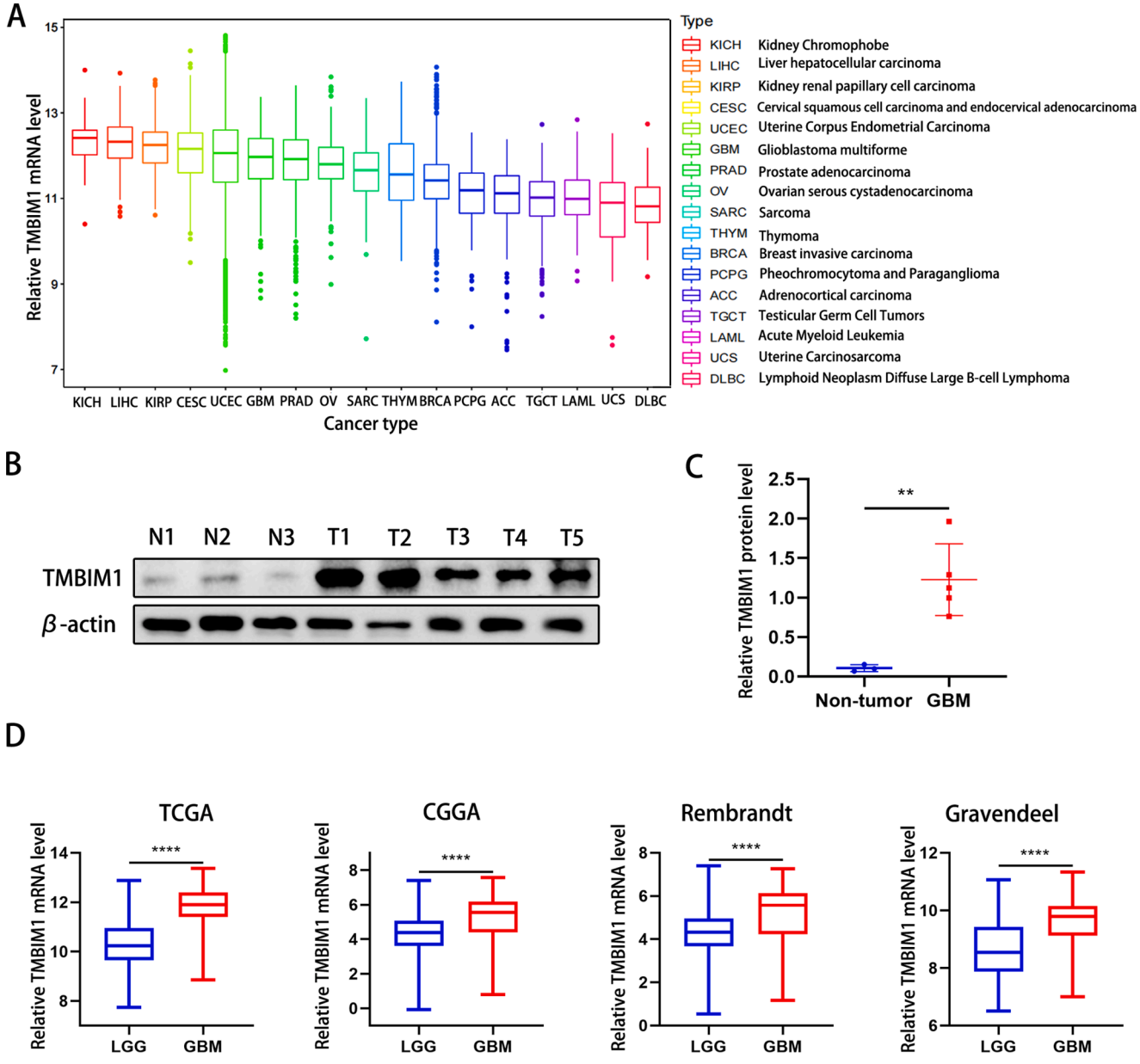

Fig. 1. TMBIM1 is upregulated in human GBM (A) Expression profiles of TMBIM1 in various human cancers from the TCGA database. **(B-C)** Western blotting was used to detect TMBIM1 protein levels in GBM ($n = 5$) tissues and nontumour ($n = 3$) tissues. N1~N3 indicate nontumour tissues, and T1~T5 indicate GBM. **(D)** TMBIM1 mRNA expression in low-grade glioma (LGG) and GBM in TCGA, CGGA, Rembrandt and Gravendeel datasets. Unpaired student *t*-test was used to measure statistical significance in nontumour group and GBM group, and LGG group and GBM group. $^{**}P < 0.01$, $^{****}P < 0.0001$.

