Immune and clinical features of CD96 expression in glioma by large-scale analysis

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Abstract

Background: Immune checkpoints target regulatory pathways in T cells which enhance antitumor immune responses and elicit durable clinical responses. As a novel immune checkpoint, CD96 is an attractive key target for cancer immunotherapy. However, there is no integrative investigation of CD96 in glioma. Our study explored the relationship between CD96 expression and clinical prognosis in glioma.

Methods: A total of 1,024 RNA and clinical data were enrolled in this study, including 325 samples from the Chinese Glioma Genome Atlas (CGGA) database and 699 samples from The Cancer Genome Atlas (TCGA) dataset. R language was used to perform statistical analysis and draw figures.

Results: CD96 had a consistently positive relationship with glioblastoma and highly enriched in IDH-wildtype and mesenchymal subtype glioma. GO enrichment and GSVA analyses suggested that CD96 was more involved in immune functions, especially related to T cell-mediated immune response in glioma. Subsequent immune infiltration analysis manifested that CD96 was positively correlated with infiltrating levels of CD4+ T and CD8+ T cells, macrophages, neutrophils, and DCs in GBM and LGG. Additionally, CD96 was tightly associated with other immune checkpoints including PD-1, CTLA-4, TIGIT, and TIM-3. Univariate and multivariate Cox analysis demonstrated that CD96 acts as an independent indicator of poor prognosis in glioma.

Conclusion: CD96 expression was increased in malignant phenotype and negatively associated with overall survival (OS) in glioma. CD96 also showed a positive correlation with other immune checkpoints, immune response, and inflammatory activity. Our findings indicate that CD96 is a promising clinical target for further immunotherapeutic in glioma patients.
Background

As the most prevalent and devastating primary intracranial tumor, glioma, especially glioblastoma multiforme (GBM, WHO grade IV) represents heterogeneity and extensive invasion, characterized by high recurrence and fatality rate [1–3]. Therefore, multiple attempts have been made to prolong life expectancies of glioma patients comprising developing effective therapy, appropriate biomarkers, and molecular targeted drugs. Numerous conventional treatment methods of central nervous system (CNS) tumors have emerged for decades, such as neurosurgical resection, radiotherapy, and chemotherapy [4, 5]. Among them, immunotherapy is reckoned an encouraging treatment resulted from evoking an anti-tumor immune response to restrain immune evasion of tumor [6]. In melanoma and non-small-cell lung cancer, immune checkpoint inhibitors like target programmed cell death protein 1 (PD-1) /programmed death ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) have been exploited and applied to clinical successfully [7–9]. Whereas many glioma patients are refractory to current immunotherapy which arouses our interest in identifying additional immune checkpoints to enhance the therapeutic efficacy of glioma [8, 10].

As a novel immune checkpoint receptor target, CD96 has recently entered the limelight to current cancer immunotherapies shown to inhibit natural killer (NK) cells [11]. Compelling evidence confirmed that blocking CD96 containment primary tumor growth in murine model systems in a CD8 + T cell-dependent manner [12, 13]. Of note, the anti-tumor activity of anti-CD96 therapy improved the efficiency in dual-combination with blockade of other immune checkpoints, like PD-1, PD-L1, TIGIT, and CTLA-4 [12]. Besides, CD96 represents several unique features that exhibit profound beneficial effects in the coming age of human cancer therapy [11, 14].

To take a systematic examination of CD96 in glioma, we gathered RNA-seq and clinical
data of 325 glioma samples dataset from the Chinese Glioma Genome Atlas (CGGA) project. Simultaneously, another data set contained 699 samples from the Cancer Genome Atlas (TCGA) cohort to further corroborate the findings. Overall, our comprehensive study of molecular and clinicopathological features of CD96 through 1,024 samples will provide a better understanding of CD96 in glioma and pave the way for developing CD96-targeted cancer immunotherapies.

Methods

Data collection from CGGA and TCGA projects

The CD96 transcriptional and clinical data of 325 glioma samples were downloaded from the CGGA cohort (http://cgga.org.cn/) ranging from WHO grade II to IV. Moreover, 699 glioma samples of all grades were obtained from TCGA mRNA-seq dataset (https://tcga-data.nci.nih.gov/repository) including RNA-seq data and clinicopathological information.

Gene Ontology (GO) and Gene Set Variation Analysis (GSVA) Analysis

After Pearson correlation analysis, gene ontology (GO) analysis of the most correlated genes was constructed by R package ‘clusterProfiler’ [15]. GSVA analysis was applied using standard settings, as implemented in the ‘GSVA’ R package [16]. Additionally, inflammatory-related metagenes were described previously[9].

