Identification of core differentially methylated genes in glioma

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Abstract. Differentially methylated genes (DMGs) serve a crucial role in the pathogenesis of glioma via the regulation of the cell cycle, proliferation, apoptosis, migration, infiltration, DNA repair and signaling pathways. This study aimed to identify aberrant DMGs and pathways by comprehensive bioinformatics analysis. The gene expression profile of GSE28094 was downloaded from the Gene Expression Omnibus (GEO) database, and the GEO2R online tool was used to find DMGs. Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the DMGs were performed by using the Database for Annotation Visualization and Integrated Discovery. A protein-protein interaction (PPI) network was constructed with Search Tool for the Retrieval of Interacting Genes. Analysis of modules in the PPI networks was performed by Molecular Complex Detection in Cytoscape software, and four modules were performed. The hub genes with a high degree of connectivity were verified by The Cancer Genome Atlas database. A total of 349 DMGs, including 167 hypermethylation genes, were enriched in biological processes of negative and positive regulation of cell proliferation and positive regulation of transcription from RNA polymerase II promoter. Pathway analysis enrichment revealed that cancer regulated the pluripotency of stem cells and the PI3K-AKT signaling pathway, whereas 182 hypomethylated genes were enriched in biological processes of immune response, cellular response to lipopolysaccharide and peptidyl-tyrosine phosphorylation. Pathway enrichment analysis revealed cytokine-cytokine receptor interaction, type I diabetes mellitus and TNF signaling pathway. A total of 20 hub genes were identified, of which eight genes were associated with survival, including notch receptor 1 (*NOTCH1*),

SRC proto-oncogene (also known as non-receptor tyrosine kinase, *SRC*), interleukin 6 (*IL6*), matrix metallopeptidase 9 (*MMP9*), interleukin 10 (*IL10*), caspase 3 (*CASP3*), erb-b2 receptor tyrosine kinase 2 (*ERBB2*) and epidermal growth factor (*EGF*). Therefore, bioinformatics analysis identified a series of core DMGs and pathways in glioma. The results of the present study may facilitate the assessment of the tumorigenicity and progression of glioma. Furthermore, the significant DMGs may provide potential methylation-based biomarkers for the precise diagnosis and targeted treatment of glioma.

Introduction

Glioma is a type of primary central nervous system (CNS) solid tumor that arises from glial cells and is aggressive and lethal (1). It comprises ~30% of all CNS tumors and 80% of all malignant brain tumors (2). According to the 2016 World Health Organization (WHO) guidelines, gliomas can be classified into grades I-IV depending on 'integrated diagnosis', combining histopathological and molecular features (3). The most malignant type is grade IV glioblastoma (GBM), the overall survival (OS) time of which is only ~14 months (4). Lower grade glioma (LGG; grades II and III) exhibits benign tendencies and leads to a favorable prognosis for patients (5). However, it has a high rate of recurrence and increases in grade over time (6). Currently, the common treatment strategies for the treatment of glioma involve surgical resection, after which adjuvant chemotherapy and/or radiotherapy are used; however, the clinical outcomes of glioma remain unsatisfactory. The pathogenesis of glioma involves multiple factors and steps, comprising genetic and epigenetic alterations. Despite a number of mechanisms of oncogenesis have been verified in glioma, including retinoblastoma, p53 and receptor tyrosine kinase signaling pathways, the genetic factors and precise mechanism of human glioma remain poorly understood (7). Therefore, vital molecular biomarkers and oncogenic pathways associated with accurate diagnosis and patient survival in glioma require further identification.

Aberrant CpG island methylation is a primary epigenetic modified form of DNA sequence in malignancy (8). The functions of key genes can be altered through methylation to modify their expression, including hypermethylation of tumor suppressor genes (TSGs) and hypomethylation of oncogenes (9,10). The methylation status of multiple TSGs may

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serve as a biomarker for the early diagnosis and prediction of prognosis (11). For example, promoter methylation-induced silencing of the O6-methylguanine-DNA methyltransferase DNA-repair gene has been demonstrated to be a powerful predictor of the sensitivity of alkylating chemotherapy in patients with glioma with long survival rates (12). The methylation level of death associated protein kinase 1 is associated with clinical features and outcomes of glioma; hypermethylation at site-1,527 or together with site-1,543 indicates good sensitivity to postoperative therapies, particularly radiotherapy (13). A number of TSGs associated with cell cycle, proliferation and DNA repair have been identified in glioma, including cyclin dependent kinase inhibitor 2A (*CDKN2A*, also known as *P16*[INK4a], *P14*[ARF]), TNF receptor superfamily member 11a (*TNFRSF11A*) and nuclear receptor binding SET domain protein 1 (*NSD1*) (14-17). Therefore, identifying differentially methylated genes (DMGs) by comparing the differences in DNA methylation status of glioma with that of normal brain tissue is crucial for elucidating the development, progression and metastasis of glioma and may be used as a guide for targeting drugs for glioma.

At present, microarray analysis based on high-throughput platforms is an efficient tool that has been applied to screen virtual genetic or epigenetic alterations in carcinogenesis, and to identify biomarkers for the diagnosis and prognosis of cancer (18). In the present study, DMGs were examined in glioma by online bioinformatics resources. The expression profile of GSE28094 from the Gene Expression Omnibus (GEO) database was downloaded to identify the DMGs between glioma and normal brain tissue. Subsequently, hierarchical clustering and functional enrichment analyses of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by Database for Annotation Visualization and Integrated Discovery (DAVID) for the DMGs were performed. A protein-protein interaction (PPI) network of DMGs was constructed and the main hub genes with high degree of connectivity were examined by Search Tool for the Retrieval of Interacting Genes (STRING), and the results were further validated by The Cancer Genome Atlas (TCGA) data to identify DMGs in the expression of glioma. This study provided comprehensive biological information for DMGs and novel targets for the diagnosis and accurate treatment of glioma.

Materials and methods

Microarray data. To identify DMGs between glioma and normal brain tissue, the GSE28094 gene expression microarray, contributed by Fernandez *et al* (19), was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/gds/) of the National Center for Biotechnology Information, a public functional genomics data repository. GSE28094 was based on the GPL9183 platform (Illumina GoldenGate Methylation Cancer Panel I). For the present study, 90 gliomas and 6 normal brain tissues were collected from GSE28094.

Data processing of DMGs. GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) online software was used to analyze GSE28094 and detect DMGs between gliomas and normal brain tissues. GEO2R is an interactive online tool in which ≥2

groups of samples can be compared in a GEO series to display differentially expressed genes under specific experimental conditions (20). The adjusted P-values were used to decrease the false positive rate using the Benjamini and Hochberg false discovery rate method. Adjusted P<0.05 and |t|>2 were used as the cut-off values for detecting DMGs. Finally, 349 DMGs were obtained, including 167 upregulated and 182 downregulated genes.

GO and KEGG pathway analyses of DMGs. GO analysis served as a crucial tool to annotate genes and gene products and to identify characteristic biological functions using high-throughput genome or transcriptome data (21), including biological process (BP), cellular component (CC) and molecular function (MF). KEGG is a collection of databases that can help to annotate genomes, biological pathways, diseases, chemical substances and drugs (22). DAVID (version 6.8; https://david.ncifcrf.gov/) was used to perform KEGG pathway enrichment analysis for the selected DMGs. P<0.05 was considered to indicate a statistically significant difference.

