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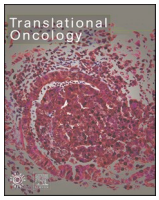

Original Research

Identification of LOX as a candidate prognostic biomarker in Glioblastoma multiforme

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ABSTRACT

Background: Glioblastoma multiforme (GBM) is the most malignant type of glioma. GBM tumors grow rapidly, have a high degree of malignancy, and are characterized by a fast disease progression. Unfortunately, there is a lack of effective treatments. An effective strategy for the treatment of GBM would be to identify key biomarkers correlating with the occurrence and progression of GBM and developing these biomarkers into therapeutic targets.

Method and Results: In this study, using integrated bioinformatics analysis, we identified differentially expressed genes (DEGs), including 130 genes that were upregulated in GBM compared to normal brain tissue, and 128 genes that were downregulated in GBM. Based on Gene Ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis, these genes were associated with regulation of tumor cell adhesion, differentiation, morphology in GBM and were mainly enriched in Complement and coagulation cascades pathway. The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to construct a Protein-Protein Interaction network. Ten hub genes were identified, including FN1, CD44, MYC, CDK1, SERPINE1, COL3A1, COL1A2, LOX, POSTN and EZH2, all of which were significantly upregulated in GBM, these results were confirmed by oncomine database exploration. Alteration analysis of hub genes found that patients with alteration in at least one of the hub genes showed shorter median survival times ($p = 0.013$) and shorter median disease-free survival times ($p = 2.488E\text{-}3$) than patients without alterations in any of the hub genes. Multiple tests for survival analysis showed that among individual hub genes only expression of LOX was correlated with patient survival ($P < 0.05$).GDS4467 data set was used to analyze the expression of LOX in gliomas with different degrees of malignancy, and it was found that the expression level of LOX was positively correlated with the malignant degree of gliomas.By analyzing GDS 4535 data set showed that the expression level of LOX was positively correlated with the differentiation degree of GBM cells

Conclusion: This research suggests that FN1, CD44, MYC, CDK1, SERPINE1, COL3A1, COL1A2, LOX, POSTN and EZH2 are key genes in GBM. However, only LOX is correlated with patient survival and promotes glioblastoma cell differentiation and tumor recurrence. LOX may be a candidate prognostic biomarker and potential therapeutic target for GBM.

Introduction

Glioblastoma multiforme (GBM), also known as glioblastoma, is one of the most malignant astrocytic tumors (WHO IV). GBM not only originates as a primary brain tumor but can also evolve from other gliomas. GBM is a common malignant neuroepithelial tumor of the central

Abbreviations: LOX, Lysyl oxidase; GBM, Glioblastoma multiforme; DEGs, differentially expressed genes; FN1, Fibronectin 1; CD44, CD44 molecule; MYC, V-myc myelocytomatosis viral oncogene homolog; CDK1, Cyclin-dependent kinase 1; SERPINE1, Serpin peptidase inhibitor, clade E, member 1; COL3A1, Collagen, type III, alpha 1; COL1A2, Collagen, type I, alpha 2; POSTN, Periostin osteoblast specific factor; EZH2, Enhancer of zeste homolog 2.

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nervous system in adults, accounting for 22.3% of neuroepithelial tumors. Despite multiple treatment options, including surgery, radiation therapy and chemotherapy, the overall survival time for patients with GBM is generally less than 2 years. [1,2] Therefore, it is critical to establish molecular targeted therapies for GBM, including the discovery of relevant biomarkers. [3]

With the development of next-generation sequencing technologies, many characteristic genes have been identified that allow a better understanding of the mechanism of the occurrence and development of GBM. [4] In recent studies, several potential diagnostic and prognostic biomarkers of GBM have been found. MGMT (O6-methylguanine DNA methyltransferase), IDH (isocitrate dehydrogenase), EGFR (epidermal growth factor receptor), and PTEN (phosphatase and tensin homolog) have been used in routine examination of GBM patients in the clinic. [5,6] More and more potential targets have been identified, such as COL8A2(Collagen type VIII alpha 2 chain), [7] PSMB8(Proteasome subunit beta type-8) [8] and IKBIP(I kappa B kinase interacting protein) [9]. Considering that the development of GBM is a complex process and that prognosis is poor, there is an urgent need for novel and sensitive molecular biomarkers and new therapeutic strategies.

In this study, we identified differentially expressed genes (DEGs) in GBM compared with normal brain tissues. Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis of these genes were performed. Subsequently, hub genes were identified from Protein-Protein Interaction (PPI) networks as key genes in GBM and were validated analysis by the oncomine database. Alteration analysis and survival analysis of these hub genes to identify the potential prognostic biomarkers. This study may help in recognizing the occurrence and progression mechanism of GBM.

Materials and methods

Data sources and data differential gene expression analysis

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was used to search for the expression level of mRNA in GBM and normal brain samples. Finally, we screened out two data sets, GSE4290 and GSE15824. GSE4290 contains 77 GBM samples and 23 normal samples, GSE15824 contains 12 GBM samples and 2 normal samples, all samples were of human origin. We used the GEO2R tool [10] to analyze the DEGs of GSE4290 and GSE15824, adjusted p value < 0.05 and $|\log FC| \geq 2.0$ were considered to be DEGs of GSE4290 and of GSE15824. A Venn diagram webtool (http://bioinformatics.psb.ugent.be/ webtools/Venn/) [11] was used to analyze the intersections of DEGs from both sources.

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

GO enrichment analysis was used to analyze the cellular component (CC), biological process (BP) and molecular function (MF) of DEGs. Pathways of DEGs were identified by KEGG pathway analysis. These analyses could help to better understand the metabolic pathways of GBM. The information of GO functional annotation and KEGG pathway analysis of DEGs in this study was performed by the “cluster Profiler” package in R. [12] p adjust <0.01 and gene counts ≥ 10 were considered to be a significant enrichment.

Construction of protein-protein interaction (PPI) network and identification of hub genes

The Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/) [13] was used to get the PPI information. The potential PPI relationship was obtained by searching the previously identified DEGs in the STRING database. Subsequently, we used Cytoscape software to construct the PPI network. [14] Nodes and edges are important parts of the entire PPI network. CytoHubba is a plugin of the Cytoscape software that was used to calculate the connectivity degree of each protein node. [15] In this study, a total of 10 hub genes were identified (connectivity degree$\geq$15).

Validation analysis

The oncomine database (https://www.oncomine.org/resource/login.html) was used to validate expression of the hub genes identified in our study. The oncomine platform was designed by doctors, scientists and software engineers at the University of Michigan. [16] It has powerful analytical capabilities, provides computational gene expression signatures, clustering and gene set modules, and automatically extracts insights from data.The screening conditions were set as differential analysis: cancer vs normal analysis, cancer type: glioblastoma, data type: mRNA, P value<0.01 and fold change>2, gene rank: Top 10%.

Alteration analysis of hub genes and effect on patient survival

cBioPortal (https://www.cbioportal. org) [17,18] was used for alteration analysis of hub genes and how this affects patient survival. The cBioPortal for Cancer Genomics provides visualization, analysis and the ability to download large-scale cancer genomics data sets, such as the data from the TCGA database. Selected was: CNS/ Brain, Glioblastoma: Glioblastoma multiforme (TCGA, Provision), genomic profiles with Mutations and mRNA expression z-scores (RNA Seq V2 RSEM), selected patient/case set:samples with mRNA data (RNA Seq V2) (166).

