



# Redox Regulator *GLRX* Is Associated With Tumor Immunity in Glioma

Yuanhao Chang<sup>1†</sup>, Guanzhang Li<sup>1†</sup>, You Zhai<sup>1</sup>, Lijie Huang<sup>1</sup>, Yuemei Feng<sup>1</sup>, Di Wang<sup>2</sup>, Wei Zhang<sup>2,3,4\*</sup> and Huimin Hu<sup>1,4\*</sup>

<sup>1</sup> Department of Molecular Neuropathology, Beijing Neurosurgical Institute, Capital Medical University, Beijing, China,

<sup>2</sup> Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing, China, <sup>3</sup> China National Clinical Research Center for Neurological Diseases, Beijing, China, <sup>4</sup> Chinese Glioma Genome Atlas Network (CGGA) and Asian Glioma Genome Atlas Network (AGGA), Beijing, China

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### \*Correspondence:

Wei Zhang  
zhangwei\_vincent@126.com  
Huimin Hu  
huhm\_bjni@163.com

<sup>†</sup>These authors have contributed  
equally to this work

### Specialty section:

This article was submitted to  
Cancer Immunity and Immunotherapy,  
a section of the journal  
Frontiers in Immunology

**Received:** 07 July 2020

**Accepted:** 26 October 2020

**Published:** 30 November 2020

### Citation:

Chang Y, Li G, Zhai Y, Huang L,  
Feng Y, Wang D, Zhang W and Hu H  
(2020) Redox Regulator *GLRX* Is  
Associated With Tumor  
Immunity in Glioma.  
*Front. Immunol.* 11:580934.  
doi: 10.3389/fimmu.2020.580934

Glutaredoxin is central to cellular redox chemistry and regulates redox homeostasis and malignant progression of many cancers. In glioma, the role of its coding gene (*GLRX*) remains unclear. We aimed to elucidate the role of glutaredoxin at the transcriptome level and its clinical prognostic value in glioma. In total, we evaluated 1,717 glioma samples with transcriptome data and corresponding clinical data as well as single-cell sequencing data from 6 glioma patients from publicly available databases. Gene set variation analysis and gene ontology analysis were performed to reveal the biological function of *GLRX*. The immune cell enrichment score was calculated by GSVA analysis. Single-cell sequencing data was visualized by *t*-distributed stochastic neighbor embedding analysis. The prognostic value of *GLRX* in glioma was verified by the Kaplan-Meier curve and multivariate COX analysis. *GLRX* was found to be highly enriched in gliomas of higher grades with wild-type *IDH*, without 1p/19q co-deletion, and with a methylated *MGMT* promoter. Moreover, *GLRX* could be a potential marker for the mesenchymal molecular subtype of gliomas. The expression of *GLRX* was closely related to the tumor immune process, immune checkpoints, and inflammatory factors with *GLRX* being specifically expressed in M0 macrophages. *GLRX* is also shown to be an independent prognostic factor in glioma. Altogether, our study outcomes show that *GLRX* is highly enriched in malignant gliomas and is closely related to the tumor immune microenvironment. Therefore, *GLRX*-targeted cell redox regulatory therapy may enhance the efficacy of glioma immunotherapy.

**Keywords:** glioma, *GLRX*, macrophage, prognosis, tumor immunity

## INTRODUCTION

Glioma is the most common malignant tumor affecting the central nervous system, and it is mainly characterized by a high recurrence rate and short survival time (1). To date, the most effective treatment for glioma is surgical resection to maximum safety extent (2), which can be followed by additional individualized treatments such as radiotherapy and chemotherapy. Even with aggressive

treatment, the prognosis for glioma patients remains very poor. Therefore, finding novel therapeutic targets and molecular targeted drugs may pave the way for an improved prognosis for these patients.

Glutaredoxin (Grx), also known as thiol transferase, is ubiquitously expressed in bacteria, viruses, and mammals. It has a relative molecular weight of approximately 12 kDa and comprises 106–107 amino acids (3). Grx is an important component of the thiol-disulfide bond oxidoreductase family and catalyzes the redox reaction between glutathione (GSH) and protein disulfide bonds that are necessary for optimal protein activity (4). Several studies have reported that Grx performs a variety of biological functions in cancer related to relieving oxidative stress, transcription regulation, and control of DNA synthesis by modulating the activity of ribonucleotide reductase (3, 5, 6). However, there are few reports on the role of Grx in glioma.

The GSH system is an essential regulator of redox balance in the brain (7), and Grx acts as a central “antioxidant” in neurons to protect them from oxidative stress injury. Previous studies have reported that Grx is also involved in glioma and metastasis development as well as in drug resistance (6, 8). Therefore, understanding the role of Grx in the context of glioma is pivotal for the development of novel therapeutic approaches targeting malignant gliomas.

We investigated the expression and function of the Grx coding gene (*GLRX*) at the transcriptome level using publicly available data sets from the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA), which included RNA sequencing (RNA-seq) data and corresponding clinical details about the cancer patients. We found that *GLRX* is associated with high tumor grade and malignant phenotypes. Moreover, gene ontology analysis and gene set variation analysis revealed, for the first time, that *GLRX* can function as a mediator of the immune response. Further CIBERSORT analysis revealed that a higher expression level of *GLRX* is correlated with enrichment of macrophages in glioma tissue. Single-cell analysis, immunohistochemical (IHC) staining, and immunofluorescent staining (IF) validated that *GLRX* may be specifically expressed in M0 macrophages. Last, we found that *GLRX* is an independent prognostic factor in glioma. Altogether, these findings suggest that *GLRX* is highly enriched in malignant gliomas and is closely related to the tumor immune microenvironment. Therefore, *GLRX*-targeted cell redox regulatory therapy may enhance response to immunotherapy in patients with glioma.

## MATERIALS AND METHODS

### Data Collection

This study was approved by the Capital Medical University Institutional Review Board. We collected transcriptome sequencing data generated by the Illumina HiSeq platform that was publicly available from the CGGA and CGGA (2019) databases (<https://www.cgga.org.cn>) for 325 and 693 samples, respectively. We evaluated the status of isocitrate dehydrogenase

(IDH) mutation, 1p/19q, and MGMT promoter methylation as described in previous studies (9–11). Overall patient survival was estimated from the date of diagnosis to the reported date of death or last follow-up. RNA-seq data were obtained from TCGA (<https://tcgadata.nci.nih.gov>), and single-cell sequencing data were retrieved from the GSE89567 data set of the Gene Expression Omnibus database. All clinical and molecular information on the samples evaluated in the present study is presented in **Table 1**. We used the online software GEPIA (<http://gepia.cancer-pku.cn>) (12) to evaluate expression differences between glioblastoma multiform (GBM) and normal brain tissues.

### Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

The biological functions and signaling pathways related to *GLRX* were explored by GO and KEGG analyses using the DAVID bioinformatics resource (version 6.7) (13). After Spearman correlation analysis, GO results on the most correlated genes were visualized by heat map.

### Gene Set Variation Analysis (GSVA)

GSVA was performed with the GSVA package (from R Project 3.5.1) of R software with default parameters. The list of GO terms was obtained from the Gene Set Enrichment Analysis database (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>). Relationships between genes and biological functions were determined using Pearson correlation analysis.

### Immune Function Analysis

The relationship between *GLRX* expression and immune function was evaluated by Pearson correlation analysis. Immune function scores (14) were calculated by GSVA analysis, and the immune function gene set was downloaded from AmiGO 2 (<http://amigo.geneontology.org/amigo/landing>). The classification of immune functions was done according to the guidelines of AmiGO 2.

### CIBERSORT

RNA-seq data were evaluated using the CIBERSORT software (<https://cibersort.stanford.edu>). The signature gene profile of 22 immune cell types was used in CIBERSORT to estimate the proportion of tumor-infiltrating immune cell types (15).

### T-Distributed Stochastic Neighbor Embedding (T-SNE) Analysis

T-SNE analysis was performed with the Rtsne package from R Project (version 3.5.1); perplexity was set to 20. Identification of cell types was based on the specific cell markers obtained from the CellMarker database (<http://biocc.hrbmu.edu.cn/CellMarker/>).

