Weighted Gene Coexpression Network Analysis Reveals Cancer Stem Cell-Associated Metabolic Gene Signature in Glioma

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Abstract

Background: Isocitrate dehydrogenase (*IDH*) mutant glioma patients have a favorable prognosis, accompanying with metabolic alterations and glioma cell dedifferentiation. Recently, mRNA expression-based stemness index (mRNAsi) characteristic relation to *IDH* status of gliomas has yet illuminated. Thus, we aimed to establish a cancer stem cell-associated metabolic gene signature for risk stratification of gliomas.

Methods: The glioma samples came from The Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CGGA) databases. Next, we performed the differential expression analysis between *IDH* mutant and *IDH* wild-type gliomas and also conducted weighted gene correlation network analysis (WGCNA) for determining the modules associated with cancer stem cell trait. Subsequently, multivariate Cox regression analysis with the Akaike information criterion (AIC) algorithm was employed to establish a stemness-related metabolic gene signature, which was validated using time-dependent receiver operating characteristic (ROC) curves and concordance index (C-index). Also, we developed a nomogram based on clinical traits and prognostic model. Additionally, according to the results of immunohistochemistry (IHC) staining, the protein levels of gene signature were consistent with the genes expression's direction.

Results: Low expression of mRNAsi was capable of predicting the unfavourable OS of gliomas with a 5-year survival rate of 14.08%. The blue module and its 1466 genes were pertinent to mRNAsi characteristic. Next, Kaplan-Meier (KM) survival curves revealed that cancer stem cell-associated metabolic genes exerted impact on gliomas' prognosis. Subsequently, univariate and multivariate Cox regression analyses were implemented, and gene signature (*LCAT*, *UST*, *GALNT13*, and *SMPD3*) was constructed, with C-index of 0.798 (95%CI: 0.769-0.827). Notably, the prognostic model presented a superior predictive value for gliomas' survival, with the area under the curve (AUC) of ROC curves at 1-year, 3-year as well as 5-year time-point of 0.845, 0.85 and 0.811, respectively. And forest plot uncovered its role as a potential independent predictor for gliomas (HR=2.840, 95%CI: 1.961-4.113, $P < 0.001$). Nomogram also presented superior predictive performance for gliomas' OS.

Conclusion: The gene signature (*LCAT*, *UST*, *GALNT13*, and *SMPD3*) can be used for risk stratification and also can serve as an independent prognostic factor of glioma patients.

Introduction

Glioma originates from glial cells or precursor cells, and is one of the most common types of primary brain tumors (1, 2), with an average annual age-adjusted incidence rate from 2010 to 2014 of 6 per 100,000 in the United States (3). According to the histopathological criteria established by the World Health Organization (WHO), glioma is classified into grade I to grade IV (4). The standard therapy for glioma patients includes maximal surgical resection (2) and radiotherapy and/or chemotherapy as early as possible (5). Although these treatments have been adopted, low grade glioma (LGG) may evolve into higher grade glioma or recur (6), with poor overall survival (OS). More recently, with the popularization of

