Identification of potential therapeutic targets for gliomas by bioinformatics analysis

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Abstract. Gliomas are primary tumors that originate in the brain or spinal cord and develop from supportive glial cells. The present study aimed to identify potential candidate molecular markers for the treatment of gliomas, and to explore the underlying mechanisms of this disease. The gene expression profile data GSE50021, which consisted of 10 specimens of normal brain tissues and 35 specimens of glioma tissues, was downloaded from Gene Expression Omnibus (GEO). The methylation microarray data GSE50022, consisting of 28 glioma specimens, was also downloaded from GEO. Differentially expressed genes (DEGs) between patients with glioma and normal individuals were identified, and key methylation sites were screened. Transcriptional regulatory networks were constructed, and target genes were selected. Survival analysis of key methylation sites and risk analysis of sub-pathways were performed, from which key genes and pathways were selected. A total of 79 DEGs and 179 key methylation sites were identified, of which 20 target genes and 36 transcription factors were included in the transcriptional regulatory network. Glutamate metabotropic receptor 2 (*GRM2*) was regulated by 8 transcription factors. Inositol-trisphosphate 3-kinase A (*ITPKA*) was a significantly enriched DEG, associated with the inositol phosphate metabolism pathway, Survival analysis revealed that the survival time of patients with lower methylation levels in cg00157228 was longer than patients with higher methylation levels. *ITPKA* was the closest located gene to cg00157228. In conclusion, *GRM2* and enriched *ITPKA,* associated with the inositol phosphate metabolism pathway, may be key mechanisms in the development and progression of gliomas. Furthermore, the present study provided evidence for an additional mechanism of methylation-induced gliomas, in which methylation results in the dysregulation of specific transcripts. The results of the present study may provide a research direction for studying the mechanisms underlying the development and progression of gliomas.

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Introduction

Gliomas are primary tumors that originate in the brain or spinal cord, and account for ~80% of all malignant brain tumors (1,2). Gliomas occur mostly in childhood, with symptoms including visual loss, pain, nausea, vomiting, weakness in the extremities, headaches and seizures (3,4). Glioma patients have a low survival rate, and of 10,000 Americans diagnosed with malignant gliomas each year, ~50% survive one year following diagnosis, and 25% two years later (5). Therefore, it is essential to explore the molecular mechanisms of glioma and develop effective methods for its treatment.

The methods used to treat gliomas at present are typically a combination of surgery, radiotherapy and chemotherapy, however, the median survival duration of patients with gliomas is only 9-12 months (6). Understanding the molecular mechanisms which underlie this disease is crucial for the development of more effective methods for its treatment (7). Previous studies revealed that methylation of CpG islands within or near promoters were associated with increased gene expression, and may contribute to tumor formation and progression (8-10). Costello *et al* (11) demonstrated that methylation of the *pl6/CDKN2* tumor suppressor gene was detected in gliomas. Other studies reported that methylation of the promoter in the DNA repair gene O-6-methylguanine-DNA methyltransferase, contributed to the progression of gliomas (12,13). Chen *et al* (14) demonstrated that the methylation of the excision repair cross-complementation group 1 promoter promoted the development of gliomas. Although previous studies have made advances in the field, the exact mechanisms of methylation-driven gliomas have not been fully elucidated.

The present study aimed to identify methylation-associated genes from differentially expressed genes between patients with glioma and normal controls, in relation to associated pathways of gliomas, to elucidate the underlying molecular mechanisms. Methylation associated genes were identified from differentially expressed genes (DEGs) by methylation analysis. Significant genes and pathways were

selected from the transcriptional regulatory network and sub-pathway enrichment analysis. Through the identification of key genes and pathways, the potential underlying molecular mechanisms and the potential biomarkers of gliomas were explored.

Materials and methods

Affymetrix microarray data. The gene expression profile data GSE50021 was downloaded from the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) (15). Gene expression profiling was based on the GPL13938 platform using the Illumina HumanHT-12 WG-DASL V4.0 expression BeadChip (Illumina Inc., San Diego, CA, USA). The array consists of 29,377 probe-sets, which it is possible to use to detect the transcription level of 20,817 human genes. A total of 45 samples, including 10 specimens of normal brain tissues and 35 specimens of glioma tissues from children with a mean age of 1.008±1.910 years were available for the expression array.