### Prognostic Analysis

Patient survival distribution and significance was evaluated by Kaplan-Meier survival curve and log-rank test. Kaplan-Meier

**TABLE 1 |** Sample information.

Characteristics (CGGA)	No. of Patients (n=325)
Age	
<45	191
≥45	134
Gender	
Male	203
Female	122
WHO Grade	
Grade II	103
Grade III	79
Grade IV	139
NA	4
TCGA Subtypes	
Proneural	102
Neural	81
Classical	74
Mesenchymal	68
Radiotherapy+TMZ Chemotherapy	
Yes	154
No	24
Radiotherapy	
Yes	258
No	51
NA	16
TMZ Chemotherapy	
Yes	178
No	124
NA	23
IDH1/2 mutation	
Mutation	175
Wild type	149
NA	1
1p/19q codeletion	
Co-deletion	67
Non-co-deletion	250
NA	8
MGMT methylation	
Methylation	130
Unmethylation	112
NA	64
<b>Characteristics [CGGA(2019)]</b>	<b>No. of Patients (n=693)</b>
Age	
<45	382
≥45	310
NA	1
Gender	
Male	398
Female	295
WHO Grade	
Grade II	188
Grade III	255
Grade IV	249
NA	1
TCGA Subtypes	
Proneural	296
Neural	167
Classical	83
Mesenchymal	147
Radiotherapy+TMZ Chemotherapy	
Yes	413
No	67
Radiotherapy	
Yes	509
No	113

(Continued)

**TABLE 1 |** Continued

Characteristics (CGGA)	No. of Patients (n=325)
NA	71
TMZ Chemotherapy	
Yes	457
No	151
NA	85
IDH1/2 mutation	
Mutation	356
Wild type	286
NA	51
1p/19q co-deletion	
Co-deletion	145
Non-co-deletion	478
NA	70
MGMT methylation	
Methylation	127
Unmethylation	73
NA	492
<b>Characteristics (TCGA)</b>	<b>No. of Patients (n=699)</b>
Age	
<45	296
≥45	340
NA	63
Gender	
Male	368
Female	268
NA	63
WHO Grade	
Grade II	223
Grade III	245
Grade IV	168
NA	63
TCGA Subtypes	
Proneural	250
Neural	115
Classical	92
Mesenchymal	105
NA	137
IDH1/2 mutation	
Mutation	443
Wild type	246
NA	10
1p/19q co-deletion	
Co-deletion	172
Non-co-deletion	520
NA	7
MGMT methylation	
Methylation	492
Unmethylation	168
NA	39

The number of glioma patients engaged in our study is listed. All patients were stratified with age, clinicopathological characteristics, and treatment options, respectively.

analysis was performed using R software (version 3.5.1, <http://www.r-project.org>). The prognostic value of GLRX was estimated by univariate and multivariate Cox proportional hazard model analysis using SPSS statistical software (version 25.0; IBM, Armonk, NY, USA). Patients with missing information were excluded from the analysis.

### IHC Staining

Paraffin-embedded samples were obtained from the CGGA sample bank. First, 5-μm sections were cut for IHC staining.

Samples were deparaffinized in an oven at 65°C for 1 h. Then the samples were rehydrated in decreasing concentrations of alcohol. IHC analysis with GLRX1 (Abcam, ab45953, 1:1000) and CD11b antibodies (Proteintech, 66519-1-ig, 1:1000) was conducted according to standard procedures. Photos were taken with an optical microscope.

## Cell Culture and Reagents

THP-1 cells (purchased from National Infrastructure of Cell Line Resource, <http://www.cellresource.cn/>) were maintained in RPMI1640 media supplemented with L-glutamine, 1% penicillin and streptomycin,  $\beta$ -mercaptoethanol (Gibco, 2169148, 0.055 mM) and 10% fetal bovine serum (FBS, Gibco) at 37°C under a humidified, 5% CO<sub>2</sub> atmosphere (16). THP-1 cells were differentiated to M0 macrophages by treatment with 25 nM phorbol 12-myristate 13-acetate (MCE, HY-18739) for 48 h, washed and incubated with normal RPMI1640 media for 24 h, and then incubated with recombinant human GM-CSF (50 ng/ml, Peprotech, 300-03) for 96 h. For M2 polarization, 50% of complete RPMI1640 medium was added, and it was incubated for 48 h. Then the M2 macrophage was obtained by removing the culture medium and culturing cells for an additional 48 h in M2 medium with recombinant human M-CSF (100 ng/ml, Peprotech, 300-25) (17).

## IF Staining

Macrophages were washed with PBS three times. Four percent fixative solution (Solarbio, P1110) was added to the Petri dish for 10 min. Then, the solution was removed and cells washed three times. Next, 0.5% Triton X-100 (Solarbio, T8200) was added to the dish for 10 min. The solution was removed and cells washed three times. Five percent BSA (Solarbio, A8010) was added to the dish, and it was incubated for 1 h at room temperature. Then, primary antibodies were added to the M0 (GLRX1: 1:500, Abcam, ab45953; CD11b: 1:100, Proteintech, 66519-1-ig) and M2 macrophages (GLRX1: 1:500, Abcam, ab45953; CD163: 1:100, Abcam, ab156769) (18), respectively, and they were incubated overnight at 4°C. The solution was removed and cells washed three times. Secondary antibodies (DyLight 488 goat antirabbit polyclonal antibody, Abcam, ab96899, 1:200; DyLight 594 goat antimouse polyclonal antibody, Abcam, ab96881, 1:200) were used for 1 h at room temperature. The solution was removed and cells washed three times. Prolong<sup>TM</sup> Diamond Antifade Mountant with DAPI (Invitrogen, P36962) was added to the dish, and photos were taken with confocal microscopy.

## Other Immune Biological Analysis

Pearson's correlation analysis was used to evaluate the relationship between *GLRX* and immune checkpoints. Inflammation-related metagenes were described as before (19).

## Statistical Analysis

A multiple group comparison was performed using Tukey's test. Other statistical computations and figure drawing were performed with several R packages, including ggplot2, pheatmap, pROC, and corrgram. All statistical tests were two-

sided, and a p-value < 0.05 was considered statistically significant in all analyses.

## RESULTS

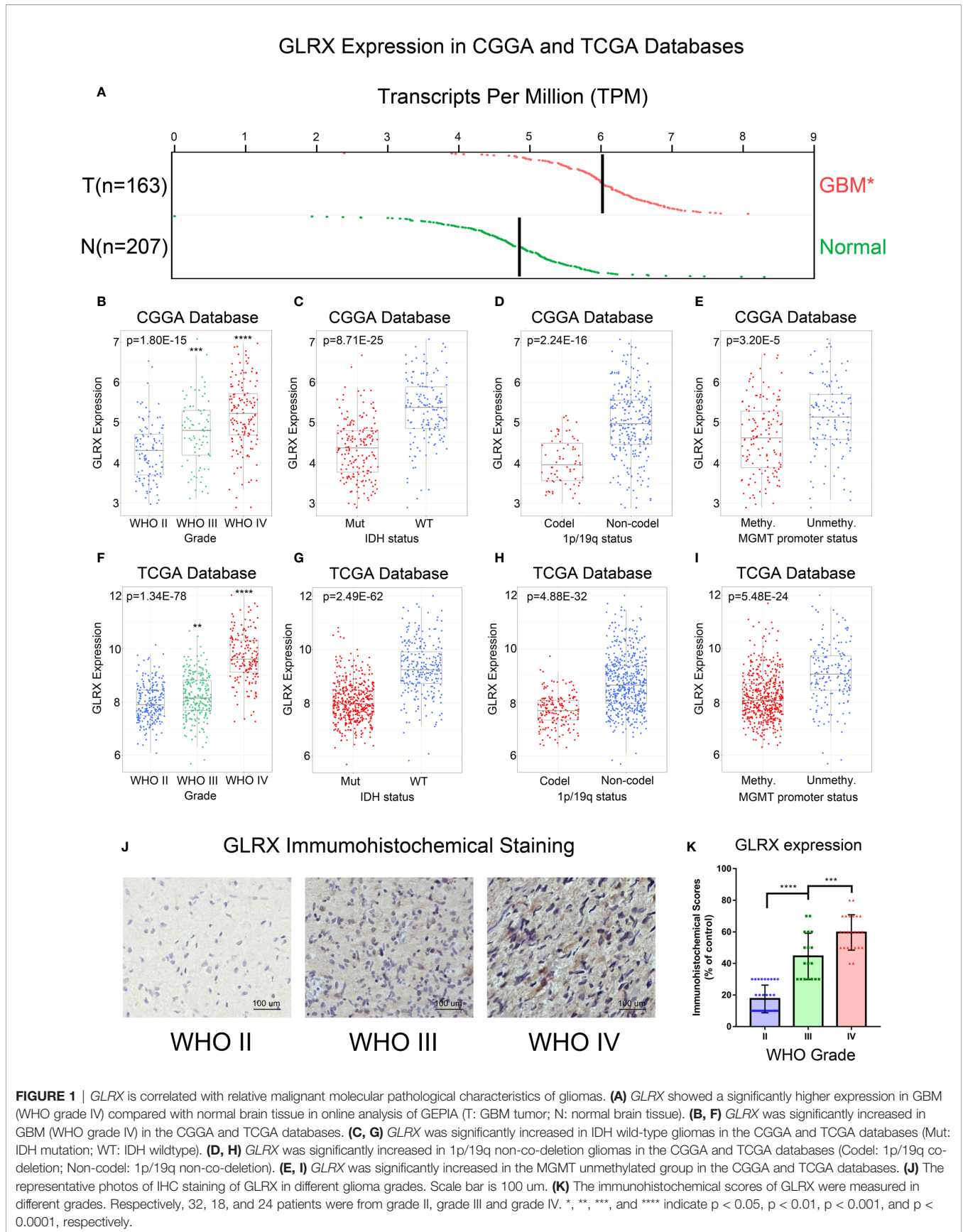
### Association of *GLRX* Expression With Clinical and Molecular Pathological Characteristics in Glioma

