

Knockdown of ETV4 promotes autophagy-dependent apoptosis in GBM cells by reducing the transcriptional activation of EMP1

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Abstract. ETS variant transcription factor 4 (ETV4) is a common cancer-promoting transcription factor and its expression has been found to be significantly upregulated in glioblastoma multiforme (GBM), as determined via analysis of the Gene Expression Profiling Interactive Analysis (GEPIA) database. In addition, our previous study demonstrated that ETV4 expression was highly positively correlated with epithelial membrane protein 1 (EMP1). The present study aimed to determine whether ETV4 could influence the activation of the PI3K/AKT/mTOR signaling pathway to affect the autophagy and apoptosis of GBM cells by regulating the transcriptional activity of EMP1. In addition to the analysis of the GEPIA database, the expression levels of ETV4 were also investigated in several different GBM cell lines. After interfering with the expression of ETV4, western blotting was used to detect the expression levels of autophagy- and apoptosis-related proteins, and a TUNEL assay was used to detect the levels of cell apoptosis. Dual luciferase reporter and chromatin immunoprecipitation assays were used to verify the potential binding site of ETV4 on EMP1. Western blotting was also used to analyze the expression levels of PI3K/AKT/mTOR signaling pathway-related proteins. The results of the current study revealed that the expression levels of ETV4 were significantly upregulated in GBM cell lines compared with those in normal glial cells. In the GBM cell line, LN-229, ETV4 was discovered to bind to the EMP1 promoter and positively regulate the expression of EMP1. The knockdown of ETV4 expression inhibited the PI3K/AKT/mTOR signaling pathway activity to promote autophagy and apoptosis, and this effect could be partially reversed by overexpressing EMP1. In conclusion,

these findings indicated that the knockdown of ETV4 in GBM cells may reduce the transcriptional activation of EMP1 and thereby inhibit PI3K/AKT/mTOR signaling pathway activity to promote autophagy and apoptosis. This provides a novel insight into potential strategies for the treatment of GBM via the induction of autophagy-dependent apoptosis.

Introduction

Glioblastoma multiforme (GBM) is a heterogeneous brain tumor with a high degree of malignancy, which can be divided into two subtypes, primary and secondary, both of which have potent invasive and proliferative abilities. GBM accounts for 70% of brain tumors diagnosed (1). GBM stem cells (GSCs) were recently discovered in GBM and are considered to be pivotal to the disease, driving tumor initiation due to their regenerative capacity, which is capable of reproducing all the biological functions of GBM tumors (2). GSCs show stronger resistance to conventional therapies than normal neural stem cells (3), resulting in a poor prognosis in patients with GBM, with a median survival rate of only 12-15 months (4). Therefore, novel effective and successful treatment methods are urgently required to eliminate the entire tumor mass.

ETS variant transcription factor 4 (ETV4) is involved in the progression of a variety of types of cancer. For example, in colorectal cancer, it was discovered to promote the epithelial-mesenchymal transition via the ERK/EGFR signaling pathway (5). In clear cell renal cell carcinoma (ccRCC), ETV4 was found to activate the metastasis-promoting gene FOS like 1, AP-1 transcription factor subunit in a PI3K/AKT-dependent manner to promote the migration and invasion of ccRCC cells (6). In addition, in non-small cell lung cancer, ETV4 promoted cancer progression by upregulating paxillin and MMP1 expression (7). However, to the best of our knowledge, the expression pattern of ETV4 in GBM has yet to be reported. Our previous study found that silencing EMP1 could inhibit the proliferation and migration of GBM cells by inhibiting the activation of the PI3K/AKT signaling pathway, which reduced the expression of the GSC stemness marker, CD44 (8). Furthermore, analysis from the Gene Expression Profiling Interactive Analysis (GEPIA) database showed that the expression of epithelial membrane protein 1 (EMP1) was highly positively associated with ETV4, and subsequent analysis from the JASPAR CORE database identified a

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binding site for ETV4 on the promoter of EMP1. Miao *et al.* (9) also reported that EMP1 could promote the proliferation and invasion of glioma cells by activating the PI3K/AKT/mTOR signaling pathway. This previous study also successfully knocked down the expression of EMP1 in athymic nude mice, which was discovered to successfully inhibit the growth of tumors *in vivo*, subsequently improving the overall survival rate of tumor-bearing animals. Furthermore, another study demonstrated that inhibiting the expression of the autophagy inhibitor, mTOR, could effectively activate and promote lethal autophagy in GBM (10). In fact, autophagy-dependent apoptosis has been suggested to be able to synergistically enhance the cytotoxicity of chemotherapy drugs towards GBM, alleviate the resistance of GBM cells to chemotherapy drugs and promote tumor mass ablation (11).

The present study aimed to determine whether ETV4 could influence the activation of the PI3K/AKT/mTOR signaling pathway to affect the autophagy and apoptosis of GBM cells by regulating the transcriptional activity of EMP1. The expression levels of ETV4 were analyzed in several GBM cell lines, and the relationship between EMP1 and EMP1 was determined. In addition, ETV4 expression was knocked down to determine its effect on the EMP1/PI3K/AKT/mTOR signaling axis and GBM cell autophagy-dependent apoptosis.

Materials and methods

Bioinformatics analysis. The expression of ETV4 in GBM was analyzed using the GEPIA database (<http://gepia.cancer-pku.cn>). Survival outcomes according to ETV4 status in primary glioma were analyzed using the Chinese Glioma Genome Atlas (CGGA) database (<http://www.cgga.org.cn/analyse/RNA-data.jsp>; dataset no. mRNAseq_325). The binding sites of ETV4 on the EMP1 promoter were predicted using the JASPAR CORE database (<http://jaspar.genereg.net>).

Cell lines and culture. A172 (cat. no. CRL-1620; American Type Culture Collection), LN-229 (cat. no. CRL-2611; American Type Culture Collection), BS149 (provided by the Clinical and Experimental Pathology Laboratory at the Xuzhou Medical University, Xuzhou, China) and U251 (serial no. TCHu 58; The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were cultured in DMEM (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% FBS (Beijing Solarbio Science & Technology Co., Ltd.) and 1% penicillin-streptomycin, and maintained at 37°C in a humidified incubator with 5% CO₂. All cell lines were used within 10 passages of their initial culture. The normal human astrocytes (NHAs) cells (cat. no. CC-2565; Lonza Group, Ltd.) were cultured according to the manufacturer's recommendations. Rapamycin (RAP; 100 nM; Beijing Solarbio Science & Technology Co., Ltd.), as a specific inhibitor of mTOR protein, was incubated with cells at 37°C for 24 h.

