Int J Clin Exp Med 2019;12(11):12679-12692
www.ijcem.com /ISSN:1940-5901/IJCEM0098630

Original Article

Identification of key pathways and candidate genes in gliomas by bioinformatics analysis

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Received June 18, 2019; Accepted October 7, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: Gliomas account for a quarter of all primary brain and central nervous system tumors, and are always followed by a high mortality rate and very low life expectancy. However, genetic alterations nor molecular pathogenesis have not been clearly defined in gliomas. Herein, we applied a bioinformatics analysis to identify diagnostic biomarkers and reveal potential therapeutic targets for gliomas. In the present study, the microarray data set GSE31095 database was downloaded from the Gene Expression Omnibus (GEO), and a total of 244 DEGs were screened out from blood samples of human glioma patients, including 183 upregulated DEGs and 61 downregulated DEGs. Of which, *CX3CR1*, *GZMB*, and *GZMA* were the top three most up-regulated DEGs; *WFDC1*, *FKBP5*, and *IL1R2* were the top three most down-regulated DEGs. Additionally, GO and KEGG analysis revealed that 244 DEGs were mainly enriched in 11 terms and 10 pathways. *GZMB*, *CD48*, and *GZMA* were screened as the top 3 hub genes in protein-protein interaction networks. Survival analysis by UALCAN showed high expression of *CD48, GZMA, GZMH, IL2RB, KLRB1, LCK, LCP1, LEF1, NKG7, RPL18, TRAF3IP3* and *ZAP70* that presented a better overall survival. Through identifying these candidate genes and pathways by bioinformatics analysis, this study sheds light on the pathogenic and prognostic molecular mechanisms of gliomas and may help us understand the underlying mechanism of gliomas, furthermore, providing clear candidates for clinical application.

Keywords: Biological markers, central nervous system diseases, computational biology, glioma, prognosis

Introduction

Gliomas are the most common primary central nervous system (CNS) tumors and account for approximately 26.5% of all CNS tumors and 80.7% of malignant tumors [1]. It is estimated that the annual incidence rate of gliomas is 6.6 per 100,000 individuals in the United States [1]. According to morphology and malignant behavior, the World Health Organization (WHO) 2007 classification recognizes subtypes and four grades (I to IV) of gliomas [2]. The most common type of glioma in adults include glioblastoma (grade IV), astrocytic tumors (grade I to III), oligodendroglial tumors (grade II to III), and ependymomas (grade I to III) [3]. Among which, the aggressive forms of grade III and all grade IV gliomas are classified as high-grade gliomas. Grade IV gliomas are referred to as glioblastoma (GBM) that are highly invasive and have the poorest overall survival (OS), with less than 5 percent of patients surviving 5 years after diagnosis [4].

The current standard therapy of newly diagnosed gliomas is surgical resection. Concomitant adjuvant radiation therapy and specific chemotherapy protocols are always follow by operation, however, this protocol is far from optimal in combating disease progression [5]. With the updated classification of WHO 2016 CNS tumors, molecular parameters and histology are first to define the main tumor categories [6]. Biomarkers like isocitrate dehydrogenase 1/2 (IDH1/2) mutations, 1p/19q codeletion, *H3F3A* mutations and *C11orf95-RELA* fusions were integrant elements for diagnosis for gliomas [6, 7]. Other diagnostically relevant bio-

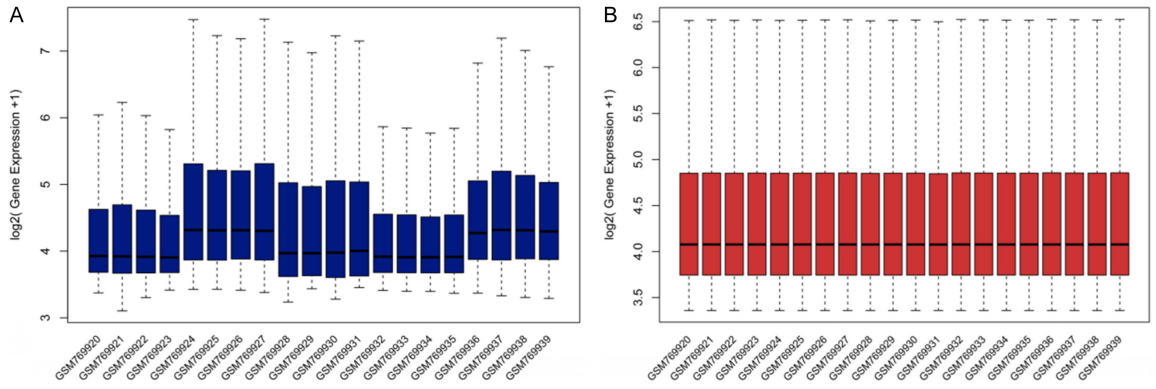
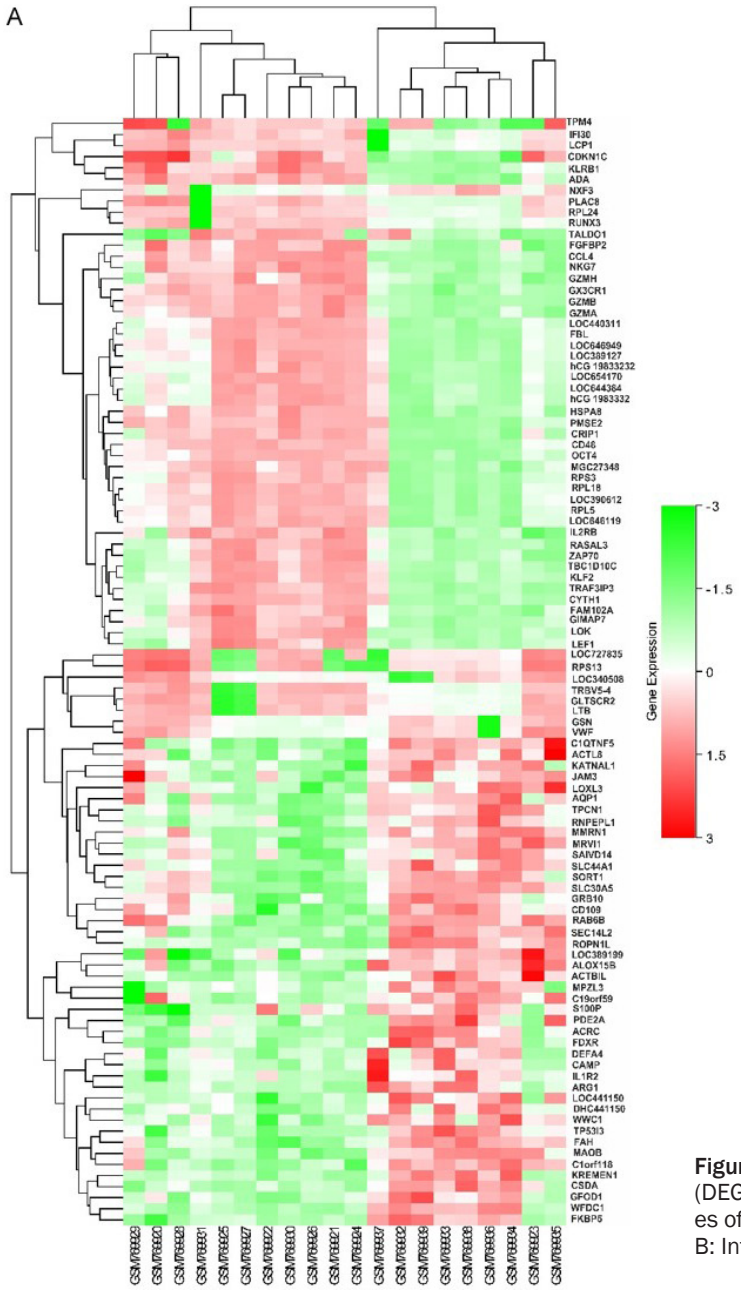


