Experimental Research

ANNALS OF MEDICINE & SURGERY

OPEN

The function of TRIML2 on the temozolomide resistance in glioblastoma

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Background: Acquired resistance to temozolomide is a major challenge for the effective treatment of glioblastoma (GBM). TRIML2, a member of the TRIM family, plays an important role in cancer genesis, progression, and treatment resistance. However, its mechanism of action in GBM resistance to temozolomide remains unclear.

Methods: RNA bulk sequencing data from temozolomide-resistant U87 cells and wild-type U87 cells were downloaded from the NCBI public database (GEO: GSE193957) and analyzed. A temozolomide-resistant cell line (U87-TR) was induced with temozolomide, and the expression of TRIML2 in temozolomide-resistant and wild-type cell lines (U87-WT) was verified by cell activity assays, wound-healing assays, and western blotting. The alteration of resistance to temozolomide was assessed following the overexpression of TRIML2 in the resistant cell line by lentiviral transfection. The differences in TRIML2 expression between primary GBM and recurrent GBM after temozolomide chemotherapy were verified by immunofluorescence, immunohistochemistry, and western blotting.

Results: The expression of TRIML2 was significantly lower in U87-TR cells than in U87-WT cells. After the TRIML2 overexpressed in U87-TR cells, their resistance to temozolomide was significantly decreased and became sensitive to temozolomide treatment. TRIML2 expression was significantly decreased in the temozolimide-resistant GBM tumors; in contrast, TRIML2 was relatively high expressed in the temozolimide-sensitive GBM tumors.

Conclusions: TRIML2 inhibits temozolomide resistance in GBM and thus may serve as a novel therapeutic target for overcoming GBM resistance to temozolomide.

Keywords: glioblastoma, temozolomide resistance, TRIML2

Introduction

Glioblastoma (GBM) is the most common malignant tumor of the central nervous system, accounting for 40–50% of all brain tumors[1], and has a 5-year overall survival rate of approximately 5%[2]. A combination of surgery, radiotherapy, and temozolomide chemotherapy is currently the first treatment option for GBM, but all GBMs can recur after treatment, with an average median survival of approximately 12–15 months[3]. Acquired resistance to temozolomide, which results from a complex, multifactorial interplay, is often observed in GBM patients after temozolomide chemotherapy[4]. After surgical resection, temozolomide, a second-generation oral DNA alkylating agent, is often used as a first-line chemotherapeutic agent for patients with GBM because it readily penetrates the blood–brain barrier and is less likely to cause significant treatment-related side effects[5]. The obstacle to the effectiveness of GBM treatment lies not only in the limitations of the extent of safe surgical resection but also in the resistance to temozolomide after surgical resection[6]. Therefore, it is clinically valuable to further explore the potential mechanisms of temozolomide resistance in GBM.

TRIML2, a member of the TRIM protein family, belongs to the unclassified group because it lacks a RING-finger domain[7]. The TRIM family is the largest class of single-protein RING-finger E3 ubiquitin ligases involved in cancer occurrence, progression, and treatment resistance and exhibits different mechanisms of action in different tumor types[8]. Several previous studies have reported that TRIML2 is involved in the physiopathological processes of a variety of diseases. During placental evolution, TRIML2 attenuates inflammation by reducing the production of proinflammatory factors in trophoblasts[9]. In human oral squamous cell carcinoma, overexpression of TRIML2 contributes to tumor growth in the G1 stage[7]. TRIML2 is implicated in the regulation of apoptosis and is involved in the development of Alzheimer's disease[10]. However, there are no reports on the relationship between TRIML2 and GBM, and the mechanism by which TRIML2 affects temozolomide resistance in GBM remains unclear. The aim of this study is to preliminarily explore the possible role of TRIML2 in GBM resistance to temozolimide and to find a potential therapeutic target on drug resistance.

In this study, for the first time, we found that TRIML2 might serve as a novel target for the treatment of GBM resistance to temozolomide, providing a new therapeutic strategy to ameliorate GBM resistance to temozolomide.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Annals of Medicine & Surgery (2025) 87:506–514

Received 3 September 2024; Accepted 9 December 2024

Published online 21 January 2025

http://dx.doi.org/10.1097/MS9.0000000000002888

Materials and methods

Collection of data and clinical samples

In this study, RNA bulk sequencing data were obtained from the NCBI public database (GEO: GSE193957). Complete glioblastoma tissue specimens, obtained from the Department of Neurosurgery of our hospital, were used as the clinical data. Freshly resected glioblastoma tissues were immediately stored at −80 °C for subsequent experiments (primary glioblastoma, n = 22; recurrent glioblastoma, n = 18). All experiments were approved by the Research Ethics Committee of our hospital.

Cell culture and lentiviral transfection

The human glioma cell line U87 was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All cells were cultured in Dulbecco's modified Eagle medium (VivaCell, C3112-0500) supplemented with 10% fetal bovine serum and stored in a humidified environment with 5% CO_2 at 37 °C. U87-TR cells were infected with lentiviruses harboring TRIML2-overexpressing or negative control (NC) U87-TR-TRIML2-LV or U87-TR-CN-LV, respectively.