Cell transfection. Small interfering RNA (siRNA/si) sequences against ETV4 (si-ETV4-1/si-ETV4-2) and a non-targeting si-negative control (NC) were synthesized and purified by Guangzhou RiboBio Co., Ltd. The sequences were as follows: si-ETV4-1, 5'-TTGGATGTTGGAGAAAATGGA-3'; si-ETV4-2, 5'-TCCAGATCATTCCTTTAGTTT-3'; and si-NC,

5'-GGCTCTAGAAAAGCCTATGC-3'. The siRNAs (10 nM) were transfected into LN-229 cells (3x10⁵ cells/well in 6-well plates) for 48 h at 37°C using Lipofectamine[®] RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols.

The plenti6/V5-DEST vector was purchased from Shaanxi Youbio Technology Co., Ltd., and used to carry ETV4 or EMP1 cDNA. Briefly, the sequences of ETV4 or EMP1 cDNA were cloned into the *Bam*HI and *Asc*I sites of the plenti6/V5-DEST vector; a non-targeting cDNA sequence [overexpression (Oe)-NC] was used as the control. After amplification and DNA sequencing, the vectors (50 nM) were transduced into LN-229 cells (3x10⁵ cells/well in 6-well plates) using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Transfection efficiency was detected via reverse transcription-quantitative PCR (RT-qPCR) at 48 h after transfection.

RT-qPCR. Total RNA was extracted from the cultured cell lines using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScript[™] RT reagent kit (Perfect Real Time) (Takara Bio, Inc.) according to the manufacturer's instructions. qPCR was subsequently performed on a StepOnePlus[™] Real-Time PCR system (Thermo Fisher Scientific, Inc.) using a One Step TB Green[®] PrimeScript[™] plus RT-PCR kit (Perfect Real Time) (Takara Bio, Inc.), according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: Initial denaturation at 42°C for 5 min and 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The following primers pairs for ETV4, EMP1 and β -actin were used for qPCR: ETV4 forward, 5'-GAAAAACAA GTCGGTGCCT-3' and reverse, 5'-GGGGGCCGAGAC CTGA-3'; EMP1 forward, 5'-ATGCCAGTGAAGATGCCC TC-3' and reverse, 5'-TG TAGATGGACACCCCA-3'; and β -actin forward, 5'-GCCGCCAGCTCACCAT-3' and reverse, 5'-TCGTCGCCACATAGGAATC-3'. β -actin was used for normalization. The relative expression levels of target genes were calculated using the 2^{- $\Delta\Delta$ C_q} method (12).

Western blotting. For western blotting, the protein samples were prepared as previously described (8). Proteins (20 μ g/lane) were separated via 10% SDS-PAGE (Beyotime Institute of Biotechnology) and subsequently transferred onto PVDF membranes (MilliporeSigma). After incubating the membranes in 5% skimmed milk powder dissolved in TBS with 0.1% Tween-20 (TBST) for 2 h at room temperature, they were incubated with a primary antibody at 4°C overnight. Following the primary antibody incubation, the membranes were washed three times with TBST and incubated with a HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using SuperSignal West Pico Plus (Thermo Fisher Scientific, Inc.) and a GS800 densitometer scanner (Bio-Rad Laboratories, Inc.). Densitometric analysis was performed using ImageJ v1.46 software (National Institutes of Health). The following primary antibodies were used: Mouse anti-ETV4 (1:1,500; cat. no. sc-113; Santa Cruz Biotechnology, Inc.), rabbit anti-EMP1 (1:1,000; cat. no. ab230445; Abcam), mouse anti-GAPDH (1:5,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.), mouse anti-Beclin-1 (1:2,000; cat. no. ab118148; Abcam),

rabbit anti-LC3B I and II (1:2,000; cat. no. ab192890; Abcam), rabbit anti-p62 (1:40,000; cat. no. ab109012; Abcam), rabbit anti-cleaved-caspase 9 (1:500; cat. no. ab2324; Abcam), rabbit anti-caspase 9 (1:10,000; cat. no. ab32539; Abcam), rabbit anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), rabbit anti-Bax (1:2,000; cat. no. ab182733; Abcam), rabbit anti-phosphorylated (p)-AKT (1:2,000; cat. no. 4060S; Cell Signaling Technology, Inc.), rabbit anti-AKT (1:1,000; cat. no. 9272S; Cell Signaling Technology, Inc.), rabbit anti-p-mTOR (1:1,000; cat. no. ab177734; Abcam) and rabbit anti-mTOR (1:3,000; cat. no. ab32028; Abcam). The following HRP-conjugated secondary antibodies were used: Goat anti-rabbit IgG H&L (HRP; 1:20,000; cat. no. ab6721; Abcam) and goat anti-mouse IgG H&L (HRP; 1:10,000; cat. no. ab6789; Abcam).

TUNEL assay. Apoptosis was analyzed using a TUNEL apoptosis detection kit (Beyotime Institute of Biotechnology). Briefly, the transfected LN-229 cells were seeded into a 24-well plate at a density of 5×10^4 cells/well, and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then washed once with PBS and incubated in 0.3% Triton-X 100 in PBS for 5 min at room temperature, before being washed again with PBS. TUNEL detection solution was prepared according to the manufacturer's protocol and 50 μ l was added to each sample and incubated at 37°C for 60 min in the dark. DAPI staining solution (10 μ g/ml) was used to visualize nuclei for 5 min at 37°C. Following the incubation, the cells were washed three times with PBS, mounted with anti-fluorescence quenching mounting solution and three random fields were visualized using a fluorescence microscope (Olympus Corporation). The apoptosis levels were estimated as the ratio of the number of TUNEL-positive cells to the number of Hoechst-positive cells, and each experiment was repeated in triplicate.

Dual luciferase gene reporter assay. A dual luciferase reporter gene assay kit (Beyotime Institute of Biotechnology) was used to detect the regulation of ETV4 on EMP1 promoter elements, according to the manufacturer's protocol. Briefly, the promoter region of EMP1 (amplified by Shanghai GenePharma Co., Ltd.) containing wild-type (WT, GGAGGAAGAC) and mutant (MUT, GAGAATTCCC) sequences, located from positions -405 to -396 upstream of the EMP1 transcription start site, were inserted as *XhoI/BglIII* fragments upstream of the luciferase gene into pGL4-luc (Promega Corporation). The luciferase reporter plasmids and regulatory factors were co-transfected into LN-229 cells using Lipofectamine 3000 reagent. After 48 h, cells were subsequently lysed with lysis buffer and incubated with firefly luciferase detection reagent. The relative light unit (RLU) was measured after mixing, and the reporter gene cell lysate was obtained as a blank control. Following this step, *Renilla* luciferase detection working solution was added to the cells and the RLU was measured after mixing. The activation degree of the reporter gene was defined as the value obtained by dividing the firefly RLU by the *Renilla* RLU, with *Renilla* luciferase acting as the internal reference. Each experiment was repeated at least three times.