Figure 1. Normalization of GSE31095. A: Before standardization; B: After standardization.



GSM ID	Title
GSM769920	Control_s1
GSM769921	Control_s2
GSM769922	Control_s3
GSM769923	Control_s4
GSM769924	Control_s5
GSM769925	Control_s6
GSM769926	Control_s7
GSM769927	Control_s8
GSM769928	Control_s9
GSM769929	Control_s10
GSM769930	Control_s11
GSM769931	Control_s12
GSM769932	Glioma_s1
GSM769933	Glioma_s2
GSM769934	Glioma_s3
GSM769935	Glioma_s4
GSM769936	Glioma_s5
GSM769937	Glioma_s6
GSM769938	Glioma_s7
GSM769939	Glioma_s8

Figure 2. Differentially expressed genes (DEGs) in 8 samples of gliomas and 12 cases of normal controls. A: Heatmap of DEGs; B: Information of 20 samples.

Table 1. Top ten up-regulated and down-regulated differentially expressed genes between gliomas and normal controls

Gene	Log_2FC	Average expression	t	*P* value	Adj. *P* value	B	Regulated
CX3CR1	2.739132	9.151235	5.242678	*3.50E-05*	0.00734	2.412201	Up-Regulated
GZMB	2.736313	9.915847	5.911169	*7.55E-06*	0.003754	3.835439	Up-Regulated
GZMA	2.427199	8.95931	4.772093	*0.000106*	0.011385	1.383474	Up-Regulated
CDKN1C	2.221356	8.422803	7.80578	*1.31E-07*	0.000636	7.526122	Up-Regulated
FAM102A	2.158081	8.481098	3.970834	*0.000709*	0.026238	-0.39081	Up-Regulated
GZMH	2.151371	9.218487	3.730074	*0.001255*	0.032638	-0.92165	Up-Regulated
IFI30	2.126464	10.14779	5.511052	*2.24E-05*	0.006189	2.838447	Up-Regulated
KLRB1	2.075754	8.922116	6.793166	*1.08E-06*	0.001571	5.622102	Up-Regulated
GIMAP7	1.982532	8.761144	4.588515	*0.000163*	0.013749	0.97816	Up-Regulated
RASAL3	1.978633	9.454504	3.990905	*0.000676*	0.025634	-0.3464	Up-Regulated
WFDC1	-3.36578	7.340504	-7.86847	*1.16E-07*	0.000636	7.638628	Down-Regulated
FKBP5	-2.60349	7.317815	-7.1631	*4.92E-07*	0.001112	6.336517	Down-Regulated
IL1R2	-2.37176	6.803303	-3.59007	*0.001747*	0.039199	-1.22817	Down-Regulated
ACRC	-2.25525	7.72429	-4.93305	*7.23E-05*	0.010228	1.737259	Down-Regulated
FDXR	-2.10694	8.155901	-6.45534	*2.25E-06*	0.002075	4.951231	Down-Regulated
C1QTNF5	-1.97927	6.979173	-7.5887	*2.04E-07*	0.000658	7.131683	Down-Regulated
DEFA4	-1.88618	6.368344	-3.89246	*0.000854*	0.028042	-0.564	Down-Regulated
S100P	-1.8096	9.215449	-3.86421	*0.000913*	0.028956	-0.62636	Down-Regulated
NXF3	-1.76503	10.0047	-5.07796	*5.94E-05*	0.00948	1.936104	Down-Regulated
MPZL3	-1.75695	9.504724	-3.91132	*0.000816*	0.027621	-0.52236	Down-Regulated

markers are; loss of nuclear ATRX expression, *TERT*-promoter mutations, *BRAF*-V600E mutation, and more that were also recommended as the rare ones [6]. This revolutionary change in glioma diagnostics indicated that genetic approaches play a complementary role to traditional histomorphology, thus, improving the diagnosis of gliomas and guided pathogenesis-based treatment.

Gene microarray and high-throughput gene databases have been widely applied in the diagnosis and treatment of human diseases. Of note, some specific genes were screened out as predictive biomarker for malignant tumors. With the rapid spread of microarray techniques, pathogenesis of several diseases has been identified. Based on these databases, this supplied an efficacious approach for researchers, which can help to elucidate the occurrence and development of carcinoma.

In the present study, we successfully identified several differentially expressed genes (DEGs) by comparing the gene expression profiles between gliomas and healthy controls. With a series of bioinformatics analysis, including identification of DEGs, functional and pathway enrichment analysis, protein-protein interaction (PPI) network integration; hub-genes were screened, which may be outstanding predictors for diagnosis, targets for treatment, and prognosticators for prognosis of gliomas in the future.

Materials and methods

Microarray data

We searched the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (available online: *http://www.ncbi.nlm. nih.gov/geo*). The raw microarray data set GSE31095 database was downloaded and selected for further study. This data set was based on GPL4133 platform and included 8 blood samples from glioma patients and 12 blood samples from normal controls, respectively.

