Bioinformatics Analysis of C3 in Brain Low Grade Gliomas as Potential Therapeutic Target and Promoting Immune Cell Infiltration

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Research Article

Keywords: cell infiltration, therapeutic, gliomas, GEPIA

Posted Date: November 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1040002/v1

Version of Record: A version of this preprint was published at Medical Oncology on January 11th, 2022.
See the published version at https://doi.org/10.1007/s12032-022-01647-6.

Abstract

Background

Low grade gliomas is the malignant nervous tumor with distinct biological and clinical characteristics. Despite advances in diagnostic and therapeutic methods, how to significantly elongate the survival of low grade gliomas is still the challenge. Complement 3, as the critical component in the innate immune system, play an essential role in local immune response and participated into the regulation of the epithelial-mesenchymal transition and tumor microenvironment.

Methods

In this study, we systematically determined the expression levels of C3 in low grade gliomas using various public databases. Then, we further identified the impact of C3 expression on immune cell infiltration compared to normal tissue, indicating the effect of cellular microenvironment on overall survival of LGG patients.

Results

We obtained transcriptional and survival of C3 in LGG from GEPIA and cBioportal database, and the differentially expressed genes were obtained. By performing the analysis of GO and protein-protein interaction network, we have identified the top-ranked 10 hub genes, which are highly associated with regulation of cell cycle. The gene set enrichment analysis demonstrated that overexpression of C3 in LGG patient is positively correlated with regulation of cell cycle. Finally, the immune cell infiltration of C3 in LGG patients was employed and clearly showed that higher neutrophil infiltration can worsen the survival of LGG patients with higher C3 expression. These results were confirmed by the Human Protein Atlas database, in which expression level of C3 protein in gliomas patients always higher.

Conclusions

This investigation implied that C3 can be as the potential targets of precise therapy for patient with low grade gliomas.

Background

Low grade gliomas (LGG) was classified as the Grade I and II by World Health Organization, accounting for approximately 17% of all primary nervous tumors[1–2]. Owing to the diverse pathology, median survival for LGG patients is only ranged from 5.6 to 13.3 years[3]. To further improve the survival of LGG patients, many new diagnostic and therapeutic methods have been well-developed in the past decade year, for

example precise therapy dependence on the specific molecular characteristics and histology[4–5], chemotherapy combined with radiation therapy[6]. Recently, LGG patients can benefit from molecular diagnosis, for example mutations of *IDH1* and *IDH2*[7–8], and further provide the precise therapeutic interventions. To determine whether more gene can be as the therapeutic targets or prognosis biomarkers, LGG patients will benefit from the molecular characteristics with precise treatment.

Tumor environment plays an important role in tumorigenesis, metastasis and affect the clinical therapy[9]. In the past decade, to target tumor environment have significantly improve the median survival of patients[10–11], including apatinib for lung cancer immunotherapy[12], immune checkpoint blockers to improve the tumor microenvironment[13] and chimeric antigen receptor T cells in refractory B-Cell non-Hodgkin's lymphoma[14]. Owing to the presentation of compact blood-brain barrier, it is hard to deliver most of therapeutic cargos into brain parenchyma[15]. To identify the indirect molecular targets (e.g. vasculature molecular targets), designation of therapeutic cargo, including siRNA or antibodies, will be useful for LGG therapy.

C3, so-called complement 3 protein, is one of critical components in complement system, which contains two subtypes of proteins, C3a and C3b, modified by C3 convertase complex[16]. The subtypes of C3 proteins (C3a and C3b) can strongly bind with many cell surface receptors to further activate downstream pathways, for example C3b binding with complement receptor 1 (CR1 or CD35) to blockade the immune adherence[17], binding with CD21 to promote the generation of B memory cell[18]. Activation of C3/C3a are potent pro-inflammatory molecules and can induce the cascade response on affect tumor microenvironment, for example recruiting neutrophils and monocytes[19]. In tumor microenvironment and cancer cells, recent reports clearly showed that cancer cell also can generate complement[20–21]. Overexpression of complement suggested that C3a/C3aR participated into the regulation of epithelial-mesenchymal transition[22]. This literature implied that complement can be as the potential therapeutic targets for cancer therapy.

In this study, we performed the comprehensive investigation to determine the correlation between C3 expression and LGG progression. By Kaplan-Meier analysis, overall survival and progression-free survival of LGG can be identified the correlation between C3 expression and other critical genes. After identifying the differentially expressed genes (DEGs), the protein-protein interaction analysis provides the hub genes and further determines the role of C3 in tumorigenesis. The gene set enrichment analysis (GSEA) was performed to explore how the C3 gene affects the cellular networks. Finally, we study the immune cell infiltration between C3 expression and immune cells using TIMER database. Our investigation identified the role of C3 in LGG tumorigenesis and provided that C3 may be as the potential therapeutic targets for efficient LGG therapy.

Methods

Database and clinical information about low grade gliomas (LGG) patients

In this investigation, the clinical information about LGG patients was obtained from TCGA database (https://portal.gdc.cancer.gov/; Data Release 29.0-March 31, 2021) and cBioportal database (http://www.cbioportal.org/). The RNA-sequencing data was analyzed using R-software package, and the mutation information was obtained from the cBioportal website. The information of C3 expression level was obtained from TIMER2.0 database.

Overall survival and progression-free survival analysis

The OS and PFS were obtained from GEPIA2.0 database (http://gepia2.cancer-pku.cn/#index), with threshold value as 50–50%. The survival information was obtained from TCGA database.

The Human Protein Atlas analysis

The protein expression level of relative biomarkers was analyzed using The Human Protein Atlas (https://www.proteinatlas.org/). The IHC staining of normal tissue was selected as cerebral cortex, and the IHC staining of tumor tissue was selected as gliomas. All the IHC images were directly downloaded from HPA database without any further modification.

Identification of differentially expression genes (DEGs)

The DEGs of C3 protein compared to normal tissues was obtained from GEPIA2.0 database, in which the threshold value considered as significant difference is |log2FoldChange|>1.0 and p value <0.01. The mRNA expression level of C3 and other critical genes compared from normal tissue (GTEx database) were also obtained from GEPIA 2.0 database.

Protein-protein interaction (PPI) network analysis

The PPI analysis of DEGs ($|\log_2 \text{FoldChange}|>1.5$ and p value <0.01) was analyzed using STRING website (https://www.string-db.org/), in which minimum required interaction score was set as 0.9 and cluster analysis parameter, number of k-mean, is set as 5. The interaction analysis was further using Cytoscape version 3.6.0.