To investigate the role of *GLRX* in gliomas, we compared the expression levels of *GLRX* between normal brain tissue and GBM (grade IV, according to the World Health Organization [WHO]). The analysis revealed that *GLRX* expression was significantly enriched in GBM samples ( $p < 0.05$ , **Figure 1A**). Due to the histopathological heterogeneity of gliomas, RNA-seq data of glioma samples from three independent databases were analyzed according to WHO guidelines, and the analysis included *IDH* mutation status, 1p/19q co-deletion status, and MGMT promoter status. Among samples from the CGGA database, *GLRX* expression was higher in GBM (grade IV) compared with glioma (grades II and III) (**Figure 1B**). This result was further validated in the RNA-seq data from TCGA and CGGA (2019) databases (**Figure 1F** and **Supplementary Figure S1A**). In addition, IHC staining was conducted to explore the expression of *GLRX* in glioma tissues. Consistent with the RNA-seq data, we found that *GLRX* was enriched in GBM tissues (**Figures 1J, K**). The *IDH* mutation status, 1p/19q co-deletion status, and MGMT promoter status play important roles in the prognosis and chemotherapy outcomes of glioma patients and vary significantly among glioma patients (20). Therefore, we explored the correlation between *GLRX* expression and these three molecular pathologic statuses. We found that *GLRX* expression was highly enriched in *IDH* wild-type glioma patients compared with those harboring *IDH* mutations (**Figures 1C, G** and **Supplementary Figure S1B**). Moreover, patients with 1p/19q non-co-deletion had a higher expression of *GLRX* in all three databases (**Figures 1D, H** and **Supplementary Figure S1C**). Regarding the MGMT promoter status in the CGGA database, we found that gliomas with a methylated MGMT promoter had lower *GLRX* expression compared to those in the unmethylated group (**Figure 1E**). A similar trend was observed in the two other databases (**Figure 1I** and **Supplementary Figure S1D**). These findings indicate that *GLRX* expression is enriched in GBM and is tightly correlated with the malignant phenotype of glioma.

### *GLRX* Is a Potential Marker for Mesenchymal Molecular Subtype Glioma

Next, we investigated the molecular expression pattern of *GLRX* in different molecular subtypes defined by TCGA network (21). *GLRX* was significantly upregulated in the mesenchymal subtype of glioma compared with the other three subtypes in the CGGA (**Figure 2A**), TCGA (**Figure 2C**), and CGGA (2019) databases (**Supplementary Figure S2A**). The IHC staining of *GLRX* in GBM tissues also verified this finding (**Figures 3E–G**). To further validate this finding, we evaluated the receiver operating characteristic (ROC) curve for *GLRX* expression and





mesenchymal subtype for gliomas of all grades. Surprisingly, the area under the curve (AUC) of *GLRX* expression was up to 90.9%, 90.2%, and 78.0% for the CGGA, TCGA, and CGGA (2019) data sets, respectively (Figures 2B, D and Supplementary Figure S2B). These results suggest that *GLRX* is highly expressed in mesenchymal subtype glioma and may play an oncogenic role in glioma progression. BMI1 and CD44 were reported to differentiate the mesenchymal molecular subtype from other gliomas (22, 23). Thus, we took these two well-studied biomarker genes as positive controls to performed ROC curve analysis (Supplementary Figure S2C–H). Through comparing the AUC of these three genes, we inferred that *GLRX* may serve as a biomarker for mesenchymal subtype gliomas.

### ***GLRX* Is Strongly Associated With Immune Functions in Glioma**

We performed GO analysis to identify the *GLRX*-related biological functions in gliomas. At first, we screened genes that were strongly correlated with *GLRX* (Pearson  $R > 0.55$  and  $p < 0.0001$ ) in all three databases. The analysis revealed a total of 479 genes in CGGA, 877 genes in TCGA, and 537 genes in CGGA (2019) that were significantly correlated with *GLRX* expression. The genes positively correlated with *GLRX* expression were mostly involved in immune response, defense response, and inflammatory response in all databases (Figures 3A, C and Supplementary Figure S3A). Additionally, we performed KEGG pathway analysis to further explore the signaling pathways associated with the abovementioned genes. As expected, the KEGG analysis identified these genes to be associated with immune response pathways, including Fc $\gamma$ R-mediated phagocytosis, the toll-like receptor signaling pathway, and complementary and coagulation cascades in the three databases (Figures 3B, D and Supplementary Figure S3B). The heat map representation of the genes (shown in Table 2) within each biological process exhibits a clear positive correlation with *GLRX* expression and the landscape of corresponding clinical patient features (Figures 3E, F and Supplementary Figure S3C). These findings suggest that *GLRX* takes part in the immune response process and may be a marker for predicting immune-related biological processes in gliomas.

### **Special Immune Function of *GLRX***

Tumor-infiltrated immune cells, including T cells, NK cells, macrophages, and other cells, mount the immune response to kill or induce apoptosis of cancer cells (24). To further clarify the role of *GLRX* in the immune response in gliomas, we first assessed the correlation between *GLRX* and GO terms downloaded from the AmiGO2 web portal (<http://amigo.geneontology.org/>). We found 84.69%, 78.90%, and 87.07% biofunction of the immune system to be positively correlated with *GLRX* in the CGGA, TCGA, and CGGA (2019) data sets, respectively (Figures 4A, B and Supplementary Figure S4A). Overall, more immune-related GO terms were positively correlated with *GLRX* than any other kind of GO term. This further illustrates that *GLRX* has a strong correlation with the immune system. Last, to understand the role of *GLRX* in the

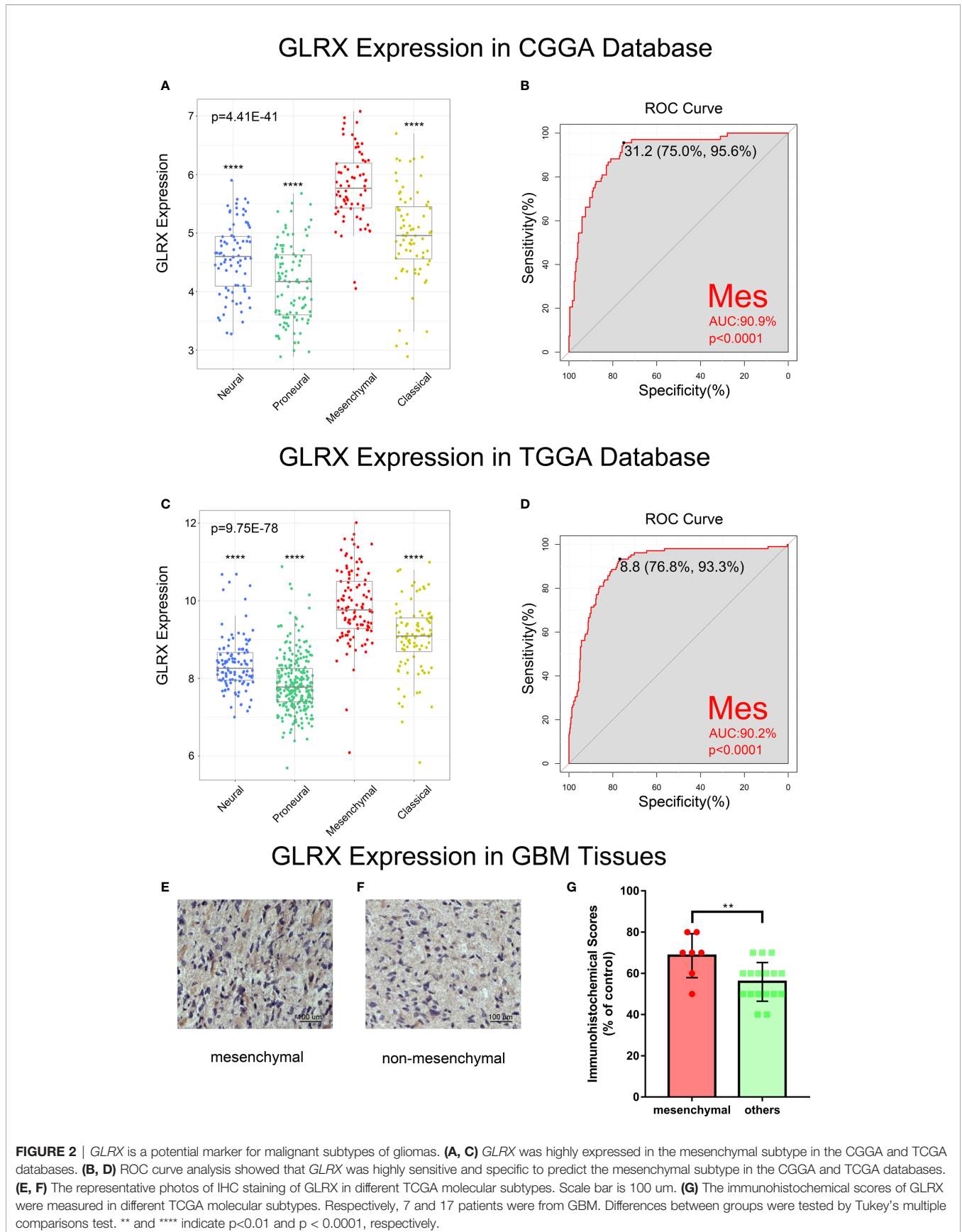
immune system, we performed a correlation coefficient analysis on data from the three databases (Figure 4C and Supplementary Figure S4B). We observed that the majority of immune functions showed positive correlation with *GLRX*; only the term “T cell-mediated immune response to tumor cell (T cell response)” was found to be negatively correlated with *GLRX*.

