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Original Article

Analysis of differentially expressed genes based on microarray data of glioma

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Abstract: Glioma represents one of the main causes of cancer-related death worldwide. Unfortunately, its exact molecular mechanisms remain poorly understood, which limits the prognosis and therapy. This study aimed to identify the critical genes, transcription factors and the possible biochemical pathways that may affect glioma progression at transcription level. After downloading micro-array data from Gene Expression Omnibus (GEO), the differentially expressed genes (DEGs) between glioma and normal samples were screened. We predicted novel glioma-related genes and carried on online software DAVID to conduct GO enrichment and transcription factor analysis of these selected genes. String software was applied to construct a PPI protein interaction network, as well as to find the key genes and transcription factors in the regulation of glioma. A total of 97 DEGs were identified associated with cancer, the GO enrichment analysis indicated these DEGs were mainly relevant to immune responses as well as regulation of cell growth. In addition, the transcription factor analysis showed these DEGs were regulated by the binding sites of transcription factors GLI2, SP1, SMAD7, SMAD3, RELA, STAT5B, CTNNB1, STAT5A, TFAP2A and SP3. PPI protein interaction network analysis demonstrated the hub nodes in the interaction network were EGFR, TGFB1, FN1 and MYC. The hub DEGs may be the most critical in glioma and could be considered as drug targets for glioma therapy after further exploration. Besides, with the identification of regulating transcription factors, the pathogenesis of glioma at transcription level might be brought to light.

Keywords: Glioma, differentially expressed genes, gene expression profiles, EGFR

Introduction

Astrocytoma refers to the tumor formed by astrocytes, which accounts for 13% to 26% of the intracranial tumors and 21.2% to 51.6% of gliomas respectively [1], and it is the most common form of gliomas. It affects more males than females and the peak onset age is 30 to 40 years old. Glioma could invade into other regions of the brain and it is easy to recurrence after surgery excision, which challenged the effectively control of glioma [2]. Although great improvements have been made in diagnostic and therapeutic procedures of glioma, the prognosis of patients with glioma is still terrible. The frequent symptom of patients with glioma is increased intracranial pressure giving rise to headache, nausea, vomiting, paropsia and so on in clinical. Glioma has been a major health problem worldwide, however our knowledge about the molecular pathogenesis of glioma. Besides, the carcinomatous change process implicated in glioma is limited comparing to other cancers.

With the aim to explore the mechanisms of tumor initiation, progression and metastasis and develop new targeted therapies for glioma, studies have focused on the signaling pathways deregulation and genes alternation related to glioma in the past few years. Gene expression profiling is a valuable tool to identify differentially expressed genes (DEGs) in human gliomas so as to find potential critical genes or transcription factors that play important roles in the regulation of glioma development and progression [3]. Numerous previous studies have identified some genes which may be used as diagnostic markers or therapy targets for glioma. For example, CD44 is found to be overexpressed in glioma and involved in many important cell functions contribute to tumorigenesis [4, 5]. CD44, a widely existed transmembrane cell-surface adhesion protein par-

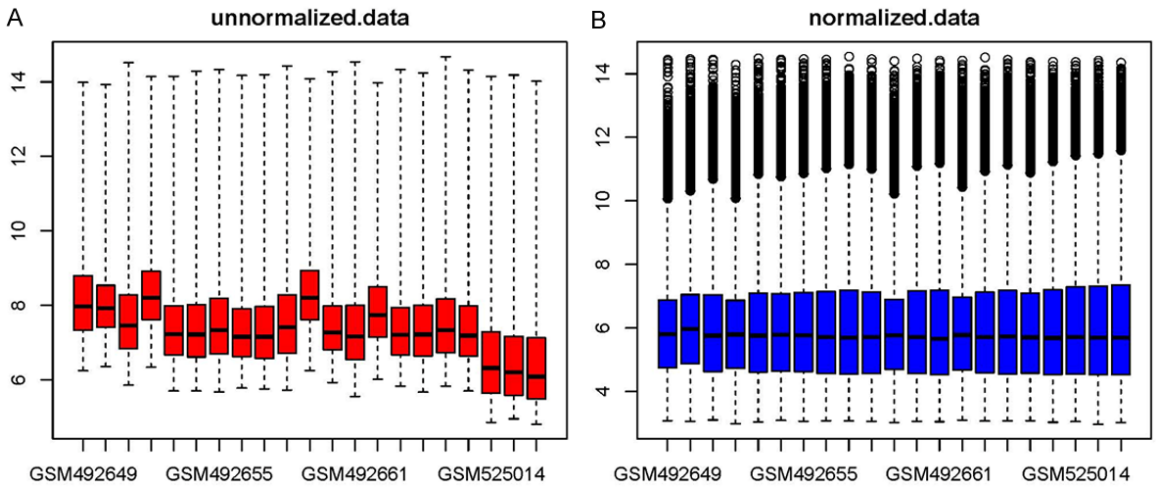

Figure 1. Cartridge of expression values data before and after standardization. The horizontal axis represented sample name, while the vertical axis represented expression value. The black lines in the boxes represented the medians of each set of data, and they were almost on the same straight line after standardization, indicating the standardization level was satisfactory.