Chromatin immunoprecipitation assay. The SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling Technology, Inc.) was used to detect the binding between ETV4 and

EMP1 promoters, according to the manufacturer's protocol. Briefly, the LN-229 cells were plated into a 15-cm plate and cross-linked at 37°C for 10 min with a final concentration of 1% formaldehyde. Glycine (0.125 M) was subsequently added to terminate the cross-linking, following which the cells were washed with PBS and centrifuged at 1,000 x g for 5 min at 4°C. The supernatant was removed and SDS lysis buffer containing a protease inhibitor complex (Beijing Solarbio Science & Technology Co., Ltd.) was added to obtain an immunoprecipitated (IP) preparation. Then, 0.5 μ l micrococcal nuclease was added to each IP preparation, which was mixed by inverting the centrifuge tube several times, and then incubated at 37°C for 20 min to digest the DNA to a length of ~150-900 bp. EDTA was subsequently added to terminate the digestion, and the cells were centrifuged at 16,000 x g for 1 min at 4°C to pellet the nuclei and remove the supernatant. A VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator (set to 6 and equipped with a 1/8-inch probe for 4 sets of 15-sec pulses) was used to lyse LN-229 cell nuclei. The lysate was centrifuged in a microcentrifuge at 9,400 x g for 10 min at 4°C to clarify the lysate and collect the supernatant. Subsequently, RNase A and Proteinase K were used for chromatin digestion and analysis of product concentration. Part of the product was set aside to act as the input group, while 1 μ g mouse anti-ETV4 (1:50; cat. no. sc-113; Santa Cruz Biotechnology, Inc.) or 1 μ g mouse anti-IgG (1:50; cat. no. sc-2025; Santa Cruz Biotechnology, Inc.) antibodies and 40 μ l protein A/G magnetic beads (MilliporeSigma) were added to the remaining product and incubated overnight at 4°C. Following the incubation, the immune complexes were precipitated and washed. After decrosslinking at 65°C overnight, DNA was recovered and subjected to RT-qPCR analysis as described above.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, Inc.). Data are presented as the mean \pm SD of at least three independent experiments. Statistical differences between two groups were determined using an unpaired Student's t-test, while those between multiple groups were determined using a one-way ANOVA followed by Tukey's post hoc test. In addition, survival rates were statistically analyzed using the Kaplan-Meier analysis followed by a log-rank test and the correlation between ETV4 and EMP1 in GBM was determined using Pearson's analysis. $P < 0.05$ was considered to indicate a statistically significant difference, and $|r| > 0.3$ (for Pearson's analysis) was considered to indicate a significant correlation.

Results

ETV4 is upregulated in GBM and associated with poor prognosis. The expression levels of ETV4 in GBM tissues were discovered to be significantly upregulated compared with those in normal tissues (Fig. 1A), according to analysis by the GEPIA database. Similar results were also observed in GBM cell lines (A172, BS149, U251 and LN-229) compared with NHAs via western blotting (Fig. 1C and D) and RT-qPCR (Fig. 1E). The expression of ETV4 was upregulated to the greatest extent in LN-229 cells; therefore, this cell line was selected for use in subsequent experiments. Analysis from the CGGA database also revealed a poor survival prognosis for