### ***GLRX* Is Associated With Inhibitory Immune Checkpoints and Inflammatory Responses**

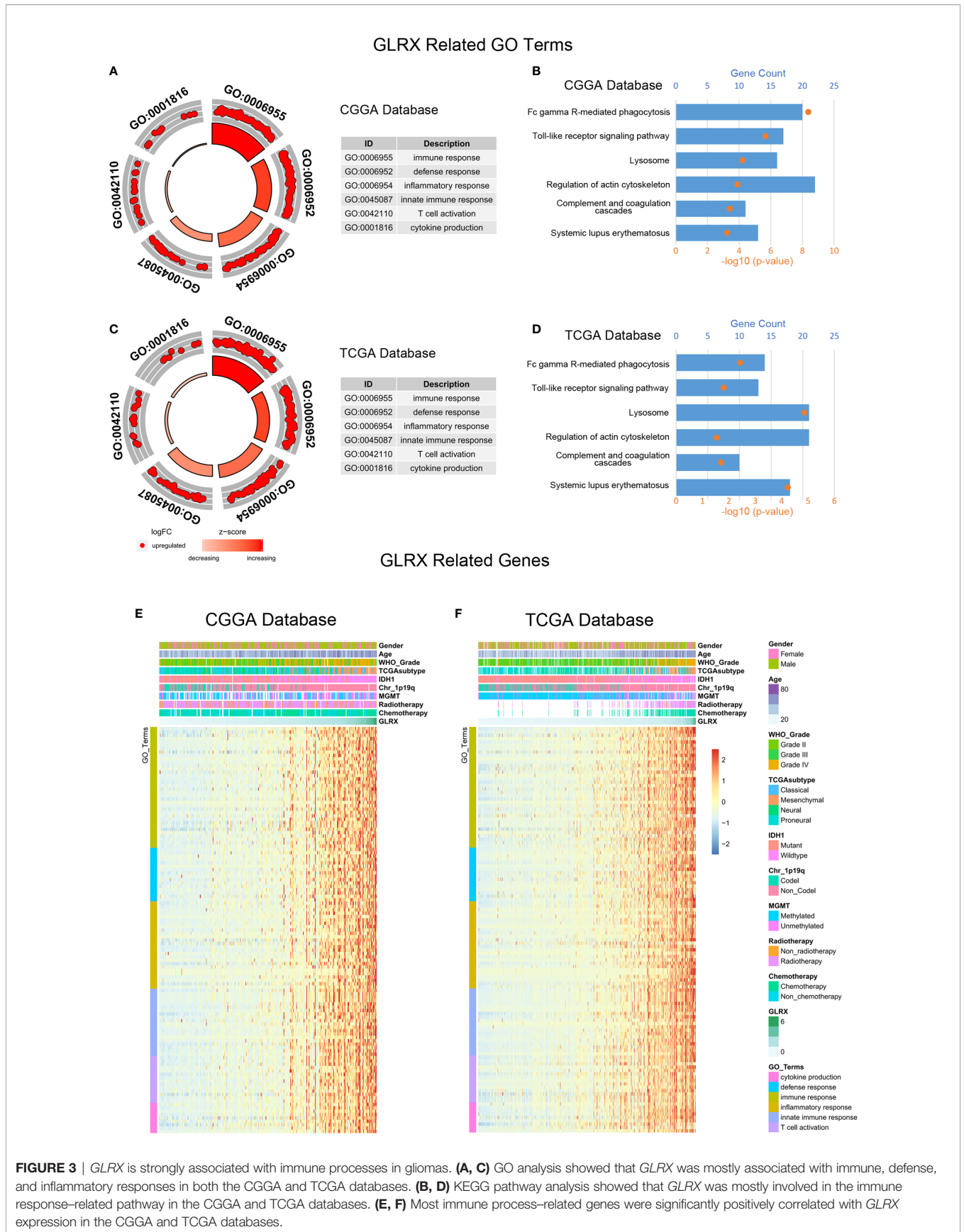
As mentioned above, most immune functions had positive correlation with *GLRX* with the exception of T cell responses. In a previous study, we reported that glioma patients with a stronger immune response had a much poorer prognosis (25). This abnormal phenomenon suggests that depletion of immune components in gliomas triggered by immune checkpoints could contribute to a malignant tumor phenotype. To validate this hypothesis, we investigated the relationship between *GLRX* and known immune checkpoint genes, including PD-1, TIM-3, PD-L1, and PD-L2, in the CGGA, TCGA, and CGGA (2019) databases (Figures 5A–H and Supplementary Figure S5A–D). The results indicated that *GLRX* had a strong positive correlation with these inhibitory immune checkpoint molecules and that *GLRX* may influence their expression to support glioma cells escaping immunological surveillance. Additionally, we also analyzed the role of *GLRX* in the glioma inflammatory response in these databases as described previously (19) (Figures 5I, J and Supplementary Figure S5E). We found that *GLRX* was positively associated with HCK, interferons, LCK, MHC-I, MHC-II, STAT1, and STAT2 expression, and it was negatively associated with IgG expression. These results suggest that upregulation of *GLRX* is involved in the activation of signal transduction in T cells, macrophages, and antigen-presenting cells, but it is negatively associated with B lymphocytes related metagenes. All these findings collectively confirm that *GLRX* plays an important role in immune response in gliomas.

### ***GLRX* Is Associated With M0 Macrophages**

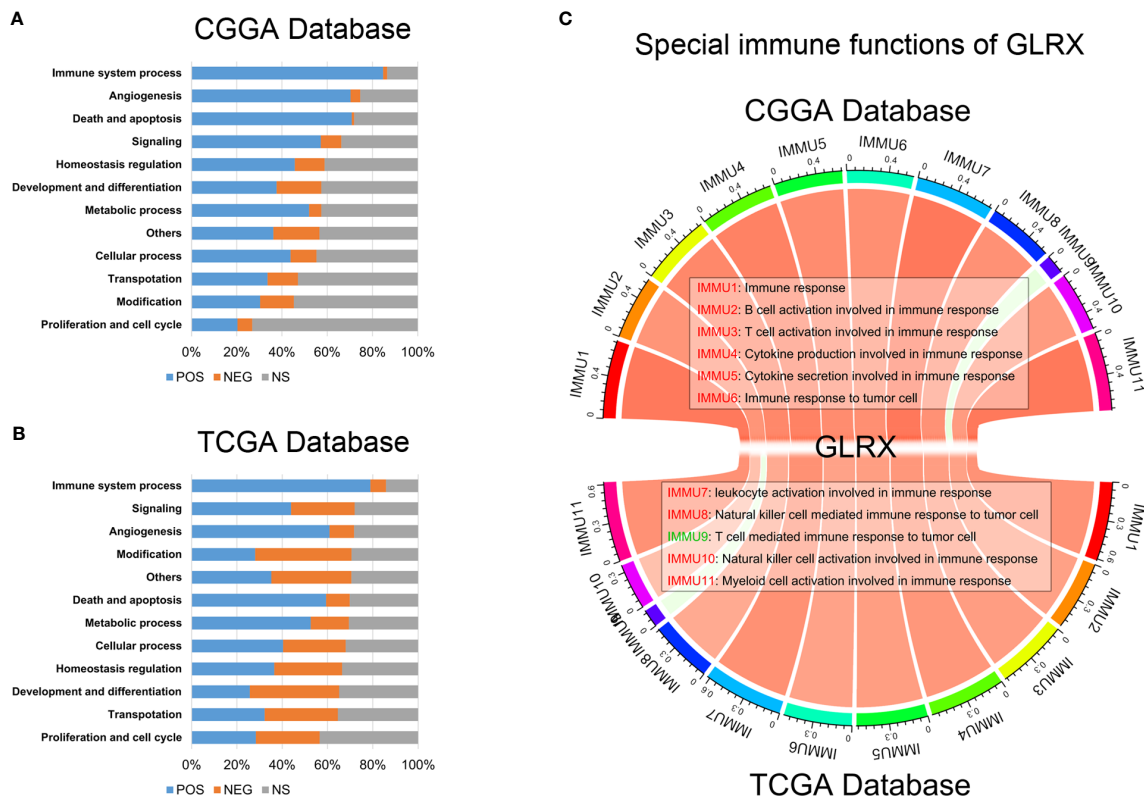
An activated immune response may promote the infiltration of immune cells into the tumor microenvironment and change its dynamics. To investigate whether *GLRX* was associated with infiltrated immune cells, we used CIBERSORT software to analyze the CGGA, TCGA, and CGGA (2019) databases (Figure 6A and Supplementary Figure S6). The analysis revealed that higher *GLRX* expression was positively correlated with enrichment of macrophages in glioma tissue. Moreover, single-cell sequencing data (Figures 6B–G) demonstrated that *GLRX* may be specifically expressed in M0 macrophages compared to other types of macrophages. To verify this finding, IHC co-localization staining was performed to explore the expression of *GLRX* in macrophages in tumor specimens. A previous study reported that CD11b was a biomarker of M0 macrophages (17). The results showed that *GLRX* was expressed in most M0 macrophages (Figures 7A, B). Furthermore, GM-CSF and PMA were used to induce THP-1 cells to differentiate



**FIGURE 2** | *GLRX* is a potential marker for malignant subtypes of gliomas. **(A, C)** *GLRX* was highly expressed in the mesenchymal subtype in the CGGA and TCGA databases. **(B, D)** ROC curve analysis showed that *GLRX* was highly sensitive and specific to predict the mesenchymal subtype in the CGGA and TCGA databases. **(E, F)** The representative photos of IHC staining of *GLRX* in different TCGA molecular subtypes. Scale bar is 100  $\mu$ m. **(G)** The immunohistochemical scores of *GLRX* were measured in different TCGA molecular subtypes. Respectively, 7 and 17 patients were from GBM. Differences between groups were tested by Tukey's multiple comparisons test. \*\* and \*\*\*\* indicate  $p < 0.01$  and  $p < 0.0001$ , respectively.







