

Characterization of IKBIP prognostic values in collaboration with immune infiltrates in glioma

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Research Article

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Abstract

Challenges remain in the prognosis prediction and treatment of glioma due to the complexity and limited understanding of the tumor immune microenvironment. However, how IKBIP protein expression relates to patient prognosis by interacting with immune cells remains uninvestigated. Here, we investigated IKBIP mRNA expression in gliomas using gene expression profiling interactive analysis. Also, we performed multiplex immunohistochemistry staining on glioma tissue microarrays to quantify IKBIP protein expression. Meanwhile, we evaluated the prognosis prediction value of IKBIP protein using clinical feature analysis and Cox regression model with Kaplan-Meier survival analysis. In addition, we applied principal component analysis to uncover the IKBIP-related immune determinants. We identified higher IKBIP mRNA and protein expression in glioma tissues than in non-tumor brain tissues. We found significant associations between IKBIP protein expression and glioma WHO grade and radiotherapy. IKBIP protein expression was closely related to patient 5-year survival. CD8⁺ and CD56⁺ immune cell was correlated with high IKBIP protein expression. Collectively, we demonstrated that high IKBIP was an immune cell related independent biomarker to predict worse patient prognosis in glioma, which could be used as future therapeutic target.

Introduction

Glioma is the most common type of central nervous system (CNS) malignancy, with a median survival time of approximately 15 months from surgical resection combined with radiotherapy and chemotherapy [1]. East Asia has the highest incidence of CNS tumors [2, 3]. Over the last decade, there has been no significant progress in glioma diagnosis and treatment [4, 5]. Although our current understanding of the molecular underpinnings of glioma has expanded substantially, patient survival remains unfavorable [6]. Thus, developing clinically relevant biomarkers for diagnosis and treatment would be essential to provide ample opportunities for glioma patient survival [7].

Most tumors comprise immune cells and tumor cells, both of which actively participate in tumor progression through constant communication [8]. Due to disruption in the blood-brain barrier (BBB) that occur during glioma-genesis, glioma areas are highly immune-infiltrated, either promoting tumor growth or suppressing immune response [9]. Tumor-infiltrating immune cells influence glioma patient survival in various ways, depending on their type and functional interactions [10]. Furthermore, the molecular heterogeneity of the tumor microenvironment (TME) became the main barrier to glioma diagnosis and treatment [11, 12]. Among which, immune cell evasion was now widely accepted as tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs) [13]. TAMs account for up to 40% of CNS tumor mass, and increased expression of TAM marker genes resulted in a suppressive immune microenvironment. Increased TILs may contribute to favorable clinical outcomes in patients with glioma [12, 14]. As a result, the composition variation of immune cells within the glioma TME was a prognosis indicator.

IKBIP (kappa B-interacting protein), also known as IKIP, is located on chromosome 12 near APAF1 (apoptotic protease-activating factor-1)[15]. Bioinformatics analysis has identified it as a hub gene in the glioma malignant process and patient prognosis [16, 17]. IKBIP protein is commonly expressed in the endoplasmic reticulum and is functionally involved in pro-apoptotic cell death [18]. Due to the mRNA translation process, the mRNA abundance differs from that of its corresponding protein, RNA signature often lacks confidence in clinical utility [19]. In glioblastoma multiforme (GBM), translational changes in mRNA are frequently more extensive than changes in transcriptional signaling pathways [20]. While prior researches were restricted to mRNA and bioinformatics[21], our study firstly validates the progressive relationship at both protein and spatial immune atlas levels.

Therefore, our current study was designed to identify the differentially expressed IKBIP protein in glioma Tissue Microarrays (TMAs) using the multiplex Immunohistochemistry (mIHC) method. We analyzed the IKBIP expression-related clinical characteristics and patient overall survival using the Cox regression model. In addition, we performed a comprehensive correlation analysis to determine the relationships between IKBIP protein expression and the infiltrated immune cells. Our research demonstrated that IKBIP was an independent prognosis-predictive biomarker for patients with glioma.

Materials and Methods

Gene Expression Profiling Interactive Analysis (GEPIA2)

GEPIA2 (<http://gepia2.cancer-pku.cn/>) is an online data analysis tool investigating RNA sequencing expression for cancer-benign comparison. We used this web-based database to retrieve IKBIP expression data from the Cancer Genome Atlas Program (TCGA) and Genotype-Tissue Expression (GTEx) programs by boxplots using GBM (n = 163), LGG (n = 518), and non-tumor brain tissue (n = 207). Meanwhile, GEPIA2 was used to assess the relevance of IKBIP mRNA expression to glioma patient prognosis.

Tumor Immune Estimation Resource (TIMER) Database Analysis

TIMER (<https://cistrome.shinyapps.io/timer/>) is a comprehensive resource for systematically analyzing immune infiltrates in various cancer types. The abundance of the infiltrated immune cell was estimated using gene expression profiles from TCGA datasets. In the glioma TME, we used TIMER to investigate correlations between IKBIP mRNA expression and tumor-infiltrating immune cells.