Survival analysis

The "TCGAbiolinks" package in R was used to download and analyze clinical data and gene expression data from the TCGA database (https://portal.gdc.cancer.gov/), [19] gene expression data type: “Gene Expression Quantification”, experimental strategy: “RNA-Seq”, workflow type: "HTSeq-FPKM" A total of 174 sample files were downloaded, of which 156 were from primary GBM patients. Patients were divided into two groups according to the median expression of each hub gene, and Kaplan-Meier survival analysis (Log rank test) was done using GraphPad Prism 7.0 software [20,21]. $P < 0.05$ was considered a statistically significant association with survival time. The results of Survival analysis were validated by GEPIA (http://gepia.cancer-pku.cn/) [22] and LinkedOmics (www.linkedomics. org). [23] GEPIA is a newly developed interactive web server for analyzing the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline. [22] The LinkedOmics database contains multi-omics data and clinical data for 32 cancer types and a total of 11,158 patients from The Cancer Genome Atlas (TCGA) project. It is also the first multi-omics database that integrates mass spectrometry (MS)-based global proteomics data generated by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) on selected TCGA tumor samples. [23] Both GEPIA and LinkedOmics provide patient survival analysis.

Analysis of target genes

The GEO database GDS4467 was downloaded to analyze the expression levels of target genes in gliomas of different malignant degrees, GDS4467 contains data on normal tissue, astrocytoma, primary glioblastoma multiforme, and secondary glioblastoma multiforme. The GDS4535 dataset was downloaded to analyze the expression levels of target genes in glioblastoma multiforme cells with different differentiation levels, GDS4535 belonged to a culture analysis of precursor cells and 4-day-differentiated glioblastoma cells (GICs) from surgical specimens.

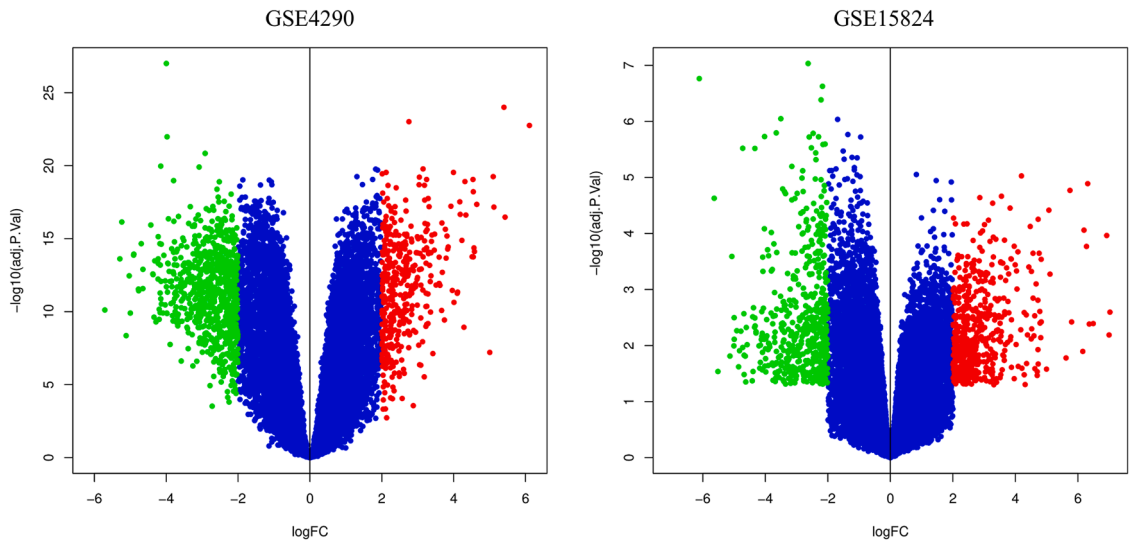


Fig. 1. Volcano plot of DEGs in GSE4290 and GSE15824, red plots represented upregulated genes with logFC≥2.0, adjusted P value < 0.05, green plots represented downregulated genes with logFC ≤−2.0, adjusted P value < 0.05,blue plots represented the rest of the genes with no significant expression change.

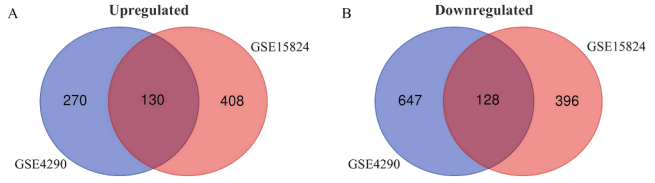


Fig. 2. The intersection of two GEO datasets, A: Genes that are upregulated in GBM relative to normal brain tissue, B: Genes that are downregulated in GBM relative to normal brain tissue.

Results

Differential expressed genes (DEGs) in GBM compared to normal brain samples

Using as criteria an adjusted P value < 0.05 and a |logFC| ≥2.0, 400 upregulated genes and 775 downregulated genes were identified in the GSE4290 of the Geo database. Based on the same criteria, 538 upregulated genes and 524 down-regulated genes were identified in GSE1582, shown as Figs. 1. The Venn diagram webtool was used to intersect the DEGs derived from these two different sources. As shown in Figs. 2A and 2B, we found a total of 130 genes that were upregulated in GBM relative to normal brain samples in both datasets and 128 genes that were downregulated in GBM relative to normal samples in both datasets.

Functional and pathway enrichment analysis of DEGs

The results of GO function annotation analysis and KEGG pathway enrichment analysis of DEGs are shown in Table 1 and Fig. 3. Criteria were p adjust< 0.01 and gene count ≥ 10. Cellular components (CC) that were significantly enriched were related to the extracellular matrix and membrane structures. Among the most affected CC were the extracellular matrix (p adjust = 1.22E-11; gene count: 31) and the proteinaceous extracellular matrix (p adjust = 1.76E-10; gene count: 26). The biological processes (BP) of DEGs that were most enriched were extracellular structure organization (p adjust = 3.53E-06; gene count: 23), extracellular matrix organization (p adjust = 3.63E-06; gene count: 21), cell-substrate adhesion (p adjust = 2.51E-05; gene count: 19) and regulation of cell morphogenesis (p adjust = 1.16E-04; gene count: 14). The Molecular Function (MF) that was most enriched was cell adhesion molecule binding (p adjust = 2.77E-03; gene count: 19). KEGG pathway analysis of DEGs revealed an enrichment in complement and coagulation cascades (p adjust = 2.03E-05; gene count: 10).

Table 1
The results of functional and pathway enrichment analysis (p adjust <0.01 and gene count≥10).

Category	ID	Description	p adjust	Count
BP	GO:0,043,062	extracellular structure organization	3.53E-06	23
BP	GO:0,030,198	extracellular matrix organization	3.63E-06	21
BP	GO:0,031,589	cell-substrate adhesion	2.51E-05	19
BP	GO:0,010,810	regulation of cell-substrate adhesion	0.000116	14
BP	GO:0,022,604	regulation of cell morphogenesis	0.000329	21
BP	GO:0,010,769	regulation of cell morphogenesis involved in differentiation	0.00104	15
BP	GO:0,010,976	positive regulation of neuron projection development	0.002328	14
BP	GO:0,050,769	positive regulation of neurogenesis	0.00326	18
BP	GO:0,001,655	urogenital system development	0.003922	15
BP	GO:0,007,160	cell-matrix adhesion	0.004246	12
BP	GO:0,010,975	regulation of neuron projection development	0.004518	18
CC	GO:0,031,012	extracellular matrix	1.22E-11	31
CC	GO:0,005,578	proteinaceous extracellular matrix	1.76E-10	26
CC	GO:0,044,420	extracellular matrix component	2.24E-09	15
CC	GO:0,005,604	basement membrane	8.99E-09	13
CC	GO:0,005,581	collagen trimer	9.46E-06	10
CC	GO:0,045,211	postsynaptic membrane	0.002135	12
CC	GO:0,005,788	endoplasmic reticulum lumen	0.0081	12
MF	GO:0,050,839	cell adhesion molecule binding	0.002766	19
MF	GO:0,005,539	glycosaminoglycan binding	0.002766	12
KEGG	hsa04610	Complement and coagulation cascades	2.03E-05	10

PPI network construction and hub gene identification

We used our analysis of DEGs in GBM to construct a PPI network. The network was found to consist of 125 nodes and 412 edges, as shown in

