

A model of molecular characteristics to classify patients with WHO Grade III gliomas

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Abstract

Background: The World Health Organization (WHO) Grade III gliomas display large within-group differences, and no studies have attempted to classify Grade III gliomas to our knowledge. We aimed to classify Grade III gliomas and complement the existing molecular classification system.

Methods: We used microarrays to profile glioma progression from normal brains (n = 5) to WHO Grade II (n = 97) to Grade III (n = 34) to Grade IV samples (n = 89). Using bioinformatics analysis to construct a model to evaluate the diagnosis and prognosis of patients with Grade III gliomas.

Results: We identified 27 gene expression patterns of glioma progression and found Grade III glioma samples had large within-group differences. There were 506 differentially expressed genes in Grade III glioma samples and used to construct a network. Three clusters were identified, we chose the first two genes with the most significant P values in each cluster, age and Isocitrate Dehydrogenase 1 (IDH1) mutations to construct a model to score and high risk scores were significantly associated with poor survival in our data and two independent datasets for patients with Grade III gliomas ($P = 1.12e-03$, $P = 2.55e-04$, $P = 5.93e-06$, respectively). The ROC area under the curve was 80.0%, 80.9% and 76.6%, respectively.

Conclusions: Our results revealed that the model was able to predict the diagnosis and prognosis of patients with Grade III gliomas, assisting with post-surgical treatment management and providing a priori molecular targeting treatment.

Keywords: Grade III gliomas, model, molecular characteristics, predictive.

Background

Glioma represents the majority of malignancies in the central nervous system and is often associated with the progressive deterioration of emotional and cognitive health and poor quality of life [1, 2]. Although multiple modes of treatment, such as surgery, chemotherapy, radiation and biotherapy, improves survival, death inevitably occurs from either recurrent or progressive disease, thus, scientific and clinical advances are desperately needed [3]. The WHO Classification of Tumors of the Central Nervous System is currently the most common classification approach. The updated 2016 revision of the WHO classification mandates the inclusion of molecular-genetic alterations in the diagnosis of diffuse glial tumors and is a significant departure from the morphology-based 2007 classification [4]. The WHO classification has been clinically applied for years and is a practical and effective approach for brain tumor analysis; it provides a means for placing tumors into specific, relevant categories [5]. Although histopathological grading is subjective and associated with significant intra-observer variability [6], it has implications not only for survival prediction but also for tailoring post-surgical management and patient selection in the evaluation of relatively new molecular targeting treatments when it is combined with molecular data [7, 8].

In recent decades, the molecular biology of gliomas has made tremendous progress. Mutations in the IDH gene are primarily located at codon R132 in IDH1 and R172 in IDH2. IDH gene mutations commonly indicate a favorable independent prognostic factor of patients with gliomas [9-11]. The combined loss of chromosomal arms 1p and 19q resulting from an unbalanced t(1;19)(q10;p10) mutation leads to loss of heterozygosity, which associated with improved prognosis and responsiveness to therapy in patients with gliomas [12]. Recently, several published studies reported that specific combinations have the ability to reclassify gliomas into rational subsets [13-15].

An increasing number of studies have reported that the WHO classification of glioma is subjective, and new classification schemes are emerging [16]. However, a new approach for WHO Grade III glioma classification has not been reported.

In this study, we used microarrays to profile glioma progression from normal brains to WHO Grade II to Grade III to Grade IV samples. We identified 27 gene expression patterns by comparing datasets from different glioma grades and found that the WHO classification could predict the prognosis of patients with gliomas and that the prognoses were worse for higher grades. However, patients with WHO Grade III gliomas had significantly different prognoses, so we divided the Grade III samples into two groups and identified their differentially expressed genes. We selected 3 clusters for further analysis based on protein-protein interaction (PPI) networks and the Molecular Complex Detection (MCODE) plugin in the Cytoscape. The most enriched Gene Ontology (GO) biological process terms in each respective cluster were cell proliferation, immune response and smooth muscle cell migration. At last, we constructed a model to score and evaluate patients' diagnosis and prognosis.