**FIGURE 4** | *GLRX* is closely related to the state of tumor immune functions. **(A, B)** *GLRX* had a positive correlation with 84.69% and 78.90% of the biological functions of immune system processes in the CGGA and TCGA databases, respectively. The scale values in the graph represent the proportions of significantly correlated biological functions in each biological function classification. **(C)** The correlation coefficient between *GLRX* and immune function scores in CGGA and TCGA databases. The red words represent a positive correlation. The green words represent a negative correlation.

toward M0 macrophages *in vitro*. Under this circumstance, M-CSF was added to medium to induce M0 macrophages to polarize to M2 macrophages (16, 17). IF staining results showed that *GLRX* was highly expressed by M0 macrophages compared to M2 macrophages (Figures 7C, D). These findings suggest that the effect of *GLRX* on the immune system is mediated by M0 macrophages, further validating that *GLRX* plays a pivotal role in the immune response.

### ***GLRX* Predicts Survival Outcome in Gliomas**

Because *GLRX* showed a robust negative correlation with the T cell response, we further investigated the prognostic value of *GLRX* by Kaplan-Meier and Cox proportional hazard model analyses. We found that patients with a higher expression of *GLRX* had a significantly shorter overall survival compared with those with lower *GLRX* expression (Figures 8A, B and Supplementary Figure S7A). Moreover, *GLRX* expression, WHO grade, age at diagnosis, *IDH* status, 1p/19q status, and *MGMT* promoter status were significantly associated with overall patient survival in all the three data sets that were evaluated. Multivariate analysis further confirmed *GLRX* as a significant predictor after adjusting for the clinical factors

mentioned above (Figures 8C, D and Supplementary Figure S7B). These findings reveal that *GLRX* may serve as an indicator for the poor prognosis in gliomas due to its suppressive effects on the T cell immune response against tumor cells.

## **DISCUSSION**

Glioma is one of the most fatal malignancies to afflict human health (1). Although temozolomide was approved for the treatment of gliomas by the U.S. Food and Drug Administration in 2005, researchers have continued to search for novel chemotherapeutic drugs with improved efficacy to treat gliomas (26). Nevertheless, no significant results have been achieved so far. Therefore, novel therapeutic approaches against gliomas remain an urgent requirement. In recent years, targeted drugs and immunotherapeutic approaches have exhibited extraordinary prospects (27, 28). Based on high-throughput sequencing, our team built the CGGA database and screened the *PTPRZ1-MET* fusion gene, which is expressed almost exclusively in secondary glioblastomas. The targeted drug PLB-1001 showed a good response rate in phase 2 clinical trials (29). Moreover, immune checkpoint blockade therapy also achieved success in treating

**TABLE 2 |** Representative genes of each biological function.

Gene	GO_Terms
NFKB2	immune response
B2M	immune response
IL4R	immune response
LILRA6	immune response
FCGR3A	immune response
LAIR1	immune response
DBNL	immune response
NCF4	immune response
STXBP2	immune response
TNFRSF14	immune response
CTSS	immune response
PDCD1LG2	immune response
BCAP31	immune response
LILRB1	immune response
LAT2	immune response
CTSC	immune response
GBP2	immune response
GALNT2	immune response
SBNO2	immune response
IFITM3	immune response
GPSM3	immune response
GPR65	immune response
FCGRT	immune response
FTH1	immune response
SQSTM1	immune response
FCER1G	immune response
MR1	immune response
ARHGDI1B	immune response
PSMB8	immune response
TNFSF8	immune response
PSMB9	immune response
IKBKE	immune response
FCGR2B	immune response
CD300A	immune response
CD274	immune response
RNF19B	immune response
TNIP1	defense response
C5AR1	defense response
CLIC1	defense response
SP140	defense response
MNDA	defense response
CLEC5A	defense response
TYROBP	defense response
TCIRG1	defense response
HCK	defense response
MAP2K3	defense response
CD300C	defense response
APOL2	defense response
CYBB	defense response
STAB1	defense response
ALOX5	defense response
CD14	defense response
CCL2	inflammatory response
NMI	inflammatory response
ADORA3	inflammatory response
S100A8	inflammatory response
AIF1	inflammatory response
CCR1	inflammatory response
S100A9	inflammatory response
ITGB2	inflammatory response
TNFRSF1B	inflammatory response
IL10RB	inflammatory response
HMOX1	inflammatory response

(Continued)

**TABLE 2 |** Continued

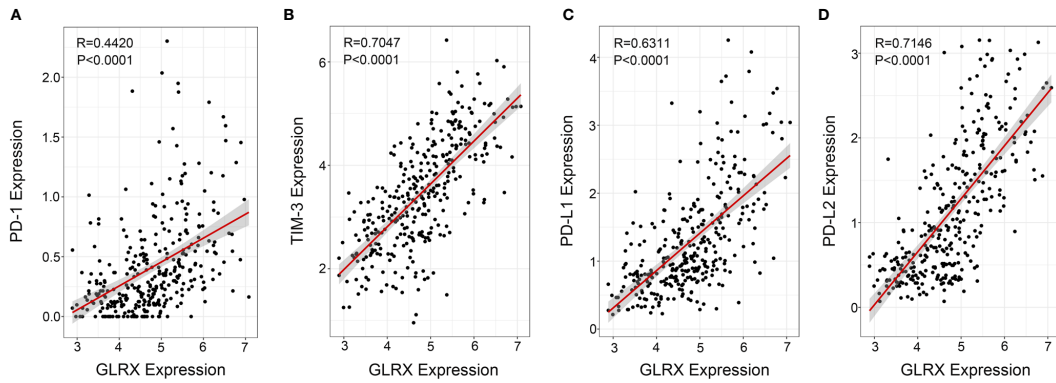
Gene	GO_Terms
TICAM2	inflammatory response
SERPINA3	inflammatory response
C2	inflammatory response
SPP1	inflammatory response
B4GALT1	inflammatory response
NFKBIZ	inflammatory response
CEBPB	inflammatory response
LY96	inflammatory response
PDPN	inflammatory response
LYZ	inflammatory response
NFAM1	inflammatory response
IL6R	inflammatory response
CD40	inflammatory response
CD163	inflammatory response
CCR5	inflammatory response
KYNU	innate immune response
IL1R1	innate immune response
TLR1	innate immune response
NCF1C	innate immune response
TLR2	innate immune response
C1R	innate immune response
APOBEC3G	innate immune response
C1S	innate immune response
C1QC	innate immune response
GCH1	innate immune response
SP100	innate immune response
NCF2	innate immune response
NCF1	innate immune response
SERPING1	innate immune response
C1QA	innate immune response
CYBA	innate immune response
C1QB	innate immune response
CORO1A	innate immune response
C1RL	innate immune response
VSIG4	innate immune response
PTPRC	T cell activation
STAT5A	T cell activation
RELB	T cell activation
PTPN22	T cell activation
VAV1	T cell activation
ITGAM	T cell activation
DOCK2	T cell activation
CD86	T cell activation
IRF1	T cell activation
CLEC7A	T cell activation
FAS	T cell activation
LCP1	T cell activation
RAB27A	T cell activation
SYK	T cell activation
SLC11A1	cytokine production
NLRC4	cytokine production
MYD88	cytokine production
LYN	cytokine production
CD4	cytokine production
CD226	cytokine production
PRKCD	cytokine production
PTAFR	cytokine production
LCP2	cytokine production

The representative genes of each biological function are which obtained from GO analysis in heat map are listed.

