Hub gene target of glioblastoma
LOX, SERPINH1 and TGFBI

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1. Introduction

Glioblastoma (GBM) is a tumor originating from glial cells, and is a malignant tumor. The long-term prognosis of the patients is poor. It is reported that the annual incidence of craniocebral malignant tumor is about 7.08 per 100,000 population. At the same time, GBM accounts for 14.6% of brain tumors, and the incidence of GBM in women is higher than that in men. What is more, the age of onset tends to be younger. The symptoms of the GBM are mainly related to the location and size of the tumor. Patients can have headaches, vomiting, hemianopsia and aphasia, which seriously reducing the quality of life. In addition, GBM progresses rapidly and is not easy for early diagnosis, resulting in a 5-year survival rate of about 35.8%. In fact, early diagnosis can effectively improve the therapeutic effect and prognosis. However, the pathogenesis of GBM has not been fully elucidated. Genetic and environmental factors may be involved in the occurrence and development of GBM. Small molecules such as circRNA and miRNAs were also involved in the progression of GBM. At present, surgery is still the main means of treatment, and the combination of chemotherapy and radiotherapy can effectively improve the curative effect. Furthermore, there is more and more evidence that biological targeted therapy can effectively improve the prognosis. Generally speaking, due to the easy recurrence of tumors and drug resistance to chemotherapeutic drugs, the
therapeutic effect is still far from satisfactory.\[^{1,10}\] Therefore, it is of important clinical value to further explore the pathogenesis and look for molecular markers for early diagnosis and targeted treatment.

Microarray technology can be used to find genetic changes in the process of tumor occurrence and development. Tang found several differentially expressed genes by mining the sequencing data of thyroid cancer tissues. What is more, further verification and multivariate analysis showed that neuropeptide Y and miR-184 could be used as molecular markers for early diagnosis and specific treatment of thyroid cancer, which provided a new idea for the study of the mechanism of thyroid cancer.\[^{11}\] Similarly, Troiano et al. found that there was a significant correlation between baculoviral IAP repeat containing 5 (BIRC5) and oral squamous cell carcinoma. Immunohistochemistry and related experiments show that BIRC5 may affect the prognosis of patients with oral squamous cell carcinoma by regulating the level of cell proliferation and methylation, suggesting that BIRC5 may be a potential therapeutic target for oral squamous cell carcinoma.\[^{12}\]

**Figure 1.** (A) Identification of DEGs and between GBM tumor tissues and normal brain tissues. (B) Common DEGs of the 2 dataset. DEGs = differentially expressed genes, GBM = glioblastoma.
Zhou et al. found that there were many differentially expressed genes between GBM tumor and normal tissues by bioinformatics analysis. Furthermore, Zhong et al. identified several key modules and genes from GBM tumor tissues by gene coexpression network analysis. Further analysis and identification of enetemed, Inc-2076, a compound that can regulate tumor cell cycle and apoptosis, provide evidence for the study of the mechanism and treatment of GBM. However, there may be false positives in the chip and sequencing, which need to be further verified.

We screened the differently expressed genes (DEGs) of GBM tumor tissue relative to normal brain tissue by bioinformatics analysis. Then the DEGs were analyzed by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG). At the same time, the protein-protein interaction (PPI) network was constructed for identification of hub genes. In addition, the cancer genome atl (TCGA) database were used to verify the expression of hub genes. Meantime, we took GBM tissues and normal brain tissues to verify the expression of hub genes. Finally, we make a preliminary analysis of the role of the hub gene in GBM.

2. Materials and methods

2.1. Access to public data

The gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) is an open source platform for the storage of genetic data. Two expression profiling datasets [GSE50161 (GPL570 platform), GSE116520 (GPL10558 platform)] were respectively downloaded from the GEO database. The GSE50161 dataset includes 13 normal brain samples and 34 GBM tumor tissues. The GSE116520 dataset includes 17 GBM tissue samples, 8 normal brain tissue samples.