Human specimens

Glioma tissue specimens were collected from 207 patients with primary glioma and 29 patients with non-tumor brain disease at Nantong University Affiliated Hospital between March 2013 and November 2017. Clinical information was obtained from the retrospective data. Glioma pathological type and WHO grade were determined regarding the 2021 World Health Organization Classification of Tumors of the Central Nervous System [22]. This study includes patients pathologically diagnosed with primary

gliomas and who underwent surgical resections. All experiments adhered to the principles outlined in the Helsinki Declaration and institutional guidelines. Written informed consent was obtained from all patients for the use of their tissue for research purposes. This study was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University (Grant No.2018-K020).

Multiplex-Immunohistochemistry (mIHC) staining

Formalin-fixed paraffin-embedded (FFPE) primary glioma tissue blocks were used to construct TMAs. Glioma TMAs (2 mm in core diameter) were produced by the manual Tissue Microarrayer System Quick Ray (UNITMA, Seoul, Korea) as previously described [23]. The mIHC staining was performed using the Opal 7-Color Manual IHC Kit (NEL810001KT, AKOYA Bioscience, USA). TMA sections were first deparaffinized and rehydrated. Microwave treatment was performed with the appropriate AR6/9 antigen retrieval buffer (AR600250ML/AR900250ML, AKOYA Bioscience, USA). The slides were blocked with tissue-blocking buffer (ARD1A01EA, AKOYA Bioscience, USA) to inhibit endogenous peroxidase activity. The primary antibodies were then incubated overnight. The slides were then incubated with Opal polymer HRP (anti-Ms+Rb) secondary antibody (ARH1001EA, AKOYA Bioscience, USA). Fluorophores (Opal 520, Opal 540, Opal 570, Opal 620, Opal 650, and Opal 690, AKOYA Bioscience, USA) were used for fluorescent visualization. Multiplex staining was accomplished by serially repeating staining cycles, with microwave treatment procedures in between each step to remove the antibody complex. Fluoromount (F6057, Sigma, USA) with DAPI was used to visualize the nuclei for the final step.

Primary antibodies used were: anti-IKBIP antibody (1:500, Orb521033, Biorbyt, UK), anti-CD3 antibody (1:500, 85061, CST, USA), anti-CD4 antibody (1:5000, ab133616, Abcam, UK), anti-CD8 antibody (1:500, 8536S, CST, USA), anti-CD20 antibody (1:1000, ab78237, Abcam, UK), anti-CD25 antibody (1:100, MA5-12680, Invitrogen, USA), anti-CD68 antibody (1:500, 76437, CST, USA), anti-CD56 antibody (1:200, 3576, CST, USA), anti-CD83 (1:1000, MAB1774, R&D, USA), anti-CD66b antibody (1:1000, ARG66287, Airgobio, CHINA), anti-IDH1(R132H) antibody (1:10, DIA-H09, DIANOVA, GER), anti-GFAP antibody (1:10000, ab33922, Abcam, UK).

Identification and expression analysis of immune cell

The fluorescence-stained slides were scanned into digital images using the Vectra 3.0 Automated Quantitative Pathology Imaging System (AKOYA Bioscience, USA). The fluorescence intensity of IKBIP and immune cell markers were then quantified within glioma regions using InForm Tissue Analysis Software (InForm 24.1, AKOYA Bioscience, USA). For IKBIP and these immune cell markers, specific algorithms were developed to automatically calculate each score based on fluorescence intensity. The expression levels of IKBIP and immune cells were scored as percentage values (positively stained cell number/nuclei number *100% of each tissue category).

Statistical analysis

The cutoff value of 0–39.85 and 39.86–100 was set to separate low and high IKBIP expressions using the X-tile software (The Rimm Lab in Yale University, USA). The medians were used as cutoff values for each immune cell expression. Pearson χ^2 test was applied to correlate clinical features with IKBIP protein expression. Both univariate and multivariate analyses were performed to assess the prognostic value of glioma patients. The survival curves were generated using the Kaplan–Meier method with a cox regression log-rank test. SPSS 22.0 (SPSS Inc, USA) software was used to conduct these statistical analyses. Statistically significant differences in between-group comparisons were defined at a significance level of P value $\leq$ 0.05.

Ethical approval

The present study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (Grant No.2018-K020). All experiments adhered to the principles outlined in the Helsinki Declaration and institutional guidelines. Written informed consent was obtained from all patients for the use of their tissue for research purposes.