PPI network and module analysis. PPI analysis was performed to illustrate the interactions and functions of the selected DMGs via STRING (version 11.0; http://string-db.org). Cytoscape is a vital workflow component for executing network visualization, analysis and publishing tasks (23). To illustrate the potential interactions among those DMGs, STRING in Cytoscape (version 3.6.0) was applied and the DMGs were mapped into STRING. A confidence score ≥0.4 and a maximum number of interactors=0 were set as the cut-off criterion. Molecular complex detection (MCODE, version 1.5.1) is a method to analyze densely connected regions in PPI networks (24). This method was used to screen modules of the PPI network in Cytoscape with a degree cut-off=2, node score cut-off=0.2, k-core=2 and maximum depth=100. The pathway analysis of genes in these modules was also performed by DAVID. In addition, the top 20 hub genes with a high degree of connectivity were inserted into STRING with a confidence score of ≥0.4 and a maximum number of interactors=0. GO and KEGG pathway analyses were also used to examine the potential interactions.

TCGA data validation of the hub DMGs. In view of the small sample size of this study, further validation analysis was performed to verify the results of TCGA data. GEPIA (http://gepia.cancer-pku.cn/index.html) is a publicly available interactive web server that can analyze the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from TCGA and GTEx projects by a standard processing pipeline (25). It supports customizable functions, including tumor and normal tissue differential expression analyses, and information can be obtained on the expression level of hub DMGs in glioma and normal brain tissue. Boxplots were displayed to visualize the association. By acquiring pathologically confirmed immunohistochemical data of glioma and normal brain tissue based on the Human Protein Atlas (HPA; https://www.proteinatlas.org/), the expression of these hub DMGs was further validated. Hub DMGs in glioma were identified using HPA. From the extensive sample collection of antibodies against the DMGs, the sample with the strongest

Table I. GO analysis of differentially methylated genes associated with glioma.

A, Hypermethylation

GO analysis	Term	Count	%	P-value
GOTERM_BP_DIRECT	GO:0008285~negative regulation of cell proliferation	23	14.7436	1.43×10^{-11}
GOTERM_BP_DIRECT	GO:0008284~positive regulation of cell proliferation	24	15.3846	5.14×10^{-11}
GOTERM_BP_DIRECT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	34	21.7949	6.75×10^{-11}
GOTERM_BP_DIRECT	GO:0046777~protein autophosphorylation	15	9.6154	6.80×10^{-10}
GOTERM_BP_DIRECT	GO:0042981~regulation of apoptotic process	16	10.2564	1.23×10^{-9}
GOTERM_CC_DIRECT	GO:0005737~cytoplasm	79	50.6410	6.58×10^{-9}
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	61	39.1026	3.46×10^{-6}
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	45	28.8462	1.77×10^{-5}
GOTERM_CC_DIRECT	GO:0048471~perinuclear region of cytoplasm	18	11.5385	2.05×10^{-5}
GOTERM_CC_DIRECT	GO:0005576~extracellular region	30	19.2308	7.41×10^{-5}
GOTERM_MF_DIRECT	GO:0005515~protein binding	121	77.5641	4.77×10^{-12}
GOTERM_MF_DIRECT	GO:0008134~transcription factor binding	16	10.2564	4.30×10^{-8}
GOTERM_MF_DIRECT	GO:0046982~protein heterodimerization activity	18	11.5385	1.01×10^{-6}
GOTERM_MF_DIRECT	GO:0005102~receptor binding	15	9.6154	3.80×10^{-6}
GOTERM_MF_DIRECT	GO:0005524~ATP binding	32	20.5128	8.54×10^{-6}

B, Hypomethylation

GO analysis	Term	Count	%	P-value
GOTERM_BP_DIRECT	GO:0006955~immune response	32	18.4971	1.89×10^{-18}
GOTERM_BP_DIRECT	GO:0071222~cellular response to lipopolysaccharide	15	8.6705	6.78×10^{-12}
GOTERM_BP_DIRECT	GO:0018108~peptidyl-tyrosine phosphorylation	16	9.2486	3.60×10^{-11}
GOTERM_BP_DIRECT	GO:0006954~inflammatory response	22	12.7168	2.12×10^{-10}
GOTERM_BP_DIRECT	GO:0022617~extracellular matrix disassembly	10	5.7804	6.50×10^{-8}
GOTERM_CC_DIRECT	GO:0005615~extracellular space	56	32.3699	3.22×10^{-22}
GOTERM_CC_DIRECT	GO:0005576~extracellular region	55	31.7919	6.24×10^{-18}
GOTERM_CC_DIRECT	GO:0009986~cell surface	22	12.7168	2.62×10^{-8}
GOTERM_CC_DIRECT	GO:0005887~integral component of plasma membrane	35	20.2312	1.66×10^{-7}
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	67	38.7283	6.33×10^{-7}
GOTERM_MF_DIRECT	GO:0005125~cytokine activity	20	11.5607	1.17×10^{-14}
GOTERM_MF_DIRECT	GO:0004713~protein tyrosine kinase activity	17	9.8266	2.94×10^{-13}
GOTERM_MF_DIRECT	GO:0008083~growth factor activity	15	8.6705	8.36×10^{-10}
GOTERM_MF_DIRECT	GO:0046934~phosphatidylinositol-4,5-bisphosphate 3-kinase activity	9	5.2023	1.74×10^{-7}
GOTERM_MF_DIRECT	GO:0004715~non-membrane spanning protein tyrosine kinase activity	8	4.6243	3.29×10^{-7}

GO, Gene Ontology; BP, biological process; CC, cell component; MF, molecular function.

intensity and quantity was selected. The normal brain tissue stained for caspase 3 (*CASP3*) expression was from a female patient (age, 45 years; patient ID, 1539; staining, not detected; intensity, negative; quantity, negative; location, none), and the glioma tissue was from a male patient (age, 56 years; patient ID, 131; staining, medium; intensity, moderate; quantity, 75-25%; location, nuclear). The normal brain tissue stained for erb-b2 receptor tyrosine kinase 2 (*ERBB2*) expression was from a female patient (age, 54 years; patient ID, 2523; staining, not detected; intensity, negative; quantity, negative; location, none), and the glioma tissue was from a male patient (age, 66 years; patient ID, 206; staining, high; intensity, strong; quantity, >75%; location, cytoplasmic/membranous).

Survival analysis of hub genes. GEPIA was used to further investigate the relapse-free survival and OS time data of the

Table II. KEGG pathway analysis of differentially methylated genes associated with glioma.