TIMER Database Analysis

Tumor Immune Estimation Resource (TIMER) serves a comprehensive resource to analyze immune infiltrates among 10,897 samples across 32 cancer types[17]. The correlations of CD96 expression with the abundance of immune infiltrates, including B cells, CD4 + T cells, CD8 + T cells, neutrophils, macrophages, and dendritic cells were detected in GBM and LGG patients on TIMER online tools (https://cistrome.shinyapps.io/timer).

Statistical computations
Statistical analysis and figures drawing were conducted using the R language, version 3.6.1 (http://www.r-project.org). Kaplan–Meier plots and Cox proportional hazard model analysis were generated using R packages ‘survminer’ and ‘survival’ [18, 19]. Receiver operating characteristic (ROC) curves were calculated by R package ‘pROC’ [20]. Area under the curve (AUC) values were depicted from the ROC curves. Corrgram and corrplot plots were drawn using the R packages ‘corrgram’, and ‘corrplot’, separately [21, 22]. All statistical tests were two-sided and p-value < 0.05 was regarded as statistical difference.

Results

CD96 was enriched in glioblastoma, IDH-wildtype, and mesenchymal glioma

To clarify differences of CD96 expression pattern in four grades of glioma malignancy, the mRNA level of CD96 was examined in CGGA and TCGA databases separately. The results revealed that glioblastoma (WHO IV) showed a higher CD96 expression in CGGA (Fig. 1A) and TCGA (Fig. 1B) cohorts than WHO grade II and grade III glioma. Compared to other pathological subsets (namely oligodendroglioma, oligoastrocytoma, and astrocytoma), CD96 expression was up-regulated relatively in glioblastoma (Figs. 1C and 1D). Isocitrate dehydrogenase (IDH) mutation distributes in almost 40% glioma which has an outsize impact on glioma development and progression[23]. To this end, the expression profile of CD96 in different IDH states was also explored. We found that CD96 was consistently enriched in IDH-wildtype (IDHWT) in CGGA as well as in TCGA database (Figs. 1E and 1F). This result suggested that CD96 expression was more prevalent without IDH mutation (IDHWT) than with IDH mutation (IDH MUT) in glioma. In order to investigate the expression pattern of CD96, the distribution of CD96 in different molecular subtypes was analyzed. Compared with the other three subtypes (classical, neural, and proneural), CD96 was
highly enriched in mesenchymal subtype in both CGGA and TCGA cohorts (Figs. 2A and 2B). To further validate our findings, we carried out ROC curves analysis of CD96 expression and mesenchymal subtype in all grade glioma. Intriguingly, we observed that area under the curve (AUC) was up to 78.6% and 92.8% in CGGA and TCGA datasets separately (Figs. 2C and 2D). These findings indicated that CD96 acted as a potential biomarker for mesenchymal subtype glioma.

**CD96 was significantly associated with immune functions in glioma**

Considering CD96 expression was closely tied to malignancy, we inferred that CD96 became an integral part of glioma progression and then performed GO analysis to unfold its role. 54 and 176 genes positively correlated with CD96 were listed by Pearson correlation analysis (Pearson |R| > 0.6) in CGGA and TCGA datasets. According to GO enrichment analysis, we found that genes most relevant to CD96 were more involved in T cell activation and regulation of lymphocyte proliferation in CGGA and TCGA databases, respectively (Figs. 3A and B). To further elucidate the immune function of CD96 in glioma, 1,540 genes from the AmiGO 2 website (http://amigo.geneontology.org/amigo) were reported to be associated with the immune response and we selected 362 and 354 genes most relevant to CD96 (Pearson |R|> 0.3) in CCGA and TCGA cohorts (Table S1) for heatmap analysis[24]. Thereinto, 357 genes were strongly positively correlated with CD96 expression, while 5 genes had a significantly negative relationship with CD96 in CGGA dataset. While 347 and 7 genes displayed directly and inversely proportional in TCGA dataset (Fig. 4). To sum up, CD96 was directly correlated with most immune responses and negatively correlated with few immune responses in glioma.

**The correlation between CD96 and T cell mediated immunity in glioma**

To fully understand the relationship between CD96 and T cell immune in glioma, we
performed gene set variation analysis (GSVA) to assess differential activities of pathways between sets of genes. As delineated in Figs. 5A and 5B, these relationships were similar in both CGGA and TCGA databases. Specifically, CD96 showed a positive correlation with T-helper 1/2 type immune response (GO:0042088 and GO:0042092), T-helper 1/2 cell cytokine production (GO:2000556 and GO:2000553), and natural killer cell mediated cytotoxicity directed against tumor cell target (GO:0002860). Conversely, CD96 was correlated negatively with T cell mediated immune response to tumor cell (GO:0002842) and T cell mediated cytotoxicity directed against tumor cell target (GO:0002852). This result re-validated that the special immune function of CD96 is to act an inhibitory role in T cell immune to tumor cells in glioma.