Results

Investigation of IKBIP mRNA expression and its prognostic value in glioma

To address a role of IKBIP in glioma, we performed broad IKBIP mRNA expression analysis through GEPIA2 using TCGA, and GTEx glioma datasets and matched normal datasets. IKBIP transcription level was relatively higher in LGG and GBM than in non-tumor brain tissues (Fig 1 A). Meanwhile, our results revealed significant correlations between high IKBIP mRNA expression and unfavorable overall survival in glioma patients (Fig 1 B).

Relationship of IKBIP protein with clinical characteristics and patient prognosis

To determine the role of IKBIP protein and patient prognosis, we investigated IKBIP protein expression using glioma TMA-mIHC. The IKBIP protein was found in the intracellular and cell membrane regions of glioma cells, subcellularly located in the endoplasmic reticulum. Consistent with its mRNA expression shown above, IKBIP protein was more abundant in glioma tissue than in non-tumor brain tissues (Fig 1 C).

Subsequently, we analyzed the associations between high IKBIP protein expression and patient clinical characteristics. We observed significant associations between IKBIP protein expression and glioma WHO grade ($\chi^2 = 11.812$, $P = 0.019$) (Table 1). The prognostic influence of IKBIP protein was then evaluated using univariate and multivariate analysis. We identified IKBIP protein expression (HR:1.516, $P = 0.01$) and WHO grade (HR:1.542, $P = 0.0001$) were closely related to patient 5-year survival (Table 2). Consistent with Cox regression analysis results, Kaplan–Meier survival curves along with the log-rank test also illustrated that high IKBIP protein expression predicted a worse patient overall survival (Fig. 1 D).

IKBIP- related Immune cell types in the glioma TME

Glioma progression was tied to the immune environment [24], thus, the role of IKBIP in the glioma immune environment remained unexplored. Then, we investigate the overall immune cell abundance associated with IKBIP mRNA using TIMER. The infiltrated immune cells showed distinct cell distribution in LGG and GBM patients. B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, DCs, and neutrophils were among the major immune cell types found to have been infiltrated into glioma TME in LGG, whereas CD4⁺ T cells, DCs, and neutrophils appeared to be the IKBIP related immune infiltrators in patients with GBM (Fig 2 A, B).

IKBIP protein related Immune cell landscape in glioma TMAs

To determine the role of immune cells interacting with IKBIP in the glioma TME, we illustrate the density and distribution of the infiltrated immune cells and their relationships to IKBIP protein expression. We found that TAMs were positioned in the perivascular area or adjacent to IKBIP expression locations (Fig 3 A, C). High macrophage (CD68⁺), neutrophil (CD66B⁺), and DC (CD83⁺) expression in relation to IKBIP protein expression were discovered. Among them, Macrophages were significantly increased in both LGG and HGG patients (Fig 3 D, E). TILs were diffusely distributed around the tumor region or near lymphatic cuffs. CD8⁺ T cells tended to be more diffuse and adjacent to glioma cells (Fig 3 A, B). We observed a relatively higher CD8⁺ T cells and CD56⁺ NK cells expression in LGG patients (Fig 3 E). Furthermore, CD4/CD8 ratios were negatively correlated with glioma grade (Fig 3 F, $P < 0.01$).

Identification of determinant immune infiltrates related to high IKBIP expression

Following that, we applied exploratory factor analysis (EFA) and principal-component analysis (PCA) to determine how IKBIP related to patient prognosis by interacting with immune cells. According to the variance decomposition table, the first eight immune cell indicators were effective (Table 3). Among them, we discovered CD56⁺ and CD83⁺ cells (NK cell and DCs unit) have higher loads in the first factor. The second factor contained higher loads of CD66B⁺ and CD20⁺ cells (neutrophil and B cell unit). In the third group, CD3⁺ and CD8⁺ cells (T cell unit) have higher loads (Fig 4 C, D). The metanalysis plot revealed that the CD8⁺, CD83⁺, and CD56⁺ immune cells exhibit protective potential in synergistic with IKBIP (Fig 4 E). Furthermore, we employed immune cell cluster analysis to analyze their enrichment status in LGG and HGG patients. We discovered extensive CD8⁺, CD56⁺, and CD83⁺ immune cell enrichment among these immune cells (Fig 4 F, G). Finally, we used mlHC to analyze CD8⁺ T cell and CD56⁺ NK cells expression in low and high IKBIP protein expression groups. IKBIP protein exhibited close relation to CD56⁺ and CD8⁺ immune cells, whereas, in low IKBIP expression group, these immune cells were less frequent and more dispersed (Supplemental Fig 1).

Our findings revealed that high IKBIP expression indicated a worse patient prognosis. We also demonstrated that CD8⁺ T cells and CD56⁺ NK cells were the major immune cell determinants correlated with high IKBIP protein expression in the glioma TME.

