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Original Article

GARNL3 identified as a crucial target for overcoming temozolomide resistance in EGFRvIII-positive glioblastoma

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Abstract: Object: Amplification of the epidermal growth factor receptor (EGFR) and its active mutant type III (EGFRvIII), frequently occurr in glioblastoma (GBM), contributing to chemotherapy and radiation resistance in GBM. Elucidating the underlying molecular mechanism of temozolomide (TMZ) resistance in EGFRvIII GBM could offer valuable insights for cancer treatment. Methods: To elucidate the molecular mechanisms underlying EGFRvIII-mediated resistance to TMZ in GBM, we conducted a comprehensive analysis using Gene Expression Omnibus and The cancer genome atlas (TCGA) databases. Initially, we identified common significantly differentially expressed genes (DEGs) and prioritized those correlating significantly with patient prognosis as potential downstream targets of EGFRvIII and candidates for drug resistance. Additionally, we analyzed transcription factor expression changes and their correlation with candidate genes to elucidate transcriptional regulatory mechanisms. Using estimate method and databases such as Tumor IMmune Estimation Resource (TIMER) and CellMarker, we assessed immune cell infiltration in TMZ-resistant GBM and its relationship with candidate gene expression. In this study, we examined the expression differences of candidate genes in GBM cell lines following EGFRvIII intervention and in TMZ-resistant GBM cell lines. This preliminary investigation aimed to verify the regulatory impact of EGFRvIII on candidate targets and its potential involvement in TMZ resistance in GBM. Results: Notably, GTPase Activating Rap/RanGAP Domain Like 3 (GARNL3) emerged as a key DEG associated with TMZ resistance and poor prognosis, with reduced expression correlating with altered immune cell profiles. Transcription factor analysis suggested Epiregulin (EREG) as a putative upstream regulator of GARNL3, linking it to EGFRvIII-mediated TMZ resistance. In vitro experiments confirmed EGFRvIII-mediated downregulation of GARNL3 and decreased TMZ sensitivity in GBM cell lines, further supported by reduced GARNL3 levels in TMZ-resistant GBM cells. Conclusion: GARNL3 downregulation in EGFRvIII-positive and TMZ-resistant GBM implicates its role in TMZ resistance, suggesting modulation of EREG/GARNL3 signaling as a potential therapeutic strategy.

Keywords: EGFRvIII, glioblastoma, GARNL3, EREG, temozolomide resistance, immune infiltration

Introduction

Glioblastoma (GBM) stands out as the most aggressive primary brain tumor, characterized by its high proliferation, invasiveness, and postoperative recurrence. Currently, the standard treatment for primary GBM involves postoperative radiotherapy combined with temozolomide (TMZ) chemotherapy [1]. However, long-term clinical trials have demonstrated that the median survival time for GBM patients undergoing standard care barely reaches 14 months [2].

Epidermal growth factor receptor (EGFR) variant III (EGFRvIII) activity mutations have been found in half of GBM patients [3] in previous high-throughput sequencing studies of primary GBM. EGFRvIII mutation entails the deletion of 801 base pairs between exons 2 and 7 of the wild-type EGFR gene, leading to the truncation

Table 1. Summary of epidermal growth factor receptor mutations among glioblastoma patients undergoing temozolomide treatment

Technology	Type	TMZ resistant (n = 159)	TMZ sensitive (n = 58)
RNA-seq	All	41	23
	EGFR mutation	10	3
	EGFR exon2-7 mutation	10	2
	EGFR change in position 6-273	5	1
WES	All	64	41
	EGFR mutation	15	4
	EGFR exon2-7 mutation	9	2
	EGFR change in position 6-273	5	1

TMZ, temozolomide; EGFR, epidermal growth factor receptor; WES, Whole exome sequencing; RNA-seq, ribonucleic acid sequencing.

of amino acids 6 to 273 in the extracellular domain. Ultimately, it disrupts the dimerization of amino acids and their ligand binding capacity [4]. In the absence of ligand binding, EGFRvIII undergoes continuous phosphorylation, thereby activating other signaling pathways and ultimately fostering malignant cell proliferation [5].

In addition, research has indicated a positive correlation between EGFRvIII and the degree of malignancy in GBM. EGFRvIII promotes GBM resistance to TMZ by promoting a malignant phenotype of the tumor and augmenting its capacity to repair DNA double-strand breaks [6]. Unraveling the mechanism of drug resistance instigated by EGFRvIII has emerged as a focal point in basic cancer research. Thus, the quest for vulnerabilities in TMZ-resistant GBM represents a promising avenue for enhancing the efficacy of TMZ treatment.

Here, we employed lentivirus library transduction to target both parental U87 cell line (U87-MG) and U87-MG cell line with EGFRvIII (U87-EGFRvIII). RNA abundance was assessed through deep sequencing under TMZ-induced chemical stress conditions. Among the candidate genes, particular attention was directed towards GTPase Activating Rap/RanGAP Domain Like 3 (GARNL3). Furthermore, we identified Epiregulin (EREG) as the upstream transcription factor regulating GARNL3. Through gene expression analysis and survival prognosis assessment in GBM patients, we elucidated pivotal role of GARNL3 in mediating TMZ resistance. In conclusion, our findings advocate for the up-regulation of GARNL3 via increased EREG expression as a promising therapeutic strategy for TMZ-resistant GBM.

Materials and methods

Acquisition and processing of EGFRvIII-positive glioblastoma-associated transcriptome sequencing datasets

Ribonucleic Acid sequencing (RNA-seq) data set (GSE112-734) of Human GBM cell line U87 infected by lentivirus expressing EGFRvIII and treated with TMZ were obtained from Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) [7]. This data set included a total of 30 samples, which were divided into 10 groups, with 3 samples in each group. We screened EGFRvIII-positive U87 cells (U87OE) cultured for 7 days or 14 days, with (U87OE_TMZ_day7, U87OE_TMZ_day14) or without TMZ treatment (U87OE_day7, U87OE_day14), respectively. In addition, we obtained RNA-seq data of GBM tissue from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), and screened the fragments per kilobase of exon model per Million mapped fragments (FPKM) counts of transcriptome of GBM patients (TCGA-GBM) treated with TMZ. From them, we obtained a total of 64 samples, including 41 TMZ-resistant cases and 23 TMZ-sensitive case (**Table 1**).

Acquisition of temozolomide resistance-related genes in EGFRvIII-positive glioblastoma

In order to obtain drug resistance-related genes, we first used the R package "limma v3.52.4" to analyze the differential expression of genes between TMZ-treated and untreated U87OE cell lines. Then, significantly differentially expressed genes (DEGs) were screened through $P < 0.05$ and $|\log_2 FC| > 1$ conditions. To further identify TMZ resistance-related DEGs, we screened EGFRvIII-positive samples from the TCGA-GBM data set and then obtained a total of 5 drug-resistant samples and 1 sensitive sample. Then the DEGs of TMZ-resistant EGFRvIII-positive GBM were obtained. We screened overlapping DEGs in U87OE cell lines and clinical GBM samples as potential resistance genes. In addition, we also used the R package "GSEABase v1.58.0" to perform gene enrichment analysis to clarify the

key functions of drug-resistant candidate DEGs in U87OE cells [8].

Assessment of immune cell infiltration in temozolomide-resistant glioblastoma tissues

We used the R package "estimate v1.0.13" to calculate the ESTIMATE scores, immune scores, stromal scores, and tumor purity of 64 samples in TCGA-GBM. We used Tumor IMmune Estimation Resource database (TIMER, https://cistrome.shinyapps.io/timer/) to analyze the infiltration of different types of immune cells, including T cells, B cells, macrophages, dendritic cells, etc. We then examined the differences in overall immune cells and infiltration of different immune cells between the TMZ-resistant and sensitive groups. The pearson correlation between the expression of candidate DEGs and the proportion of key immune cell infiltration was examined.

Correlation between drug resistance candidate genes and patient prognosis

In this step, we counted the differences in overall survival (OS) levels at different expression levels of candidate DEGs, and used the R package "survival v3.5-7" to analyze the survival prognosis based on the Kaplan-Meier method. DEGs significantly related to prognosis were screened and regarded as key factors for TMZ resistance. Then, we obtained GBM-related driver genes from CellMarker database (http://xteam.xbio.top/CellMarker/) and analyzed the Pearson correlation between the expression of key drug resistance factors and driver genes to clarify their relevance in promoting tumor occurrence and progression.

Transcriptional regulators of candidate genes associated with temozolomide resistance in EGFRvIII-positive glioblastoma

Gene promoter sequence-bound transcription factors (TFs) were retrieved from online databases (Cister, AliBaba2.1, MIM-chip, and JASPAR). Then, we analyzed the Pearson correlation between the expression levels of key drug resistance factors and these TFs. Also, the differences in transcription level of TFs in patients with EGFRvIII mutation GBM with TMZ resistance was analyzed.

Acquisition, culture, and processing of glioblastoma cells in vitro

The EGFRvIII-positive GBM cell line U87 (U87-EGFRvIII) (Vigen, Zhanjiang, Guangdong, China) and its wild-type counterpart (WT, U87-MG) (BIOSPECIES, Guangzhou, Guangdong, China), as well as the TMZ-resistant GBM cell line U251 (U251-TMZ) and its parental strain (U251-MG) (BIOSPECIES, Guangzhou, Guangdong, China), were routinely seeded in 6-well plates at a density of 1.2 × 10^6 cells and cultured in DMEM (BIOSPECIES, Guangzhou, Guangdong, China) complete medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were maintained at 37°C with 5% CO_2. Cell lines were cultured until reaching the logarithmic growth phase, after which they were treated with DMEM containing 0, 200, and 400 μM/L TMZ and continuously cultured for 7 days. Each concentration was applied to cells in triplicate. Throughout this period, the culture medium was refreshed every 3 days with new medium containing the respective concentrations of TMZ.

Detection of drug sensitivity and cell viability

Cells were seeded at a density of 50,000 cells per well in 96-well plates and cultured in media containing varying concentrations of TMZ (0, 1, 10, 100, 200, 400, 800, 1600, and 3200 μM/L). After 7 days of incubation, 10 μl of Cell-Counting-Kit-8 (CCK8) (Abbkine, Wuhan, Hubei, China) was added to each well, and the cells were further incubated for 4 h. Subsequently, the absorbance value at a wavelength of 450 nm was measured using a microplate reader. Cell viability (%) was calculated using the formula: (OD of cells in the treatment group - OD of blank wells)/(OD of cells in the control group - OD of blank wells) * 100. Additionally, the cell inhibition rate (%) was calculated as follows: (OD of the control group - OD of the administration group)/(OD of the control group - OD of the blank well) * 100.

Detection of target gene transcription levels

Total cellular RNA was extracted using the Trizol method, followed by conversion into cDNA using a reverse transcription kit (GenStar, Beijing, China). The resulting cDNA was thoroughly mixed with specific primers for GARNL3 (For-

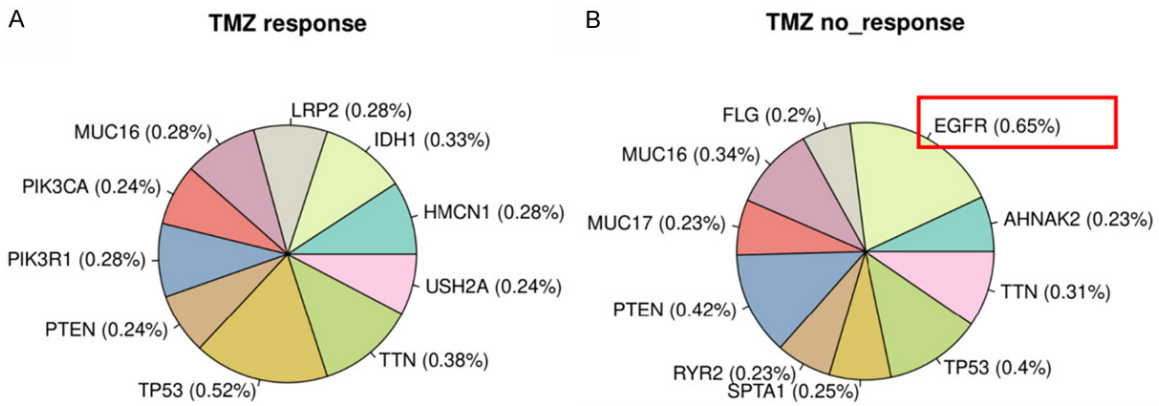

Figure 1. The distribution of gene mutations among glioblastoma (GBM, n = 105) patients undergoing temozolomide (TMZ) treatment, with a total mutation frequency of 5668. A, B. Top 10 of genes ranked by mutation frequency are depicted for TMZ-sensitive (n = 2125) and resistant (n = 3543) GBM patients, respectively. TMZ-resistant patients exhibit a notably higher frequency of epidermal growth factor receptor (EGFR) mutations, accounting for 0.64% (23/3543) of cases. MUC16, mucin 16; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PTEN, phosphatase and tensin homolog; TP53, tumor protein p53; TTN, titin; USH2A, usher syndrome type-2A protein; HMCN1, hemicentin-1; IDH1, isocitrate dehydrogenase 1; LRP2, low-density lipoprotein receptor-related protein 2; FLG, filaggrin; MUC17, mucin 17; RYR2, ryanodine receptor 2; SPTA1, spectrin alpha, erythrocytic 1; AHNAK2, AHNAK nucleoprotein 2.

ward: 5'-AACAATCAACGTGTCCCTCAAT-3'; Reverse: 5'-TTTGTCCAGATTCATGGCACTT-3') and GAPDH (Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3'; Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'), along with qPCR reagents (GenStar, Beijing, China), and subjected to amplification in a qPCR instrument. Fluorescence values were monitored during amplification to determine the amplification threshold (Cq). The mRNA level of GARNL3 relative to GAPDH was calculated using the formula 2^(-ΔΔCq).

