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*Original Article*

# Screening feature genes of astrocytoma using a combined method of microarray gene expression profiling and bioinformatics analysis

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**Abstract:** The aim of our study was to find feature genes associated with astrocytoma and correlative gene functions which can distinguish cancer tissue from adjacent non-tumor astrocyte tissues. Gene expression profile GSE15824 was downloaded from Gene Expression Omnibus database which included 8 astrocytoma tissues and 3 adjacent non-tumor astrocyte samples. The raw data were first transformed into probe-level data and the differentially expressed genes (DEGs) between tissues of patients with astrocytoma and normal specimen were identified using T-test in samr package of R. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was applied to analyze the gene ontology (GO) enrichment on gene functions and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Finally, corresponding protein-protein interaction (PPI) networks of DEGs was constructed using the Cytoscape based on the data collected from STRING online datasets. A total of 3072 genes, including 1799 up-regulated genes and 1273 down-regulated genes, were filtered as DEGs, and we learnt that the DEGs including AQP4, PMP2, SRARCL1 and SLC1A2CAMs etc and that AQP4 was most significantly related to cell osmotic pressure. Three feature genes in KEGG pathway are highly enriched in cancer specimen while two genes are in the normal tissues. The discovery of featured genes significantly related to the regulation of cell osmotic pressure, has the potential to use in clinic for diagnosis of astrocytoma in future. In addition, it has a great significance on studying mechanism, distinguishing normal and cancer tissues, and exploring new treatments for astrocytoma. However, further experiments were needed to confirm our result.

**Keywords:** Astrocytomas, differentially expressed genes (DEGs), protein-protein interaction

## Introduction

Astrocytoma is one of the most common cancer of the brain, affecting people of any age but with a preference for the males at their 30 s to 40 s. A particular kind of glial cells, called astrocyte, which shaped like star, is where they originate. Generally speaking, they can occur in most parts of the brain and occasionally in the spinal cord, accounting for 21.2%~51.6% of all glioma and 13%~26% of intracranial tumor. Children or young adults are prone to be affected with the low grade, while the high-grade type is more prevalent in adults. Patients with astrocytoma have a tendency of high intracranial pressure, with clinical symptom of altered mental status, cognitive impairment, headaches, nausea and vomiting, even with visual disturbances, motor impairment, seizures, sensory anomalies, ataxia, etc. Astrocytoma has an intrinsic tendency to progress from low-grade to more advanced grades. Besides, astrocytoma often shows high rates of local invasion that lead to local recurrence of the disease [1]. Thus, research on the pathogenesis and development on astrocytoma has a profound and lasting significance on clinical medicine.

In recent years, considerable researches about the molecular genetics and growth regulation of the astrocytoma [2, 3] have made remarkable progress. It was found that sex-steroid hormone, androgens and estrogens played regulatory roles in the progression of astrocytoma. Meanwhile, there are some small molecules which can treat astrocytoma, such as the epidermal growth factor receptor (EGFR), insulin-like growth factor-binding protein 2 (IGFB*P2*), a potential new therapeutic target [4]. However, patients with astrocytoma can't be treated effi-

ciently and selectively just with these molecules. Therefore, the need for screening feature genes of astrocytoma is highlighted, in the hope for new methods to elucidate the mechanism of astrocytoma and new therapeutics for the disease.

In this paper, microarrays were utilized to identify differentially expressed genes (DEGs) between cancer and normal astrocyte cells. Significance of differential expression was tested by Limma and adjusted for multiple testing with the Benjamini and Hochberg (BH) procedure. The functions of DEGs were investigated by annotating to biological process and pathways, helping us to elucidate the mechanism of the astrocytoma on a molecular level. In addition, candidate small molecules were identified for their potential use in the treatment of astrocytoma.