A, Hypermethylation

KEGG analysis	Term	Count	%	P-value
KEGG_PATHWAY	hsa05200:Pathways in cancer	35	22.4	1.73×10^{-17}
KEGG_PATHWAY	hsa04550:Signaling pathways regulating pluripotency of stem cells	15	9.6	2.32×10^{-8}
KEGG_PATHWAY	hsa04151:PI3K-AKT signaling pathway	21	13.5	2.17×10^{-7}
KEGG_PATHWAY	hsa04510:Focal adhesion	16	10.3	4.91×10^{-7}
KEGG_PATHWAY	hsa05218:Melanoma	10	6.4	1.17×10^{-6}

B, Hypomethylation

KEGG analysis	Term	Count	%	P-value
KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	25	14.5	1.68×10^{-12}
KEGG_PATHWAY	hsa04940:Type I diabetes mellitus	12	6.9	6.46×10^{-11}
KEGG_PATHWAY	hsa05332:Graft-vs.-host disease	11	6.4	9.97×10^{-11}
KEGG_PATHWAY	hsa05323:Rheumatoid arthritis	15	8.7	1.94×10^{-10}
KEGG_PATHWAY	hsa04668:TNF signaling pathway	15	8.7	2.79×10^{-9}

KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, *Homo sapiens*.

DMGs by Kaplan-Meier survival curves. The hazard ratio (HR) with 95% confidence intervals and log-rank P-value were calculated and indicated in the plot. Log-rank P<0.05 was considered to indicate a statistically significant difference in the survival curve.

Results

Identification of DMGs in glioma. After the analysis of GSE28094, a total of 349 DMGs were obtained between glioma and normal brain tissue, including 167 upregulated (hypermethylated) and 182 downregulated (hypomethylated) genes (Table SI).

GO functional enrichment analysis. In order to further understand the underlying mechanism of these DMGs in glioma, all DMGs were imported to the DAVID software. GO function results showed that upregulated DMGs were particularly enriched in BP, including positive and negative regulation of 'cell proliferation', 'positive regulation of transcription from RNA polymerase II promoter', 'protein autophosphorylation' and 'regulation of apoptosis' (Table I). As for CC, the DMGs were enriched in 'cytoplasm', 'plasma membrane', 'nucleoplasm', 'perinuclear region of cytoplasm' and 'extracellular region' (Table I). In addition, MF was enriched predominantly in 'protein binding', 'transcription factor binding', 'protein heterodimerization activity', 'receptor binding' and 'ATP binding' (Table I).

For downregulated DMGs, enriched BP included 'immune response', 'cellular response to lipopolysaccharide', 'peptidyl-tyrosine phosphorylation', 'inflammatory response' and 'extracellular matrix disassembly' (Table I). CC analysis revealed enrichment in 'extracellular space and region', 'cell surface', 'integral component of plasma membrane' and 'plasma membrane' (Table I). In addition, MF showed enrichment in 'cytokine activity', 'protein tyrosine kinase activity', 'growth factor activity', 'phosphatidylinositol-4,5-bisphosphate 3-kinase activity' and 'non-membrane spanning protein tyrosine kinase activity' (Table I). This information suggested that DMGs may serve a crucial function in the tumor immune microenvironment in glioma.

Pathway functional enrichment analysis. KEGG pathway enrichment analysis identified that upregulated DMGs were significantly enriched in pathways in 'cancer', 'signaling pathways regulating pluripotency of stem cells', 'PI3K-AKT signaling pathway', 'focal adhesion' and 'melanoma'. However, downregulated DMGs exhibited enrichment in the pathways of 'cytokine-cytokine receptor interaction', 'type I diabetes mellitus', 'graft-vs.-host disease', 'rheumatoid arthritis' and 'TNF signaling pathway'. These screened pathways suggested that DMGs may have a vital function in the etiology and pathogenesis of glioma (Table II).

PPI network construction and module analysis. Based on the information in the STRING protein query and the data downloaded from public databases, a PPI DMG network was constructed with 349 nodes and 3,796 edges (Fig. 1). To examine the significant modules in this PPI network, MCODE in Cytoscape was performed. Interactions with a score of >0.9 were considered significant. The top four modules with the highest scores in the network were selected (Table III). The four modules were mapped into STRING (Fig. 2), and these four core modules were mainly associated with 'cytokine-cytokine receptor interaction', 'proteoglycans in cancer', 'basal cell carcinoma', 'Jak-STAT signaling pathway' and 'cancer pathway' by KEGG pathway enrichment analysis (Table IV).

Table III. Modules of the protein-protein interaction networks.

Category	Score	Nodes	Edges	Genes
1	27.056	37	487	*IL10, IL1B, IFNG, MMP9, CREB1, CASP8, TNFSF10, IL13, LCK, IL8, BAX, AKT1, NOS2, PTEN, SPP1, KRAS, JAK2, TNFRSF1A, TNF, CD34, CD86, PDGFRB, IL11, IL6, CD40, CASP3, EGF, NOTCH1, ERBB2, CCL3, CCR5, BCR, SRC, CSF1, CDKN2A, IL3, CSF2*
2	8	28	108	*TNC, CSF3, ITGB1, IL1A, CD2, FAS, OSM, MPO, TNFRSF10A, PLG, MMP7, STAT5A, THY1, CD36, IGF1R, CTLA4, SERPINE1, SPI1, PLAU, LIF, MMP14, IL16, NGF, IL12B, MMP3, PI3, MMP2, TIMP2*
3	4	6	10	*SMO, PTCH1, GLI2, WNT5A, PTHLH, FGF9*
4	4	4	6	*HLA-DQA2, HLA-DPA1, HLA-DPB1, HLA-DOB*

Score defined as the product of the complex subgraph, density and the number of vertices in the complex subgraph (24).
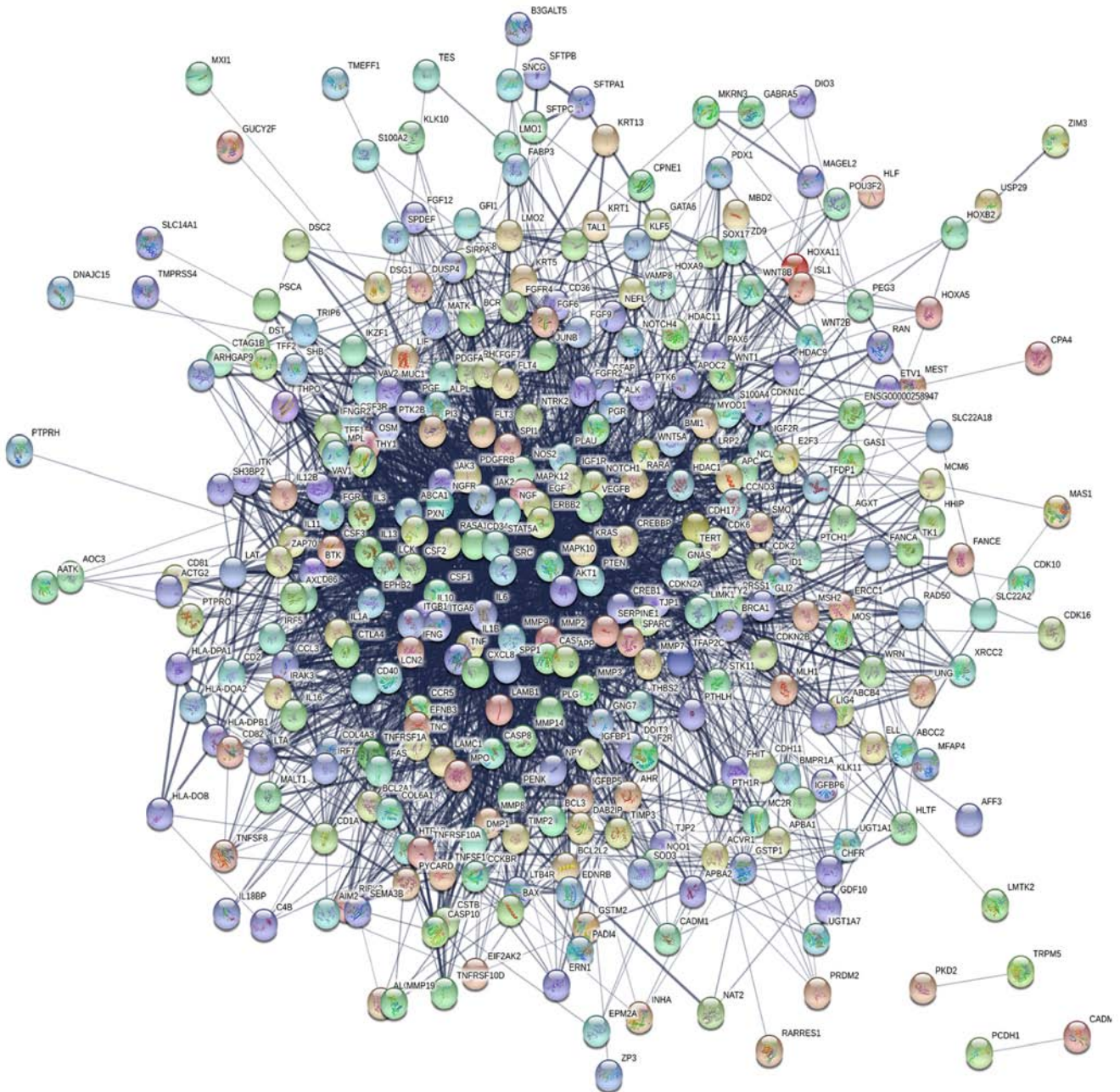