2.2. DEGs identified using GEO2R

GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) is an interactive online tool used to identify DEGs in datasets from the GEO. GEO2R may also be used to distinguish DEGs between GBM and normal brain tissues. The rule of statistical significance is that P-value < .05 and Fold change (FC) > 2 or FC < -2. Volcano diagrams were delineated by SangerBox software (http://sangerbox.com/). Venn diagram was delineated by FunRich software (http://www.funrich.org).

2.3. Functional annotation of DEGs using KEGG and gene body (GO) pathway enrichment analysis

The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/home.jsp; version 6.8) is an online suite of analysis tools with an integrated discovery and annotation function. The GO resource is widely used in bioinformatics, and covers 3 aspects of biology, including biological process, cellular component and molecular function. To perform the GO and KEGG analysis of DEGs, Database for Annotation, Visualization and Integrated Discovery online tool was implemented. The rule of statistical significance is that P < .05. Furthermore, the pathway and process enrichment analysis were performed by Metascape (http://metascape.org/gp/index.html).

2.4. Construction of a PPI network

Search Tool for the Retrieval of Interacting Genes (http://string.embli.de/), an open source online tool, was used to construct a PPI network of the identified DEGs, and Cytoscape visualization software version 3.6.1 was used to present the network. A confidence score > 0.4 was considered as the criterion of judgment.

2.5. Identification and analysis of hub genes

The Cytoscape plug-in Molecular Complex Detection (MCODE) (version 1.5.1) identified the most important module of the network map. The criteria of MCODE analysis are that degree cutoff = 2, Max depth = 100, MCODE scores > 5, node score cutoff = 0.2. Then, cytoHubba, a free plug-in of Cytoscape, was performed to identify the hub genes when degrees ≥ 10. The pathway and process enrichment analysis of the hub genes were performed by Metascape.

Table 1

| Term | Description | Count in gene set | P-value |
|------|-------------|------------------|---------|
| GO:0050877 | Neurological system process | 57 | 6.13E-06 |
| GO:0007267 | Cell-cell signaling | 46 | 5.09E-11 |
| GO:0007242 | Intracellular signaling cascade | 46 | 0.01048 |
| GO:0008111 | Ion transport | 44 | 7.90E-07 |
| GO:0019226 | Transmission of nerve impulse | 42 | 1.30E-16 |
| GO:0007268 | Synaptic transmission | 39 | 1.08E-16 |
| GO:0006796 | Phosphate metabolic process | 34 | 0.05138 |
| GO:0005886 | Plasma membrane | 154 | 2.71E-08 |
| GO:0044459 | Plasma membrane part | 107 | 1.99E-09 |
| GO:0005570 | Extracellular region | 68 | 0.05972 |
| GO:0005850 | Cytoskeleton | 59 | 8.31E-04 |
| GO:0040202 | Synapse | 53 | 1.57E-23 |
| GO:0005099 | Calcium ion binding | 47 | 8.33E-06 |
| GO:0015267 | Channel activity | 29 | 2.48E-06 |
| GO:0022803 | Passive transmembrane transporter activity | 29 | 2.60E-06 |
| GO:0022838 | Substrate specific channel activity | 28 | 3.89E-06 |
| GO:004672 | Protein kinase activity | 28 | 0.00364 |
| GO:0005198 | Structural molecule activity | 28 | 0.00658 |
| hsa04020 | Calcium signaling pathway | 16 | 2.69E-05 |
| hsa04080 | Neuroactive ligand-receptor interaction | 16 | 0.00163 |
| hsa04510 | Focal adhesion | 12 | 0.01123 |
| hsa04512 | ECM-receptor interaction | 11 | 3.82E-05 |
| hsa04114 | Oocyte meiosis | 11 | 3.74E-04 |