microarray chips and high-throughput sequencing, tumor stratification has transformed from morphology to molecular traits (7). At present, due to the presence of the limitations in the early diagnosis of gliomas and prediction of its prognosis, specifically for high-grade glioma (HGG), it is imperative to illuminate the molecular mechanisms of gliomas. In 2006, the WHO have established some molecular biomarkers for sub-classification, including the co-deletion of chromosomal arms 1p and 19q, isocitrate dehydrogenase (*IDH*) mutations, methylated status of the O-(6)-methylguanine-DNA methyl transferase (*MGMT*) promoter, alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) mutations or loss, and telomerase reverse transcriptase (*TERT*) promoter mutations (6). *IDH* mutations appear to confer favorable OS in glioma patients, regardless of WHO grades (8, 9). It may be of interest to note that *IDH1* (R132H) mutation alters the metabolic flux of isocitrate and α -ketoglutarate (α -KG), and induces widespread metabolic reprogramming, thought to be common characteristics of tumorigenesis and serve roles in gene expression, cell differentiation and the tumor microenvironment (10), and provokes some compensatory metabolic changes for maintaining biosynthetic requirements during glioma cells proliferation (11–13). The metabolic alterations are considered to propel tumorigenesis and block differentiation by influencing epigenetics (14–16). Additionally, a population of undifferentiated cells with stemness in glioma has been determined as a main factor affecting tumor initiation, development, recurrence and invasion (17). In essence, the development of cancer involves in cells gradual loss of the differentiated phenotype and acquisition of progenitor cell-like, or stem cell-like characteristics, which more readily cause the tumor cell migration to distant organs, resulting in unfavorable prognosis and chemotherapy-resistance (18–20). Recently, it has been reported that the characteristics of tumor stem cells were investigated by bioinformatics methods. Malta et al. employed a novel one-class logistic regression machine-learning algorithm (21) to identify transcriptome and epigenetic traits derived from normal pluripotent stem cells and their differentiated progeny in The Cancer Genome Atlas (TCGA) database (22). They reported two stemness indices, including a gene expression-based stemness index (mRNAsi), representative of the degree of dedifferentiation of tumors (23), and an epigenetically regulated-mRNAsi (EREG-mRNAsi) (24). The scope of these two indices ranges from 0 to 1, where the closer the value to 1, the stronger the stem cell characteristics of tumors are. In the previous study, mRNAsi and EREG-mRNAsi values were demonstrated to be negatively correlated with gliomas' pathology and clinical traits. They ascribed the phenomenon in high frequency of *IDH* mutations in glioma patients. Since *IDH* mutations reduce cell differentiation, mRNAsi values were higher in the *IDH* mutant subset. However, the cancer stem cell-related metabolic genes associated with *IDH* mutations in gliomas have yet specifically elucidated. Thus, we aimed to establish a prognostic model pertinent to metabolic reprogramming and dedifferentiated status in *IDH* mutant glioma, for risk stratification in gliomas.

In the present study, we have determined that glioma patients with higher mRNAsi score had poor prognosis via Kaplan-Meier (KM) method and log-rank test. Next, we performed differential expression analysis between *IDH*-mutant and *IDH*-wild-type gliomas after merging the TCGA-GBM and TCGA-LGG cohorts and removing their batch effects. Subsequently, weighted gene correlation network analysis (WGCNA) was employed to identify the modules closely associated with the mRNAsi trait. Then, we

selected genes related to metabolism and cancer stem cell characteristic as core genes. Next, univariate Cox regression analysis revealed their relationship to gliomas' prognosis, where the genes with P -value less than 0.05 were used to perform multivariate Cox regression analysis with Akaike information criterion (AIC) algorithm for constructing a prognostic model (including *LCAT*, *UST*, *GALNT13* and *SMPD3* genes). In addition, we developed a nomogram for clinicians and validated its predictive performance using calibration curves based on bootstrap method. Our findings may provide deep insight into risk stratification of gliomas, illuminate the potential molecular traits of *IDH* mutant gliomas from metabolic reprogramming and cancer stem cell aspects, and be conducive to target therapies for glioma patients in the near future.

Materials And Methods

Data Sources and Processing

The RNA-sequencing data (FPKM) of glioblastoma (GBM) ($n = 169$) and LGG ($n = 529$) as well as its clinical data were downloaded from the TCGA database (<https://portal.gdc.cancer.gov>). Subsequently, the Ensembl database was employed to integrate the Ensembl ID into the official gene symbols, and expression data was log2 converted. The values of mRNAsi and EREG-mRNAsi of all glioma patients, including LGG and GBM, were obtained from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5902191/> (24). Furthermore, the clinical information (gender, age, status, overall survival, IDH status, tumor grade, histological subtypes, MGMT status and other mutation information) of TCGA-GBM and TCGA-LGG patients was downloaded from the cBioPortal for Cancer Genomics (www.cbioportal.org). Additionally, external cohorts of mRNAseq_693 (25, 26) and mRNAseq_325 (27, 28) from the CGGA database (www.cgga.org.cn/) were acted as external validation datasets, where only primary glioma samples were selected. The combat function of the “sva” package was employed to remove batch-batch differences (29).