The dual channel methylation microarray data GSE50022, was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) (15). Gene expression profiling was based on the platform of GPL16304 using the Illumina HumanMethylation450 BeadChip (UBC enhanced annotation v1.0; Illumina, Inc.). The array consisted of 485,512 probe-sets, which detect >485,000 methylation sites per sample at single-nucleotide resolution. Methylation data of 28 samples from patients with glioma (mean age, 0.943±0.782 years) were analyzed in the present study. The methylation index matrix was processed with GenomeStudio v2011 software (Illumina, Inc.) which indicated the methylation ratios of the probes.

Identification of differentially expressed genes. The raw expression profile data were initially preprocessed using the impute package in R (16). The processed data were normalized using the preprocess Core in R (17). DEGs, between normal brain tissues and glioma tissues were analyzed by limma package in R (18). The fold change (FC) of the expression of individual genes was also calculated for differential expression test. All genes with a P-value <0.05 and log2 FC >1 were considered significant and selected as DEGs.

Screening of key methylation sites. The raw methylation index matrix were initially preprocessed using the impute package in R (16). The methylation sites located around the DEGs were screened according to the methylation chip annotation information. Methylation sites which had a methylation index >0.8 in >80% of samples were selected. Key methylation sites which were located 50 kb upstream and/or downstream of the transcription start site were screened.

Transcriptional regulatory network construction. The selected key methylation sites were mapped to the transcription factor binding site data predicted by the University of California Santa Cruz (UCSC) genome browser (19), and the methylation information in the transcription factor binding site was obtained. The transcriptional regulatory network was constructed using Cytoscape software (version 3.2.0; Institute for Systems Biology, Seattle, WA, USA) (20).

Survival analysis of key methylation sites. Survival analysis of methylation sites was performed based on the methylation index. The samples were divided into two parts according to the mean of the methylation index: One part had high methylation index (>0.87); another part had lower methylation index (≤0.87). A Kaplan-Meier curve based on the survival time of the two parts was constructed, and the log-rank test was used to test for a significant difference between the groups with P<0.05 considered to indicate a statistically significant difference.

Analysis of risk sub-pathway. Gene Ontology (GO) analysis is a commonly used method for functional studies of large-scale genomic or transcriptomic data (21). Kyoto Encyclopedia of Genes and Genomes (KEGG) (22) is a primary information-based database which stores information concerning how molecules and genes are networked. The Database for Annotation Visualization and Integrated Discovery (DAVID) (23) was used to systematically extract biological meaning from large gene or protein lists. GO function and KEGG pathways of downregulated DEGs, regulated by transcription factors were analyzed using DAVID 6.7, with a false discovery rate <0.05.

With in metabolic pathways, the closer the proximity of components in the network, the greater the potential for similarity of the biological functions. Therefore, identification of the sub-pathway of diseases is critical. The K-clique was used to divide the metabolic pathway into sub-pathways through the iSubpathwayMiner package in R (24). Sub-pathways with P<0.05 were considered significant.

Results

Identification of differentially expressed genes. Following analysis of the expression profile data, the expression information of 20,727 genes in 45 samples was obtained. The normalized results revealed that the expression median following normalization was a straight line (Fig. 1). From all the genes recorded, 79 significantly downregulated DEGs were selected. However, no upregulated DEGs were identified.

Screening of key methylation sites. Following preprocessing of the methylation index matrix, 382,049 methylation sites in 28 samples were detected. A total of 79 significantly downregulated DEGs overlapped with the methylation data, and 1,474 methylation sites associated with DEGs were identified. The methylation signals of 1,187 methylation sites were detected in the methylation chip. A total of 204 methylation sites, which had a methylation index >0.8 in >80% of samples were selected. A total of 179 key methylation sites in 65 genes, which were located 50 kb upstream or downstream of the transcription start site, were selected.

Analysis of the transcriptional regulatory network. According to the UCSC genome browser (19), 26 methylation sites were revealed to be located in 42 transcription factor binding sites (Table I). A total of 20 target genes and 36 transcription factors were included in the transcriptional regulatory network (Fig. 2). Based on this, the glutamate metabotropic receptor 2 (*GRM2*) gene was regulated by

Table I. Key methylation site information.