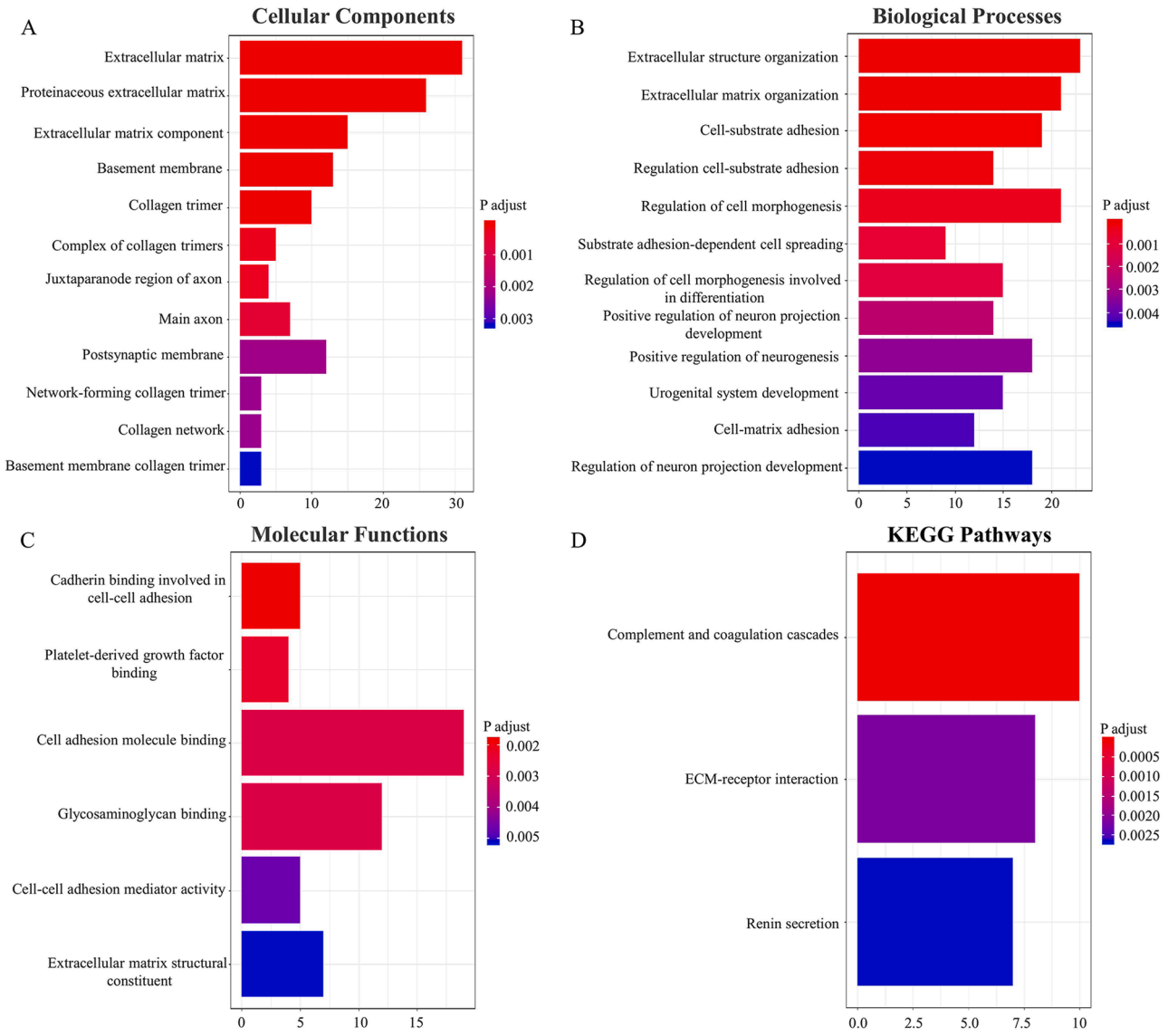


Fig. 3. Results of GO enrichment analysis and KEGG pathway analysis (p adjust <0.01). A: The main Cellular Components of the DEGs. B: The main Biological Processes of the DEGs. C: The main Molecular Functions of the DEGs. D: The main KEGG pathways of the DEGs.

Fig. 4. 10 genes with the highest (≥15) connectivity degrees in the PPI network were identified as hub genes. According to Table 2, FN1 displayed the highest connectivity degree (degree=40), followed by CD44 (degree=32), MYC (degree=23), CDK1 (degree=21), SERPINE1 (degree=18), COL3A1 (degree=18), COL1A2 (degree=17), LOX (degree=17), POSTN (degree=16), EZH2 (degree=15). These 10 hub genes were all upregulated.

Validation analysis of all hub genes

The 10 hub genes that were identified in our PPI network were submitted to the oncomine database. The screening conditions were set as differential analysis: cancer vs normal analysis, cancer type: glioblastoma, data type: mRNA, P value<0.01 and fold change>2, gene rank: Top 10%. Based on the oncomine database, the 10 hub genes were upregulated in most studies on CNS tumors (Fig. 5). All of these genes were upregulated in GBM(Table 3). [24–29] Fig. 6 illustrates the most significant upregulated data for each of the 10 hub genes that were identified in Table 3.

Analysis of genetic alterations in hub genes and association with patient overall survival and disease-free survival

A group of 160 patients with GBM were analyzed for mutations in the 10 hub genes. According to the cBioPortal, genetic alterations were found in all 10 hub genes (Fig. 7). 69 patients had an alteration in one or more hub gene(s). In contrast,91 patients did not have alterations in any of the hub genes. We compared the group of patients that had at least one genetic alteration in a hub gene to the group that had no alterations in any of the hub genes. Fig. 8A shows, that patients without alterations in any of the hub genes had a median survival of 14.72 months, while patients with at least one alteration had a median survival of 12.65 months. This difference is statistically significant (Log-rank test; p = 0.039). Likewise, disease/ progression-free survival was longer for the alteration-free group (median = 8.61 months) compared to the group with one or more alterations (median = 4.96 months) (Fig. 8B). This difference is also statistically significant (Log-rank test; p = 2.488E-3) (Fig. 9).

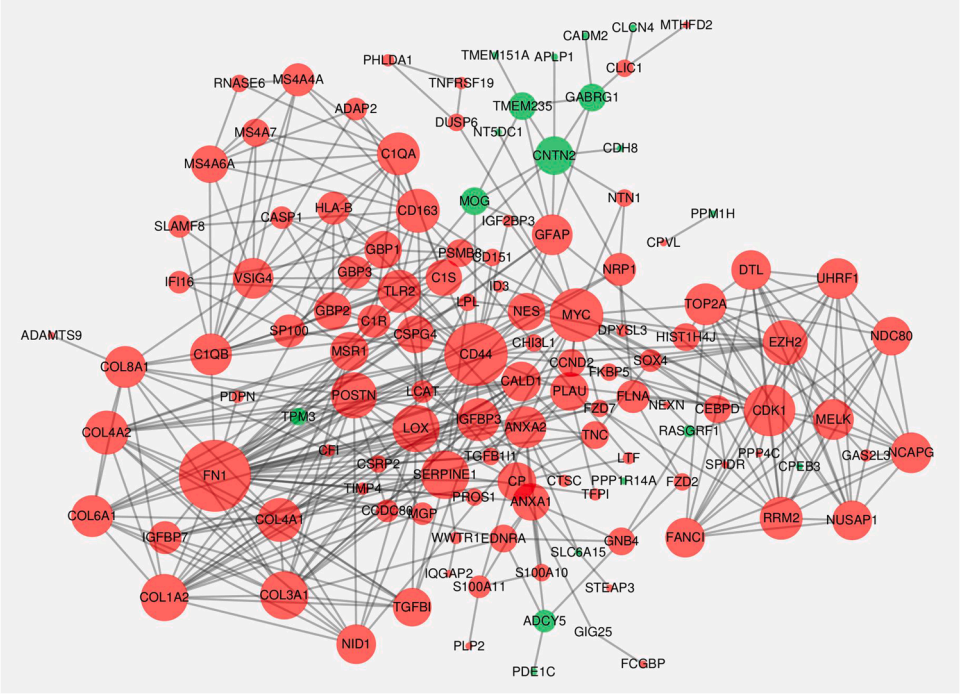


Fig. 4. PPI network constructed for the DEGs in GBM. Red nodes indicate upregulated genes, green nodes indicate downregulated genes and node size is correlated with connectivity of the gene by degree.