Methods

Patients and samples

The glioma samples were obtained from Beijing Tiantan Hospital between January 2005 and December 2009. The samples included 5 cases of normal brain tissues samples (hereafter referred to as "grade0"), 97 cases of WHO Grade II samples (hereafter referred to as "grade2"), 34 cases of Grade III samples (hereafter referred to as "grade3") and 89 cases of Grade IV samples (hereafter referred to as "grade4"). Patients were graded according to the WHO classification guidelines by two neuropathologists (detailed information about the tissue samples is presented in table S1). Overall survival (OS) was calculated from the date of diagnosis until death or the end of follow-up, while progression-free survival (PFS) was defined as the time between diagnosis and the first unequivocal clinical or radiological sign of disease progression. All of samples were rinsed with normal saline after surgical resection and divided into two parts, one of which was placed in liquid nitrogen immediately and then stored at -80°C until use, and the other was placed in formaldehyde solution and for hematoxylin-eosin stained to assess the percentage of tumor cells.

RNA isolation and microarray expression profiling

Total RNA was extracted using the mirVana miRNA Isolation kit (Ambion 1561) according to the manufacturer's protocol. Only samples with >80% tumor cells were selected for RNA extraction and subsequent experiments. The Agilent 4×44K Whole Human Genome Oligo Microarrays were implemented and normalized as our previous description [17]. The raw and processed data are publicly available at the Gene Expression Omnibus (GEO) website under the accession number GSE109857.

Expression patterns of multi-stage glioma samples

The tissue samples were divided into the following four stages: grade0, grade2, grade3 and grade4. Gene expression patterns were defined by expression levels changed relative to those changes found in neighboring stages. There are three scenarios for differences in gene expression between adjacent stages: upregulation (u), downregulation (d), and no significant change (n). Accordingly, the genes were divided into 27 groups defined by the four stages. The group in which gene expression increasing steadily occurred was named as “uuu”, the group of genes with sharply decreasing expression was referred to as “ddd”, and the group consisting of genes with no significant changes were called as “nnn”.

PPI network construction and subnetwork analysis

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; <https://string-db.org>) was employed to construct PPI networks for differentially expressed genes [18]. The tab-delimited format of the PPI network was exported from STRING and imported into Cytoscape, which was used to visualize the network [19]. Then, we used the MCODE plugin in Cytoscape to identify discrete clusters from the former PPI network [20]. The GO functional enrichment analysis of clusters were conducted in the Database of Annotation Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>).

Prognostic analysis

We proposed a model that considered several factors to generate a score for each patient to predict the prognosis of patients with WHO Grade III gliomas. In detail, we used univariate Cox regression analysis to evaluate the association between survival time and the expression levels of selected genes, IDH1 mutations and age. A mathematical formula was constructed for the model [21]. The risk score of each patient was calculated as follows:

$$\begin{aligned} \text{Risk_Score} &= i * \exp(\text{gene}(i)) \\ &+ \beta * \text{mutation}(\text{IDH1}) \\ &+ \gamma * (\text{Age}) \end{aligned}$$

α , β and γ represent the regression coefficients of genes expression values, IDH1 mutations and age. All patients in the dataset were thus assigned to high- risk and low-risk groups using the median risk score as the cut-off point. The Kaplan-Meier method was used to estimate the overall survival time for the two subgroups, and differences in survival time were analyzed using the log rank test (R package “survival”).

Analysis of public datasets

Two independent datasets of WHO Grade III glioma transcriptome data were used for validation, namely, the Chinese Glioma Genome Atlas RNA sequencing dataset (CGGA data) and GEO microarray dataset (GSE16011), as well as their corresponding clinical information (Table S2-S3). Because the raw data were not provided for CGGA data, the two sets of normalized data were directly downloaded and analyzed.

Statistical analysis

All statistical analyses in this study were performed using R software (<http://www.r-project.org>). T-Distributed Stochastic Neighbor Embedding (t-SNE) were implemented in the R package Rtsne. Gene expression pattern plots, circus plots and ROC curves were generated by the R packages ggplot2, OmicCircos, pROC, respectively. All statistical tests were two-sided, and a *P* value less than 0.05 was considered to be statistically significant.

