Overexpression of Tripartite Motif-Containing 48 (TRIM48) Inhibits Growth of Human Glioblastoma Cells by Suppressing Extracellular Signal Regulated Kinase 1/2 (ERK1/2) Pathway

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Background:
Herein, we found that tripartite motif-containing 48 (TRIM48) was reduced in human glioblastoma (GBM) cell lines. We investigated whether and how TRIM48 functions in human GBM in vitro.

Material/Methods:
Human GBM cells (U87 MG and U138 MG) were infected with lentivirus to overexpress TRIM48, and 1 human GBM cell line (T98G) was infected with siRNAs to knock down TRIM48 expression. Techniques used included cell proliferation assay, measured by CCK-8 and BrdU-ELISA method, and cell cycle assay, determined using flow cytometry. Curcumin, a specific activator of extracellular signal regulated kinases (ERK1/2), or PD98059, a specific inhibitor of ERK1/2, was used to activate or block the ERK1/2 pathway, respectively. Expression of phosphorylated (p)-ERK1/2, and its downstream targets (Cyclin D1) were measured to assess the mechanism.

Results:
Our data suggest that overexpression of TRIM48 reduces the viability of U87 MG and U138 MG and leads to cell cycle arrest (in G0-G1 phase), which is associated with blockade of the ERK1/2 pathway and reduction of Cyclin D1. In contrast, knockdown of TRIM48 resulted in the opposite effects. Interestingly, the inhibitory effect of TRIM48 overexpression on human GBM cell growth and the inactivation of ERK1/2 were significantly alleviated with additional curcumin treatment, while it promoted the effect of siTRIM48 on human GBM cell growth, and the activation of ERK1/2 was significantly alleviated with additional PD98059 treatment.

Conclusions:
TRIM48 suppressed the growth of human GBM cell via the prevention of ERK1/2 activation.

MeSH Keywords:
Biochemistry • Cell Growth Processes • Gene Targeting

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Background

Glioblastoma multiforme (GBM) accounts for about 80% of all glioblastomas [1], and is the most invasive and deadly form of human malignant brain cancer [2], with an average survival of only 14 months after diagnosis [3]. GBM treatment is limited and insufficiently studied [4] due to poor prognosis, undesirable surgical resection, and radio- or chemoresistance [5]. Thus, a better understanding of GBM and its underlying pathogenic mechanisms is necessary to manage this disease.

The tripartite motif-containing (TRIM) protein family plays an important role in cancer progression and is characterized by a RING-finger motif, a B-box domain, and a coiled-coil domain. Among TRIM protein family members, TRIM8 [6], TRIM14 [7], TRIM24 [8], and TRIM28 [9] have been found to be involved in human GBM. TRIM48, known as a TRIM protein, lacks the coiled-coil domain and facilitates death of human lung cancer cells (A549) in a mouse xenograft model, probably through enhancing ASK1 activation [10]. Notably, TRIM48 is one of the 7 human-specific genes that are predicted to encode proteins that function as E3 ubiquitin ligases, thus mediating a wide range of biological functions, such as cell proliferation and cell cycle [11]; however, to date, there have been no reports regarding the molecular or biological functions of TRIM48, and it is unclear how TRIM48 contributes to GBM tumorigenesis. Meanwhile, based on our unpublished work, TRIM28 expression has been found to be decreased in GBM in the TCGA database and is associated with the survival time of patients with GBM, suggesting its important role in GBM. Therefore, we investigated the roles of TRIM48 in regulating GBM.

Extracellular signal regulated kinase 1 (ERK1) (44 kDa) and its homologous subtypes ERK2 (42 kDa) are involved in regulation of various cellular physiological activities (such as cell growth and differentiation), in particularly, in brain tissue, which makes them critical in the development of neurons and astrocytes [12]. Evidence suggests that the ERK1/2 pathway is aberrantly activated in GBM [13], and preventing its activation is important to prevent this disease. TRIM family member TRIM65 exerts oncogenic activity in human lymphoma malignancies via favoring ERK1/2 activation [14]. However, data on the roles of TRIM48 in regulating ERK1/2 pathway in GBM are very scarce.

In this study, we found that TRIM48 was downregulated in GBM cell lines (A172, T98G, U138MG, U251MG, U373, and U87MG). We established lentivirus-mediated TRIM48 overexpression in U87 MG and U138 MG, and siRNA-mediated TRIM48 knock-down in T98G. Our data suggest that overexpression of TRIM48 prevents human GBM cell growth, and shows the mechanisms involved in blocking the ERK1/2 pathway.