DEGs = differentially expressed genes, GBM = glioblastoma, GO = gene ontology, KEGG = Kyoto encyclopedia of genes and genomes.
2.6. Expression analysis of hub genes and survival analysis
UCSC Xena (https://xena.ucsc.edu/welcome-to-ucsc-xena/) could integrate the public genomic data sets to analyze and visualize the expression level of hub genes. Then, the clustering analysis of expression level of hub genes was performed using heatmaps. Gene expression profiling interactive analysis, a web server for cancer and normal gene expression profiling and interactive analyses (http://gepia.cancer-pku.cn/).[19] Survival analysis was performed in GEPIA. What is more, the expression of hub genes in GBM were verified again in GEPIA. Expression on Box Plots |Log 2FC| Cutoff is 1, P-value Cutoff is .01, jitter size is 0.4, match TCGA data. Then, the median expression of tumor and normal samples in bodymap was performed. At the same time, cBioPortal is useful in integrative analysis of complex cancer genomics and clinical profiles (http://cbioportal.org).[20] The Genomic Alteration Types and putative copy-number alterations from GISTIC of the hub genes were performed in cBioPortal.

2.7. Establishment of animal models
The C57 black 6 (C57BL/6) mice (8 ± 1 weeks) were weighed, and this information was recorded. They were then numbered and assigned to groups according to the random number table method. The rats were divided into 4 groups, 6 rats in each group. Group A: Con; Group B: OV; Group C: OV/TTK-OE; Group D: OV/TTK-KO. GBM model was established in mice by inoculation of tumor cell suspension: The mice were anesthetized by intraperitoneal injection of 1% barbital (0.01 mL/g) on a super-clean bench, and a small amount of ether was dipped in a cotton ball and placed in front of the nose of the mice. After routine disinfection, the position of the needle was determined, and 6 µL tumor cell suspension was sucked vertically and slowly into the mouse brain parenchyma with a 50 µL microsyringe, and the needle was retained for 5 minutes after gentle injection. The needles were slowly withdrawn, sterilized, and kept in a Specific Pathogen Free environment. The growth status of the nude mice was observed daily and recorded.

2.8. Western blot
Extracting total protein from the tissue, after the concentration was determined by UV method, 1/4 of the protein sample volume of 5 x protein loading buffer (reduced) was added to each tissue, boiled at 100°C for 10 minutes, cooled, packed and frozen in ~80°C refrigerator until use. Protein samples were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel electrophoresis, membrane transformation.

Figure 2. The enrichment analysis of DEGs by DAVID and Metascape. (A) Heatmap of enriched terms across input differently expressed gene lists, colored by p-values, via the Metascape. (B) Network of enriched terms colored by cluster identity. (C) Network of enriched terms colored by P-value, where terms containing more genes tend to have a more significant P-value. DEGs = differentially expressed genes.
and other operations. Block 5% skim milk at room temperature for 1 hour. The primary antibody was added, and the samples were incubated overnight at 4°C. After shaking tris buffered saline tween for 3 times (5 minutes/time), rabbit secondary antibody was added. After incubation for 1 hour at room temperature, tris buffered saline tween was shaken 3 times (5 minute/time). The results were analyzed after developing the chemiluminescence solution.

2.9. Real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) assay verification

A total of 20 GBM participates were recruited. After surgery, 20 GBM tumor samples from GBM patients and 20 adjacent normal brain tissues samples were obtained. The research conformed to the Declaration of Helsinki and was authorized by the Human Ethics and Research Ethics Committees of the Zhejiang Cancer Hospital. The informed consents were obtained from all participates.

Total RNA was extracted from 20 GBM tumor samples and 20 adjacent normal brain tissues samples by the RNAiso Plus (Trizol) kit (Thermofisher, Massachusetts, America), and reverse transcribed to cDNA. RT-qPCR was performed using a Light Cycler® 4800 System with specific primers for genes. The RQ values ($2^{-\Delta\Delta C_t}$, where $C_t$ is the threshold cycle) of each sample were calculated, and are presented as fold change in gene expression relative to the control group. GAPDH was used as an endogenous control.

