

DNA methylation profiling identifies potentially significant epigenetically-regulated genes in glioblastoma multiforme

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Abstract. Glioblastoma multiforme (GBM) is one of the most lethal and damaging types of human cancer. The current study was conducted to identify differentially methylated genes (DMGs) between GBM and normal controls, and to improve our understanding of GBM at the epigenetic level. The DNA methylation profile of GBM was downloaded from the Gene Expression Omnibus (GEO) database using the accession number GSE50923. The MethyAnalysis package was applied to identify DMGs between GBM and controls, which were then analyzed by functional enrichment analysis. Protein-protein interaction (PPI) networks were constructed using the hypermethylated and hypomethylated genes. Finally, transcription factors (TFs) that can regulate the hypermethylated and hypomethylated genes were predicted, followed by construction of transcriptional regulatory networks. Furthermore, another relevant dataset, GSE22867, was downloaded from the GEO database for data validation. A total of 476 hypermethylated and 850 hypomethylated genes were identified, which were mainly associated with the functions of 'G-protein-coupled receptors ligand binding', 'cytokine activity', 'cytokine-cytokine receptor interaction', and 'D-glutamine and D-glutamate metabolism'. The hypermethylated gene neuropeptide Y (*NPY*) and the hypomethylated gene tumor necrosis factor (*TNF*) demonstrated high degrees in the PPI network. Forkhead box protein A1 (*FOXA1*), potassium voltage-gated channel subfamily C member 3 (*KCNC3*) and caspase-8 (*CASP8*) exhibited high degrees in the transcriptional regulatory networks. In addition, the methylation profiles of *NPY*, *TNF*, *FOXA1*, *KCNC3* and *CASP8* were confirmed by another dataset. In summary, the

present study systematically analyzed the DNA methylation profile of GBM using bioinformatics approaches and identified several abnormally methylated genes, providing insight into the molecular mechanism underlying GBM.

Introduction

Glioblastoma multiforme (GBM), which develops from astrocytes, is the most common primary brain tumor in adults and one of the most damaging types of human cancer. Despite aggressive multimodal treatment with surgery, radiotherapy and chemotherapy, the prognosis of GBM is extremely poor (1,2). Additionally, GBM demonstrates a high potential to infiltrate the brain parenchyma, which poses a challenge to the available treatment strategies. Typically, GBM results in mortality at 12-15 months post-diagnosis (1). Therefore, novel therapies for GBM are urgently required.

Identifying the mechanisms underlying the development of GBM is important for the development of new treatments. The genetic alterations that affect genes controlling cell growth, apoptosis and invasion have been widely examined in GBM (3). Epigenetic alterations have also been identified to be involved in GBM by affecting the expression of cancer-associated genes alone or in combination with genetic mechanisms (4). Aberrant methylation of gene promoters is the most widely studied epigenetic change that occurs during oncogenesis. It is understood that increased methylation, termed hypermethylation, in the CpG island promotes carcinogenesis by silencing tumor suppressor genes, while loss of methylation, termed hypomethylation, enhances the transcriptional activation of oncogenes and induces chromosomal instability (5,6). Decreased expression of tumor suppressor genes, including retinoblastoma gene, phosphatase and tensin homolog, and TP53, is associated with CpG island promoter hypermethylation and has been reported in GBM (7-9). Epigenetic silencing of O6-methylguanine-DNA methyltransferase, a DNA repair gene that can protect cancer cells from chemotherapeutic alkylating agents, has been revealed to be significantly associated with longer survival times in patients with GBM who are treated with alkylating agents (10,11). Such epigenetic changes may be promising targets for epigenetic anticancer treatments. Indeed, the DNA-demethylating agents 5-azacitidine and 5-aza-2'-deoxycytidine have been approved by the Food and Drug Administration for the treatment of myelogenous leukemia and myelodysplastic syndromes (12).

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Bioinformatics tools and algorithms assist the processing and analysis of high-throughput DNA methylation data (13,14). For example, a previous study used a joint analysis of DNA methylation and gene expression data of GBM to demonstrate that changes in DNA methylation can be associated with survival outcome (15). In addition, a recent study used a computational approach to integrate gene expression and genomic or methylation data to investigate biological networks in GBM (16). The current study used bioinformatics approaches to reanalyze the DNA methylation data deposited by Lai *et al* (17). The present results may improve understanding of the epigenetic regulation mechanism of GBM and provide potential gene methylation biomarkers for GBM, which may contribute to the development of treatments.

Materials and methods

Microarray data. DNA methylation data from the GBM study by Lai *et al* (17) were retrieved from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/projects/geo/>) with the accession number GSE50923. The DNA methylation profiles of 54 GBM samples and 24 control brain samples were previously investigated using Illumina HumanMethylation27 BeadChip. The patient details are presented in the original study by Lai *et al* (17). Only data from 31 GBM and 24 control brain samples were present in the GEO database, therefore, only these data were analyzed in the current study.