Screening for differential expressed genes (DEGs)

The downloaded gene package and series of matrix file(s) were converted for analysis by the

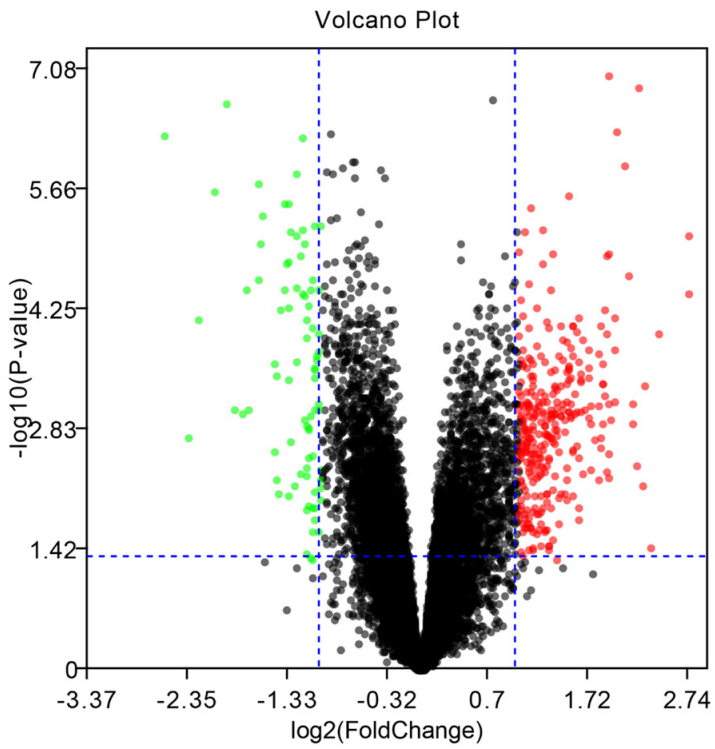


Figure 3. Volcano plot of all the DEGs. (Black points mean genes no significant difference. Red points mean upregulated genes screened on the basis of |fold change|>1.0 and a corrected *P*-value of <0.05. Green points mean downregulated genes screened on the basis of |fold change|>1.0 and a corrected *P*<0.05).

R language software and annotation package. Then, data were normalized by the normalize between array function from R package "LIMMA" (available online: http://www.bioconductor.org/). After normalization of data, the student's *t*-test was introduced to compare the difference between glioma group and control group. *P*-value <0.05 and |fold change (FC)|>1 were chosen as the thresholds for screening differential expressed genes (DEGs). Further validated procedures for the DEGs were performed using the online GEO2R analysis tool from the GEO database.

Gene ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of DEGs

The FunRich version 3.1.3 (available online: http://funrich.org/) is a foundational tool for function analysis of high-throughput gene analysis [8]. It is widely used for analysis, annotation, and displays the biological functional and pathway of genes. Gene ontology (GO) annotations were performed using FunRich on the screened DEGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs was also performed by using the FunRich. In the present study, we analyzed the DEGs that were significantly upregulated and downregulated in the GSE31095 gliomas data, and used *P*<0.05 as the threshold for statistical significance.

Protein-protein interaction (PPI) network integration

To analyze the connection among proteins, predicted target genes of the top 25 most upregulated and downregulated DEGs were uploaded to STRING database (available online: http://string-db.org/), and the results were visualized in Cytoscape 3.7.1 [9]. Furthermore, we identified and screened out the top 25 hub genes according to degree, after that DEGs networks were established by Cytoscape 3.7.1.

Kaplan-Meier survival analysis associated with target genes for patients with glioma

UALCAN is an interactive web resource for analyzing cancer transcriptome data. The top 25 hub genes were uploaded to UALCAN (available online: http://ualcan.path.uab.edu/). Effects of hub genes expression level on glioma patient survival were achieved, and visualized by Kaplan-Meier. The statistical test was two sided and *P*<0.01 was considered as statistically significant.

Results

Microarray data and Identification of DEGs

The downloaded microarray dataset GSE31095 from the Gene Expression Omnibus (GEO) online database included 8 cases of glioma (GSM769932-GSM769939) and 12 cases of normal controls (GSM769920-GSM769931). The data before and after normalization were presented in **Figure 1**. Then, the data were further processed by unpaired *t*-test (*P*<0.05, $|\log_2$