Differential Expression Analysis

After eliminating batch effects, the RNA-seq data of TCGA-GBM and TCGA-LGG were integrated into a single glioma group. According to *IDH* mutation status, we used “limma” package to implement differential expressed analysis between IDH wild-type and *IDH* mutant gliomas. The differential expressed genes (DEGs) should met the following criteria: $|\log_2\text{Fold-Change}(\log_2\text{FC})| > 1$ and adjusted $P < 0.05$.

The Relationship Between mRNAsi and Clinical Traits

An unpaired t-test was applied to compare differences in the mRNAsi values of glioma samples. One-way analysis of variance (ANOVA) used to compare comparison with significant differences in the mRNAsi among variable groups. X-tile software (Version 3.6.1) (30) was applied to determine the optimal cut-off values in survival data, then we classify glioma patients into high and low expression groups. This software undertook enumeration method, meaning it groups different values as cut-off for statistical analysis, and adopted the result with the smallest P -value as the optimal truncation value. Based on the

best cut-off values, KM survival curves of two groups were depicted using the “survminer” package, and the *P*-value was calculated using the log rank test.

Weighted Gene Coexpression Network Analysis

Establishment of a Coexpression network

Construction of significant modules was completed using the “WGCNA” package, which was used to find the correlations between genes (31). We selected a total of 2441 DEGs to establish a coexpression network. The formula of the constructed adjacency matrix was as follows: $a_{ij} = |\text{cor}(i,j)|^\beta$ (22), where $\text{cor}(i,j)$ represents the Pearson correlation coefficients between gene *i* and gene *j*, a_{ij} represents the adjacency between gene *i* and gene *j* and β is the soft threshold. The “pickSoftThreshold” function of the “WGCNA” package was employed to determine the value of β , making the network close to the scale-free network distribution on the basis of connectivity (31). Subsequently, an adjacency matrix and topological overlap matrix (TOM) was constructed according to the β value.

Identification of significant modules

Next, the dynamic shear method was applied to classify consistently expressed genes into the same modules (32), with a minimum of 50 genes in a module. To improve the merging of modules with high similarity, we selected 0.25 as a cut-off value (33). Additionally, we aimed to identify the modules with the highest relation to sample features. Then, the correlation coefficients and *P* value between modules and clinical traits (mRNAsi and EREG-mRNAsi values) were calculated, and the top ranking modules were selected as the most essential modules. Subsequently, we calculated the gene significance (GS) and module member (MM) (22). The higher the GS value of genes, the stronger their association with the stem index.

Functional enrichment analysis of the modules

We extracted the genes in the most significant modules and performed functional enrichment analysis using the clusterProfiler package (34), including gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analyses. An adjusted $P < 0.05$ acted as a cut-off value.

Metabolism-related Genes

The seventy metabolism-related gene sets derived from the KEGG pathway database and a total of 1466 metabolic genes were obtained from <https://figshare.com/s/d2a968666f6f4c9ea224> (35). To investigate the genes closely related to metabolic reprogramming and cancer stem cell trait, we screened out the intersected region in the most significant modules and KEGG metabolic pathway gene lists, which were regarded as core genes, and Venn plot showed these genes.

Survival and univariate Cox analyses

Adopting optimal truncation values calculated by X-tile software, we divided glioma into high and low core genes expression groups. Next, KM survival curves revealed that their expression levels' exerted impact on gliomas' OS. And univariate Cox regression analysis was used to identify core genes significantly influencing glioma patients' prognosis, where genes with $P < 0.05$ were selected for further analysis.