ticipated in many physiological and pathological processes such as lymphocyte homing and activation, cell survival and migration, and tumor growth and metastasis [6, 7]. It is reported CD44 is expressed at both high transcriptional and translational levels in glioma and its expression is related to the degree of malignancy [8]. Furthermore, the up-regulation of CD44 in malignant gliomas may be an indication of tumor cell growth and migration. Many other glioma-related genes like FN1 [9], TGF-ß [10], SPP1 and EGFR [11] have all been studied and their possibilities to be used as targets for diagnosis and therapy of glioma have also been evaluated. However these studies just reported a few DEGs and the correlations among these genes were still unknown.

This study screened the DEGs in glioma samples systematically based on micro-array data, and predicted novel glioma-related genes by DAVID software, finally analyzed these selected critical genes through constructing a PPI protein network. We hoped to find the key genes and transcription factors associated with the pathogenesis of glioma, furthermore they may be considered as molecular targets for early diagnosis or therapy of glioma.

Materials and methods

Data resources and preprocesses

The gene expression profile of glioma GSE19728 [12] was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). A total of 21 samples were available for further analysis, including 17 glioma samples and 4 normal disease samples. RMA function [13] in Affy package of R software was applied to convert the original data in CEL format into a matrix of probe expression value.

Screening of differential genes

Firstly, the probe numbers of the preprocessed data were converted into gene names combining with the chip platform (GPL570) of GSE19728 data. The probes corresponding to more than one gene were removed, and the average expression values of a plurality of probes corresponding to the same gene were calculated. Then, SAM package [14] was used to filter DEGs between 17 glioma samples and 4 normal samples. The 4 normal samples were considered as a control. The conditions for DEGs screening were as follows: the difference values between genes should be more than 2-fold with $q<0.05$. At last, a hierarchical clustering map was constructed for the DEGs using R software.

Prediction of novel glioma-related genes

The screened up-regulated and down-regulated DEGs were subjected to perform GENETIC_ASSOIATION_DB_DISEASE_CALSS analysis with the online software DAVID [15] (*p*

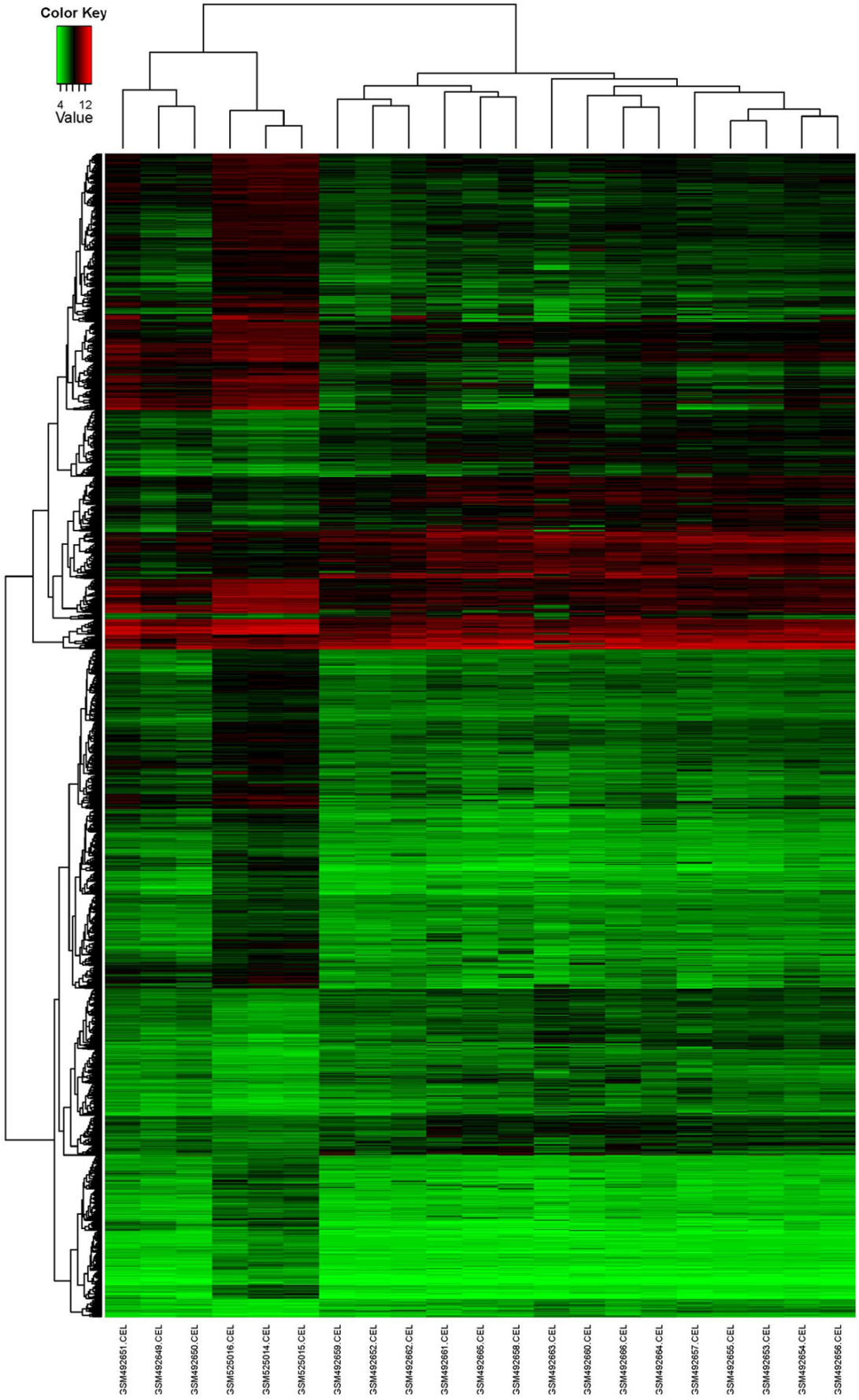
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Figure 2. The hierarchical clustering map for the differentially expressed genes in glioma. The horizontal axis below showed sample name, while the horizontal axis above showed sample clustering, the left vertical axis represented gene clustering.