Results

Discovery of different outcomes of patients with Grade III gliomas

The gene expression profiles of all 225 samples were analyzed. After we normalized and filtered the probes, 20,358 genes were used in subsequent analyses. These genes were divided into 27 groups according to their expression, ranging from normal to Grade II to Grade III to Grade IV samples (Table S4 and Fig.1a). The expression patterns “uuu” (n=119), “nuu” (n=216), “ddd” (n=97), “ndd” (n=190) were used for further analysis. We performed a principle component analysis (PCA) with the 622 genes and found that patients with WHO Grade II gliomas and patients with Grade IV gliomas were clearly separated; however, patients with Grade III gliomas were mixed with patients in the other two grades (Fig.1b). Further analysis indicated that the prognoses of patients with Grade II or Grade IV were not significantly different within their group (Figure S1a-d); however, Grade III glioma samples displayed large within-group differences (Fig.1c-d). Consideration of the prognostic outcomes of WHO grading could be a good indicator for predicting the survival of patients with gliomas (Fig.1e-f). However, patients with Grade III gliomas had a range of outcomes, so we attempted to establish a model to assist the diagnosis and treatment of patients with Grade III gliomas.

Identification of differential genes in Grade III glioma samples

We employed t-SNE and unsupervised clustering to divide the Grade III samples into two groups. Group1 had 22 samples and Group2 had 12 samples (Fig.2a). Then, we identified 506 differentially

expressed genes through an unpaired Student's t-test; 298 genes were highly expressed in Group1, and 208 genes were highly expressed in Group2. The distribution of the differentially expressed genes on chromosomes is shown in Fig.2b. Based on the GO analysis, the significant biological processes of genes in Group1 were primarily associated with cell proliferation, cell adhesion, angiogenesis; however, highly expressed genes in Group2 were significantly enriched in cerebellar granule cell differentiation, central nervous system development and adult behavior. Survival analysis of the groupings of Grade III samples is shown in Fig.S1e-f.

PPI analysis of the differentially expressed genes in patients with Grade III gliomas

We used all differentially expressed genes in patients with Grade III gliomas to construct a PPI network in STRING. The largest connected subnetwork contained 340 nodes and 1,830 edges (Fig.3a). The nodes degree distribution in the subnetwork displayed a scale-free feature, and the power was 0.8 (Fig.S2). Then, we extracted discrete clusters from the subnetwork using the MCODE plugin in Cytoscape software. Three clusters were identified in the subnetwork, which included a total of 82 genes. The respective clusters had 44, 15, and 23 genes, and the corresponding gene numbers are listed in Table S5. Three clusters interaction networks are shown in Fig.3b-d. Then, we examined the functions of these 3 clusters using GO enrichment analysis. Up to 10 of the most significantly enriched GO biological process terms corresponding to each cluster are shown in Fig.3e-g. The most significantly enriched terms in Cluster1 were DNA repair and cell cycle, including microtubule-based movement, chromosome segregation, mitotic G2 DNA damage checkpoint, and mitotic chromosome condensation and so on; in Cluster2, the most enriched terms were chemotaxis, cell-cell signaling, inflammatory response and immune response, and in Cluster3, the terms were smooth muscle cell migration and protein ubiquitination, and patterning of blood vessels.

A model to evaluate the diagnoses and prognoses of patients with Grade III gliomas

We performed univariate Cox regression analysis to evaluate the association between survival time and all genes in the 3 clusters. In each cluster, we chose the first two genes with the most significant P values. We ultimately chose CENPE and DEPDC1 in Cluster1, VIM and ANXA1 in Cluster2, and PLAU and ASB1 in Cluster3. In addition to these 6 genes, age and IDH1 mutations were used to construct a model to evaluate the diagnosis and prognosis of patients with Grade III gliomas (detailed information about the model is presented in Table 1). Regression coefficients with a plus sign indicate that increased expression was associated with decreased survival (risk factors), and conversely, a minus sign indicates that increased expression was associated with increased survival (protective factors). Survival analysis indicated that the model could predict the prognosis of patients with Grade III gliomas ($P = 1.12e-03$), and high risk scores were associated with poor survival (Fig.4a). In addition, we used two independent datasets of WHO Grade III transcriptome data to validate this model. The results revealed that high risk scores were significantly associated with poor survival in the CGGA data for patients with Grade III gliomas ($P = 2.55e-04$) and in GSE16011 for patients with Grade III gliomas ($P = 5.93e-06$) (Fig.4b-c). ROC curve analysis for the model in patients with Grade III gliomas was performed, and the area under

the curve was 80.0% (Fig.4d). The ROC area under the curve was 80.9% and 76.6%, respectively, in CGGA data and GSE16011 (Fig.4e-f). These results suggested that the model was able to predict the diagnosis and prognosis of patients with Grade III gliomas, assisting with post-surgical treatment management and providing a priori molecular targeting treatment.