Correlation Between CD96 And Other Immune Checkpoints

As therapeutic targets, a growing number of immune checkpoints have been emerged and examined in clinical trials or clinical situations [25, 26]. Thereby, we analyzed the relationship between CD96 and other immune checkpoints, such as PD-1, CTLA-4, TIGIT, TIM-3, NR2F6, and GITR. Pearson correlation analysis revealed that CD96 was tightly associated with PD-1, CTLA-4, TIGIT, and TIM-3. Co-expression of PD-1 with CD96 was consistent with the previous research [12]. These results were validated in CGGA and TCGA datasets mutually (Figs. 5C and 5D), implying the possible synergistic effects of CD96 with these checkpoint members. Accordingly, we postulated CD96 may contribute significantly to the inflammatory response in glioma and used the previous method to test[9]. As shown in Figure S1, CD96 was positively associated with HCK, MHC-I, MHC-II, STAT1, STAT2, and FCGR2A, especially with LCK. This finding additionally evidenced the vital immune function of CD96 in glioma.

Correlation between CD96 and immune infiltration level in GBM and LGG
Tumor-infiltrating lymphocytes are an independent predictor of sentinel lymph node status and survival in cancers. Hence, we investigated whether CD96 expression was connected with immune infiltration levels in GBM (glioblastoma multiforme) and LGG (Low Grade Glioma) by TIMER website tools. Our findings manifested that CD96 expression related positively with infiltrating levels of dendritic cell (r = 0.504), neutrophil (r = 0.487), macrophage (r = 0.431), and CD8 + T cell (r = 0.406) in LGG. Simultaneously, CD96 had a marginal positively association with B cell (r = 0.329) and CD4 + T cell (r = 0.37) infiltration level in LGG. On the other hand, CD96 expression has no significant relationships with tumor purity (r = -0.117) infiltrating levels of B cell (r = 0.101), CD8 + T cell (r = -0.157), CD4 + T cell (r = -0.1), and so on in GBM (Fig. 6). These differences implied that CD96 would make more contribution to immune infiltration in LGG than GBM, especially in dendritic cells.

**CD96 predicted worse survival in glioma**

Owing to the high relevance between CD96 and immune suppressor in glioma, the prognostic impact of CD96 was verified via Kaplan–Meier method. Overall survival analysis in glioma and GBM patients demonstrated that high expression of CD96 predicted relatively poor survival in CGGA and in TCGA cohorts (Fig. 7). Eventually, we evaluated the independence of the clinicopathological significance of CD96 in glioma by univariate and multivariate Cox regression analyses. The results indicated that CD96, age, gender, WHO grade, IDH mutation, and h1p19q codeletion status were closely associated with overall survival and revealed CD96 is an independent prognosticator for glioma patients (Table S2).

**Discussion**

Recent studies about tumor immunotherapy continue to soar and bring hope to glioma
patients. Among those immunotherapeutic strategies, immune checkpoint blockade offers remarkable benefits to the therapies of various tumor types by increasing anti-tumor immunity[27]. However, researches on the field of neural tumor immunotherapy mainly focus on CTLA-4 and PD-1/PD-L1 blockade currently. Nonetheless, potential immune-related adverse events hindered the wide clinical application of immunotherapy[27]. The identification of alternative checkpoint targets may facilitate the solution of this predicament and furtherance therapeutic benefits for cancer treatment.