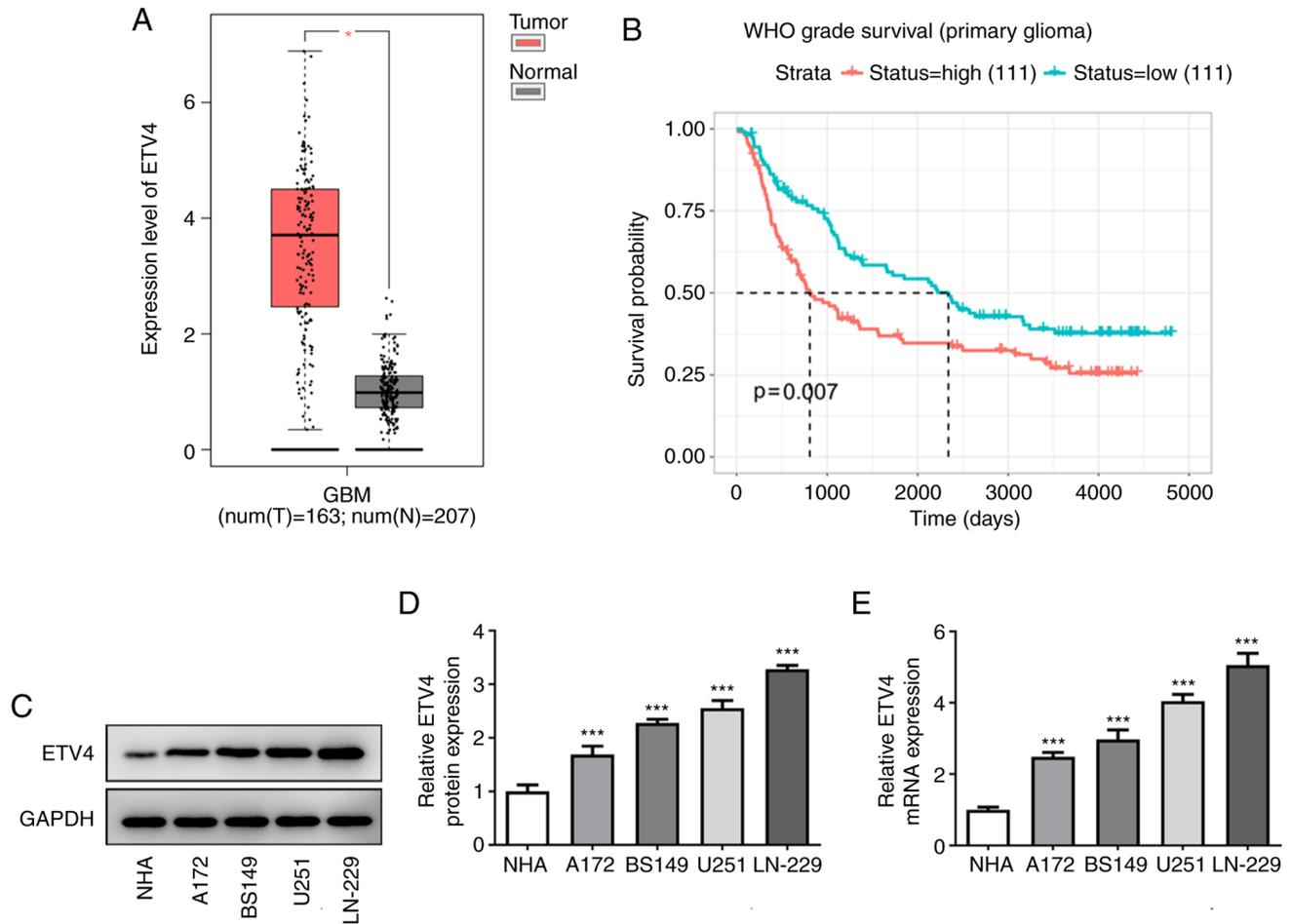


Figure 1. Expression of ETV4 in GBM tissues and cell lines and its influence on the survival probability of GBM. (A) Gene Expression Profiling Interactive Analysis database found that the expression of ETV4 in GBM tumor tissues (n=163) was upregulated compared with that in normal tissues (n=207). *P<0.05 vs. normal tissue. (B) Chinese Glioma Genome Atlas database analysis found that the survival probability of patients with primary glioma with high expression levels of ETV4 was significantly reduced. P=0.007 vs. status=Low. (C) Representative western blots of ETV4 expression levels in NHAs and several GBM cell lines (A172, BS149, U251 and LN-229). GAPDH served as the loading control. Histogram comparing the (D) protein and (E) mRNA expression levels of ETV4 in different cell lines. n=3. ***P<0.001 vs. NHAs. ETV4, ETS variant transcription factor 4; GBM, glioblastoma multiforme; T, tumor; N, normal; NHAs, normal human astrocytes.

patients with high ETV4 expression (Fig. 1B), which suggested that ETV4 may play an important role in promoting GBM progression.

si-ETV4 promotes autophagy and apoptosis in LN-229 cells. siRNA was used to silence ETV4 expression in LN-229 cells. Following the analysis of the transfection efficiency (Fig. 2A), si-ETV4-1 was found to exert the most effective interference effect. In order to reduce the possibility of an off-target effect, si-ETV4-1 and si-ETV4-2 were both selected for use in follow-up experiments to explore the effect of ETV4 on autophagy and apoptosis.

The protein expression levels of the autophagy marker, Beclin-1, were first analyzed, and it was found that, compared with those observed in the si-NC group, the expression levels of Beclin-1 in the si-ETV4 group were significantly upregulated (Fig. 2B and C). In addition, following the knockdown of ETV4 expression, the expression levels of LC3B II/I (the ratio of LC3B II/LC3B I) were discovered to be upregulated, while p62 expression levels were downregulated (Fig. 2B, D and E), indicating that

silencing ETV4 expression may activate autophagic flux in LN-229 cells.

Silencing ETV4 expression also increased the number of TUNEL-positive cells (green fluorescence) (Fig. 2F). The cell apoptosis rate was significantly higher in the si-ETV4-1 group compared with the si-NC group (Fig. 2G). These results were validated via western blotting. In more detail, compared with the si-NC group, the expression levels of cleaved-caspase 9 were found to be upregulated in the si-ETV4 groups (Fig. 2H and K). Notably, the activation of caspase was not caused by the increase in the concentration of zymogen (Fig. 2H). Furthermore, the expression levels of the anti-apoptotic protein, Bcl-2, were found to be downregulated (Fig. 2H and I), while the expression levels of the proapoptotic protein, Bax, were upregulated (Fig. 2H and J). Since, similar results were obtained using si-ETV4-1 and si-ETV4-2 specific knockdown of ETV4 in LN-229 cells, it was concluded that the knockdown of ETV4 may activate the endogenous apoptotic pathway of LN-229 cells, which is regulated by the caspase 9 cascade. si-ETV4-1 was selected for subsequent experiments due to its more effective interference effect.