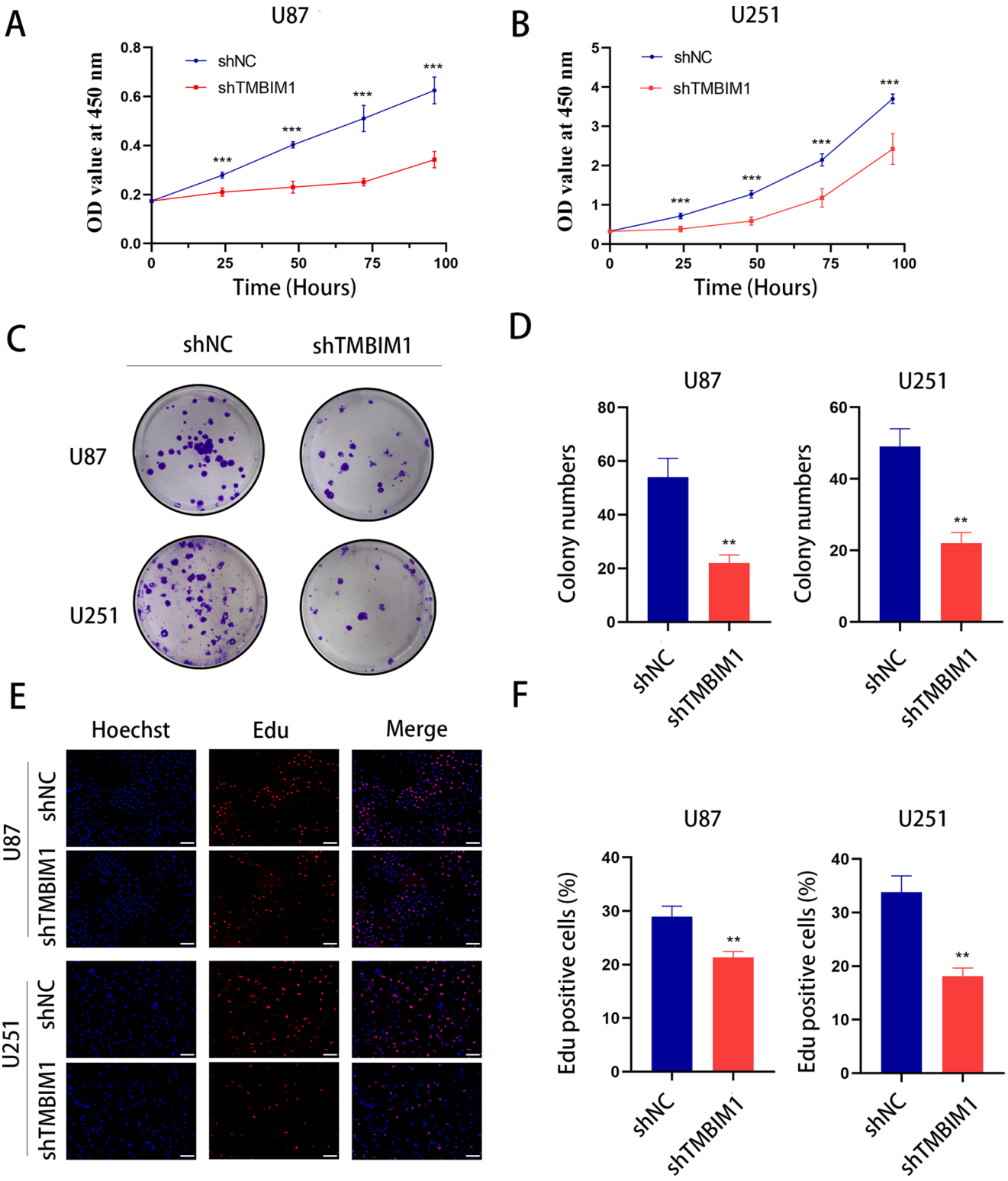

Fig. 2. TMBIM1 promotes cell proliferation in human GBM cells. (A-B) CCK-8 assays were used to measure the viability of U87 and U251 cells. **(C-D)** TMBIM1 knockdown inhibited U87 and U251 cell colony formation. **(E-F)** EdU assays were used to evaluate cell proliferation. Representative images are shown in E. Quantification data showed that the percentage of EdU-positive U87 and U251 cells with TMBIM1 knockdown was downregulated. Scale bars: 50 μm. Unpaired student *t*-test was used to measure statistical significance in shNC group and shTMBIM1 group. $^{**}P < 0.01$, $^{***}P < 0.001$.

TMBIM1 knockdown arrests the cell cycle at G2/M phase in GBM cells

The deregulation of the cell cycle usually plays an important role in proliferation. So, we conducted a GSEA using glioma patient gene profiling data and found that TMBIM1 was related to the cell cycle (Fig. 3A). We then detected whether the cell cycle of the two cell lines was affected by flow cytometry. The results showed that the cell cycle of the knockdown group was blocked at G2/M (Fig. 3B, C, and D). Cyclin B1 plays an important role in the transition from G2/M phase to G0 phase, so western blotting was used to detect the expression of Cyclin B1