Table 1. The first 10 differentially expressed genes identified by limma package

Gene	logFC	AveExpr	t	*P.* Value	adj.P.Val	B
NWD2	-3.25748	4.738948	-18.1227	1.29E-13	2.44E-09	20.90525
SLC26A4-AS1	-3.69876	4.61058	-17.3635	2.83E-13	3.55E-09	20.2083
NEGR1	-1.66088	4.026083	-15.9566	1.32E-12	8.25E-09	18.81946
LINC00889	-2.04929	4.17295	-16.2629	9.32E-13	8.25E-09	19.13318
TMEM132D	-2.32697	5.877129	-16.0793	1.14E-12	8.25E-09	18.9459
C11orf87	-2.51614	3.902093	-15.3996	2.50E-12	1.02E-08	18.23165
CDH12	-2.69386	4.905664	-15.1948	3.17E-12	1.02E-08	18.00976
KRT222	-2.91881	4.434116	-15.4358	2.39E-12	1.02E-08	18.27056
KCNV1	-2.87572	4.378165	-14.8366	4.87E-12	1.24E-08	17.61394
SERTM1	-3.71885	4.655747	-14.8275	4.92E-12	1.24E-08	17.60379

Table 2. Cancer-related genes

Term	*P* value	Genes
CANCER	0.0096	EGFR, COL1A1, TOP2A, LGALS3BP, FN1, GNAS, HLA-B, CCND2, AKAP9, ID3, APOE, TGFB1, TGFBR1, AKAP10, CDK6, EZH2, BCL6, NOTCH1, MMP2, IGFBP7, SLC40A1, C1QA, HLA-A, HLA-DRB1, MYC, HLA-G, NUMA1, HLA-DPB1, NF1, CFLAR, TIMP3, SMAD4, SH2B2, LRP1, AKAP13, TCF7L2

value <0.05). The identified DEGs were further mapped to String [16] database, and the pairs of interactions with high confidence (combined_score >0.9) were selected for analysis. Following that, cytoscape [17] was utilized to transform the genes of interactions into graphic.

Analysis of GO enrichment and transcription factors

The selected and new predicted glioma-related genes were all subjected to conduct GO enrichment analysis using online software DAVID with FDR <0.05. In addition, TfactS [18] database was taken to do transcription factor analysis of the selected and new predicted glioma-related genes respectively.

PPI protein interaction network analysis

Finally, the online software String (combined_score >0.4) was carried on to perform protein interaction network analysis.

Results

Data preprocessing

The results of data processed before and after normalization were shown in **Figure 1**. The black lines in the boxes represented the medians of each set of data, from the distribution of which, the standardization degree of data could be judged. In **Figure 1**, the black lines in the boxes were almost on the same straight line after standardization, indicating the standardization level was satisfactory.

DEGs screening

The DEGs between 17 glioma samples and 4 normal samples were screened by SAM package. A total of 3647 DEGs were selected, including 1174 up-regulated genes and 2473 down-regulated genes. A hierarchical clustering diagram for 21 DEGs was constructed using R software (**Figure 2**). Since **Figure 2** was mainly divided into two clusters as glioma and normal samples, however two glioma samples named GSM492650 and GSM492651 were located in control sample cluster, therefore they were excluded in the subsequent analysis. Then limma package [19] in R software was applied to screen the remaining DEGs between 15 glioma samples and 4 normal samples with screening conditions q<0.05 and |logFC| >1.5.

