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. The present study may provide a research direction for studying the mechanisms underlying the development and progression of gliomas.

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 gliomas 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 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 Human Methylation450 BeadChip (Illumina Inc., San Diego, CA, USA.). The array consists 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
8 transcription factors; the rhomboid-like 3 (*RHBDL3*) gene was regulated by 4 transcription factors and rhomboid 5 homolog 2 (*RHBD2*) had 2 methylation sites in the transcription factor binding sites.
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...
metabotropic glutamate receptors and functions as the main excitatory neurotransmitter in the central nervous system.

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.
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.
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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