ID	Chromosome	MAPINFO	tfbs_start	tfbs_end	tf	Distance_ closest_ TSS	Closest_ TSS_ gene_name
cg06191091	chr17	30583855	30583848	30583862	*USF*	-9339	*RHBDL3*
cg02629157	chr9	138670609	138670546	138670568	*TCF11*	25013	*KCNT1*
cg11709150	chr1	2440438	2440431	2440444	*TCF11*	10256	*PLCH2*
cg04585209	chr11	6292311	6292257	6292272	*TAXCREB*	306	*CCKBR*
cg07125541	chr12	113534668	113534663	113534687	*STAT5A*	38736	*RASAL1*
cg10707626	chr3	51747098	51747027	51747051	*STAT5A*	6018	*GRM2*
cg06191091	chr17	30583855	30583849	30583860	*SREBP1*	-9339	*RHBDL3*
cg12603173	chr11	64508421	64508409	64508423	*RREB1*	-66	*RASGRP2*
cg11025960	chr3	51749188	51749177	51749195	*RFX1*	8108	*GRM2*
cg10692302	chr3	51747227	51747224	51747245	*PPARG*	6147	*GRM2*
cg02629157	chr9	138670609	138670558	138670569	*POU6F1*	25013	*KCNT1*
cg11014582	chr6	76333727	76333675	76333696	*PAX6*	-852	*LMO7*
cg04341461	chr1	2410006	2409978	2410006	*PAX5*	-1616	*PLCH2*
cg05289873	chr17	40321636	40321576	40321597	*PAX4*	11660	*KCNH4*
cg10692302	chr3	51747227	51747222	51747252	*PAX4*	6147	*GRM2*
cg04625615	chr15	41788368	41788310	41788330	*P53*	2313	*ITPKA*
cg07200386	chr8	22079169	22079113	22079135	*OLF1*	10682	*PHYHIP*
cg11014582	chr6	76333727	76333676	76333683	*NKX25*	-852	*LMO7*
cg09864712	chr16	726786	726720	726749	*MYOGNF1*	712	*RHBDL1*
cg06191091	chr17	30583855	30583848	30583862	*MYCMAX*	-9339	*RHBDL3*
cg00810908	chr3	13612319	13612306	13612320	*MEIS1AHOXA9*	2080	*FBLN2*
cg11025960	chr3	51749188	51749181	51749190	*LMO2COM*	8108	*GRM2*
cg03358506	chr8	22058702	22058688	22058703	*ISRE*	31149	*PHYHIP*
cg07776629	chr16	57989122	57989116	57989129	*IRF2*	15898	*CNGB1*
cg07776629	chr16	57989122	57989116	57989129	*IRF1*	15898	*CNGB1*
cg10692302	chr3	51747227	51747225	51747244	*HNF4*	6147	*GRM2*
cg06632557	chr11	61313548	61313495	61313505	*HMX1*	-3678	*SYT7*
cg00155846	chr9	138011566	138011506	138011522	*HAND1E47*	14081	*OLFM1*
cg11025960	chr3	51749188	51749181	51749190	*GATA3*	8108	*GRM2*
cg11025960	chr3	51749188	51749179	51749193	*GATA1*	8108	*GRM2*
cg04625615	chr15	41788368	41788310	41788321	*GATA3*	2313	*ITPKA*
cg05392169	chr9	138011814	138011802	138011816	*FOXO3*	14329	*OLFM1*
cg05289873	chr17	40321636	40321585	40321597	*CREB*	11660	*KCNH4*
cg12309456	chr17	74475402	74475346	74475357	*CP2*	2225	*RHBDF2*
cg12163800	chr17	74475355	74475346	74475357	*CP2*	2272	*RHBDF2*
cg07012189	chr14	93408043	93408038	93408062	*COMP1*	18599	*CHGA*
cg04585209	chr11	6292311	6292251	6292269	*CMYB*	306	*CCKBR*
cg05392169	chr9	138011814	138011806	138011824	*CMYB*	14329	*OLFM1*
cg05934090	chr22	38823188	38823137	38823161	*BRACH*	949	*KCNJ4*
cg10368536	chr16	67518179	67518168	67518184	*ARP1*	-463	*AGRP*
cg06191091	chr17	30583855	30583847	30583863	*ARNT*	-9339	*RHBDL3*
cg03358506	chr8	22058702	22058694	22058703	*AREB6*	31149	*PHYHIP*

ID, probe number in methylation chip; MAPINFO, methylation position; tfbs_start, the starting point in transcription factor binding sites; tfbs_end, the end point in transcription factor binding sites; TF, transcription factor; Distance_closest_TSS, the nearest transcription start point position; Closest_TSS_gene_name, the nearest gene.