Discussion

IKBIP was an endothelial expressing gene with pro-apoptosis functions [18, 21]. Bioinformatics analyses revealed high IKBIP mRNA expression was associated with poor patient prognosis in hepatocellular carcinoma [25]. Consistently, IKBIP mRNA could predict an inferior survival of glioma in Chinese Glioma Genome Atlas and TCGA databases [26]. To date, there have been vast differences in glioma risk globally, with the highest incidence occurred in East Asia [27, 28]. Meanwhile, numerous genetic association studies have been conducted widely in order to identify risk factors for prognosis prediction [28]. However, recent clinical trial results have yet to show significant improvements in the prognosis of glioma patients [29]. Thus, facilitating early discovery of promising biomarkers in clinical populations holds real promise for improving patient survival [30]. In this study, we discovered a high IKBIP protein expression in glioma. We categorized differentially expressed IKBIP protein for clinical characteristic and overall survival analysis using glioma TMA-mIHC. Simultaneously, we investigated the contents of immune cells to identify IKBIP correlated immune infiltrators. The demonstrated role of IKBIP protein was an independent prognostic biomarker for patient survival, and its high protein presence on glioma cells has been associated with worse patient prognosis with infiltrated immune cells.

Increasing evidence points to the significance of immune cell-mediated anti-tumor therapy [31]. Although immune therapies have extended lifespan for multiple cancer patients, one notable exception is glioma [32]. There were only 4 approved drugs for GBM to date, none involved immunotherapy [33]. Partly because the severe immunosuppressive TME has resulted in inadequate immune cell intratumoral trafficking, rendering glioma highly resistant to therapy [34]. Therefore, the relationship between potential glioma biomarkers and immune cells must be discovered to assess its efficacy of target therapy. Our findings revealed an increased immune cell infiltration in glioma TME than in non-tumor brain tissue. In HGG, the infiltrated CD8⁺ T cells and CD56⁺ NK cells were relatively lower than in LGG. However, when CD8⁺ T cell infiltrated in "non-supportive" niches, like GBM, T cells were enriched for features of hypo-functionality distinct from canonical exhaustion, in situ spatial mapping of GBM highlighted dysfunctional CD4⁺ and CD8⁺ T cell interactions [35]. Hence, our results demonstrated that high IKBIP expression predict worse patient prognosis and indicated a "non-supportive" glioma TME. Even with strong IKBIP expression, dysfunctional CD8⁺ T cells and CD56⁺ NK cells demonstrated poorer tumor suppression. T cell and NK cell-based combination strategies have already been identified as effective in targeting multiple clinical mechanisms of GBM progression [34, 36]. Radiotherapy (RT) is known to modulate the immune microenvironment of glioma through the modulation of RT-triggered anti-tumor immune responses [37]. Genetic and epigenetic adaptation of cancer cells by radiotherapy prolong the life span of glioma-bearing animals [38]. Our results demonstrated a close relation between IKBIP expression and radio therapy sensitivity ($P = 0.022$). However, there has been a limited appreciation to date of the dynamic and longitudinal alterations elicited in infiltrating immune cells within the TME during radiation response. Our findings demonstrated that gliomas with high IKBIP expression can be treated with immunotherapy targeting CD8⁺ T cells and CD56⁺ NK cells in combination with radiation therapy.

Nonetheless, there still exist limitations to our research. The underlying mechanisms of IKBIP in the development and prognosis of glioma were not specified. Subsequent research should be conducted to discover the mechanisms regarding its role in glioma development using in vivo and in vitro models. In addition, Deep immune cell expression profiling methods such as mass cytometry will be required to compare specific immune cell subtypes within glioma immune microenvironment.

In conclusion, we demonstrated that high expressed IKBIP was an independent prognostic factor for worse patient prognosis in glioma. More importantly, it predicts glioma patient prognosis by interacting with infiltrated CD8⁺ and CD56⁺ immune cells. It should be considered when applying combined cell-based glioma immune therapy in order to benefit human health.

Abbreviations

Blood Brain Barrier (BBB)

Central nervous system (CNS)

Epithelial-mesenchymal transition (EMT)

Exploratory factor analysis (EFA)

Gene Expression Profiling Interactive Analysis (GEPIA)

Genotype-Tissue Expression (GTEx)

Glioblastoma Multiforme (GBM)

Higher-grade glioma (HGG)

Immunohistochemistry (IHC)

Isocitrate dehydrogenase 1 R132H (IDH1R132H)

Lower-grade glioma (LGG)

Multiplex-Immunohistochemistry (mIHC)

Principal-component analysis (PCA)

The Cancer Genome Atlas Program (TCGA)

Tissue microarrays (TMAs)

Tumor-associated macrophages (TAMs)

Tumor-infiltrating lymphocytes (TILs)

Declarations

Ethical Approval Statement: This study was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University (Grant No.2018-K020), Jiangsu, China.

Consent for publication: This publication has been approved by all co-authors.