Identification of differentially methylated regions (DMRs). The original methylation data were processed using the Bioconductor lumi package version 2.18.0, which is designed to process the Illumina microarray data (18,19). Following quality control and background correction, these data were scaled by quantile normalization as implemented in the lumi package. The methyAnalysis package version 1.8.0 (20) was applied to identify DMRs between GBM and controls. $P < 0.001$ and a difference in methylation levels, calculated as the M-value (19), of >1 or <-1 were considered to indicate a statistically significant DMR. The identified DMRs were then annotated using the methyAnalysis package and DMRs located at gene promoter regions, within 2 kb upstream of the transcription start site, were selected for further analysis.

Functional enrichment analysis. Functional enrichment analysis was conducted to explore the most significant differentially methylated genes (DMGs) with relevant biological functions. Gene Ontology (GO) is a bioinformatics tool for annotating genes, gene products and sequences using defined GO terms (21). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive database linking genomic data, stored in the GENES database, with higher order functional information, stored in the PATHWAY database (22). Reactome is a free online database of biological pathways (23). The KEGG pathway, Reactome pathway and GO functional terms for the DMGs were identified using the following cut-off criteria: $P < 0.05$ and number of over-represented genes > 2 .

Construction of a protein-protein interaction (PPI) network. The PPI data were retrieved from the STRING database

(<http://string.embl.de/>). Subsequently, the DMGs were mapped into these interactions, and DMG pairs with an interaction score > 0.9 were selected to construct the PPI networks, which were visualized using Cytoscape software version 3.1.0 (24).

Classification of DMGs. TSGene (25) is a database for tumor suppressor genes (TSGs) and the TAG database (26) provides information on well-characterized oncogenes and TSGs. The known TSGs and oncogenes were identified from the list of DMGs based on information retrieved from the TSGene and TAG databases.

Identification of transcription factors (TFs) regulating DMGs and construction of a transcriptional regulatory network. The TF regulation data were downloaded from the Encyclopedia of DNA Elements data portal (27,28), and TFs that regulate the hypermethylated genes and hypomethylated genes were identified. The hypergeometric distribution was used to assign a P-value for the prediction of these TFs and an adjusted P-value of < 0.05 was considered significant. Furthermore, transcriptional regulatory networks of hypermethylated genes and hypomethylated genes were constructed using Cytoscape (version 3.6.0) (24).

Data validation. To validate the identified DMGs from GSE50923, another relevant DNA Methylation Profiling dataset, GSE22867 (29), was downloaded from the GEO database and used for data validation. GSE22867 included 55 GBM samples and 3 control brain samples. The platform was GPL8490 Illumina HumanMethylation27 BeadChip (HumanMethylation27_270596_v.1.2). The β -value was calculated based on the methylated and unmethylated signal of the sample data, and a t-test was implemented using the genefilter package (version 1.56.0) (30) to identify significant differentially methylated CpGs ($P < 0.05$). In addition, selected CpG sites exhibited a mean methylation (β -value) difference ≥ 0.05 between the disease group and the control group. Subsequently, the genes covering the differentially methylated CpGs were identified and compared with the identified DMGs from GSE50923.

Results

Identification of DMGs. Based on the methyAnalysis package, a total of 2,407 DMRs were identified between the GBM samples and normal controls. Among these DMRs, 476 hypermethylated and 850 hypomethylated regions were located in gene promoters, which clearly distinguished GBM from normal samples (Fig. 1).

Functional enrichment analysis. To gain insight into the dysregulated biological processes induced by DMGs, separate functional enrichment analyses were performed for the hypermethylated and hypomethylated genes. The data indicated that certain KEGG pathways, including 'neuroactive ligand-receptor interaction', and 'D-glutamine and D-glutamate metabolism', were enriched by hypermethylated genes, while 'cytokine-cytokine receptor interaction' and 'hematopoietic cell lineage' were enriched by hypomethylated genes (Table I). GO terms were grouped into the following three categories:

Table I. Top five KEGG pathways enriched by hypermethylated genes and hypomethylated genes.

A, Hypermethylated genes		
KEGG ID	KEGG pathway	P-value
4080	Neuroactive ligand-receptor interaction	1.18×10^{-3}
471	D-Glutamine and D-glutamate metabolism	3.62×10^{-3}
4114	Oocyte meiosis	6.76×10^{-3}
250	Alanine, aspartate and glutamate metabolism	7.87×10^{-3}
4971	Gastric acid secretion	1.02×10^{-2}
B, Hypomethylated genes		
KEGG ID	KEGG pathway	P-value
4060	Cytokine-cytokine receptor interaction	1.22×10^{-10}
4640	Hematopoietic cell lineage	1.49×10^{-5}
5150	Staphylococcus aureus infection	1.35×10^{-4}
4610	Complement and coagulation cascades	2.72×10^{-4}

KEGG, Kyoto Encyclopedia of Genes and Genomes.