Table 2
10 hub genes with connectivity degree≥15 identified from DEGs in GBM.

Gene symbol	Gene title	Degree
FN1	Fibronectin 1	40
CD44	CD44 molecule (Indian blood group)	32
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	23
CDK1	Cyclin-dependent kinase 1	21
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	18
COL3A1	Collagen, type III, alpha 1	18
COL1A2	Collagen, type I, alpha 2	17
LOX	Lysyl oxidase	17
POSTN	Periostin osteoblast specific factor	16
EZH2	Enhancer of zeste homolog 2 (Drosophila)	15

Association of expression of individual hub genes with patient survival

For each of the individual hub genes, 156 GBM patients were divided into two groups according to mRNA expression level (top 78 vs. bottom 78) and the effect of mRNA expression on patient survival was analyzed by Kaplan-Meier analysis. According to the results of survival analysis (Fig. 8), only the expression levels of COL1A2 and LOX were correlated with the overall survival time of GBM (Log-rank (Mantel-Cox) test; $P<0.05$). For these two genes, low expression was associated with increased patient survival. However, after the survival analysis validation, using either GEPIA or LinkedOmics, COL1A2 was no longer associated with overall survival (Fig. 10). The survival validation results for LOX are shown in Figs. 11A (GEPIA) and 11B (LinkedOmics), respectively. Using either test, LOX remained associated with overall survival. Therefore, out of all 10 hub genes, only LOX was associated with overall survival by multiple tests.

Level of LOX in glioma of different malignant degrees and in different differentiated GBM cells

Based on the analysis of GDS4467 dataset, it was found that the expression level of LOX was positively correlated with the malignant degree of glioma(Fig. 12A). LOX expression was higher in astrocytoma (grade II) than in normal brain tissue, higher in GBM(grade IV) than in astrocytoma(grade II), and higher in recurrent GBM than in primary GBM. The analysis of GDS 4535 dataset showed that the expression level of LOX was positively correlated with the differentiation degree of GBM cells(Fig. 12B).

Discussion

In this study, we used the publicly available GEO datasets to identify key DEGs in GBM. The DEGs that we identified were associated with regulation of tumor cell adhesion, differentiation, morphology, and the main pathways involved in complement and coagulation cascades, what is also the main pathway of lung adenocarcinoma, [30] pancreatic ocarcinoma [31] and astrocytoma [32]. This pathway is associated with the regulation of tumor microenvironment and tumor development relies upon the essential contributions from the tumor microenvironment alterations. Complement and coagulation cascades pathway was a significant pathway involved in the progression of GBM.

Upon PPI network construction 10 hub genes were identified that were closely associated with GBM, including FN1, CD44, MYC, CDK1, SERPINE1, COL3A1, COL1A2, LOX, POSTN and EZH2. All of these hub genes were upregulated in GBM, which was verified using the oncomine database, and supported by previous research. [24–29] According to the cBioPortal, genetic alterations exist for all 10 hub genes and having genetic alterations in at least one of the hub genes negatively affects patient overall survival and disease/progression-free survival. We also correlated the expression of individual hub genes with patient survival time based on data obtained from the TCGA database. Only COL1A2 and LOX were found to be closely associated with overall survival of patients with GBM, with low expression levels leading to increased survival time. However, expanding our survival analysis to GEPIA and LinkedOmics, only LOX expression correlated to patient survival. Therefore, only LOX was associated with overall survival when multiple tests were considered.

Lysyl oxidase (LOX), also known as protein-lysine 6-oxidase, is a protein that, in humans, is encoded by the LOX gene. [33] Up-regulation of the LOX gene in tumor cells can promote tumorigenesis, [34] tumor

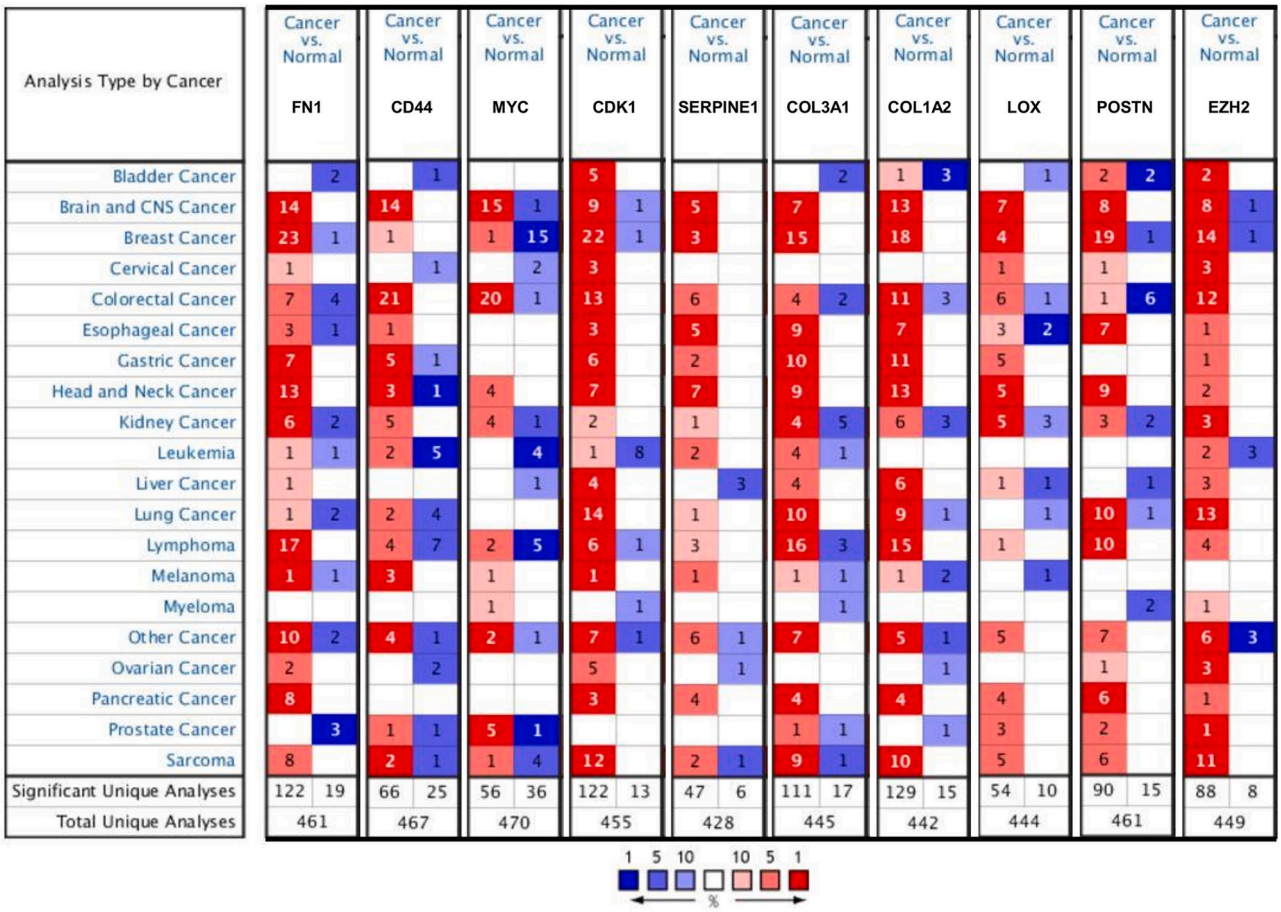


Fig. 5. The mRNA expression pattern of 10 hub genes of GBM identified in our study in different tumor types. The fold change was defined as>2 and the p value was set at <0.01. All 10 hub genes were overexpressed in most studies on CNS tumors. Red indicates overexpression, blue indicates downregulation. Cell color is determined by the best gene rank percentile for the analyses within the cell. Numbers in each cell indicate the number of studies related to the gene.