Consequently, a total of 1685 DEGs were obtained, including 402 up-regulated and 1283 down-regulated genes. The first ten DEGs were displayed in **Table 1**.

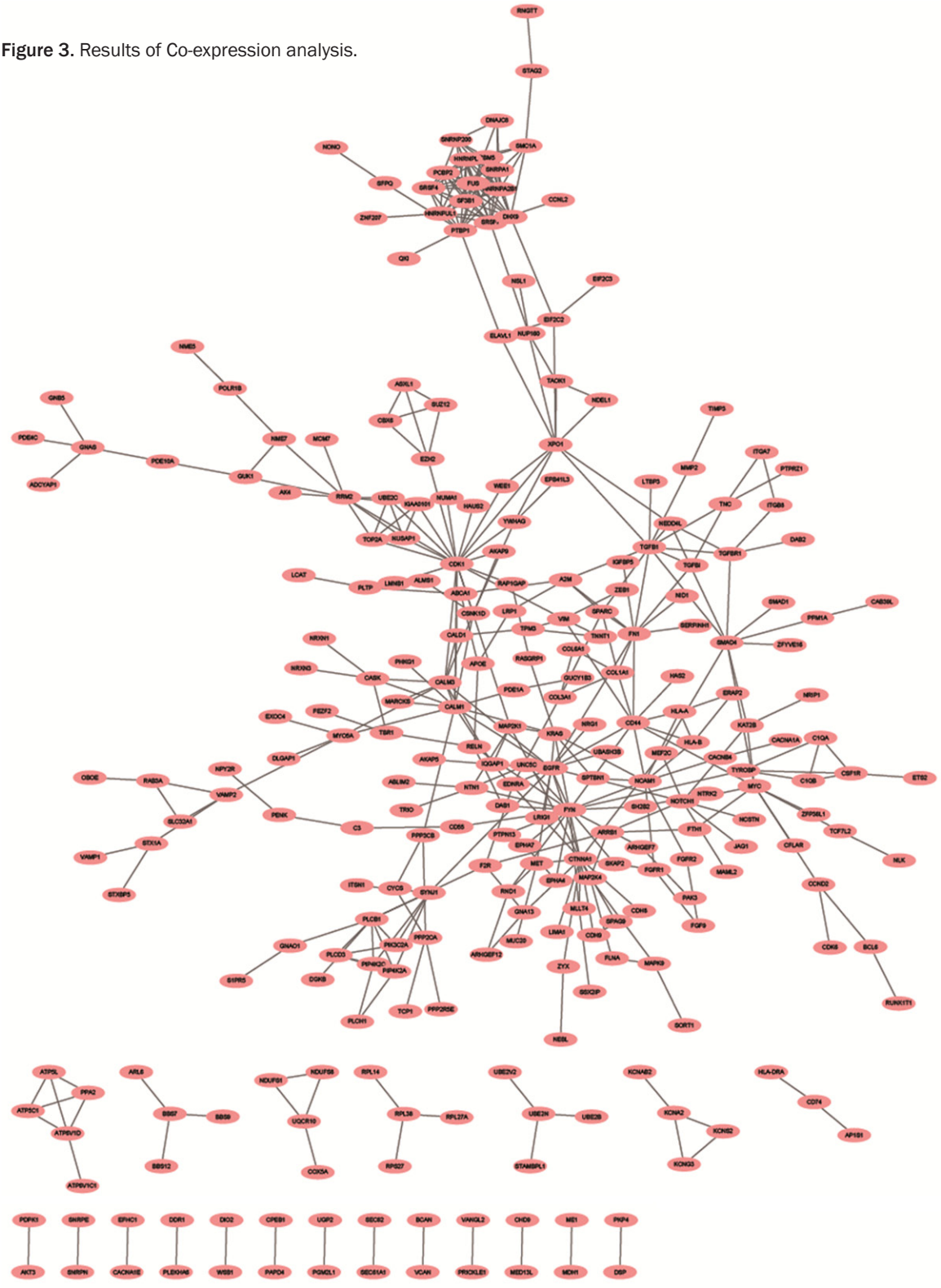

Figure 3. Results of Co-expression analysis.