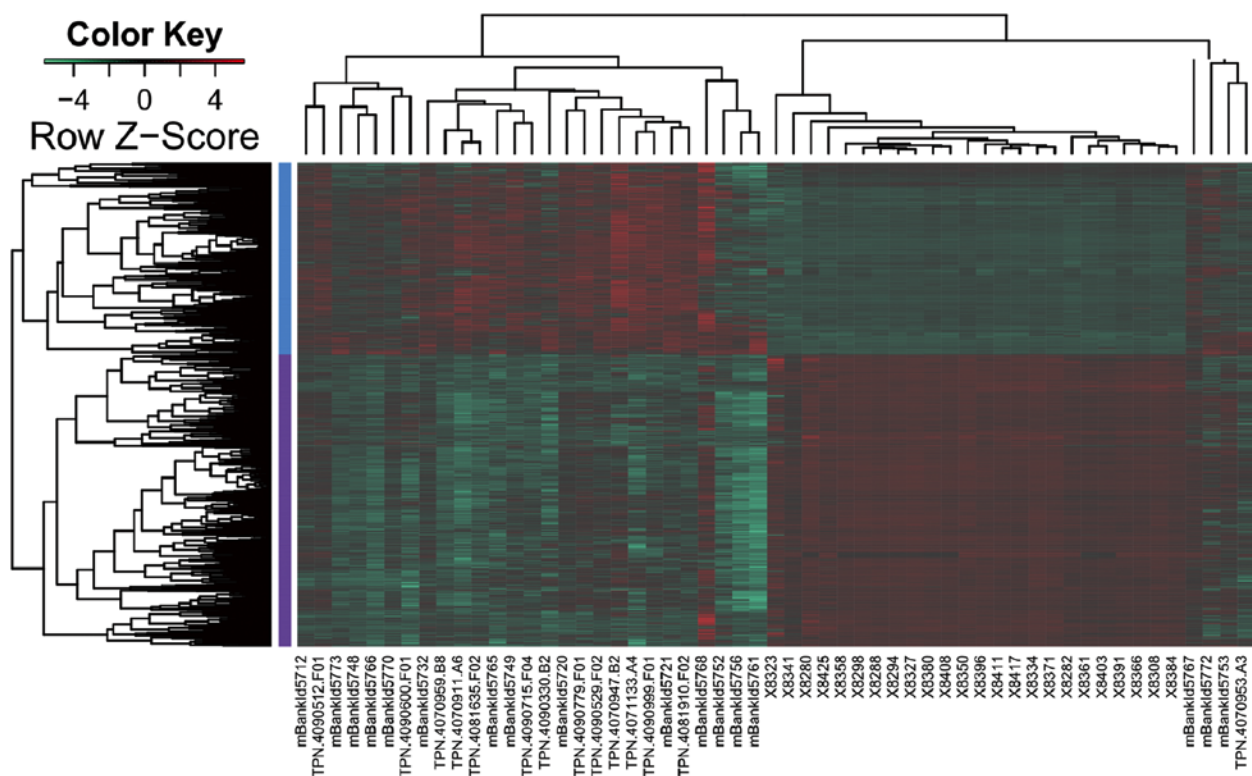


Figure 1. Cluster analysis of differentially methylated genes between patients with glioblastoma multiforme and controls. Each row represents a single gene and each column represents a single sample. Red represents hypermethylated genes and green indicates hypomethylated genes.

Biological process, molecular function and cellular component. The most significantly enriched GO biological process and molecular function terms were 'anatomical structure development' and 'sequence-specific DNA-binding transcription factor activity', respectively, while no statistically significant enriched cellular component terms were identified (Table II).

For hypomethylated genes, the most significant GO terms were 'defense response', 'cytokine activity' and 'extracellular region' (Table II). Reactome pathway analysis demonstrated that both hypermethylated and hypomethylated genes were closely associated with the 'G-protein-coupled receptor (GPCR) ligand binding pathway' (Table III).

Table II. Top five GO terms enriched by hypermethylated genes and hypomethylated genes.

A, Hypermethylated genes		
GO ID	GO term	P-value
Biological process		
GO:0048856	Anatomical structure development	3.19×10^{-8}
GO:0032502	Developmental process	4.06×10^{-8}
GO:0044707	Single-multicellular organism process	4.85×10^{-8}
GO:0032501	Multicellular organismal process	8.26×10^{-8}
GO:0007275	Multicellular organismal development	3.47×10^{-7}
Molecular function		
GO:0003700	Sequence-specific DNA binding transcription factor activity	5.04×10^{-9}
GO:0001071	Nucleic acid binding transcription factor activity	5.51×10^{-9}
GO:0043565	Sequence-specific DNA binding	1.76×10^{-8}
GO:0044212	Transcription regulatory region DNA binding	6.04×10^{-7}
GO:0000975	Regulatory region DNA binding	9.99×10^{-7}
Cellular component		
GO:0044459	Plasma membrane part	2.37×10^{-1}
GO:0019897	Extrinsic to plasma membrane	2.61×10^{-1}
GO:0008076	Voltage-gated potassium channel complex	4.53×10^{-1}
GO:0034705	Potassium channel complex	4.53×10^{-1}
GO:0019898	Extrinsic to membrane	5.85×10^{-1}
B, Hypomethylated genes		
GO ID	GO term	P-value
Biological process		
GO:0006952	Defense response	8.19×10^{-12}
GO:0006954	Inflammatory response	9.55×10^{-12}
GO:0050896	Response to stimulus	6.68×10^{-11}
GO:0002376	Immune system process	8.73×10^{-11}
GO:0009611	Response to wounding	6.82×10^{-10}
Molecular function		
GO:0005125	Cytokine activity	3.13×10^{-7}
GO:0005126	Cytokine receptor binding	1.30×10^{-5}
GO:0004872	Receptor activity	6.25×10^{-5}
GO:0005102	Receptor binding	1.39×10^{-3}
GO:0005506	Iron ion binding	8.86×10^{-3}
Cellular component		
GO:0005576	Extracellular region	$<1.00 \times 10^{-3}$
GO:0005615	Extracellular space	$<1.00 \times 10^{-3}$
GO:0044421	Extracellular region part	$<1.00 \times 10^{-3}$
GO:0071944	Cell periphery	3.84×10^{-9}
GO:0005886	Plasma membrane	5.56×10^{-9}