Discussion

Using of gene expression data from glioma tissues to determine better treatment options is becoming increasingly common in clinical practice [22]. In our study, transcriptomic data from multiple stages in a total of 225 samples were used to analyze glioma progression.

Survival analysis of all glioma samples using only the WHO classification could clearly distinguish different grades. We found that WHO Grade III samples were mixed with Grade II and Grade IV samples and that the survival times of patients with Grade III gliomas were clearly different. That is, patients with Grade III gliomas may have completely different prognoses. Due to urgent need for individualized treatment, a method to assist with diagnosis and treatment is needed, especially for tumors that appear to be different within the same group.

We discovered that highly expressed genes in Group1 were associated with cell proliferation, immune response and angiogenesis; these biological behaviors are similar to those of malignant cells. In contrast, highly expressed genes in Group2 were mainly enriched in nervous development and neurological function, their molecular phenotype partially retained the characteristics of normal glial cells. Furthermore, the median OS of Group2 patients was 1,412 days with a 10-year survival rate of 50%, whereas the median OS of Group1 patients was 651 days with a 10-year survival rate of only 18%. Thus, the better the differentiation of tumor cells, the closer they are to the characteristics of normal cells, and the smaller the impact on patients.

For patients with Grade III gliomas, we proposed a model that included 6 genes, age and IDH1 mutations to evaluate diagnoses and prognoses. By consulting literatures, we know that age is associated with glioma patients' prognosis [23, 24] and IDH1 mutations or not commonly is thought to be a favorable independent prognostic indicator of glioma patients [25], although the regression coefficients of age and IDH1 mutations in our study were not significant, the sample size of Grade III was probably small. CENPE, a cell cycle related gene, has been reported to significantly increase with increasing WHO grades [26]. DEPDC1 expression is increased in glioma cell lines, tissues, and brain tumor initiating cells, and the suppression of endogenous DEPDC1 expression by small interfering RNA inhibited glioma cell viability and induced apoptosis through NFκB signaling [27]. VIM encodes a type III intermediate filament protein and has been reported participate in the epithelial-to-mesenchymal transition phenotype in human glioblastoma [28]. ANXA1, an invasion-associated gene, exhibits increased expression levels in glioblastoma multiforme (GBM) [29, 30]. PLA1 induced increased expression by Oncostatin M, which is involved in cell matrix remodeling, migration, proliferation control and angiogenesis [31]. ASB1 encodes a

protein that contains an ankyrin repeat sequence and a SOCS box domain and is related to the innate immune system, but as far as we know, it has not been reported to be associated with gliomas.

To the best of our knowledge, this is the first report to combine WHO classifications with a molecular model to evaluate the diagnosis and prognosis of patients with Grade III gliomas. Furthermore, multiple, rather than single, factors are considered to drive the disease process in complex glioma tumors. Of course, the transcriptome represents only one level of biology, and we should account for other “-omics” data types, such as proteomics and epigenomics data.

Conclusions

Our results provide suggestions for the future development of a mechanistically based molecular risk estimation model for Grade III gliomas. The signature might better lend itself and be accessible to translation into clinical practice than other classification approaches, which could aid in the implementation of individualized medicine and treatment with optimal regimens.

Abbreviations

WHO: World Health Organization

IDH1: Isocitrate Dehydrogenase 1

PPI: Protein-protein interaction

MCODE: Molecular Complex Detection

GO: Gene Ontology

OS: Overall survival

PFS: Progression-free survival

t-SNE: T-Distributed Stochastic Neighbor Embedding

GBM: Glioblastoma multiforme

Declarations

Ethics approval and consent to participate

All patients signed informed consent forms and the consent was obtained in written. The use of human tissue samples and experimental procedures for this study were reviewed and approved by the Ethics Committee of the Cancer Institute and Hospital, Chinese Academy of Medical Sciences. The ethics committee approved this procedure. The dataset was anonymized prior to analysis.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in the current study are not publicly available. Anonymous data is available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Botao Zhang and Dan Lu analyzed and collected the data and were the main contributors in writing the manuscript. They contributed equally to this work. Rongfang Shen interpreted the patient data. Shujun Cheng and Lin Feng critically reviewed the manuscript and gave important input. All authors have approved the final version of the manuscript.

Acknowledgment

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Neurogenetics

Not applicable.