Availability of data and material: Raw data were generated at the Clinical Bio-bank of the Affiliated Hospital of Nantong University. Derived data supporting the findings of this study were available from the corresponding author Guihua Wang upon request for the protection of patient privacy.

Competing interests: The authors declare no potential conflicts of interest.

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Author contributions

Concept design and literature search: Chunmei Zhao

Experimental studies: Luyun Gu

Data acquisition and analysis: Xudong Wang, Shaoqing Ju

Manuscript preparation: Chunmei Zhao, Guihua Wang

Manuscript review: Guihua Wang

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Tables

Tables 1 to 3 are available in the supplementary files section.

Figures

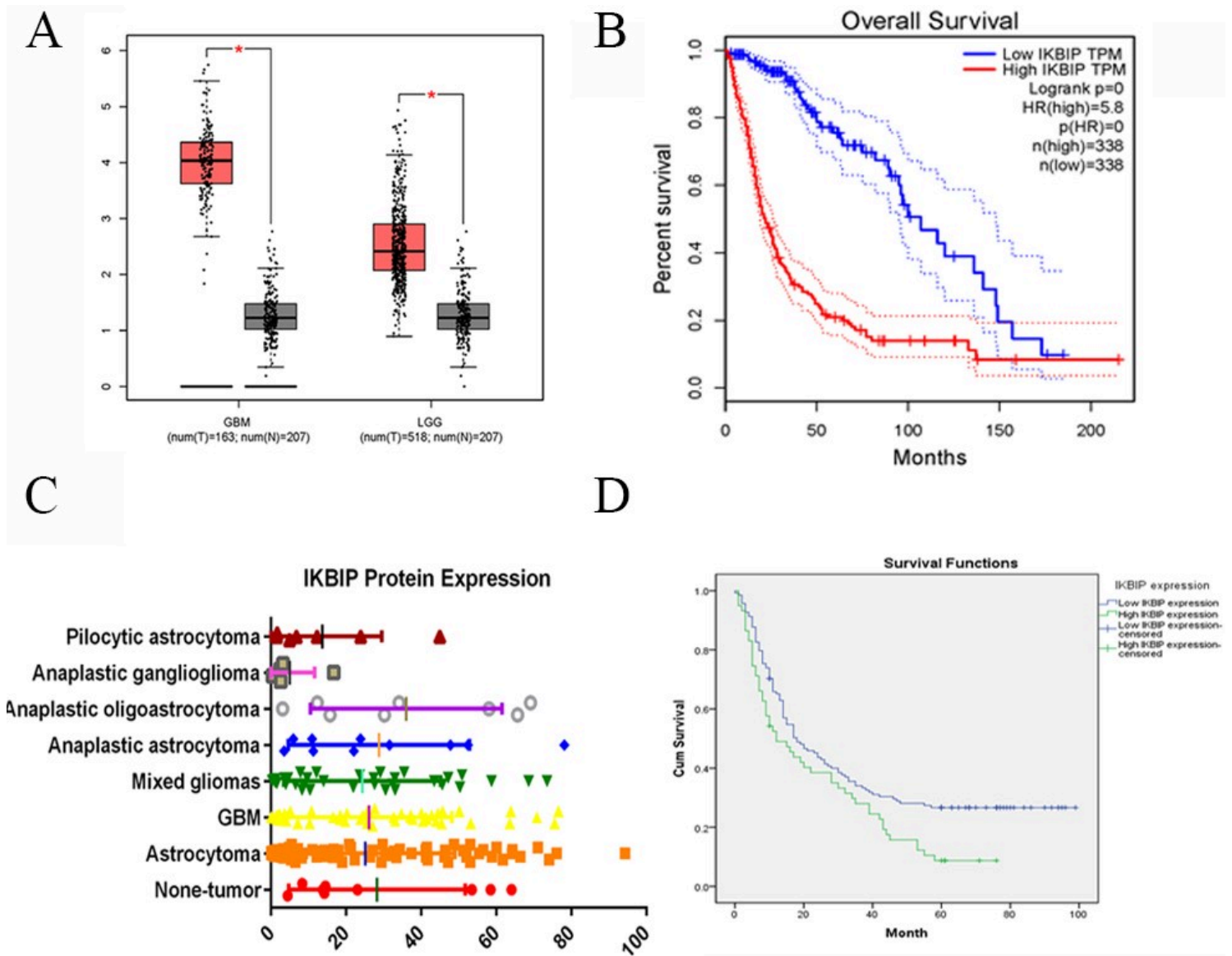


Figure 1

IKBIP expression is associated with glioma patient survival through interactions with immune cells

A. The red cells indicate that the IKBIP mRNA level was higher in glioma tissue than in normal brain tissue, while the blue cells indicate that the IKBIP mRNA level was lower in glioma tissue than in normal brain tissue.