Puromycin drug screening assay

Puromycin (Gibco, A1113803) was added to the culture dish of the U87-TR cell line, and different concentrations (0, 2, 6, 8, and 16 μg/mL) were added. The cell state under different concentrations of puromycin was observed and photographed under a light microscope after 5 days to determine the optimal puromycin concentration. The optimal concentration of puromycin (6 μg/mL) was determined, and this concentration was subsequently used to screen for TRIML2 gene-transfected U87-TR cells.

Generation of temozolomide-resistant U87 cell lines

U87 cells were inoculated into 96-well plates, and the half-maximal inhibitory concentration (IC50) of temozolomide (Aladdin, T127425) was determined by a cell activity assay. According to IC50, temozolomide (TMZ) was prepared at six different concentrations in the media (25, 50, 100, 200, 400, and 1000 μM/L). U87 cells were cultured starting from the lowest concentration of temozolomide (25 μM/L), and the temozolomide concentration was increased exponentially when cells grew stably. Each concentration was maintained for 1 month until the end of the sixth month, and a cell activity assay was performed to verify resistance to temozolomide. The induced temozolomide-resistant cells were named U87-TR (1000 μM/L resistant).

Cell viability assay and cell colony formation assay

Cells were inoculated into 96-well plates at a density of 2000 cells/well, and low to high concentrations of TMZ medium (25, 100, 400, 1000, 2000, or 4000 μM/L) were added to each well. Three replicate wells were set up for each concentration, and the plates were incubated at 37 °C. Then, CCK-8 (Biosharp, BS350B) was added to each well after 3 days. Cell viability was assessed by measuring the absorbance at 450 nm of an enzyme marker after 2 h of incubation at 37 °C. Cell proliferation was assessed using an EdU assay kit (Beyotime Biotech, Nanjing, China) according to the manufacturer's instructions, and the IC50 value was determined as a measure of cell sensitivity to TMZ treatment.

Cells were inoculated in 6-well culture plates at a density of 5000 cells per well. The medium was replaced with a fresh medium every 3 days. The cells were incubated for 2 weeks, washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 minutes at room temperature, and stained with 0.5% crystal violet. They were rinsed with running water following air drying and then counted under a microscope. Cell colonies were photographed with a digital camera.

Western blotting

The TRIML2 antibody used in this study was purchased from Affinity Biosciences. Glioma tissues or cells were lysed with RIPA buffer (Beyotime Biotechnology, China) for 20 min. Total protein was collected from the cell lysate supernatant after centrifugation at 12 000 rpm for 10 min. The protein concentration was determined with a BCA protein assay kit (Thermo Fisher, USA). Twenty micrograms of protein per lane was separated by SDS–PAGE and then transferred to a PVDF membrane. The PVDF membranes were incubated with 5% skim milk for 2 h and then incubated with the TRILM2 primary antibody (Affinity, DF14787; rabbit anti-TRIML2 antibody, dilution 1:100) overnight. After washing with PBS, the membranes were incubated with secondary antibodies for 2 hours. Signals from the PVDF membranes were detected using chemiluminescent reagents. We calculated the area of reference GAPDH and target protein TRIML2 in all WB results by ImageJ software, divided the area of TRIML2 by the area of GAPDH as the relative expression of TRIML2, and finally produced a bar graph to visualize the relative expression of TRIML2.

Wound-healing assay

Wound scratches were produced by scraping confluent monolayer cultures using a T200 pipette tip loaded with temozolomide. Images were taken at 0, 12, and 24 h, and the images were analyzed using software. Three wound-healing areas were photographed for the experimental and control groups.

Immunohistochemistry

Paraffin specimens were obtained from the First Affiliated Hospital of Xinjiang Medical University, and the tissues were cut to a thickness of 4 μm. The tissue slides were incubated with TRILM2 primary antibody (Affinity, DF14787; rabbit anti-TRIML2 antibody, dilution 1:200) overnight at 4 °C. The slides were then incubated with a biotin-labeled secondary antibody (ZSBG-BIO, pv-6000) for 1 hour at 37 °C. Then, the slides were stained with diaminobenzidine (DAB) and observed under a light microscope.

Immunofluorescence

Primary antibodies TRIML2 (Affinity, DF14787, rabbit anti-TRIML2 antibody, dilution 1:100) and GFAP (ABclonal, A0237, mouse anti-GFAP antibody, dilution 1:100) were incubated overnight at 4 °C. Fluorescent secondary antibodies (goat-anti-rabbit, Abcam, ab150077, 488 nm, dilution 1:300; donkey-anti-mouse, Beyotime, A0460, 555 nm, dilution 1:200) were added and incubated in the dark at 37 °C for 1 h. PBS was used as the primary antibody. After three washes, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI). The fluorescence field was observed using a confocal microscope (Olympus FV-1000).

Statistical analysis

GraphPad Prism 9 was used for statistical analysis. A one-way ANOVA test was used to analyze the significance of differences between groups. $P < 0.05$ was considered to indicate statistical significance.