Gene set enrichment analysis (GSEA)

The overlaps of DEGs with MigDB gene set was performed using GSEA website (http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp), selecting hallmark gene sets, KEGG gene sets, reactome gene sets and WikiPathways gene sets (FDR q-value < 0.05 considered as significant difference). GSEA profile was analyzed using GSEA software (GSEA v4.1.0) using Molecular Signatures Database (MSigDB, http://www.gsea-msigdb.org/gsea/msigdb/index.jsp). The parameters of GSEA was set as default. Number of permutations was set as 2000 and collapse to gene symbols was set as No_collapse. Normalized enrichment score (NES) more than 0 was considered as up-regulation, and NES less than 0

was considered as down-regulation. The NOM p-value <0.05 & FDR q-value <0.25 is considered as significant difference.

Immune cell infiltration analysis

The immune cell infiltration analysis was performed using TIMER 2.0 database (http://timer.cistrome.org/). Correlation between gene expression and immune infiltration was performed using Gene blocks with purity adjustment, which contains 21 types of immune cells. The outcome of immune cell infiltration with clinical and gene expression was performed using TIMER 2.0 outcome block: Z-score>0 & p<0.05 was considered as increased risk; Z-score <0 & p<0.05 was considered as decreased risk.

Statistical analysis

All the information obtained from database was analyzed. The OS (overall survival) and PFS (progression-free survival) were defined as clinical endpoints. The GSEA analysis and images were generated using Graphpad_prism version 5.0. p value < 0.05 is the cutoff value to identify whether significance.

Results

Expression level and genetic status of C3 in LGG

C3 is the critical component in complement system and plays an important role in immune response. We firstly examine the cop number of C3 genes in LGG using cBioportal website (Figure 1A). There are four types of genetic status identified in LGG, containing deep deletion, shallow deletion, diploid, gain and amplification. Among these genetic statuses in LGG, diploid and gain are the major status, implying the overexpression levels of C3. By analyzing the mutation frequency of C3 dependence on sub-types of LGG, mutation and amplification are major status, i.e. oligoastrocytoma, oligodendrogliomas and astrocytoma, which confirmed this genetic status is ordinary in LGG. By performing the analysis of mRNA expression level, we found that the C3 expression level in LGG is significantly higher than normal brain tissue, about 2 times, implying that overexpression of C3 may play an important role in LGG tumorigenesis.

Gene mutation always plays the critical role in genetic network regulation. For C3 protein, it always contains six sub-domains, i.e. A2M_N, A2M_N_2, A2M, A2M_comp, A2M_re and NTR. Among these domains, there are 13 sites identified as mutation, especially for S770R. This mutation in C3 can affect the FGFR2 IIIb C3 transfroming activity, causing aberrant receptor recycling and persistent FRS2-dependent signaling[23]. To further identify the role of C3 in various cancers, we found that C3 always displays higher expression level and may highly related with patient prognosis.

Effect of higher C3 expression on OS and PFS

Although the expression level of C3 in LGG tissue is significantly higher in normal tissue, the protein level is still unknown. Here, we utilized the Human Protein Atlas to study the protein level in LGG patient

tissues. As shown in Figure 2A, we can observed the expression level of C3 protein in LGG is significantly higher than normal nervous tissue, consistent with mRNA level. To further investigate the impact of overexpression C3 in LGG, we performed the Kaplan-Meier analysis utilizing GEPIA2 database. As shown in Figure 2B-C, the medium survival time (OS and PFS) of LGG patients with low expression of C3 is significantly better than higher expression level of C3 (logrank p value are 0.0031 and 0.0055, respectively). These results implied that overexpression of C3 protein in tumor tissue worsen the survival time of patients. If C3 protein levels can be inhibited by specific therapeutic methods, e.g. antibody or siRNA silencing, the overall survival of LGG patients may benefit from these intervention.

Hub genes of C3 overexpression in LGG

To investigate the molecular mechanism of C3 overexpression in LGG, we utilized the protein-protein interaction network to identify the hub genes. The differentially expressed genes (DEGs, |log2FoldChange|>1.5 & FDR q-value <0.01) were obtained from GEPIA2 website, compared to normal tissue (GTEx data). These DEGs was analyzed using STRING website and the interaction network was regenerated using CytoScape software, as shown in Figure 3. The top 10 hub genes are C3AR1, CDK1, ITGAM, UBE2C, CCNB1, THBS1, CXCL12, POMC, CCNB2 and ADCY4, respectively. The biological function of these hub gens are listed in Table 1, which always are related with regulation of cell cycle. To further analyze expression levels of these hub genes, we utilized the GEPIA2 database to obtain the expression level compared to normal tissue. As shown in Figure 3, we can find that C3AR1, CDK1, ITGAM, UBE2C, CCNB1, THBS1, CXCL12 and CCNB2 are significantly higher than normal tissue, while ADCY4 and POMC are lower than normal tissue.

Correlation between critical biomarkers and C3

By analyzing the transcriptome levels of GBM, Cameron W Brennan et al.[24] have identified several critical pathways to affect the tumorigenesis of glioblastoma, i.e. RTK pathway, PI3K pathway, MAPK pathway, p53 pathway, RB1 pathway and ChrMod regulation, respectively. Here, we utilized cBioportal database to analyze the correlation between critical genes of these pathways and C3 expression level. As shown in Figure 4, these results only displayed the correlation with significant difference. Among these genes, MET, FGFR3, RB1, IDH1, CDK6 and CDKN2C display the positively correlation with C3 expression level in LGG (Spearman co-efficiency >0 & p-value <0.05), meanwhile PI3KR1, PDFFRA, PTEN, ND1, BRAF and ATRX display the negatively correlation with C3 (Spearman co-efficiency <0 & p-value <0.05).

Gene set enrichment analysis of DEGs

To explore the potential molecular mechanism for LGG tumorigenesis, we firstly analyze the transcription factors dependence on genetic statuses. As shown in Figure 5A-B, we can find that the major transcription factor of up regulated DEGs are TP53 and E2F1, related with the proliferation pathway and implied that overexpression of C3 may promote the tumor growth. Meantime, the major transcription factors of down-regulated genes are NFKB1, RELA, STAT3 and SP3. NFKB1, RELA and STAT3 are attributed as the NFκB pathway-related factors, and SP3 also can strongly interact with NR1 NF-κB site.[25]

The major transcription factors of down-regulated genes can be attributed as NFκB-related pathway, implying down-regulation of these genes may owing to the inhibition of NFκB pathway.