Statistical analysis

The results of cell experiments were analyzed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Data were assessed for normal distribution and are presented as mean ± standard deviation. In the CCK8 detection, the IC50 value was obtained using the nonlinear fitting curve method. Differences between groups were assessed using Student's *t*-test. For bioinformatics analysis, statistical analysis and visualization were conducted using R software (version 4.1.0). Gene differential expression was assessed using a generalized linear model with a likelihood ratio test. Differences in immune infiltration scores, Pearson correlation coefficient (*r*) values, and overall survival (OS) between groups were determined using the Wilcoxon rank sum test. The Benjamini & Hochberg (False discovery rate, FDR) method was employed to correct the *p*-values. Results with $P < 0.05$ were considered statistically significant.

Results

EGFR mutations in temozolomide-resistant glioblastoma

In the TCGA database, clinical data from 217 GBM patients treated with TMZ were collected. Among them, RNA-seq data were available for 64 cases, while gene mutation information was obtained for 105 cases (**Table 1**). Among GBM patients with genetic mutations, TP53 was found to be predominant in those who responded to TMZ (0.518%, **Figure 1A**), whereas EGFR mutation frequency was highest among TMZ-resistant GBM patients (0.649%, **Figure 1B**), suggesting a potential association between EGFR mutations and TMZ resistance in GBM patients.

Further statistical analysis was performed on the EGFR mutation types of GBM patients. We analyzed EGFR mutation data from 19 GBM patients (**Figure 2A**) and observed that missense mutations constituted the primary variant classification (85.19%, **Figure 2B**), with single nucleotide polymorphisms (SNPs) being the predominant variant type (88.89%, **Figure 2C**). The most frequent mutation involved a

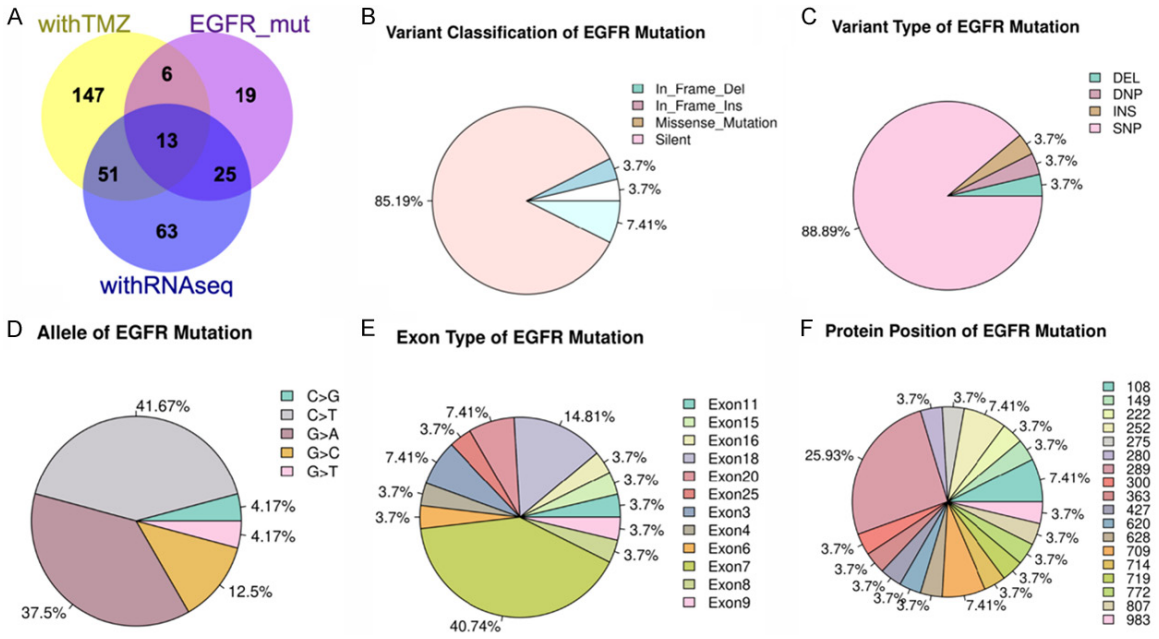

Figure 2. Distribution of epidermal growth factor receptor (EGFR) mutation types in glioblastoma (GBM) patients. A. Data distribution of 271 GBM patients treated with temozolomide (TMZ), among whom 19 patients exhibited EGFR mutations, with transcriptome sequencing data available for 64 patients; B. Variant classifications of EGFR mutations in GBM patients treated with TMZ (with a total mutation frequency of 27), among which missense mutations are accounted for the highest frequency at 85.19% (23/27); C. Variant types of EGFR mutations with predominantly single nucleotide polymorphisms (SNPs) at 88.89% (24/27); D. Single nucleotide base mutations of EGFR, with C>T mutations comprising the majority at 41.67% (10/24), followed by G>A mutations at 37.50% (9/24); E. Mutation frequencies of different exons of EGFR, where exon 7 is accounted for the highest frequency at 40.74% (11/27); F. Amino acid changes following EGFR mutations, with amino acid 289 exhibiting the most prevalent change at 25.93% (7/27). withRNAseq, glioblastoma tissues tested with ribonucleic acid sequencing; with TMZ, glioblastoma patients treated with temozolomide; EGFR_mut, glioblastoma patients with EGFR mutation; In_Frame_Del, bases deletion mutations in reading frame; In_Frame_Ins, bases insertion mutations in reading frame; DEL, deletion mutation; DNP, double nucleotide polymorphism; INS, insertion mutation; C, cytosine; G, guanine; T, thymine; A, adenine.

Table 2. Overview of epidermal growth factor receptor mutation patterns in epidermal growth factor receptor variant III-positive glioblastoma patients

Tumor_Sample_Barcode	Variant_Classification	Variant_Type	HGVS.c	HGVS.p	HGVS.p_Short	Protein_position	Codons	Exon	TMZ resistance
TCGA-06-2563	Missense_Mutation	SNP	C>T	p.Arg252Cys	p.R252C	252	Cgc/Tgc	7	Yes
TCGA-12-3652	Missense_Mutation	SNP	G>C	p.Arg252Pro	p.R252P	252	cGc/cCc	7	No
TCGA-27-2528	Missense_Mutation	SNP	C>T	p.Arg222Cys	p.R222C	222	Cgc/Tgc	6	No
TCGA-06-2561	Missense_Mutation	SNP	C>T	p.Arg149Trp	p.R149W	149	Cgg/Tgg	4	No
TCGA-06-2565	Missense_Mutation	SNP	G>A	p.Arg108Lys	p.R108K	108	aGa/aAa	3	No
TCGA-27-1831	Missense_Mutation	SNP	G>A	p.Arg108Lys	p.R108K	108	aGa/aAa	3	No

HGVS.c, human genome variation society complementary deoxyribonucleic acid; HGVS.p, human genome variation society protein; SNP, single nucleotide polymorphism; C, cytosine; G, guanine; T, thymine; A, adenine; TMZ, temozolomide.

C-to-T base change (41.67%), followed by a G-to-A base change (37.50%, **Figure 2D**). Additionally, EGFR mutations in GBM mainly occurred in exons 2-7 (55.56%, **Figure 2E**) and at amino acid positions 108-273 (22.22%) and 289 (25.93%, **Figure 2F**), corresponding to EGFRvIII (**Table 2**). Compared to TMZ-sensitive patients, the variety of EGFR mutations in the resistant group increased, particularly in terms of amino acid mutations in exons 2-7 and 6-273, which rose to 48.15% (vs. 7.41%) and 18.52% (vs. 3.70%, **Figure 3**), respectively.

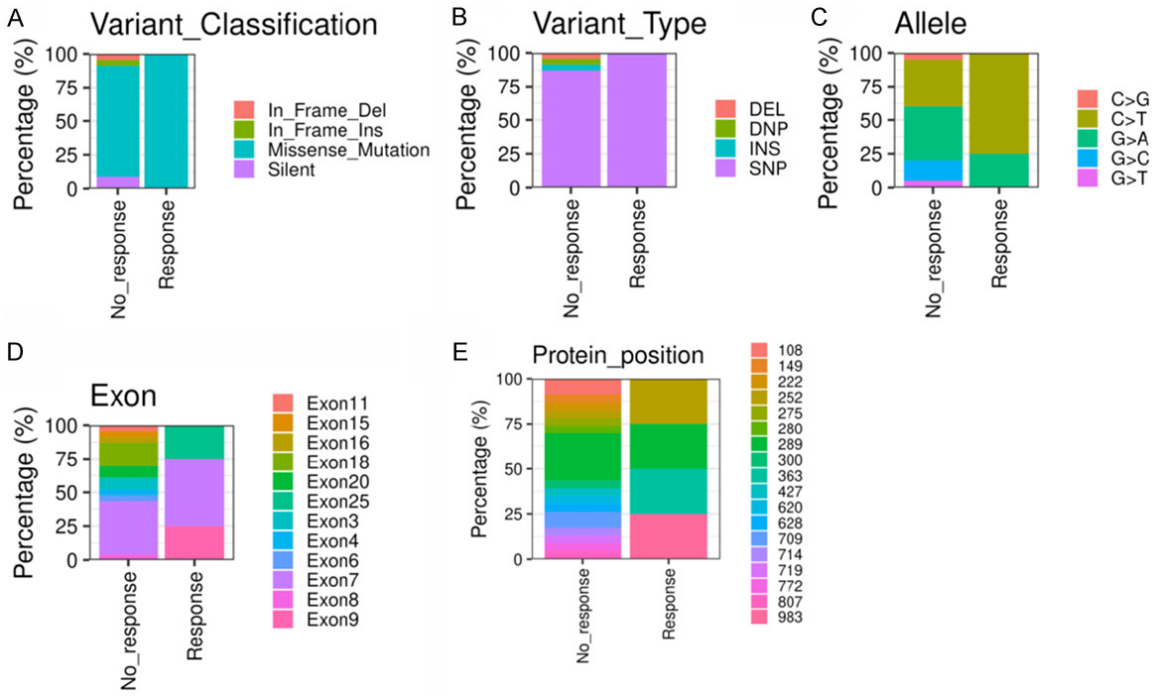

Figure 3. Frequency changes of different epidermal growth factor receptor (EGFR) mutation types in patients with temozolomide (TMZ)-resistant glioblastoma (GBM). (A-E) Proportions of variant classifications (A), variant types (B), single nucleotide polymorphisms (C), exon mutations (D), and amino acid mutations (E) frequencies of EGFR in TMZ-resistant or sensitive GBM samples. Compared to the TMZ-sensitive group, TMZ-resistant samples exhibit an increased diversity of EGFR mutation types, as well as elevated frequencies of mutations in exons 2-7 (48.15% vs. 7.41%) and amino acid positions 6-273 (18.52% vs. 3.70%). In_Frame_Del, bases deletion mutations in reading frame; In_Frame_Ins, bases insertion mutations in reading frame; DEL, deletion mutation; DNP, double nucleotide polymorphism; INS, insertion mutation; SNP, single nucleotide polymorphisms; C, cytosine; G, guanine; T, thymine; A, adenine; Response, glioblastoma patients responding to temozolomide treatment; No_response, glioblastoma patients have no or weak response to temozolomide treatment.