Table 3
Results of verification analysis from the oncomine database (P value<0.01 and fold change>2, gene rank: Top 10%).

Genes	Studies	Fold change	P value	Genes	Studies	Fold change	P value
FN1	Liang Brain [24]	2.535	3.95E-17	COL3A1	Bredel Brain [25]	27.463	5.36E-17
	Bredel Brain [25]	9.193	1.27E-14		Liang Brain [24]	5.116	3.55E-04
	Shai Brain [26]	5.918	1.27E-08		Sun Brain [28]	12.692	2.58E-13
	Lee Brain [27]	7.356	1.33E-09		Lee Brain [27]	119.244	5.46E-04
	Sun Brain [28]	4.692	3.91E-19	COL1A2	Lee Brain [27]	34.116	3.25E-13
	TCGA	6.8	4.73E-11		Liang Brain [24]	3.065	3.26E-05
CD44	TCGA	8.395	3.00E-03		Sun Brain [28]	9.4	2.52E-16
	Bredel Brain [25]	7.243	1.17E-15		Shai Brain [26]	2.318	2.71E-05
	Liang Brain [24]	4.142	3.36E-09		Murat Brain [29]	2.037	5.22E-06
	Sun Brain [28]	6.456	3.30E-23		Bredel Brain [25]	9.942	1.80E-04
	Shai Brain [26]	3.592	3.46E-08	LOX	TCGA	4.766	4.13E-22
	Murat Brain [29]	2.591	7.09E-18		Murat Brain [29]	4.584	2.17E-13
	Lee Brain [27]	3.14	1.47E-04		Sun Brain [28]	8.577	3.62E-19
MYC	Bredel Brain [25]	4.012	1.29E-11		Liang Brain [24]	4.827	9.63E-05
	Liang Brain [24]	2.676	3.00E-03		Lee Brain [27]	4.309	5.44E-05
	Sun Brain [28]	3.651	7.98E-12		Shai Brain [26]	2.22	1.46E-04
CDK1	Murat Brain [29]	8.576	2.25E-29	POSTN	TCGA	15.394	2.49E-16
	TCGA	4.275	4.03E-14		Murat Brain [29]	3.534	6.82E-11
	Sun Brain [28]	5.007	9.39E-19		Liang Brain [24]	3.546	1.00E-03
	Bredel Brain [25]	3.124	4.32E-05		Bredel Brain [25]	3.765	1.30E-05
SERPINE1	Lee Brain [27]	7.899	7.50E-11		Sun Brain [28]	5.869	7.11E-11
	Shai Brain [26]	4.634	8.93E-07		Lee Brain [27]	12.925	2.00E-03
	Bredel Brain [25]	2.877	1.73E-06	EZH2	TCGA	9.853	2.80E-38
	Sun Brain [28]	6.875	5.35E-10		Sun Brain [28]	7.432	3.03E-18
					Murat Brain [29]	10.372	1.64E-08

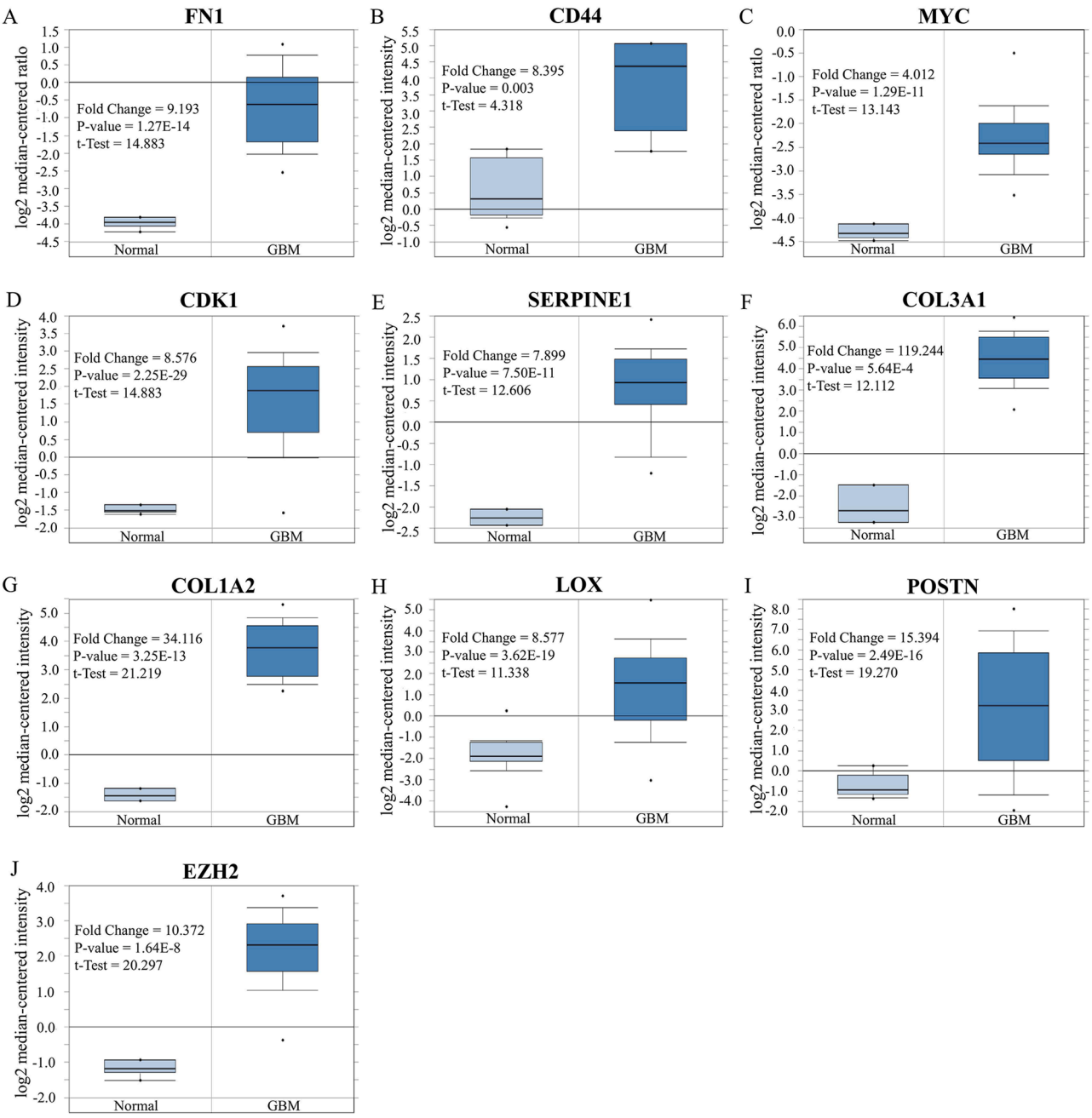


Fig. 6. Each hub gene was most significantly overexpressed in different studies (ONCOMINE database). Box plots derived from gene expression data in ONCOMINE comparing expression of 10 hub genes in normal and GBM tissue. The fold change was defined as >2 and p value was set at < 0.01. A: Comparison of FN1 mRNA expression (fold change=9.193, $P = 1.27E-14$). B: Comparison of CD44 mRNA expression (fold change=8.395, $P = 0.003$). C: Comparison of MYC mRNA expression (fold change=4.012, $P = 1.29E-11$). D: Comparison of CDK1 mRNA expression (fold change=8.576, $P = 2.25E-29$). E: Comparison of SERPINE1 mRNA expression (fold change=7.899, $P = 7.50E-11$). F: Comparison of COL3A1 mRNA expression (fold change=119.244, $P = 5.64E-4$). G: Comparison of COL1A2 mRNA expression (fold change=34.116, $P = 3.25E-13$). H: Comparison of LOX mRNA expression (fold change=8.577, $P = 3.62E-19$). I: Comparison of POSTN mRNA expression (fold change=15.394, $P = 2.49E-16$). J: Comparison of EZH2 mRNA expression (fold change=10.372, $P = 1.64E-8$).