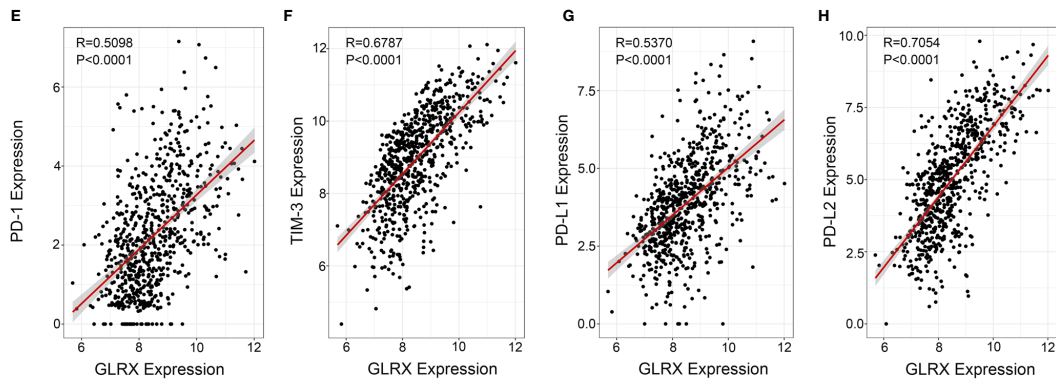
gliomas. Cloughesy et al. reported that neoadjuvant anti-PD-1 immunotherapy, which enhances T cell-mediated antitumor immunity, could significantly extend overall survival of patients