8 transcription factors; the rhomboid-like 3 (*RHBDL3*) gene was regulated by 4 transcription factors and rhomboid 5 homolog 2 (*RHBDF2*) had 2 methylation sites in the transcription factor binding sites.

Table II. GO analysis of the differentially expressed genes.

Category	Term	Count	P-value	FDR
GOTERM_BP_FAT	GO:0006813-potassium ion transport	3	0.013	14.588
GOTERM_BP_FAT	GO:0007242-intracellular signaling cascade	5	0.044	41.371
GOTERM_BP_FAT	GO:0015672-monovalent inorganic cation transport	3	0.047	43.728
GOTERM_BP_FAT	GO:0006811-ion transport	4	0.050	45.428
GOTERM_CC_FAT	GO:0034703-cation channel complex	3	0.012	10.880
GOTERM_CC_FAT	GO:0044459-plasma membrane part	8	0.012	11.321
GOTERM_CC_FAT	GO:0005886-plasma membrane	10	0.023	20.748
GOTERM_CC_FAT	GO:0034702-ion channel complex	3	0.026	23.371
GOTERM_MF_FAT	GO:0005509-calcium ion binding	7	<0.001	0.864
GOTERM_MF_FAT	GO:0005261-cation channel activity	4	0.005	5.578
GOTERM_MF_FAT	GO:0022836-gated channel activity	4	0.007	7.698
GOTERM_MF_FAT	GO:0046873-metal ion transmembrane transporter activity	4	0.008	8.936
GOTERM_MF_FAT	GO:0030955-potassium ion binding	3	0.012	12.513
GOTERM_MF_FAT	GO:0005267-potassium channel activity	3	0.013	13.401
GOTERM_MF_FAT	GO:0005216-ion channel activity	4	0.013	13.593
GOTERM_MF_FAT	GO:0022838-substrate specific channel activity	4	0.014	14.678
GOTERM_MF_FAT	GO:0015267-channel activity	4	0.015	15.992
GOTERM_MF_FAT	GO:0022803-passive transmembrane transporter activity	4	0.016	16.088
GOTERM_MF_FAT	GO:0046872-metal ion binding	11	0.020	20.008
GOTERM_MF_FAT	GO:0043169-cation binding	11	0.021	21.264
GOTERM_MF_FAT	GO:0043167-ion binding	11	0.024	23.361
GOTERM_MF_FAT	GO:0004435-phosphoinositide phospholipase C activity	2	0.032	30.645
GOTERM_MF_FAT	GO:0031420-alkali metal ion binding	3	0.035	32.899
GOTERM_MF_FAT	GO:0004629-phospholipase C activity	2	0.040	36.483

GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; counts, numbers of DEGs; FDR, false discovery rate.

Survival analysis of key methylation sites. Survival analysis of the 204 methylation sites demonstrated that cg00157228 significantly affected the survival time of patients. The survival time of patients with lower methylation levels in cg00157228 was increased compared with patients with higher methylation levels in cg00157228 (Fig. 3). Inositol-triphosphate 3 kinase A (*ITPKA*) was the gene located closest to cg00157228.

Analysis of risk sub-pathways. GO analysis of 20 target genes confirmed that specific DEGs were significantly enriched in different GO categories, which were associated with biological processes including potassium ion transport, monovalent inorganic cation transport and ion transport (Table II). However, the 20 target genes were not significantly enriched in any pathways. A total of 8 glioma related sub-pathways were mined from the inositol phosphate metabolism pathway. *ITPKA* was the DEG enriched in these 8 sub-pathways (Fig. 4).