Prediction of new glioma-related genes

GENETIC_ASSOIATION_DB_DISEASE_CALSS analysis was performed on the up-regulated and down-regulated DEGs, respectively. Finally 36 novel cancer-related genes were found in the up-regulated genes (**Table 2**). Through analysis of interactions with high confidence, we

Table 3. Novel cancer-related genes

Term	New Genes
Cancer	A2M, ABCA1, ADCYAP1, ARRB1, ASXL1, C1QB, CALM1, CALM3, CBX6, CD44, CDK1, COL3A1, COL6A1, CSF1R, CSNK1D, CTNNA1, DAB2, EDNRA, ERAP2, FTH1, FYN, GNB5, IGFBP5, IQGAP1, ITGB8, JAG1, KAT2B, KIAA0101, KRAS, LRIG1, LTBP3, MAML2, MEF2C, MET, NCAM1, NCSTN, NEDD4L, NID1, NLK, NRG1, NTRK2, NUSAP1, PDE10A, PDE4C, PPM1A, RELN, RRM2, RUNX1T1, SERPINH1, SMAD1, SPARC, SUZ12, SYNJ1, TGFBI, TNC, TYROBP, UBASH3B, UBE2C, XPO1, ZFP36L1, ZFYVE16

obtained 420 pairs of interactions containing 271 genes. Then the interacted genes were transformed into graphic (**Figure 3**). The genes associated with the cancer-related genes were listed in **Table 2**. There were 61 novel genes possibly being related to glioma were identified. The detailed results were indicated in **Table 3**.

Results of GO enrichment analysis

The up-regulated and down-regulated cancer-related genes in **Table 2** and novel glioma-related genes in **Table 3** were all taken to perform GO enrichment analysis. And we found these DEGs were mainly relevant to immune responses as well as regulation of cell growth (**Figure 4**). The concrete GO enrichments were listed in **Table 4**.

Transcription factors analysis

Altogether, there were 81 transcription factors involved in cancer-related genes and novel glioma-related genes. And the relationships between up-regulated and down-regulated genes were displayed in **Figure 5**. From which, There were 17 common transcription factors, while the specific transcription factors for the up-regulated and down-regulated genes were 60 and 4, respectively. In addition, the glioma-related genes were mainly regulated by the binding sites of transcription factors GLI2, SP1, SMAD7, SMAD3, RELA, STAT5B, CTNNB1, STAT5A, TFAP2A and SP3 (**Table 5**).

Construction of PPI protein interaction network

The PPI protein interaction network was constructed by online software string (**Figure 6**). In order to predict the critical genes in glioma, we draw a histogram for the number of contiguous genes in the interaction network (**Figure 7**). We could obviously determine that the hub nodes of the network were EGFR, TGFB1, FN1 and MYC, as they interacted with most of the genes. Hence, we predicted they may be the key genes associated with glioma.

Discussion

In this study, we predicted a total of 97 cancer-related genes and they were all differentially expressed in our study, indicating they might be closely associated with glioma. After GO enrichment and transcription factor analysis of these cancer-related genes, we found these genes were mainly regulated by the binding sites of transcription factors (GLI2, SP1, SMAD7, SMAD3, RELA, STAT5B, CTNNB1, STAT5A, TFAP2A and SP3). As we know, the transforming growth factor-ß (TGF-ß) played an important role in the pathogenesis of glioma, and it could turn from a tumor suppressor to a tumor promoter during carcinogenesis [10]. However, the Smad pathway was one of the most important pathways for TGF-ß related intracellular signal transduction to regulate various pathological and physiological effects. Smad3 and Smad7 belonged to receptor-regulated R-Smads and inhibitory I-Smads group respectively. Smad proteins were activated by phosphorylation [20], and its phosphorylation caused nuclear translocation, moreover Smad could interact with other transcription factors to medicate their target genes [21, 22]. It was reported in a recent study, the promoting effect of TGF-ß in glioma was associated with abnormal regulation of Smad pathway, and antagonizing of TGF-ß may be a potential method for glioma therapy. STAT was a family consisting of cytoplasmic transcription factors transmitting signals to the nucleus where STATs could bind to specific DNA promoters and regulate gene expression [23]. A number of studies had demonstrated that STATs were critical in some cellular processes including innate and adaptive immune function, regulation of cell differentiation, growth and apoptosis [24, 25], in particular elevated activity of Stat5 was found in a huge variety of human tumors like glioma [26]. In addition, strong evidence showed that aberrant STAT signaling, especially Stat3 and Stat5, were involved in the development and progression of several types of cancers such as glioma through apoptosis induction or cell proliferation