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Tables

Table 1. Clinical characteristics of the all glioma patients (220 patients)

Characteristic	No. patients (%)
Age (year)	
< 40	100 (45)
≥ 40	120 (55)
Gender	
Male	124 (56)
Female	96 (44)
WHO Grading	
II	97 (44)
III	34 (15)
IV	89 (41)
IDH1 mutation	
Yes	99 (45)
No	119 (54)
NA	2 (1)

Table 2. Detailed information of the model.

Variable	Coefficient	<i>P</i> value
CENPE	1.366	0.001
DEPDC1	2.078	0.001
VIM	0.836	0.002
ANXA1	0.467	0.003
PLAU	0.546	0.006
ASB1	-1.336	0.010
Age	0.013	0.379
IDH1 mutation	-0.413	0.314

Figures

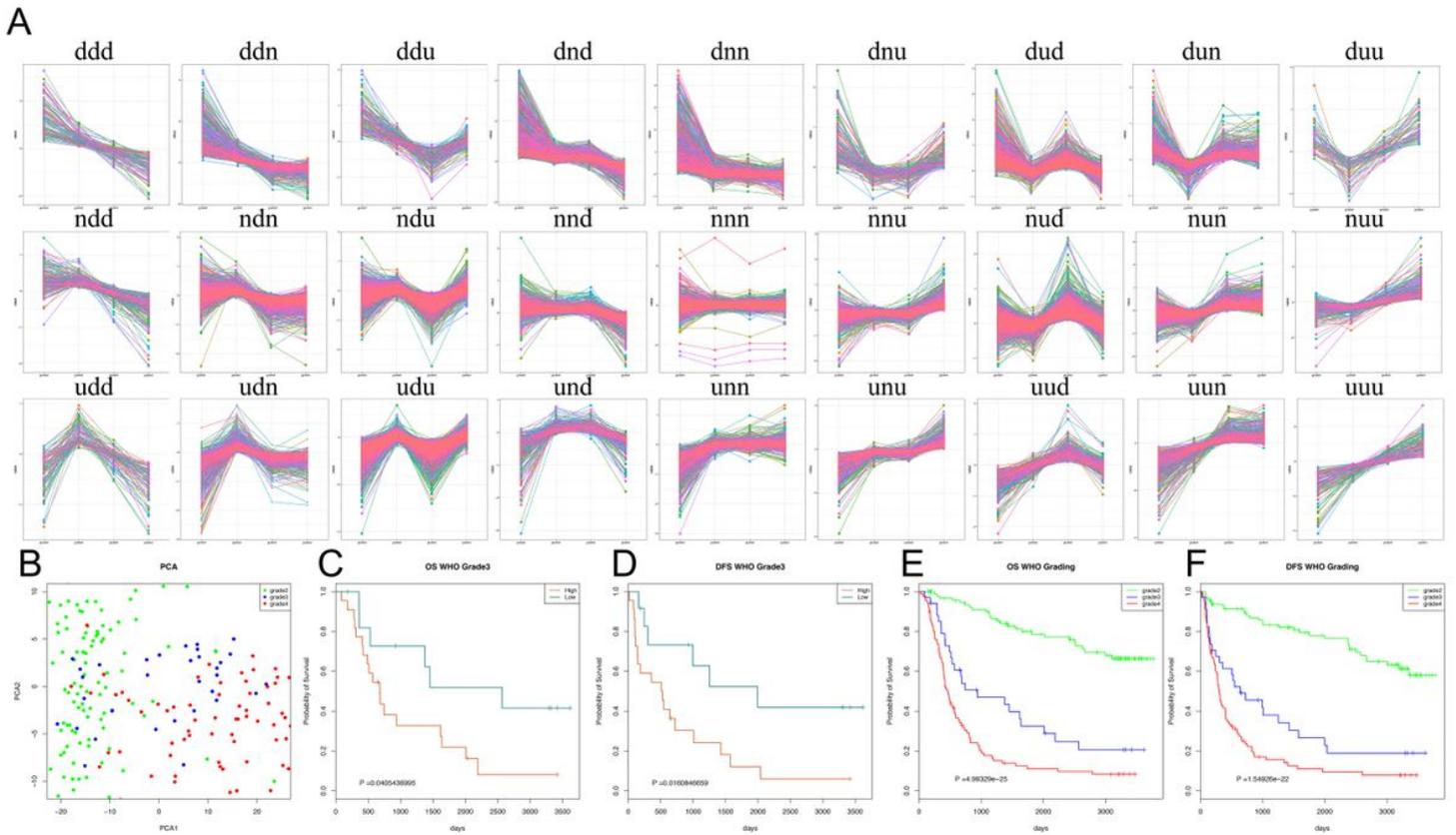


Figure 1

Global gene classification and survival results. (a) The genes were classified into 27 expression patterns according to their expression dynamics throughout the glioma development process. The stages of progression were plotted on the x-axis, and the normalized gene