Discussion

Gliomas are the most common malignant tumors of the brain, but the molecular mechanisms underlying the progression of gliomas remain unclear (25). In the present study, a bioinformatics approach was used to predict potential therapeutic targets and explore the possible molecular mechanisms involved. A total of 79 DEGs associated with caspase inhibition were identified. By constructing a transcriptional regulatory network and performing analysis of risk sub-pathways and survival analysis of key methylation sites, we identified key genes and pathways were identified, including *GRM2*, *ITPKA* and inositol phosphate metabolism.

GRM2 is a protein-coupled receptor, and is associated with diseases that include schizophrenia (26). *GRM2* is expressed in the foetal and the adult brain, and is associated with inhibition of the cyclic adenosine monophosphate pathway (27). Meldrum *et al* (28) demonstrated that L-glutamate activates

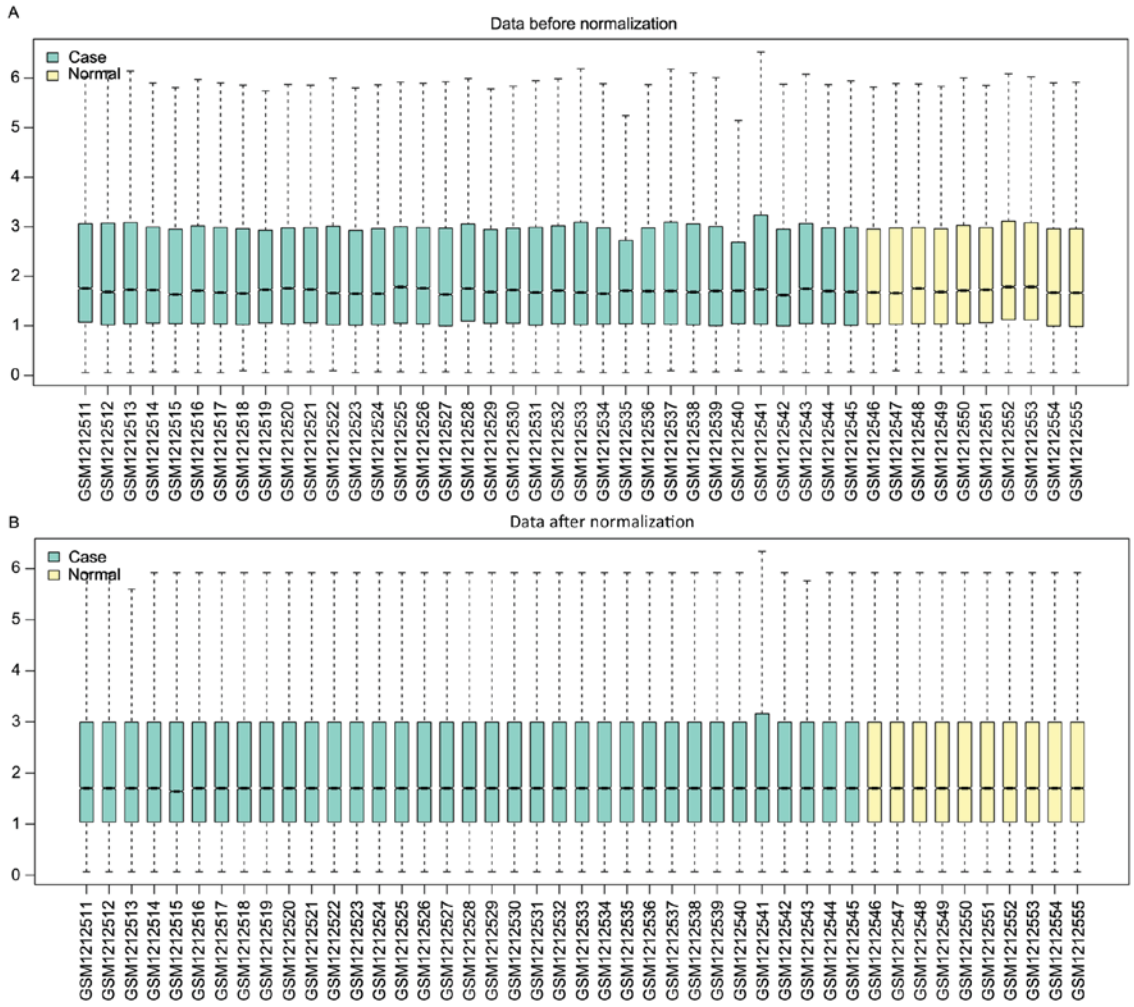

Figure 1. Boxplot of normalized expression values for the dataset. The dotted line in the middle of each box represents the median of each sample, and its distribution among samples indicates the level of normalization of the data, with a straight line revealing a fair normalization level. (A) Data before normalization. (B) Data after normalization.