Then, we also analyzed the impact of DEGs on pathways using GSEA database. As shown in Figure 5C, we can find that up-regulated DEGs are highly associated with Reactome_signaling_by_receptor_tyrosine_kinase, Hallmark_hypoxia, Reactome_neutrophil_degranulation and Hallmark_TNFA_signaling_via_NFKB pathways. Moreover, we also observed that the down-regulated DEGs are highly related with Hallmark_E2F_targets, Reactome_cell_cycle, Reactome_cell_cycle_mototic and Reactome_cell_cycle_checkpoints pathways, as shown in Figure 5D.

To further explore the impact of these DEGs, we analyzed the GSEA profile of DEGs using GSEA software with Reactome gene sets, which can identify the impact of DEGs in LGG, as shown in Figure 6. The top 5 up-regulated pathways are Reactome_cell_cycle, Reactome_cell_ccle_mitotic, Reactome_cell_cycle_checkpoints, Reactome_mitotic_metaphase_and_anaphase and Reactome_M_phase. These pathways are highly related with tumor growth and cell cycle regulation. Moreover, the top 5 down regulated pathways are Reactome_atimicrobial_peptides, Reactome_phase_I_functionalization_of_compounds, Reactome_disease_of_glycosylation, Reactome_diseases_of_metabolism and Reactome_disoders_of_transmembrane_transporters. These inhibited pathways are highly associated with metabolism, indicating overexpression of C3 may affect the energy metabolism of cancer cells. We also observed the inhibition of _TNFA_signaling_via_NFKB (NES=-1.555 with FDR q-value =0.216), which is consistent with the results of transcription factor analysis (Figure 5B). Among these pathways, regulation of cell cycle pathway plays the central role in LGG tumorigenesis.

Immune cell infiltration analysis

Tumor microenvironment always plays an critical roles in tumorigenesis and affects the prognosis of LGG patients[9]. To explore how overexpression of C3 gene affects the immune cell filtration in LGG, we further analyzed the correlation between immune cells and C3 expression level. We analyzed the correlation between C3 expression level and 20 types of immune cells using TIMER2.0 database. As shown in Figure 7, we can find that major types of immune cells are highly associated with C3 expression in LGG patients. Among these immune cell infiltration status, we found that the immune cell infiltration of neutrophil is positive correlation with C3 expression (Cor=0.808 & p value=1.26e-111), indicating that higher infiltration level of immune worsens the survival of LGG patients (Figure 7G-H).

To our knowledge, the biomarkers of neutrophil cells are MPO, CD11b, CD66b and CD16, respectively. To further analyze protein expression of neutrophil biomarkers in LGG patients, we utilized HPA database to analyze the expression levels of these biomarkers. As shown in Figure 8, IHC staining results clearly showed that the expression levels of these biomarkers are highly expressed in gliomas tissues, confirming the immune cell infiltration results.

Discussion

Complement system is the major immune response system in blood circulation system, involving in host innate immune response[26]. In recently investigation, the innate immune system may play an important role in tumorigenesis and proliferation, indirectly affecting the survival of cancer patients[27–28]. In complement system, C3 is the central role in activation of complement system by cleaving C3 molecules to C3a through C3 convertase complex[16].Binding of C3 molecules to targeted cell surface can further recruit the immunological cells to infiltrate tumor tissue, including neutrophils[29]. How C3 protein to affect the progression of LGG should be determined.

In this investigation, we firstly explored the C3 expression levels in various tumor tissue (Figure 1E), clearly demonstrated that the C3 expression level in LGG is significantly higher than normal tissue and also displays the significantly up-regulated status in most of cancers. Then, we analyzed the C3 copy number, frequency and mutation sites in LGG, which confirmed the overexpression of C3 in LGG.

To explore the impact of C3 on survival of LGG patients, the OS and PFS results (Figure 2B) corroborated the higher expression of C3 in LGG worsens the survival time, confirming the negative correlation between C3 expression and survival. Then, we examined the protein expression of C3 protein in normal tissue and tumor tissue. In the normal tissue, the C3 proteins mainly expressed around the vasculature. While, the C3 expression in gliomas tissue are highly expressed, not only endothelial cells (Figure 2A). These results confirmed that higher expression of C3 protein in tumor tissue is positively associated with tumorigenesis.

To explore the intrinsic molecular mechanism of C3 overexpression to promote tumor proliferation, we obtained the DEGs in LGG compared to normal tissues. The PPI analysis (Figure 3) clearly showed the top 10 hub genes, i.e. *C3AR1*, *CDK1*, *ITGAM*, *UBE2C*, *CCNB1*, *THBS1*, *CXCL12*, *POMC*, *CCNB2* and *ADCY4*. These genes are mainly related with cell cycle, implying that regulation of cell cycle may play the critical role in tumorigenesis. By analyzing the correlation between C3 gene and critical biomarkers (Figure 4), these results implied that the inhibition of PI3K and MAPK pathways, while the RTK, p53 and RB1 pathways are over-activated in LGG.

To explore the molecular mechanism in LGG tumorigenesis, we firstly analyzed the transcription factors of up-regulated and down-regulated genes. As shown in Figure 5, the transcription factors of up-regulated genes are TP53 and E2F series, demonstrating the activation of p53 pathway and regulation of cell cycle. Moreover, the transcription factors of down-regulated genes is *NFKB1*, *RELA*, *STAT3* and *SP* series. *NFKB1*, *RELA* and *STAT3* is highly associated with TNFα/NFκB pathway, implying the inhibition of TNFα/NFκB pathway. Then, the GSEA overlaps (Figure 5C-D) and profile analysis (Figure 6) clearly showed that cell cycle-related pathways are activated in LGG and confirmed the inhibition of TNFα/NFκB pathway.

Most of affected pathways are relative with immune response. For tumor microenvironment, it always participated into the regulation of tumor progression. The immune cell infiltration in tumor tissue is highly

related with tumor proliferation, drug resistance and epithelial-mesenchymal transition (EMT)[9]. Consequently, we analyzed the immune cell infiltration in LGG tissue. By analyzing the 21 types of immune cells in LGG tissue, we find that the neutrophil infiltration is highly associated with C3 expression (Figure 7). Higher neutrophil infiltration can worsen the survival of LGG patients, and inhibition of C3 expression may improve the survival of LGG patients. To validate the neutrophil infiltration in gliomas tissue, we analyzed the neutrophil biomarkers using HPA database. The IHC results clearly demonstrated that the biomarkers of neutrophil in LGG patients is highly expressed, consistent with neutrophil infiltration.