Construction of PPI networks. A total of 476 hypermethylated and 850 hypomethylated genes were mapped to the STRING database and significant interactions with scores >0.9 were selected. A total of 83 hypermethylated genes and 204 hypomethylated genes were screened to separately construct PPI networks involving hypermethylated genes (Fig. 2) and

hypomethylated genes (Fig. 3). The DMGs adenylate cyclase type 2 (*ADCY2*; degree, 18), neuropeptide Y (*NPY*; degree, 11) and somatostatin (*SST*; degree, 11) demonstrated the highest degrees, determined by the number of interactions in the hypermethylated gene network. Kininogen 1 (*KN1G1*; degree, 27), proto-oncogene tyrosine protein kinase (*SRC*; degree, 18) and

A, Hypermethylated genes

Reactome ID	Name	P-value
500792	GPCR ligand binding	8.88x10 ⁻²
112316	Neuronal system	1.61x10 ⁻¹
373076	Class A/1 (Rhodopsin-like receptors)	3.55x10 ⁻¹
418597	G α (z) signaling events	4.98x10 ⁻¹
1296072	Voltage-gated potassium channels	9.92x10 ⁻¹

Reactome ID	Name	P-value
500792	GPCR ligand binding	5.94x10 ⁻⁴
211897	Cytochrome P450-arranged by substrate type	1.05x10 ⁻³
373076	Class A/1 (Rhodopsin-like receptors)	5.68x10 ⁻³
211945	Phase 1-Functionalization of compounds	6.96x10 ⁻³

GPCR, G-protein-coupled receptor.

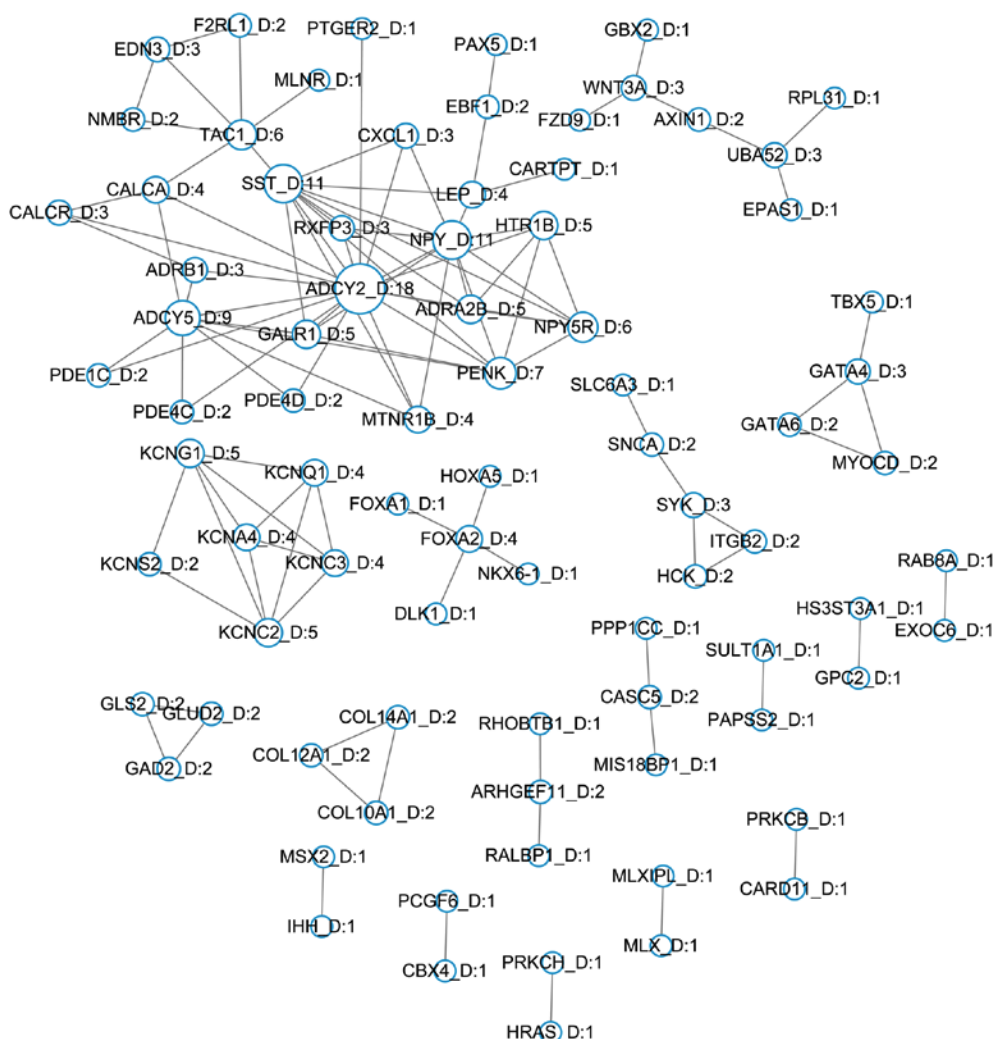


Figure 2. Protein-protein interaction networks for hypermethylated genes. White nodes with blue circles represent the hypermethylated genes and the node size indicates the degree value (larger nodes possess a higher degree). The degree display format of the node is gene_D: num, where 'gene' indicates the gene name, 'D' stands for degree and 'num' is the degree value. Lines/edges represent interactions.