B. Kaplan-Meier survival analysis of LGG and GBM patients using TCGA datasets.

C. IKBIP protein expression across different histological types of gliomas and non-tumor brain tissues.

D. Kaplan-Meier curve showing the overall survival of patients with glioma in the high and low IKBIP expression groups within tumor cells.

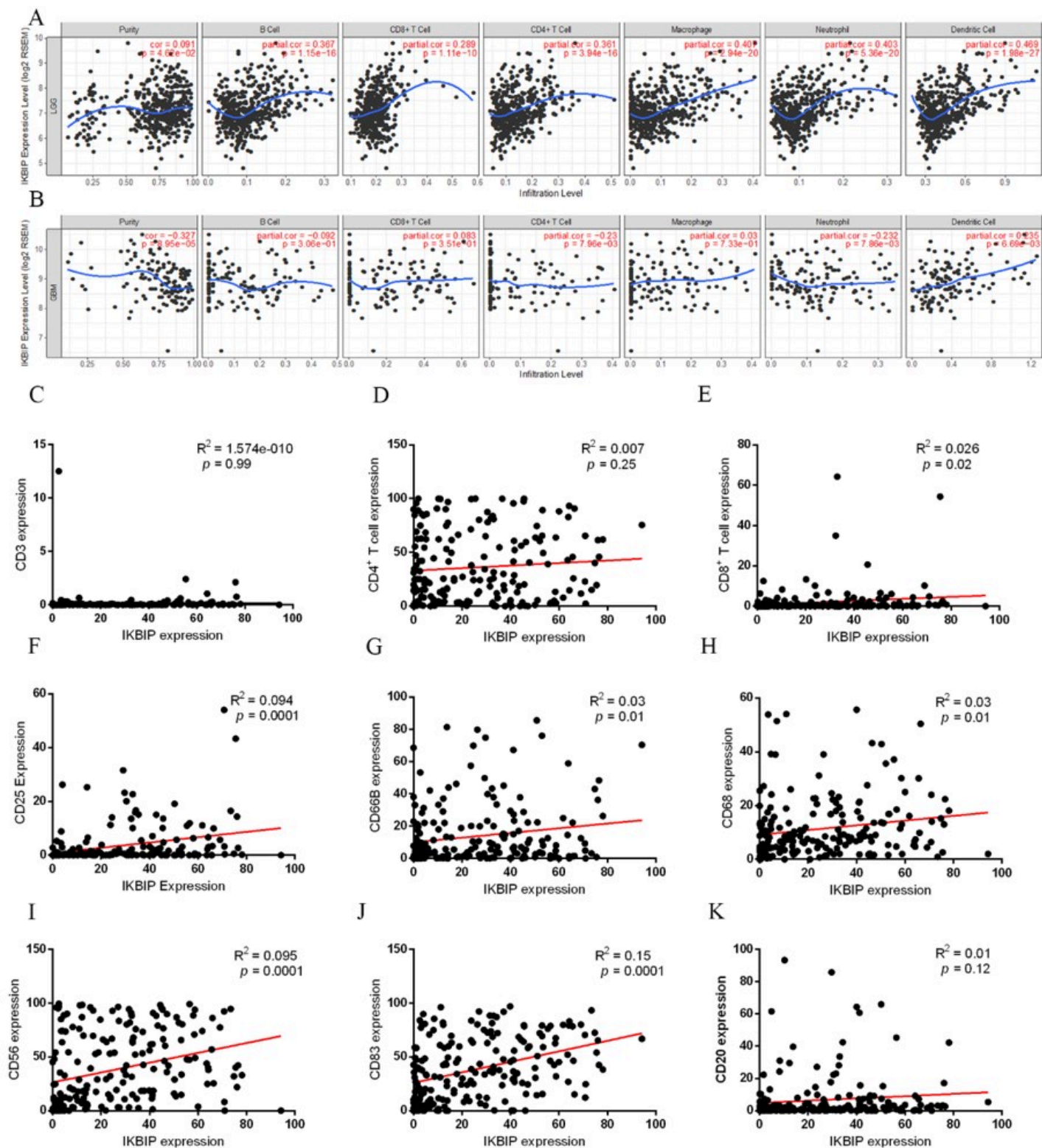


Figure 2

Infiltrated immune cells are correlated with IKBIP expression in the glioma TME

A. In LGG, B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and DCs were found to be closely related to high IKBIP expression.

B. In GBM, CD4+ T cells, neutrophils, and DCs were found to be closely related to high IKBIP expression.C-K. Spearman's correlation analysis of IKBIP and immune cells marked by (C) CD3, (D) CD4, (E) CD8, (F) CD25, (G) CD66B, (H) CD68, (I) CD56, (J) CD83, and (K) CD20 (n=207).L, M. Major immune cell infiltration levels in LGG and GBM with different IDH1R132H mutation statuses.