## Materials and methods

### *Affymetrix microarray analysis*

The gene expression profile data GSE15824 were downloaded from NCBI (National Center for Biotechnology Information) GEO (Gene Expression Omnibus) (http://www.ncbi.nlm.nih.gov/geo/) database based on platform GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array). Only 11 chips are available, including 8 astrocytoma tussues and 3 normal specimens. The annotation information of all probe sets was provided by Affymetrix Company where we downloaded the raw data file [5].

Firstly, the raw probe-level data in CEL files were converted into recognizable expression measures profiling [6], in which the probe expression data were standardized by (Robust Multi-array Average) RMA function. Then, we converted the probe numbers into corresponding gene names by R/Bioconductor annotation package and Affymetrix Human Gene 2.0 ST Array. Probes without gene annotation were dismissed. For each sample, the expression values of all probes for a given gene were reduced to a single value by taking the average expression value [15].

### *DEG screening and hierarchical cluster analysis between astrocytoma and normal astrocyte cell*

Differentially expressed genes (DEGs) between tissues of patients with astrocytoma and normal specimen were identified using T-test in samr package of R language. Changes of gene expression higher than quadruple and q>0.05 were selected out using significant analysis of microarrays (SAM) [7]. Hierarchical cluster analysis was used to ensure the confidence level of the selected DEGs. Pearson coefficient was for the samples while spearman coefficient was for the gene expression. The sample grouping information was testified by hierarchical cluster figure and meanwhile some unreasonable specimens were filtered out.

### *Gene ontology and pathway enrichment analysis*

DAVID (Database for Annotation, Visualization and Integrated Discovery) [8], bioinformatics resources consisting of an integrated biological knowledgebase and analytic tools to extract biological meaning from large gene or protein lists systematically were applied to identify over-represented GO categories in biological process with the *p*-value <0.01 and count >2, and to identify significant pathways with the *p*-value <0.05 and count >2.

Gene expression profiling of astrocytoma and normal tissues were re-obtained after hierarchical clustering and sample filtering. DEGs between astrocytoma and normal tissues were filtered out by Limma package [9]. adj. P Val<0.01 and |logFC|>4 were used as the cut-off criterion to screen the DEGs. GO and KEGG pathway enrichment analysis were performed on down- and up- regulated genes respectively with DAVID. All the DEGs in the interaction network were analyzed by GO terms (P<1e-5) and KEGG pathways. FDR (False Discovery Rate) less than 0.05 were used as the cut-off criterion.

### *Principal components analysis (PCA) of the top 10 DEGs*

Principal component analysis (PCA), a statistical procedure, is used to convert a set of values of possibly correlated variables into a set of values of linearly uncorrelated variables, that is, principal components [10, 11]. Method of PCA (principal components analysis) was performed on the top 10 DEGs with the smallest *p*-value, in order to distinguish normal tissue from astrocytoma tissues. In the method, the number of original variables is larger than or equal to the

