Screening and authentication of molecular markers in malignant glioblastoma based on gene expression profiles

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Abstract. Glioblastoma (GBM) is a malignant tumor of the central nervous system with high mortality rates. Gene expression profiling may determine the chemosensitivity of GBMs. However, the molecular mechanisms underlying GBM remain to be determined. To screen the novel key genes in its occurrence and development, two glioma databases, GSE122498 and GSE104291, were analyzed in the present study. Bioinformatics analyses were performed using the Database for Annotation, Visualization and Integrated Discovery, the Search Tool for the Retrieval of Interacting Genes, Cytoscape, cBioPortal, and Gene Expression Profiling Interactive Analysis softwares. Patients with recurrent GBM showed worse overall survival rate. Overall, 341 differentially expressed genes (DEGs) were authenticated based on two microarray datasets, which were primarily enriched in ‘cell division’, ‘mitotic nuclear division’, ‘DNA replication’, ‘nucleoplasm’, ‘cytosol, nucleus’, ‘protein binding’, ‘ATP binding’, ‘protein C-terminus binding’, ‘the cell cycle’, ‘DNA replication’, ‘oocyte meiosis’ and ‘valine’. The protein-protein interaction network was composed of 1,799 edges and 237 nodes. Its significant module had 10 hub genes, protein-protein interaction network was composed of 1,799 edges and 237 nodes. Its significant module had 10 hub genes, DLGAP5, CDK1, TOP2A, ASPM, MELK, BUB1B,idity, nuclear division, and Gene Expression Profiling Interactive Analysis softwares. Patients with recurrent GBM showed worse overall survival rate. Overall, 341 differentially expressed genes (DEGs) were authenticated based on two microarray datasets, which were primarily enriched in ‘cell division’, ‘mitotic nuclear division’, ‘DNA replication’, ‘nucleoplasm’, ‘cytosol, nucleus’, ‘protein binding’, ‘ATP binding’, ‘protein C-terminus binding’, ‘the cell cycle’, ‘DNA replication’, ‘oocyte meiosis’ and ‘valine’. The protein-protein interaction network was composed of 1,799 edges and 237 nodes. Its significant module had 10 hub genes, DLGAP5, CDK1, TOP2A, ASPM, MELK, BUB1B, CENPB, CCNB1, NDC80, and MTLK.

Introduction

Gliomas are one of the common primary neoplasms of the brain, and it is caused by carcinogenesis of the brain and spinal glial cells (1). The annual incidence is 3-8 cases per 100,000. Just as with other types of tumor (such as gastric, breast and colorectal cancer), gliomas are also induced by the interaction between genetic high-risk factors and environmental factors, including air pollution and ionizing radiation (2). Certain known diseases are also genetic susceptibility factors of gliomas, such as neurofibromatosis (type I) and tuberculous sclerotic diseases (3). According to the grading system set by the World Health Organization (WHO) (4), gliomas are classified into four grades: Grade 1, the slightest with the most favorable prognosis; to grade 4, the most severe with the worst prognosis of all grades. The anaplastic gliomas, in terms of traditional cytology, are classified as grade 3, and glioblastoma (GBM) is classified as grade 4 (5).

GBM is the most common and lethal malignant primary brain tumor in adults, and is a member of a group of tumors known as gliomas (6). GBM is evolved from astrocytic neuroglial cell lineage that supports neurocytes, and it accounts for 12-15% of intracranial tumors, and 50-60% of astrocytic tumors (7). The molecular mechanism underlying GBM progression remains unclear, however there is an increasing number of studies suggesting genetic mutations (8-10).
Numerous previous studies have performed bioinformatic analyses to investigate differentially expressed genes (DEGs) in patients with GBM, as well as their roles in different pathways, molecular functions and biological processes (11-13). The overall survival rate is different in patients with GBM to those with different mutation statuses of isocitrate dehydrogenase (IDH1), and a previous study has demonstrated that patients with GBM that possess mutated IDH1 have an improved prognosis (14). A total of 23 differently expressed microRNAs (miRNAs) were selected in patients with GMB that possessed mutated and wild IDH1, and these miRNAs were identified as IDH1 mutation miRNAs (11). A molecular marker consisting of 10 miRNAs was identified and validated in the GBM in a previous study; among these, 7 were considered as IDH1 mutation miRNAs (11). To identify molecular markers, these genetic molecular markers have been used to store genetic data (23). Two expression profiling datasets [GSE122498 (GPL570 platform) (24) and GSE104291 (GPL570 platform) (25)] were obtained from the GEO. The datasets of GSE122498 contained 1 normal sample and 16 GBM samples. Similarly, GSE104291 consisted of 2 normal samples and 24 GBM samples.