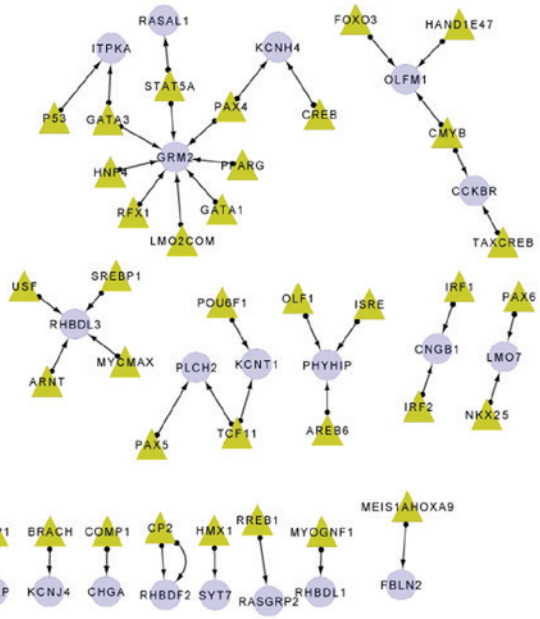

Figure 2. Transcriptional regulatory network analysis. Yellow triangle nodes represent transcription factors, purple circle nodes represent target genes, arrows represent the transcriptional regulation relationship, and repeated connection lines represent 2 methylation sites in the transcription factor binding sites.

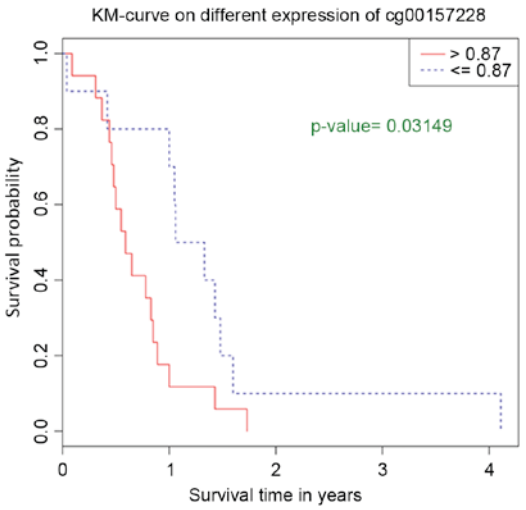

Figure 3. Prediction of survival probabilities based on cg00157228 methylation, as assessed using KM analysis. The significance was determined using the log-rank test. KM, Kaplan-Meier.

metabotropic glutamate receptors and functions as the main excitatory neurotransmitter in the central nervous system.