### GLRX Related Immune Checkpoints

#### CGGA Database

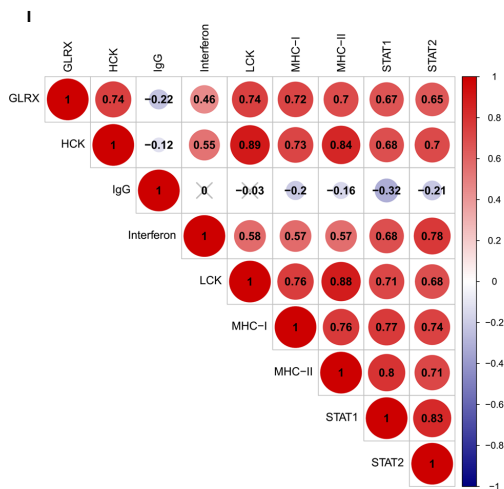


#### TCGA Database

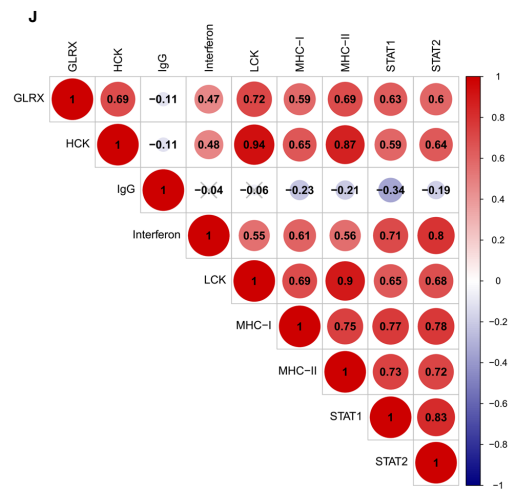


### Correlogram of GLRX and Inflammatory Activities

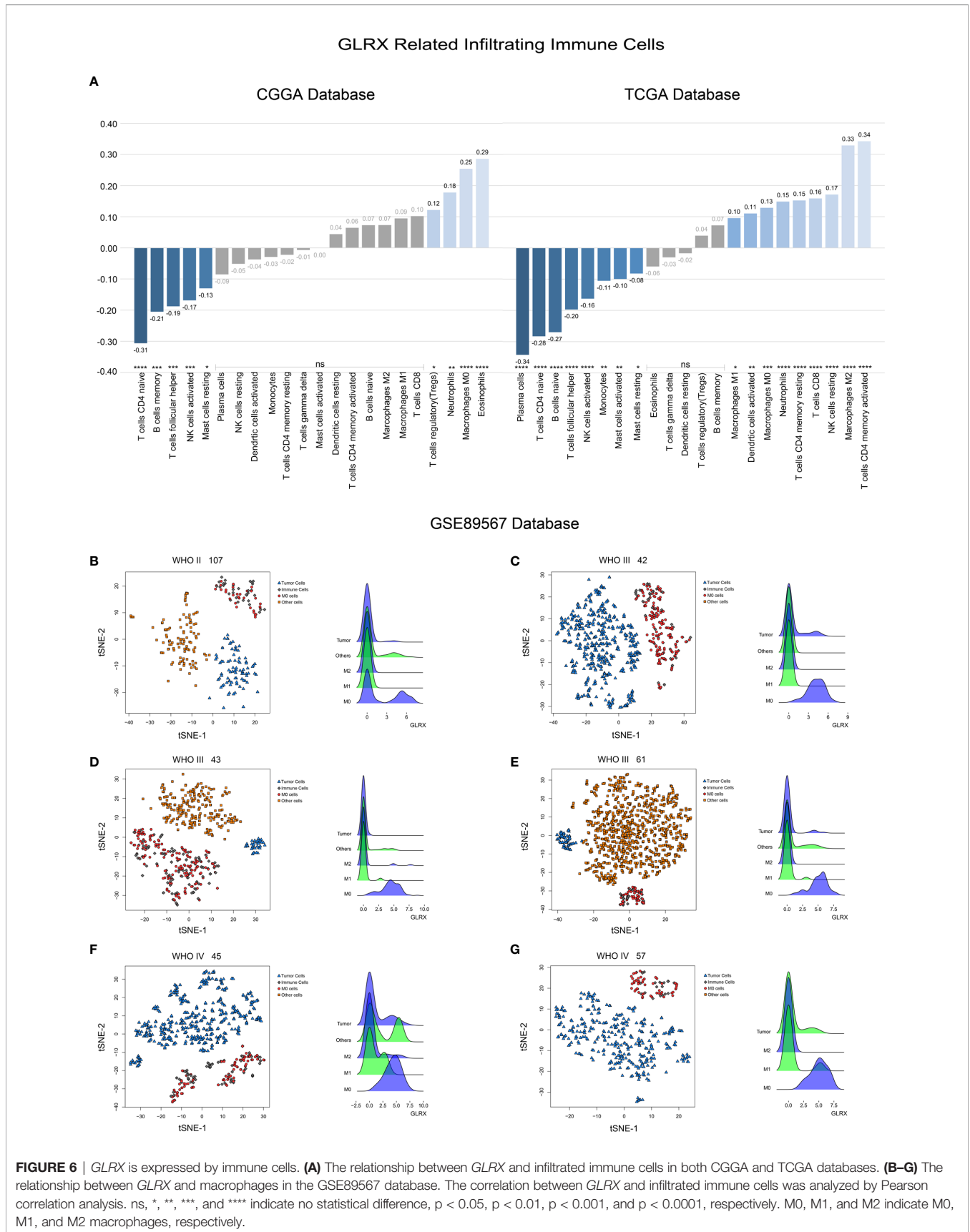
#### CGGA Database



#### TCGA Database

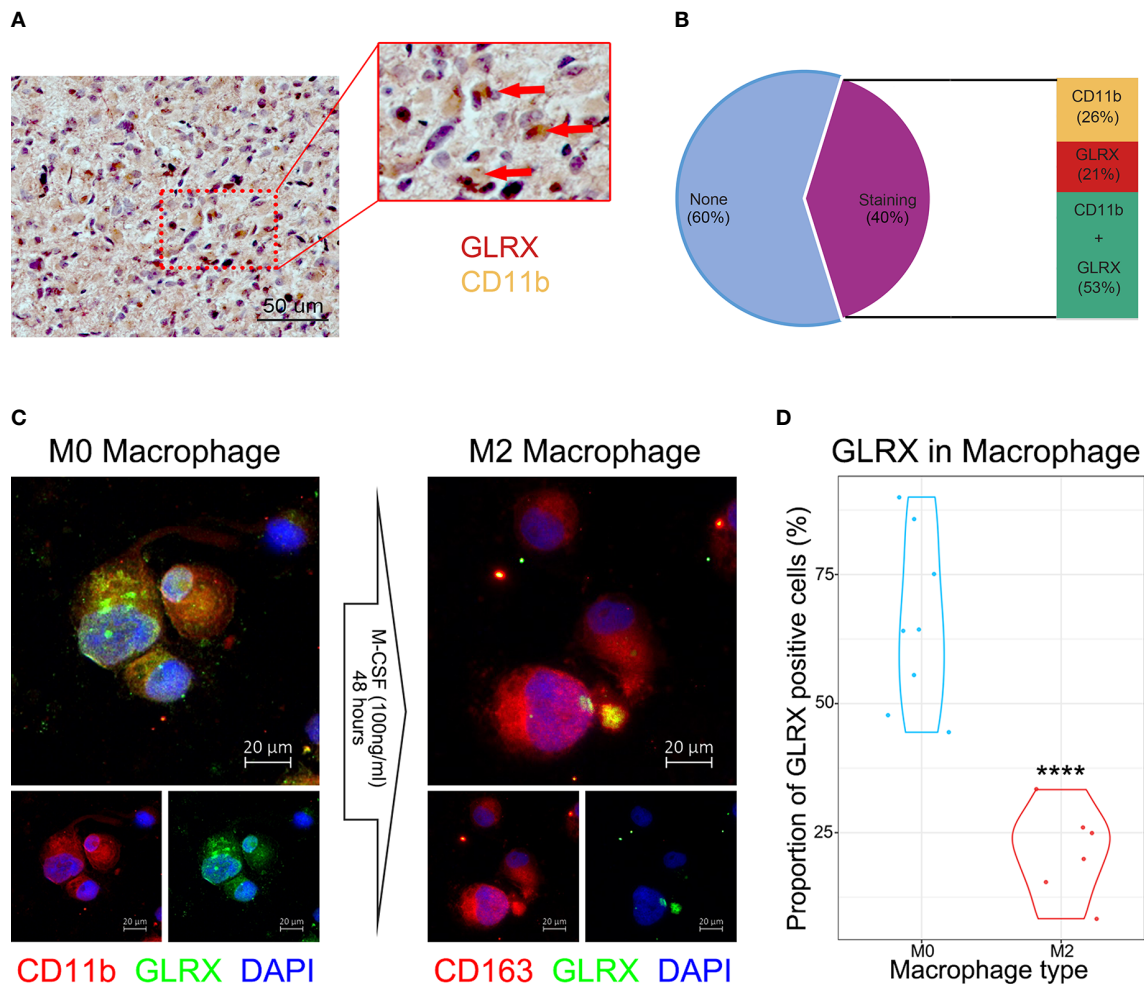


**FIGURE 5** | *GLRX* is associated with inhibitory immune checkpoints and inflammatory activities. **(A–H)** *GLRX* was synergistic with inhibitory immune checkpoints in tumor-induced immune responses. A strong correlation between *GLRX* and inhibitory immune checkpoint expression was found in both the CGGA and TCGA databases. **(I, J)** The correlation coefficient between *GLRX* and inflammatory activity function scores in gliomas. The red circle represents a positive correlation. The blue circle represents a negative correlation. The grey “x” represents no significant correlation. Similar results are found in both CGGA and TCGA databases.





## GLRX Expressed in M0 Macrophage

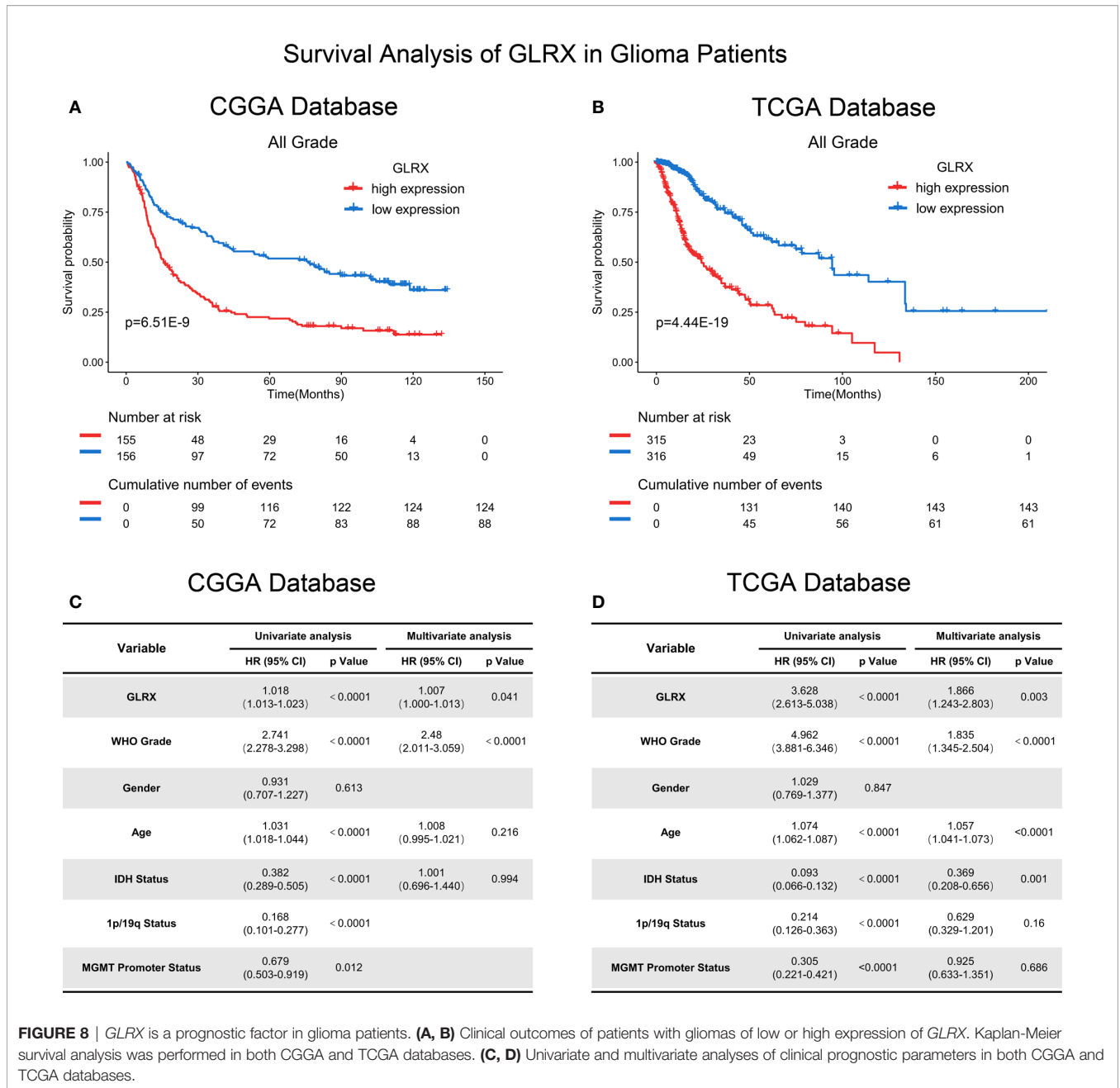


**FIGURE 7 |** *GLRX* is specifically expressed by M0 macrophages. **(A)** The IHC co-localization staining of CD11b and *GLRX*. The red point represents *GLRX*, and the yellow area represents CD11b. Scale bar is 50  $\mu\text{m}$  **(B)** The proportion of stained cells. None represents cells without expression of CD11b and *GLRX*. Staining represents cells expressing CD11b and/or *GLRX*. CD11b represents cells expressing CD11b. *GLRX* represents cells expressing *GLRX*. CD11b + *GLRX* represents cells expressing CD11b and *GLRX*. **(C)** The left three photos are IF staining of M0 macrophages. Green fluorescence is *GLRX*, and red fluorescence is CD11b. Above is the merged photo of the below two. The right three photos are IF staining of M2 macrophages. Green fluorescence is *GLRX*, and red fluorescence is CD163. Above is the merged photo of the below two. Cell nuclei are stained with DAPI. Scale bar is 20  $\mu\text{m}$ . **(D)** The violin graph shows the proportion of *GLRX*-positive cells in M0 or M2 macrophage IF images. \*\*\*\*indicates  $p < 0.0001$ .

with recurrent glioblastomas (30). Despite the promising clinical results, these therapeutic approaches can benefit just a fraction of patients with gliomas. Therefore, exploring therapeutic approaches or a multifunctional small molecule that could benefit most glioma patients is of great interest.

The thioredoxin and glutathione systems are the key cellular redox systems involved in gliomas (8, 31, 32). Glioma proliferation is associated with parenchymal alterations and oxidative stress that further leads to the impairment of brain homeostasis (4). Tumor cellular respiration produces hyperoxides, such as  $\text{H}_2\text{O}_2$ , and reactive oxygen species (ROS),

which, when present at high levels, damage the DNA. This process is considered to be a pernicious factor in malignant glioma development (33). Hence, functional antioxidant systems that can scavenge these hyperoxides hold promise to keep the cell cycle of glioma cells under control. Simultaneously, a better understanding of the antioxidant system can pave the way for finding new therapeutic approaches to fight gliomas. Because glioma cells are more susceptible to oxidative stress induced by hyperoxides, inhibition of antioxidant systems or their components can prevent them from performing oxidative scavenging, thereby exposing the glioma cells to intense



**FIGURE 8 |** *GLRX* is a prognostic factor in glioma patients. **(A, B)** Clinical outcomes of patients with gliomas of low or high expression of *GLRX*. Kaplan-Meier survival analysis was performed in both CGGA and TCGA databases. **(C, D)** Univariate and multivariate analyses of clinical prognostic parameters in both CGGA and TCGA databases.

oxidative stress and blocking their proliferation, leading to their death. The endogenous antioxidative molecule Grx plays an important role in the glutathione system (7, 34). Grx expression is associated with tumor proliferation and therapy resistance in several cancers. Previous studies report that Grx is overexpressed in pancreatic ductal carcinoma compared to normal pancreatic tissue and that Grx overexpression increases MCF-7 adenocarcinoma cell resistance to doxorubicin (35, 36). However, little is known about the role of Grx in gliomas. Therefore, as a potential therapeutic target, it is imperative to explore the unique role of Grx and how it works in gliomas.