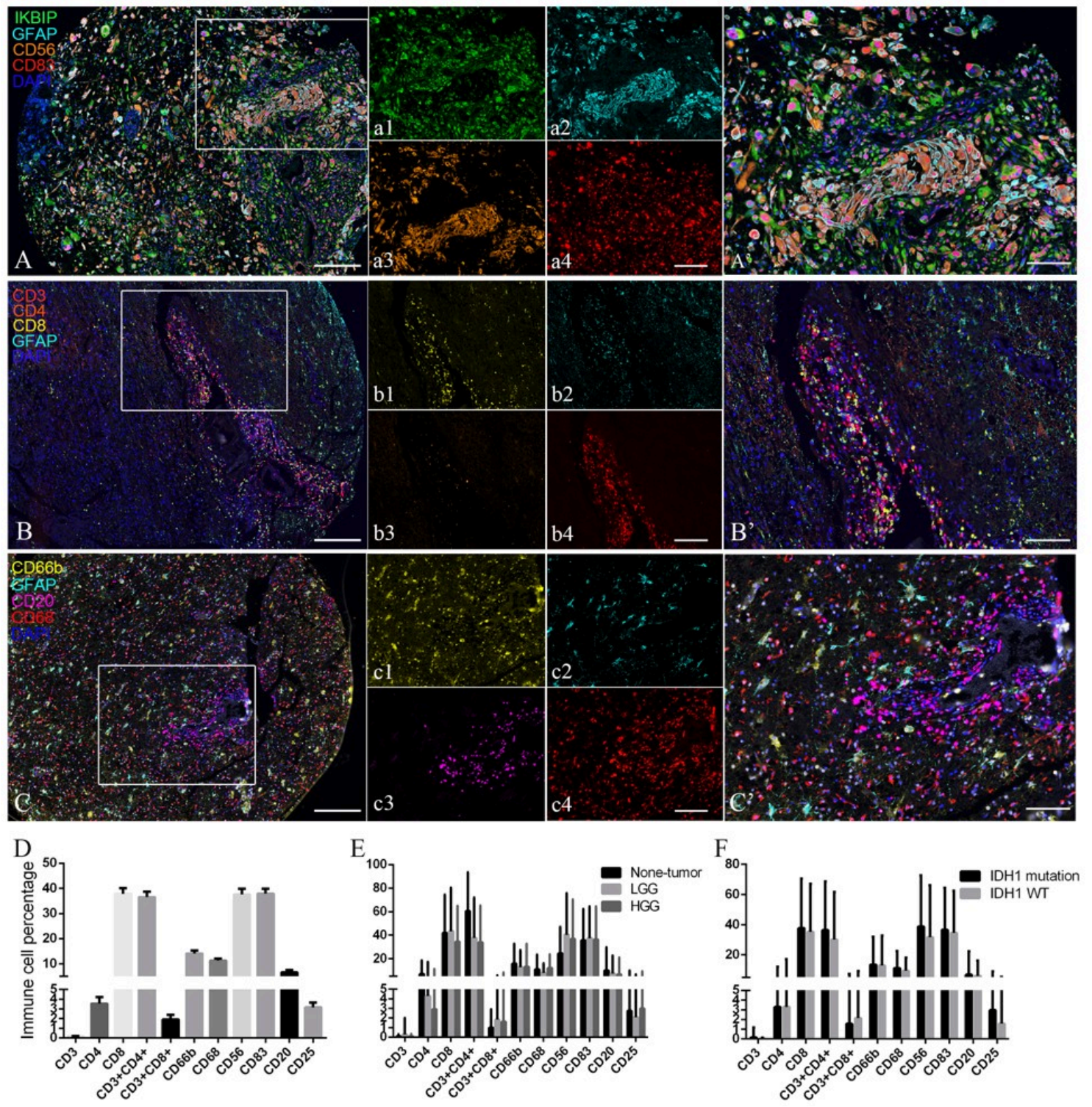


Figure 3

IKBIP protein expression related immune cell landscape in the glioma TME

A. Representative mIHC images of IKBIP and CD56+ and CD83+ immune cells near lymphatic cuffs. GFAP labels glioma cells, and DAPI labels nuclei (bar = 50 μ m).

B. Representative mIHC images of CD3+, CD4+, CD8+ immune cells in WHO grade III astrocytoma. GFAP labels glioma cells, and DAPI labels nuclei (bar = 50 μ m).

C. Representative mIHC images of CD66b+, CD20+, and CD68+ immune cells near a lymphatic cuff. GFAP labels glioma cells, and DAPI labels nuclei (bar = 50 μ m).

D. Immune cell percentage within the glioma TME presented by mIHC analysis.

E. Differential expression of immune cells in non-tumor brain tissue, LGG, and HGG patient tissues.F. CD4+ cell /CD8+ cell ratios of none-tumor, LGG, and HGG patients (****P < 0.01).G. Expression of immune cells with different IDH1R132H mutation statuses.