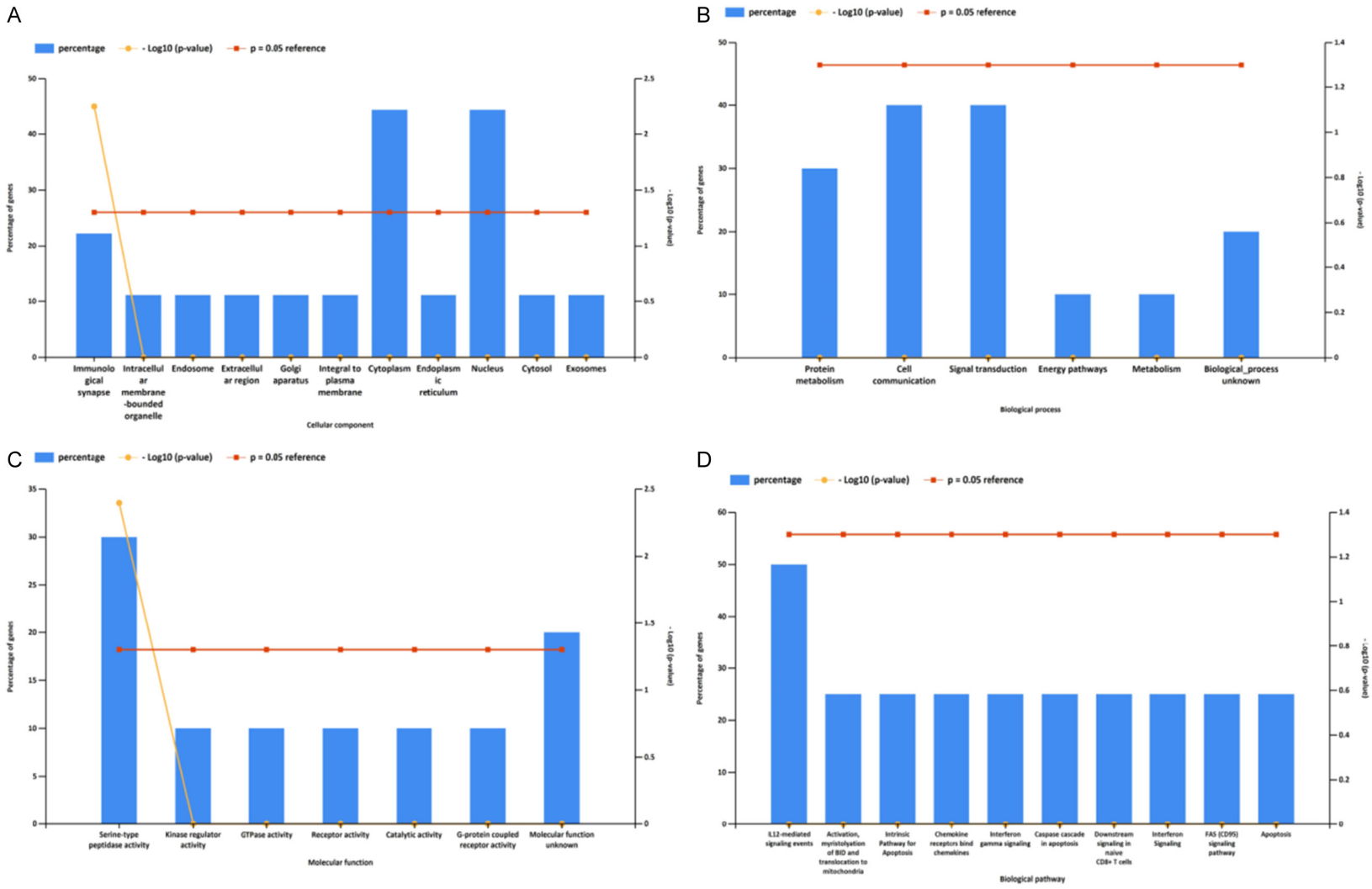


Figure 4. GO annotation and KEGG pathway enrichment analysis of top 10 most upregulated DEGs.

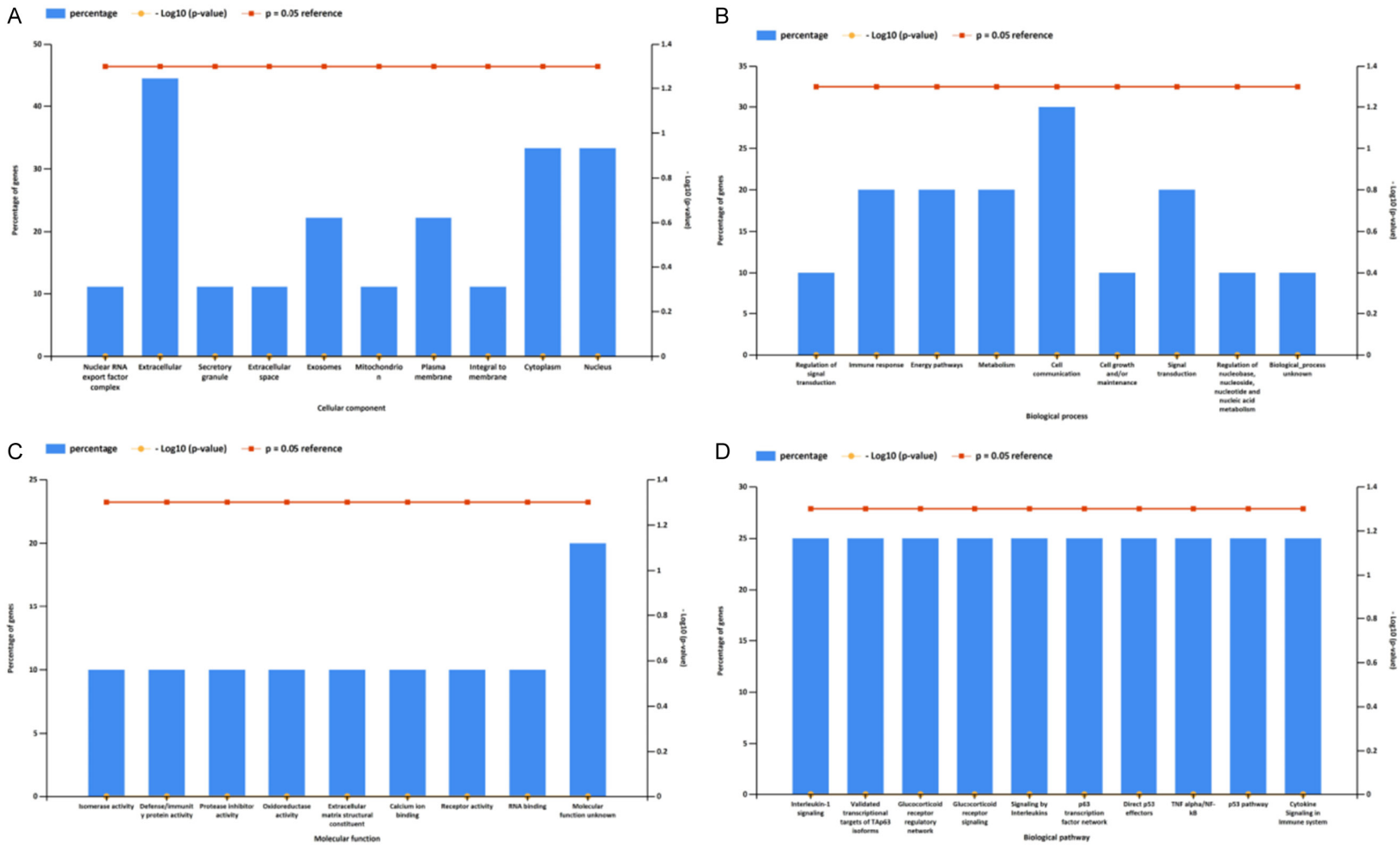


Figure 5. GO annotation and KEGG pathway enrichment analysis of top 10 most downregulated DEGs.