**DEGs identified by GEO2R.** GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) is an interactive online tool to identify DEGs from GEO series (26). GEO2R could be applied to distinguish DEGs between normal brain tissue and GBM tissue samples. According to the method by Benjamini and Hochberg (false discovery rate) (27), the tool could alter the P-values, so as to obtain the adjusted P-values (adj. P), and to maintain a balance between the possibility of false-positives and detection of statistically significant genes. If one probe set does not have the homologous gene, or if one gene has numerous probe sets, the data will be removed. The rule of statistical significance is that adj. P≤0.01 and fold change (FC) ≥2 were considered to indicate a statistically significant result.

**Functional annotation for DEGs with the KEGG and GO analysis.** DAVID (https://david.ncifcrf.gov/home.jsp; version 6.8), is an online analysis tool suite with the function of Integrated Discovery and Annotation (28). GO is an ontology tool widely used in bioinformatics, which covers three aspects of biology, including ‘biological process (BP)’, ‘cellular component (CC)’ and ‘molecular function (MF)’ (29). KEGG (www.kegg.jp), is one of the most commonly used biological information databases in the world (30). To analyze GO and the biological pathway information of the DEGs, the DAVID online tool was implemented. P<0.05 was considered to indicate a statistically significant result. The UCSC Genome Browser (genome.ucsc.edu) is a graphical viewer for exploring genome annotations and was used to hierarchically cluster key genes.

**Construction of the PPI network and identification of significant modules.** Search Tool for the Retrieval of Interacting Genes (STRING; string.embl.de), an online open tool, was applied to construct the PPI network (31). Cytoscape (version 3.6.1), a free visualization software, was used to present the network (32). A confidence score >0.4 was considered the criterion of judgment. The Molecular Complex Detection (MCODE) (version 1.5.1; a plug-in of Cytoscape) subsequently identified the most important module of the network map (33). The criteria of the MCODE analysis is that the degree of cut-off=2, MCODE scores >5, Max depth=100, node score cut-off=0.2, and k-score=2.

**Analysis and identification of hub genes.** When the degrees were set (degrees ≥10), the hub genes were excavated. Subsequently, with the KEGG and GO analysis in the DAVID database, functional annotation for the hub genes was performed. One co-expression network of these hub genes and a survival analysis was obtained using eBioPortal (www.ebioportal.org) (34). Furthermore, The Biological Networks Gene Oncology tool (version 3.0.3) was used to analyze and visualize the hub genes’ ‘CC’, ‘BP’ and ‘MF’ (35). The clustering analysis of hub genes was performed using OmicShare.
(version: 2015-2019; www.omicshare.com/tools/index.php), and the University of California Santa Cruz (UCSC) Xena software (xena.ucsc.edu/welcome-to-ucsc-xena) was used to securely analyze and visualize the hub genes in the scope of public genomic datasets. The expression profiles of these hub genes were analyzed and displayed using the online database Gene Expression Profiling Interactive Analysis (GEPIA; gepia.cancer-pku.cn). The unpaired Student’s t-test was used to make the comparisons between the normal sample and GBM samples. P<0.05 was used to indicate statistically significant results. The Kaplan-Meier plotter (www.kmplot.com) was used to perform the overall survival rate analysis. The log rank test was used to compare survival curves.