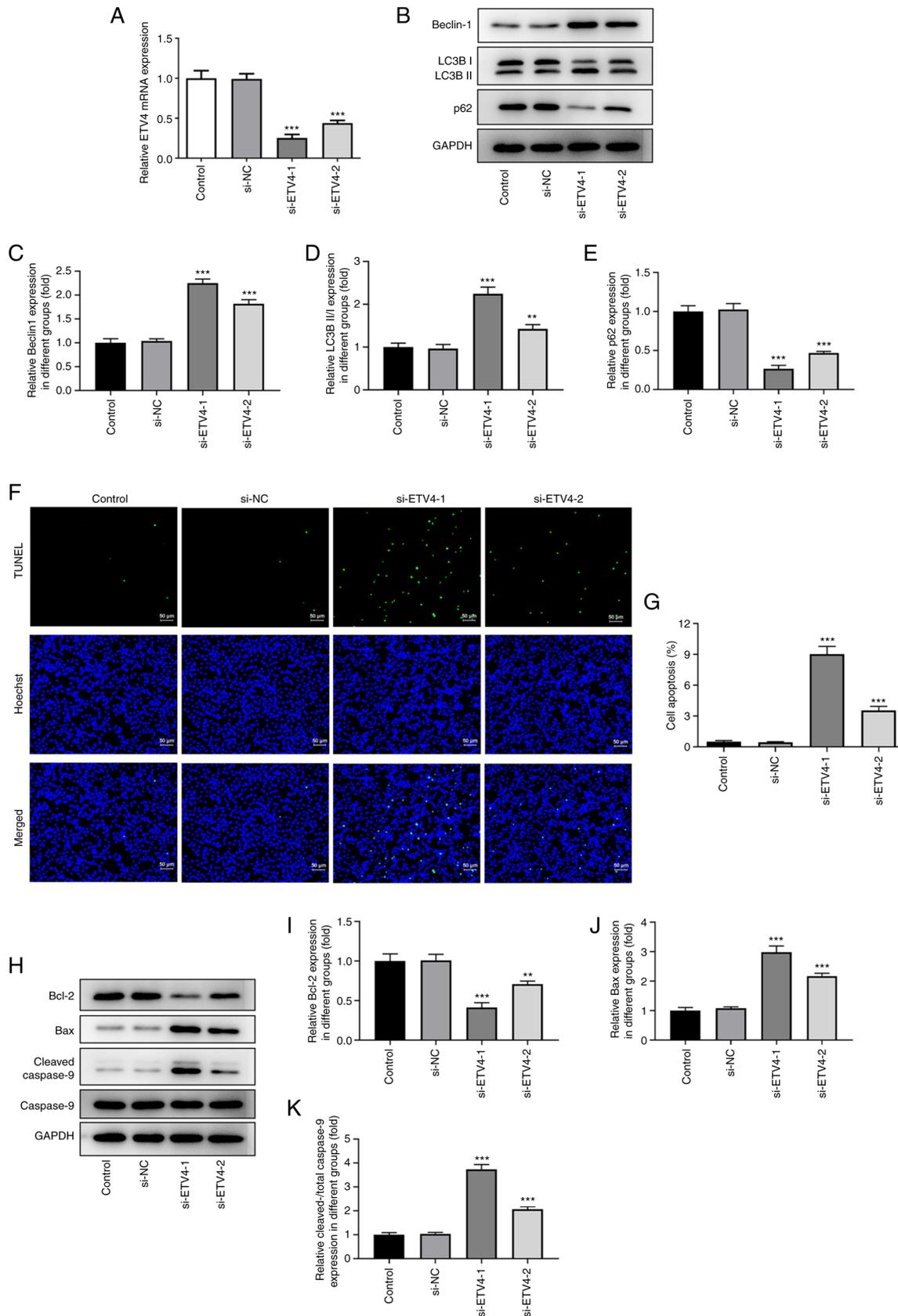


Figure 2. Low expression of ETV4 in LN-229 cells promotes autophagy and apoptosis. (A) Knockdown efficiency of si-ETV4-1/2 was analyzed via reverse transcription-quantitative PCR (n=3). (B) Western blotting was used to analyze Beclin-1, LC3B II/I and p62 expression levels in different groups (control, si-NC, si-ETV4-1 and si-ETV4-2; n=3). GAPDH served as the loading control. Semi-quantification of (C) Beclin-1, (D) LC3B II/I and (E) p62 protein expression levels. (F) TUNEL assay was used to determine the effect of ETV4 on LN-229 cell apoptosis (n=3; TUNEL, green; Hoechst, blue). Scale bar, 50 μm. (G) Percentage of TUNEL-positive cells. (H) Representative western blots demonstrating Bcl-2, Bax, cleaved-caspase 9 and caspase 9 expression levels in different groups (control, si-NC, si-ETV4-1 and si-ETV4-2; n=3). GAPDH served as the loading control. Semi-quantification of (I) Bcl-2, (J) Bax, (K) cleaved-caspase 9 protein expression levels. Untransfected cells were used as the control. **P<0.01, ***P<0.001 vs. si-NC. ETV4, ETS variant transcription factor 4; LC3B II/I, LC3B II/LC3B I; si, small interfering RNA; NC, negative control.

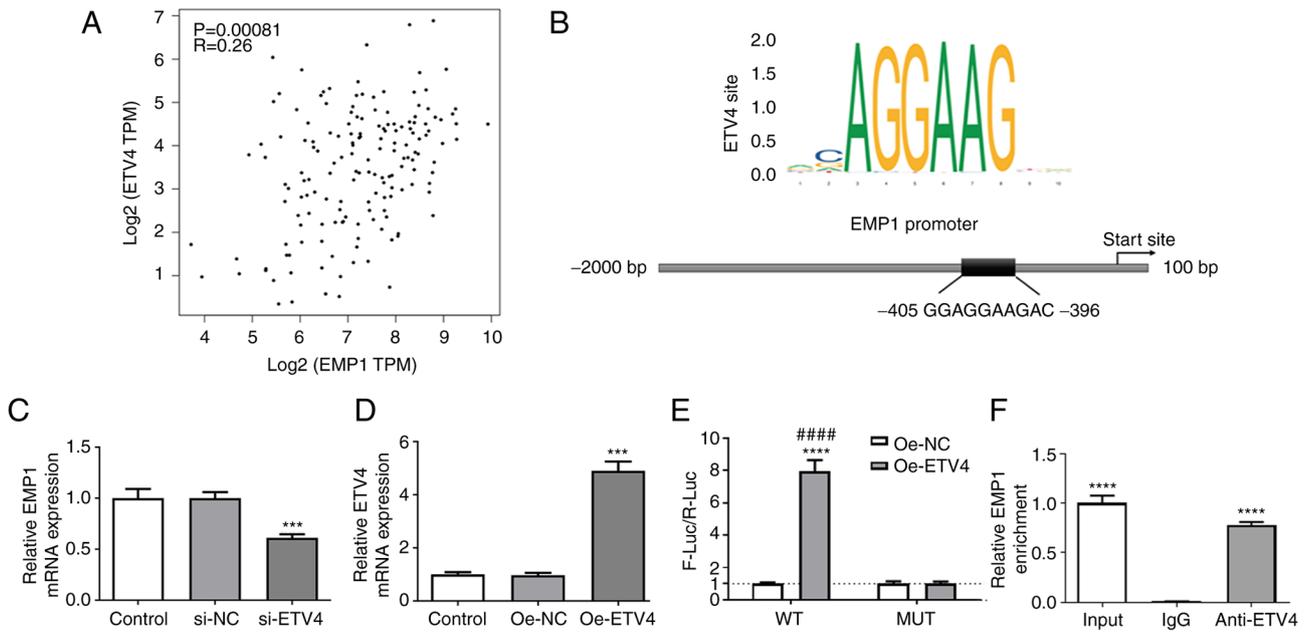


Figure 3. ETV4 binds to the promoter region of EMP1 to positively regulate its expression. (A) Gene Expression Profiling Interactive Analysis database revealed a weak positive correlation between the expression of ETV4 and EMP1 in glioblastoma multiforme tissues ($P=0.00081$; $R=0.26$). (B) JASPAR database predicted the binding site of ETV4 in the EMP1 promoter region. (C) EMP1 expression levels in LN-299 cells following the transfection with si-NC or si-ETV4 were determined via RT-qPCR. $n=3$. $***P<0.001$ vs. si-NC. (D) Efficiency of ETV4 overexpression was analyzed via RT-qPCR. Untransfected cells were used as the control. $n=3$. $***P<0.001$ vs. Oe-NC group. (E) Dual luciferase reporter gene assay was used to determine the interaction between ETV4 and the EMP1 promoter region in LN-229 cells. $n=3$. $****P<0.0001$ vs. WT-EMP1 + Oe-NC group; $####P<0.0001$ vs. MUT-EMP1 + Oe-ETV4 group. (F) Chromatin immunoprecipitation assay was used to reveal that ETV4 could bind to the promoter region of EMP1. IgG was used as the negative control immunoprecipitate. $n=3$. $****P<0.0001$ vs. IgG. ETV4, ETS variant transcription factor 4; EMP1, epithelial membrane protein 1; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA; NC, negative control; Oe, overexpression; WT, wild-type; MUT, mutant.

ETV4 activates EMP1 transcription in LN-229 cells. A previous study revealed that the upregulated expression of EMP1 promoted the proliferation and invasion of glioma cells by activating the PI3K/AKT signaling pathway (7). The JASPAR database was used to predict the binding site of ETV4 in the EMP1 promoter region (Fig. 3B). Therefore, it was hypothesized that ETV4 may participate in the regulation of GBM disease progression by activating the transcription of EMP1. To test this hypothesis, the effect of silencing ETV4 expression in LN-229 cells on the expression of EMP1 was first analyzed. Although, using GEPIA database analysis, the correlation coefficients ($R=0.26$) of EMP1 and ETV4 indicated that they were negligibly correlated in GBM (Fig. 3A). The results revealed that the mRNA expression levels of EMP1 were significantly downregulated following the knockdown of ETV4 (Fig. 3C). Next, an ETV4 overexpression vector was successfully constructed *in vitro* (Fig. 3D), and the results of the dual luciferase reporter gene assay, which was used to detect the promoter activity of EMP1, found that the overexpression of ETV4 increased luciferase expression driven by an EMP1 promoter fragment containing a putative ETV4 binding site (Fig. 3E). The results of the chromatin immunoprecipitation assay in LN-229 cells also demonstrated that ETV4 bound to the promoter region of EMP1 (Fig. 3F). These results indicated that ETV4 may target the promoter region of EMP1 to positively regulate the expression of EMP1 in LN-229 cells.