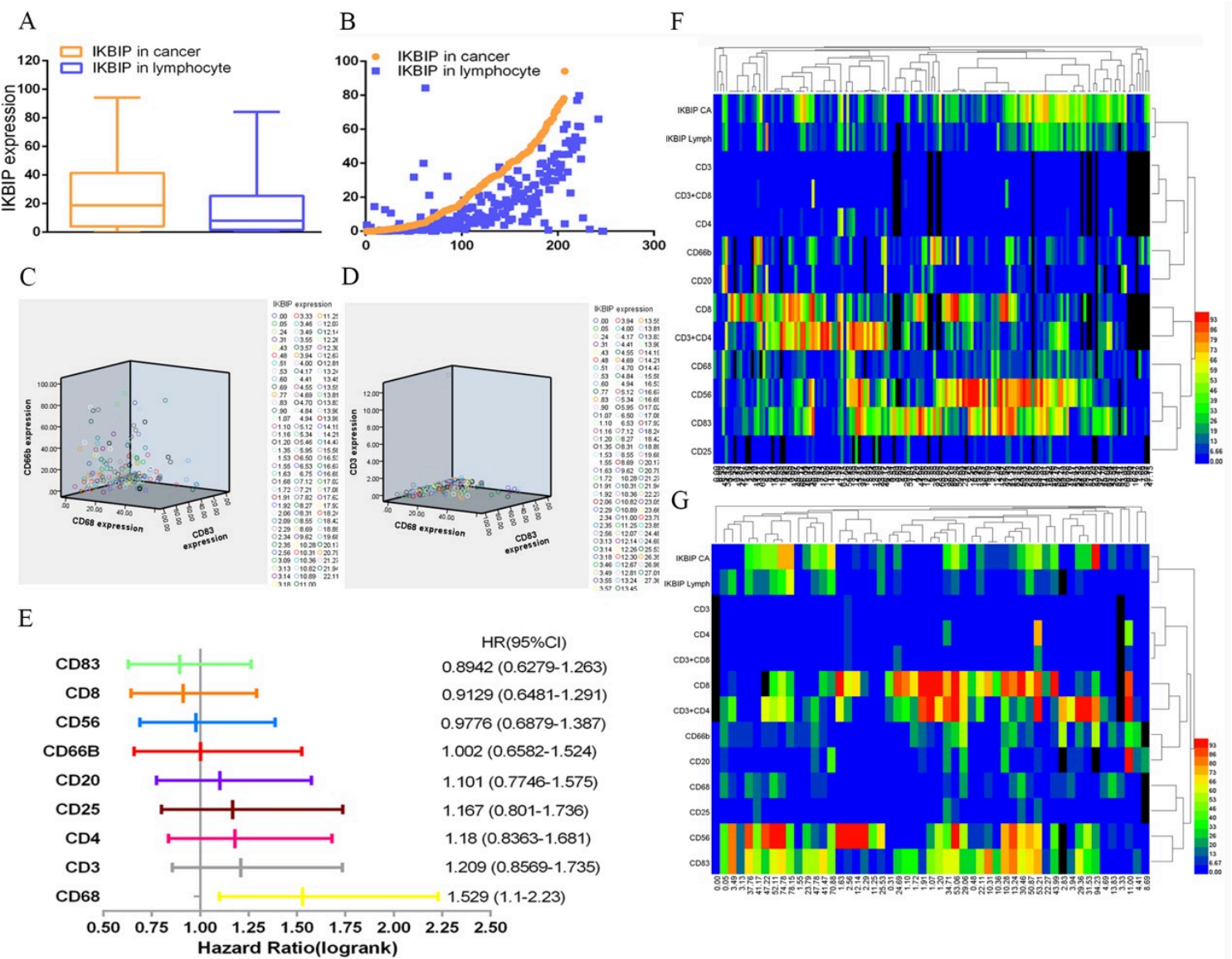


Figure 4

CD8+ and CD56+ immune cells are determinant immune infiltrates related to high IKBIP expression A. Relative expression of IKBIP with a significant difference between glioma cells and lymphocytes ($P < 0.1$). B. Scatter plot showing the monotonic positive relationship between IKBIP expression in glioma cells and that in lymphocytes ($P = 0.007$). C, D. PCA 3D dot plot of IKBIP expression data annotated in terms of the correlation with four major factors, including CD66B, CD68, CD3, and CD83 expression, in patients with glioma. E. Illustration of the hazard ratios of IKBIP expression-related immune cells for overall survival. Log-rank hazard ratios and 95% confidential intervals (HRs and 95% CIs, shown with plots and numbers) for the parameters used for predicting overall survival, including myeloid lineage cells and lymphocytes. F, G. Immune-associated bulk tissue heatmap of the leading immune cell expression clusters in LGG and HGG patients revealed by TMA mIHC (tile color indicates significance).

Supplementary Files

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