cell proliferation and tumor metastasis, [35] tumor microenvironment (TME) remodeling,tumor invasion,immunomodulation [36] and canceration. [37] LOX regulates the progression of various human malignant tumors and plays an important role in cancer cell mitosis. [38] Studies have shown that LOX plays an important role in glioma, the LOX gene promotes glioma migration, invasion infiltration and angiogenesis, [39] and has the same effect in GBM. [40,41] KuA inhibits the growth and migration of human GBM cells in vitro and in vivo by down-regulating the expression of LOX. [42] Han et al. showed that in the Chinese population certain variants of the LOX gene were associated with increased risk of glioma and with shorter patient survival times. [43] Glioma patients with high LOX expression showed a higher enrichment fraction of immune cell infiltration and a higher immune checkpoint level, and were associated with multiple chemotherapy agents indicate that LOX have potent predictive value for prognosis, chemotherapy and immunotherapy in glioma patients. [44] The role of the LOX gene in glioma and GBM suggests that it may be a potential target for the treatment of GBM. In our study, LOX was significantly upregulated and closely correlated with the prognosis of GBM, In addition, the expression level of LOX was positively correlated with the

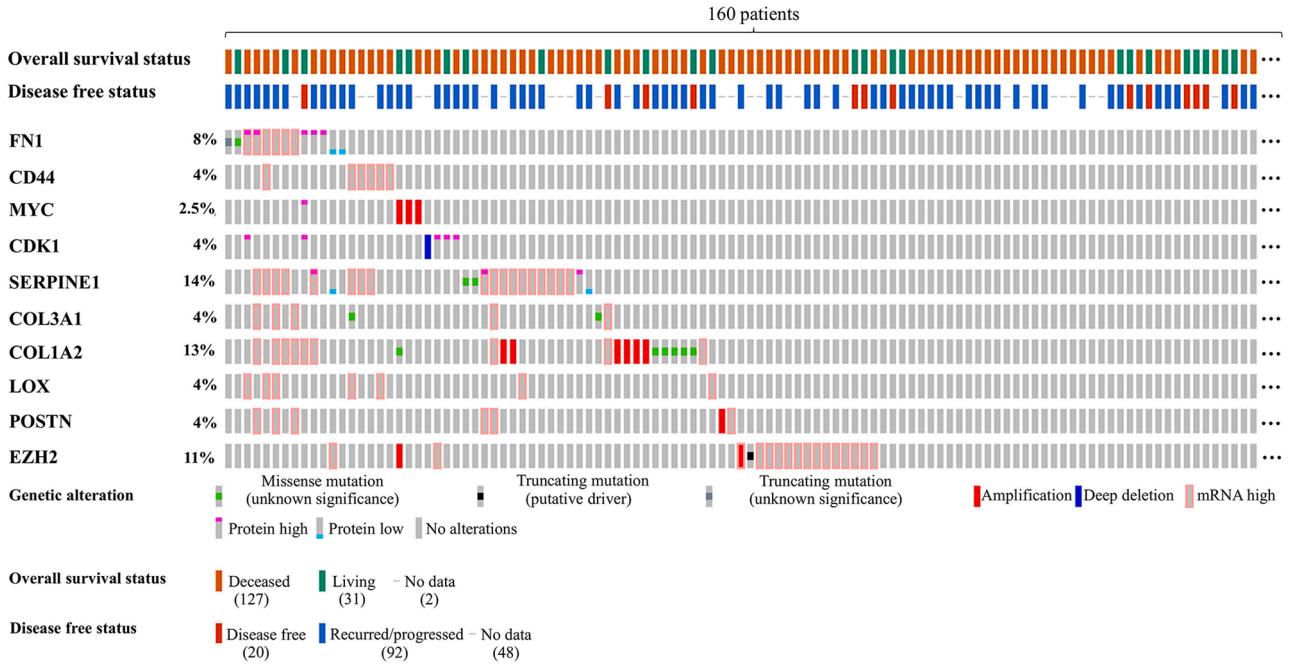
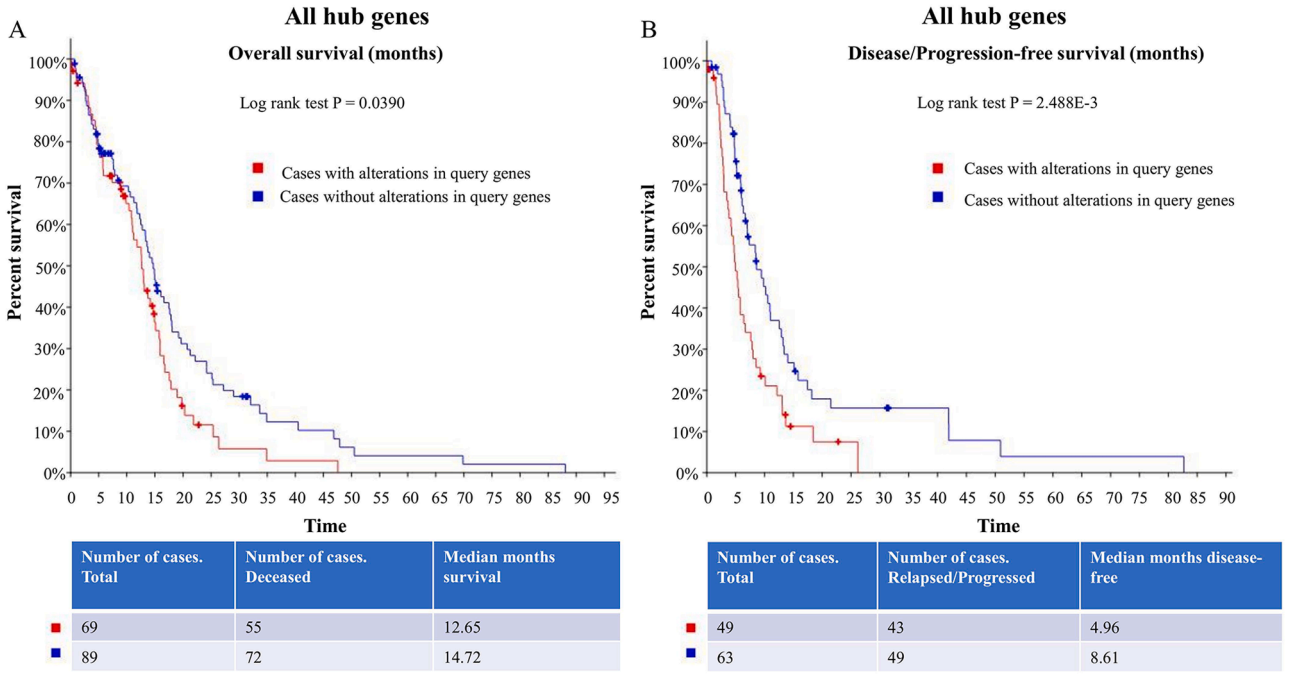


Fig. 7. Genetic alterations of 10 hub genes identified in this study. Every column represents a sample/patient ($n = 160$). 69 patients had alterations in one or more of the hub genes. 91 patients had no alterations in any of the hub genes.