3. Results

3.1. Screening of DEGs between GBM and normal brain tissues.

Volcano diagrams show the DEGs of GSE50161 (Fig. 1A) and GSE116520 (Fig. 1B). A Venn diagram revealed 467 common DEGs between the 2 datasets (Fig. 1C).

3.2. Functional annotation for DEGs using KEGG and GO analysis.

The results of GO analysis revealed that variations in the biological process were predominantly enriched in neurological system process, cell-cell signaling, intracellular signaling cascade, ion transport, synaptic transmission, phosphate metabolic process and so on. Changes in cellular component were primarily enriched in plasma membrane, plasma membrane part, extracellular region, cytoskeleton, synapse and so on. Variations in molecular function were enriched in calcium ion binding, channel activity, passive transmembrane transporter activity, substrate specific channel activity, protein kinase activity, structural molecule activity and so on (Table 1). KEGG analysis demonstrated that DEGs were largely enriched in pathways in Calcium signaling pathway, Neuroactive ligand-receptor interaction, Focal adhesion, ECM-receptor interaction, Oocyte meiosis and so on (Table 1). Pathway and process enrichment analysis by Metascape. Heatmap of enriched terms across input differently expressed gene lists, colored by p-values, via the Metascape. Network of enriched terms colored by cluster identity. Network of

![Figure 3](image)

**Figure 3.** The PPI network of DEGs, the significant MCODE module and hub genes. (A) The PPI network consists of 2995 edges and 412 nodes. (B) The significant MCODE module. (C) 10 hub genes. (D) Pathway and process enrichment analysis of hub genes. DEGs = differentially expressed genes, MCODE = matrix metalloproteinase-2, PPI = protein-protein interaction.
enriched terms colored by $P$-value, where terms containing more genes tend to have a more significant $P$-value. (Fig. 2A–C).

### 3.3. Construction and analysis of PPI networks

Construction of a PPI network revealed 2995 edges and 412 nodes in the PPI network (Fig. 3A). The network possessed significantly more interactions than expected. Such enrichment indicates that the identified proteins are at least partially associated.

### 3.4. Hub gene selection and functional annotation

MCODE were found in Figure 3B. 10 hub genes were found in Figure 3C. Pathway and process enrichment analysis by Metascape were shown in Figure 3D.

### 3.5. Overall survival analysis

The overall survival rate analysis of the GBM which content 20-month, 40-month, 60-month overall survival were displayed (Fig. 4A–J). LOX, serpin family H member 1 (SERPINH1) and transforming growth factor beta induced (TGFBI) were negatively correlated with the overall survival rate in patients with GBM ($P < .05$) (Fig. 4F, I, J).

### 3.6. Analysis of hub genes

The expression level of hub genes and the clustering analysis of expression level of hub genes indicated that the hub genes are high in GBM tumor tissues (Fig. 5A). Genetic alteration of the hub genes in GBM were shown in Figure 5B. Putative copy number alterations from GISTIC of lysyl oxidase (LOX), SERPINH1 and TGFBI were shown in Figure 5C.

Body maps were shown in Figure 6 (A-F, LOX, SERPINH1, TGFBI, collagen type I alpha 1 chain, collagen type I alpha 2 chain, lumican). The expression of the hub genes in GEPIA were shown in Figure 7A–I.

### 3.7. Verification of LOX, SERPINH1 and TGFBI

As presented in Figure 8, the relative expression levels of LOX, SERPINH1 and TGFBI were significantly higher in GBM samples, compared with the normal brain tissues groups. The result demonstrated that LOX, SERPINH1 and TGFBI might be considered as biomarkers for GBM.

### 3.8. Western blot

Western blot analysis showed that LOX, SERPINH1 and TGFBI were highly expressed in GBM. ($P < .05$).