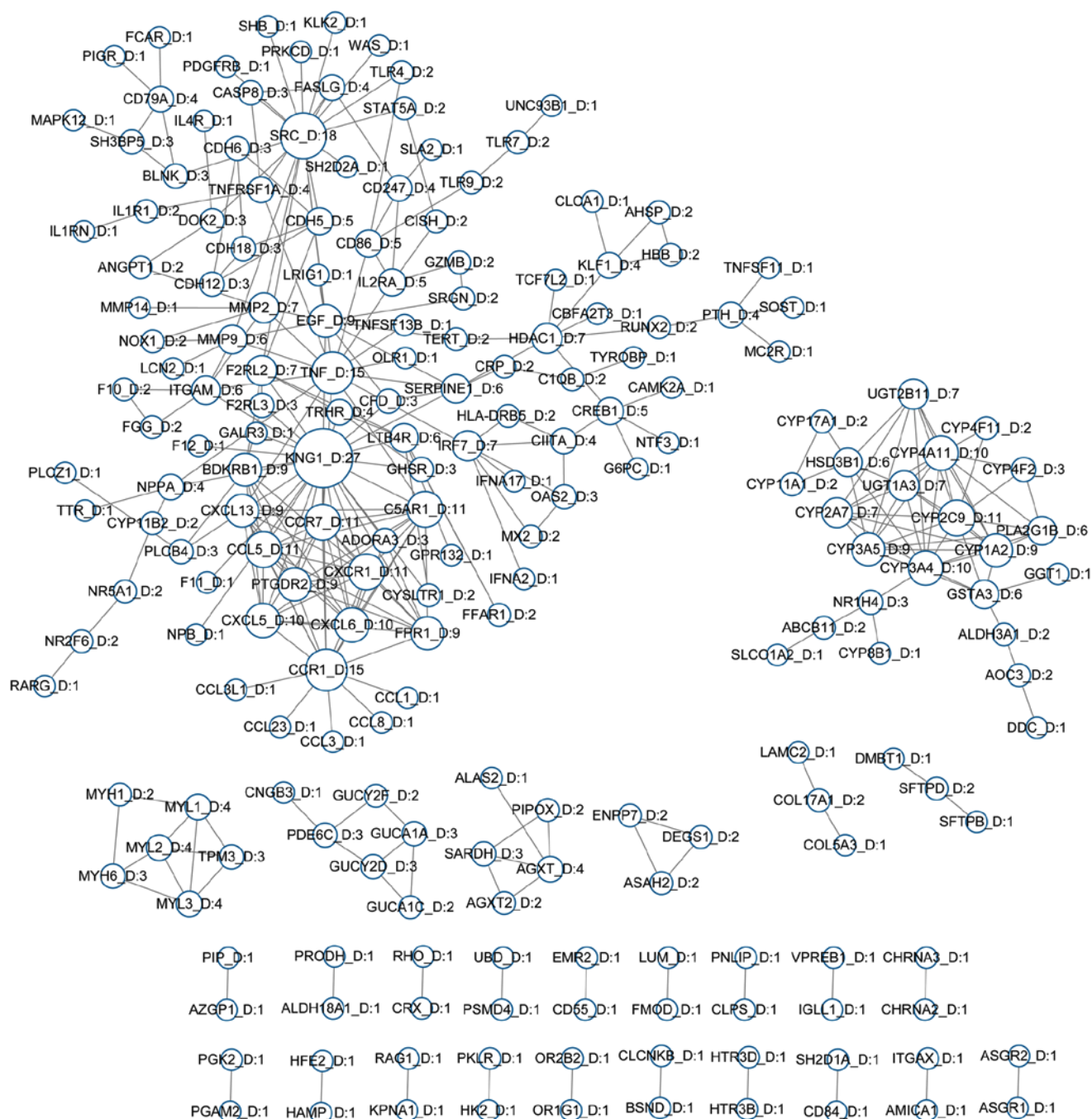


Figure 3. Protein-protein interaction network for hypomethylated genes. White nodes with blue circles represent the hypermethylated genes and the node size indicates the degree value (larger nodes possess a higher degree). The degree display format of the node is gene_D: num, where 'gene' indicates the gene name, 'D' stands for degree and 'num' is the degree value. Lines/edges represent interactions.

tumor necrosis factor (*TNF*; degree, 15) exhibited the highest degrees in the hypomethylated gene network.

Classification of the DMGs. A total of 43 cancer-associated genes were identified among the hypermethylated genes, including 8 oncogenes and 25 TSGs, while 41 hypomethylated genes were revealed to be cancer-associated genes, including 10 oncogenes and 24 TSGs.