Transcriptome sequencing data screened EGFRvIII GBM drug resistance candidate genes to TMZ

Comparative analysis revealed 2303 (**Figure 4A**) and 1619 (**Figure 4B**) DEGs in U87OE cells following 7 and 14 days of TMZ treatment respectively, compared to the untreated group. Functionally, these candidate genes were predominantly enriched in focal adhesion, cytokine receptor interaction, Extracellular Matrix (ECM) receptor interaction, p53 signaling pathway, and so on (**Figure 5**). Previous studies have underscored the critical role of focal adhesion in conferring chemoresistance in various cancers [9]. Additionally, research by Y Rajesh et al. indicated that ECM remodeling in TMZ-resistant GBM cells [10], suggesting that exploring ECM-related pathway genes could be a promising therapeutic avenue for combating GBM with TMZ resistance. The degree of alteration in the p53 signaling pathway has been closely associated with efficacy of TMZ in treating GBM [11], further substantiating the relevance of these findings in identifying candidate genes associated with TMZ resistance.

In addition, to validate these candidate genes further, we conducted an analysis on an external data set (TCGA-GBM) where we compared 434 dysregulated genes (**Figure 4C**). These genes exhibited significant expression differences between TMZ non-responsive and TMZ-sensitive patients. Among them, 18 candidate genes displayed consistent expression patterns indicative of anti-TMZ drug properties (**Figure 4D**, **4E**).

GARNL3 prolonged the median survival of GBM patients treated with TMZ

Survival analysis conducted at 1 year, 3 years, and OS intervals for the 18 resistant candidate genes revealed that only GARNL3 gene expres-

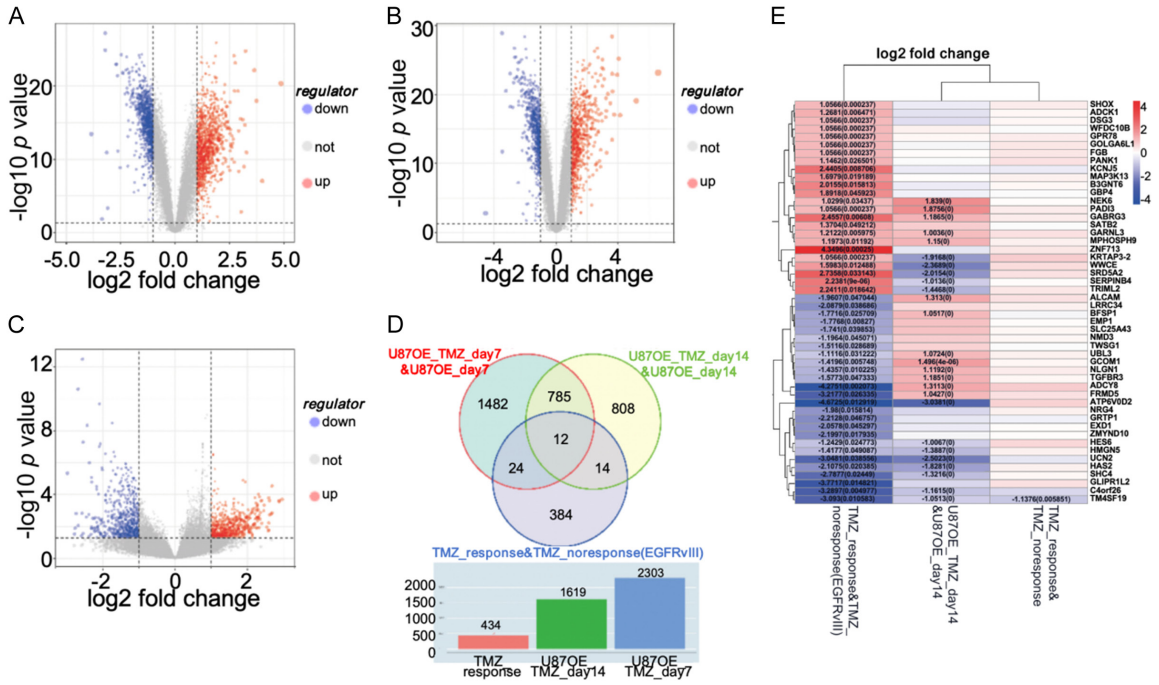

Figure 4. Difference in transcriptome expression among temozolomide (TMZ)-resistant epidermal growth factor receptor variant III (EGFRvIII)-positive glioblastoma (GBM). (A, B) Differential gene expression in EGFRvIII-positive U87 cells (U87OE) following 7-day (A) or 14-day (B) treatment with TMZ in GSE112734 data set; (C) Differential gene expression between EGFRvIII-positive GBM tissue experiencing relapse following TMZ treatment and the group that responds to TMZ treatment, as observed in The Cancer Genome Atlas Program (TCGA) database; (D) Overlapping distribution of significant differentially expressed genes (DEGs) between GSE112734 gene set and TCGA-GBM data set; (E) Expression difference fold of 50 common DEGs in GSE112734 and TCGA-GBM datasets. Red dots in the volcano map represent significantly up-regulated DEGs fold multiples and significant *P*-value distributions ($P < 0.05$ and $\log_2FC > 1$), blue dots indicate significantly down-regulated DEGs distribution ($P <$ and $\log_2FC < -1$), and gray dots indicate no DEGs distribution ($P > 0.05$, or $P < 0.05$ and $|\log_2FC| < 1$). U87OE_TMZ_day7/U87OE_TMZ_day14, EGFRvIII-positive U87 cell line treated with temozolomide for 7 or 14 days; U87OE_day7/U87OE_day14, EGFRvIII-positive U87 cell line conventional cultured for 7 or 14 days; TMZ_response, EGFRvIII-positive glioblastoma patients responding to temozolomide; TMZ_noresponse, EGFRvIII-positive glioblastoma patients have no or weak response to temozolomide.

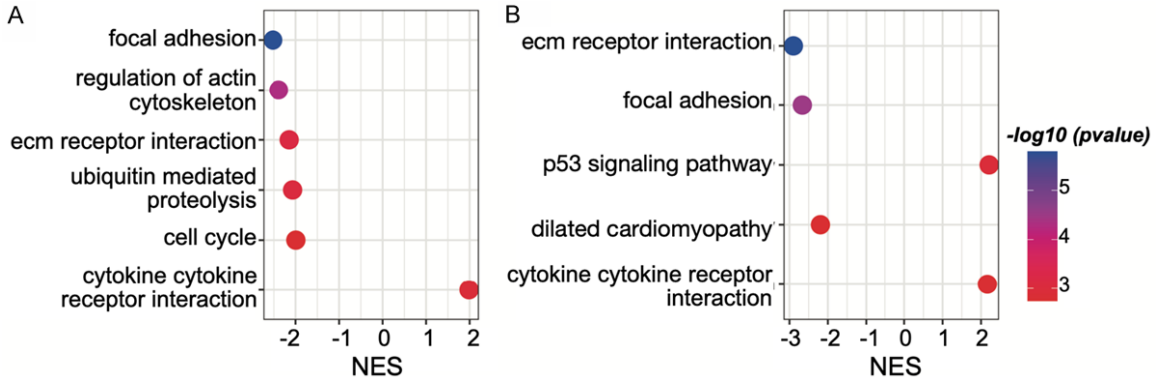

Figure 5. Gene set expression analysis (GSEA) pathway analysis of common significantly differentially expressed genes (DEGs). (A) GSEA pathway analysis of drug-resistant candidate DEG after temozolomide treatment for 7 (A) or 14 (B) days. NES, normalization Enrichment score. The higher the |NES| value, the more active the change degree of the pathway, with positive values representing activation and negative values representing inhibition.

sion levels exhibited significant differences in OS ($P < 0.05$, **Figure 6A**). Conversely, the expression of the remaining 17 resistant candidate genes showed no significant impact on

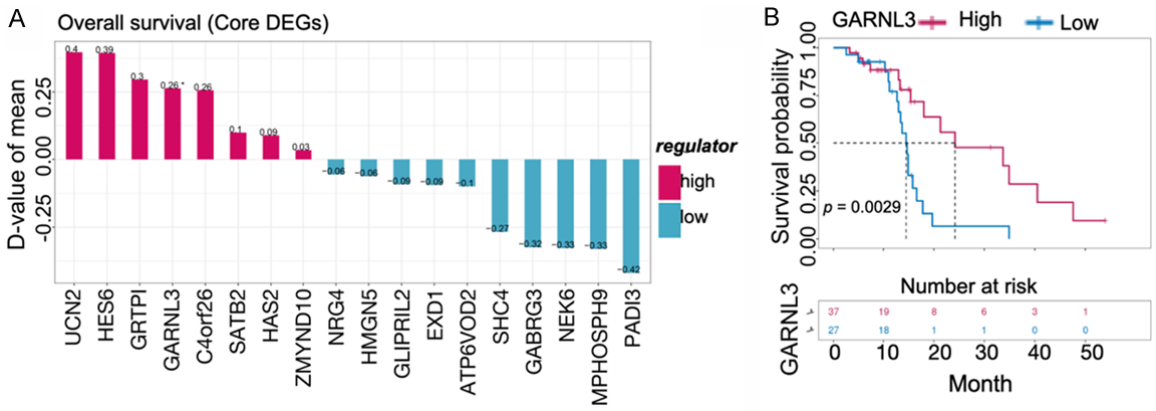

Figure 6. Results of survival analysis of 18 resistant genes. A. Overall survival (OS, year) difference results between samples with high and low expression of drug-resistant genes. Log-rank test shows the significance of OS values in samples with different expression levels; B. Kaplan-Meier survival curves of high and low expression levels of GTPase activating rap/ranGAP domain like 3 (GARNL3) gene, and differences between them are tested (*P < 0.05 compared with low level of GARNL3). DEGs, significantly differentially expressed genes.

Table 3. Correlation analysis between GTPase activating rap/ranGAP domain like 3 expression level and survival prognosis of patients

GARNL3	1-year OS			3-year OS			5-year OS	OS		
	Number	Mean ± SD	*p*	Number	Mean ± SD	*p*	Number	Number	Mean ± SD	*p*
High level	17	2.07±1.09	0.001556	3	3.95±0.56	/	0	37	1.29±1.04	0.002926
Low level	14	1.38±0.47		0	/		0	27	1.02±0.53	

GARNL3, GTPase activating rap/ranGAP domain like 3; OS, overall survival (year); SD, standard deviation.

the prognosis of patients (Supplementary Table 1; Supplementary Figure 1). In GBM patients treated with TMZ, lower GARNL3 expression levels correlated with shorter average survival of patients (Supplementary Table 2). It indicated heightened mortality of patients (**Figure 6B**). These results suggested a potential role for GARNL3 in mediating mechanisms of TMZ resistance. Thus, the effect of clinical chemotherapy may be greatly reduced. In shorter survival periods such as 1 or 3 years, UCN2 also significantly impacted the survival prognosis of patients (Supplementary Table 1), with lower gene expression levels correlating with reduced mean survival of patients (Supplementary Table 2).

We also observed a sharp decline in survival rates among patients with low GARNL3 expression at approximately 15 months post-treatment, with subsequent long-term survival rates approaching 100% (**Figure 6B**). This suggested that following TMZ treatment, low GARNL3 expression potentially contributed to short-term overall survival in GBM patients treated with TMZ (GBM-TMZ) but not to long-term overall survival (**Table 3**; **Figure 6B**). Compared to the low GARNL3 expression group (1-year OS: 14/31, 1.38±0.47; 3-year OS: 0/3), the high GARNL3 expression group exhibited higher 1-year (17/31, 2.07±1.09) and 3-year (3/3, 3.95±0.56) OS rates (**Table 3**). To facilitate smooth follow-up experiments, we also assessed expression of GARNL3 in GBM cells following 7 and 14 days of TMZ treatment. It was found that after 7 days of TMZ treatment, the downregulation trend of GARNL3 was not significant compared to untreated conditions (Supplementary Table 3). Therefore, we believed that U87OE treated with TMZ for 14 days may offer a more effective model for replicating TMZ resistance in EGFRvIII mutant GBM.