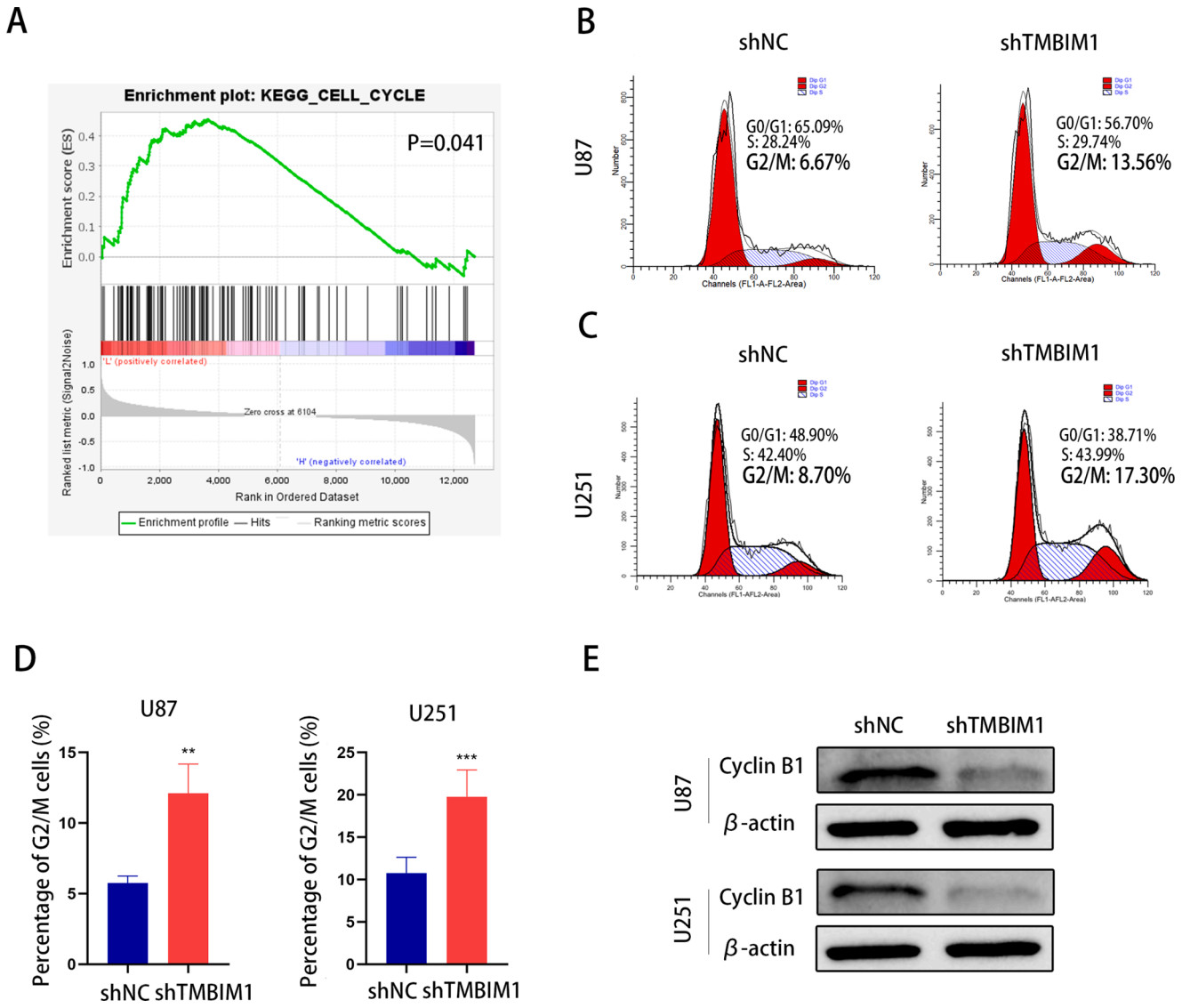

Fig. 3. TMBIM1 knockdown arrests the cell cycle at G2/M phase in GBM cells. A. GSEA showed that TMBIM1 regulated biological processes associated with the cell cycle. **(B-D)** U87 and U251 cells accumulated at the G2/M phase in the knockdown group, as measured by flow cytometry. **E.** Cell cycle protein expression levels in U87 and U251 cells detected by western blot. Unpaired student *t*-test was used to measure statistical significance in shNC group and shTMBIM1 group. $^{**}P < 0.01$, $^{***}P < 0.001$.

in the U87 and U251 cell lines. We found that the expression level of Cyclin B1 protein in the knockdown group was much lower than that in the control group (Fig. 3E). These results indicate that TMBIM1 knockdown leads to arrest of the GBM cell cycle.

TMBIM1 knockdown induces GBM cell apoptosis

Since the TMBIM protein family play a crucial role in the regulation of apoptosis[10–12], we further explored the effect of TMBIM1 on apoptosis evasion in GBM cells. First, we performed TUNEL staining, and we observed that in both U87 cells and U251 cells, the proportion of TUNEL-positive cells in the knockdown groups was significantly higher than that in the control group (Fig. 4A and B). Next, flow cytometry was used to detect the apoptotic proportion of the two cell lines stained by Annexin V-FITC/PI. In the two cell lines, the proportion of apoptotic cells in the knockout group was much higher than that in the control group (Fig. 4C and D). Moreover, immunofluorescence staining assays showed that in the two cell lines, the knockdown group had a significantly higher level of cleaved Caspase 3; in contrast, the expression level of BCL-2 in the knockdown groups was lower than that in the control groups (Fig. 4E and F).

To further confirm our conjecture, we performed western bolt analysis to detect the expression levels of the apoptosis-related proteins, including Bad, Bax, BCL-2, cleaved Caspase 3 and total Caspase 3. In the two GBM cell lines, the decrease in the expression level of TMBIM1 led to increased expression levels of Bad, Bax, and cleaved Caspase 3, with no significant alteration in the total Caspase 3 level. In contrast, BCL-2 reduced with the decreased expression level of TMBIM1 (Fig. 4G and H). These results indicate that TMBIM1 expression can negatively regulate apoptosis of GBM cells.

TMBIM1 regulates the sensitivity of GBM cells to TMZ

Lang F et al. reported that a apoptosis evasion usually contributes to TMZ resistance in gliomas[21]. To further investigate the role of TMBIM1 in glioma, public TCGA datasets were used to analyse the effect of TMBIM1 expression on the survival time of glioma patients with different treatments. We found that among GBM patients receiving TMZ treatment at any time, patients with high TMBIM1 expression had significantly shorter survival times than patients with low TMBIM1