Increasing researches demonstrated that CD96 emerges as a potent modulator of anti-tumor immune responses [11]. CD96 has been shown to regulate negatively NK cell-mediated immune surveillance and intervene multidimensional adhesion, inhibition, and activation of participating cells[11, 28-30]. The special effect of CD96 has also been reported in some tumors. Hepatocellular carcinoma (HCC) patients with the high expression level of CD96 within tumor are strongly correlated with deteriorating disease situations, shorter disease-free survival, and overall survival times[31]. Targeting host CD96 appears as an innovative strategy for clinical application combination with current immunotherapies. Herein, we probed the biologic functions of CD96 in glioma through the large scale and in-depth analyses. CD96 expression was markedly enriched in higher malignant pathological type gliomas. Moreover, high expression of CD96 was observed in the malignant molecules phenotype, including IDH wildtype and mesenchymal subtype. Collectively, CD96 exhibited a malignant biological property in glioma. Meanwhile, through the analysis of the relationship between CD96 and biological processes, we noted that CD96 had a positive association with immune response and inflammatory activities. A range of immunotherapy targeting checkpoint inhibitors and blocking monoclonal antibodies (mAbs) have been widely adopted and several of them are ongoing in glioblastoma [8, 32]. Compared to monotherapy treatments, combination approaches were
reported to be more effective and associated with substantially longer progression-free survival[33, 34]. In our research, CD96 showed a high concordance with immune checkpoints, PD-1, CTLA-4, TIGIT, TIM-3, NR2F6, and GITR, indicating the potential synergistic effects of these markers. We inferred that CD96 collaborating with other checkpoint members especially PD-1 may increase effect glioma immunotherapy. Indeed, the previous studies have demonstrated that concurrent blockade of CD96 and PD-1 increased anti-tumor immunity over targeting PD-1 alone without inducing serious immune-related toxicities potentially[35, 36]. This provided support to our research. We also discovered that higher CD96 expression predicted worse survival rates in glioma and GBM patients. This significant prognostic signature implied that CD96 blockade may significantly improve the prognosis of glioma patients, especially GBM patients.

Conclusions

To sum up, we initially explored the genetic and clinical characteristics of CD96 based on CGGA and TCGA datasets. Our results highlighted CD96 may be a promising biomarker and therapeutic target for glioma which present favorable application prospects.

Additional Files

Additional file 1 Figure S1. The relationship between CD96 and inflammatory activities in glioma.

Additional file 2 Table S1. The most relevant immune genes to CD96 in CGGA and TCGA datasets.

Additional file 3 Table S2. Univariate and multivariate analyses of clinical prognostic parameters in CGGA and TCGA datasets.

Abbreviations
AUC: Area under the curve; CGGA: Chinese Glioma Genome Atlas; GBM: Glioblastomas; GO: Gene ontology; GSVA: The gene set variation analysis; IDH: Isocitrate dehydrogenase; OS: Overall survival; PD-L1: Programmed death-ligand 1; TCGA: The Cancer Genome Atlas dataset; TIM3: T cell immunoglobulin mucin-3; WHO: World Health Organization

Declarations

Consent for publication

All authors have reviewed the manuscript and consented for publication.

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Availability of data and materials

All the data in this study were obtained from The Cancer Genome Atlas dataset (TCGA; https://tcga-data.nci.nih.gov/repository) and Chinese Glioma Genome Atlas dataset (CGGA; http://www.cgga.org.cn/).

Authors’ contributions

Shengtao Y and Fang C conceptualized and designed this study. Hua Z performed the data collection and analysis. Qiang Z wrote the manuscript. Yinchun F, Qian L, and Jiancheng S participated in drawing diagrams and revision. All authors gave final approval of the manuscript.

Ethics approval and consent to participate

All the procedures in this study were approved by the ethics committees of all hospitals, and written informed consent was obtained from all patients.

Competing interests
The authors declare that they have no competing interests.

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Figures
Figure 1

Relationship between CD155 expression and clinical glioma parameters in the CGGA and TCGA cohorts. (A, B) Correlation of CD96 transcript levels and WHO grade. (C, D) CD96 expression pattern in different WHO grades. (E, F) Association between CD96 expression and IDH-wildtype for all grade glioma. **, *** and **** represent p < 0.05, p < 0.01 and p < 0.0001, respectively.
CD96 was highly enriched in mesenchymal molecular subtype glioma. (A, B) CD96 had a strong expression pattern in mesenchymal subtype glioma in CGGA and TCGA datasets. (C, D) CD96 showed high sensitivity to predict mesenchymal subtype glioma in ROC curve analysis.
Figure 3

Gene ontology (GO) analysis about genes most relevant to CD96 in CGGA database (A) and TCGA database (B).
The heatmap of CD68 related immune genes in glioma in CGGA and TCGA cohorts.
Figure 5

CD96-related T cell immunity and immune checkpoint markers in glioma. (A, B) The relationship between CD96 and T cell immunity in glioma in CGGA and TCGA datasets. (C, D) Corrgram map of CD68 and immune checkpoint markers in glioma in CGGA and TCGA databases.
Figure 6

Correlation of CD96 expression with immune infiltration level in GBM and LGG.
Survival analysis of glioma based on CD96 expression. (A, B) Survival analysis of CD96 in whole grade glioma. (C, D) Survival analysis of CD96 in glioblastoma based on data from CGGA and TCGA cohorts, respectively.

Supplementary Files
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