Overexpression of EMP1 reverses the promoting effect of si-ETV4 on autophagy and apoptosis. To verify whether the pro-autophagic and proapoptotic effects of si-ETV4 in LN-229

cells were achieved by downregulating the expression of EMP1, an EMP1 overexpression vector was successfully established *in vitro* (Fig. 4A-C). The vector was transfected into LN-299 cells and western blotting was used to determine the expression levels of autophagy- and apoptosis-related proteins. The expression levels of Beclin-1 and LC3B II/I were significantly downregulated and p62 was upregulated in LN-229 cells co-transfected with si-ETV4 + Oe-EMP1 compared with in cells co-transfected with si-ETV4 + Oe-NC (Fig. 4D-G). Moreover, it was found that si-ETV4-induced apoptosis was partially reversed in the si-ETV4 + Oe-EMP1 group compared with the si-ETV4 + Oe-NC group, with a decreased number of TUNEL-positive cells along with the downregulation of Bax and cleaved-caspase 9 expression levels and the upregulation of Bcl-2 expression (Fig. 4H-M). These findings suggested that the effect of si-ETV4 on promoting autophagy and apoptosis may be achieved by reducing the transcriptional activation of EMP1 by ETV4.

Interfering with the ETV4/EMP1 axis promotes autophagy-dependent apoptosis and inhibits the mTOR signaling pathway. Rapamycin (RAP) is a specific inhibitor of the mTOR protein; it can bind to the intracellular receptor, FK506 binding protein (FKBP)-12, to form a complex and directly act on the FKBP-12-RAP binding domain of mTOR to inhibit protein activity (13). In the present study, LN-229 cells were co-incubated with RAP and si-ETV4, and the results of the TUNEL staining assay showed that the co-treatment could upregulate apoptosis compared with the RAP + si-NC group, while the concurrent overexpression of EMP1 could