Correlation between TMZ resistance and tumor-infiltrated immune cells

The relationship between TMZ resistance and the immune environment in the tumor microenvironment was further discussed. We found that only immune scores were significantly

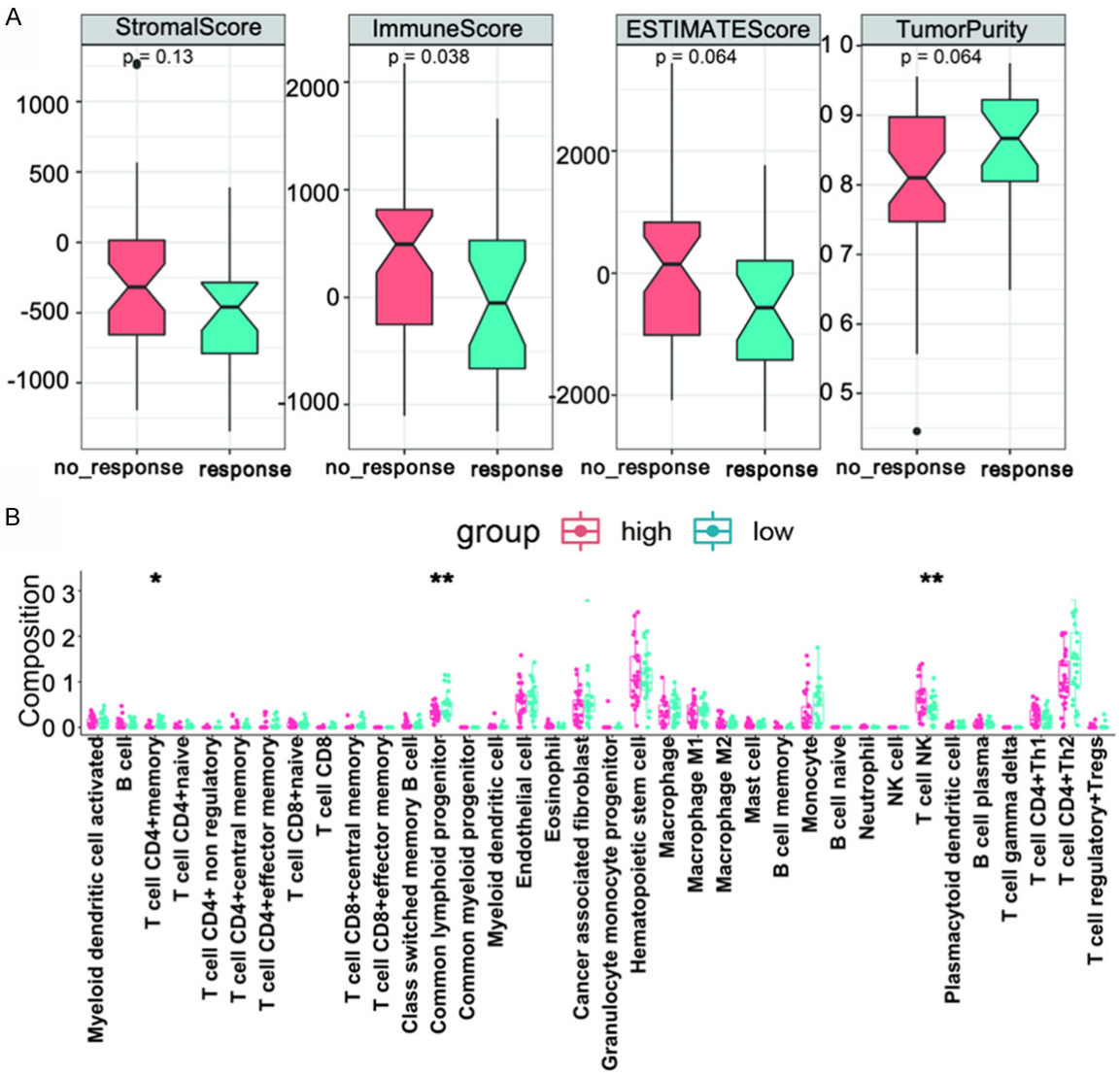

Figure 7. Difference of immunoinfiltration between temozolomide (TMZ)-sensitive and resistant glioblastoma (GBM). A. Total immune score of GBM tissues are evaluated based on the estimate algorithms and tested by Wilcox method; B. Differences in infiltration fractions of different immune cells in GBM samples with high GTPase activating rap/ranGAP domain like (GARNL3) expression compared with the low-level of GARNL group (*$P < 0.05$, **$P < 0.01$ compared control group). no_response, glioblastoma patients have no or weak response to temozolomide; response, glioblastoma patients response to temozolomide.

associated with TMZ resistance in GBM patients (**Figure 7A**). However, the mechanism underlying this resistance suggested that the tumor microenvironment mediated immune escape of tumor cells, enabling tumor cells to resist chemotherapy-induced apoptosis [12]. Therefore, further analysis was the warranted to elucidate the mechanism linking immune infiltration and TMZ resistance.

To investigate the correlation between GARNL3 expression and immune infiltration, we compared the differences in immune cell infiltration in GBM-TMZ samples. Here, the relative infiltration proportion of memory CD4+ T cells and common lymphoid progenitor cells significantly increased, while NK-T cells significantly decreased (**Figure 7B**) in the low GARNL3 expression group. These results suggested that decreased GARNL3 expression may contribute to increased immune cell infiltration. Additionally, the downregulation of NK-T cells associated with low GARNL3 expression in the tumor microenvironment, cells known for their

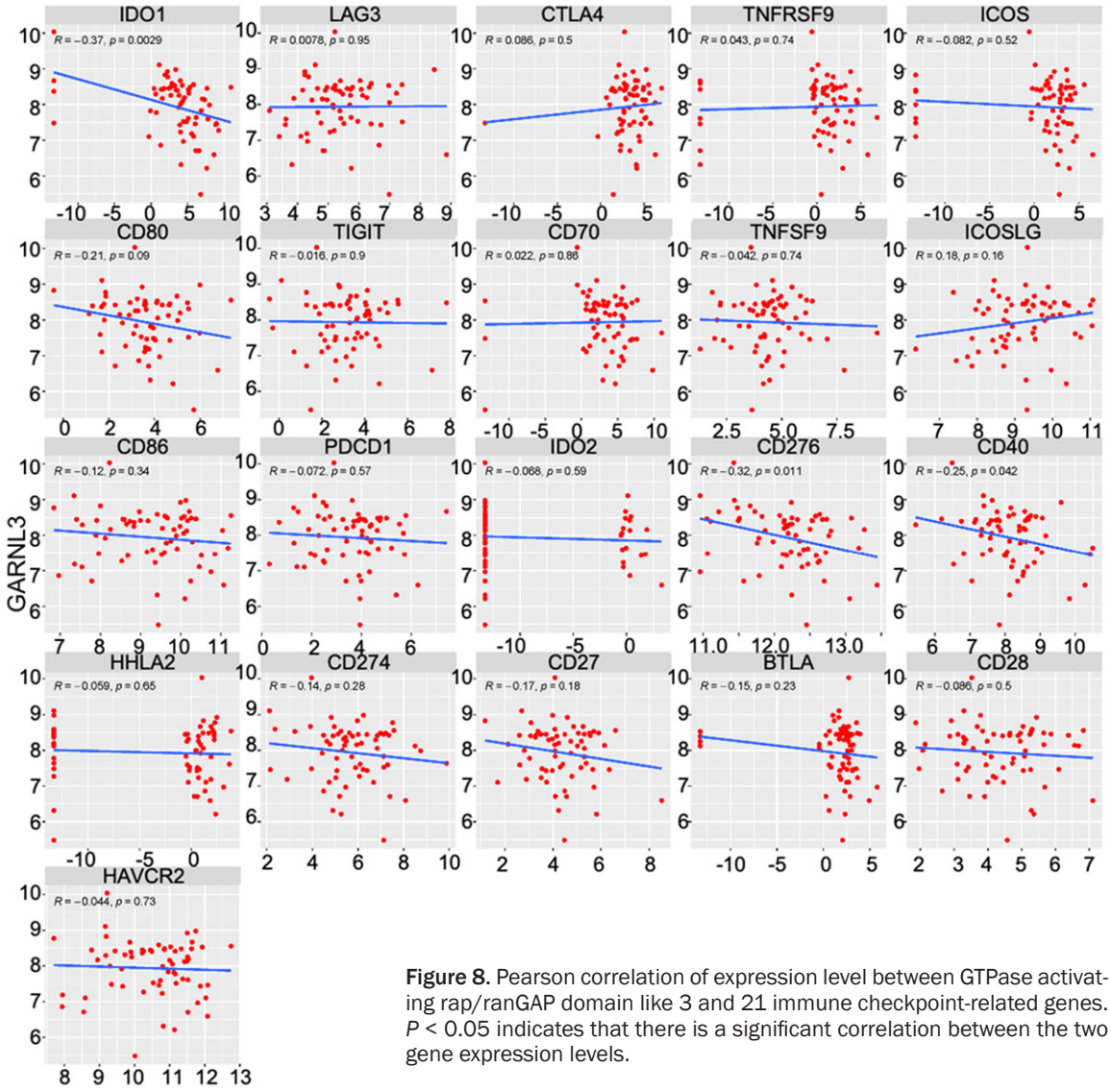

Figure 8. Pearson correlation of expression level between GTPase activating rap/ranGAP domain like 3 and 21 immune checkpoint-related genes. $P < 0.05$ indicates that there is a significant correlation between the two gene expression levels.

tumor-killing mechanisms [13], may compromise the anti-tumor response, potentially promoting tumor escape. We also observed significant downregulation of GNLY in TMZ-sensitive patients, indicating its potential role as a key marker gene for NK T cell-mediated GBM tumor escape (Supplementary Figure 2). In addition, survival analysis of NK T cell marker genes revealed no significant effect on survival of GBM-TMZ patients (Supplementary Figure 3).

Moreover, immune checkpoint-related genes played a crucial role in immune evasion [14]. Thus, we explored the correlation between GARNL3 expression and immune checkpoint-related genes, including 21 genes [15] (Supplementary Table 4). Our analysis revealed that GARNL3 expression was negatively associated with IDO1 (R = -0.37, P = 0.0029), CD40 (R = -0.25, P = 0.042) and CD276 (R = -0.32, P = 0.011). These immune checkpoint-related genes were negatively correlated with statistical significance (**Figure 8**). IDO1 has emerged as a promising immune target for treatment of GBM owing to its high expression level and effective immunosuppressive activity [16]. As co-stimulative molecules, CD40 and CD276 served as co-stimulatory molecules that can impeded T cell infiltration. These results indicated that decreased GARNL3 expression may participate in the formation of the tumor immunosuppressive microenvironment, potentially resulting in TMZ resistance.

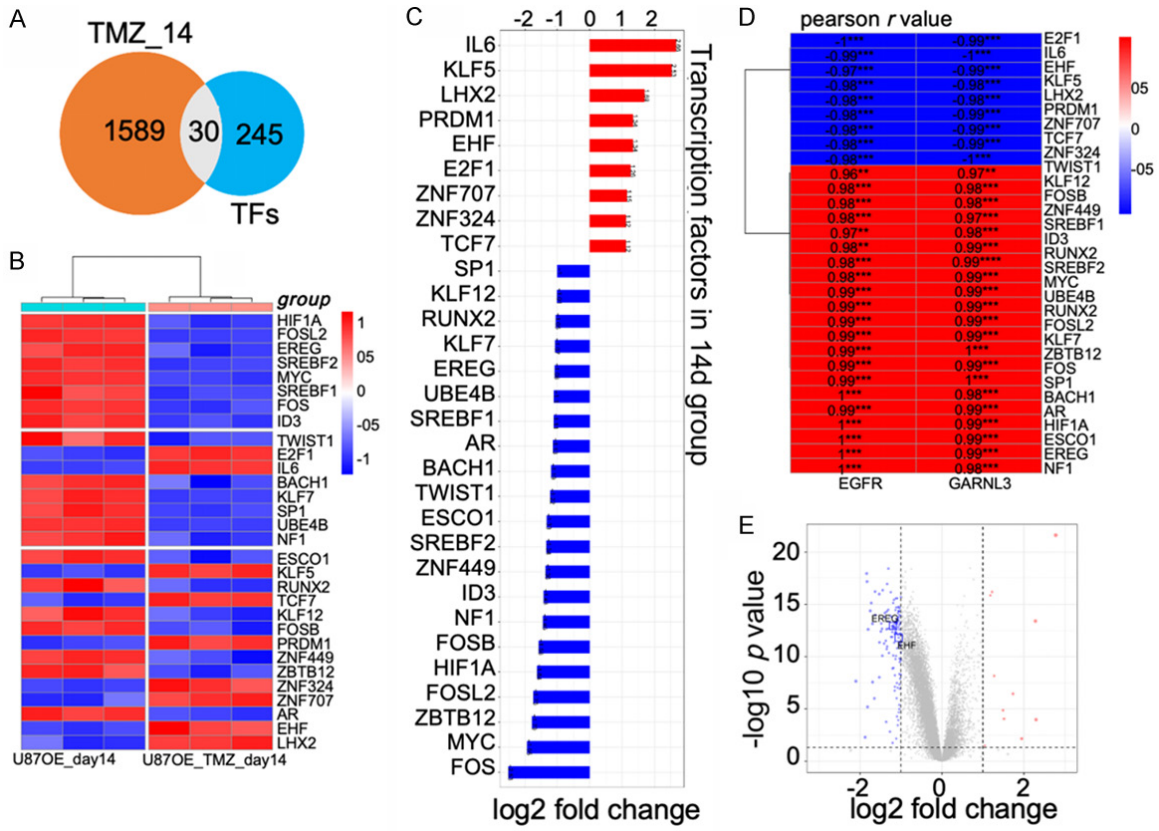

Figure 9. Acquisition of transcription factors (TFs) associated with GTPase activating rap/ranGAP domain like 3 (GARNL3) expression in temozolomide (TMZ)-resistant glioblastoma (GBM). A. Overlap between 275 TFs and significantly differentially expressed genes (DEGs) in epidermal growth factor receptor variant III (EGFRvIII)-positive U87cell line (U87OE) cultured without/with TMZ for 14 days (U87OE_day14/U87OE_TMZ_day14), indicating 30 of 275 TFs are significantly differentially expressed; B. Expression levels of 30 TFs in U87OE cell line; C. Differential expression folds of 30 TFs between U87OE_TMZ_day14 and U87OE_day14 groups; D. Pearson correlation analysis results between GARNL3, epidermal growth factor receptor (EGFR) and 30 TFs; E. Differential genes distribution between U87 cell line with or without EGFRvIII expression after treating with TMZ for 14 days (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). TMZ_14, significantly differentially expressed genes in EGFRvIII-positive U87 cell line treated with/without temozolomide for 14 days.