Data availability

RNA bulk sequencing data can be found in the NCBI public database (GEO: GSE193957).

Results

TRIML2 is associated with temozolomide resistance in U87 cells

We downloaded the RNA bulk sequencing data (GEO: GSE193957) associated with temozolomide resistance in a human glioma cell line (U87) from the GEO public database and performed in-depth biological information mining. The results revealed that the expression of the TRIML2 gene was significantly downregulated in the temozolomide-resistant glioma cell line (U87-TR) compared to the temozolomide-sensitive glioma cell line (U87-WT) (Fig.1A-B), which suggested that TRIML2 was closely related to temozolomide resistance in U87 cells. However, the mechanism by which TRIML2 affects temozolomide resistance in GBM remains unknown. Therefore, we studied the mechanism by which TRIML2 affects temozolomide resistance in GBM, which in turn provides a new therapeutic target for the clinical treatment of temozolomide-resistant GBM.

Culturing and validation of temozolomide-resistant U87 cells

We induced the U87 wild-type glioma cell line (U87-WT) to generate temozolomide-resistant U87-TR cells (see Materials and Methods for details). A comparison of cell morphology between the two cell lines under a light microscope revealed that U87-WT cells had a high number of axons, while U87-TR cells exhibited a lower number of axons and longer axons with strip-shaped cell bodies (Fig.2A). We generated a temozolomide sensitivity curve via a cell viability assay. Compared with that of U87-TR cells, the activity of U87-WT cells decreased significantly at temozolomide concentrations less than 1000 μmol/L (Fig.2B), suggesting that the cultivated U87-TR cells were significantly resistant to temozolomide at concentrations less than 1000 μmol/L. To further confirm the temozolomide resistance of U87-TR cells, we conducted wound-healing experiments on U87-WT and U87-TR cells cultured in media supplemented with 1000 μM/L temozolomide. The results were photographed and observed under a microscope at 0, 12, and 24 h. The U87-TR cells had basically migrated through the scratches after 24 h; however, the U87-WT cells had not migrated through the scratches (Fig.2C). The colony formation assay showed that there was a large amount of colony formation in U87-TR cells at a temozolomide concentration of 1000 μmol/L; however, there was almost no colony formation in U87-WT cells at the same temozolomide concentration (Fig.2D). Western blotting experiments showed that the expression of TRIML2 was significantly lower in U87-TR cells than in U87-WT cells (Fig.2E). The above results suggested that cultivated U87-TR cells were significantly resistant to temozolomide at a concentration of 1000 μmol/L or less. Thus, the cultivation and validation of