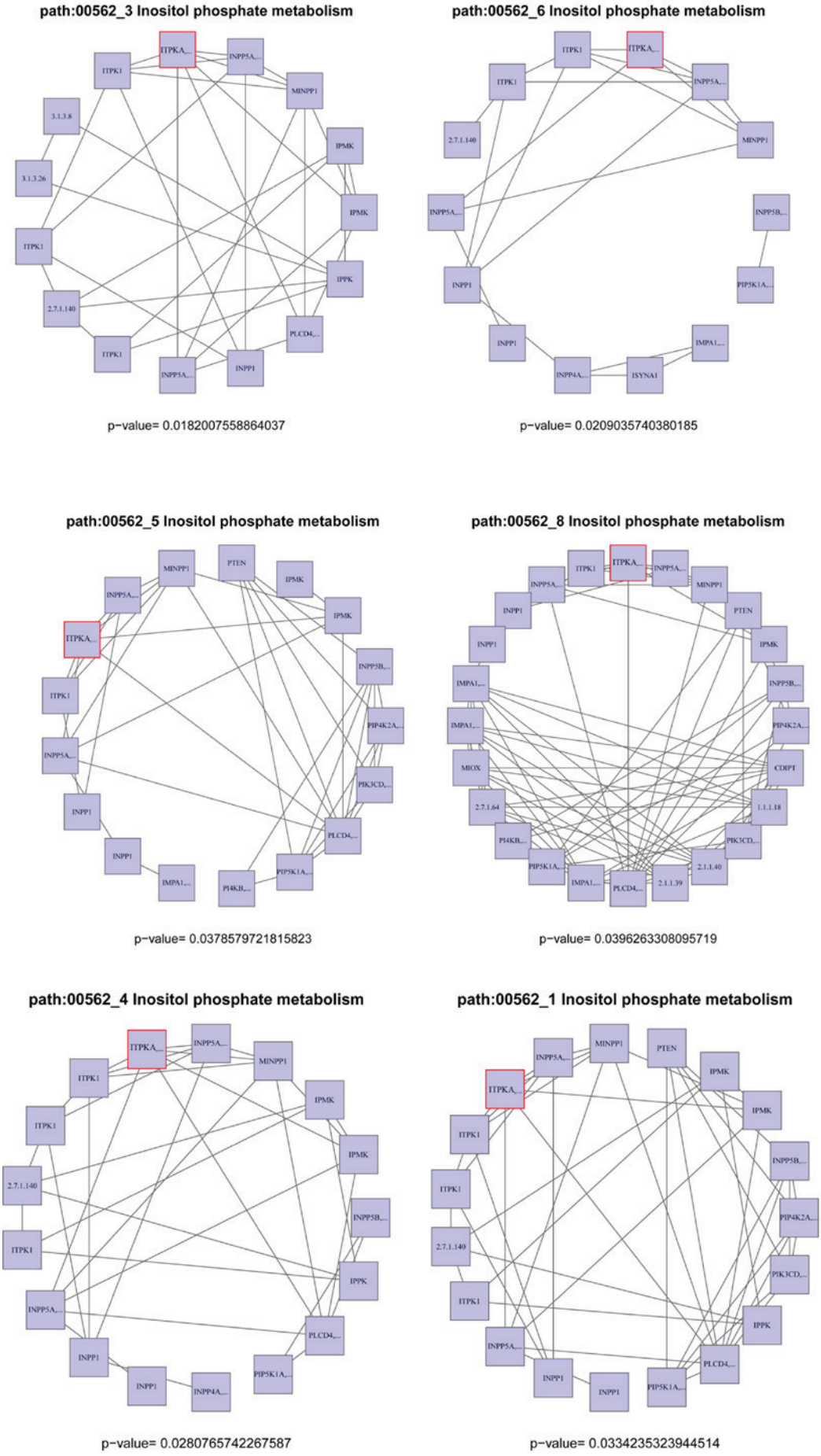

Figure 4. Sub-pathway enrichment analysis of differentially expressed genes. Digital nodes refer to enzymes; letter nodes refer to genes; node with red borders refer to DEGs enriched in the pathway; lines represent the interactions of genes in the networks.