Conclusions

In summary, our study showed that the overexpression of C3 in LGG can worsen survival of patients. The overexpression of C3 in LGG can lead the over-activation of cell cycle-related pathways, highly associated with tumorigenesis. LGG patients may benefit from the improvement of neutrophil infiltration. These results indicated that LGG patients may benefit from the inhibition of C3 in LGG, and the results displayed C3 can be as the excellent therapeutic targets for LGG therapy.

Abbreviations

LGG
Low grade gliomas
GSEA
Gene set enrichment analysis
DEGs
Differentially expressed genes
IHC
Immunohistochemistry
HPA
Human protein atlas

Declarations

Acknowledge

Not applicable.

Funding

Funding was provided by Henan University for Young Teachers.

Availability of data and materials

All data and materials are available at TCGA, TIMER, GEPIA2.0 database.

Authors' contributions

Jiliang Yan together performed the analysis and generated all the figures in this manuscript; Kaiting Miao, Lijing Wang, Yuanyuan Ma participated into the preparation of manuscript and revised the manuscript; Haigang Wu designed and supervised this assays, writing this manuscript and further revision.

Competing interests

Not applicable

Ethics approval and consent to participate

Not applicable

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Tables

Table 1
Biological functions of top 10 hub genes in LGG

Gene name	Biological function	Gene name	Biological function
C3AR1	Binding of C3a by the encoded receptor activates chemotaxis, granule enzyme release, superoxide anion production, and bacterial opsonization	THBS1	an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions
CDK1	essential for G1/S and G2/M phase transitions of eukaryotic cell cycle	CXCL12	functions as the ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4, and plays a role in many diverse cellular functions, including embryogenesis, immune surveillance, inflammation response, tissue homeostasis, and tumor growth and metastasis
ITGAM	The alpha M beta 2 integrin is important in the adherence of neutrophils and monocytes to stimulated endothelium, and also in the phagocytosis of complement coated particles	POMC	exhibits antibacterial and antifungal activity
UBE2C	required for the destruction of mitotic cyclins and for cell cycle progression, and may be involved in cancer progression	CCNB2	essential components of the cell cycle regulatory machinery
CCNB1	necessary for proper control of the G2/M transition phase of the cell cycle	ADCY4	couple to olfactory receptors and that there may be multiple receptor-mediated mechanisms for the generation of cAMP signals

Table 2
Cox Proportional Hazard Model of LGG

	Coef	HR	Se(coef)	95%CI_l	95%CI_u	z	p value
T cell CD8+	5.672	290.667	2.00	5.769	14645.18	1.836	0.005
Age	0.011	1.011	0.01	0.988	1.035	0.949	0.343
C3 level	-0.280	0.756	0.067	0.663	0.862	-4.188	0.000

Figures

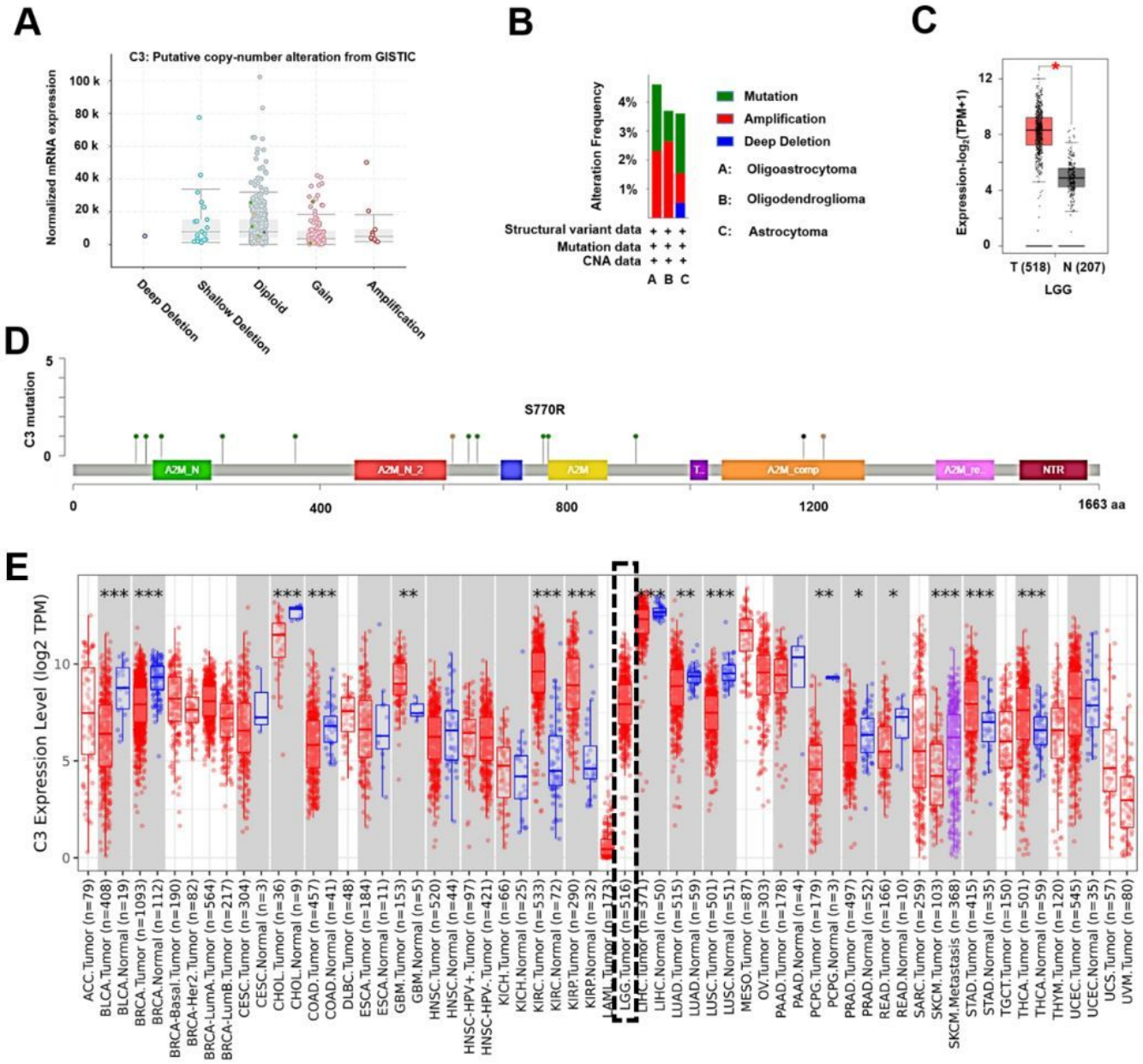

Figure 1

A. Copy number alterations of C3 genes in brain low grade gliomas. B. Mutation frequency of C3 genes dependence on subtypes of LGG. C. mRNA expression levels of C3 genes compared to normal tissue. D. Mutation sites of C3 genes in LGG. E. mRNA expression levels of C3 genes dependence on various cancer types.