Establishment of a Prognostic Signature

Multivariate Cox regression analysis based on AIC algorithm (36) was used to construct a prognostic model. Additionally, the risk score formula was composed of the product of expression level of each core gene and their coefficients derived from multivariate Cox regression analysis. As shown in Eq. 1, G_i is the expression level of gene i , coefficient_i is the coefficient of gene i and n is the number of core genes in the prognostic model. Given that 5-year survival rate is of significance for patients suffering from tumors, we utilized optimal cut-off value of risk score on time-dependent receiver operating characteristic (ROC) curve of evaluating 5-year survival to classify patients into high and low risk groups (37). Subsequently, we used KM curves to show the relationship between the risk score and OS of gliomas.

$$\text{Risk score} = \sum_{i=1}^n G_i \times \text{coefficient}_i \quad (1)$$

Evaluation of the Prognostic Value of a Four-gene-based Signature

Next, time-dependent ROC curves (38) (1-year, 3-year and 5-year) were employed to evaluate the predictive capability of the risk score model for glioma patients' prognosis. Also, the concordance index (C-index) was used to assess the predictive accuracy of this prognostic model, namely the discrepancy between the true value and predicted value of model (39). The C-index ranges from 0.5 to 1.0, where 0.50–0.70 refers to low accuracy, 0.71–0.90 represents medium accuracy, and higher than 0.90 is representative of high accuracy. Risk score, clinical characteristics and molecular factors, including gender, age, glioma grade, *IDH* mutation, methylation of *MGMT* promoter and histological subtypes were analyzed using multivariate Cox regression analysis. The factors with $P < 0.05$ were considered to be independent prognostic indicators. Subsequently, a nomogram of 1-year, 3-year and 5-year OS was developed using the "rms" package (40) in the RStudio platform (41). Similarly, calibration curves was used to verify the accuracy of the nomogram in predicting glioma survival via the bootstrap resampling method (42).

External Validation of Prognostic Value

We merged the expression data of mRNAseq_693 and mRNAseq_325 cohorts and removed the batch effects, then verified the prognostic value of this prognostic model. The risk score was calculated based on Eq. 1, where glioma patients were divided into high and low risk groups according to optimal cut-off values on the ROC curve at 5-year time-point. Additionally, we also constructed a nomogram and perform calibration analysis for validation of its predictive value, beneficial for clinicians to make decisions.

Immunohistochemistry

The human protein atlas database (HPA, <https://www.proteinatlas.org/>) (43) was employed to detect the protein expression of core genes, where the researchers used highly specific antibodies and adopted immunohistochemistry (IHC) method to compare the protein levels between normal and tumor tissues. We chose the cerebral cortex tissues, LGG and HGG samples to visualize their expression from the protein aspect.

Statistical Analysis

All analyses were implemented in RStudio software (Version 3.5.0). Firstly, selection of DEGs was performed via the Wilcoxon test (44). Second, X-tile software was used for means testing using a standard ANOVA calculation on two-population (high vs. low groups) cut-points (30). Thirdly, Pearson's chi-square tests were employed to investigate the correlation among core genes. Additionally, KM curves were generated by the "survminer" package and *P*-values were calculated via the Wilcoxon log rank test. Two-tailed *P*-values less than 0.05 were considered statistically different.

Results

Clinical Characteristics of Datasets

A total of 624 glioma samples from the TCGA database with sufficient IDH status were included for differential expression analysis. However, 616 samples were used for construction of the prognostic model due to eight patients without sufficient survival data. Meanwhile, there were 588 primary glioma samples from the mRNAseq_693 and mRNAseq_325 cohorts as an external validation cohort. And Table 1 showed the clinical characteristics of glioma patients in two databases. The flow diagram of the present study was provided in Fig. 1.