GARNL3 was regulated by transcription factor EREG during TMZ resistance in EGFRvIII GBM patients

We obtained 275 TFs bound to the GARNL3 promoter region. Among these, 30 TFs exhibited significant differential expression following 14 days of TMZ treatment (**Figure 9A-C**). Pearson correlation analysis revealed a significant positive correlation between GARNL3, EGFR and the expression levels of these 30 TFs (such as EREG, NF1, AR, etc.), while a significant negative correlation was observed with the transcription levels of E2F1, IL6, EHF, etc. (**Figure 9D**). It was suggested that these TFs may regulated the role of GARNL3 in EGFRvIII-positive GBM resistant to TMZ.

Further investigation into the correlation between EGFRvIII mutation and EGFR expression levels involved comparing the expression of these 30 TFs after 14 days of TMZ treatment. The results showed that only 2 genes (EREG: logFC = -1.19, P < 0.001; EHF: logFC = -1.06, P < 0.001) were significantly underexpressed in the TMZ-treated U87-EGRFVIII cell line at 14 days (**Figure 9E**).

In addition, we found that the expression of EHF increased after 14 days of TMZ treatment. This result was contrary to the previous expression trend. Thus, we focused on EREG. In summary, it can be concluded that EGFRvIII mutation may lead to the decrease of EREG. This would reduce the GARNL3 transcription level.

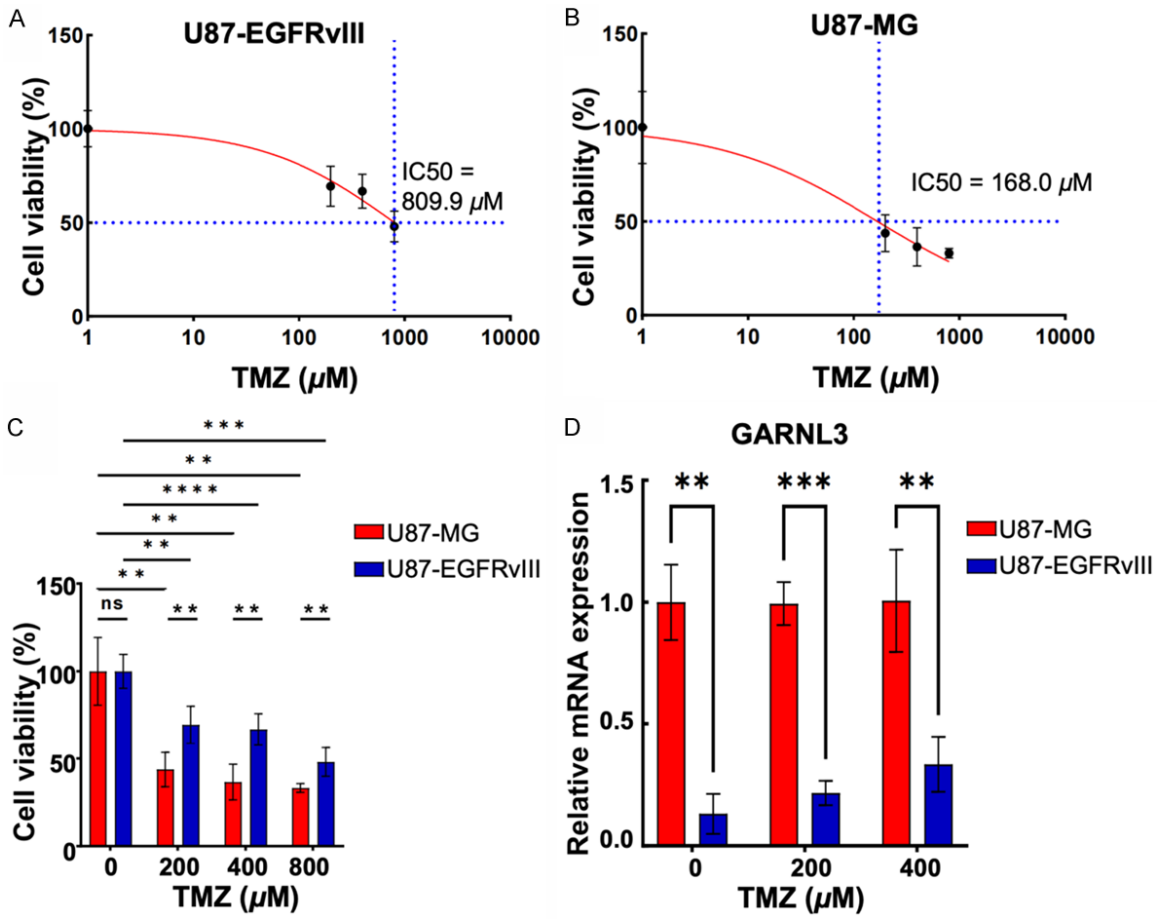

Figure 10. Effects of epidermal growth factor receptor variant III (EGFRvIII) on temozolomide (TMZ) sensitivity and GTPase activating rap/ranGAP domain like 3 (GARNL3) transcript levels in glioblastoma (GBM). (A, B) Half inhibitory concentration (IC50) of TMZ for U87 cell line expressing EGFRvIII (U87-EGFRvIII, A) and its wild type (U87-MG, B); (C, D) Changes in cell viability (C) and GARNL3 transcript levels (D) of U87-EGFRvIII cell line after intervention with different concentrations (0, 200, 400 and 800 μM/L) of TMZ (ns P>0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Finally, it played a role in promoting TMZ resistance. Importantly, these results are not conducive to the clinical benefit of patients. Additionally, differences in immunohistochemistry staining between tumor samples and normal cerebral cortex were observed in the Human Protein Atlas (HPA, https://www.proteinatlas.org/) database. The results showed that in GBM samples, the translation expression level of GARNL3 was down-regulated in GBM samples and negatively correlated with disease status [17].

Significantly low expression of GARNL3 in EGFRvIII-positive glioblastoma

Subsequently, we conducted in vitro experiments using U87 cell lines to elucidate the association between GARNL3 expression and TMZ resistance in GBM. Initially, our tests revealed a significantly higher IC50 value for U87-EGFRvIII cells (809.9 μM, **Figure 10A**) when exposed to TMZ compared to the wild-type (WT) cell line (U87-MG group, 168.0 μM, **Figure 10B**), showing a 4.8-fold increase in TMZ-resistant GBM. Following interventions with varying concentrations of TMZ, the viability of both U87-EGFRvIII and U87-MG cells markedly decreased compared to pre-intervention levels (P < 0.01 or 0.001, **Figure 10C**). However, even after TMZ treatment, the viability of U87-EGFRvIII cells remained significantly higher than that of the U87-MG drug intervention group (P < 0.01, **Figure 10C**), suggesting a potential role of EGFRvIII in promoting TMZ resistance in GBM.

Table 4. Expression level of GTPase activating rap/ranGAP domain like 3 in glioblastoma cell after intervention with temozolomide (compared to the cell line without treatment)

Temozolomide concentration	0 μM/L	200 μM/L	400 μM/L
U87-MG	1.008±0.156	7.906±1.558**	12.842±6.676*
U87-EGFRvIII	1.123±0.699	17.022±3.95**	28.519±9.597**

*, ** indicate $P < 0.01$ or 0.001 compared with GBM cell lines treated without temozolomide (0 μM/L group). U87-MG, uppsala 87 malignant glioma; U87-EGFRvIII, U87-MG with epidermal growth factor receptor variant III.

Concurrently, we observed a substantial decrease in GARNL3 expression levels in the U87-EGFRvIII cell line compared to the U87-MG cell line ($P < 0.01$, **Figure 10D**). Moreover, following TMZ administration (200 and 400 μM), the GARNL3 levels in the U87-EGFRvIII cell line remained significantly lower compared to the U87-MG cell line ($P < 0.01$ or 0.001, **Figure 10D**), indicating involvement of EGFRvIII in the downregulation of GARNL3 in GBM. Additionally, GARNL3 expression in both U87-EGFRvIII and U87-MG cell lines significantly increased after TMZ treatment (200, 400 μM) compared to pre-treatment levels (0 μM) ($P < 0.05$ or 0.01, **Table 4**). When considered alongside the cell viability test results depicted in **Figure 10**, these findings further suggested a potential role of GARNL3 expression in enhancing the inhibitory effect of TMZ on GBM cell viability.

Significantly low expression of GARNL3 in temozolomide-resistant glioblastoma

Additionally, we investigated the role of GARNL3 in U251 cells treated with TMZ. The CCK8 assay revealed a markedly elevated IC50 for TMZ-resistant U251-TMZ cells compared to U251-MG cells, indicating a 47.75-fold increase (590.2 μM/12.36 μM, **Figure 11A**, **11B**). This confirmed the establishment of a TMZ-resistant GBM cell line (U251-TMZ). Notably, after exposure to TMZ concentrations ranging from 1 to 1800 μM/L, the cell viability of U251-MG cells was consistently lower than that of U251-TMZ cells ($P < 0.05$, 0.001, or 0.0001, **Figure 11C**), highlighting the suppressive effect of TMZ on U251-MG cells compared to the TMZ-resistant U251-TMZ cells.

Furthermore, when U251-TMZ cells were co-cultured with GARNL3-expressing U251-MG cells after TMZ treatment (200 μM or 400 μM), a significant difference in cell viability was observed ($P < 0.001$, **Figure 11D**). This indicated a contrasting effect between GARNL3 expression and TMZ-mediated suppression of cell viability. Collectively, these findings corroborated the lack of involvement of GARNL3 in promoting GBM and TMZ resistance.

Discussion

Currently, TMZ is the most commonly used and effective drug for treating GBM. TMZ administration alone can improve the median survival of GBM patients by 8 months [18]. However, almost all patients eventually develop resistance. There was growing evidence that other proteins mediated tumor resistance to TMZ. In this study, we performed transcriptome screening on TMZ-treated EGFRvIII type GBM cells. Our's results determined that low GARNL3 expression was associated with promoting TMZ resistance in EGFRvIII type GBM cells. More importantly, GARNL3 was significantly lowly expressed in U87OE_TMZ_day14 with TMZ resistance. Our findings indicated that TMZ treatment with U87OE_14 d was more effective in replicating the EGFRvIII mutant GBM TMZ resistance model.