	Number of cases. Total	Number of cases. Deceased	Median months survival
Cases with alterations in query genes	69	55	12.65
Cases without alterations in query genes	89	72	14.72

	Number of cases. Total	Number of cases. Relapsed/Progressed	Median months disease-free
Cases with alterations in query genes	49	43	4.96
Cases without alterations in query genes	63	49	8.61

Fig. 8. Association between alterations of the 10 hub genes and overall survival or disease/Progression-Free survival in 160 patients with GBM. A: Alterations in one or more of 10 hub genes was correlated with decreased overall GBM patient survival ($P = 0.0390$). Red line: 69 patients with alterations in at least one of the hub genes; blue line: 89 patients with no alterations in any of the hub genes. For 2 patients without hub gene alterations, no survival data were available. B: Alterations in one or more of the 10 hub genes were correlated with decreased disease/ progression-free survival ($P = 2.488E-3$). Red line: 49 patients with alterations in at least one of the hub genes; blue line: 63 patients with no alterations in any of the hub genes. For 48 patients no data on progression-free survival were available.

malignant degree of glioma and the differentiation of GBM cells, the upregulation of LOX may play an important role in the deterioration of GBM.

We also identified nine other hub genes, including FN1, CD44, MYC, CDK1, SERPINE1, COL3A1, COL1A2, POSTN and EZH2. FN1 has been reported to be associated with tumor growth and angiogenesis in GBM. [45] CD44 expression was found to be significantly increased in more severe types of GBM. Its expression was higher in the central regions of the tumor than in the peripheral regions, and correlated with survival, supporting the candidacy of CD44 as a prognostic and therapeutic biomarker for GBM. [46] Studies have shown that COL3A1 and COL1A2, were independent factors for clinical prognosis of GBM, while mir-29b, which inhibits the expression of COL1A2 and COL3A1 in GBM, has an obvious anticancer effect. [47,48] The expression of COL3A1 has been

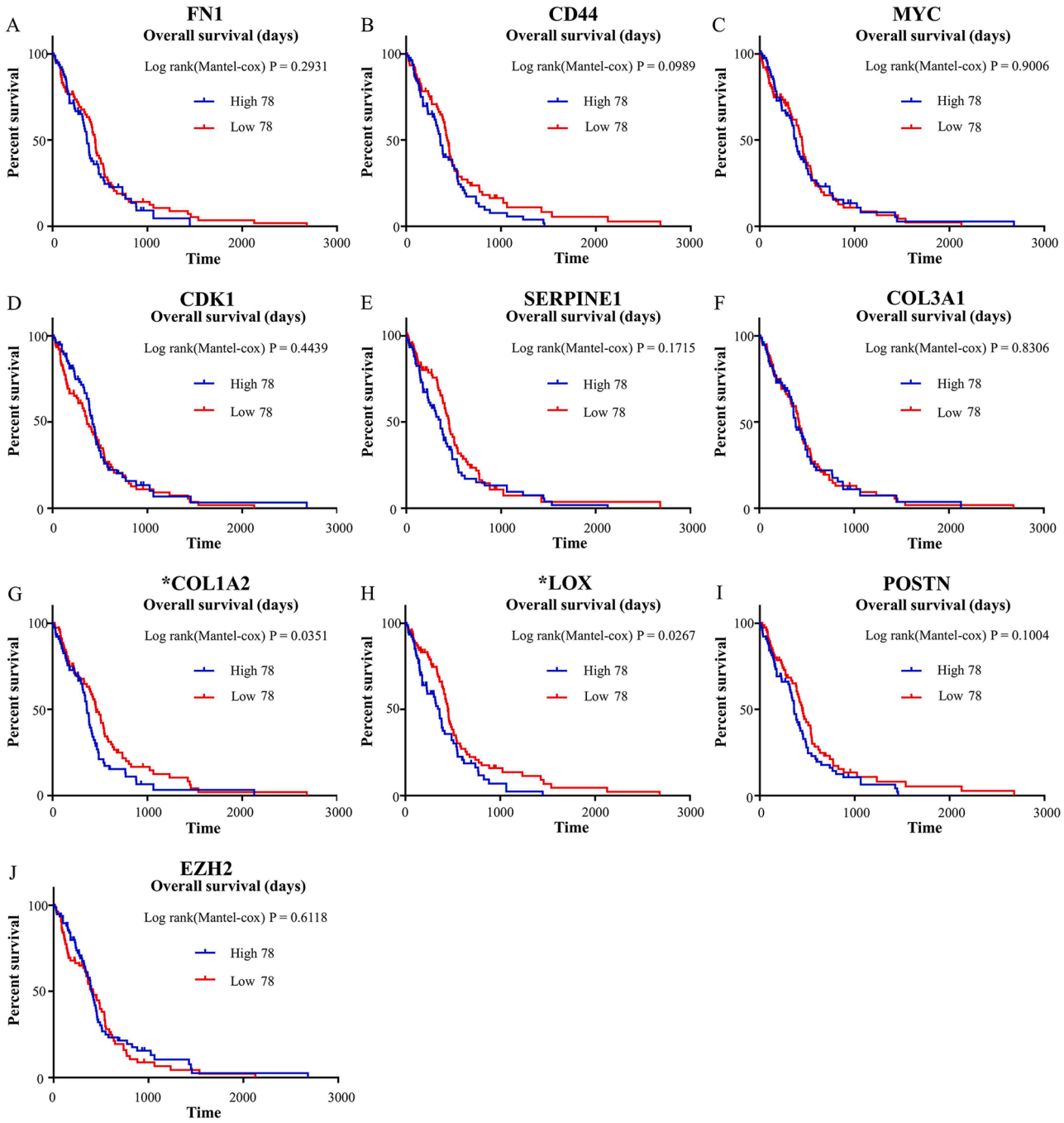


Fig. 9. Relationship between each hub gene and overall survival time (days) in GBM. A: The expression level of FN1 mRNA was not correlated with the overall survival time in GBM($P = 0.2931$,P>0.05). B: The expression level of CD44 mRNA was not correlated with the overall survival time in GBM($P = 0.0989$,P>0.05).C: The expression level of MYC mRNA was not correlated with the overall survival time in GBM($P = 0.9006$,P>0.05).D: The expression level of CDK1 mRNA was not correlated with the overall survival time in GBM($P = 0.4439$,P>0.05).E: The expression level of SERPINE1 mRNA was not correlated with the overall survival time in GBM($P = 0.1715$,P>0.05).F: The expression level of COL3A1 mRNA was not correlated with the overall survival time in GBM($P = 0.8306$,P>0.05). G: The expression level of COL1A2 mRNA was correlated with the overall survival time in GBM($P = 0.0351$,P<0.05), indicated by the asterisk. . H: The expression level of LOX mRNA was correlated with the overall survival time in GBM($P = 0.0267$,P<0.05), indicated by the asterisk. I: The expression level of POSTN mRNA was not correlated with the overall survival time in GBM($P = 0.1004$,P>0.05).J: The expression level of EZH2 mRNA was not correlated with the overall survival time in GBM($P = 0.6118$,P>0.05).

reported not only to be directly related to the grade of glioma, but also to be associated with survival. [49] Thus, the FN1, CD44, COL1A2, and COL3A1 genes have previously been identified as key genes for GBM. [50] Based on the role of MYC in cell hypoxia and glucose metabolism in glioblastoma, it could also be a potential target for the treatment of GBM. [51–53] SERPINE1 is a target gene for TGF-β, and the strong association of statin activity with TGF-β makes it possible to treat TGF-β-dependent GBM with statin therapy. [54] In addition, promoting POSTN gene expression can increase the invasiveness of GBM, while silencing POSTN gene expression inhibited the growth of GBM tumor cells. [55,56] These studies fully confirm the feasibility of POSTN as a therapeutic target for GBM. Increased expression of the EZH2 gene is involved in the tumorigenesis of GBM, EZH2 to participate in the β-catenin-USP1-EZH2 signaling axis is the key factor to cause GBM. [57,