Table 2. Details of KEGG pathway information

KEGG pathway	No. of genes in the background dataset	Fold enrichment	*P*-value	Genes mapped
IL-12 mediated signaling events	111	28.401476	0.0018094	GZMB; GZMA
Activation, myristolyation of BID and translocation to mitochondria	5	316.22093	0.0031766	GZMB
Intrinsic pathway for apoptosis	31	51.088902	0.0195732	GZMB
Chemokine receptors bind chemokines	42	37.711661	0.026449	CX3CR1
Interferon gamma signaling	47	33.700635	0.0295623	IFI30
Caspase cascade in apoptosis	52	30.460812	0.0326682	GZMB
Downstream signaling in naive $CD8^+$ T cells	65	24.369587	0.0407087	GZMB
Interleukin-1 signaling	39	40.611814	0.0245774	IL1R2
Validated transcriptional targets of TAp63 isoforms	54	29.332843	0.0339085	FDXR
Glucocorticoid receptor regulatory network	80	19.800861	0.0499237	FKBP5

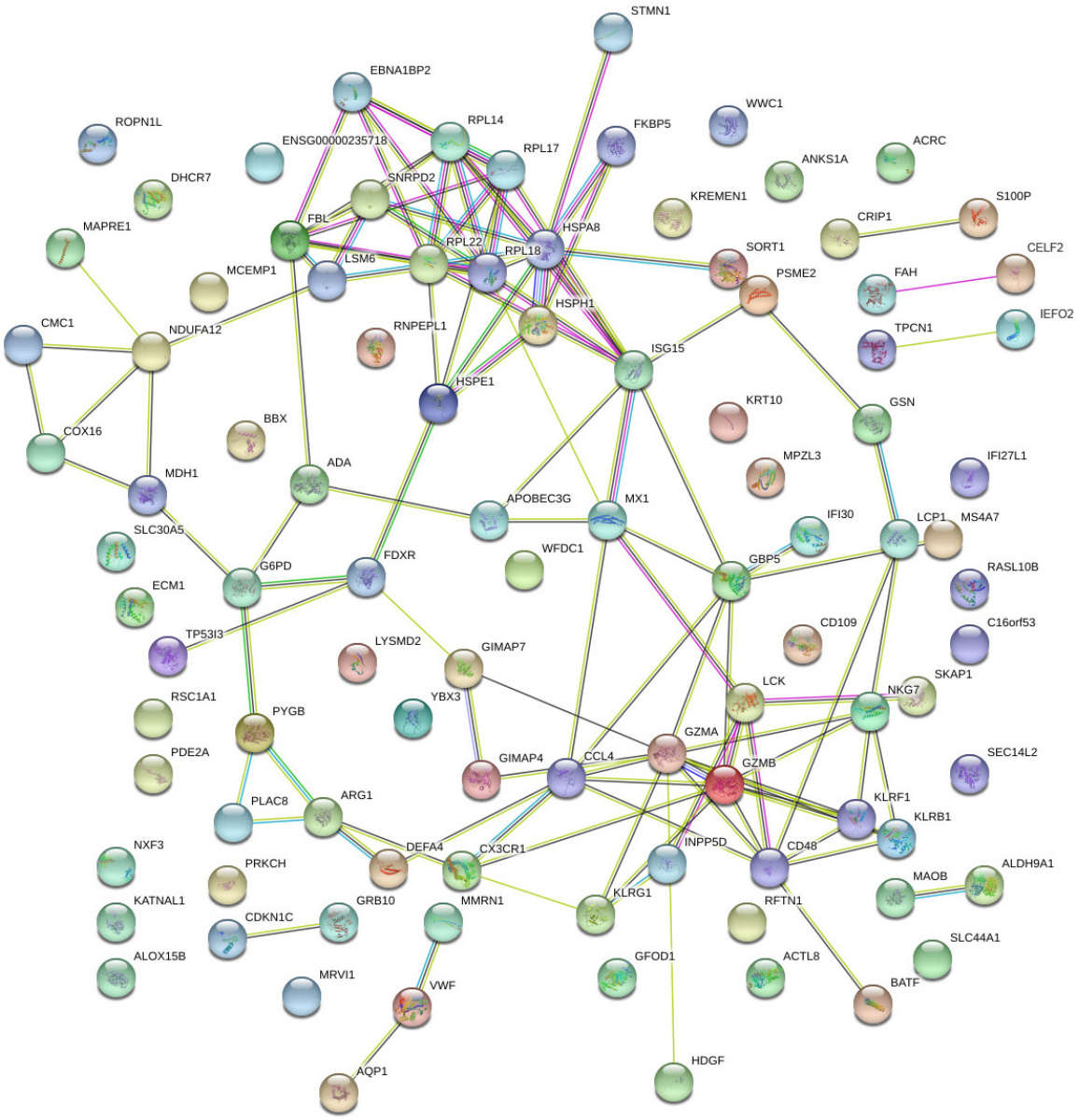


Figure 6. Protein-protein interaction (PPI) network of DEGs.

Table 3. Top 25 hub genes with higher degree of connectivity

Node name	Degree	Regulate
GZMB	11	Up
CD48	9	Up
GZMA	9	Up
ZAP70	9	Up
IL2RB	8	Up
CCL4	7	Up
KLRB1	6	Up
NKG7	6	Up
GZMH	5	Up
LCK	5	Up
RASAL3	4	Up
TRAF3IP3	3	Up
GIMAP7	3	Up
ARG1	3	Down
HSPA8	3	Up
CX3CR1	3	Up
DEFA4	3	Down
RPL5	3	Up
ADA	2	Up
LEF1	2	Up
LCP1	2	Up
FDXR	2	Down
KLF2	2	Up
CAMP	2	Down
RPL18	2	Up

FC|>1). In all, 244 DEGs were successfully screened out (see **Figure 2**). Among the 244 DEGs, 183 DEGs were significantly upregulated and 61 DEGs were downregulated. The top ten DEGs of most upregulated and downregulated were listed in **Table 1** for better visualization. According to fold change (FC), *CX3CR1*, *GZMB*, and *GZMA* were the top three most upregulated DEGs; *WFDC1*, *FKBP5*, and *IL1R2* were the top three most downregulated DEGs. **Figure 3** shows the volcano plot and the scatter plot of all the DEGs.

Enrichment analysis of gene ontology and KEGG pathway

For further analysis of the functions of these selected genes, we subsequently conducted GO functional annotation and KEGG pathway enrichment analysis. Three GO categories, including cellular component (CC), biological process (BP), and molecular function (MF) were selected in the functional annotation. The GO terms of the top 10 most upregulated DEGs were shown in **Figure 4A-C**, including immunological synapse, intracellular membrane-bounded organelle, endosome *et al.* in the CC category; serine-type peptidase activity, kinase regulator activity, GTPase activity *et al.* in the MF category; protein metabolism, cell communication, signal transduction, *et al.* in the BP category, and KEGG pathway analysis including IL12-mediated signaling events, activation, myristolyation of BID and translocation to mitochondria, *et al.* (**Figure 4D**). Subsequently, the top 10 most downregulated DEGs were analyzed the same way, the results are shown in **Figure 5A-D**. KEGG pathway information of both up regulated and downregulated DEGs were listed in **Table 2**.