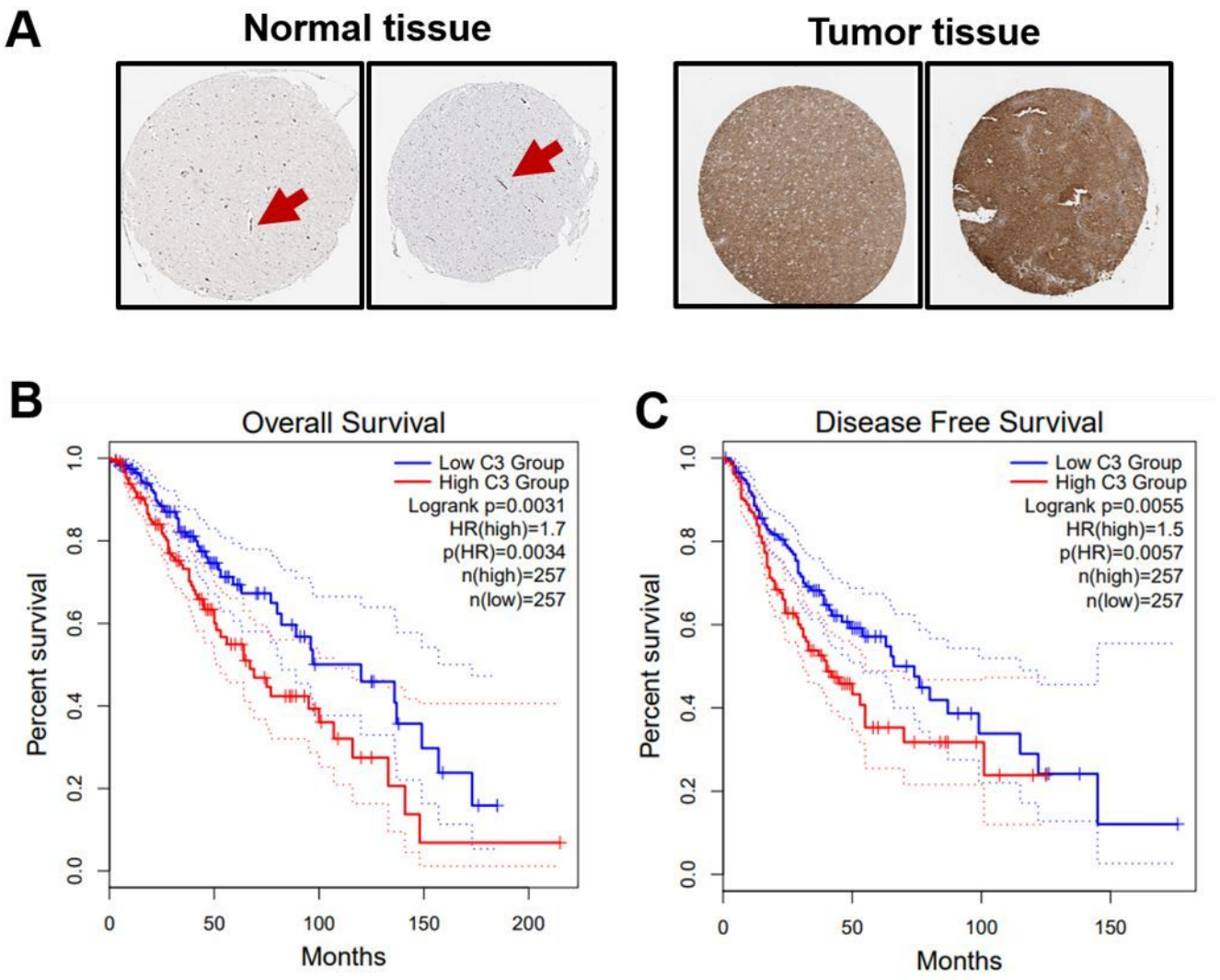

Figure 2

A. IHC levels of C3 protein in brain low grade gliomas compared to cortex tissue. Overall survival (B) and disease free survival (C) of LGG.

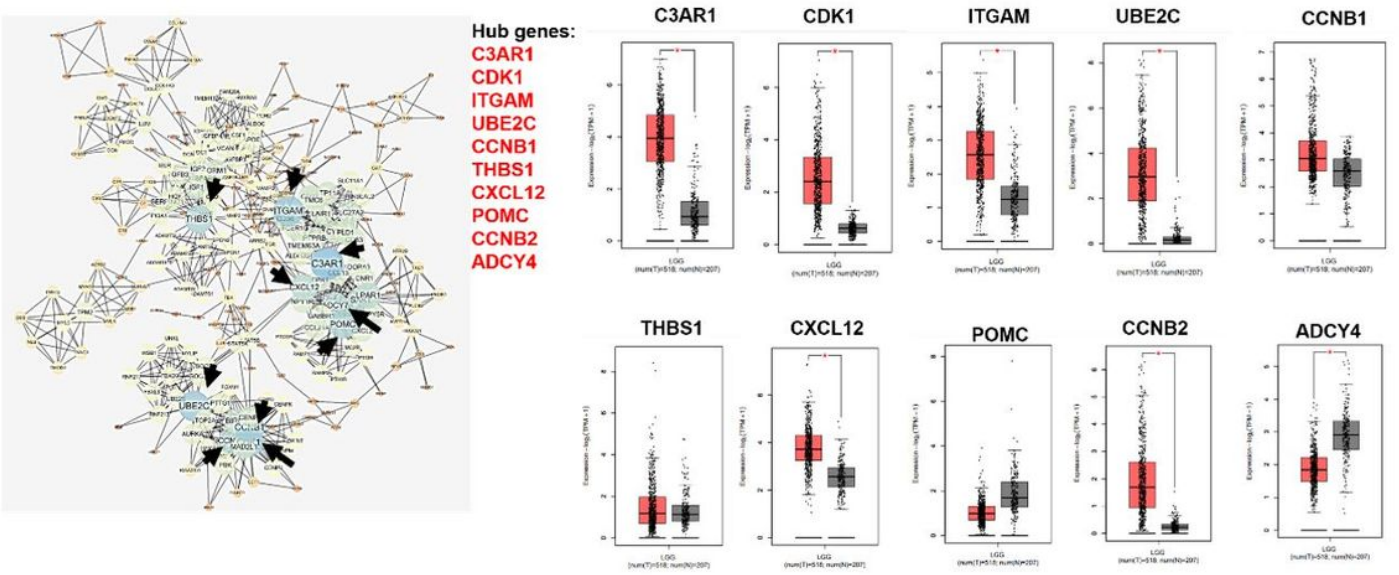

Figure 3

Protein-protein interaction network of DEGs and expression levels of top 10 hub genes in LGG.