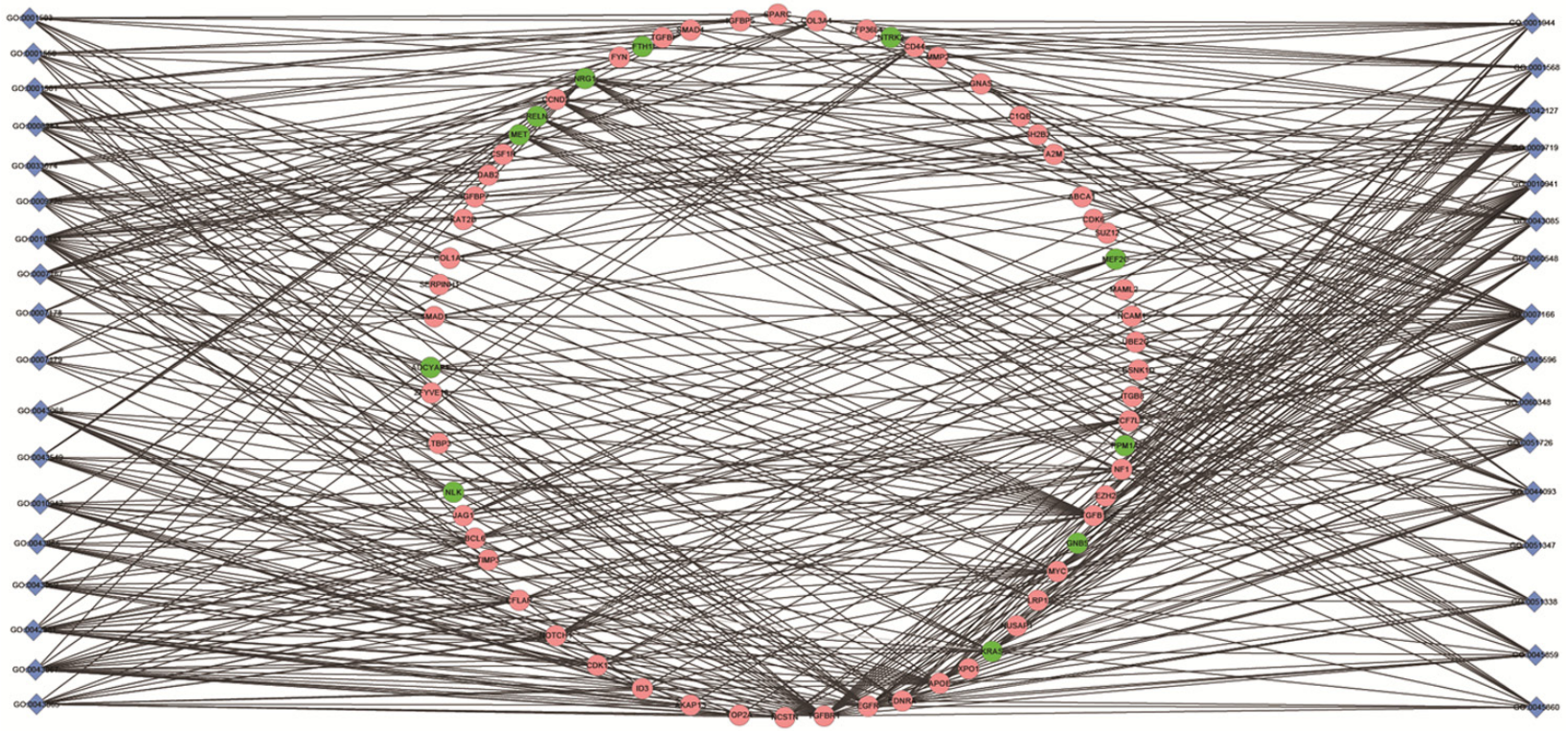

Figure 4. GO enrichment analysis. Diamonds represented GO term; circles represented genes, red circles showed up-regulated genes, green circles showed down-regulated genes.

Table 4. GO enrichment analysis

Go Term	FDR
GO:0010033~response to organic substance	7.74E-06
GO:0043085~positive regulation of catalytic activity	8.36E-06
GO:0042127~regulation of cell proliferation	3.60E-05
GO:0044093~positive regulation of molecular function	5.32E-05
GO:0007167~enzyme linked receptor protein signaling pathway	6.65E-05
GO:0001568~blood vessel development	9.71E-05
GO:0001944~vasculature development	1.27E-04
GO:0042981~regulation of apoptosis	0.001252
GO:0001503~ossification	0.001307
GO:0043067~regulation of programmed cell death	0.001454
GO:0010941~regulation of cell death	0.001537
GO:0007166~cell surface receptor linked signal transduction	0.001617
GO:0001501~skeletal system development	0.001666
GO:0060348~bone development	0.002183
GO:0007179~transforming growth factor beta receptor signaling pathway	0.004043
GO:0043065~positive regulation of apoptosis	0.0065
GO:0043068~positive regulation of programmed cell death	0.007013
GO:0010942~positive regulation of cell death	0.007374
GO:0001558~regulation of cell growth	0.007695
GO:0007178~transmembrane receptor protein serine/threonine kinase signaling pathway	0.007764
GO:0051726~regulation of cell cycle	0.015945
GO:0045596~negative regulation of cell differentiation	0.018367
GO:0009719~response to endogenous stimulus	0.019434
GO:0045859~regulation of protein kinase activity	0.023518
GO:0045860~positive regulation of protein kinase activity	0.023711
GO:0043066~negative regulation of apoptosis	0.029893
GO:0033674~positive regulation of kinase activity	0.031394
GO:0043549~regulation of kinase activity	0.032327
GO:0043069~negative regulation of programmed cell death	0.034044
GO:0060548~negative regulation of cell death	0.034932
GO:0008283~cell proliferation	0.040462
GO:0009725~response to hormone stimulus	0.04173
GO:0051347~positive regulation of transferase activity	0.042479
GO:0051338~regulation of transferase activity	0.047261