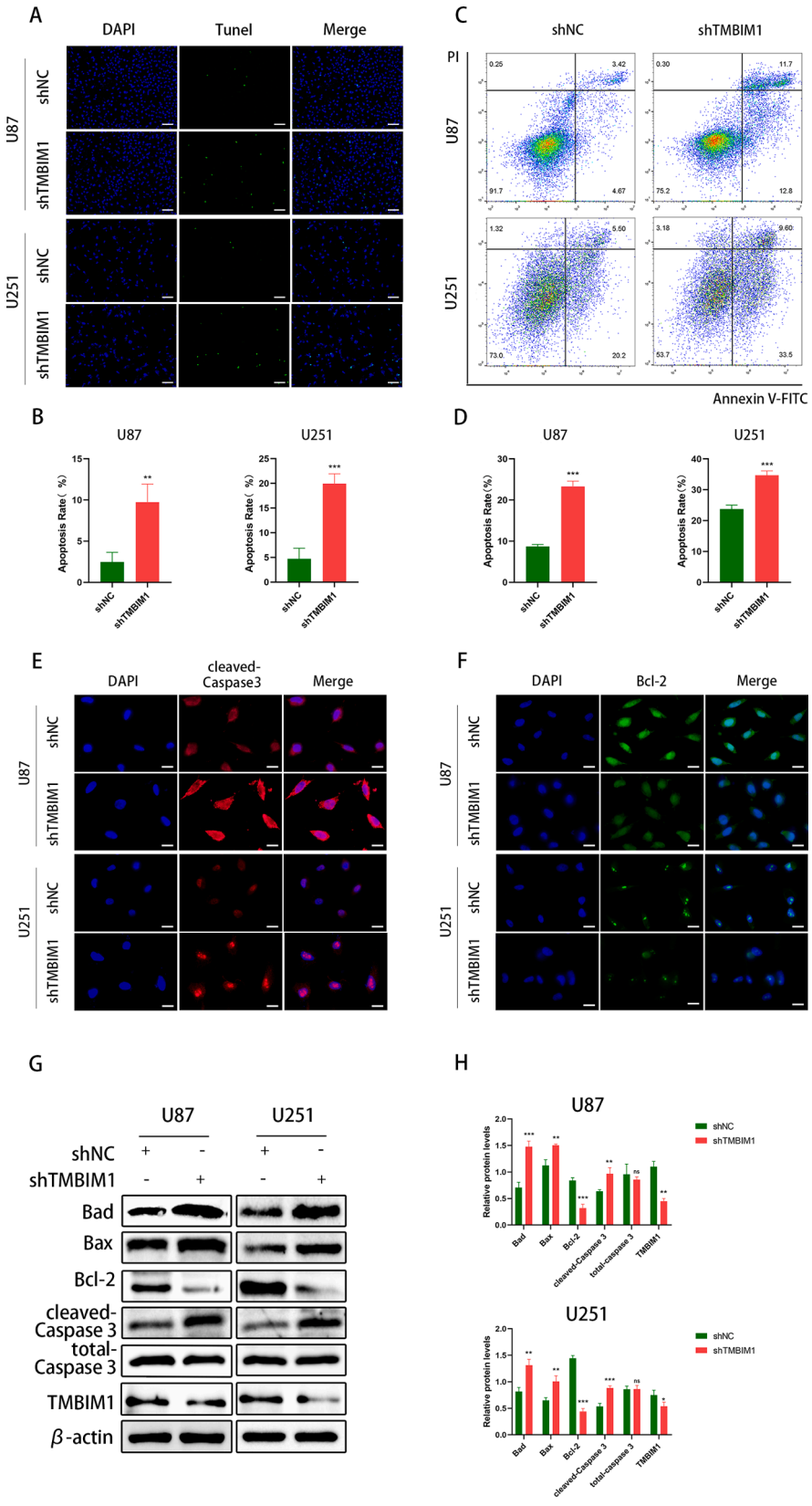

Fig. 4. TMBIM1 knockdown induced GBM cell apoptosis. (A-B) U87 and U251 cell apoptosis was detected by TUNEL staining. Scale bars: 50 μm. The statistical analysis results of the positive rates are shown. **(C-D)** Annexin V-FITC/PI staining and flow cytometric analysis were used to evaluate the apoptosis in U87 and U251 cells transfected with shNC or shTMBIM1. **(E-F)** Effects of TMBIM1 knockdown on cleaved Caspase 3 and Bcl-2 expression in U87 and U251 cells. Scale bars: 10 μm. **(G-H)** Cell apoptosis-related protein expression quantified by western blot. Unpaired student *t*-test was used to measure statistical significance in shNC group and shTMBIM1 group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression (Fig. 5A). Consistent with this finding, among LGG patients, patients with high TMBIM1 expression had a shorter survival time than patients with low TMBIM1 expression if they had received TMZ treatment at any time (Fig. 5C). In patients treated with ionizing radiation (IR) alone, TMBIM1 expression was not significantly associated with survival time (Fig. 5B and D). Furthermore, the similar results were obtained from CGGA datasets (Supplementary Fig. S2).

Since the methylation status of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) promoter is closely related to TMZ resistance in GBM[22], we further explored the relationship

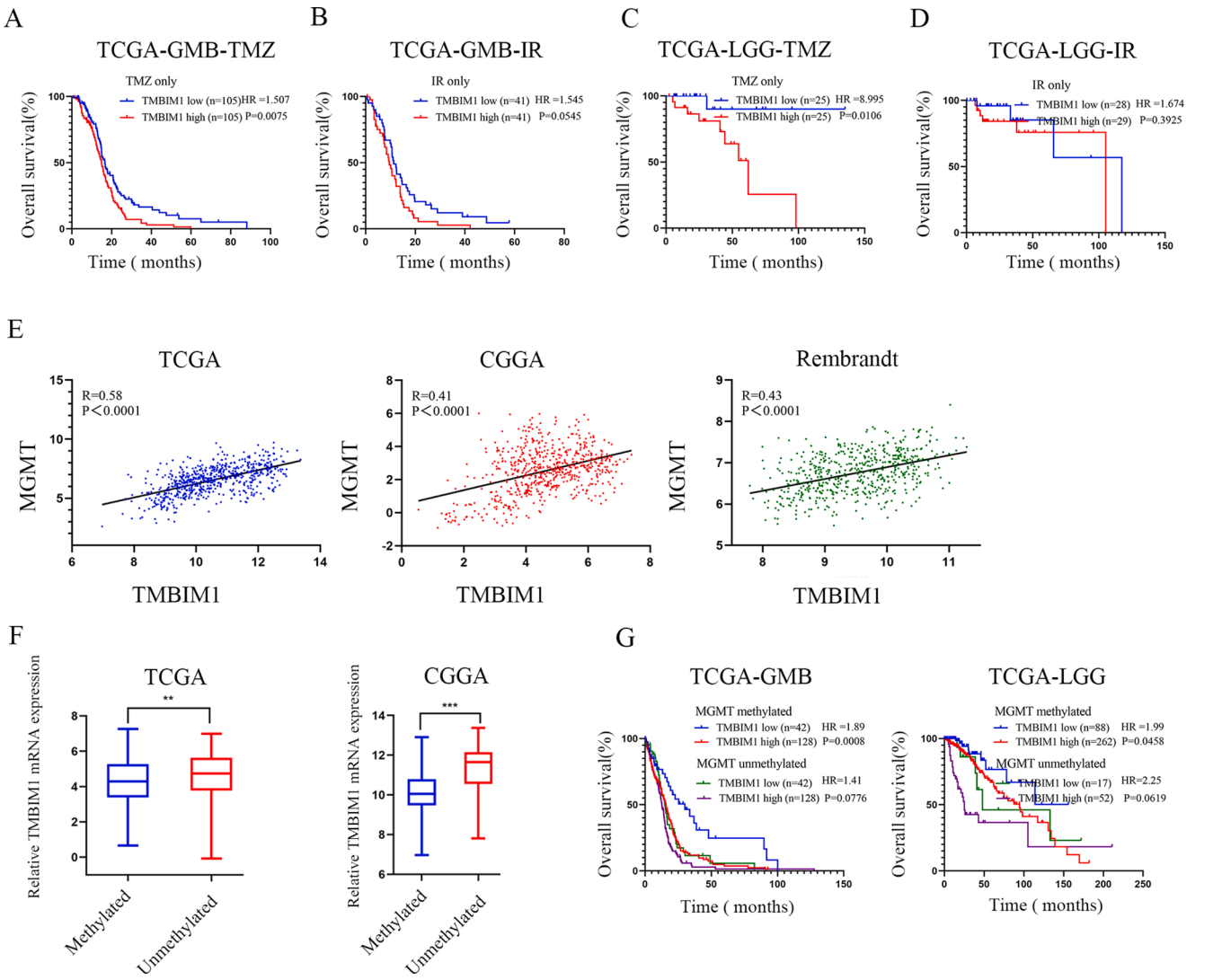

Fig. 5. TMBIM1 regulates the sensitivity of GBM cells to TMZ. (A-B) Kaplan–Meier overall survival curves in TCGA-GBM patients. Patients were divided into groups according to the median TMBIM1 expression and treatment modality (TMZ at any time vs. IR only). **(C-D)** TCGA-LGG patients were separated by the median TMBIM1 expression and treatment modality (TMZ at any time vs. IR only). **(E)** The correlation between TMBIM1 and MGMT in the TCGA, CGGA and Rembrandt datasets. **(F)** The relationship between TMBIM1 expression and MGMT status in TCGA and CGGA, $^{**}P < 0.01$, $^{***}P < 0.001$. **(G)** Overall survival amongst patients with gliomas. The patients were divided into groups according to the upper quartile TMBIM1 expression and different methylation statuses of the MGMT promoter in TCGA-GBM and TCGA-LGG. The log-rank test was used to measure survival differences in TMBIM1 low group and TMBIM1 high group. Pearson's test was used to analyse the correlation between TMBIM1 and MGMT. Unpaired student *t*-test was used to measure statistical significance in methylated group and unmethylated group.