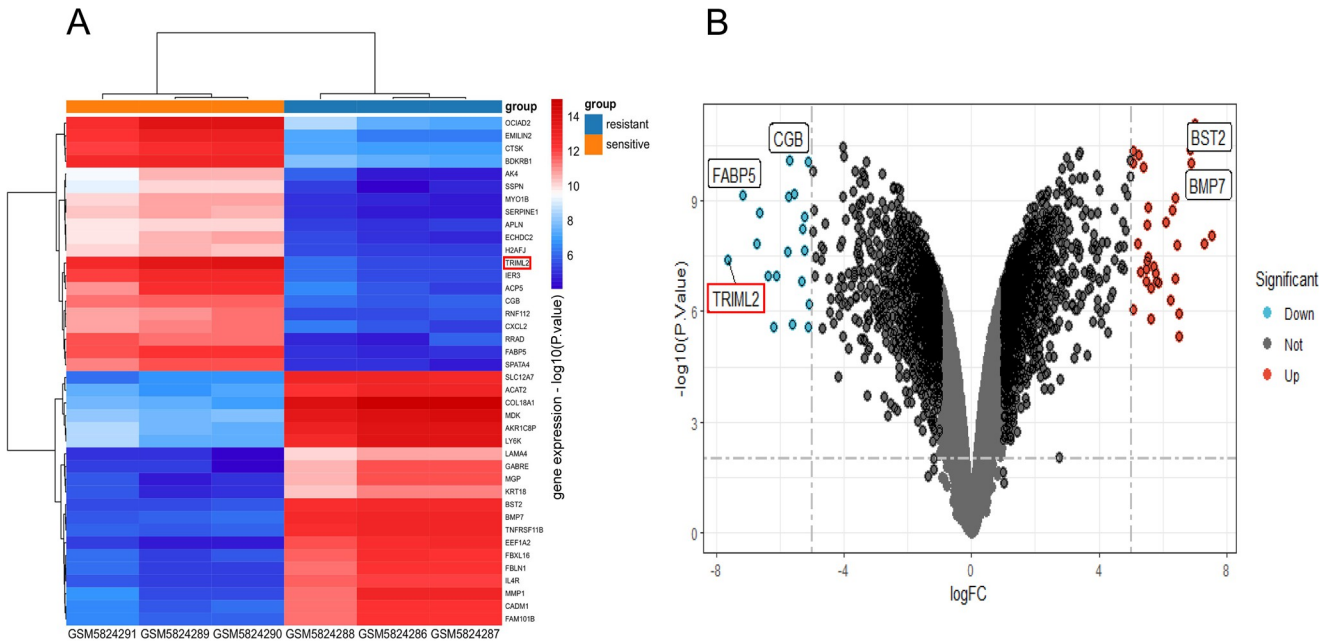

Figure. 1. RNA bulk sequencing data (GEO: GSE193957) of temozolomide-resistant U87 cells and wild-type U87 cells (temozolomide-sensitive) were downloaded from the NCBI public database and analyzed. (A) Heatmap of gene expression in the temozolomide-resistant ($n = 3$) and temozolomide-sensitive ($n = 3$) groups. TRIML2 was significantly downregulated in the resistant group compared with the sensitive group. (B) Volcano plot of gene expression in the temozolomide-resistant group ($n = 3$) relative to the temozolomide-sensitive group ($n = 3$). TRIML2 expression was significantly downregulated in the resistant group compared with the sensitive group.

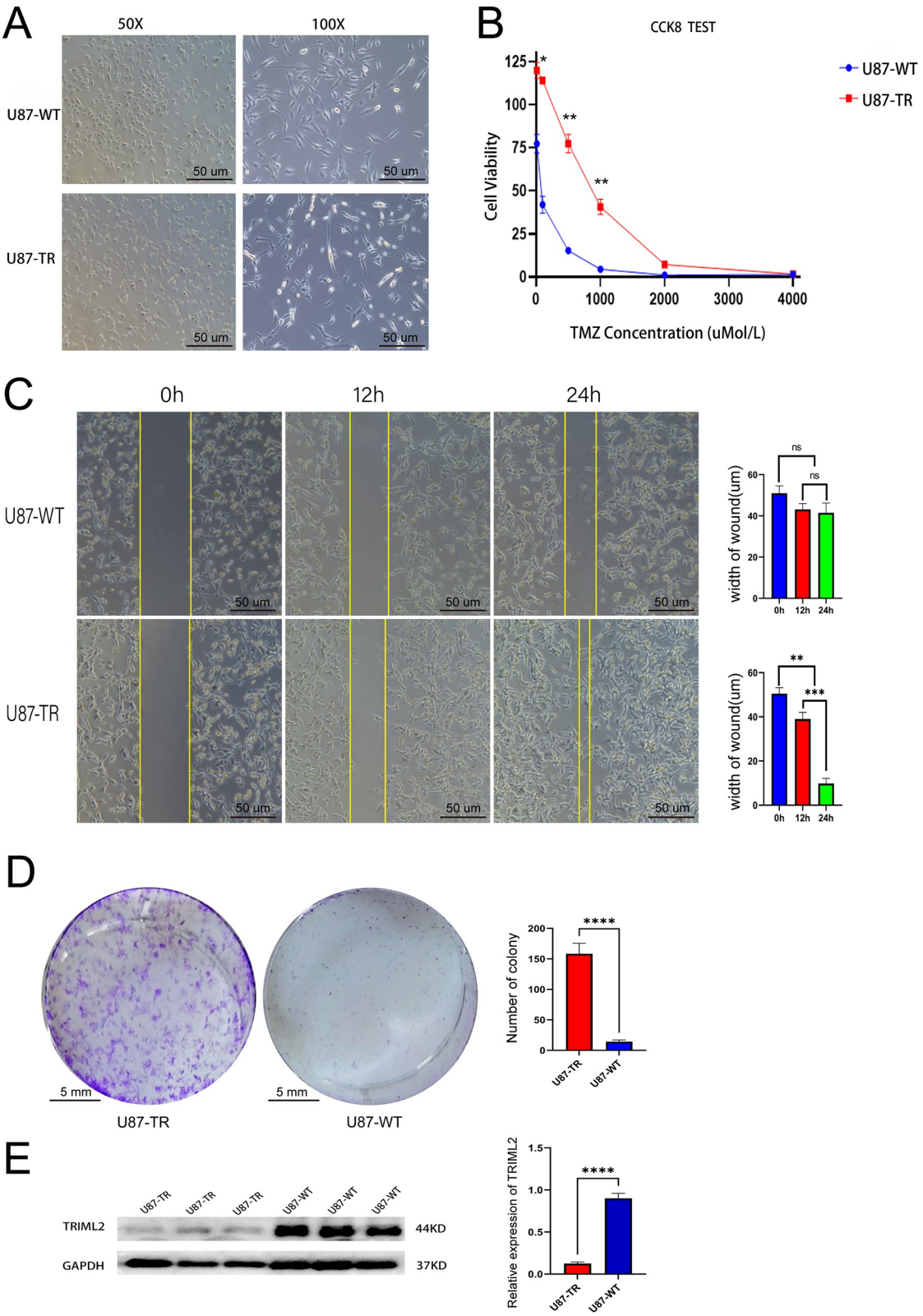

Figure. 2. Establishment and validation of the temozolomide-resistant U87 cell line (U87-TR). (A) Morphological characteristics of U87-TR and U87-WT cells under different magnifications (50X, 100X) relative to U87-WT cells. U87-TR cells showed the morphological characteristics of longer axons with strip-shaped cell bodies. (B) After 72 hours of incubation in temozolomide medium at different concentrations, U87-TR cells exhibited higher IC50 values than did U87-WT cells. (C) After incubation with 1000 μM/L temozolomide for 24 hours, U87-TR cells showed a significantly faster wound healing rate than U87-WT cells. (D) After culturing in media supplemented with 1000 μM/L temozolomide for 24 hours, U87-TR cells formed significantly more colonies than did U87-WT cells. (E) The protein expression of TRIML2 in U87-TR cells was significantly lower than that in U87-WT cells.