Material and Methods

siRNAs transfection

Three siRNA sequences targeting TRIM48 were designed (Table 1). Cells were transfected with 20 μmol/l of siRNA-TRIM48 (siTRIM48) or a negative control (siNC) via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to a previously reported study [15]. After 48 h, silencing efficacy of TRIM48 was detected by RT-PCR and Western blot.

Design of lentiviruses-mediated TRIM48 overexpression and its transfection

The designed primers of human TRIM48 gene (AF521869.1) were: 5’-GGGAGAAGCTGTTAAAGAA-3’, 5’-CCGGATCGAGGAGACAAATCAGAACTCGAGTTCTGATTTGTCTCTGGTCTTTTTG-3’ (reverse), and 5’-GGATCCAGGAGACAAATCAGAACTCGAGTTCTGATTTGTCTCTGGTCTTTTTG-3’ (reverse), which was inserted into Plasmid pLVX-Puro (Clontech), and 2000 ng/ml of pLVX-Puro-TRIM48 was transfected into U87 MG and U138 MG using Lipofectamine 2000. pLVX-Puro without TRIM48 expression was used as a control vector.

Table 1. siRNA- TRIM48 sequences.

| Label 1 | TRIM48 sequence | Forward primer: 5’-GGGAGAAGCTGTTAAAGAA-3’, start point 551-569 |
|---|---|---|
| siRNA sequence | 5’-CCGGGTGGGAGAAGCTGTTAAAGAACTCGAGTTCTTTAACAGCTTCTCCCTTTTTG-3’ (reverse) |

| Label 2 | TRIM48 sequence | Forward primer: GGATCCAGGAGACAAATCAGAACTCGAGTTCTGATTTGTCTCTGGTCTTTTTG-3’ (reverse) |
|---|---|---|
| siRNA sequence | 5’-GGGAGAAGCTGTTAAAGAA-3’, start point 551-569 |

| Label 3 | TRIM48 sequence | Forward primer: 5’-GGGAGAAGCTGTTAAAGAA-3’, start point 551-569 |
|---|---|---|
| siRNA sequence | 5’-GGGAGAAGCTGTTAAAGAA-3’, start point 551-569 |
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**Cells culture and treatment**

U87, U138, and T98G cells (ATCC, Manassas, VA, USA) were maintained at 37°C under 5% CO₂ in a medium of HyClone DMEM (SH30243.01) supplemented with 10% of fetal bovine serum ( Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin solution (100X, Solarbio, Beijing, China) until growing to 80% confluence. Then, cells were digested and seeded in 96-well plates (3×10⁴ cells) and cultured overnight for further experiments.

To study whether the ERK1/2 pathway was involved, U87MG cells transfected with pLVX-Puro-TRIM48 were treated with 10 μmol/l of curcumin, while T98G cells transfected with siTRIM48 were treated with 10 μmol/l of PD98059 or vehicle (phosphate-buffered saline, PBS), and then cells were cultured normally as mentioned above.

**Cell proliferation analysis**

After treatment, proliferation of U87, U138, and T98G cells at 0, 24, 48, and 72 h was determined by use of the Cell Counting Kit-8 (CCK-8) (CP002, SAB, Shanghai, China) and BrdU Cell Proliferation ELISA kit (colorimetric), respectively, according to the manufacturer’s instructions.

**Cell cycle analysis**

For cell cycle assay, after treatment, cells were fixed in 70% ethanol at 4°C for 24 h, and then re-suspended with 1 mg/ml of RNase (Solarbio). The sample was treated with propidium iodide (PI) (50 μg/ml, 400 μl) (7Sea Biotech, Shanghai, China). DNA content within GBM cells was quantified using flow cytometry (BD Biosciences, San Jose, USA), and cell cycle phase was assessed using FlowJo software (Tree Star, Ashland, OR, USA).

**Western blotting**

After quantification, using a BCA protein assay kit (Thermo), total protein (25 μg) in supernatant of the lysate of brain tissue or GBM cell samples was separated using 15% SDS-PAGE, then transferred onto nitrocellulose (NC) membranes (HATF00010, Millipore) and maintained with antibody against TRIM48 (Abcam, Ab171619), antibody against Cyclin D1 (Abcam, Ab16663), antibody against ERK1/2 (CST, #4695), antibody against p-ERK1/2 (CST, #4370), and anti-GAPDH antibody (CST, #5174) at 4°C overnight followed by secondary antibodies (A0208, Beyotime, Shanghai, China) for 1 h at 25°C. Immunoreactive bands were determined in the ECL system (GE Healthcare/Amersham Biosciences).