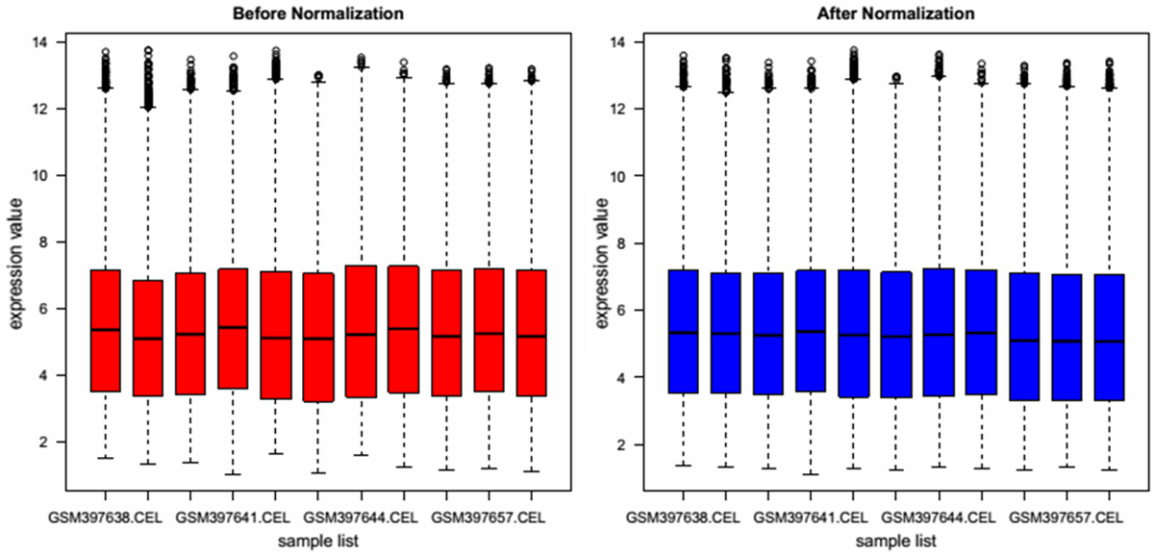


**Figure 1.** The cassette figures of expression data before and after standardization of all normal tissues and astrocytoma tissues. The horizontal axis stands for the names of samples while the vertical axis represents the expression value. The black line in the cassette is the median of every sample, which indicates the level of standardization of data. The medians of the samples were almost on the second dotted line, which indicated that the degree of standardization was very well. Figure on the left is the cassette figure of expression value of every sample before normalization while the figure on the right is cassette figures after normalization.

number of principal components. The principal components are orthogonal and sensitive to the relative scaling of the original variables. Therefore, PCA is a powerful tool when using several variances instead of hundreds of thousands of data in the listing of samples.

*Protein-protein interaction network analysis*

The protein-protein interactions (PPIs) is a research tool used to reveal the functions of proteins which can also help discover the rules of cellular activities including growth, development, metabolism, differentiation and apoptosis at the molecular level. The identification of protein interactions in a genome-wide scale is an important step for the interpretation of the cellular control mechanisms. It was proved that many function-associated genes can co-express and the gene expression levels are varied according to cell type and state. Thus, STRING (Search Tool for the Retrieval of Interacting Genes) (http://string-db.org/) database [12] was used to build the PPI network to predict the interactions of the top 10 DEGs. The interaction with combination score >0.4 was defined as the selection criteria. Subsequently, the PPI network we obtained was input into Cytoscape.

## Results

*Data preprocessing*

The standardized expression profiling data after preprocessing were shown in **Figure 1**, which clearly showed the differences before and after the standardization. The volatility of data after standardization is much stable than data before the process, for the median of expression value of all genes is on one same line. For dataset GSE15824, a total of 38000 expression values from three normal astrocyte tissue samples and eight astrocytoma specimens were obtained.

*Hierarchical clustering results of gene expression*

Total 3072 DEGs between normal tissues and those with astrocytoma were screened out by t-matching test of samr in R software, including 1799 up-regulated and 1273 down-regulated genes. The eight astrocytoma specimens were on the astrocytoma tissue cluster while the three control samples were on the normal astrocyte tissue cluster (**Figure 2**), which is reasonable. Astrocytoma can be divided into two sub-clusters according to hierarchical clustering.