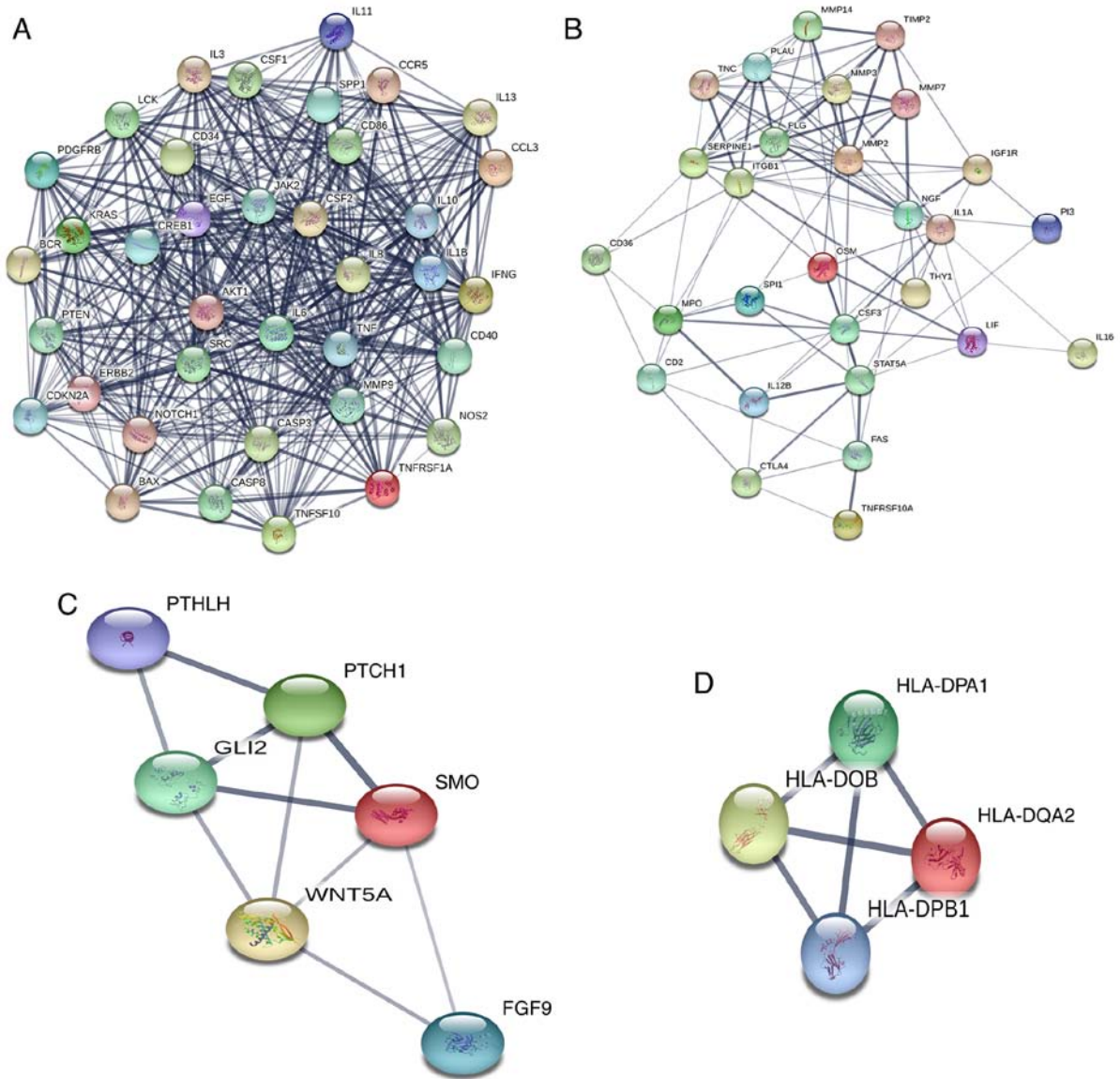Figure 1. The PPI network of differentially methylated genes.

Figure 2. Top four modules for differentially methylated genes were selected. (A) Module 1. (B) Module 2. (C) Module 3. (D) Module 4.

Validation of the hub genes in TCGA database. The top 20 DMGs with the highest degree of connectivity were selected as hub genes in the PPI network. The top 20 hub genes were annotated as AKT serine/threonine kinase 1 (*AKT1*), SRC proto-oncogene (also known as non-receptor tyrosine kinase; *SRC*), epidermal growth factor (*EGF*), tumor necrosis factor (*TNF*), *IL6*, *NOTCH1*, interleukin 8 (*IL8*), *MMP9*, *IL10*, *CASP3*, interleukin 1 β (*IL1B*), colony stimulating factor 2 (*CSF2*), phosphatase and tensin homolog (*PTEN*), *ERBB2*, *CDKN2A*, CD34 molecule (*CD34*), Janus kinase 2 (*JAK2*), interferon gamma (*IFNG*), KRAS proto-oncogene (*KRAS*) and cAMP responsive element binding protein 1 (*CREB1*). In addition, GEPIA was used to determine the expression levels of hub genes between glioma and normal brain tissue. Compared with that in normal brain tissue, the expression level of the four hub genes, including *NOTCH1*, *CASP3*, *IL1B* and *CREB1*, was significantly elevated both in LGG and GBM (Fig. 3). Boxplots were used to visualize the expression levels of these four hub genes. Furthermore, the immunohistochemical data in glioma and normal brain tissue based on

HPA data showed that the expression of *CASP3* and *ERBB2* increased in glioma (Fig. 3). In addition, the expression level of hypomethylated genes increased in GBM compared with that in normal brain tissue, including *IL6*, notch receptor 1 (*NOTCH1*), matrix metallopeptidase 9 (*MMP9*), *IL10*, *IL1B* and *CREB1* (Table V). For the aforementioned hub genes, the significant consistency of methylation and expression status partially confirmed the reliability and stability of the results.