To determine whether GARNL3 had a significant impact on the prognosis of patients, we performed survival analysis on multiple candidate genes. We found no significant difference between the expression of other genes except GARNL3. It was possible that these genes were not closely related to the survival prognosis of GBM patients after TMZ treatment. It was found that the low expression of GARNL3 may contribute to the short-term overall survival of the patients themselves but not to the long-term overall survival. The mean survival time of 3 years was significantly lower than 1 year. Therefore, GARNL3 can be used as a predictor of poor prognosis in patients. Also, it would be a key target for TMZ resistance in GBM patients. The function and structure of GARNL remain understudied, but current understanding suggests its role as a GTPase activating

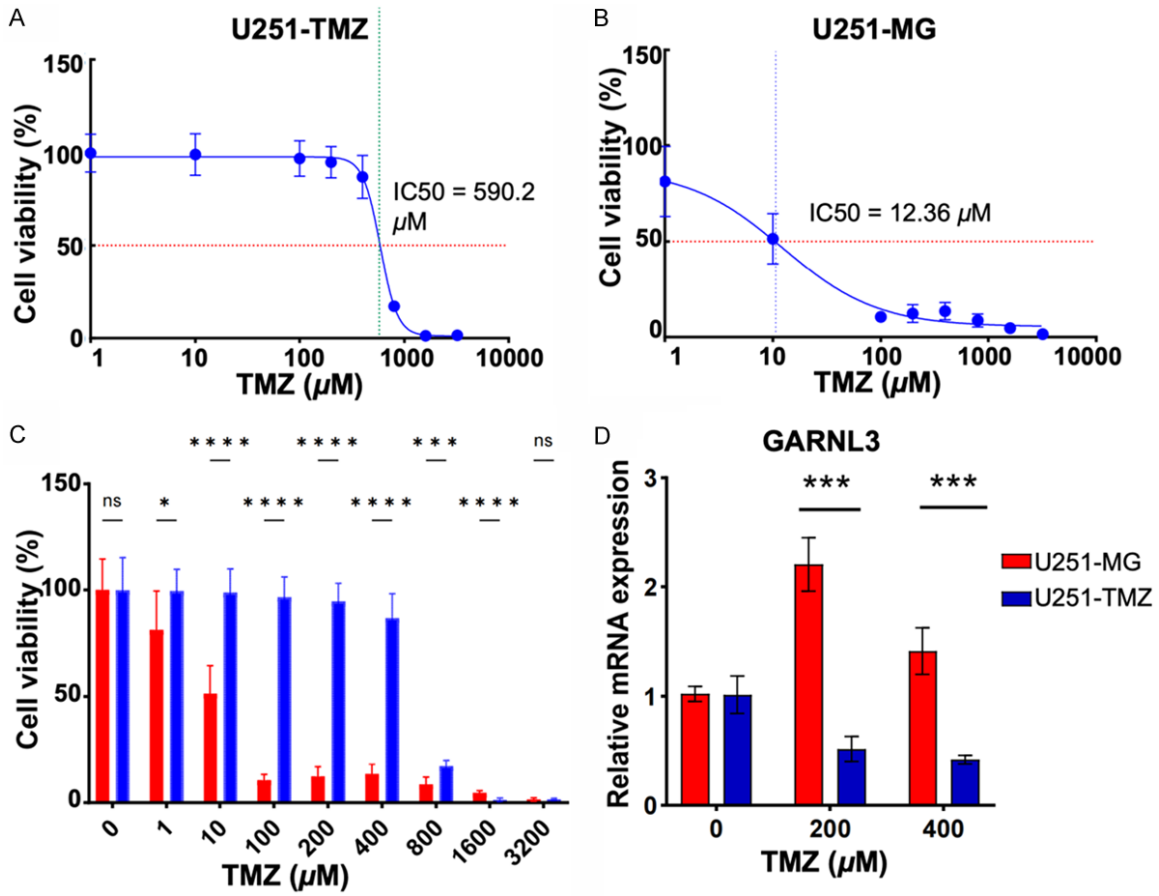

Figure 11. Expression changes of GTPase activating rap/ranGAP domain like 3 (GARNL3) in temozolomide (TMZ)-resistant glioblastoma (GBM). (A, B) Half inhibitory concentration (IC50) of TMZ for TMZ-resistant U251 cell strain (U251-TMZ, A) and its parent strain (U251-MG, B); (C) Changes in cell viability of U251-TMZ cell line after intervention with 0, 1, 10, 100, 200, 400, 800, 1600, 3200 μM/L TMZ; (D) Changes in GARNL3 expression levels of U251 drug-resistant cell lines after intervention with 0, 200 and 400 μM/L TMZ (ns $P>0.05$, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$).

protein (GAP), facilitating the conversion of GTP to GDP. Consequently, low GARNL3 expression may lead to the accumulation of GTP, activating downstream signaling cascades implicated in tumor development and drug resistance [19]. Although the understanding of GARNL3 is limited, our analysis underscores its significance in predicting tumor patient prognosis and implicates it as a gene worthy of further exploration for its potential therapeutic implications.

Chemotherapy resistance in GBM, often encountered with TMZ treatment, underscores the pivotal role of the tumor microenvironment in modulating tumor cell responses to chemotherapy. Previous studies have highlighted TMZ's potential to induce immune escape in GBM cells [20], suggesting its influence on the tumor immune microenvironment and immune checkpoint expression. To investigate this hypothesis, we analyzed the relationship between the expression of GARNL3, various immune cell infiltration, and 21 immune checkpoints. In the low GARNL3 expression group, the infiltration of NK T cells was significantly decreased, while the lymphoid precursor cells and CD4+ T cells were significantly increased. Previous studies had shown that GBM patients receiving TMZ standard chemoradiotherapy could induce significant, sustained activation of NK cells. The increase in NK cell response was significantly correlated with the prolongation of patient survival. However, CD4+ cells failed to produce a memory state after TMZ administration [21]. Therefore, we believed that the sensitivity of GBM patients to TMZ may be related to the low-

level infiltration of NK T cells, but not T cells. NK cells can spontaneously kill tumor cells by recognizing different ligands through a variety of activating receptors. In the study by Murakami et al., EGFRvIII-positive GBM cell lines were constructed and co-cultured with NK cell [22] for evaluating the anti-tumor effect of NK cell line in vitro. The findings indicated that this NK cell line effectively suppressed the growth of human GBM cells expressing EGFRvIII, including the U87MG cell line. These results suggest a negative correlation between higher NK cell activity and the aggressiveness of EGFRvIII-positive GBM cells. In addition, our analysis revealed a positive correlation between expression of GARNL3 and the infiltration level of NK cells. Therefore, it can be inferred that in EGFRvIII GBM patients exhibiting low GARNL3 expression, NK T cells may play a pivotal role in mediating communication between GBM cells and the tumor microenvironment.

To further demonstrate the key role of immune escape in GBM cells under TMZ treatment, we analyzed the correlation between GARNL3 and immune checkpoint genes. We found that GARNL3 was negatively correlated with the expression of IDO1, CD40, and CD276. Their high expression levels were all associated with poor prognosis in GBM patients [23, 24]. This finding is consistent with our current data. IDO1, an interferon-induced tryptophan catabolic enzyme, promotes immunosuppression in cancer by modulating T-cell-mediated inhibition of cytolytic CD8 effector T cells [25]. In anti-tumor immune response, increased IDO1 expression can induce baseline immunosuppression of monocytes via GBM extracellular vesicles [26]. CD40, a member of the TNF receptor family, acts as a co-stimulatory protein that plays a key role in the pro-inflammatory immune activation of antigen-presenting cells such as dendritic cells in cancer [27]. Previous studies have shown that CD40 stimulates the activation of tumor-associated T cells, inhibiting tumor progression in melanoma, lymphoma, and pancreatic cancer [28]. The study of Yang et al. [23] demonstrated that promoting CD40 therapy maximizes the activation of anti-tumor immunity, suggesting that CD40 therapy can reprogram the tumor microenvironment and enhance the therapeutic effect of GBM. CD276, an immune checkpoint molecule belonging to the CD28 family, plays a pivotal role in inhibiting T-cells in GBM. CD276 expression may vary in different subtypes of GBM such as IDH mutant GBM [29], H3 mutant GBM [30], suggesting its potential influence on biological behaviors, clinical features, and prognoses. Low GARNL3 expression predicts poor prognosis in GBM patients treated with TMZ, while high GARNL3 expression prolongs median survival and enhances the TMZ sensitivity. Depletion of GARNL3 in TMZ-sensitive GBM cells may effectively inhibit the expressions of IDO1, CD40, and CD276, thus enhancing the immune escape of tumor. These results suggest that these immune checkpoints may play a key role in inhibiting ability of GARNL3 in GBM patients after TMZ attack.

Of particular significance, EREG emerged as a pivotal transcription factor upstream of GARNL3 at both the protein and mRNA levels, potentially playing a crucial role in inducing TMZ resistance mediated by GARNL3. Initially expressed in a transmembrane form, EREG binds to EGFR, initiating downstream signaling cascade and playing essential roles in physiological stress, inflammation, and angiogenesis regulation. Normally, EREG is expressed at low levels in healthy tissues, but its upregulation can activate EGFR, promoting tumor progression, as observed in various malignancies including bladder cancer, brain cancer and breast cancer. Paradoxically, low EREG expression combined with gene mutations has been associated with tumor drug resistance and poor prognosis [31]. Our results showed that EREG was significantly underexpressed in TMZ-resistant EGFRvIII-positive GBM. In colorectal cancer (CRC), low EREG expression in macrophages coupled with RAS gene mutation was shown to promote resistance to EGFR-tyrosine kinase inhibitor, suggesting that low EREG expression in GBM patients with EGFRvIII mutation may indicate reduced sensitivity to TMZ. Further TFs analysis found that EREG positively regulated the transcription of GARNL3. Low expression of GARNL3 may be the key pivotal in TMZ resistance in EGFRvIII GBM.

This study revealed a significant underexpression of GARNL3 in EGFRvIII GBM clinical samples unresponsive to TMZ treatment. Moreover, in vitro experiments demonstrated a marked decrease in GARNL3 expression in TMZ-resistant EGFRvIII-positive GBM cell lines, a find-

ing significantly associated with adverse prognosis in GBM patients with EGFRvIII mutation. These observations strongly indicate that the EREG/GARNL3 axis represents a critical molecular mechanism underlying TMZ resistance in EGFRvIII mutant GBM. However, our study does have certain limitations. In our in vitro experiments, we did not explore the impact of interfering with the expression of GARNL3 and EREG to elucidate the regulatory relationship between EREG and GARNL3. Additionally, direct evidence confirming the role of low GARNL3 expression in promoting GBM resistance to TMZ was lacking. Addressing these aspects will be part of our future research agenda.

Conclusion

Our study presents a novel approach leveraging transcriptome sequencing systems to determine the drivers of TMZ resistance in GBM, leading to the identification of promising therapeutic targets for GBM patients. We demonstrated the pivotal roles of EREG and GARNL3 in mediating TMZ resistance, particularly in GBM harboring EGFRvIII mutations. Augmenting EREG/GARNL3 signaling largely eliminated EGFRvIII-associated TMZ resistance, suggesting that promoting EREG/GARNL3 expression could be a viable strategy for developing therapeutics. Assessment of EREG/GARNL3 expression levels may serve as valuable predictive biomarkers for treatment response in GBM patients.

Disclosure of conflict of interest

None.

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References

[1] Han L, Liu C, Qi H, Zhou J, Wen J, Wu D, Xu D, Qin M, Ren J, Wang Q, Long L, Liu Y, Chen I, Yuan X, Lu Y and Kang C. Systemic delivery of monoclonal antibodies to the central nervous system for brain tumor therapy. Adv Mater 2019; 31: e1805697.

[2] Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114: 97-109.

[3] Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, Beroukhim R, Bernard B, Wu CJ, Genovese G, Shmulevich I, Barnholtz-Sloan J, Zou L, Vegesna R, Shukla SA, Ciriello G, Yung WK, Zhang W, Sougnez C, Mikkelsen T, Aldape K, Bigner DD, Van Meir EG, Prados M, Sloan A, Black KL, Eschbacher J, Finocchiaro G, Friedman W, Andrews DW, Guha A, Iacocca M, O'Neill BP, Foltz G, Myers J, Weisenberger DJ, Penny R, Kucherlapati R, Perou CM, Hayes DN, Gibbs R, Marra M, Mills GB, Lander E, Spellman P, Wilson R, Sander C, Weinstein J, Meyerson M, Gabriel S, Laird PW, Haussler D, Getz G and Chin L; TCGA Research Network. The somatic genomic landscape of glioblastoma. Cell 2013; 155: 462-477.

[4] Rutkowska A, Stoczynska-Fidelus E, Janik K, Wlodarczyk A and Rieske P. EGFR(vIII): an oncogene with ambiguous role. J Oncol 2019; 2019: 1092587.

[5] Sangar V, Funk CC, Kusebauch U, Campbell DS, Moritz RL and Price ND. Quantitative proteomic analysis reveals effects of epidermal growth factor receptor (EGFR) on invasion-promoting proteins secreted by glioblastoma cells. Mol Cell Proteomics 2014; 13: 2618-2631.