Table 1
Clinic pathological characteristics of glioma samples from the TCGA and CGGA databases

Characteristics	Training cohort	Validation cohort
	(TCGA, N = 616)	(CGGA, N = 588)
Age (%)		
≤ 46	321 (52.11)	353 (60.03)
> 46	295 (47.89)	234 (39.80)
NA	-	1 (0.17)
Gender (%)		
Female	267 (43.34)	241 (40.99)
Male	349 (56.66)	347 (59.01)
Grade (%)		
G2	239 (38.80)	208 (35.37)
G3	256 (41.56)	169 (28.75)
G4	121 (19.64)	211 (35.88)
<i>IDH</i> _status (%)		
<i>IDH</i> _mut	411 (66.72)	309 (52.55)
<i>IDH</i> _wt	205 (33.28)	279 (47.45)
<i>MGMT</i> _status (%)		
Methylated	459 (74.51)	187 (31.80)
Unmethylated	149 (24.19)	332 (56.46)
NA	8 (1.3)	69 (11.74)
TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas; G2, grade 2; G3, grade 3; G4, grade 4; <i>IDH</i> , Isocitrate dehydrogenase; <i>IDH</i> _mut, <i>IDH</i> mutation; <i>IDH</i> _wt, <i>IDH</i> wild type; NA, not available.		

Correlation Between mRNasi and Clinical Traits in Glioma Patients

The relationships between mRNasi and some clinical information were investigated. As shown in Fig. 2C, mRNasi was higher in glioma patients with *IDH* mutations ($P < 0.0001$). Additionally, patients older than 60 years old were associated with low levels of miRNasi (Fig. 2A, $P < 0.0001$), while there was no obvious difference in gender (Fig. 2B, $P = 0.69$). Intriguingly, mRNasi levels gradually decreased as the glioma

grade increased (Fig. 2D, $P < 0.0001$). In addition, we observed that glioma patients with low mRNAsi score had an unfavorable prognosis (Fig. 2E, $P < 0.0001$), with an optimal cut-off value of 0.30 calculated by X-tile software. However, the difference in EREG-mRNAsi was not statistically significant (Fig. 2F, $P = 0.13$). Hence, we selected mRNAsi as the main trait for the next analysis.

Identification of Differentially Expressed Genes (DEGs)

Considering that mRNAsi was closely associated with glioma OS and the difference in *IDH* wild-type and *IDH* mutant glioma was significant, our aim was to investigate the DEGs between the two groups. Subsequently, the “limma” package was adopted to conduct differential expression analysis in 207 cases with wild-type *IDH* and 417 cases of *IDH* mutant gliomas after removing batch-batch differences. Using the absolute value of $\log_2\text{FoldChange}$ ($|\log_2\text{FC}| > 1.0$) and an adjusted P -value ($\text{adj.}P < 0.05$) as the cut-off value, we identified 2441 DEGs (Supplementary Table 1), with up-regulation of 1616 and down-regulation of 825 DEGs.

WGCNA

Essential modules

After eliminating four outlier samples (Supplementary Figure 1), a total of 2441 DEGs were used to establish a coexpression network using the “WGCNA” package. The value of β was six based on the scale-free topology distribution, making the R^2 reach 0.88 (Figure 3A). We selected 0.25 as the MEDissThres value, resulting in eight modules (black, blue, brown, green, grey, pink, red and yellow), with the gene number contributing to each ranging from 67 to 1132 (Figure 3B). Each color module represents a common gene expression module. Next, in a correlation analysis of these modules and glioma traits (mRNAsi and EREG-mRNAsi), we found that the blue ($R^2=0.70$, $P<0.0001$, Figure 3C) module was obviously positively correlated with mRNAsi and the brown ($R^2=-0.6$, $P<0.0001$, Figure 3C) module was negatively correlated with mRNAsi. Then, by application of intramodular analysis, we determined that the blue module was characterized by higher GS and MM as the most significant module ($\text{cor}=0.83$, $P=1.3\text{e-}96$, Figure 3D). Ultimately, the Venn diagram showed that a total of seven genes, including *LCAT*, *UST*, *IPMK*, *NEU4*, *GALNT13*, *PDE6B* and *SMPD3* genes, were cancer stem cell-pertinent metabolic genes (Figure 3E), designated as core genes. In addition, the correlation analysis among these genes revealed that they had medium positive correlations with each other at the mRNA expression level except *PDE6B*, which was negatively correlated with other genes (Supplementary Figure 2A). Meanwhile, higher *PDE6B* expression was observed in *IDH* wild-type glioma patients via a hierarchical clustering heatmap (Supplementary Figure 2B).