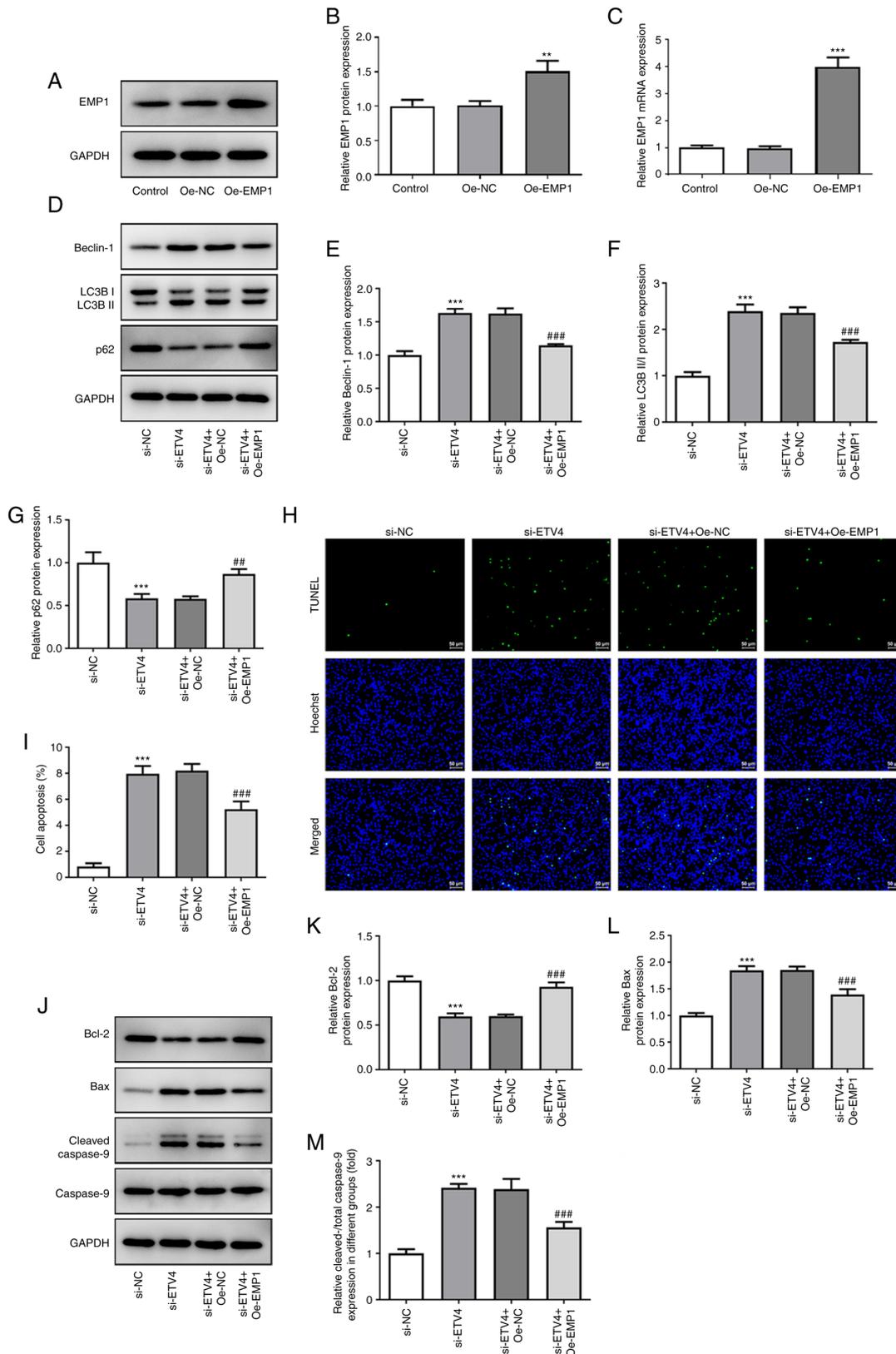


Figure 4. Overexpression of EMP1 in LN-229 cells reverses the promoting effects of si-ETV4 on autophagy and apoptosis. (A) Representative western blots showing EMP1 expression after transfection with Oe-EMP1. Untransfected cells were used as the control. GAPDH served as the loading control. (B) Histograms showing the statistical results from part (A). (C) Reverse transcription-quantitative PCR was used to determine the transfection efficiency of Oe-EMP1. n=4. **P<0.01, ***P<0.001 vs. Oe-NC. (D) Representative western blots demonstrating Beclin-1, LC3B II/I and p62 expression levels in different groups (si-NC, si-ETV4, si-ETV4 + Oe-NC and si-ETV4 + Oe-EMP1). GAPDH served as the loading control. Semi-quantification of (E) Beclin-1, (F) LC3B II/I and (G) p62 protein expression. (H) TUNEL assay was used to determine the effect of EMP1 on LN-229 cell apoptosis (TUNEL, green; Hoechst, blue). Scale bar, 50 μ m. (I) Percentage of TUNEL-positive cells. (J) Representative western blots demonstrating Bcl-2, Bax, cleaved-caspase 9 and caspase 9 expression levels in different groups (si-NC, si-ETV4, si-ETV4 + Oe-NC and si-ETV4 + Oe-EMP1). GAPDH served as the loading control. Semi-quantification of (K) Bcl-2, (L) Bax and (M) cleaved-caspase 9 protein expression. n=3. ***P<0.001 vs. si-NC; ##P<0.01, ###P<0.001 vs. si-ETV4 + Oe-NC. EMP1, epithelial membrane protein 1; ETV4, ETS variant transcription factor 4; si, small interfering RNA; Oe, overexpression; NC, negative control; LC3B III/I, LC3B II/LC3B I.

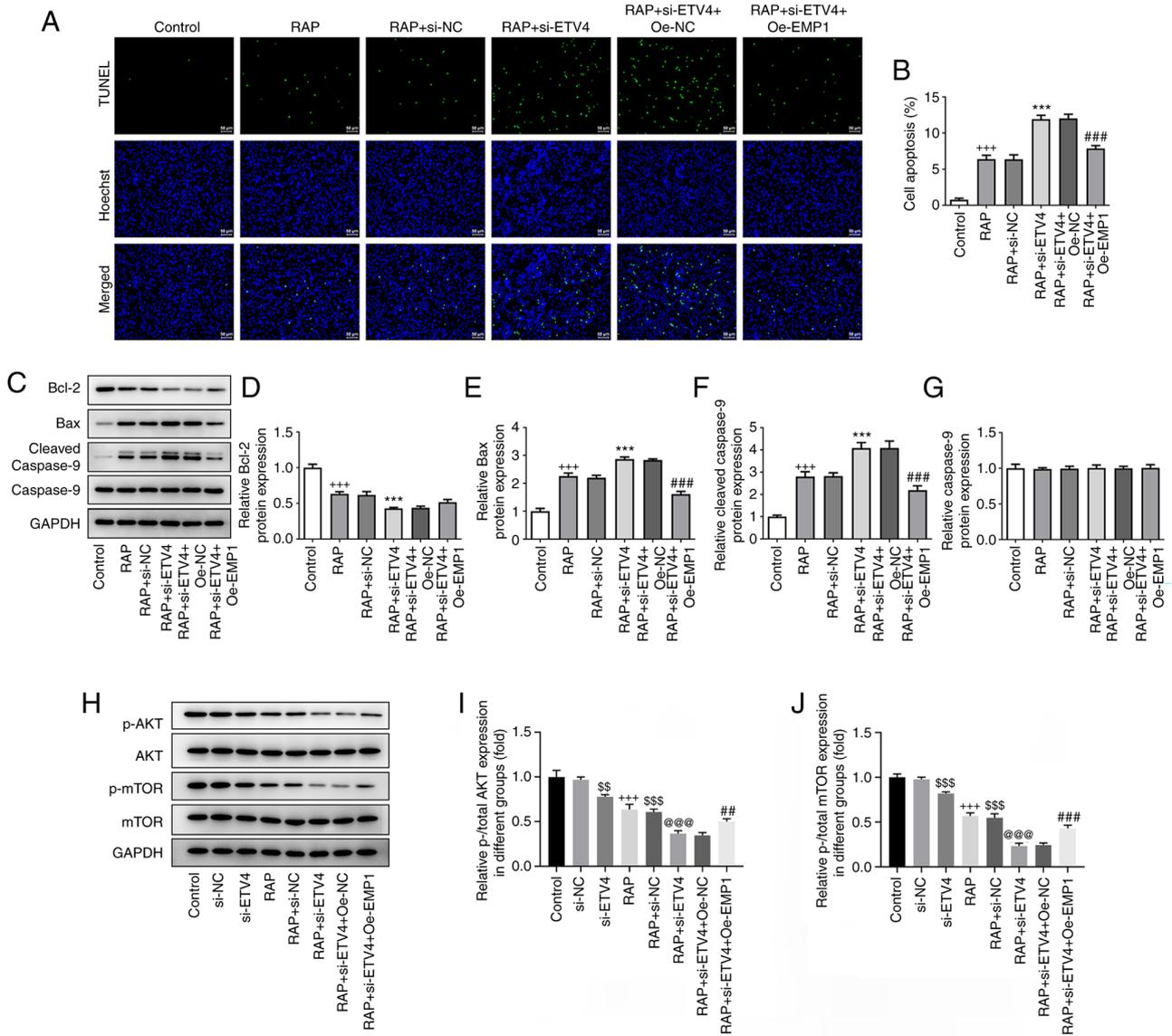


Figure 5. Interference of the ETV4/EMP1 axis in LN-229 cells promotes autophagy-dependent apoptosis and inhibits the mTOR signaling pathway. (A) TUNEL assay was used to detect apoptosis in LN-229 cells transfected with si-NC, si-ETV4, si-ETV4 + Oe-NC or si-ETV4 + Oe-EMP1 after 3 h of incubation with 100 nM RAP or treatment with RAP only (TUNEL, green; Hoechst, blue). Untreated/untransfected cells were used as the control. Scale bar, 50 μ m. (B) Percentage of TUNEL-positive cells. (C) Representative western blots demonstrating Bcl-2, Bax, cleaved-caspase 9 and caspase 9 expression levels in different groups (control, RAP, RAP + si-NC, RAP + si-ETV4, RAP + si-ETV4 + Oe-NC and RAP + si-ETV4 + Oe-EMP1). GAPDH served as the loading control. Semi-quantification of (D) Bcl-2, (E) Bax, (F) cleaved-caspase 9 and (G) caspase 9 protein expression. Untransfected cells were used as the control. (H) Representative western blots demonstrating p-AKT, AKT, p-mTOR and mTOR expression levels in different groups (control, si-NC, si-ETV4, RAP, RAP + si-NC, RAP + si-ETV4, RAP + si-ETV4 + Oe-NC and RAP + si-ETV4 + Oe-EMP1). GAPDH served as the loading control. Semi-quantification of (I) p-/total AKT and (J) p-/total mTOR protein expression levels. Untransfected cells were used as the control. $n=3$. $^{***}P<0.001$ vs. control; $^{SS}P<0.01$, $^{SSS}P<0.001$ vs. si-NC; $^{@@}P<0.001$ vs. si-ETV4; $^{***}P<0.001$ vs. RAP + si-NC; $^{##}P<0.01$, $^{###}P<0.001$ vs. RAP + si-ETV4 + Oe-NC. EMP1, epithelial membrane protein 1; ETV4, ETS variant transcription factor 4; si, small interfering RNA; NC, negative control; Oe, overexpression; p-, phosphorylated; RAP, rapamycin.