Feature genes of astrocytoma

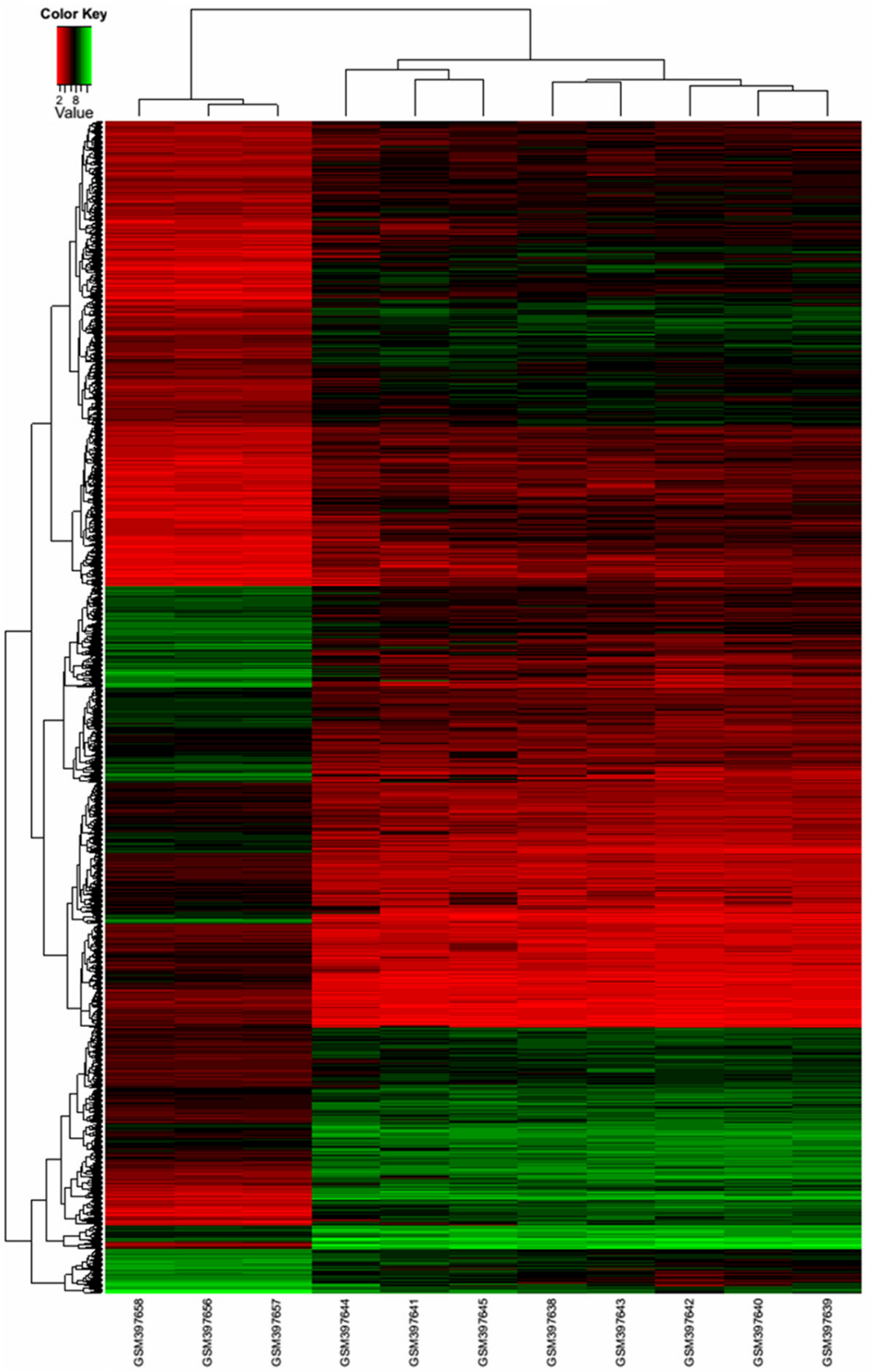

**Figure 2.** Hierarchical Clustering Figure of Astrocytoma. The horizontal axis below is the names of the samples. (GSM397656-GSM397658 are three normal astrocyte tissue samples while GSM397638-397645 are eight astrocytoma specimens). The vertical axis on the left shows the cluster situation of the samples horizontal axis above reveals the situation of genes. The red color stands for the situation of down-regulated while the green color stands for the up-regulated situation. Astrocytoma tissues and normal astrocyte tissues are mainly involved in two clusters according to hierarchical clustering among the DEGs.