### 4. Discussion

GBM is the most common malignant tumor of the brain. As the tumor is easy to recur, the long-term prognosis of the patients is poor. What is more, GBM can involve anywhere in the central nervous system and progress rapidly, which seriously affecting...
the work and life of patients. However, the mechanism of GBM has not been fully clarified, and the existing treatment methods and therapeutic effects are far from satisfactory. Through the sequencing analysis of brain tumor tissue by microarray technology, Griesinger et al. found that there was a significant correlation between multiple immune infiltration and the pathogenesis of brain tumor, which provided new evidence for the exploration of the mechanism and treatment of brain tumor. In addition, Kruthika et al. found that PBK can regulate cell proliferation and apoptosis, and is related to tumor recurrence and chemotherapeutic drug resistance. Furthermore, biological immunotherapy may also effectively improve the prognosis of patients. At the same time, more clinical evidence is needed.

In this study, bioinformatics technology was used to screen hub genes (collagen type IV alpha 1 chain, SERPINH1, collagen type III alpha 1 chain, collagen type I alpha 1 chain, collagen type I alpha 2 chain, collagen type V alpha 1 chain, lumican, TGFBI, LOX, periostin) from GBM tumor tissues, which may be used as therapeutic targets and biomarkers of GBM. Among them, LOX, SERPINH1 and TGFBI deserve more attention.

Figure 5. Expression analysis of hub genes in UCSC. (A) Hub genes were high expressed in GBM tumor tissues. (B) Genetic alteration in cBioportal. (C) Putative copy number alterations. GBM = glioblastoma.
LOX (lysyl oxidase), is mainly involved in regulation of protein-lysine 6-oxidase activity, protein binding, protein phosphorylation, gene expression and apoptotic process. Abnormal expression of LOX is involved in the occurrence and development of many diseases. De Donato found that LOX was highly expressed in serous ovarian cancer by immunohistochemical quantitative detection. At the same time, in vitro experiments showed that LOX may be involved in the occurrence and development of ovarian cancer by regulating cell proliferation, cell migration and specific gene expression, suggesting that LOX may be a therapeutic target. [23] What is more, Ma found several differentially expressed genes related to pancreatic cancer through biological sequencing analysis of patients with pancreatic cancer. At the same time, multivariate analysis showed that LOX may be used as a prognostic marker of pancreatic cancer, which provides a new idea for the study of the mechanism of pancreatic cancer. [24] In addition, Zhao et al showed that the expression of LOX was significantly high in gastric cancer tissues by immunohistochemistry. Animal experiments have shown that LOX may be involved in the occurrence and development of gastric cancer by regulating the expression of matrix metalloproteinase-2 and matrix metalloproteinase-9. [25] Furthermore, Da Silva et al detected several molecules abnormally expressed in astrocytoma by PCR detection. At the same time, correlation analysis and survival analysis proved that LOX and HIF1α were related to the progression of astrocytoma, and functional experiments showed that LOX may participate in the occurrence and development of astrocytoma by affecting tumor cell proliferation and angiogenesis. [26] Consistent with the above study, we found that LOX was highly expressed in patients with GBM. Survival analysis showed that the prognosis of patients with GBM with high expression of LOX was poor. At the same time, we verified the high expression of LOX in patients with GBM through the clinical data of TCGA. Finally, we verified the high expression of LOX in GBM patients by reverse transcriptase polymerase chain reaction. We speculate that LOX may be involved in the occurrence and development of GBM by affecting cell cycle and apoptosis. LOX may be used as a target for early diagnosis and specific treatment of GBM, and the related mechanism is worthy of further exploration.