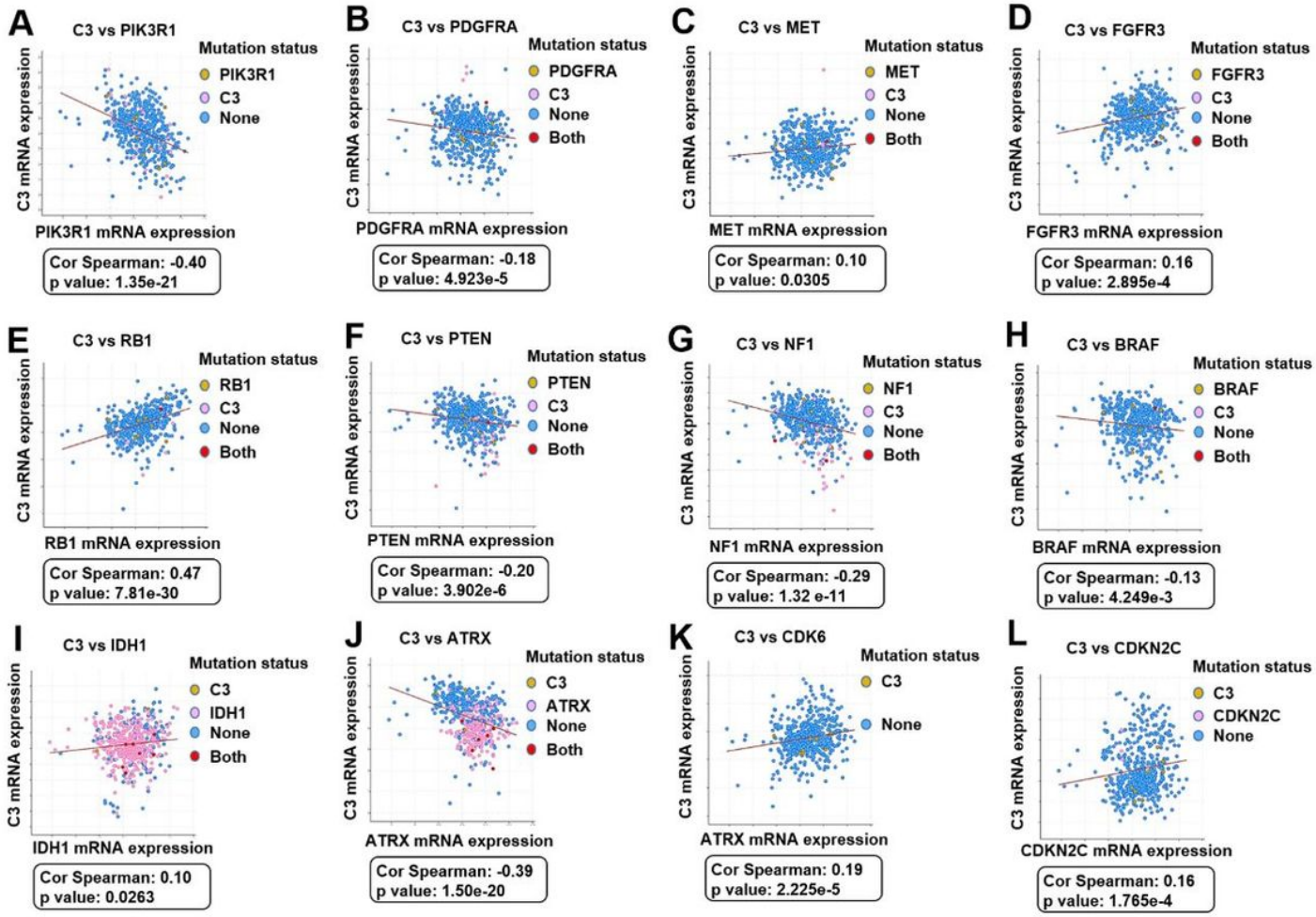

Figure 4

Correlation plot of critical biomarkers with C3 genes in LGG.