Prediction of TFs that target the DMGs and construction of transcriptional regulatory networks. A total of 55 TFs that could regulate the hypermethylated genes were

identified, including polycomb repressive complex 2 subunit, neuron-restrictive silencer factor and cohesin complex component. Additionally, 55 TFs that could regulate the hypomethylated genes were identified, including signal transducer and activator of transcription 3, and forkhead box protein A1 (*FOXA1*). Notably, *FOXA1* was also revealed to be hypermethylated. The transcriptional regulatory networks of hypermethylated genes and hypomethylated genes are presented in Figs. 4 and 5, respectively. The top 10 hypermethylated genes and hypomethylated genes with high node degrees in the regulatory networks are listed in Table IV, including the hypermethylated genes, *FOXA1* (degree, 35),

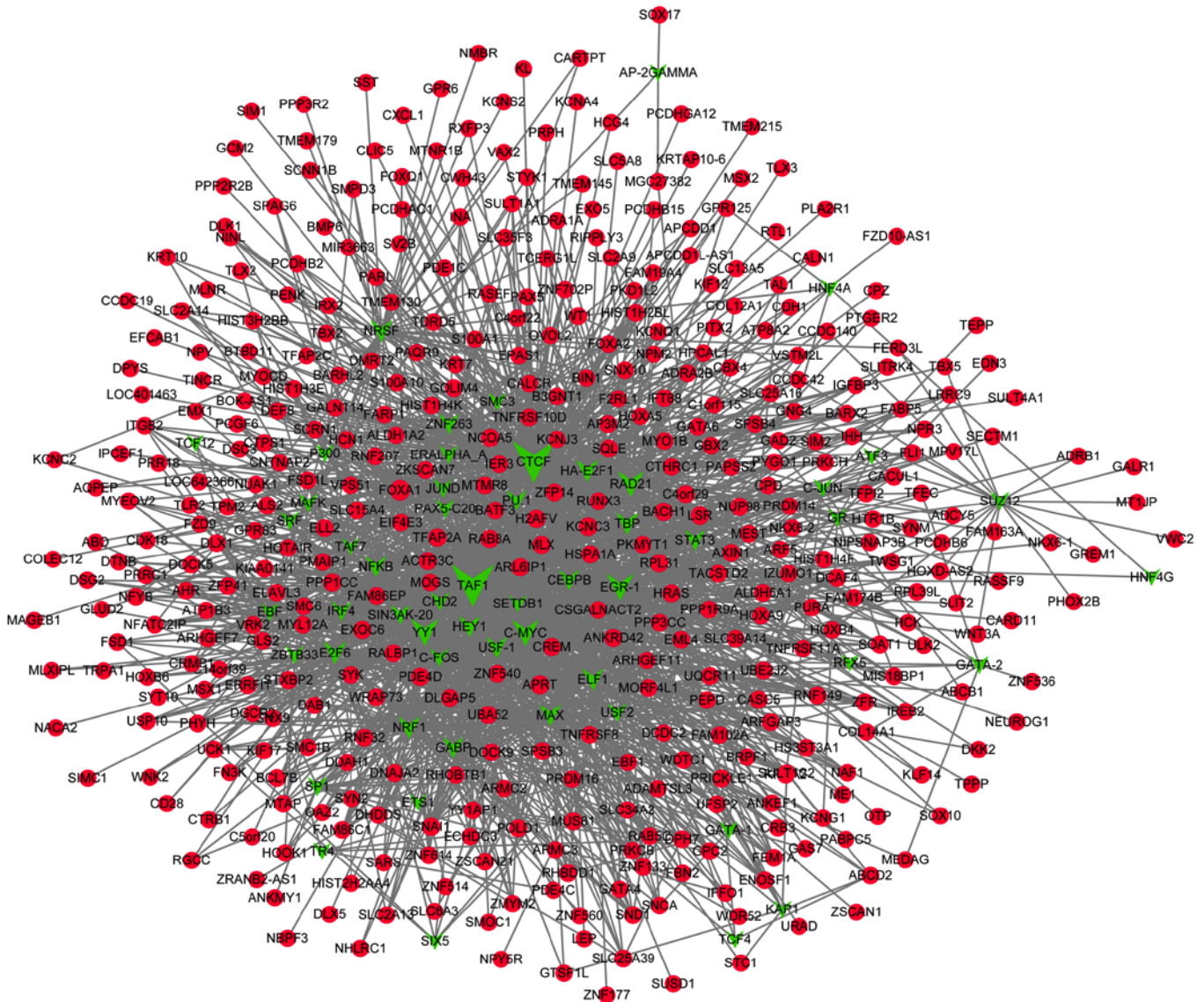


Figure 4. Transcriptional regulatory network of hypermethylated genes. Red nodes represent the hypermethylated gene, green arrows represent transcription factors and the size of node indicates the degree value. Lines/edges represent interactions.

adenine phosphoribosyltransferase (degree, 17) and potassium voltage-gated channel subfamily C member 3 (*KCNC3*; degree, 15), and the hypomethylated genes, proliferating cell nuclear antigen-associated factor (degree, 14) and caspase-8 (*CASP8*; degree, 12).