between TMBIM1 expression and MGMT promoter methylation. First, through three public datasets (TCGA, CGGA and Rembrandt), we found that MGMT expression was positively correlated with TMBIM1 expression in glioma tissues (Fig. 5E). In TCGA and CGGA, patients with unmethylated MGMT promoters had higher levels of TMBIM1 than those with methylated MGMT promoters (Fig. 5F). In addition, among GBM and LGG patients with MGMT promoter methylation, the survival time of patients with high TMBIM1 expression was shorter than that of patients with low TMBIM1 expression. However, in patients with MGMT promoter unmethylation, high TMBIM1 expression and low TMBIM1 expression were not significantly associated with survival time (Fig. 5G). In summary, these results indicated that TMBIM1 might regulate the sensitivity of GBM cells to TMZ.

TMBIM1 inhibits p38 phosphorylation, which can be reversed by the p38 inhibitor BIRB796

Since the p38/MAPK pathway plays an important role in regulating cell proliferation and apoptosis[13], we used western blotting to detect the protein expression levels of p38 and p-p38 in the U87 and U251 cell lines to determine whether TMBIM1 has an effect on the p38/MAPK pathway. The results showed that in the two cell lines, when the expression of TMBIM1 decreased, the expression of p-p38 increased significantly, while the expression level of p38 remained unchanged (Fig. 6A and B). We know that the phosphorylation of p38 is an indicator of the activation of the p38/MAPK signalling pathway [14]. Therefore, we speculate based on these results that TMBIM1 prevents GBM cell apoptosis by blocking p38 phosphorylation. To further verify the hypothesis, we used the p38 phosphorylation inhibitor BIRB796 to conduct a rescue experiment, and the effect of the inhibitor was verified by western blot (Fig. 6G). Through flow cytometry, we found that the apoptosis rate of cells has no significant difference between the TMBIM1 knockdown group and the normal control group when added BIRB796, which suggests that BIRB796 reversed the pro-apoptotic effect of TMBIM1 knockdown (Fig. 6E and F). Similarly, the colony formation assay demonstrated that BIRB796 also rescued the pro-proliferative