**Table 1.** GO enrichment analysis of the DEGs

| Terms for biological process and KEGG pathways | Gene count | Fold enrichment | FDR |
|---|---|---|---|
| GO:0006954 inflammatory response | 23 | 3.8 | 2.50E-04 |
| GO:0006952 defense response | 32 | 2.8 | 5.70E-04 |
| GO:0009611 response to wounding | 29 | 3 | 8.70E-04 |
| GO:0050778 positive regulation of immune response | 14 | 5.2 | 4.70E-03 |
| GO:0051969 regulation of transmission of nerve impulse | 13 | 4.8 | 3.00E-02 |
| GO:0006955 immune response | 30 | 2.4 | 4.80E-02 |
| GO:0048584 positive regulation of response to stimulus | 16 | 3.7 | 5.50E-02 |
| GO:0002253 activation of immune response | 10 | 5.8 | 9.80E-02 |
| GO:0002526 acute inflammatory response | 10 | 5.5 | 1.40E-01 |
| GO:0006959 humoral immune response | 9 | 6.2 | 1.70E-01 |
| GO:0002684 positive regulation of immune system process | 15 | 3.4 | 2.30E-01 |
| GO:0002252 immune effector process | 11 | 4.4 | 3.20E-01 |
| Cell adhesion molecules (CAMs) | 14 | 4.7 | 6.40E-03 |
| Graft-versus-host disease | 8 | 9.1 | 2.10E-02 |
| Intestinal immune network for IgA production | 8 | 7.3 | 9.90E-02 |
| ECM-receptor interaction | 16 | 15.1 | 3.3E-11 |
| Focal adhesion | 20 | 7.9 | 1.3E-9 |

Notes: Red ones represent pathways enriched in up-regulation genes and the yellow represents pathways enriched in down-regulation genes.

*DEGs screening and enrichment analysis after flitration*

The grouping of the 11 samples is reasonable by clustering of DEGs. Limma package was reused to screen the DEGs between the eight cancer tissue sample and the three normal tissue specimens, in order to explore the information of these DEGs. Genes with .p.Val<0.01 and |logFC|>4 were screened out. Finally, total 723 DEGs were screened out, including 476 up-regulated genes and 247 down-regulated genes. With online enrichment tool of DAVID, we observed that these up-regulated genes were obviously enriched in process of 12 immune reactions and biological process coping to trauma, three signal pathways as well (**Table 1**). No specific biological process was found among the down-regulated genes, but two specific pathways were highly enriched.

*Principal components analysis (PCA) of DEGs*

We can distinguish the normal astrocyte tissues from the astrocytoma tissues by the top 10 DEGs based on their principal components analysis (PCA) (**Figure 3**). The first principal component account for the 98.57% of variance while the second principal component account for 0.61%, with the cumulative variance is 99.18% all together. Besides, the effect of variation pointing of the ten genes is larger on astrocytoma tissues. Gene expression value of these top ten genes in astrocytoma tissue is much higher than those of normal tissues.

*PPI network of the top 10 genes*

Protein information of two genes among the top 10 is not available in the NCBI database (**Table 2**). STRING online was used to find interaction network among proteins and the PPI network of the eight most significant genes was obtained by STRING (**Figure 4**). As we can see from **Figure 4**, two gene, NTRK and CX3CR1 has not been found to be associated with other protein while the other four DEGs was found to form the four protein interaction pairs centered on AQP4. AQP4 was the hub gene in this PPI network and its function was to form the specific water chan-