drug-resistant cell lines were completed. These findings further confirmed that TRIML2 expression may be associated with temozolomide resistance in GBM.

After TRIML2 overexpressed in U87-TR cells, their resistance to temozolomide was significantly decreased and became sensitive to temozolomide treatment

To clarify whether TRIML2 affects temozolomide resistance in GBM, we transfected U87-TR cells with a lentivirus overexpressing TRIML2, used a control lentivirus and an empty virus at the same time, and confirmed the success of transfection by immunofluorescence. To further confirm successful transfection, we performed U87-TR puromycin screening experiments with different puromycin concentrations (0, 2, 4, 6, 8, and 16 μg/mL), and we found that the concentration of puromycin that was sufficient to inhibit the nontransfected cells was 6 μg/mL (Fig.3A). TRIML2-overexpressing U87-TR cells (named U87-TR-TRIML2-OE) and empty virus-transfected U87-TR cells (named U87-TR-NC or U87-TR-TRIML2-NC) were successfully screened (Fig.3B). Western blotting experiments revealed that U87-TR-TRIML2-OE cells expressed significantly more TRIML2 than did U87-TR-TRIML2-NC cells and approached the TRIML2 expression level in U87-WT cells (Fig.3C), suggesting that U87-TR-TRIML2-OE cells stably express TRIML2. We derived the temozolomide sensitivity curves of the above three cell types via a cell viability assay. The activity of U87-TR-TRIML2-OE cells was significantly lower than that of U87-TR-TRIML2-NC cells when the temozolomide concentration was less than 1000 μmol/L and was close to that of U87-WT cells (Fig.3D). Cell colony formation assays revealed that the number of colonies formed by U87-TR-TRIML2-OE cells was significantly lower than that formed by U87-TR-TRIML2-NC cells and approached the number of colonies formed by U87-WT cells when the temozolomide concentration was 1000 μmol/L (Fig.3E). Wound-healing assays revealed that the migration ability of U87-TR-TRIML2-OE cells was similar to that of U87-WT cells after incubation with 1000 μM/L temozolomide for 24 h (Fig.3F). The above results suggested that the overexpression of TRIML2 may reverse the U87-TR cells from a temozolomide-resistant state to a temozolomide-sensitive state.

The expression of TRIML2 was significantly downregulated in GBM which was resistant to temozolomide chemotherapy

To further understand the expression of TRIML2 in temozolomide-resistant GBM, tumor samples from patients with primary GBM, which is often sensitive to the temozolomide chemotherapy, and recurrent GBM, which is usually resistant to the temozolomide chemotherapy, were collected and pathologically examined. Immunohistochemistry revealed that the expression of TRIML2 was significantly lower in recurrent GBM than in primary GBM (Fig.4A). Western blotting experiments revealed that the expression of TRIML2 was significantly lower in recurrent GBM than in primary GBM (Fig.4B). Immunofluorescence results also showed that the expression of TRIML2 was significantly lower in recurrent GBM than in primary GBM (Fig.4C). These results showed that TRIML2 may inhibit the resistance of GBM to temozolomide.

Discussion

Glioblastoma (GBM) is one of the most common intracranial tumors and has high mortality and recurrence rates[11]. Surgical resection with radiotherapy and chemotherapy is currently the main treatment for GBM; even so, the clinical prognosis of GBM patients remains poor, with an average survival time of 12–18 months[12,13]. Previous studies have shown that effective chemotherapy after surgical resection can significantly delay tumor recurrence in GBM patients[14]. However, acquired resistance to temozolomide in GBM is often observed in clinical practice, which limits the efficacy of temozolomide chemotherapy in GBM patients[15]. Resistance to temozolomide in GBM is a major obstacle to its treatment, which underscores the urgency and necessity of interventions for temozolomide resistance. Therefore, elucidating new mechanisms of GBM resistance to temozolomide is of great value and significance for prolonging the survival of GBM patients. This study analyzed the potential relationship between TRIML2 expression and temozolomide resistance in U87 glioma cell lines and human GBM tumor samples and further explored the role of TRIML2 in GBM resistance to temozolomide.