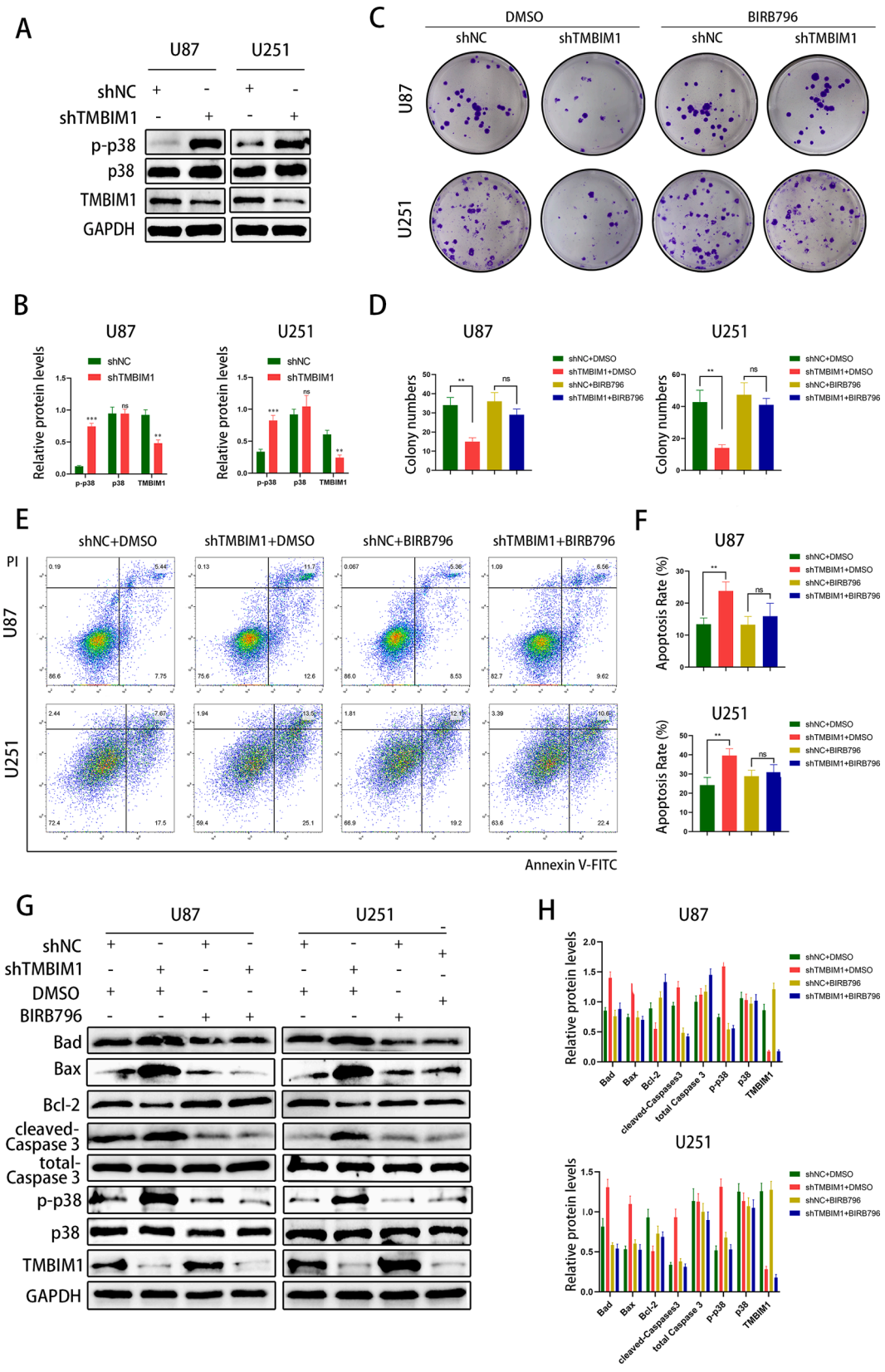

Fig. 6. TMBIM1 inhibits p38 phosphorylation, which can be reversed by the p38 inhibitor BIRB796. (A-B) Western blotting was used to detect the p38, p-p38 and TMBIM1 protein expression levels. **(C-D)** Clone formation assays showed that the inhibitory effect of TMBIM1 knockdown on cell proliferation was reversed by BIRB796 in the U87 and U251 cell lines. **(E-F)** The p38 phosphorylation inhibitor BIRB796 rescued the cell apoptosis induced by TMBIM1 knockdown in U87 and U251 cells. Cell apoptosis was determined by a CytoFLEX flow cytometer. **(G-H)** The p38 phosphorylation inhibitor BIRB796 reversed the effects of TMBIM1 knockdown on apoptosis-related proteins in U87 and U251 cells. Western blot analyses were used to determine the levels of Bad, Bax, Bcl-2, cleaved Caspase 3, total caspase-3, p38, p-p38, TMBIM1 and GAPDH. Unpaired student *t*-test was used to measure statistical significance in shNC group and shTMBIM1 group, shNC +DMSO group and shTMBIM1+DMSO group, and shNC +BIRB796 group and shTMBIM1+BIRB796 group. $^{**}P < 0.01$, $^{***}P < 0.001$.

effect of TMBIM1 (Fig. 6C and D).

Next, we conducted western blot assays to detect apoptosis-related proteins, including Bad, Bax, BCL-2, cleaved Caspase 3, and total Caspase 3. The results showed that BIRB796 prominently reversed the expression level of apoptosis-related proteins (Fig. 6G and H). Taken together, we can conclude that TMBIM1 is involved in the inhibition of p38 phosphorylation, which plays a crucial role in promoting cell proliferation and attenuating cell apoptosis in GBM.

TMBIM1 knockdown suppressed tumour growth in an intracranial xenograft model

To further explore the role of TMBIM1 in vivo, we constructed an intracranial xenograft model with stable TMBIM1 knockdown U87 cells. As expected, HE staining revealed that the volume of tumours in the nude mice was significantly smaller in the TMBIM1 knockdown group than in the control group (Fig. 7A and Supplementary Fig. S4). Immunohistochemical staining showed that TMBIM1 knockdown significantly increased the expression levels of p-p38, Bad, Bax, and cleaved-caspase3 and reduced the levels of Bcl-2 and cyclinB1, which was consistent with our results in vitro (Fig. 7B and Supplementary Fig. S3). In addition, Kaplan–Meier analysis showed that the mice in the TMBIM1 knockdown group survived significantly longer than those in the control group (Fig. 7C). Taken together, these results demonstrated that TMBIM1 knockdown suppressed tumour growth and induced cell apoptosis *in vivo* .

Discussion

GBM, the highest grade intracranial malignant tumour as defined by the World Health Organization, accounts for approximately one-sixth of all brain tumours and one-half of gliomas. Although current standard therapies, such as surgery, chemotherapy radiotherapy and immunotherapy, have developed rapidly, the 5-year survival rate after diagnosis is still very low. Since both apoptosis resistance and increased survival signalling are major regulators of cancer cell survival, targeting only one of these compartments may not be sufficient to obtain therapeutic effects [23]. In GBM, the highly mutated genome caused the deregulation of many key signalling pathways involving growth, proliferation, survival, and apoptosis [4], which suggests we could explore the molecular mechanism underlying the development and progression of GBM to find a potential therapeutic gene target. TMBIM1, localized in late endosomes and lysosomes, was regarded as a membrane protein that can regulate Ca^{2+} homoeostasis, cell death and disease progression, including cancer [7,24]. A meta-analysis showed that TMBIM1 was a higher risk gene for colon cancer [25]. Zhao Gn et al. proposed that TMBIM1 was an effective inhibitor of nonalcoholic steatohepatitis, the main cause of liver cancer and cirrhosis [26]. However, research on TMBIM1 in tumour is only superficial and the mechanistic and role of TMBIM1 in GBM tumour genesis is totally unexplored. Our research identified for the first time that TMBIM1 promotes cell proliferation and decreases apoptosis of GBM cells by activating the p38/MAPK signalling pathway. And we also found TMBIM1 regulates the sensitivity of GBM cells to TMZ (Figure 8).