**Results**

**Screening of DEGs in GBM samples.** Following the analysis of the datasets (GSE122498 and GSE104291) with GEO2R, the differences between control and GBM tissues were presented in volcano plots (Fig. 1A and B). The analysis of GSE122498 and GSE104291 identified 1,079 and 4,202 DEGs, respectively (Fig. 1C). The Venn diagram revealed that the common part between the 2 datasets included 341 DEGs.

**Functional annotation for DEGs with the KEGG and GO analysis.** The results of the GO analysis demonstrated that variations in the BP were primarily enriched in ‘cell division’, ‘mitotic nuclear division’ and ‘DNA replication’. Changes in CC were primarily enriched in the nucleoplasm, cytosol and nucleus. The variations in MF were enriched in protein binding, ATP binding and protein C-terminus binding. The KEGG analysis demonstrated that DEGs were prevalently enriched in the ‘cell cycle’, ‘DNA replication’, ‘oocyte meiosis’, ‘valine, leucine and isoleucine degradation’, ‘fanconi anemia pathway’, ‘GABAergic synapse’, ‘dopaminergic synapse’, and ‘endocrine and other factor-regulated calcium reabsorption’ (Table I).

**Construction of the PPI network and identification of significant module and hub genes.** The PPI network was constructed and significant modules were identified, with 1,799 edges and 237 nodes in the PPI network (Fig. 1D), and 884 edges and 44 nodes in the significant module (Fig. 1E). Degrees ≥10 were considered as the criterion of judgment, which was the criterion to determine significance. Overall, 10 genes were identified as hub genes within Cytoscape: CDK1, BUB1B, NDC80, NCAFG, BUB1, CCNB1, TOP2A, DLGAP5, ASPM and ME2 (Fig. 1F). Among the hub genes, CDK1 and BUB1B had the highest scores, suggesting that they may play important roles in the occurrence or development of GBM. With DAVID, the KEGG and GO analyses of DEGs involved in hub genes were analyzed. The results revealed that these genes were prevalently enriched in ‘cell division’, ‘mitotic nuclear division’, ‘cell proliferation’, ‘condensed nuclear chromosome outer kinetochore’, ‘kinetochore’, ‘condensed chromosome kinetochore’, ‘protein kinase activity’, ‘protein serine/threonine kinase activity’ and ‘histone kinase activity’. Analyses of the KEGG pathway indicated that significant genes were primarily enriched in the ‘cell cycle’, ‘progesterone-mediated oocyte maturation’, ‘oocyte meiosis’ and the ‘p53 signaling pathway’ (Table II).

**Hub genes analysis.** According to the Gene cards, summaries for the function of the 10 hub genes were obtained (Table III). A co-expression network of these significant genes was obtained using cBioPortal (Fig. 1G). The BP, CC and MF analysis for these genes is presented in Fig. 2A-C. Then, a Kaplan-Meier plotter was used to perform the survival analysis. Patients with recurrent GBM demonstrated worse overall survival rate (Fig. 3A). According to the UCSC analysis, hierarchical clustering indicated that these hub genes may differentiate those individuals with GBM from the normal individuals (Fig. 3B). The hub genes were identified between non-GBM samples and GBM samples. It was demonstrated that the expressions of hub genes were upregulated in the GBM (including recurrent and primary GBM), when compared with the solid tissue, non-GBM samples (Fig. 3B). Therefore, the expression patterns of hub genes did not seem to demonstrate any significant difference between primary tumors and recurrent tumors, although relapsed tumors often demonstrated higher aggressiveness compared with primary tumors. The authors suggested that if a differentiation between the recurrent tumor and the primary tumor is required, the initiatory groups should be set as recurrent and primary GBM in the GEO database. Heat maps revealed that the hub genes could differentiate the GBM samples from the non-GBM samples (Fig. 3C and D). Fig. 3C primarily presents the expression levels of hub genes in the GSE122498, but it could also demonstrate that the expression levels of all hub genes in the non-GBM samples were downregulated when compared with the GBM samples. This study may further verify the aforementioned differences in the expression levels of hub genes between non-GBM samples and GBM samples (Fig. 3D).