**Table 2.** The top ten differentially genes with significant differences between normal and astrocytoma group

| Gene | logFC | t | adj.P.Val | Gene description |
|---|---|---|---|---|
| T15657 | 9.444096688 | 39.44034236 | 1.88E-10 | IB1702 Infant brain |
| HBB | 9.219704692 | 23.17316535 | 1.45E-08 | HBB hemoglobin, beta |
| SPARCL1 | 9.130504959 | 27.08821081 | 3.98E-09 | SPARC-like 1 (hevin) |
| NTRK2 | 9.045312864 | 33.96836612 | 6.02E-10 | Neurotrophic tyrosine kinase, receptor, type 2 |
| PMP2 | 8.308515609 | 34.27961572 | 5.60E-10 | Peripheral myelin protein 2 |
| CX3CR1 | 8.680306819 | 18.40722783 | 9.19E-08 | Chemokine (C-X3-C motif) receptor 1 |
| SLC1A2 | 8.588852018 | 38.61164865 | 2.15E-10 | Solute carrier family 1 (glial high affinity glutamate transporter), member 2 |
| BE046923 | 8.520085202 | 21.26313075 | 2.84E-08 | CDNA FLJ37059 fis, clone BRACE2014746 |
| HBA1 | 8.443034807 | 28.78510934 | 2.27E-09 | Hemoglobin, alpha 1 |
| AQP4 | 7.982807338 | 17.77619934 | 1.23E-07 | Aquaporin 4 |

Notes: The red represents genes whose prtotein information hadn't been found in NCBI.

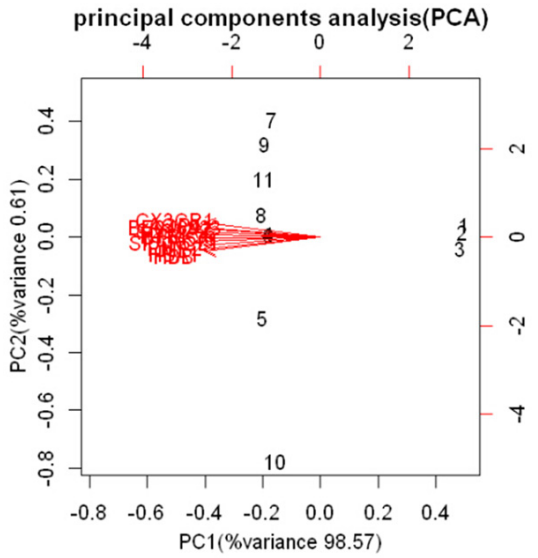


**Figure 3.** Principal components analysis (PCA) of the top ten DEGs. The horizontal axis stands for the score of every first principal component while the vertical axis represents the score of the second principal component. The first principal component account for the 98.57% of variance while the second principal component account for 0.61%, with the cumulative variance is 99.18% all together. 1-3 are the normal samples (three in all) while the 4-11 represents the astrocytoma specimens (eight all together). The red line represents the extent of effect of the gene variation on the astrocytoma tissues, among which the ten genes are highly expressed in the astrocytoma tissues.

nel associated with the balance of osmotic pressure regulation The up-regulation of these genes can disturb the osmotic pressure, thus resulting in the increase of intracranial pressure of patients with astrocytoma. In addition, HBA1 and HBA2 are involved in the process of hemoglobin formation.

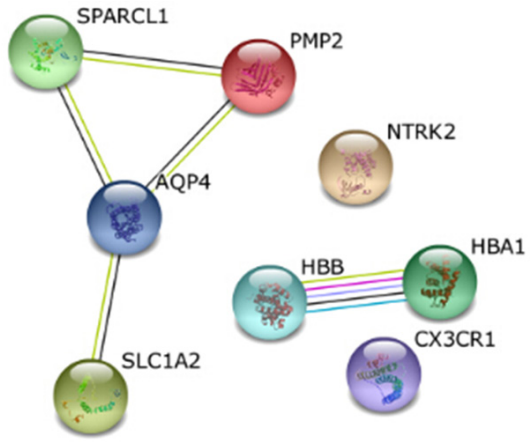


**Figure 4.** PPI network ofthetop ten DEGs. Six among the ten were on the nodes and the lines stand for the corresponding protein-protein interaction pairs.

## Discussion

Astrocytomais, one of the most common glioma, originate in a particular kind of glial cells, star-shaped brain cells in the cerebrum called astrocytes. If not treated in time, patients with astrocytoma may face the risk of death. However, in recent years, clinical treatment of the disease is limited for the little knoeledge of disease-associated genes. Thus, further research on pathogenesis of astrocytoma is quite essential and necessary.