expression level in each panel was plotted on the y-axis. Each gene is depicted with a colored line according to its relative expression level at the corresponding stages. (b) The PCA showed that WHO Grade III samples were mixed with Grade II and Grade IV samples. Green points represent Grade II samples, blue points represent Grade III samples, and red points represent Grade IV samples. (c, d) The OS and DFS curves for patients with Grade III gliomas. (e, f) The OS and DFS curves for all patients with gliomas.

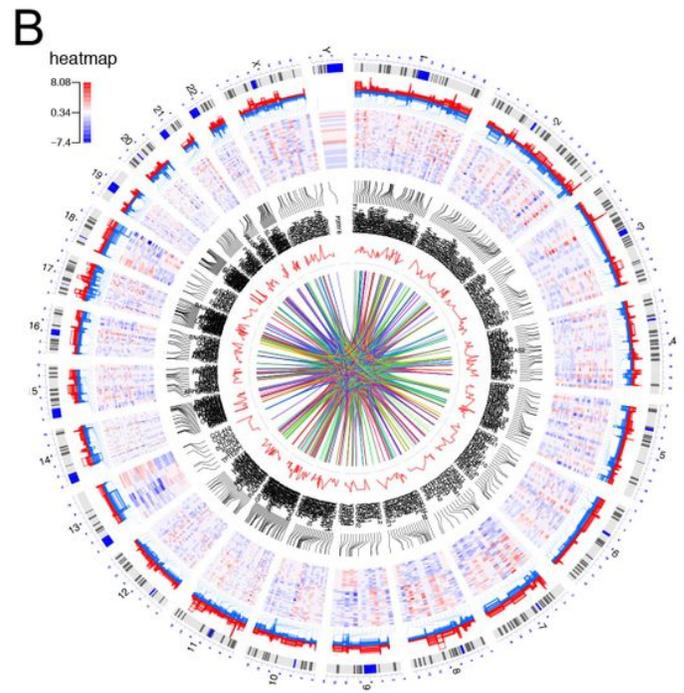
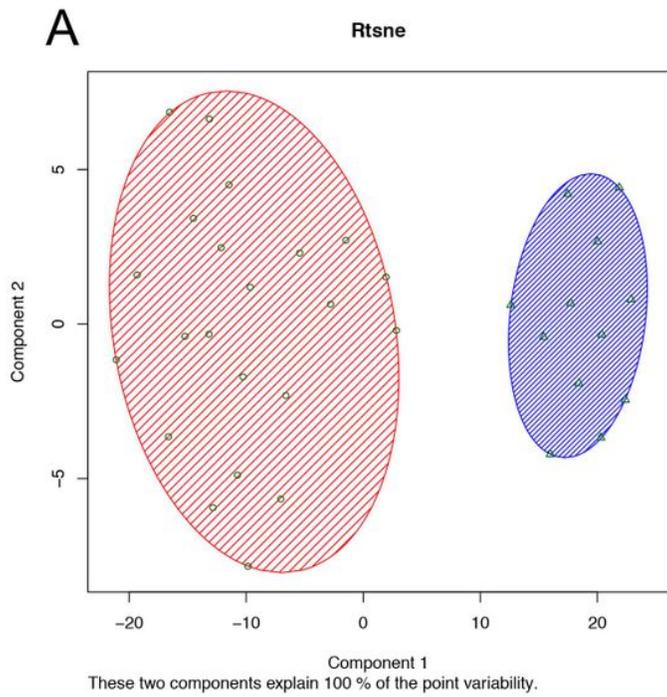


Figure 2

The aberrations of genes in Grade III samples. (a) Grade III samples were divided into two groups. The red shaded part represents Group1, 22 samples, and the blue shaded part represents Group2, 12 samples. (b) The distribution of the different genes between Group1 and Group2 on chromosomes.