**Patients**

To study whether TRIM48 was involved in human GBM, a total of 12 healthy people and 25 patients with GBM (WHO grade IV) were recruited at Xi’an DaXing Hospital. Our study protocol was approved by the Ethics Committee of Xi’an DaXing Hospital, and all participants (aged 14–71 years old) provided written informed consent.

**Statistical analysis**

Data are presented as means of 3 trials, and values were recorded as mean ± standard error of the mean. Two-sided statistical method was conducted using the t test, with a significant difference being P<0.05.

**Results**

**Expression of TRIM48 in human GBM cell lines**

To study whether TRIM48 is involved in human GBM, mRNA and protein levels of TRIM48 expression were determined in normal brain tissue and several GBM cell lines (A172, T98G, U138MG, U251MG, U373, and U87MG). Our data demonstrated that TRIM48 was significantly downregulated in GBM cells in comparison to normal brain tissue (Figure 1, P<0.05).

**Overexpression of TRIM48 inhibited proliferation and led to cell cycle arrest of human GBM cells via ERK1/2 pathway**

To study the roles of overexpression of TRIM48 in cell growth of human GBM in vitro, we injected lentivirus expressing TRIM48 into human GBM cell lines, and then cell proliferation and cell cycle were assessed. As shown in Figure 2A, expression of TRIM48 was significantly increased in the TRIM48 group in comparison to vector, suggesting successful establishment of
TRIM48 overexpression. CCK-8 and BrdU-ELISA methods showed that TRIM48 reduced the viability of U138 cells (Figure 2B, 2C) when compared with vector. Besides, Figure 2D shows that TRIM48 obviously induced cell cycle arrest of U138 cells at G0/G1 phase.

Cyclin D1 is a regulatory factor in G1/GS progression and is a downstream target of the ERK1/2 pathway. To investigate the participation of ERK1/2 in the inhibitory effect of TRIM48 on GBM cell growth, protein levels of Cyclin D1 and p-ERK1/2 were detected. Our data showed that TRIM48 overexpression reduced Cyclin D1 and p-ERK1/2, but had no obvious effect on total ERK1/2 expression (Figure 2E), suggesting the prevention of ERK1/2 activation induced by TRIM48 overexpression in human GBM cells.

To substantiate that the ERK1/2 pathway was the mechanism by which TRIM48 regulated GBM cell growth, we injected lentivirus expressing TRIM48 into U87MG in the absence or presence of curcumin to activate ERK1/2 signaling. As shown in Figure 1, TRIM48 was downregulated in human GBM cells. mRNA and protein levels of TRIM48 in human normal brain tissue and GBM cell lines (A172, T98G, U138MG, U251MG, U373MG, and U87MG) were assessed by Western blotting and RT-PCR, respectively. The experiments were repeated 3 times. ** P<0.01 versus normal cells.
Figure 3. Overexpression of TRIM48 inhibited growth of human GBM cells via inactivating ERK1/2 pathway. mRNA and protein levels of TRIM48 in human (A). U87MG, assessed by Western blotting and RT-PCR. After transfection for 48 h, proliferation of U87MG were assessed at 0, 24, 48, and 72 h by (B). CCK8 and (C). BrdU-ELISA method, respectively (D, E). Cell cycle analysis was assessed by flow cytometry (F). Protein levels of Cyclin D1, ERK1/2, and p-ERK1/2. Cyclin D1 was quantified using GAPDH-1. ERK1/2 and p-ERK1/2 were quantified using GAPDH-2. The experiments were repeated 3 times. ** P<0.01 versus Vector. * P<0.05, ## P<0.01 versus TRIM48 overexpression.