As there were different samples or individuals between the GSE122498 and GSE104291 databases, the names of the different samples are presented at the bottom of Fig. 3C and D. In addition, individual variation exists between the different samples. Therefore, the clustering patterns between datasets GSE122498 and GSE104291 were similar, but not identical. The expression profile of hub genes in human tissue was demonstrated using GEPIA. It was revealed that these genes in GBM were present in higher levels when compared with the matched normal samples (Fig. 4).

**Discussion**

Gliomas are one of the most common primary malignant tumors of the brain, and there are different histological grades and classifications for it (19). According to the WHO, gliomas are classified into four grades: Grade I-IV; and into three pathological types: Astrocytoma, oligodendroglioma and mixed (astrocytoma and oligodendroglioma) gliomas (19). GBM belongs to grade IV glioma with high fatality rate and different severity and histological subtypes (36,37). It is not sensitive to radiotherapy or chemotherapy, and is prone to malignant progression; it lacks clear molecular classification, therapeutic targets and associated targeted drugs (19). The standard treatment for GBM is surgery, followed by radiotherapy, or radiotherapy combined with chemotherapy. If surgery is not practical, radiotherapy or radiotherapy/chemotherapy could be given (38). GBM is capable of extensively invading and infiltrating the normal
Figure 1. Identification of DEGs and PPI network construction. (A) Volcano plot presents the difference between non-GBM and GBM tissues after analysis of the datasets GSE122498 with GEO2R. Downregulated genes are presented in green, and upregulated genes are presented in red. (B) Volcano plot presents the difference between non-GBM and GBM tissues after analysis of the datasets GSE104291 with GEO2R. The green presents the down-regulated genes, and the red presents the up-regulated genes. (C) Venn diagram indicated that 341 genes were contained in the GSE122498 and GSE104291 datasets simultaneously. (D) The PPI network of DEGs was constructed using Cytoscape. (E) Most significant module was obtained from PPI network of DEGs using MCODE, including 44 nodes and 884 edges. (F) Network of hub genes. (G) Hub genes and their co-expression genes were analyzed using cBioPortal. Nodes with a bold black outline represent hub genes. Nodes with a thin black outline represent the co-expression genes. *P<0.05. GBM, glioblastoma; DEGs, differentially expressed genes; PPI, protein-protein interaction; GEO, Gene Expression Omnibus; MCODE, Molecular Complex Detection.
surrounding brain tissue, making it impossible to completely remove the tumor tissue (5). Following surgery, radiotherapy could kill the remaining tumor cells and prevent recurrence, but it can damage a large number of normal brain cells (38). Even with the best treatment, the recurrence rate of GBM remains high, and was estimated to be 3.20/100,000 worldwide in 2018 (39). Therefore, research into an accurate understanding of the underlying molecular mechanism and reliable therapeutic targets of GBM has generated wide concern.