[6] Vengoji R, Atri P, Macha MA, Seshacharyulu P, Perumal N, Mallya K, Liu Y, Smith LM, Rachagani S, Mahapatra S, Ponnusamy MP, Jain M, Batra SK and Shonka N. Differential gene expression-based connectivity mapping identified novel drug candidate and improved temozolomide efficacy for glioblastoma. J Exp Clin Cancer Res 2021; 40: 335.

[7] Dong F, Eibach M, Bartsch JW, Dolga AM, Schlomann U, Conrad C, Schieber S, Schilling O, Biniossek ML, Culmsee C, Strik H, Koller G, Carl B and Nimsky C. The metalloprotease-disintegrin ADAM8 contributes to temozolomide chemoresistance and enhanced invasiveness of human glioblastoma cells. Neuro Oncol 2015; 17: 1474-1485.

[8] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A,

Pomeroy SL, Golub TR, Lander ES and Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.

[9] Berrazouane S, Doucet A, Boisvert M, Barabe F and Aoudjit F. VLA-4 induces chemoresistance of T cell acute lymphoblastic leukemia cells via PYK2-mediated drug efflux. Cancers (Basel) 2021; 13: 3512.

[10] Rajesh Y, Biswas A, Kumar U, Das S, Banerjee I, Banik P, Bharti R, Nayak S, Ghosh SK and Mandal M. Targeting NFE2L2, a transcription factor upstream of MMP-2: a potential therapeutic strategy for temozolomide resistant glioblastoma. Biochem Pharmacol 2019; 164: 1-16.

[11] Lan Y, Lou J, Hu J, Yu Z, Lyu W and Zhang B. Downregulation of SNRPG induces cell cycle arrest and sensitizes human glioblastoma cells to temozolomide by targeting Myc through a p53-dependent signaling pathway. Cancer Biol Med 2020; 17: 112-131.

[12] Castells M, Thibault B, Delord JP and Couderc B. Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death. Int J Mol Sci 2012; 13: 9545-9571.

[13] Maskalenko NA, Zhigarev D and Campbell KS. Harnessing natural killer cells for cancer immunotherapy: dispatching the first responders. Nat Rev Drug Discov 2022; 21: 559-577.

[14] Topalian SL, Drake CG and Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell 2015; 27: 450-461.

[15] Zhao Y, Zhang M, Pu H, Guo S, Zhang S and Wang Y. Prognostic implications of pan-cancer CMTM6 expression and its relationship with the immune microenvironment. Front Oncol 2021; 10: 585961.

[16] Lukas RV, Juhasz C, Wainwright DA, James CD, Kennedy E, Stupp R and Lesniak MS. Imaging tryptophan uptake with positron emission tomography in glioblastoma patients treated with indoximod. J Neurooncol 2019; 141: 111-120.

[17] Li C, Pu B, Gu L, Zhang M, Shen H, Yuan Y and Liao L. Identification of key modules and hub genes in glioblastoma multiforme based on co-expression network analysis. FEBS Open Bio 2021; 11: 833-850.

[18] Back MF, Ang EL, Ng WH, See SJ, Lim CC, Chan SP and Yeo TT. Improved median survival for glioblastoma multiforme following introduction of adjuvant temozolomide chemotherapy. Ann Acad Med Singap 2007; 36: 338-342.

[19] He H, Huang J, Wu S, Jiang S, Liang L, Liu Y, Liu W, Xie L, Tao Y, Jiang Y and Cong L. The roles of GTPase-activating proteins in regulated cell death and tumor immunity. J Hematol Oncol 2021; 14: 171.

[20] Wang S, Yao F, Lu X, Li Q, Su Z, Lee JH, Wang C and Du L. Temozolomide promotes immune escape of GBM cells via upregulating PD-L1. Am J Cancer Res 2019; 9: 1161-1171.

[21] Pellegatta S, Eoli M, Cuccarini V, Anghileri E, Pollo B, Pessina S, Frigerio S, Servida M, Cuppini L, Antozzi C, Cuzzubbo S, Corbetta C, Paterra R, Acerbi F, Ferroli P, DiMeco F, Fariselli L, Parati EA, Bruzzone MG and Finocchiaro G. Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not CD8(+) T cell activation in the presence of adjuvant temozolomide. Oncoimmunology 2018; 7: e1412901.

[22] Murakami T, Nakazawa T, Natsume A, Nishimura F, Nakamura M, Matsuda R, Omoto K, Tanaka Y, Shida Y, Park YS, Motoyama Y, Nakagawa I, Yamada S, Tamura K, Takeshima Y, Takamura Y, Wakabayashi T and Nakase H. Novel human NK cell line carrying CAR targeting EGFRvIII induces antitumor effects in glioblastoma cells. Anticancer Res 2018; 38: 5049-5056.

[23] Yang F, He Z, Duan H, Zhang D, Li J, Yang H, Dorsey JF, Zou W, Nabavizadeh SA, Bagley SJ, Abdullah K, Brem S, Zhang L, Xu X, Byrne KT, Vonderheide RH, Gong Y and Fan Y. Synergistic immunotherapy of glioblastoma by dual targeting of IL-6 and CD40. Nat Commun 2021; 12: 3424.

[24] Zhang C, Zhang Z, Li F, Shen Z, Qiao Y, Li L, Liu S, Song M, Zhao X, Ren F, He Q, Yang B, Fan R and Zhang Y. Large-scale analysis reveals the specific clinical and immune features of B7-H3 in glioma. Oncoimmunology 2018; 7: e1461304.

[25] Zhai L, Ladomersky E, Lauing KL, Wu M, Genet M, Gritsina G, Gyorffy B, Brastianos PK, Binder DC, Sosman JA, Giles FJ, James CD, Horbinski C, Stupp R and Wainwright DA. Infiltrating T cells increase IDO1 expression in glioblastoma and contribute to decreased patient survival. Clin Cancer Res 2017; 23: 6650-6660.

[26] Jung MY, Aibaidula A, Brown DA, Himes BT, Cumba Garcia LM and Parney IF. Superinduction of immunosuppressive glioblastoma extracellular vesicles by IFN-gamma through PD-L1 and IDO1. Neurooncol Adv 2022; 4: vdac017.

[27] Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y and Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev 2009; 229: 152-172.

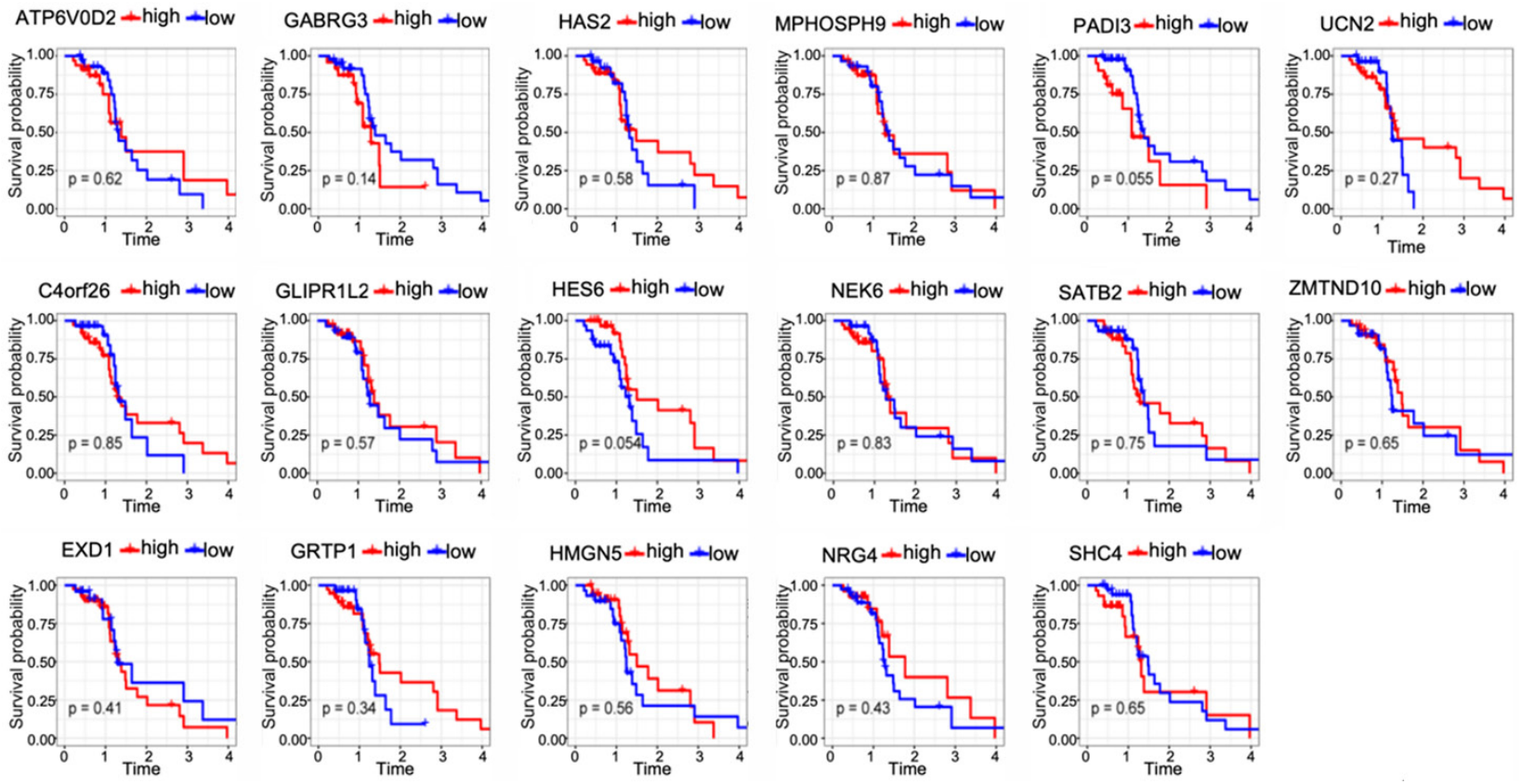
[28] von Leoprechting A, van der Bruggen P, Pahl HL, Aruffo A and Simon JC. Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lympho-

cyte-mediated lysis and induces apoptosis. Cancer Res 1999; 59: 1287-1294.
[29] Zhang M, Zhang H, Fu M, Zhang J, Zhang C, Lv Y, Fan F, Zhang J, Xu H, Ye D, Yang H, Hua W and Mao Y. The inhibition of B7H3 by 2-HG accumulation is associated with downregulation of VEGFA in IDH mutated gliomas. Front Cell Dev Biol 2021; 9: 670145.
[30] Zhou Z, Luther N, Ibrahim GM, Hawkins C, Vibhakar R, Handler MH and Souweidane MM. B7-H3, a potential therapeutic target, is expressed in diffuse intrinsic pontine glioma. J Neurooncol 2013; 111: 257-264.
[31] Cheng WL, Feng PH, Lee KY, Chen KY, Sun WL, Van Hiep N, Luo CS and Wu SM. The role of EREG/EGFR pathway in tumor progression. Int J Mol Sci 2021; 22: 12828.

Supplementary Table 1. Summary of significant *p* values of 18 hub genes related to the prognosis of glioblastoma patients treated with temozolomide

SYMBOL	one year_pval	OS_pval	three year_pval
ATP6V0D2	0.160933401	0.622196536	0.157299207
C4orf26	0.247337567	0.851760058	NA
EXD1	0.218658879	0.41300334	0.808365156
GABRG3	0.638540089	0.135901243	NA
GARNL3	0.001555912	0.002926491	NA
GLIPR1L2	0.747965746	0.568032906	0.225252906
GRTP1	0.090406499	0.33743911	NA
HAS2	0.462551576	0.578976114	NA
HES6	0.321569458	0.054103581	0.808365156
HMGN5	0.905464561	0.56346019	0.157299207
MPHOSPH9	0.676291013	0.869573179	0.808365156
NEK6	0.789989275	0.827212263	0.808365156
NRG4	0.441646079	0.433593433	0.225252906
PADI3	0.66053993	0.054739511	NA
SATB2	0.939967904	0.750194575	0.225252906
SHC4	0.330208711	0.650418028	0.808365156
UCN2	0.021961183	0.266720608	NA
ZMYND10	0.692446629	0.646483412	0.225252906

*OS, overall survival.