We started by checking the expression of *GLRX* in glioblastoma tissue compared with normal brain tissue. Our

results showed that, similar to other tumor types, glioblastoma samples exhibited higher expression of *GLRX*. Next, we analyzed RNA-seq data of 1,717 glioma patients compiled from the CGGA, CGGA (2019), and TCGA databases. As expected, *GLRX* expression was significantly upregulated in higher malignant pathological grades of gliomas. Moreover, we also found that *GLRX* expression was significantly higher in glioma patients with malignant molecular phenotypes, including those harboring the *IDH* wild-type state, 1p/19q non-codeletion state, and *MGMT* unmethylated promoters. Furthermore, *GLRX* was highly enriched in mesenchymal subtype gliomas. The mesenchymal subtype is characterized by stronger immunosuppression, and aggressive phenotype, and malignant

proliferation due to the mesenchymal differentiation triggered by *NF1* mutations (21). A previous study has reported enhanced expression of immune checkpoints in mesenchymal subtype cancers compared with the other three transcriptional characteristic subtypes (37). Therefore, *GLRX* may be upregulated and involved in the immunosuppressive microenvironment of gliomas *via* modulation of the cellular component of the immune system. These findings suggest that *GLRX* expression is associated with the malignant behavior of gliomas. Thus, unraveling the mechanism of *GLRX* in gliomas may pave the way for the development of novel therapeutic approaches to fight this deadly malignancy.

To gain an in-depth understanding of the biological functions of *GLRX*, a series of analyses were performed. GO analysis revealed that *GLRX* plays a crucial role in immune and inflammatory responses in gliomas. Consistent with these results, KEGG and GSEA analyses also show that *GLRX* and related genes are involved in several immune response pathways, and *GLRX* is positively correlated with most immune functions with the exception of T cell response. Furthermore, *GLRX* was found to be significantly enriched in the mesenchymal glioma subtype with *GLRX* negatively mediating the T cell response and playing a suppressive role in the antitumor immune response. Taken together, these results suggest that *GLRX* may upregulate the expression of immune checkpoints to perform these functions. Upon analysis of the relationship between *GLRX* and known immune checkpoint genes, we confirm that *GLRX* is positively correlated with most inhibitory immune checkpoints, including PD-1, TIM-3, PD-L1, and PD-L2, which are involved in the regulation of the PD-1/PD-L1 pathway. These immune checkpoints are major negative immune regulators and are involved in regulating T cell activation, tolerance, and exhaustion (19). Our findings demonstrate that *GLRX* may exert antiglioma immune roles by affecting the expression of these inhibitory immune checkpoints. Additionally, *GLRX* is involved in inflammatory activities known to promote glioma progression *via* activation of tumor-associated macrophages (14, 38). To further validate the role of *GLRX* in the immune response, we used CIBERSORT software to calculate the percentage of each type of infiltrated immune cell. Our results show that *GLRX* is positively correlated with macrophages but negatively correlated with different subgroups of T cells. This further confirms the conclusions of our study. Last, single-cell sequencing analysis and IHC co-localization staining were performed to identify the exact components of the immune system that express *GLRX*. RNA-seq data and cellular molecular biomarkers reveal that *GLRX* is enriched in immune cells, particularly in M0 macrophages. M0 macrophages are a subgroup of resting immune cells that can undergo a directional polarization (17) to classically activated M1 macrophages and alternatively activated M2 macrophages. Macrophages in glioma tissue are prone to M2-like phenotypes, which are considered to be tumor-supporting macrophages (39). A previous study also reported that patients with higher expression of M0 macrophages had a poorer prognosis (17). Thus, we suspected that *GLRX* may play a role

in M0 polarization and have an immuno-suppressive function. Based on the results of our present study, we hypothesize that *GLRX* is a potential target for redox and immunotherapy of gliomas.

Importantly, high levels of *GLRX* were associated with poor patient prognosis. Univariate and multivariate analyses indicated that high expression of *GLRX* predicted significantly lower survival. As a result, *GLRX* may serve as a potential prognostic predictor for glioma patients.

Redox therapy is being increasingly explored in tumor therapy (40, 41). Studies on breast, liver, pancreatic, and non-small cell lung cancers report that blocking the glutathione system could prevent tumor cell proliferation *in vitro* and *in vivo* (33, 35, 36, 42). As gliomas have access to abundant oxygen as well as to cellular respiration products, glioma cells become more dependent on the antioxidant system to survive and proliferate. Even a slight reduction in antioxidant levels could lead to glioma cell death (43). Meanwhile, cancer immunotherapy has also shown potential benefits for glioma patients. CAR-T, anti-PD-1, and anti-PD-L1 immunotherapies have shown higher immune response rates and longer survival in patients with brain metastases (2, 24, 27, 30). Our study suggests that *GLRX* is a key regulator of immune checkpoints and the immune response. Therefore, as a co-regulator of both redox and immune systems, inhibiting Grx could not only kill glioma cells through directly enhancing oxidative stress, but also downregulate the expression of inhibitory immune checkpoints and enhance the immune response. Thus, our study establishes *GLRX* as a novel potential target to enhance the efficacy of anticancer therapies, thereby paving the way for novel therapeutic approaches for treating gliomas.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.cgga.org.cn>, CGGA.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Capital Medical University Institutional Review Board (IRB). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

YC, GL: data analysis and editing the manuscript. YZ, LH: data collection and organization of CGGA database. YF, DW: data collection and organization of TCGA database. WZ, HH: conception, supervision, and design of the manuscript.

All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 81972816, 81672479).

## ACKNOWLEDGMENTS

We thank Ms. Shuqing Sun and Hua Huang for tissue sample collection and clinical data retrieval.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.580934/full#supplementary-material>

**SUPPLEMENTARY FIGURE 1** | GLRX is correlated with the relative malignant molecular pathological characteristics of glioma. **(A)** GLRX was significantly increased in GBM (WHO grade IV) in the CGGA (2019) database. **(B)** GLRX was significantly increased in IDH wild-type gliomas in the CGGA (2019) database (Mut: IDH mutation; WT: IDH wild type). **(C)** GLRX was significantly increased in 1p/19q non-co-deletion gliomas in the CGGA (2019) database (Codet: 1p/19q co-deletion; Non-codet: 1p/19q non-co-deletion). **(D)** GLRX was significantly increased in the MGMT unmethylated group in the CGGA (2019) database. ns and \*\*\*\* indicate no statistical difference and  $p < 0.0001$ , respectively.

**SUPPLEMENTARY FIGURE 2** | GLRX is a potential marker for malignant subtypes of gliomas. **(A)** GLRX was highly expressed in the mesenchymal subtype in the CGGA (2019) database. **(B)** ROC curve analysis showed that GLRX was highly sensitive and specific to predict the mesenchymal subtype in the CGGA (2019) database. **(C–E)** ROC curve analysis showed that BMI1 was less sensitive

and specific to predict the mesenchymal subtype in the CGGA, TCGA, and CGGA (2019) databases, respectively. **(F–H)** ROC curve analysis showed that CD44 was highly sensitive and specific to predict the mesenchymal subtype in the CGGA, TCGA, and CGGA (2019) databases, respectively. Differences between groups were tested by Tukey's multiple comparisons test. \*\*\* and \*\*\*\* indicate  $p < 0.001$  and  $p < 0.0001$ , respectively.

**SUPPLEMENTARY FIGURE 3** | GLRX is strongly associated with immune processes in gliomas. **(A)** GO analysis showed that GLRX was mostly associated with immune, defense, and inflammatory responses in the CGGA (2019) database. **(B)** KEGG pathway analysis showed that GLRX was mostly involved in the immune response-related pathway in the CGGA (2019) database. **(C)** Most immune process-related genes were significantly positively correlated with GLRX expression in the CGGA (2019) database.

**SUPPLEMENTARY FIGURE 4** | GLRX is closely related to the state of tumor immune functions. **(A, B)** GLRX had positive correlation with 87.07% of the biological functions of the immune system process in the CGGA (2019) database. The scale values in the graph represent the proportions of significantly correlated biological functions in each biological function classification. **(B)** The correlation coefficient between GLRX and the immune function scores in the CGGA (2019) database. The red words represent a positive correlation. The green words represent a negative correlation.

**SUPPLEMENTARY FIGURE 5** | GLRX is associated with immune checkpoints and inflammatory activities. **(A–D)** GLRX was synergistic with inhibitory immune checkpoints in tumor-induced immune responses. A strong correlation between GLRX and inhibitory immune checkpoint expression was found in the CGGA (2019) database. **(E)** The correlation coefficient between GLRX and inflammatory activity function scores in the CGGA (2019) database. The red circle represents a positive correlation. The blue circle represents a negative correlation. The grey "x" represents no significant correlation.

**SUPPLEMENTARY FIGURE 6** | The relationship between GLRX and infiltrated immune cells in the CGGA (2019) database. ns, \*, \*\*, \*\*\*, and \*\*\*\* indicate no statistical difference,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , and  $p < 0.0001$ , respectively.

**SUPPLEMENTARY FIGURE 7** | GLRX is a prognostic factor in glioma patients. **(A)** Clinical outcomes of patients with gliomas of low or high expression of GLRX. Kaplan-Meier survival analysis was performed in the CGGA database. **(B)** Univariate and multivariate analyses of clinical prognostic parameters in CGGA database.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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