Mapping of PPI network and DEGs-target network

Physiologically, proteins rarely function alone but function in networks. In this study, PPI networks were identified for potential target genes to identify the top ten most down-regulated and upregulated DEGs using the STRING database of known and predicted PPIs (**Figure 6**). After processing the data from STRING using Cystoscope software, we screened out the top 25 hub nodes according to degree (**Table 3**). The 10 nodes with highest degree were granzyme B (*GZMB*), cluster of differentiation 48 (*CD48*), granzyme A (*GZMA*), zeta-chain-associated protein kinase *70 (ZAP70),* interleukin-2 receptor subunit beta (*IL2RB*), c-c motif chemokine 4 (*CCL4*), killer cell lectin-like receptor subfamily B member 1 (*KLRB1*), natural killer cell granule protein 7 (*NKG7*), granzyme H (*GZMH*), tyrosine-protein kinase Lck; among which *GZMB* exerted the highest node degree (degree =11). For better visualization of interaction of these DEGs, we additionally constructed networks based on the top 25 DEGs in PPI network, as presented in **Figure 7**.

Survival analysis

Survival analysis of potential target genes showed that among 511 glioma patients, whom had high expression of *CD48, GZMA, GZMH, IL2RB, KLRB1, LCK, LCP1, LEF1, NKG7, RPL18, TRAF3IP3*, and *ZAP70,* had a better overall survival (P<0.01) (**Figure 8**).

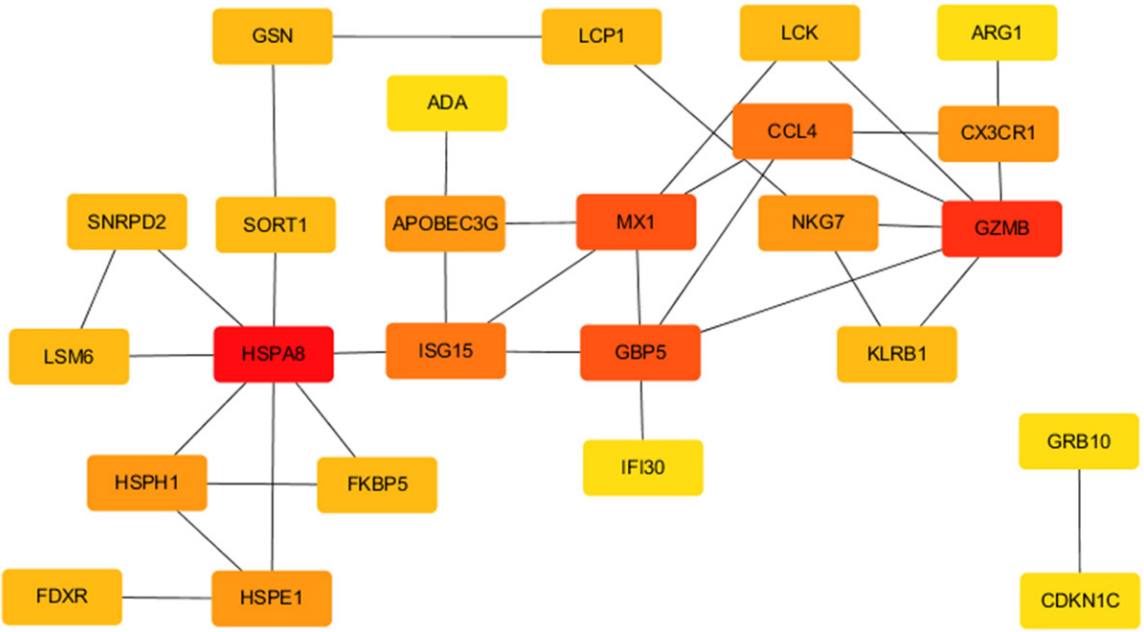


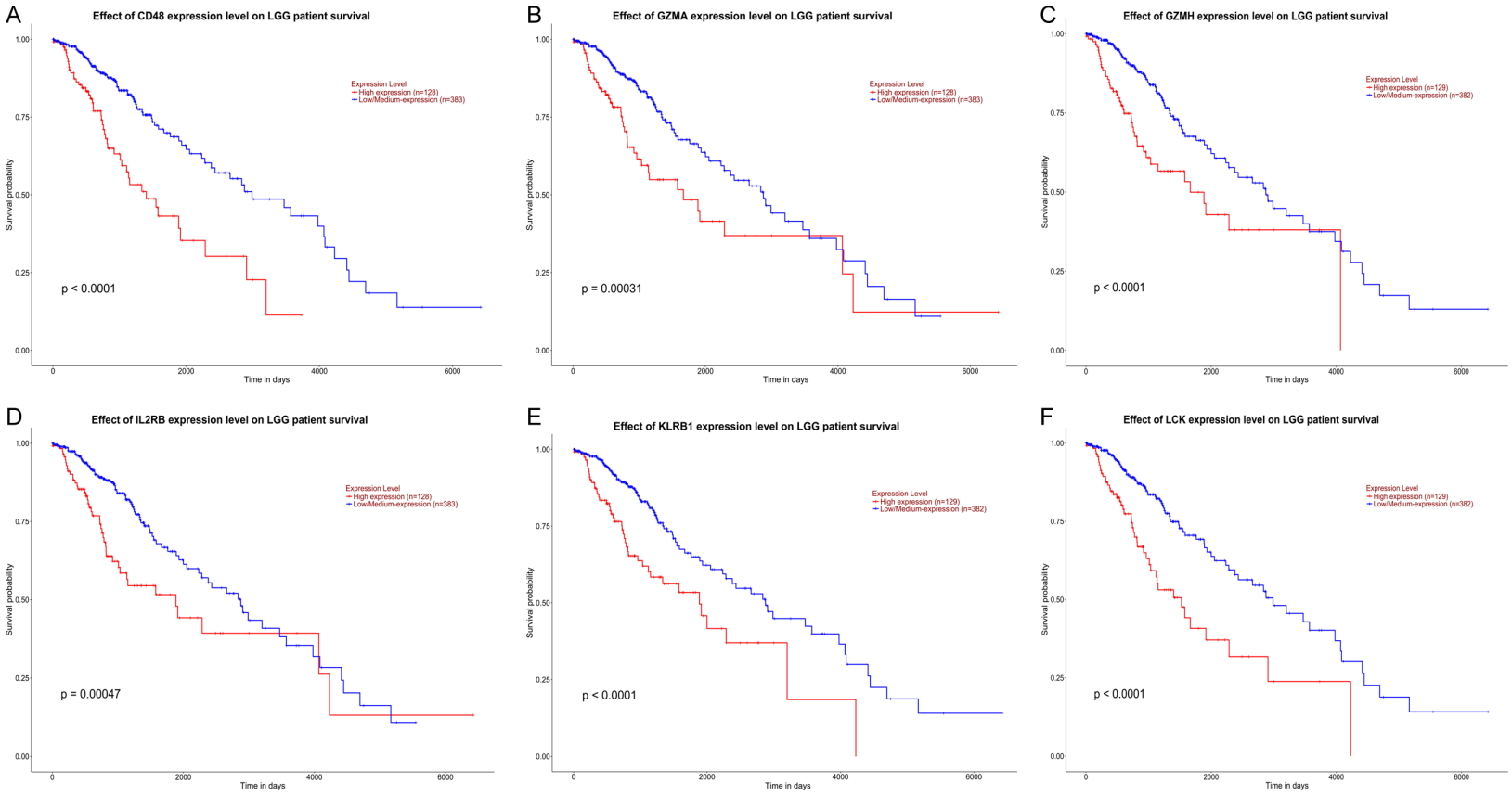
Figure 7. The top 25 hub genes with significant connectivity.