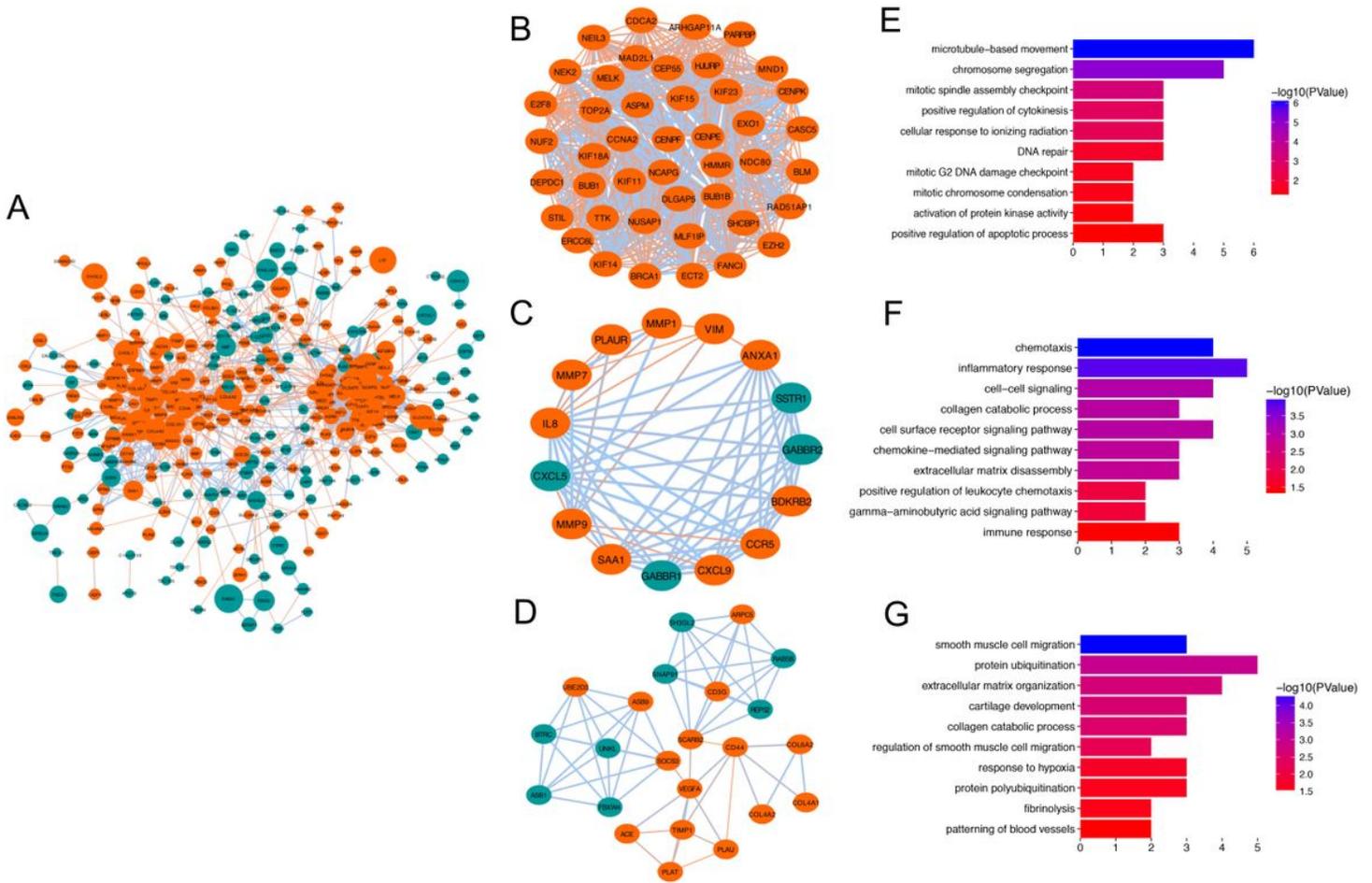


Figure 3

PPI network analysis of the differentially expressed genes in Grade III samples. (a) PPI network of associated proteins encoded by the differentially expressed genes. The blaze orange nodes and the persian green nodes represent genes that were highly expressed in Group1 and Group2, respectively. The node size represents the fold change of genes. The edges represent correlation coefficients, and the colors of the edges represent the combined scores from STRING, where blue represents a high combined score and atomic tangerine represents a low combined score. (b-d) Three discrete clusters identified from the network. (e-g) The most significantly enriched GO terms in each cluster.