Knockdown of TRIM48 promoted human GBM cell growth via ERK1/2 pathway

To further investigated the role of the ERK1/2 pathway in TRIM48-regulated GBM cell growth, siTRIM48 was transfected into human GBM T98G cell lines, and then cell proliferation, cell cycle, and protein levels of Cyclin D1 and p-ERK1/2 were assessed. Figure 4A shows that expression of TRIM48 was...
Figure 4. siTRIM48 promoted growth of human GBM cells via activating ERK1/2 pathway. Significant reduction in protein and mRNA levels of TRIM48 in human (A). T98G cells were obtained, indicating successful establishment of TRIM48 deficiency. T98G cells transfected with siTRIM48 were treated with 10 μmol/l of PD98059, and then cell proliferation was measured using (B), CCK8 and (C). BrDU-ELISA method, respectively (D, E). Cell cycle analysis was assessed by flow cytometry (F). Protein levels of Cyclin D1, ERK1/2, and p-ERK1/2. Cyclin D1 was quantified using GAPDH-1. ERK1/2 and p-ERK1/2 were quantified using GAPDH-2. The experiments were repeated 3 times. * P<0.05, ** P<0.01 versus siNC; && P<0.01 versus siNC+PD98059.
significantly decreased in the siTRIM48 group in comparison to siNC, confirming the depletion of TRIM14 within GBM cells. We found that siTRIM48 significantly enhanced cell proliferation (Figure 4B, 4C), reduced cell counts in G0/G1 phase, increased cell counts in S phase (Figure 4D, 4E), and increased the expressions of Cyclin D1 and p-ERK1/2 (Figure 4F) when compared with siNC group. However, the siTRIM48-induced changes in the events above were significantly reversed with additional PD98059 treatment, suggesting that siTRIM48 enhanced the growth of GBM cells via activating the ERK1/2 pathway.

### Expression of TRIM48, Cyclin D1, and p-ERK1/2 in human GBM in vivo

To confirm the involvement of TRIM48 and its association with the ERK1/2 pathway in human GBM in vivo, mRNA expression of TRIM48 and Cyclin D1 in brain tissue of GBM patients (n=25) and healthy people (n=12) were assessed using RT-PCR. Protein levels of TRIM48, Cyclin D1, and p-ERK1/2 in 3 pairs of healthy and tumorous brain tissue were assessed using Western blotting. As shown in Figure 5, significantly reduced TRIM48 but enhanced Cyclin D1 and p-ERK1/2 were observed in GBM patients when compared with healthy people, substantiating the involvement of TRIM48 and its correction with the ERK1/2 pathway in human GBM in vivo.

### Discussion

The TRIM protein family members play various roles in GBM. TRIM8 is a cancer suppressor and is downregulated in human GBM, but TRIM14, TRIM24, and TRIM28 are cancer promoters and are up-regulated in human GBM [6–9]. In this study, our data showed that TRIM48 was downregulated in human GBM brain tissue and cell lines, suggesting the involvement of TRIM48 in human GBM.

TRIM48 was shown to contribute to human lung cancer cell death in a mouse xenograft model [10]. However, whether and how TRIM48 functions in the growth of human GBM cells has been unclear. In this study, our data showed that overexpression of TRIM48 significantly inhibited the viability of U87 and U138 cells, and its inhibitory effect on cell growth was probably mediated by cell cycle arrest (G0-G1 phase) in parallel with reduction in protein levels of Cyclin D1. On the contrary, knock-down of TRIM48 promoted cell growth of human GBM cells.

ERK1/2 is strongly activated in GBM [13]. The constant activation of ERK1/2 is critical to sustained cancer cell growth and is closely linked to the inductive cyclin D1 and cell cycle progression [16]. Some studies have reported that the enhanced cyclin D1 in G1 phase of the cell cycle depends on constant activation of the ERK1/2 pathway [17,18]. Here, we confirmed that ERK1/2 was activated in the brain tissue of human GBM, as shown by enhanced p-ERK1/2 and Cyclin D1. We demonstrated the roles of TRIM48 in regulating ERK1/2 in human GBM in vitro. Our data showed that overexpression of TRIM48 blocked the ERK1/2 pathway, while knockdown of TRIM48 activated the ERK1/2 pathway in human GBM cells. Interestingly, the inhibitory effect of TRIM48 overexpression on human GBM cell growth and the inactivation of ERK1/2 were significantly alleviated by additional treatment with curcumin, while the promoting effect of siTRIM48 on the growth of human GBM cells was remarkably attenuated with additional treatment of PD98059, suggesting that ERK1/2 is the mechanism by which TRIM48 regulates cancer cell growth in human glioblastoma in vitro. Further in vivo research is needed to confirm the therapeutic efficacy of TRIM48 in GBM.
Conclusions

Our data suggest that TRIM48 is downregulated in GBM patients. TRIM48 overexpression inhibits the growth of human GBM cells after inhibition of ERK1/2 pathway activation.

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Conflict of interest

None.