With the progress of gene-sequencing technology, a large number of DEGs have been identified in a number of other types of tumor (such as gastric, breast and colorectal cancer) (21,22). DEGs may serve a variety of functions in the occurrence and development of diseases, such as transcription, post-transcriptional processing and the regulation of protein expression. The present study aimed to identify the DEGs that play a key role in the occurrence and malignant process of gliomas and that may serve as molecular markers and therapeutic targets for GBM.
CDK1 is a cell cycle regulatory gene (40). According to the results from the present study, the expression levels of CDK1 in GBM tissues were significantly increased compared with normal tissues. The occurrence of tumors is a complex process with multiple damages to normal cell genomes (41). These damages include not only oncogene activation, but also inactivation or deletion of tumor suppressor gene (42). A previous study has revealed that the functional effects of polygene would eventually aggregate into the cell cycle mechanism (43). Among them, the two key checkpoints of the G1/S and G2/M phases of the cell cycle are the primary causes of malignant proliferation (44). However, a compound formed by combining CDK1 with Cyclin B1, the mitotic promoting factor, plays an important role in the G2/M checkpoint of the cell cycle (45). A previous study indicated that the positive degree of CDK1 expression could reflect the malignant degree of tongue squamous cell carcinoma (46). This in turn suggests that CDK1 overexpression may induce genetic mutations and chromosome structural abnormalities, leading to failure of checkpoint regulation of the cell cycle G2/M, which accelerates the progression of the cell cycle and excessive cell proliferation, resulting in tumor development (46). Overexpression of CDK1 was also observed in pancreatic cancer and lung cancer (47,48). Therefore, CDK1 has been indicated to play an important role in tumor occurrence as it may be associated with the occurrence and development of GBM, and the result may provide potential novel insights for further research into the association between GBM and CDK1 expression.

According to the results of the present study, compared with normal tissues, the expression of BUB1B in GBM tissues increased significantly. Mitosis is the process by which a eukaryotic cell divides into two identical cells, and it plays a crucial role in the evolution and homeostasis of multicellular organisms (49). The mitotic checkpoint is the core regulator during this process, acting as a signal-regulating mechanism that prevents the cell from entering the late stage of mitosis before all chromosomes adhere to the spindle (50). Abnormalities in mitotic checkpoints have been observed in numerous different types of tumor (such as gastric, breast and colorectal cancer), and one of the consequences of abnormalities of mitotic checkpoints is chromosomal instability that make cells more susceptible to malignancy (51). BUB1B is an important constituent protein of the mitotic checkpoint, and is a multidomain protein kinase that responds to centromere tension (52,53). Studies have demonstrated that BUB1B is overexpressed in various different types of tumor, such as renal and breast carcinoma, and its mutation and overexpression are associated with chromosomal instability (54-56). Therefore, further investigation on BUB1B may lead to a greater understanding of its importance in the GBM process, and novel ideas for investigating its molecular mechanisms and establishing more effective treatments.