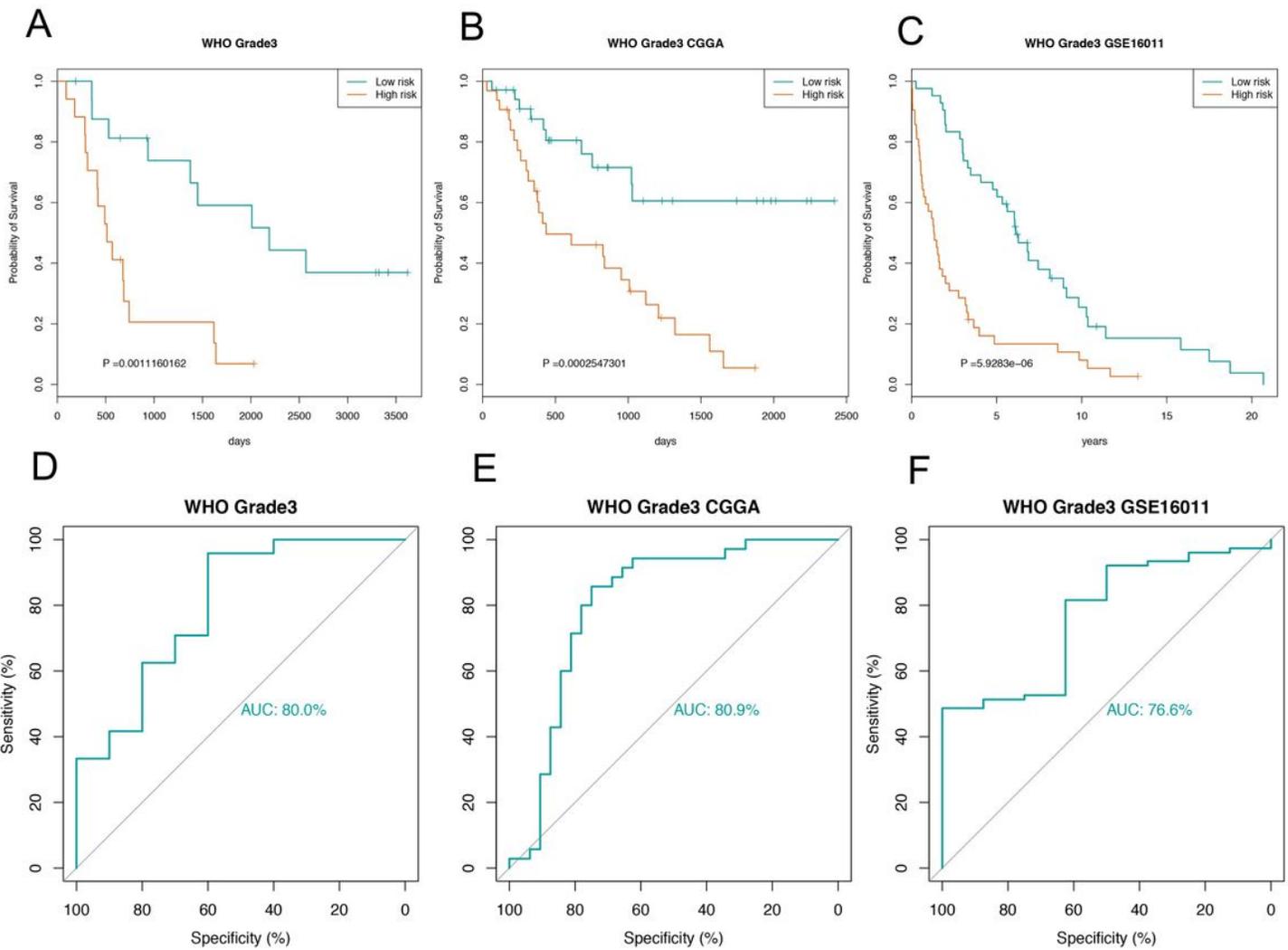


Figure 4

The model associated with patients with Grade III glioma survival and predictive values. (a-c) Kaplan–Meier estimates of OS of patients according to the model. (d-f) The ROC curves of our dataset and two independent datasets.

Supplementary Files

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