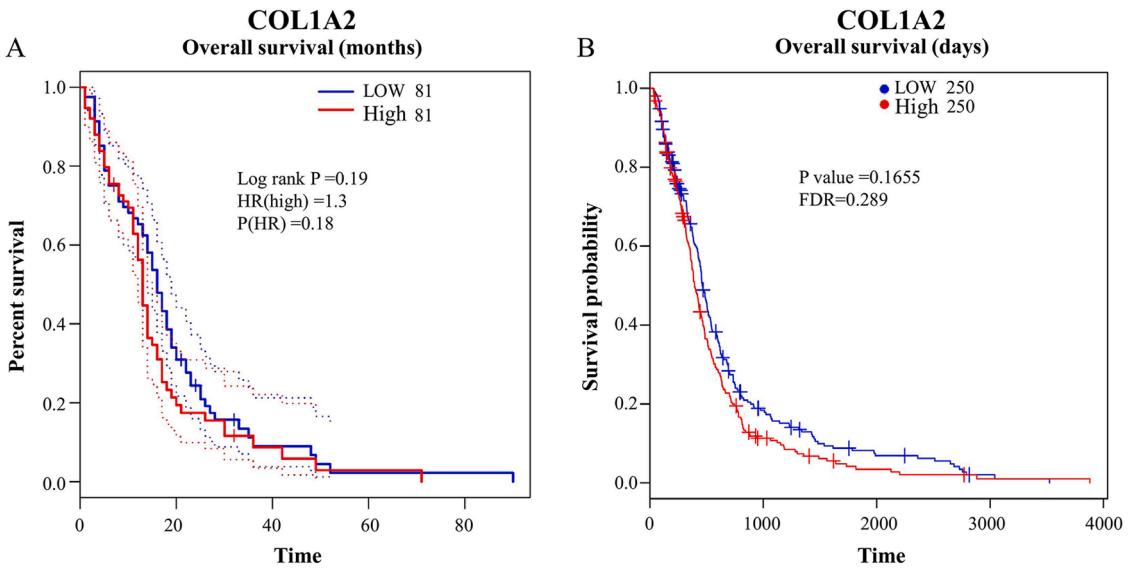


Fig. 10. Survival analysis verification showing that COL1A2 is not associated with overall survival using multiple tests. A: Results from GEPIA suggest that in a group of 162 patients COL1A2 is not associated with overall survival (logrank $P = 0.19$). B: Results from LinkedOmics indicate that in a group of 500 GBM patients COL1A2 is not associated with overall survival ($P = 0.165$). Patients were divided into two groups according to the median mRNA expression levels. Red line: patients with highest expression levels; blue line: patients with lowest expression levels.

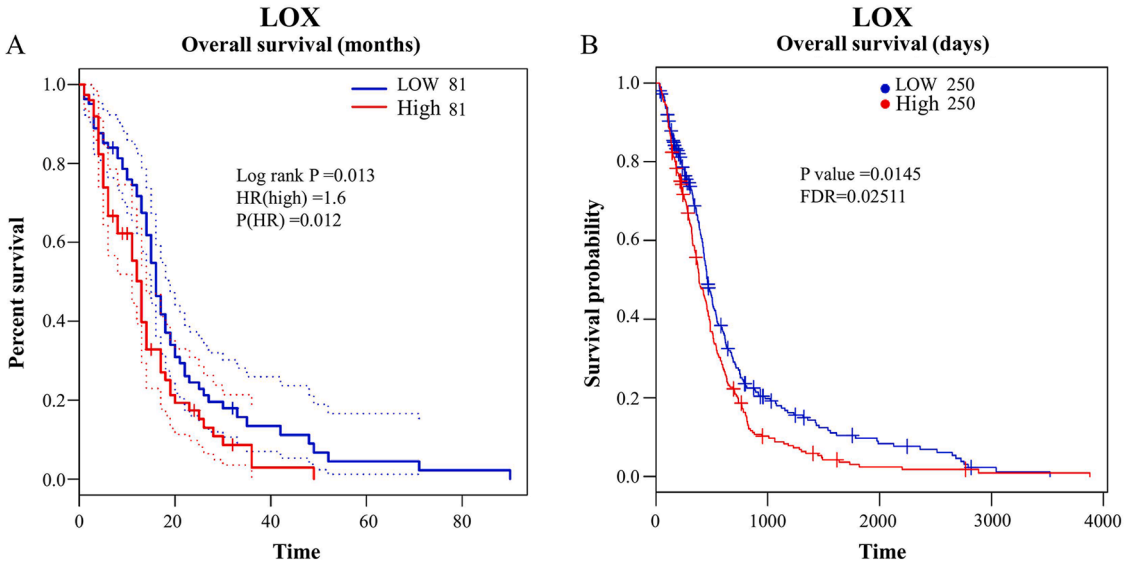


Fig. 11. Survival analysis verification showing that LOX is associated with overall survival using multiple tests. A: Results from GEPIA suggest that in a group of 162 GBM patients LOX is associated with overall survival (logrank $P = 0.013$). B: Results from LinkedOmics indicate that in a group of 500 patients with GBM LOX is associated with overall survival ($P = 0.0145$). Patients were divided into two groups according to the median mRNA expression levels. Red line: patients with highest expression levels; blue line: patients with lowest expression levels.

58] The EZH2 gene is expected to be a new therapeutic target for GBM. Thus, all 10 hub genes identified by us have sufficient evidence to prove their important role in GBM, which fully confirms the results of our study, however, in our study, these nine hub genes were not associated with overall survival time.

Conclusion

In this study, we found the complement and coagulation cascades pathway is an important pathway in GBM. In addition, a total of 10 hub genes were identified, that were all significantly upregulated in GBM, including FN1, CD44, MYC, CDK1, SERPINE1, COL3A1, COL1A2, LOX, POSTN and EZH2. Of these, only LOX gene expression was significantly associated with patient survival and promotes glioblastoma cell differentiation and tumor recurrence. It may represent a prognostic biomarker and potential therapeutic target in GBM. However, more research is needed to apply molecular targeted therapy of these genes in GBM and to elucidate the mechanism of these genes. Furthermore, the relationship between expression of these genes and clinical prognosis of GBM needs to be confirmed in additional studies.

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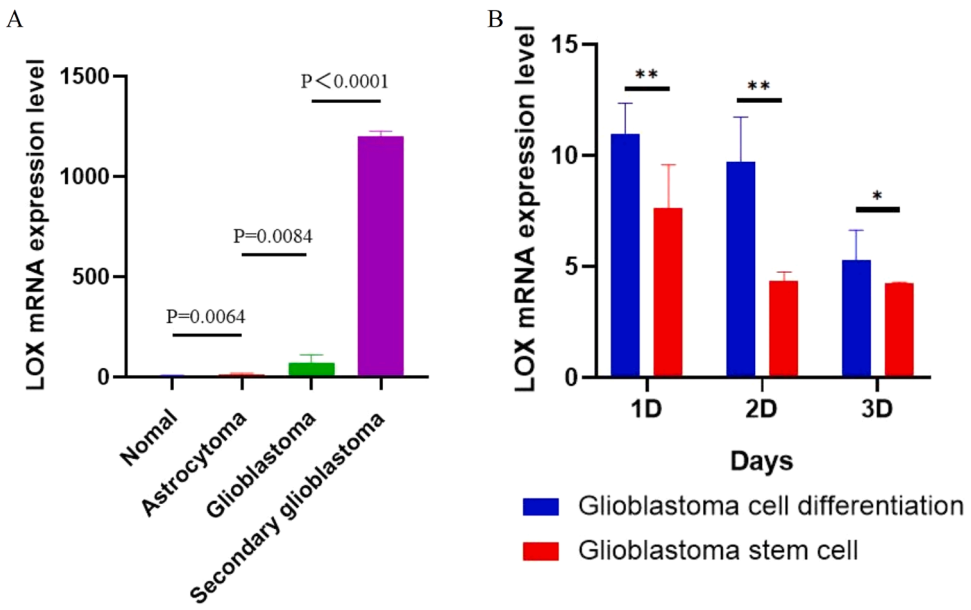

Fig. 12. A: The expression level of LOX was positively correlated with the malignant degree of glioma,.

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Declaration of Competing Interest

All authors have no conflict of interest and all patients agree to submit this manuscript.

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