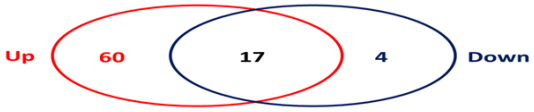

Figure 5. The common and specific transcription factors of the up-regulated and down-regulated genes.

prevention [27], indicating targeted Stat3 or Stat5 might provide a potential novel strategy for cancer intervention.

Through protein interaction network analysis of all these cancer-related genes, we obtained the hub nodes (EGFR, TGFβ1, FN1 and MYC) in the interaction network. As these genes may be the most critical genes glioma, they could be considered as drug targets for glioma therapy. Among these crucial genes, epidermal growth factor (EGF) receptor (EGFR), a receptor tyrosine kinase, was the most commonly reported to be altered in gliomas [28], thus leading to abnormally regulated kinase activity and excessive downstream signaling transduction, which may contribute to the invasion and aggravation of gliomas [29, 30]. The amplification and overexpression of EGFR were two apparent features

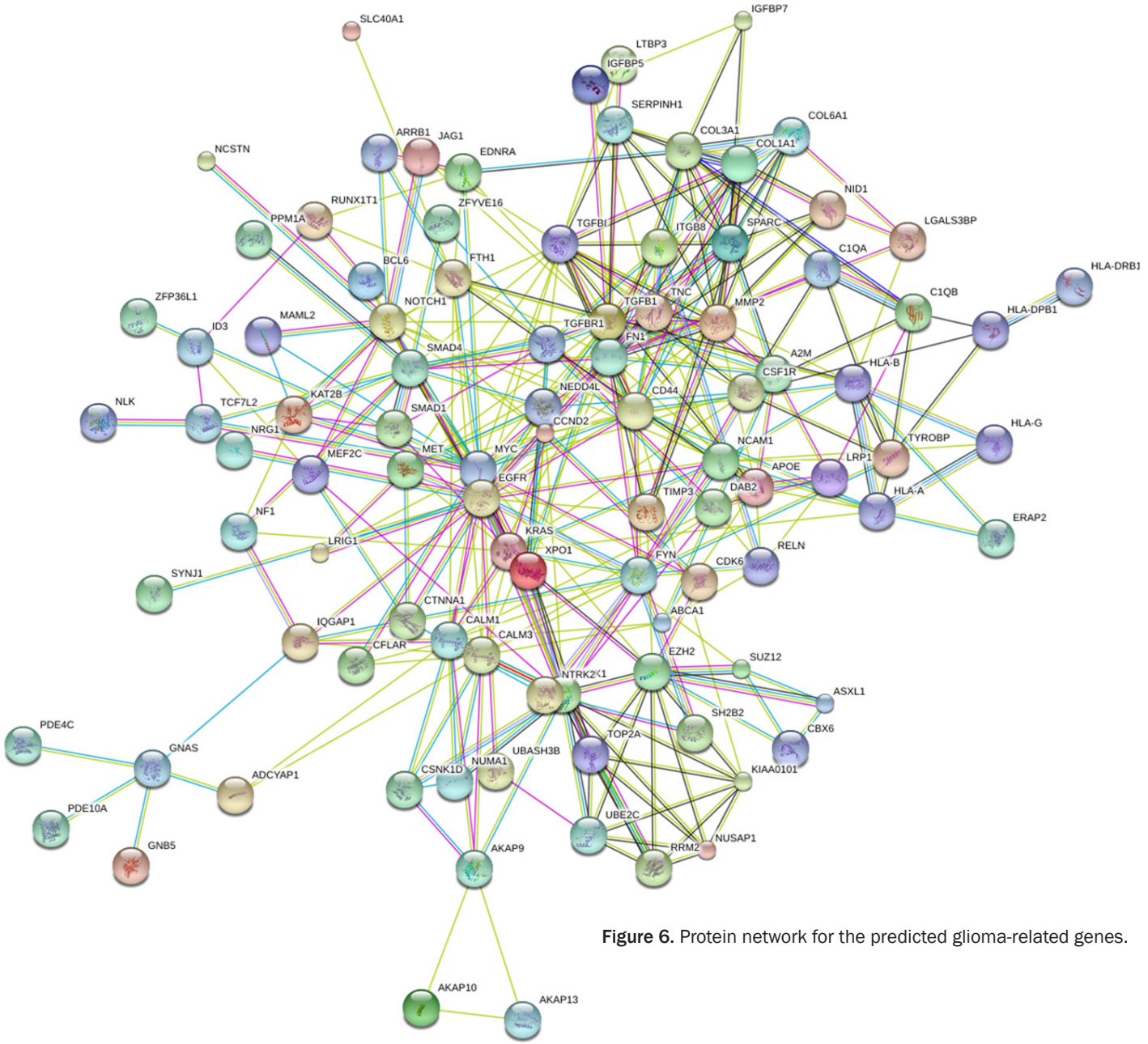

Figure 6. Protein network for the predicted glioma-related genes.

Table 5. The first 10 transcription factors of glioma-related genes

Transcription Factor	*P*. value	E. value	Q. value	FDR control (B-H)
GLI2	0.00E+00	0.00E+00	0.00E+00	6.17E-04
SP1	0.00E+00	0.00E+00	0.00E+00	1.24E-03
SMAD7	0.00E+00	0.00E+00	0.00E+00	1.85E-03
SMAD3	0.00E+00	0.00E+00	0.00E+00	2.47E-03
RELA	2.00E-05	1.62E-03	2.25E-05	3.09E-03
STAT5B	3.00E-05	2.43E-03	2.41E-05	3.70E-03
CTNNB1	3.00E-05	2.43E-03	2.41E-05	4.32E-03
STAT5A	4.00E-05	3.24E-03	2.81E-05	4.94E-03
TFAP2A	1.20E-04	9.72E-03	7.50E-05	5.56E-03
SP3	2.60E-04	2.11E-02	1.46E-04	6.17E-03

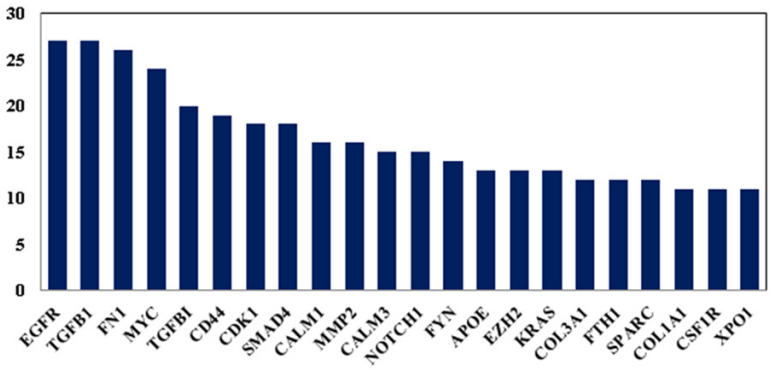

Figure 7. The histogram for the number of contiguous genes in the interaction network. The horizontal axis showed gene name while the horizontal axis showed the number of contiguous genes interacted with.