Data validation. From the GSE22867 validation dataset, 2,151 DMGs were identified. As demonstrated in Fig. 6, a total of 690 overlapping DMGs were revealed. In particular, the hypermethylated genes *NPY*, *FOXA1* and *KCNC3*, as well as the hypomethylated genes *TNF* and *CASP8*, were among the overlapping DMGs.

Discussion

The current study systemically analyzed the DNA methylation profile of GBM samples to improve our understanding of GBM at the epigenetic level and identify potential biomarkers as therapeutic targets for GBM.

Differential analysis revealed that the methylation levels of 1,326 genes were altered in patients with GBM compared with those in the controls. The biological functions most associated with the DMGs were explored by functional enrichment analysis. The results revealed that DMGs were closely associated with the functions of 'GPCR ligand binding', 'cytokine activity', 'cytokine-cytokine receptor interaction', and 'D-glutamine and D-glutamate metabolism'. Both 'cytokine activity' and 'cytokine-cytokine receptor interaction' are associated with inflammatory responses, and there is a close association between inflammation and cancer. Unresolved inflammation resulting from a failure in the regulation of the immune response creates a tumor microenvironment, an important aspect of tumorigenesis proliferation, survival and migration (31,32). GPCR ligands bind to GPCRs and activate the downstream signaling that regulates cellular physiology. Aberrant G-protein signaling is closely associated with cancer development and progression (33).

To investigate whether abnormally methylated genes are associated with GBM, PPI networks of the screened genes

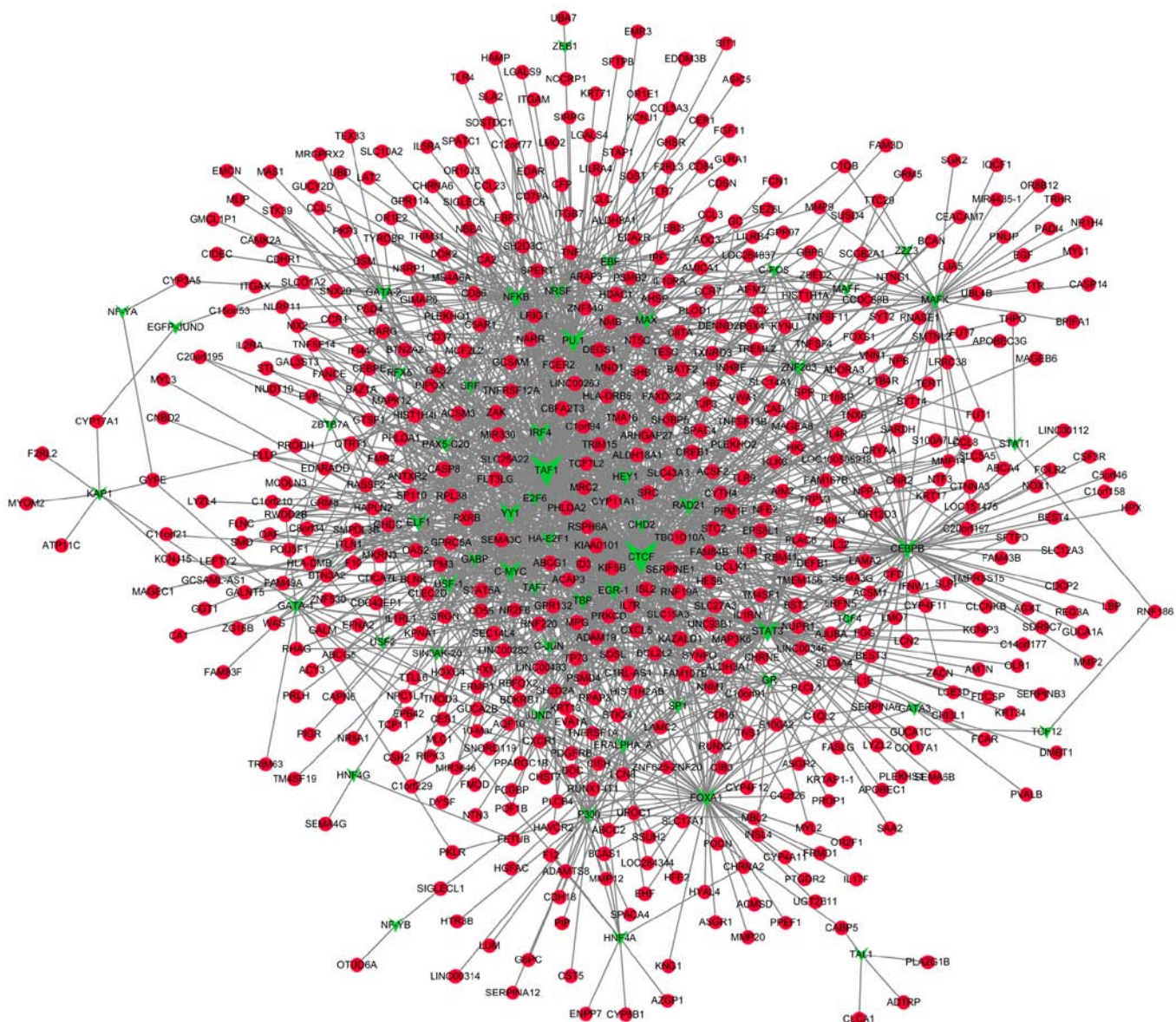


Figure 5. Transcriptional regulatory network of hypomethylated genes. Red nodes represent the hypermethylated gene, green arrows represent transcription factors and the size of node indicates the degree value. Lines/edges represent interactions.