Many previous studies have reported that TRIM family proteins are involved in a variety of cellular activities and processes, including RNA-binding activities such as mRNA repression and mRNA localization, autophagy, apoptosis, cell cycle progression and mitosis, DNA damage response, viral infection, immune activation, and inflammatory processes[16-24]. TRIM family proteins are also involved in a variety of cellular signaling and biological processes that are closely related to cancer development, progression, and therapeutic resistance and exhibit tumor-promoting or tumor-suppressing functions in different human cancer types[8,25], suggesting that whether the TRIM family acts as a pro-oncogene or antioncogene during tumorigenesis and development is dependent on the tumor type and the specific signaling pathway. Therefore, TRIM family members have great potential as biomarkers for cancer diagnosis and prognosis. TRIML2, one of the largest members of the TRIM family, is a P53-associated tumor suppressor whose biological function remains largely unknown[22,26]. Previous studies have shown that TRIML2 is negatively correlated with inflammatory responses during placental evolution and is positively correlated with the development of Alzheimer's disease, the growth of human oral squamous cell carcinoma, and apoptosis[7,9,10,27]. However, no relevant studies have reported the mechanism by which TRIML2 affects GBM resistance to temozolomide. In this study, we found that the TRIML2 gene enrichment in U87-TR cells was significantly lower than that in U87-WT cells, indicating that TRIML2 may play a role in the mechanism of GBM resistance to temozolomide. We found that the expression of TRIML2 was significantly lower in U87-TR cells than in U87-WT cells, which further suggested that TRIML2 might inhibit temozolomide resistance in U87 cells.

Based on the above experimental findings, we further explored the possible mechanism by which TRIML2 affects temozolomide resistance in U87 cells. Our findings suggest that after TRIML2 overexpressed in U87-TR cells, their resistance to temozolomide was significantly decreased and became sensitive to temozolomide treatment, implying that TRIML2 may inhibit temozolomide resistance in glioma cells. We explored the expression of TRIML2 in human GBM tissues for