of gliomas occurring in nearly 50% of cases [31] and the up-regulation of EGFR may correlated with a unfavorable prognosis in several types of cancer [32]. Furthermore, Kaiming Xu et al. [28] demonstrated that EGFR signaling could alters the expression of COX-2 and the expression of COX-2 was induced by stimulation of EGF in human glioma cell lines. They also investigated the mechanisms how EGFR regulated COX-2 expression and demonstrated that the main signaling pathway contributed to EGFR-dependent COX-2 induction was the p38 mitogen-activated protein kinase (p38-MAPK). And the p38 pathway increased COX-2 activity by phosphorylation and activation of the Sp1/Sp3 transcription factors [28]. In view of the prominent function of EGFR in glioma, targeting this receptor might be explored as a diagnostic method or potential therapy for these tumors. TGF-β played important roles during cancer development processes including cell invasion, immune suppression, and microenvironment modification [33]. And TGF-β was an critical immunosuppressive factor expressed in glioma. Studies indicated that TGF-β could suppress the proliferation of cytotoxic T cells isolated from glioma patients [34]. The high activity of the TGF-β signaling pathway in glioma was also reported to be associated with a poor prognosis [35]. Furthermore, Christian et al. found that the mRNA levels of 3 TGF-β isoforms were all up-regulated in glioma, which correlated with the degree of malignancy [36]. All told that TGF-β was a potential molecular target for the prognosis or treatment of glioma.

To conclude, we analyzed the DEGs between glioma samples and normal samples based on micro-array expression data, and identified several critical genes and transcription factors including EGFR, TGFB1, FN1 and MYC that may play significant regulation roles in glioma. Although some of these genes were reported in previous studies such as EGFR, most of the results obtained in our study have not been found before, which may help us better elucidate the potential molecular pathogenesis of glioma and develop novel targets for glioma therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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