Discussion

Gliomas are highly malignant primary brain cancers with a dreadful overall survival and for which treatment options are limited. Therefore, prediagnosis is quite essential for the prognosis of patients. Recently, with the emergence of high-throughput sequencing technologies based on microarray analysis, several potential biomarkers of human carcinomas were screened out. This will help to determine clinical strategy in diagnosis, therapeutic options, and progression of diseases.

In this study, we screened out 266 DEGs in glioma samples compared to normal controls by performing a differential expression analysis on a gene array downloaded from GEO database. Among these DEGs, *CX3CR1* and *WFDC1* were shown to be the most upregulated and downregulated in gliomas. There have been some studies concerning the characteristics of *CX3CR1* in human carcinoma. In gastric cancer, *CX3CR1* appeared to be significant higher compared with control subjects, and it was linked to lymph node metastasis, higher clinical TNM stage and larger tumor size [10]. Meanwhile, overexpressed *CX3CR1* could promote gastric cancer cell migration, invasion, proliferation and survival *in vitro* [10]. Shen *et al.* reported that *CX3CR1* was overexpressed in human breast tumors and bone metastases, and when administering *CX3CR1* antagonists to breast cancer mice, dramatic reduction of tumors were found in both skeleton and visceral organs [11]. The presence of the *CX3CL1/CX3CR1* is a central underlying mechanism in the tumourigenesis process. It had been proven that human glioblastoma tumors and neural cancer stem cells express the chemokine *CX3CL1* and its receptor *CX3CR1* [12]. *WFDC1* (whey acidic protein four disulfide core 1) acts as a metastasis suppressor, belongs to a family of whey acidic protein (WAP) which often modulates cancer genes that encodes ps20 (20 kDa prostate stromal protein) [13]. Previous studies have shown that *WFDC1* expression was remarkably downregulated in highly prolific mesenchymal cells and in various cancers including fibrosarcomas and in tumors of the brain, bladder, prostate, ovary and lung [14, 15]. Expression of *WFDC1* in the prostate could induce apoptosis by regulation of *PTGS2/COX-2*, thus, restricting development and progression of neoplasms [16].

We further identified that biological pathways for DEGs were involved in the regulation of gliomas by bioinformatics analysis. Of note, granzyme B (*GZMB*), an apoptosis-inducing protease, participated the most in biological pathway regulation and presented the highest degree in


A
Effect of CD48 expression level on LGG patient survival
Expression Level
High expression (n=128)
Low/Medium-expression (n=383)
p < 0.0001
Survival probability
Time in days
B
Effect of GZMA expression level on LGG patient survival
Expression Level
High expression (n=128)
Low/Medium-expression (n=383)
p = 0.00031
Survival probability
Time in days
C
Effect of GZMH expression level on LGG patient survival
Expression Level
High expression (n=129)
Low/Medium-expression (n=382)
p < 0.0001
Survival probability
Time in days
D
Effect of IL2RB expression level on LGG patient survival
Expression Level
High expression (n=128)
Low/Medium-expression (n=383)
p = 0.00047
Survival probability
Time in days
E
Effect of KLRB1 expression level on LGG patient survival
Expression Level
High expression (n=129)
Low/Medium-expression (n=382)
p < 0.0001
Survival probability
Time in days
F
Effect of LCK expression level on LGG patient survival
Expression Level
High expression (n=129)
Low/Medium-expression (n=382)
p < 0.0001
Survival probability
Time in days