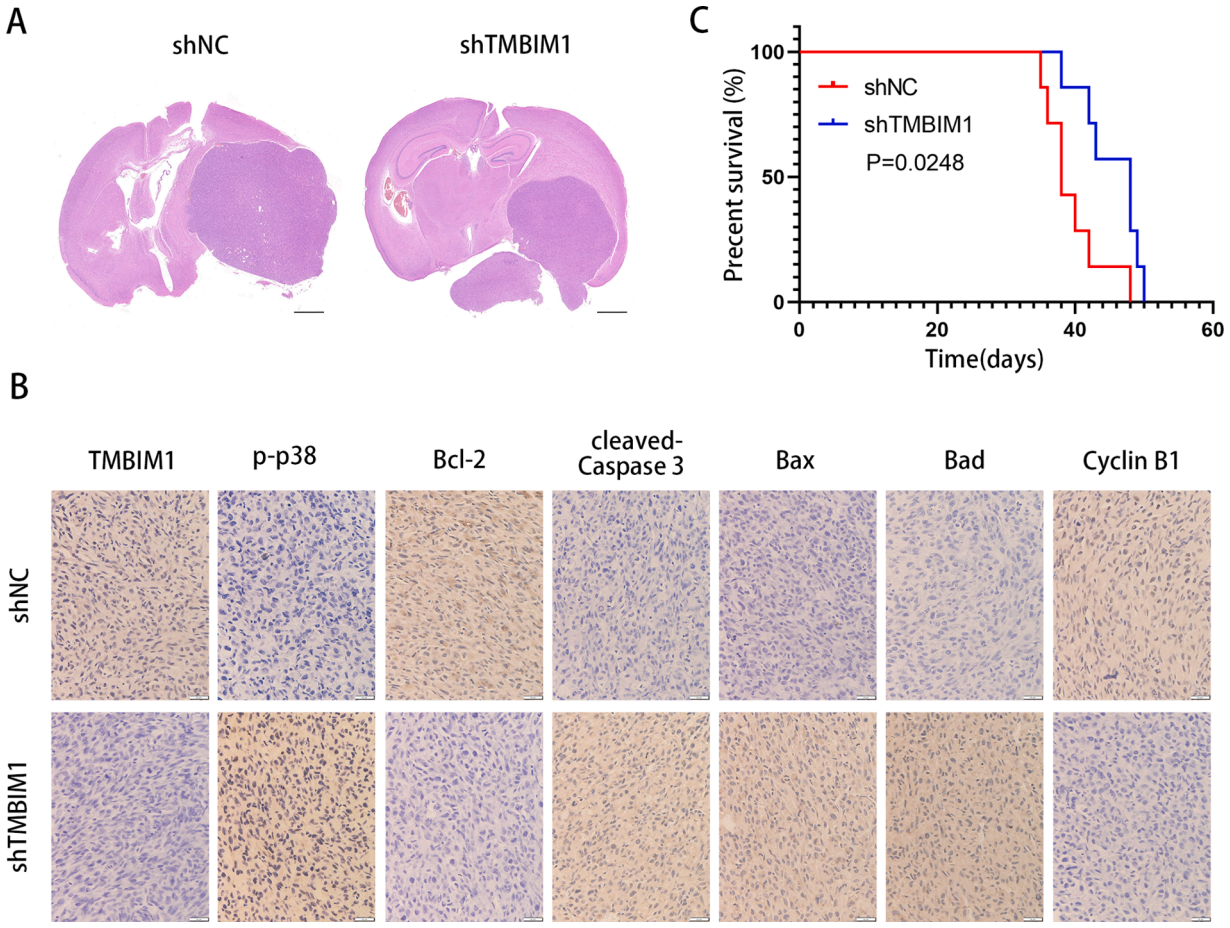

Fig. 7. TMBIM1 knockdown induced cell apoptosis and suppressed tumour growth in intracranial Xenograft model. (A) Representative images of HE stained mouse brain sections. shNC groups, $n = 7$; shTMBIM1 groups, $n = 7$. Scale bars: 1000 μm. **(B)** IHC analyses of TMBIM1, p-p38, Bcl-2, cleaved Caspase 3, Bax, Bad and cyclin B1in mouse tumour sections. Scale bars: 20 μm. **(C)** Mouse survival is shown by Kaplan-Meier curves. shNC groups, $n = 7$; shTMBIM1 groups, $n = 7$. The log-rank test was used to measure survival differences in shNC and shTMBIM1 group.

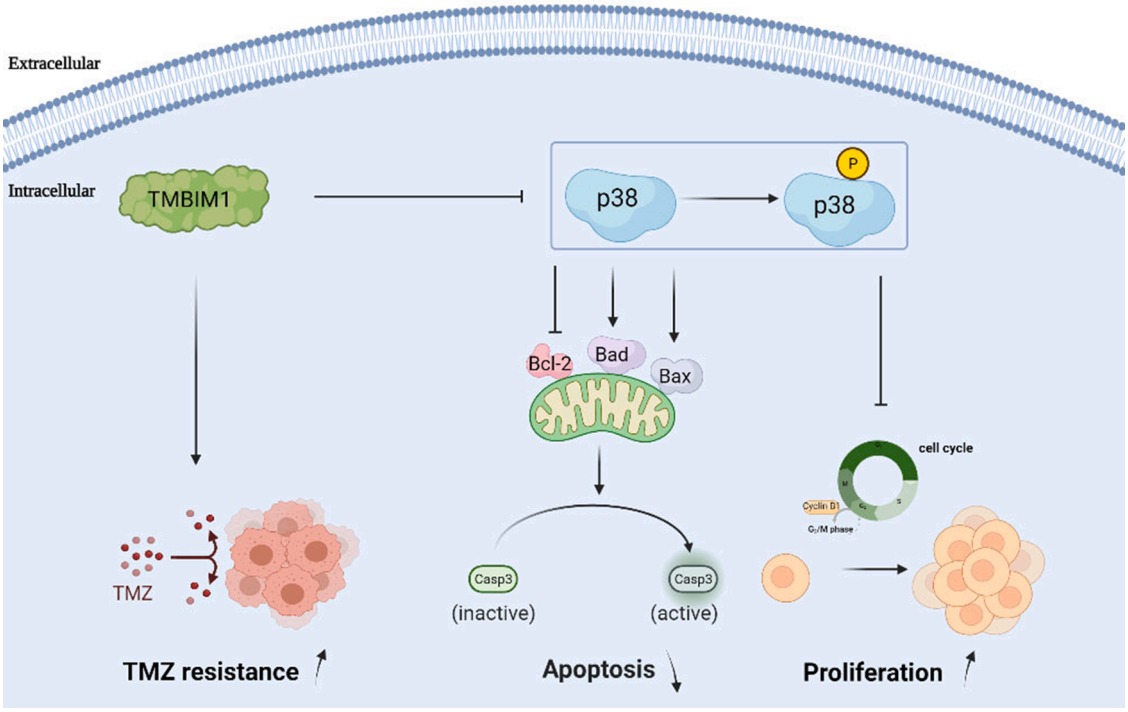

Fig. 8. Mechanistic model for the TMBIM1 regulation of cell proliferation, apoptosis, cell cycle and TMZ resistance in GBM.

First, we found that TMBIM1 was highly expressed in GBM and associated with glioma malignancy, so we speculated that TMBIM1 might be an oncogenic gene in GBM. Considering that excessive proliferation of cells is one of the hallmarks of tumours [27], we explored whether TMBIM1 was related to the proliferation of GBM cells and found that TMBIM1 knockdown could indeed inhibit proliferation in GBM cells. Meanwhile, TMBIM1 knockdown suppressed tumour growth, which was confirmed in an intracranial xenograft model. The cell cycle is usually closely related to cell proliferation. Harashima H et al. mentioned that the cell cycle in eukaryotes has four phases, G0/G1, S, G2, and M, controlled by a series of complex signal pathways, checkpoints, kinases and other proteins [28]. The synthesis of the CDK1/CyclinB1 complex is crucial in regulating the transition of the G2/M phase [29]. Similarly, our results showed that TMBIM1 knockdown arrested the cell cycle in the G2/M phase and downregulated cyclin B1 expression levels in GBM. Based on these results, we could conclude TMBIM1 might regulate the cell cycle in G2/M phase to promote GBM cells proliferation.