Kaplan-Meier plots and expression level of hub DMGs. The prognostic data of the 20 hub DMGs were obtained from GEPIA. The results indicated that overexpression of *MMP9* (HR, 3.6; P<0.001), *EGF* (HR, 2.0; P=6.1x10^{-8}), *CASP3* (HR, 4.3; P<0.001), *IL6* (HR, 2.7; P=1.4x10^{-13}), *IL10* (HR, 2.8; P=4.1x10^{-13}) and *ERBB2* (HR, 2.1; P=1x10^{-8}) was significantly associated with shorter OS time. By contrast, the high expression of *NOTCH1* (HR, 0.53; P=9x10^{-7}) and *SRC* (HR, 0.62; P=0.00026) was associated with longer OS time in glioma (Fig. 4).

Table IV. The enriched pathways of modules.

Module	Term	P-value	FDR	Genes
1	hsa05152:Tuberculosis	2.06×10^{-12}	2.45×10^{-9}	*AKT1, TNFRSF1A, CASP3, IL6, TNF, BAX, CREB1, CASP8, IFNG, IL1B, JAK2, NOS2, IL10*
1	hsa05145:Toxoplasmosis	1.12×10^{-11}	1.33×10^{-8}	*AKT1, TNFRSF1A, CASP3, TNF, CCR5, CASP8, IFNG, JAK2, CD40, NOS2, IL10*
1	hsa05142:Chagas disease (American trypanosomiasis)	2.27×10^{-10}	2.69×10^{-7}	*AKT1, TNFRSF1A, IL6, CCL3, TNF, CASP8, IFNG, IL1B, NOS2, IL10*
1	hsa04060:Cytokine-cytokine receptor interaction	1.68×10^{-9}	2.00×10^{-6}	*TNFRSF1A, IL6, TNFSF10, CCL3, TNF, CCR5, IFNG, IL1B, IL13, CD40, IL10, IL11*
1	hsa05161:Hepatitis B	4.48×10^{-9}	5.33×10^{-6}	*AKT1, CASP3, IL6, TNF, KRAS, BAX, CREB1, MMP9, CASP8, PTEN*
2	hsa04060:Cytokine-cytokine receptor interaction	1.44×10^{-4}	1.57×10^{-1}	*OSM, LIF, TNFRSF10A, CSF3, FAS, IL12B, IL1A*
2	hsa05205:Proteoglycans in cancer	5.35×10^{-4}	5.82×10^{-1}	*IGF1R, FAS, IL12B, MMP2, ITGB1, PLAU*
2	hsa05162:Measles	1.05×10^{-3}	1.14	*TNFRSF10A, STAT5A, FAS, IL12B, IL1A*
2	hsa04630:Jak-STAT signaling pathway	1.45×10^{-3}	1.57	*OSM, LIF, CSF3, STAT5A, IL12B*
2	hsa05202:Transcriptional misregulation in cancer	2.44×10^{-3}	2.62	*IGF1R, SPI1, MPO, MMP3, PLAU*
3	hsa05217:Basal cell carcinoma	1.82×10^{-6}	1.29×10^{-3}	*WNT5A, SMO, PTCH1, GLI2*
3	hsa05200:Pathways in cancer	1.05×10^{-5}	7.46×10^{-3}	*WNT5A, SMO, FGF9, PTCH1, GLI2*
3	hsa04340:Hedgehog signaling pathway	8.86×10^{-5}	6.29×10^{-2}	*SMO, PTCH1, GLI2*
3	hsa05205:Proteoglycans in cancer	4.86×10^{-3}	3.40	*WNT5A, SMO, PTCH1*
3	hsa04390:Hippo signaling pathway	8.50×10^{-2}	46.77	*WNT5A, GLI2*
4	hsa05310:Asthma	7.49×10^{-8}	5.78×10^{-5}	*HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DOB*
4	hsa05332:Graft-vs.-host disease	1.01×10^{-7}	7.77×10^{-5}	*HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DOB*
4	hsa05330:Allograft rejection	1.43×10^{-7}	1.11×10^{-4}	*HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DOB*
4	hsa04940:Type I diabetes mellitus	2.12×10^{-7}	1.63×10^{-4}	*HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DOB*
4	hsa04672:Intestinal immune network for IgA production	2.99×10^{-7}	2.31×10^{-4}	*HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DOB*

FDR, false discovery rate; hsa, *Homo sapiens*.