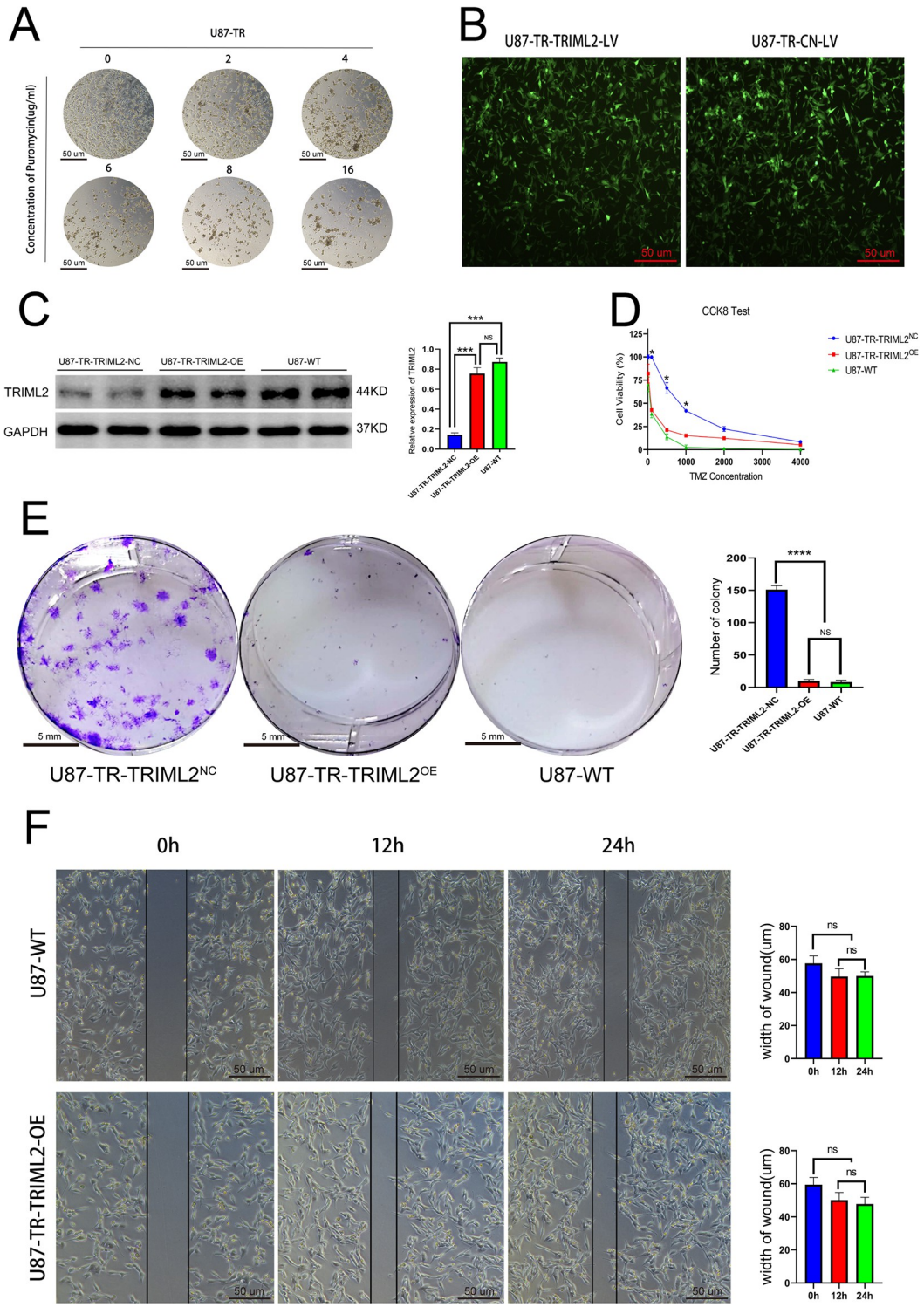

Figure. 3. Overexpression of TRIML2 reversed the U87-TR cells from a temozolomide-resistant state to a temozolomide-sensitive state. (A) The optimal puromycin concentration (6 μg/ml) required to cultivate stable U87-TR cells was determined by puromycin drug screening assays (0, 2, 4, 6, 8, and 16 μg/ml). (B) Fluorescence microscopy images of U87-TR cells after transfection with TRIML2-LV or NC-LV. (C) TRIML2 expression in TRIML2-LV-transfected U87-TR cells (U87-TR-TRIML2-OE) was significantly greater than that in NC-LV-transfected U87-TR cells (U8T-TR-TRIML2-NC). U87-WT cells were used as a blank control. (D) After 72 h of incubation in media supplemented with different concentrations of temozolomide, the U87-TR-TRIML2-OE cells exhibited lower IC50 values than did the U8T-TR-TRIML2-NC cells, and these values were close to the IC50 values of the U87-WT cells. (E) The number of colonies formed by U8T-TR-TRIML2-OE cells was significantly lower than that formed by U87-TR-TRIML2-NC cells after incubation in media supplemented with 1000 μM/L temozolomide for 24 h. U87-WT cells were used as a blank control. (F) After 24 hours of incubation with 1000 μM/L temozolomide, the wound-healing rate of U87-TR-TRIML2-OE cells was similar to that of U87-WT cells.