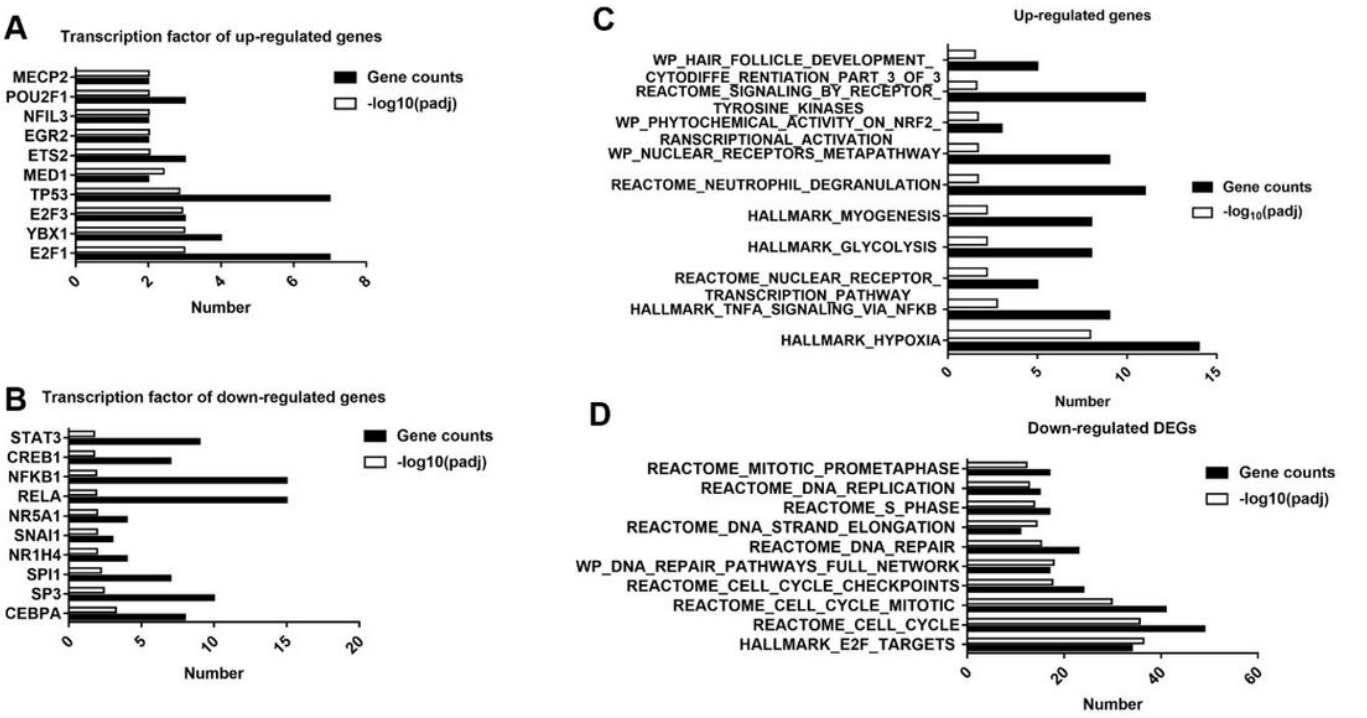

Figure 5

Transcription factors of up-regulated (A) and down-regulated (B) genes in LGG. Enriched annotation of up-regulated (A) and down-regulated (B) genes in LGG.

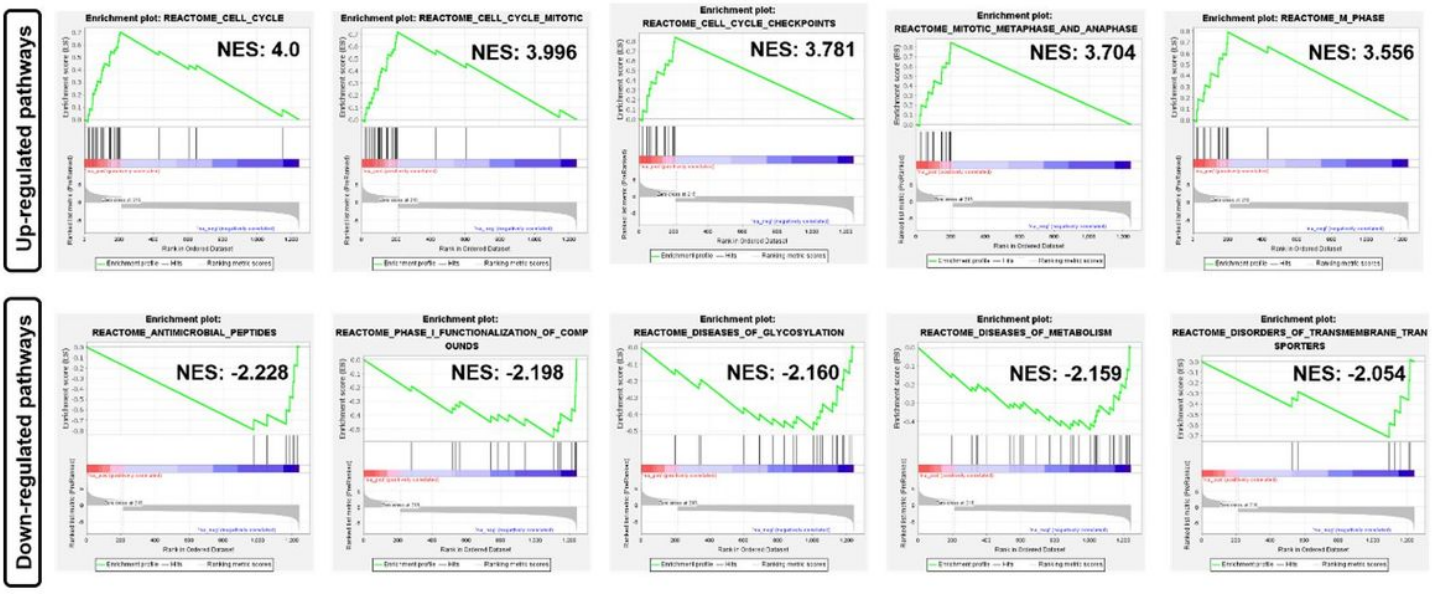

Figure 6

GSEA profile of up-regulated and down-regulated pathways in LGG.