Discussion

Glioma is the most common malignant tumor of the CNS (26). Despite drastic treatment, including neurosurgical resection after which radiotherapy in combination with temozolomide is used, a poor prognosis and high recurrence rate remains (27). Genetic and epigenetic alterations play a vital role in cellular transformation and therapy resistance (28). Therefore, potential specific molecular biomarkers and core therapeutic targets require further investigation. Global genomic hypomethylation and promoter hypermethylation of key genes is a hallmark of various types of cancer, such as lymphoma, breast and ovarian cancer (29). DNA methylation can alter the expression level of genes, which can further influence the proliferation and
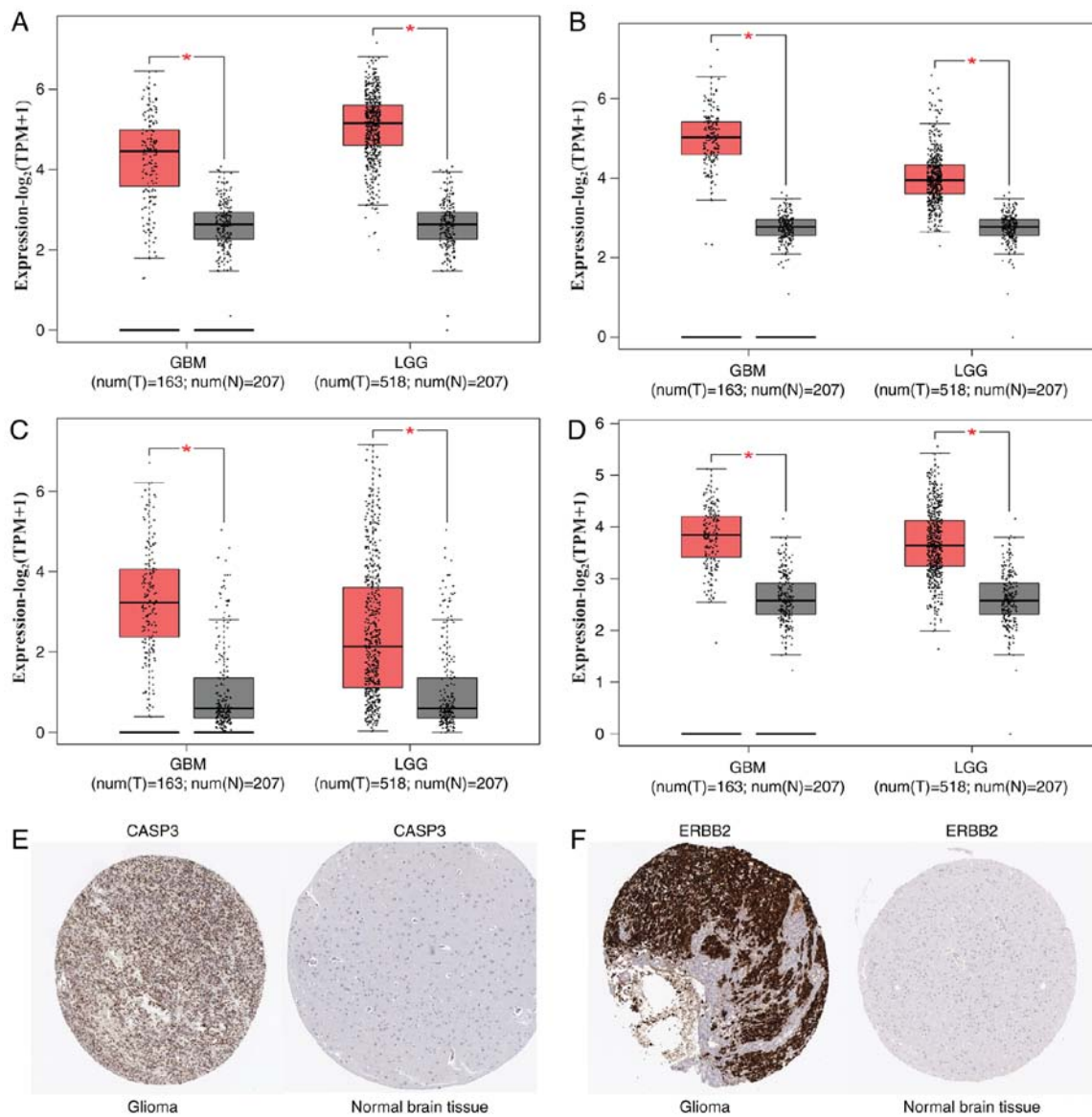
Figure 3. Expression level of the hub genes in glioma and normal brain tissues. (A-D) Expression level of (A) *NOTCH1*, (B) *CASP3*, (C) *IL1B* and (D) *CREB1* in glioma and normal brain tissues. Red indicates glioma, gray indicates normal brain tissue. (E) CASP3 protein was strongly upregulated in glioma tissues compared with normal brain tissues based on the Human Protein Atlas database. (F) ERBB2 protein was strongly upregulated in glioma tissues compared with normal brain tissues based on the Human Protein Atlas database. *P<0.05. GBM, glioblastoma multiforme; LGG, brain lower grade glioma; TPM, transcripts per million. *NOTCH1*, notch receptor 1; *CASP3*, caspase 3; *IL1B*, interleukin 1 β; *CREB1*, cAMP responsive element binding protein 1; *ERBB2*, erb-b2 receptor tyrosine kinase 2.

migration of tumor cells (30). Hypomethylation has been verified to result in chromosome instability by activating the transcription of the commonly silenced transposons, similar to repetitive sequences (31). Aberrant gene promoter region hypermethylation of certain tumor suppressor genes, such as *MLH1*, *MGMT*, and *CDKN2A* genes, can lead to epigenetic silencing of such gene expression, which is associated with vital biological processes, including DNA repair and cell cycle control (32). Therefore, in order to gain a further understanding of the underlying mechanisms that affect the incidence and development of glioma, epigenetic alterations require further investigation. Bioinformatics analysis provides objective data for this purpose. In the present study, the GSE28094 dataset, containing 90 gliomas and 6 normal brain tissues, was extracted from GEO as a discovery set. Numerous bioinformatics tools detected a total of 349 DMGs, including 167 hypermethylated genes and 182 hypomethylated genes,

which may be associated with the tumorigenicity and progression of glioma.

As revealed by GO analysis, hypomethylated genes in glioma were enriched in BPs of 'immune response', 'cellular response to lipopolysaccharide', 'peptidyl-tyrosine phosphorylation', 'inflammatory response' and 'extracellular matrix (ECM) disassembly'. MF indicated enrichment in 'cytokine activity', 'protein tyrosine kinase activity', 'growth factor activity', 'phosphatidylinositol-4,5-bisphosphate 3-kinase activity' and 'non-membrane spanning protein tyrosine kinase activity'. In addition, for hypermethylated genes in glioma, GO analysis indicated that enriched BPs were positive and negative regulation of 'cell proliferation', 'positive regulation of transcription from RNA polymerase II promoter', 'protein autophosphorylation' and 'regulation of apoptotic process'. MF enrichment revealed 'protein binding', 'transcription factor binding', 'protein heterodimerization activity', 'receptor binding' and 'ATP binding'. These

Table V. Top 20 hub genes with the highest degree of connectivity, and validation of the hub genes in The Cancer Genome Atlas database.

Gene	Degree of connectivity	Methylation status	Adjusted P-value	P-value	Expression status
AKT1	121	Hypermethylation	1.09×10^{-5}	4.71×10^{-7}	Upregulation
SRC	112	Hypomethylation	2.08×10^{-3}	2.27×10^{-4}	No change
EGF	112	Hypomethylation	1.35×10^{-2}	2.54×10^{-3}	No change
TNF	110	Hypomethylation	4.62×10^{-2}	1.43×10^{-2}	No change
IL6	108	Hypomethylation	4.76×10^{-2}	1.47×10^{-2}	Upregulation
NOTCH1	88	Hypomethylation	5.76×10^{-13}	4.59×10^{-15}	Upregulation
IL8	81	Hypomethylation	1.30×10^{-3}	1.26×10^{-4}	No change
MMP9	77	Hypomethylation	2.64×10^{-2}	6.41×10^{-3}	Upregulation
IL10	76	Hypomethylation	4.29×10^{-2}	1.31×10^{-2}	Upregulation
CASP3	75	Hypermethylation	2.70×10^{-2}	6.66×10^{-3}	Upregulation
IL1B	74	Hypomethylation	1.43×10^{-2}	2.78×10^{-3}	Upregulation
CSF2	73	Hypomethylation	3.50×10^{-2}	9.68×10^{-3}	No change
PTEN	72	Hypermethylation	1.90×10^{-2}	4.13×10^{-3}	No change
ERBB2	70	Hypermethylation	1.18×10^{-2}	2.10×10^{-3}	Upregulation
CDKN2A	67	Hypermethylation	3.58×10^{-2}	1.00×10^{-2}	Upregulation
CD34	66	Hypermethylation	8.65×10^{-4}	7.93×10^{-5}	No change
JAK2	63	Hypermethylation	2.23×10^{-5}	1.05×10^{-6}	No change
IFNG	63	Hypomethylation	3.77×10^{-2}	1.07×10^{-2}	No change
KRAS	59	Hypermethylation	9.14×10^{-3}	1.49×10^{-3}	No change
CREB1	58	Hypomethylation	1.53×10^{-5}	7.03×10^{-7}	Upregulation

results suggested that dysregulation of cell proliferation and apoptosis may serve an important role in the occurrence and development of tumors, which was in agreement with previous studies (33,34). As a major component of tumor local microenvironment, the ECM affects tumor progression by promoting cell transformation and metastasis (35). Notably, ECM anomalies also lead to stromal cell deregulation and facilitate tumor-associated angiogenesis and inflammation, which comprise the tumorigenic microenvironment (35). It has been reported that active but dysfunctional immune responses are associated with cancer (36). Clinical data have indicated that immune stimulation and immune suppression simultaneously occur in patients with cancer, with increased concentrations of TNFα, interleukin (IL)6, IL8 and IL10 (37), which is in accordance with the results of the present study. Therefore, tumor-promoting immune disorder may serve a pivotal role in glioma oncogenesis, suggesting that cancer immunotherapy is likely to become a key part of the clinical management of glioma.