Most malignant tumours have the characteristics of evading apoptosis [30]. Apoptosis involves a variety of pathways, including exogenous pathways induced by death receptors and endogenous pathways mediated by mitochondria, ultimately achieved by activating caspase-3. among them, the pro-apoptotic factors Bax and Bad and the anti-apoptotic factor Bcl-2 in the Bcl-2 family can significantly regulate the activation of caspases in the mitochondria-mediated pathway [31, 32]. In previous studies, it has been shown that the TMBIM protein family is involved in the inhibition of endogenous and exogenous programmed cell death in mammals [10–12]. TMBIM1, as one of its members, has not been further studied in GBM. In our research, the results demonstrated that TMBIM1 knockdown could induce cell apoptosis. Western blot analysis showed that the pro-apoptotic proteins (Bax and BAD) of the Bcl-2 family were significantly increased, while the anti-apoptotic protein Bcl-2 was significantly decreased in the TMBIM1 knockdown groups, and the apoptotic terminal protein cleaved-caspase3 was significantly increased. In addition, these apoptosis-related proteins were similarly altered in intracranial xenograft model. In summary, these results indicated that TMBIM1 regulates caspase3 by regulating apoptosis-related proteins in the mitochondrial-mediated pathway to attenuate GBM cell apoptosis in vitro and in vivo. These results not only filled the gap in the role of TMBIM1 in apoptosis and GBM to some extent but also provided ideas for the subsequent study of TMBIM1 in tumour.

Numerous studies have revealed the roles of the dysregulation of apoptosis pathways in chemoresistance of gliomas, and the BCL-2 pathway confers resistance against TMZ in GBM cell treatment [21]. Therefore, we explored the role of TMBIM1 in TMZ resistance in glioma. Our results showed that TMBIM1 segregated LGG and GBM patients who received TMZ treatment at any time into different survival groups, which suggested that TMBIM1 might be related to TMZ resistance. The DNA repair enzyme MGMT is a recognized predictor for tumour response to temozolomide and MGMT promoter methylation is the key mechanism of MGMT gene silencing [33]. Our results showed that the prognosis of patients with high TMBIM1 expression who had methylated MGMT was similar to that of patients with unmethylated MGMT. among those patients, patients with methylated MGMT and low TMBIM1 expression had the best prognosis. Taken together, these results demonstrated that TMBIM1 might be able to regulate the sensitivity of GBM cells to TMZ. Based on our results, subsequent studies could also further explore whether TMBIM1 can serve as a novel biomarker of response to TMZ in glioma. However, these results were just found from public datasets, and a large number of molecular biological experiments are needed to verify and further explore the role of TMBIM1 in regulating the sensitivity of GBM cells to TMZ.

The p38/MAPK pathway plays an important role in cell death and survival. The Cascades of phosphorylation events can activate the p38 signalling pathway and activated phosphorylated p38 further activates a variety of substrates to mediate functions of p38 signalling in diverse cellular processes such as cell cycle regulation, proliferation, survival, and death [34]. Our results showed that TMBIM1 knockdown activated the p38/MAPK pathway by phosphorylating p38. Thornton TM and Hui L et al. revealed that the activation of the p38/MAPK pathway induced cell cycle arrest through the establishment of G2/M cell cycle checkpoint and inhibited cell proliferation in cancer [35,36]. Consistent with these studies, our results revealed that TMBIM1 promoted proliferation and

that TMBIM1 knockdown promoted cyclin B1-mediated G2/M cell cycle arrest, which suggested that the Cell cycle-mediated proliferation effect of TMBIM1 might be related to the p38/MAPK pathway in GBM. The p38/MAPK pathway can promote apoptosis and regulate pro-apoptotic and anti-apoptotic proteins, such as Bcl-2 family proteins and caspase family proteins [13]. Similarly, our results showed that Bcl-2 family proteins (Bad and Bax) and caspase family proteins (cleaved-Caspase 3) were increased, while Bcl-2 was decreased when the p38/MAPK pathway was activated both in vitro and in an intracranial xenograft model. These results showed that TMBIM1′s attenuation of mitochondrial-mediated apoptosis might be achieved through the p38/MAPK pathway.

To further demonstrate our conclusions, we performed rescue experiments. There are four isomers of p38/MAPK, including p38α, p38β, p38γ and p38δ. p38α is highly abundant in most cell types and it plays a major role in p38/MAPK. Therefore, we used BIRB796, a highly effective p38α inhibitor, to conduct rescue experiments. Our results showed that BIRB796 reversed the cell apoptosis and anti-proliferation induced by TMBIM1 knockdown in two GBM cell lines. In summary, our results demonstrated that TMBIM1 regulated GBM cell apoptosis and proliferation through the p38/MAPK pathway both in vitro and in vivo. Our results enriched the regulation of p38 MAPK signalling in GBM and potential molecular mechanism of TMBIM1 in GBM. However, the specific interaction between TMBIM1 and p38 is still unclear. Since TMBIM1 is not a phosphatase, it cannot activate p38 directly, and it might activate p38 indirectly by interacting with other molecules, which would be the highlight of our future research.

In conclusion, TMBIM1 has multiple tumour-promoting effects in GBM, including promoting proliferation, decreasing apoptosis, and regulating TMZ resistance. Unlike many anticancer agents targeting only one of these compartments, therapy targeting TMBIM1 might inhibit GBM growth and progression in multiple ways, which provides a new idea for the treatment of GBM. Although our results were only validated on the level of two GBM cell lines and mice without the validated in more patient tissues, our research would provide an important basis for future intensive molecular mechanism research in GBM and present a potential therapeutic target for treating GBM .

Availability of data and material

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

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Ethics approval and consent to participate China

The following information was supplied related to ethical approvals (i.e., approving body and any reference numbers): Institutional Ethics Committee of the Faculty of Medicine at Renmin Hospital of Wuhan University approval (2012LKSZ (010) H) to carry out the study within its facilities.

CRediT authorship contribution statement

Jiayang Cai: Conceptualization, Methodology, Writing – review & editing. **Lun Gao:** Conceptualization, Methodology, Writing – review & editing. **Yixuan Wang:** Methodology, Writing – review & editing. **Yong Li:** Formal analysis, Writing – original draft, Writing – review & editing. **Zhang Ye:** Formal analysis, Writing – original draft, Writing – review & editing. **Shiao Tong:** Formal analysis, Writing – original draft, Writing – review & editing. **Tengfeng Yan:** Formal analysis, Writing – original draft, Writing – review & editing. **Qian sun:** Formal analysis, Writing – original draft, Writing – review & editing. **Yang Xu:** Formal analysis, Writing – original draft, Writing – review & editing. **Hongxiang Jiang:** Formal analysis, Writing – original draft, Writing – review & editing. **Si Zhang:** Formal analysis, Writing – original draft, Writing – review & editing. **Linyao Zhao:** Formal analysis, Writing – original draft, Writing – review & editing. **Ji'an Yang:** Conceptualization, Writing – review & editing. **Qianxue Chen:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors have declared that no competing interest exists.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101391.

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