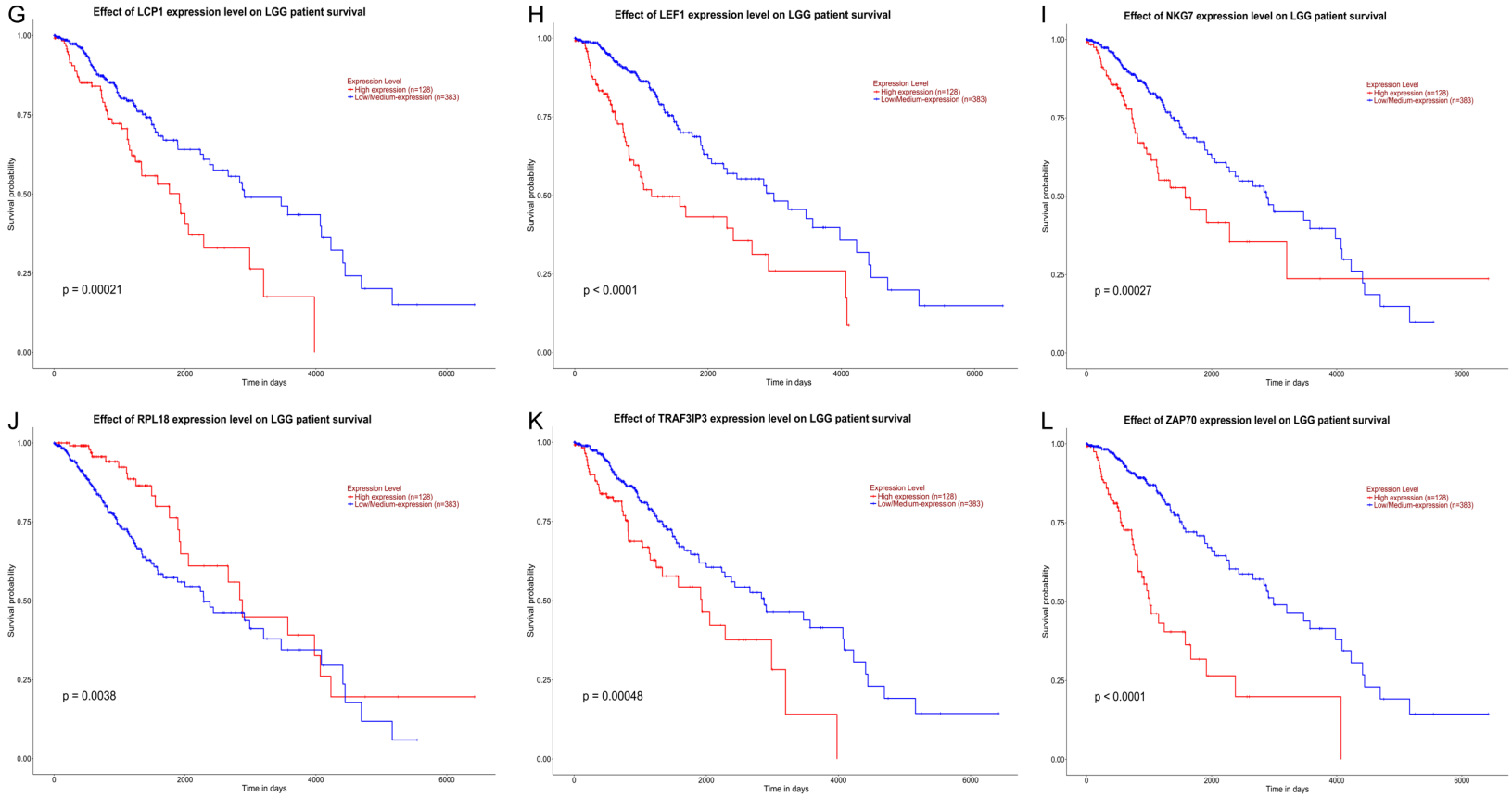


Figure 8. The survival prognostic value of top 25 hub genes in gliomas. Effort of A. CD48 (P<0.0001), B. GZMA (P=0.00031), C. GZMH (P<0.0001), D. IL2RB (P=0.00047), E. KLRB1 (P<0.0001), F. LCK (P<0.0001), G. LCP1 (P=0.00021), H. LEF1 (P<0.0001), I. NKG7 (P=0.00027), J. RPL18 (P=0.00038), K. TRAF3IP3 (P=0.00048), and L. ZAP70 (P<0.0001) expression on Brain lower grade glioma (LGG) patients survival based on UALCAN database showed a better overall survival.

PPI networks. Mice deficient in *GZMB* were protected from the development of some types of tumors, and this was related to impaired regulatory function of T or NK cells [17]. In the literature, another study provided evidence that through blocking NK-mediated target cell apoptosis, *GZMB* was involved in natural killer (NK)-mediated cell death in breast adenocarcinoma and melanoma tumor cells [18]. Lung cancer was associated with decreased expression of granzyme B, furthermore, genomic sequence analyses showed *GZMB* was decreased in atypical adenomatous hyperplasia (AAH, a premalignant lesion in the development of lung adenocarcinomas) relative to matched normal controls [19]. *GZMB* was released by $CD8^+$ T cells and natural killer cells during the cellular immune response, and mediated cancer cell death [20, 21]. By establishing a novel *GZMB*-targeted peptide and applying in non-invasive PET imaging assessment, Larimer *et al.* found that high-uptake tumors subsequently regressed, and low-uptake tumors progressed with immunotherapy in colon carcinoma mice models. Also, this result was demonstrated in melanoma patients by *GZMB* imaging probes [20]. A *COX* regression study examined 468 colorectal cancer patients indicating that *GZMB* was inversely correlated with stage of colorectal cancer patients at diagnosis, but associated with improved all-cause and cancer-specific survival [22]. As well, a small sample study on non-Hodgkin lymphoma also showed that less expression of *GZMB* transcripts meant a lower survival rate [23]. *GZMA* acted as a pro-inflammatory cytokine and participated in the apoptosis of abnormal cells that might contribute to cancer development. Like *GZMB*, it was high expressed in several human carcinomas. Survival analysis base on UALCAN showed that high expression of the *GZM* family, including *GZMA* and *GZMH* meant a better overall survival.

In KEGG pathway enrichment analysis, interleukin-12 (IL-12) mediated signaling events were most related among DEGs. IL-12 has been extensivly investigated in previous studies and was proved to be a potent inducer of antitumor immunity [24]. *In vitro*, IL-12-conditioned cellular media could improve the anti-tumor efficacy of $CD8^+$ T cells for about 10 to 100-fold in melanoma mouse models [25]. Furthermore, clinical trials of adoptive cell therapy in human melanoma cases found that after being modified with a tyrosinase-specific T-cell receptor, functional activity of $CD8^+$ T cells were enhanced when conditioned with IL-12, and this was indicated by heightened *GZMB* expression [25]. Other pathways in this study may indicate the way of future research of gliomas.

Conclusion

In summary, the present bioinformatics analysis identified 2 key DEGs (*CX3CR1* and *WFDC1*) from the GSE31095 database in glioma patients with respect to normal controls. Furthermore, *GZMB* was presented as the highest connective degree among hub genes of gliomas, which also participated in several tumor biological pathways. These preliminary findings may highlight promising strategies for studying the underlying mechanism of gliomas and provide a clear candidate for clinical application. However, it still needs further molecular and clinical experiments to validate these assumptions.

Acknowledgements

This work was supported by Fundamental Research Funds for the Central Universities under Grant xzy012019129; Xi'an Science and Technology program SF1510(4); Xi'an Science and Technology program under Grant J20170219II; and the Natural Science Foundation of Sichuan Province (2019YJ0067).

Disclosure of conflict of interest

None.

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