KEGG analysis revealed that DMGs were significantly enriched in pathways including 'cancer', 'signaling pathways regulating pluripotency of stem cells', 'PI3K-AKT signaling pathway', 'focal adhesion' and 'melanoma'. Activated AKT modulates the function of numerous substrates, such as mTOR, glycogen synthase kinase 3 β or forkhead box protein O1, which are involved in the regulation of cell metabolism, proliferation, cell cycle progression and survival (38). In recent years, the PI3K/AKT signaling pathway has been associated with tumorigenesis and development of GBM (39). Pluripotent mouse embryonic stem cells can be expanded in large numbers in vitro by symmetrical self-renewal (40). Self-renewal entails proliferation with a concomitant suppression of differentiation,

which is responsible for the occurrence and progression of cancer (40). Furthermore, differentially methylated regions in induced pluripotent stem cells, derived by epigenetic reprogramming, are significantly enriched in specific tissues and cancer, demonstrating that hypermethylation and hypomethylation of cytosine-phosphate-guanine islands are broadly involved in tissue differentiation, epigenetic reprogramming and cancer (41).

In addition, focal adhesion is associated with tumor cell migration and invasion (42). A previous study have shown that the expression and activity of focal adhesion kinase, an intracellular non-receptor tyrosine kinase, is frequently associated with cell adhesion, migration, survival, proliferation, differentiation and angiogenesis in cancer (43). These findings demonstrated the reliability of the present results, indicating the critical role of the PI3K/AKT signaling pathway, pluripotency of stem cells and focal adhesion kinase in the tumorigenicity of glioma, providing a promising therapeutic target for glioma.

The PPI network for DMGs uncovered the overview of their functional connections. The top 20 hub genes were also selected, in which five DMGs with a high degree of connectivity were identified: AKT1, SRC, EGF, TNF and IL6 (gene count >100). Furthermore, TCGA database analysis was used to validate the top 20 hub genes. The results revealed that the hypomethylation of IL6, NOTCH1, MMP9, IL10, IL1B and CREB1 was associated with high expression levels in glioma. Moreover, immunochemistry images of these hub genes were obtained from HPA. Compared with that of the other eight hub genes (AKT1, IL6, NOTCH1, MMP9, IL10, IL1B, CDKN2A and CREB1), the expression intensity and quantity of CASP3 and ERBB2 were higher in glioma. Therefore, the immunochemistry images of CASP3 and ERBB2 were selected to visualize their
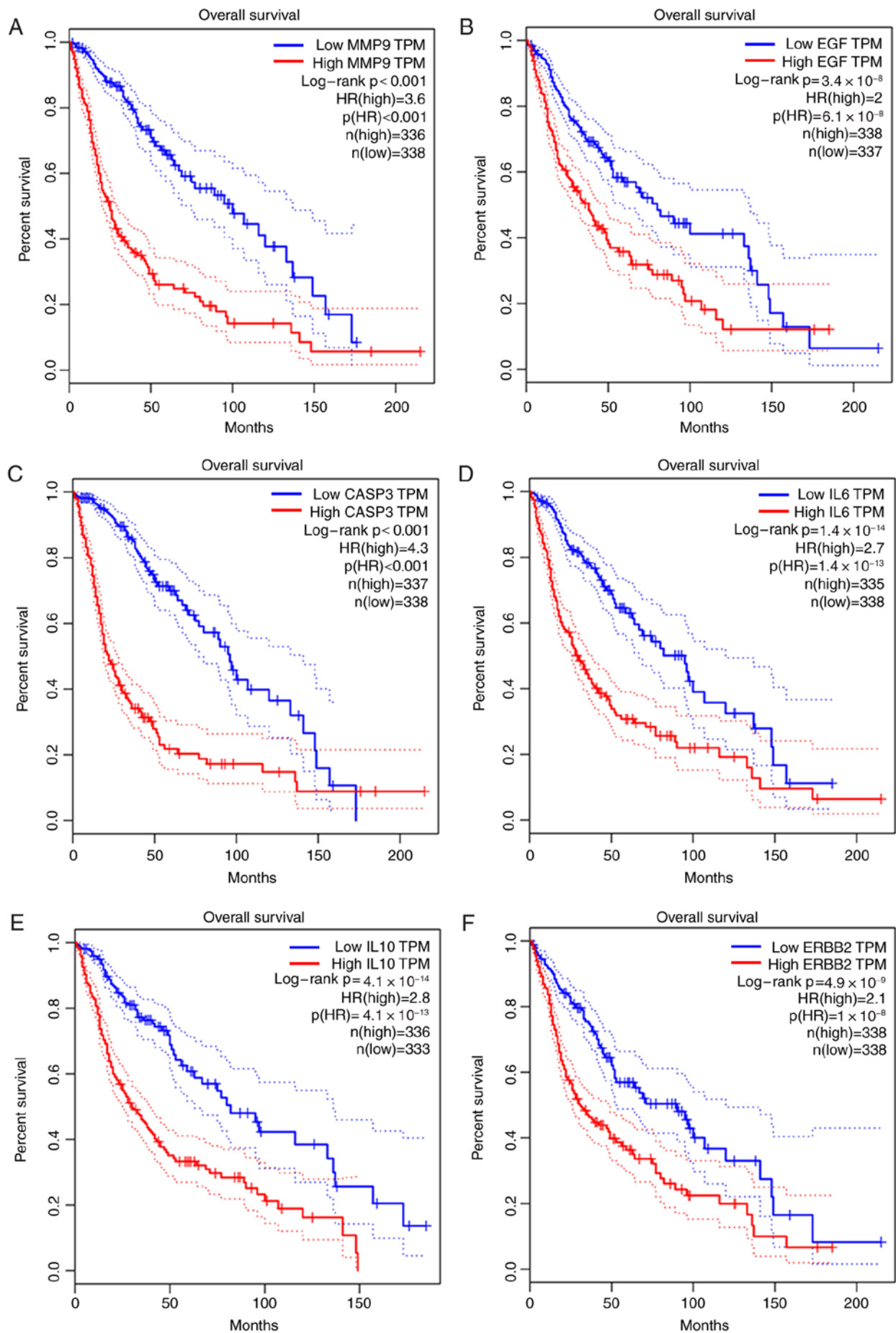
Figure 4. Prognostic value of eight differentially methylated genes in patients with glioma. (A-F) High expression of (A) *MMP9*, (B) *EGF*, (C) *CASP3*, (D) *IL6*, (E) *IL10* and (F) *ERBB2* was significantly associated with poor prognosis in glioma patients.
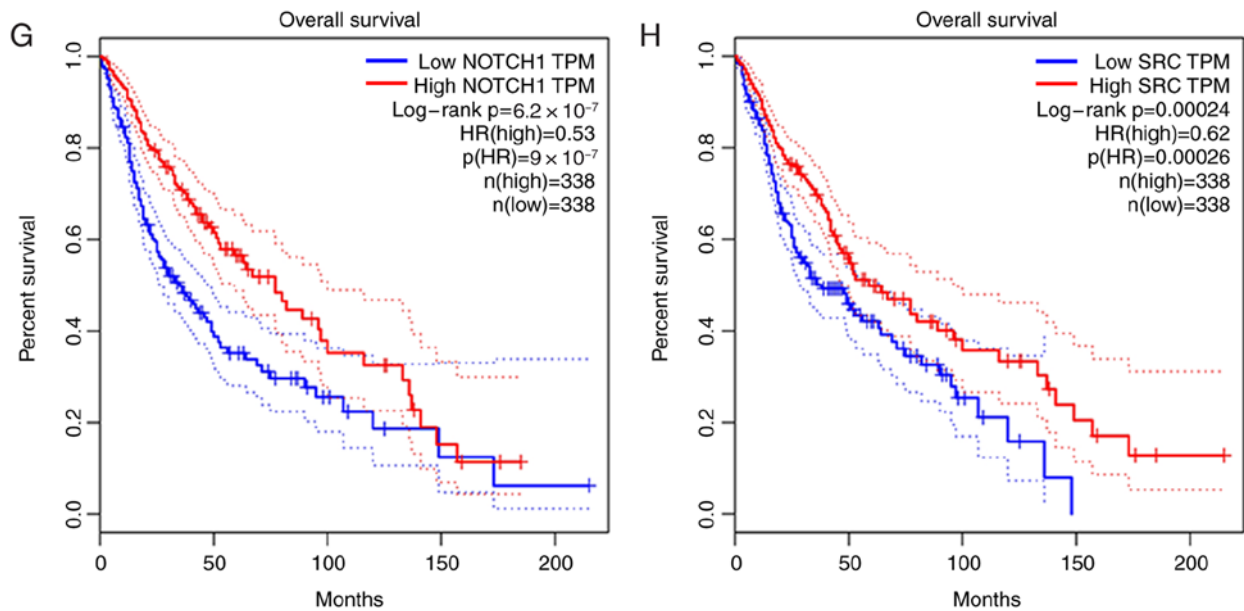
Figure 4. Continued. (G and H) High expression of (G) *NOTCH1* and (H) *SRC* was associated with improved prognosis in glioma patients. HR, hazard ratio; TPM, Transcripts per million; *MMP9*, matrix metallopeptidase 9; *EGF*, epidermal growth factor; *CASP3*, caspase 3; *IL6*, interleukin 6; *IL10*, interleukin 10; *ERBB2*, erb-b2 receptor tyrosine kinase 2; *NOTCH1*, notch receptor 1; *SRC*, SRC proto-oncogene.