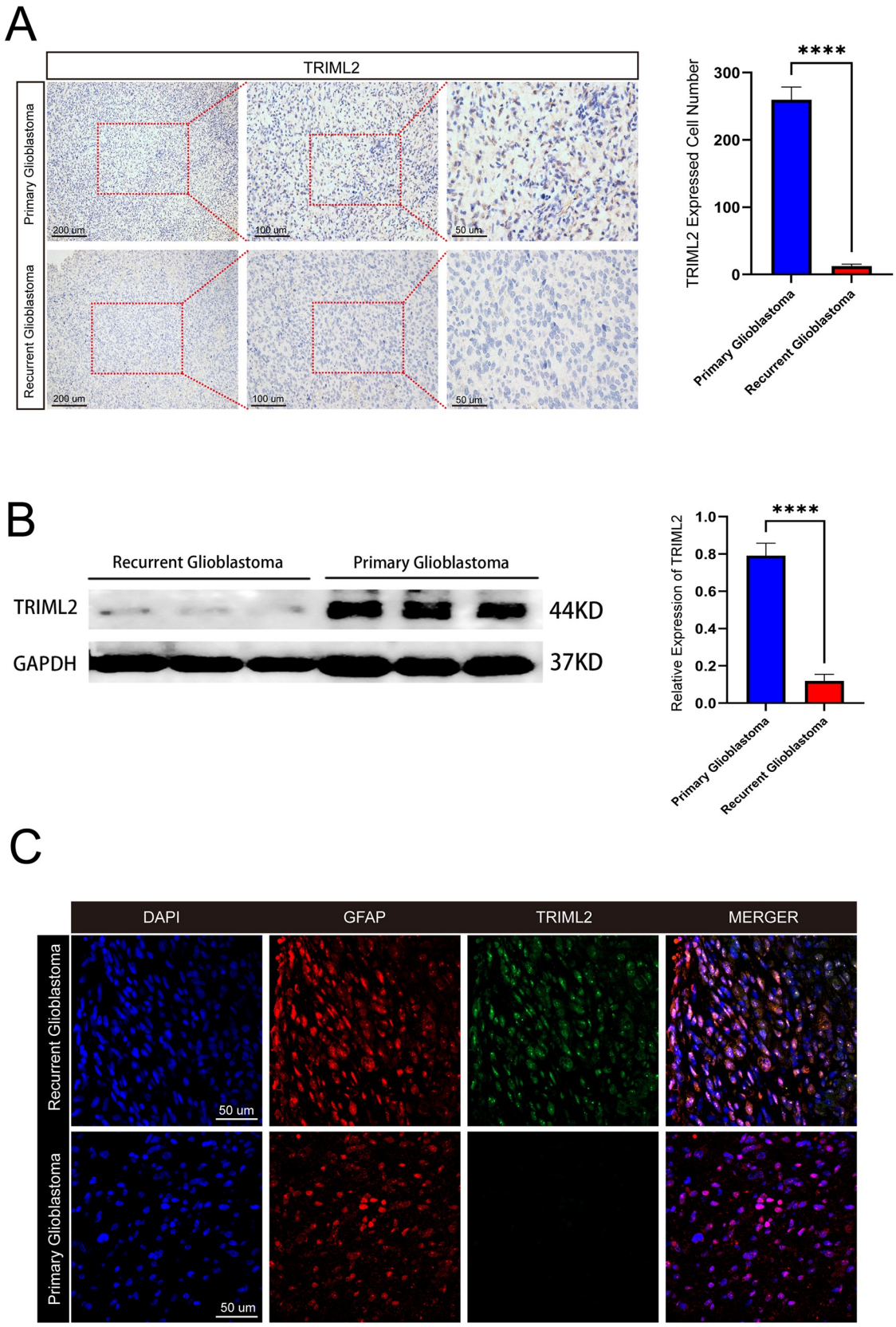

Figure. 4. The expression of TRIML2 was significantly downregulated in GBM which is resistant to temozolomide chemotherapy. (A) Immunohistochemical results showed that the expression of TRIML2 in recurrent GBM tissue was significantly lower than that in primary GBM tissue. (B) Western blotting results showed that the expression of TRIML2 in recurrent GBM tissue was significantly lower than that in primary GBM tissue. (C) Immunofluorescence results showed that the expression of TRIML2 in recurrent GBM tissue was significantly lower than that in primary GBM tissue.

the first time and found that the expression of TRIML2 was significantly lower in temozolomide-resistant GBM, suggesting that TRIML2 may inhibit the resistance of GBM to temozolomide. Previous studies have shown that TRIML2 expression is significantly greater in tumor tissues than in adjacent paracancerous tissues in human breast cancer, and its expression in tumor cells is also significantly greater than that in normal breast cells[28]. In human oral squamous cell carcinoma, TRIML2 expression was upregulated and significantly correlated with tumor growth, and TRIML2-knockdown cells exhibited cell cycle arrest in the G1 phase and inhibited cell growth; however, overexpression of TRIML2 promoted tumor growth in the G1 phase[7]. These studies all suggest that TRIML2 plays a procarcinogenic role in the development of the abovementioned tumors. Conversely, cancer microarray data collected from the ONCOMINE database showed that TRIML2 expression was frequently downregulated in gastric, colorectal, lung, and hepatocellular carcinomas, suggesting that TRIML2 has distinct functions in different tumors[27]. In this study, TRIML2 inhibited the resistance of GBM to temozolomide. Therefore, restoring the sensitivity of GBM to temozolomide through the overexpression of TRIML2 through targeted therapy may be a promising therapeutic strategy. Our study provides new insights into GBM resistance to temozolomide, potentially highlighting TRIML2 as a new option for future interventional therapy in the clinic.

Conclusion

We reported for the first time that TRIML2 may inhibit GBM resistance to temozolomide and that TRIML2 may serve as a new potential target for the treatment of GBM .

Limitation

The present study is small basic research, aimed to explore the possible role of TRIML2 in GBM resistance to temozolimide and to find a potential therapeutic target on drug resistance. Unfortunately, however, we only found a phenomenon that RIML2 may inhibit GBM resistance to temozolomide, and we were limited by the conditions to carry out mechanistic studies, so we are still unable to answer the question about how TRIML2 inhibits temozolomide resistance in GBM.

Suggestions for future studies

We found, for the first time, that TRIML2 may inhibit GBM resistance to temozolomide and that TRIML2 may serve as a new potential target for the treatment of GBM. Although we were not able to do a mechanistic exploration, we shared this phenomenon to our colleagues in related fields, introducing TRIML2 into the research field of treatment of GBM for the first time. This may be potentially helpful to scholars who are studying TRIML2 and GBM resistance to temozolomide, and we hope that this study will make a valuable contribution to the treatment of GBM.

Ethical approval

The subjects of this study were glioblastoma cell lines and paraffin specimens; the cell lines were purchased from Biologics and the paraffin specimens were obtained from the specimen bank; no patients were involved.

Consent

No patients or volunteers were involved in this study.

Sources of funding

The funding for this research comes from: 1. Xinjiang Key Laboratory of Neurological Disorder Research, Uygur Autonomous Region, China (grant No. XJDX1711-2202); 2. the National Natural Science Foundation of Xinjiang, Uygur Autonomous Region, China (grant No. 2021D01C312).

Author's contribution

Q.F.: methodology, Resources, Writing the original draft, Writing – review & editing. P.C.: collected the data of Revision. Z.W.: supported statistical software. B.L.: supported statistical method. Q.Z.: supported statistical software. I.A.: supervision, data validation, revision. Y.W.: conceptualization, funding acquisition, investigation, methodology.

Conflicts of interest disclosure

All authors declared that there is no potential conflict of interest and agreed to publish the article.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Professor Yongxin Wang and Dr. Ilhamjan Anwar.

Provenance and peer review

The content of the manuscript has not been published, or submitted for publication elsewhere.

Data availability statement

RNA bulk sequencing data can be found in the NCBI public database (GEO: GSE193957).

Acknowledgements

None.

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