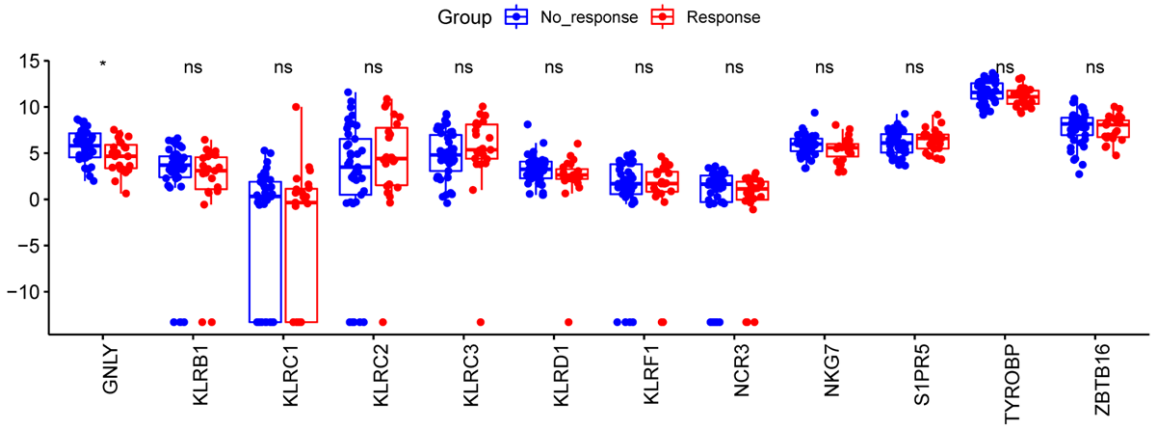


Supplementary Figure 1. Survival curves of the other 17 candidate genes.

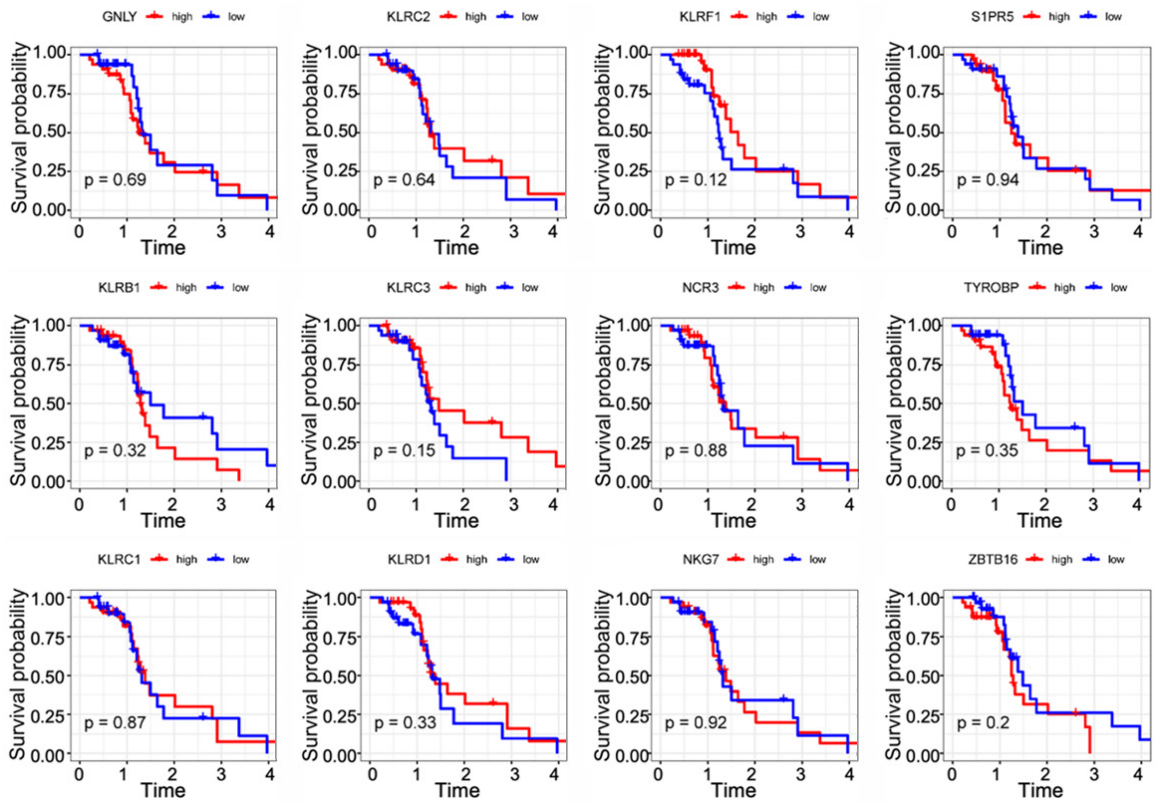
Supplementary Table 2. Statistical results of 1-year, 3-year and overall survival rate of glioblastoma patients treated with temozolomide with different expression levels of 18 key genes

symbol	one year OS_SD	one year OS_mean	one year OS_number	three year OS_SD	three year OS_mean	three year OS_number	OS_ SD	OS_ mean	OS_ number
ATP6V0D2_High	1.23	2.02	12.00	0.38	4.23	2.00	1.00	1.13	33.00
ATP6V0D2_Low	0.65	1.60	19.00	NA	3.38	1.00	0.71	1.23	31.00
C4orf26_High	1.12	2.02	17.00	0.56	3.95	3.00	1.04	1.29	36.00
C4orf26_Low	0.49	1.45	14.00	NA	NA	NA	0.56	1.03	28.00
EXD1_High	0.81	1.70	19.00	NA	3.97	1.00	0.78	1.14	40.00
EXD1_Low	1.11	1.86	12.00	0.80	3.94	2.00	1.01	1.23	24.00
GABRG3_High	0.47	1.41	9.00	NA	NA	NA	0.48	0.97	24.00
GABRG3_Low	1.03	1.90	22.00	0.56	3.95	3.00	1.02	1.30	40.00
GARNL3_High	1.09	2.07	17.00	0.56	3.95	3.00	1.04	1.29	37.00
GARNL3_Low	0.47	1.38	14.00	NA	NA	NA	0.53	1.02	27.00
GLIPR1L2_High	0.89	1.74	17.00	0.42	3.67	2.00	0.85	1.13	36.00
GLIPR1L2_Low	0.99	1.78	14.00	NA	4.50	1.00	0.90	1.23	28.00
GRTP1_High	1.08	1.98	19.00	0.56	3.95	3.00	1.05	1.30	37.00
GRTP1_Low	0.43	1.41	12.00	NA	NA	NA	0.49	1.00	27.00
HAS2_High	1.16	1.97	16.00	0.56	3.95	3.00	1.03	1.21	36.00
HAS2_Low	0.53	1.54	15.00	NA	NA	NA	0.61	1.13	28.00
HES6_High	1.01	1.93	18.00	0.80	3.94	2.00	0.97	1.37	33.00
HES6_Low	0.76	1.53	13.00	NA	3.97	1.00	0.70	0.97	31.00
HMGN5_High	0.75	1.70	17.00	NA	3.38	1.00	0.76	1.15	35.00
HMGN5_Low	1.12	1.83	14.00	0.38	4.23	2.00	0.99	1.21	29.00
MPHOSPH9_High	0.90	1.65	14.00	NA	3.97	1.00	0.77	1.03	35.00
MPHOSPH9_Low	0.96	1.85	17.00	0.80	3.94	2.00	0.95	1.36	29.00
NEK6_High	0.85	1.68	15.00	NA	3.97	1.00	0.77	1.04	37.00
NEK6_Low	1.01	1.83	16.00	0.80	3.94	2.00	0.97	1.36	27.00
NRG4_High	1.05	1.94	10.00	0.42	3.67	2.00	0.91	1.14	26.00
NRG4_Low	0.87	1.68	21.00	NA	4.50	1.00	0.85	1.20	38.00
PADI3_High	0.65	1.54	7.00	NA	NA	NA	0.61	0.89	21.00
PADI3_Low	0.99	1.82	24.00	0.56	3.95	3.00	0.94	1.31	43.00
SATB2_High	0.94	1.84	17.00	0.42	3.67	2.00	0.89	1.22	35.00
SATB2_Low	0.93	1.66	14.00	NA	4.50	1.00	0.85	1.12	29.00
SHC4_High	0.97	1.84	10.00	NA	3.97	1.00	0.83	1.03	29.00
SHC4_Low	0.92	1.72	21.00	0.80	3.94	2.00	0.89	1.30	35.00
UCN2_High	1.09	2.03	19.00	0.56	3.95	3.00	1.06	1.34	37.00
UCN2_Low	0.22	1.33	12.00	NA	NA	NA	0.41	0.95	27.00
ZMYND10_High	0.94	1.85	15.00	0.42	3.67	2.00	0.90	1.19	32.00
ZMYND10_Low	0.92	1.68	16.00	NA	4.50	1.00	0.85	1.16	32.00

*OS, Overall survival; SD, standard deviation.



Supplementary Figure 2. Differences in expression levels of 12 marker genes of natural killer-T cells in glioblastoma tissues with temozolomide (TMZ) (ns $P>0.05$, $*P < 0.05$ compared with TMZ response group). No_response, glioblastoma patients have no or weak response to temozolomide; Response, glioblastoma patients response to temozolomide.



Supplementary Figure 3. Prognosis survival curve of 12 marker genes of natural killer-T cells in glioblastoma patients.

Supplementary Table 4. List of genes enriched in negative immune regulation function and pathways

gene	function	Expressed cells
BTLA	It plays a negative regulatory role in the body's anti-tumor immune response and is related to the immune escape mechanism of tumor	B cells, T cells, NK cells, dendritic cells and macrophages
CD27	It plays an important role in T cell activation, CTL differentiation and cytotoxic function, B cell differentiation and Ig production, and the regulation of NK cytotoxic activity	Adolescent T cells, memory B cells, NK cells, hematopoietic stem cells (HSCs) and progenitor cells
CD274	It is the key to T cell proliferation and production of IFNγ and IL-10. The interaction with PDCD1 can inhibit T cell proliferation and cytokine production	Activated T and B cells, dendritic cells, keratinocytes and monocytes
CD276	It leads to increased NF-κB activity, increased expression of VEGF and IL-8, and further promotes tumor-related angiogenesis and tumor invasion	Resting fibroblasts, endothelial cells, osteoblasts
CD28	It is a costimulatory molecule expressed on the surface of T cells and plays an important role in the activation of T cells	T cells
CD40	A surface antigen related to the function of T cells and B cells, which is essential for mediating a variety of immune and inflammatory reactions, including t cell-dependent immunoglobulin conversion, memory of b cell development and germinal center formation	B cells, thymic epithelial cells, activated monocytes/macrophages, dendritic cells
CD70	It induces the proliferation of costimulatory t cells, promotes the production of cytolytic t cells, and contributes to the activation of t cells	B cells, T cells
CD80	It is involved in the activation and proliferation of costimulatory T cells, and plays an important role in autoimmunity, humoral immunity and transplantation rejection	Activated B cells, activated T cells, macrophages, peripheral blood mononuclear cells and dendritic cells
CD86	It is involved in the activation and proliferation of costimulatory T cells	Monocytes, activated B and T cells, and endothelial cells
CTLA4	Acting as an immune checkpoint and down-regulating immune response	Regulatory T cells (Treg)
HAVCR2	It is a Th1-specific cell surface protein activated by macrophages, which inhibits Th1-mediated autoimmune and alloimmune responses, and promotes immune tolerance	Th1, Th17, Treg, NK, NK-T, dendritic cells, monocytes, mast cells and macrophages
HHLA2	Exert the function of co-inhibition and co-stimulation	B cells
ICOS	It plays an important role in the re-immunization stage of the body, inflammatory reaction, autoimmune diseases, tumor immunity and transplantation immunity	Activated T cells
ICOSLG	It is involved in the positive regulation of T cell receptor signal pathway and interleukin-4 production	Peripheral blood B cells, monocytes, dendritic cells
IDO1	It exerts antibacterial and anti-tumor defense, neuropathology, immune regulation and antioxidant activities	Dendritic cells, monocytes and macrophages
IDO2	It can inhibit the proliferation of CD4+T cells and CD8+T cells, which is closely related to body immunity and tumor	Dendritic cells
LAG3	Its combination with MHC II molecules down-regulates the activity of T cells	Activated T cells, NK cells, B cells and plasma cell dendritic cells
PDCD1	It plays a role in the late stage of T cell reaction (immune effect stage)	B cells, NK cells, dendritic cells, macrophages
TIGIT	Suppress immune cells in multiple steps of tumor immune cycle	cells, regulatory CD4+T cells, follicle-assisted CD4+T cells, effector CD8+T cells and NK cells
TNFRSF9	Transmit activation, proliferation or apoptosis signals between immune cells to regulate T-cell-mediated immune response	CD4+ and CD8+ T cells
TNFSF9	Transmit activation, proliferation or apoptosis signals between immune cells to regulate T-cell-mediated immune response	CD4+ and CD8+ T cells