were constructed and the degrees of the nodes were analyzed in the present study. The results demonstrated that the top three genes with the highest node degrees were *ADCY2*, *NPY* and *SST* in the hypermethylated genes network, and *KNG1*, *SRC* and *TNF* in the hypomethylated genes network. Among these genes, *NPY* and *TNF* were validated in the alternative dataset. *NPY* encodes a 36-amino acid neuropeptide that acts as a neurotransmitter in the brain and autonomic nervous system of humans. Using bioinformatic analysis, abnormal methylation of *NPY* has been observed in numerous types of cancer (34-36). Furthermore, a previous study reported that *NPY* is expressed in various types of intracranial tumor in humans and that *NPY* mRNA is detectable in the temporal lobe in higher quantities compared with that in tumors (37). In addition, the present study identified that *TNF* was hypomethylated, which may lead to upregulation of the gene. The expression of *TNF-α* has been identified to be increased in GBM (38), which corresponds with the current study. Therefore, *NPY* and *TNF* may

be involved in GBM due to their abnormal methylation and may result in the disturbance of biological processes.

The hypermethylated genes *FOXA1* and *KCNC3*, as well as the hypomethylated gene *CASP8*, exhibited high degrees in the transcriptional regulatory networks, which was also confirmed by the second dataset. *FOXA1* acts as a TF and has been demonstrated to be a potential regulator of human glioma progression (39). *KCNC3*, encoding the Kv3.3 voltage-gated potassium channel, is expressed in various neuronal cell types that are involved in motor function (40). Previous studies have demonstrated an association between *KCNC3* expression and the poor prognosis of patients with GBM (41). Patients with GBM with higher *KCNC3* expression exhibit improved survival times (42). Genomic loss of *CASP8* by DNA methylation may result in tumor resistance to therapies targeting *TNF*-related apoptosis-inducing ligand-associated apoptosis pathways (43). In summary, the methylation of *FOXA1*, *KCNC3* and *CASP8* in GBM should be investigated in future

Table IV. Top 10 hypermethylated and hypomethylated genes with high node degree in the transcriptional regulatory networks.

Gene	Node degree
Hypermethylated	
<i>FOXA1</i>	35
<i>APRT</i>	17
<i>KCNC3</i>	15
<i>LSR</i>	15
<i>SNAI1</i>	13
<i>NUP98</i>	13
<i>PDE4D</i>	12
<i>RPL31</i>	12
<i>RPL31</i>	12
<i>CREM</i>	12
Hypomethylated	
<i>KIAA0101</i>	14
<i>CASP8</i>	12
<i>TMA16</i>	12
<i>FAM107B</i>	11
<i>LINC00263</i>	11
<i>RXRβ</i>	11
<i>PHLDA1</i>	10
<i>DEGS1</i>	10
<i>KAZALD1</i>	10
<i>CIRL-AS1</i>	9

studies, as this may promote the development of therapeutic approaches.

In conclusion, the current study comprehensively analyzed the DNA methylation profile of GBM using bioinformatics approaches and identified several abnormally methylated genes, including *NPY*, *TNF*, *FOXA1*, *KCNC3* and *CASP8*. The findings of the present study improve the understanding of the molecular mechanism underlying GBM, and provide potential biomarkers for GBM and the development of novel treatment strategies. However, the number of samples included in this study was relatively small. Experimental verification and additional studies with larger sample sizes are required to confirm the present results.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available as datasets GSE50923 and GSE22867 from the GEO database (<http://www.ncbi.nlm.nih.gov/projects/geo/>).

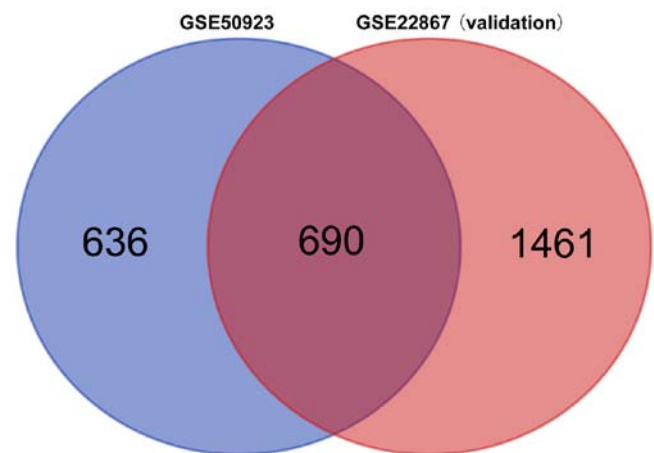


Figure 6. A Venn diagram illustrating data validation using another dataset.

Authors' contributions

SK and SC conceived and designed the study, and drafted the manuscript. WC acquired, analyzed and interpreted the data, and conducted the statistical analysis. BY acquired and interpreted data, and revised the manuscript for important intellectual content. 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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