SERPINH1 (serpin family H member 1) is mainly involved in regulation of RNA binding, collagen binding, collagen biosynthetic process and negative regulation of endopeptidase activity. Christiansen et al found that the abnormal mutation of SERPINH1 may affect the synthesis and function of collagen, which may lead to osteogenic insufficiency and seriously affect the quality of life of patients. [28] What is more, Kamikawaji et al found that microRNA-29a plays an important role in the occurrence and development of (IPF) in idiopathic pulmonary fibrosis. At the same time, luciferase reports and related experiments showed that microRNA-29a may act on SERPINH1 to regulate fibroblast migration and collagen synthesis, thus inducing idiopathic pulmonary fibrosis. [29] In addition, Qi et al found several differentially expressed genes through sequencing analysis of clear cell renal cell carcinoma tumor and normal tissues. Further functional analysis showed that SERPINH1 may participate in the progression of clear cell renal cell carcinoma by regulating epithelial to mesenchymal transformation, suggesting that SERPINH1 may be used as a target for early diagnosis and specific treatment of clear cell renal cell carcinoma. [30] Furthermore, Wu et al found that SERPINH1 was significantly up-regulated in hepatocellular carcinoma tissues by quantitative polymerase chain reaction and Western blot quantitative analysis. Further Transwell and flow cytometry showed that SERPINH1 promoted the proliferation of hepatocellular carcinoma cells by regulating cell cycle and apoptosis. [31] Consistent with the
above study, we found that SERPINH1 was highly expressed in patients with GBM. Survival analysis showed that the prognosis of patients with high expression of SERPINH1 was poor. And we verified the high expression of SERPINH1 in patients with GBM. We speculate that SERPINH1 may be involved in the occurrence and development of GBM by regulating cell proliferation and collagen synthesis. SERPINH1 may be used as a target for early diagnosis of GBM.
TGFBI (transforming growth factor beta induced), is mainly involved in regulation of integrin binding, extracellular matrix structural constituent, collagen binding, angiogenesis, cell adhesion, cell proliferation and extracellular matrix organization. Through bioinformatics analysis, Yan found that TGFBI could regulate the proliferation of non-small cell lung cancer cells and affect the prognosis of patients. What is more, Wang et al. found that TGFBI was abnormally expressed in oral squamous cell carcinoma. Functional experiments showed that TGFBI may be involved in the occurrence and development of oral squamous cell carcinoma by affecting cell proliferation, migration and regulating inflammation. In addition, through quantitative detection and multiple regression analysis, Lang et al. found that there was a significant correlation between TGFBI and high-grade urothelial carcinoma, suggesting that TGFBI may be used as an early diagnostic marker of high-grade urothelial carcinoma. Furthermore, Lin et al. found that TGFBI may be involved in the occurrence and development of GBM by regulating TGF-β signaling pathway. Similarly, Guo et al. proved that TGFBI may regulate apoptosis and proliferation, and then affect the prognosis of patients GBM by immunohistochemistry and cell experiment. Consistent with the above study, we found that TGFBI was highly expressed in patients with GBM. Survival analysis showed that the prognosis of patients with high expression of TGFBI was poor. And we verified the high expression of TGFBI in GBM patients by reverse transcriptase polymerase chain reaction. We speculate that TGFBI may be involved in the occurrence and development of GBM by regulating cell proliferation, collagen synthesis and interstitial environment. TGFBI may be used as a target for early diagnosis and specific treatment of GBM, and the related mechanism is worthy of further exploration.

Although this study has carried out rigorous bioinformatics analysis, there are still some shortcomings. Firstly, the sample size in the dataset is small, and it is still necessary to further expand the sample size to get more accurate results. Secondly, this paper only carried out a small sample verification. Animal experiments for comprehensive verification and functional experiments in order to further understand the molecular mechanism of GBM were necessary.

In conclusion, bioinformatics technology could be a useful tool to predict progression of GBM and to explore the mechanism of GBM. In addition, there are DEGs between GBM tumor and normal brain tissues. These molecules may be involved in the mechanism of the occurrence and development of GBM, and may be used as molecular targets for early diagnosis and specific treatment.

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Shuyuan Zhang and Weiwei Zhang analyzed the data and were major contributors in writing. Caixing Sun and Liang Xia were involved in critically revising manuscript for important intellectual content. Bin Wu, Liwen Li, Kai Jin and Yangfan Zou made substantial contributions to research conception and designed the draft of the research process. All authors read and approved the final manuscript.

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