differential expression level in glioma vs. normal brain tissue. It was revealed that eight genes were associated with survival, which were either protective or risky, based on HR. *NOTCH1* and *SRC* were defined as protective with HR<1, whereas *IL6*, *MMP9*, *IL10*, *CASP3*, *ERBB2* and *EGF* were defined as risky with HR>1. The aforementioned genes identified in the present study may serve as valuable prognostic biomarkers in glioma.

In previous studies, a number of these hub genes have already been examined as promising predictors of glioma. For instance, ILs, including *IL6* and *IL10*, two important Th2-associated cytokines, are often co-expressed in glioma and further participate in malignancy progression and lead to an unfavorable prognosis (44,45). *MMP9* contributes to the degradation of ECM, angiogenesis and the invasiveness of glioma (46). AKT1 kinase is a crucial member of activated proliferation and survival pathways in cancer, which has the ability to suppress apoptosis, deregulate the cell cycle and alter the angiogenic potential (47). Increased levels of AKT in ~80% of all GBM cases suggested that activation of the AKT pathway was strongly implicated in the development of GBM (48). Epidermal growth factor receptor (EGFR) is activated by binding of its extracellular ligand, EGF, resulting in enhanced cell proliferation, growth and survival (49). A series of signaling pathway cascades activated by EGF/EGFR are associated with GBM progression, including activation of K-RAS and AKT signaling and mammalian target of rapamycin, together with PI3K pathways (50). Accumulating evidence shows that *NOTCH1* is upregulated in classical and proneural subtypes of GBM, and is associated with tumor grade (51). *NOTCH1* inhibition suppresses the biological behavior of metastasis, invasion and epithelial-mesenchymal transition (52). SRC is one of several oncogenic tyrosine kinases, which is activated in various types of cancer to influence survival and metastasis, such as lung cancer, breast cancer and melanoma (53). The SRC-linked STAT3/MMP pathway is responsible for glioma

stem cell (GSC) maintenance and growth (54); therefore, the inhibition of the SRC/STAT3 pathway may be a novel therapeutic target in glioma. The *CREB1* gene plays a crucial role in the development and progression in various types of cancer, such as colon cancer, ovarian cancer and bladder cancer (55). Overexpression of *CREB1* is significantly associated with advanced WHO grade, Karnofsky Performance Scale and short survival time of patients with glioma (56). Furthermore, inhibition of *CREB1* activation can prevent GSC self-renewal and suppress neoplastic growth (57). The *ERBB2* proto-oncogene (also known as *HER2*) is one of the most useful molecules for classification and prognosis in cancer, and its activation and downstream signals are highly dependent on dimerization with *EGFR* (58). *ERBB2*, in combination with survivin and CD99 molecule, has the ability to form branched multipeptides, which stably bind with T2 cells to exhibit 40-60 and 60-80% cytotoxic activity against the U251 GBM and primary cells, respectively; therefore, they are promising candidates for immunotherapeutic GBM treatment (59). Caspases are well-renowned proteases that play a central role in initiating and executing cell apoptosis (60). A number of human cancer cell lines, such as MCF7, HCC1500, CRL1500, MDA-MB-231, A549, DU145, HepG2, HeLa and HGC27, upregulate *ERBB2* expression, which promotes tumor survival via inhibiting the apoptotic activity of *CASP3/8* (61). Therefore, apoptosis-associated *CASP3* is an important gene in human carcinogenesis. According to previous studies, the underlying mechanism of *IFNG* in glioma remains unclear, and further research is necessary (62).

In summary, through bioinformatics analysis of glioma microarray gene expression data, a total of 349 DMGs were obtained between glioma and normal brain tissue, including 167 hypermethylated and 182 hypomethylated genes. The results showed that *IL6*, *MMP9*, *IL10*, *CASP3*, *ERBB2* and *EGF*, which were associated with the tumor immune

microenvironment, may act in the progression of malignant glioma. Molecular biological experiments are further required to elucidate the function and mechanism of these identified candidate DMGs in glioma, thereby offering new implications on glioma immune microenvironment and immune-related therapy.

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Availability of data and materials

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

Authors' contributions

All authors participated in the preparation of the manuscript. WZ conceived and designed the study. JX, HXG and WS conducted the research. YZ and QW standardized the procedure for sequence search. WLC, ML and MBW contributed to data analysis and revised the manuscript. JX wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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