### Table III. Summaries for the function of 10 hub genes.

| No. | Gene symbol | Full name                                          | Function                                                                 |
|-----|-------------|----------------------------------------------------|--------------------------------------------------------------------------|
| 1   | CDK1        | Cyclin dependent kinase 1                          | Plays a key role in the control of the eukaryotic cell cycle by modulating the centrosome cycle as well as mitotic onset; promotes G2-M transition, and regulates G1 progression and G1-S transition via association with multiple interphase cyclins. |
| 2   | BUB1B       | BUB1 mitotic checkpoint serine/threonine kinase B | Implicated in triggering apoptosis in polyploid cells that exit aberrantly from mitotic arrest. May play a role for tumor suppression. |
| 3   | NDC80       | NDC80, kinetochore complex component               | Acts as a component of the essential kinetochore-associated NDC80 complex, which is required for chromosome segregation and spindle checkpoint activity. |
| 4   | NCAPG       | Non-SMC condensin I complex subunit G              | Regulatory subunit of the condensin complex, a complex required for conversion of interphase chromatin into mitotic-like condense chromosomes. |
| 5   | BUB1        | BUB1 mitotic checkpoint                           | Serine/threonine-protein kinase that performs 2 crucial functions during mitosis: It is essential for spindle-assembly checkpoint signaling and for correct chromosome alignment. |
| 6   | CCNB1       | Cyclin B1                                         | Essential for the control of the cell cycle at the G2/M (mitosis) transition. |
| 7   | TOP2A       | Topoisomerase II alpha                            | Diseases associated with TOP2A include Female Breast Cancer and Malignant Peripheral Nerve Sheath Tumor. Among its related pathways are Cell Cycle, Mitotic and SUMOylation. |
| 8   | DLGAP5      | DLG associated protein 5                          | Potential cell cycle regulator that may play a role in carcinogenesis of cancer cells. Mitotic phosphoprotein regulated by the ubiquitin-proteasome pathway. |
| 9   | ASPM        | Abnormal spindle microtubule assembly             | Involved in mitotic spindle regulation and coordination of mitotic processes |
| 10  | MELK        | Maternal embryonic leucine zipper kinase          | Diseases associated with MELK include Uterine Corpus Endometrial Carcinoma. Among its related pathways are Neuroscience. |
Figure 2. Enrichment analysis via The Biological Networks Gene Oncology tool. (A) Biological process, (B) cellular components, and (C) molecular function analysis of hub genes was constructed. The color depth of nodes refers to the corrected P-value of ontologies, and the deepest shade represented the most significant enrichment term. The size of nodes refers to the numbers of genes that are involved in the ontologies. P<0.01 was considered to indicate a statistically significant difference.
The NDC80 complex is located on the outer layer of the kinetochore, linking the kinetochore and microtubules (57). It is involved in regulating the normal separation of chromosomes in mitosis, and is also crucial for spindle assembly checkpoints (58). NDC80 is the main component of the NDC80 compound and is highly expressed in actively dividing cells, such as tumor cells. NDC80 plays an important role in normal mitosis, the assembly of kinetochore, the spindle checkpoint, maintenance of chromosomal stability and the occurrence and development of tumors (59,60). According to the results from the present study, the expression levels of NDC80 in GBM tissues were significantly increased compared with normal tissues. Another study indicated that overexpression of NDC80 could result in sustained hyperactivation of mitotic checkpoints and therefore induce tumor formation (61). The expression levels of Mad2 were also significantly increased in mice with high expression of NDC80, and a previous report has demonstrated that overexpression of the Mad2 gene would cause hyperactivation of mitotic checkpoints, resulting in the production of aneuploid chromosomes, which induces tumor formation (62). In summary, the present study revealed that NDC80 is highly expressed in GBM, and high expression
levels of NDC80 may play an important role in the occurrence and development of GBM. Studies on the molecular mechanism of NDC80 in the occurrence and development of GBM are useful for investigating the role of NDC80 as a target of intervention for GBM treatment. Future studies could observe tumor changes in a GBM animal model following knockdown...
of BUB1B (or NDC80). Upregulation of BUB1B expression (or NDC80) could then be observed and variations in the tumor could be recorded. If the knockdown of BUB1B (or NDC80) could decrease the size of GBM and upregulation of BUB1B (or NDC80) could deteriorate the GBM, then it could be suggested that a high expression level of BUB1B (or NDC80) is a risk factor for GBM development. Clinical trials could then be performed in order to verify the curative effect of gene therapy via downregulation of BUB1B (or NDC80) in patients with GBM.

The present study, however, has certain limitations. The screening of 10 key genes is based on bioinformatics analysis, which is an observational study that could only provide clues for further studies on the mechanisms underlying the occurrence and development of GBM. The screened target genes were validated by sequencing databases, such as The Cancer Genome Atlas and GEPIA. In addition, clinical samples need to be collected in order to verify the aforementioned conclusions. However, the present study only provides a potential theory or idea for the mechanism and/or development of a treatment strategy for GBM. Finally, the sample size of this research is small, and it maybe cause the false positive result. Further investigation on the association between mutations in IDHs or the methylation status of the MGMT promoter and the expression levels of hub genes identified in the present study is required.

In conclusion, the present study aimed to identify differentially expressed genes that may be present in the occurrence or development of GBM. Finally, 341 DEGs and 10 hub genes were identified between GBM samples and normal samples, which could be used as diagnostic and therapeutic biomarkers for GBM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YFZ and LBM performed the experiment, and were major contributors in writing and submitting the manuscript. XY made substantial contributions to research conception and also drafted the research protocol. ZKH made substantial contributions to analysis and interpretation of data. MJS made substantial contributions to research conception and analysis and interpretation of data. In addition MJS was involved in drafting the manuscript. CHH and DYW were responsible for analyzing the gene expression data and generating the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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