According to the result of gene profiling expression and the PCA (principal component analysis), top ten DEGs closely related to astrocytoma have been linked by our method, which can help to identify astrocytoma tissues among normal samples. In the PPI network, we found

that proteins encoded by the genes ranging top ten have close relationship with each other. AQP4 and SLC1A2 were defined as marker for astrocytes [13, 14], indicating their possible interaciotns with each other. Secreted protein acidic and rich in cysteine (SPARC), which can promote glioma invasion and delays tumor growth, is frequently found to be over-expressed in gliomas [15, 16]. Recurrent activating mutations Neurotrophic tyrosine kinase, receptor, type 2 (NTRK2) fusion genes were identified in non-cerebellar tumors as pilocytic astrocytoma [17]. Holmseth found that Solute carrier family 1 (glial high affinity glutamate transporter), member 2 (SLC1A2), is expressed at high levels in brain astrocytes and at lower levels in neurons [18]. HBA1 and HBA2, which are also expressed at high level, are related to the formation of hemoglobin. All these findings indicate their possible relationship with astrocytoma, thus revealing their potential therapeutic role in clinic medicine.

Above all, the protein AQP4 acted as hub nodes in PPI network, in response to local shifts in extracellular osmolarity. This result suggests that the expression profile of these genes may play an important role in the occurrence and development of astrocytoma. AQP4 (aquaporin 4) belongs to the aquaporin family of integral membrane proteins that conduct water through the cell membrane. In human body, there are thirteen water channels but their expression patterns are tissue specific. AQP4, constitutively expressed in the basolateral cell membrane of principal collecting duct cells in the kidney, is reported to provide a pathway for water to exit these cells [19, 20]. AQP4 is expressed in astrocytes and up-regulated by direct insult to the central nervous system [3, 21]. It may mediate rapid changes in cell volume in response to local shifts in extracellular osmolarity [22].

It is reported that AQP4, the highly conserved water channel protein, is up-regulated in high-grade astrocytoma where water homeostasis is disrupted [23]. In Tani's study, it suggested that some complicated mechanism for control of water content relevant to AQP4 was within the brain, indicating it may be an important drug target for treatment of cerebral edema [23]. Besides, the results of PPI network of our study also showed that AQP4, in the center of the network, is essential in the regulation of cell osmotic pressure. Furthermore, astrocytoma can cause the increase of intracranial pressure, which may be resulted from the up-regulation of osmotic pressure associated genes in patients with astrocytoma.

Additionally, researches on AQP4 at mRNA and protein levels in human astrocytoma tissues may be of great help in the treatment of astrocytoma [23, 24]. The expression of AQP4 at mRNA and protein levels decreased in low-grade of astrocytoma tissues, but increased in high-grade astrocytoma versus normal brain tissues [20, 25]. Besides, it also suggested that AQP4 may play essential roles during water-transporting in the growth and proliferation of malignant astrocytoma.

In conclusion, our data provides a comprehensive bioinformatics analysis of genes and pathways which may be involved in the progression of astrocytoma. Total 1700 DEGs from GSE-19824 was obtained, and protein-protein interaction networks of these DEGs were constructed. We found that up-regulated genes in cancer tissues are mainly involved in the immune system. Three feature genes in KEGG pathway are highly enriched in cancer specimen while two are in the normal tissues. The top ten up-regulated genes in astrocytoma tissue can be used to distinguish cancer samples from normal specimens from the results of PCA. Furthermore, we confirmed the association between AQP4 and astrocytoma. However, as for other critical genes as PMP2, SRARCL1 and SLC1A2 in CAMs (Cell adhesion molecules (CAMs) signal pathway, further analyses are still required to unravel their mechanism in the process of malignant progression in astrocytoma.

## Disclosure of conflict of interest

None.

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