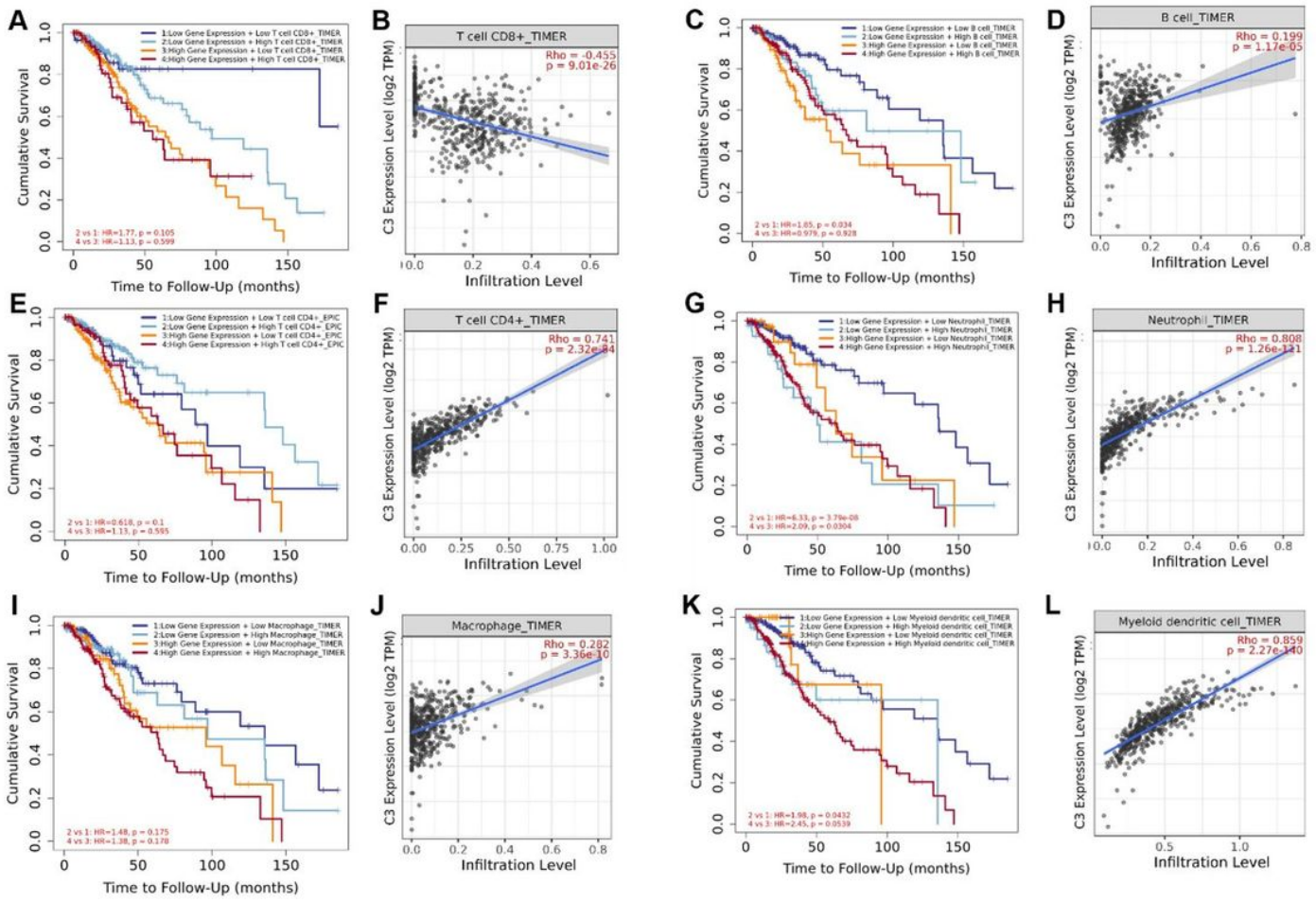

Figure 7

Prognostic values of C3 mRNA level and immune cell infiltration levels: A-B, T cell CD8+; C-D, B cell; E-F, T cell CD4+;G-H, neutrophil; I-J, macrophage; K-L, myeloid dendritic cell.

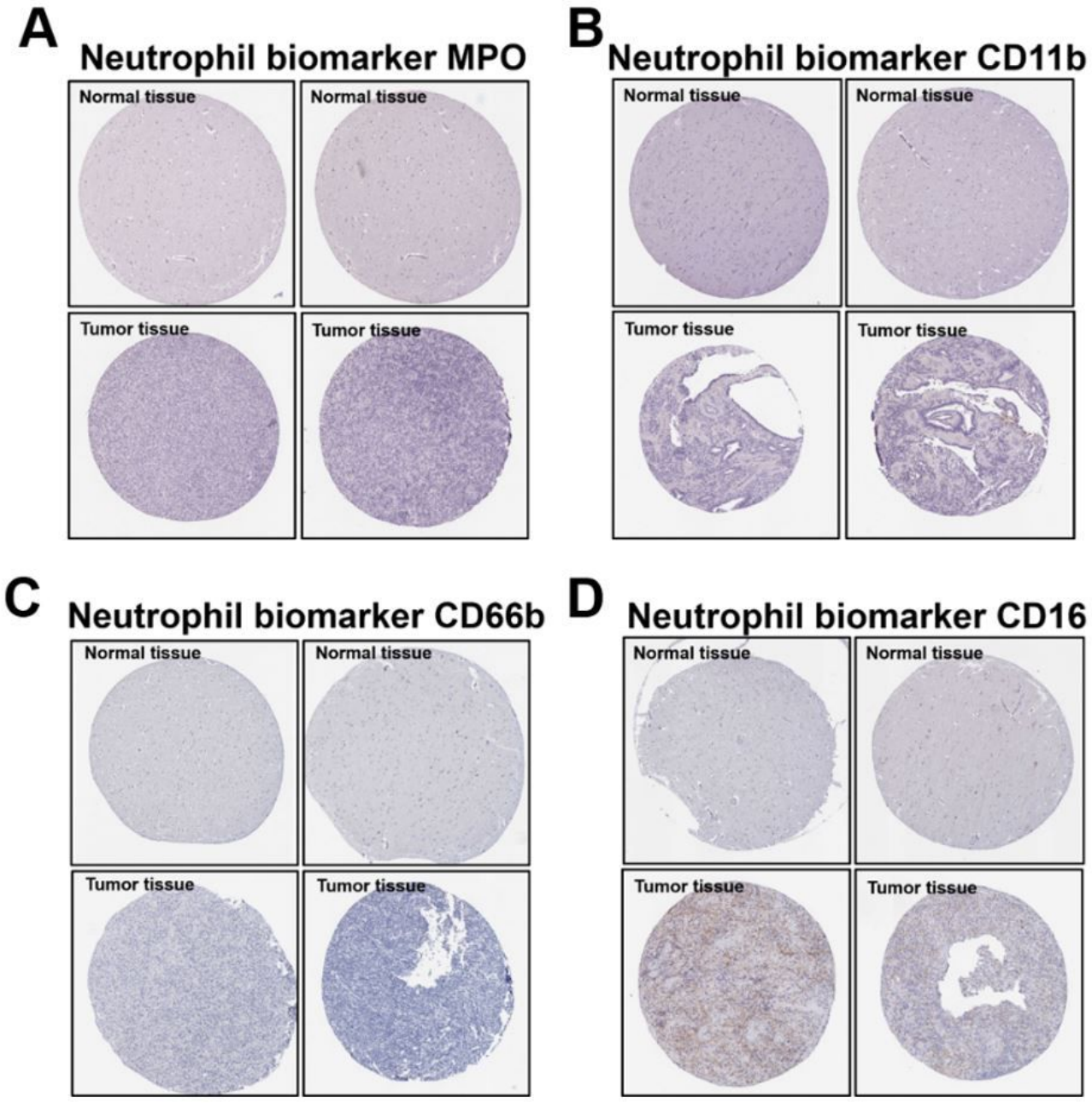

Figure 8

Expression levels of neutrophil biomarkers in LGG compared to normal cortex tissue: A, MPO; B, CD11b; C, D66b; D, neutrophil.