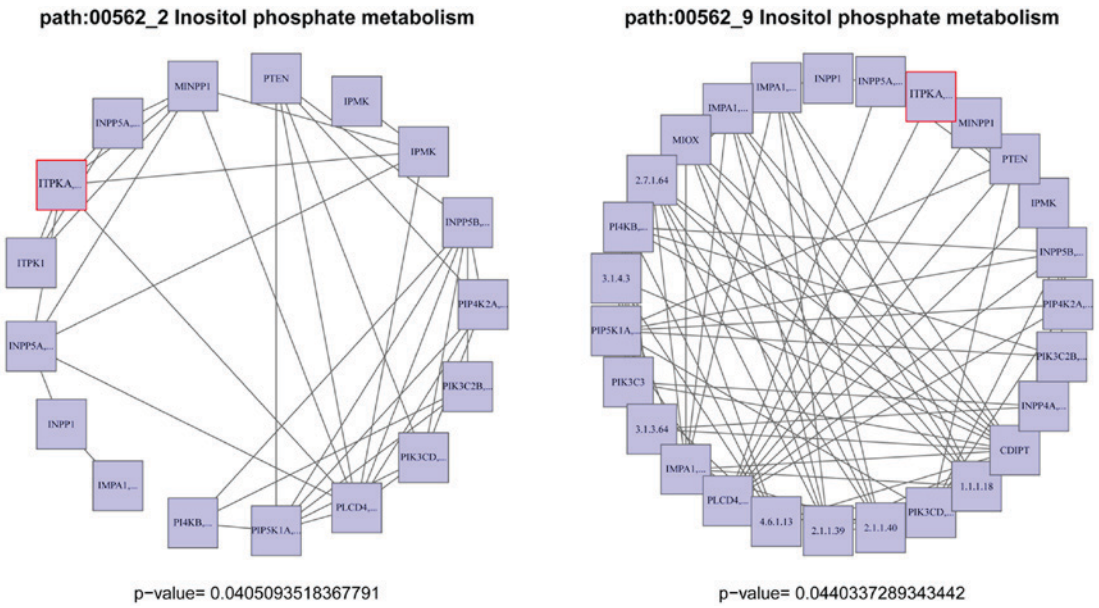

Figure 4. Continued. Sub-pathway enrichment analysis of differentially expressed genes. Digital nodes refer to enzymes; letter nodes refer to genes; node with red borders refer to DEGs enriched in the pathway; lines represent the interactions of genes in the networks.

Ullian *et al* (29) revealed that glutamate receptors may be involved in synaptogenesis or synaptic stabilization. Glutamatergic neurotransmission has been reported to participate in the majority of normal brain functions (30). Furthermore, previous studies have demonstrated that glioma is a primary central nervous system associated cancer (31,32). According to a previous study, the downregulation of *GRM2* may be caused by methylation in the promoter, and *GRM2* downregulation may promote the progression of gliomas (33). In the present study, *GRM2* was downregulated in glioma cells, and 8 methylation sites were identified in the promoter region of *GRM2*. Transcriptional regulatory networks revealed that methylation in the promoter of *GRM2* may influence the binding of 8 transcription factors. Furthermore, *GRM2* may be a potential therapeutic target in the treatment of gliomas. Arcella *et al* (34) revealed that pharmacological blockade of group II metabotropic glutamate receptors reduced the growth of glioma cells *in vivo*.

Inositol phosphate metabolism was the selected sub-pathway in the present study. Tilly *et al* (35) demonstrated that stimulation of human epidermoid carcinoma cells using bradykinin, results in very rapid release of inositol phosphates. Lee *et al* (36) revealed that changes in inositol phosphate metabolism are associated with neoplasia in mouse keratinocytes. Mishra *et al* (37) demonstrated that inositol phosphates trigger numerous cellular processes by regulating calcium release from internal stores. Another previous study revealed that calcium imbalance is associated with gastric cancer (38). The results of the present study provide evidence that inositol phosphate metabolism was the enriched pathway associated with methylation-induced gene silencing. Thus, inositol phosphate metabolism may be a potential candidate pathway for the treatment of gliomas.

ITPKA is responsible for regulating a large number of inositol polyphosphates that are important in cellular signaling (39). Kato *et al* (39) indicated that *ITPKA* was downregulated in oral squamous cell carcinoma, and may be a potential novel molecular target. Windhorst *et al* (40) demonstrated that *ITPKA* was a novel cell motility-promoting protein that increased the metastatic potential of tumor cells. In the present study, *ITPKA* was downregulated and was enriched in the inositol phosphate metabolism pathway. Survival analysis revealed the survival time of patients with lower methylation levels in cg00157228 was longer than patients with higher methylation levels in cg00157228. *ITPKA* was the nearest gene to cg00157228. Taken together, these results indicated that downregulation of *ITPKA* due to methylation in cg00157228 may be a potential molecular mechanism involved in the development of gliomas, and may be a potential therapeutic target for novel treatments.

In conclusion, *GRM2*, *ITPKA* and inositol phosphate metabolism may contribute to the progression of gliomas. Furthermore, the present study provides an additional mechanism underlying methylation-induced gliomas, which is that methylation results in the dysregulation of specific transcripts. However, further experiments are required to confirm these results.

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