downregulate apoptosis compared with the RAP + si-ETV4 + Oe-NC group (Fig. 5A and B). At the protein level, the co-incubation of RAP with si-ETV4 was discovered to upregulate the expression levels of cleaved-caspase 9 and Bax, and downregulate the expression levels of Bcl-2 compared with the RAP + si-NC group. Following the concurrent overexpression of EMP1, these effects were partially reversed (Fig. 5C-G). These findings suggested that the effect of the ETV4/EMP1 axis on the autophagy and apoptosis of LN-229 cells may be associated with the mTOR signaling pathway. Next, western blotting was used to measure the expression levels of PI3K/AKT/mTOR signaling pathway-related proteins. The

results demonstrated that the activation of AKT and mTOR was inhibited after the incubation of RAP and/or si-ETV4, while the concurrent overexpression of EMP1 could partially reduce the degree of inhibition (Fig. 5H-J). These results indicated that interference with the ETV4/EMP1 signaling axis may promote autophagy-induced apoptosis and inhibit the activation of the mTOR signaling pathway in LN-229 cells.

Discussion

GBM is a heterogenous malignant brain tumor that accounts for ~80% of primary brain tumors and is extremely aggressive

and lethal (14), seriously affecting the survival and cognitive function of patients. The treatment regimen for GBM comprises standard surgery, radiotherapy and chemotherapy; however, once the disease begins to deteriorate, most patients succumb to the disease within 2 years of treatment (15). The results of the present study demonstrated that si-ETV4 promoted the autophagy and apoptosis of LN-229 GBM cells. Thus, this study further explored the potential underlying molecular mechanism of ETV4 in GBM.

As a common cancer-promoting transcription factor, ETV4 has been shown to promote the occurrence and development of a variety of types of cancer (6,7,16-18), including non-small cell lung, prostate and endometrial cancer. In the current study, the analysis of the CGGA database revealed that patients with primary glioma and high expression levels of ETV4 had a poor survival rate. Current research into the role of ETV4 in glioma has focused on its ability to promote tumor cell proliferation and invasion. Padul *et al* (19) found that in oligodendroglioma, ETV4 promoted tumor cell aggressiveness by regulating the Notch signaling pathway. Jiang *et al* (20) also suggested that the pro-invasive effect of ETV4 in glioma may also be regulated by the co-transcriptional complex formed between itself and the transcription factor, Sp1. However, to the best of our knowledge, there are currently no studies reporting the role of ETV4 in GBM. In the present study, the expression of ETV4 was found to be upregulated in 163 patients with GBM from the GEPIA database, which was validated in several GBM cell lines *in vitro*.

Autophagy is a common method used by tumors to generate metabolic precursors and ATP to survive in extreme environments. If the homeostatic autophagic balance is disrupted, inadequate repair or major cellular stress will trigger autophagic death (21). Autophagy-regulated tumor cell death has been a research hotspot in recent decades, and there have been numerous studies on GBM that have found that this may occur via the involvement of the mTOR, Src and ERK signaling pathways (11,13,22). In the present study, the knockdown of ETV4, using siRNA, was discovered to promote autophagy and apoptosis in LN-229 cells. Through analysis of the GEPIA database, it was discovered that ETV4 was weakly positively correlated with EMP1. Meanwhile, si-ETV4 was found to significantly decrease the expression of EMP1 in LN-229 cells. Thus, it was hypothesized that the transcription of ETV4 may positively regulate the expression of EMP1. The results of the dual luciferase reporter and chromatin immunoprecipitation assays further verified this hypothesis, as ETV4 was identified to bind to the EMP1 promoter (-405-396) region. In GBM, EMP1 has been found to upregulate CD44 by activating the PI3K/AKT/mTOR signaling pathway to maintain stemness and promote cell proliferation and invasion (8). Subsequently, tumor suppression and a longer lifespan were found in tumor-bearing mice that had knocked out EMP1 expression (8,9). In the current study, an EMP1 overexpression vector was constructed to explore whether the effect of ETV4 on the biological behavior of LN-299 cell autophagy and apoptosis occurred via EMP1. The results of the western blotting and TUNEL staining analyses revealed that the overexpression of EMP1 may partially reverse autophagy and apoptosis induced by ETV4 knockdown. These findings indicated that inhibiting the ETV4/EMP1 signaling axis may play an important role in promoting autophagy and apoptosis in LN-229 GBM cells.

mTOR has been reported to be upregulated in GBM, and the PI3K/AKT/mTOR signaling pathway was discovered to play a crucial role in regulating cell proliferation, apoptosis and autophagy (22). In the present study, LN-229 cells were co-incubated with RAP, si-ETV4 and Oe-EMP1 alone or in combination, and the expression levels of p-AKT and p-mTOR, which indicate that the PI3K/AKT/mTOR signaling pathway has been activated, were measured. Furthermore, the effect of the ETV4/EMP1 axis on the PI3K/AKT/mTOR signaling pathway was explored. The results found that the inhibition of the ETV4/EMP1 axis induced autophagy and apoptosis in LN-229 cells, which was discovered to occur via the inhibition of the PI3K/AKT/mTOR signaling pathway.

In conclusion, the findings of the present study suggested that ETV4 may be upregulated in GBM tissues and several GBM cell lines (A172, BS149, U251 and LN-229), which was found to reduce the survival probability of patients with primary GBM. Mechanistically, silencing ETV4 gene expression in LN-229 cells was discovered to promote autophagy-induced apoptosis. This effect was found to be associated with EMP1. ETV4 was identified to target the promoter region of EMP1 to positively regulate its expression, and interference of the ETV4/EMP1 axis promoted autophagy-dependent apoptosis and inhibited the mTOR signaling pathway. These findings are of great significance to the current understanding of the progression of GBM, and provide a novel insight and theoretical foundations for the treatment of GBM via regulation of autophagy-dependent apoptosis. However, most of the experimental data obtained in this study are based on the LN-229 cell line. Although it is a commonly used cell line for studying gliomas, the usage of only one cellular model may weaken the data because they could result from just the genotypic/phenotypic features of this specific cell line. Therefore, in future studies we will verify the results of this experiment in other glioma cell lines and explore the significance of silencing ETV4 for the treatment of GBM in *in vivo* animal models.

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Availability of data and materials

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

Authors' contributions

JW, CS, JL and MG conceived and designed the study. JW, CS, JL, HJ and YQ collected and analyzed the data. All authors were involved in the writing of the manuscript and revised the manuscript. JW and MG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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