Functional enrichment analyses

To further describe the functions of the genes in the blue module, gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were undertaken. As shown in Figure 4, the GO chord plots of GO term and KEGG pathway enrichment analyses indicated that the blue module's genes were mainly enriched in regulation of the membrane potential, postsynapse, ion

channel activity (GO terms, Figure 4A-C), neuroactive ligand-receptor interaction and the cAMP signaling pathway (KEGG, Figure 4D).

Survival Analysis

To explore the prognostic value of core genes in glioma, the best truncation values were determined by X-tile software and glioma patients were classified into high/low expression groups. Of note, KM survival curves denoted that most of the core genes with low expression were associated with unfavorable OS ($P < 0.0001$, Figure 5A-F). By contrast, high expression of *PDE6B* was associated with a worse prognosis for glioma patients ($P < 0.0001$, Figure 5G). Next, we included all core genes for univariate Cox regression analysis, where the results demonstrated that six core genes (HR < 1.0 , $P < 0.0001$, Table 2) were protective factors of glioma, other than *PDE6B*, which had an HR value of 1.257 (95%CI: 1.187-1.33) ($P < 0.0001$, Table 2), functioning as a risk factor.

Construction of a Prognostic Signature

Multivariate Cox proportional hazard regression analysis with the AIC algorithm had identified a four gene-based signature, including *LCAT*, *UST*, *GALNT13* and *SMPD3* genes (Table 2, Figure 6A), with the coefficients of the four core genes used to calculate the risk score according to equation 1. The risk score formula was as follows: risk score = $(-0.028787) * LCAT$ expression level + $(-0.024918) * UST$ expression level + $(-0.031937) * GALNT13$ expression level + $(-0.26647) * SMPD3$ expression level, which had the minimal AIC value of 2158.69 and C-index of 0.798 (95%CI: 0.769-0.827). Additionally, survival curve revealed that glioma patients with high risk score had worse OS than low risk patients (Figure 6B, HR=5.992, 95%CI: 4.373-8.209, $P < 0.0001$). The 5-year survival rates were separately 8.79% and 69.2% in high and low score groups classified by an optimal cut-off value of 1.659003. Furthermore, a GO chord plot demonstrated that four genes were enriched in ammonium ion metabolic process, glycoprotein biosynthetic process, dermatan sulfate biosynthetic process, steroid esterification (GO term, category = "BP", Figure 6C) and O-acyltransferase activity (GO term, category = "MF", Figure 6C).

Assessment of the Prognostic Value of the Four-gene-based Signature

We used the TCGA cohort to internally verify the prognostic performance of the risk score model. Firstly, the area under the curve (AUC) of the time-dependent ROC curves were 0.845, 0.85 and 0.811 for 1-year, 3-year and 5-year OS, respectively (Figure 6D). Second, we evaluated whether the four-gene-based signature was an independent prognostic indicator of the glioma. According to the result of univariate Cox regression analysis, we did not take the gender trait (HR=1.113, 95%CI: 0.849-1.458, $P=0.439$, Table 3) into consideration for performing multivariate Cox regression analysis. Then we used a forest plot to show that the risk score (HR=2.840, 95%CI: 1.961-4.113, $P < 0.001$, Figure 6E, Table 3) was an independent prognostic factor via multivariate Cox regression analysis combining significant clinical traits, such as age (≥ 60 vs. < 60), tumor grade (HGG vs. LGG), *MGMT* status (unmethylated vs. methylated) as well as histological subtypes (ASTR vs. GBM; ODG vs. GBM; mixed glioma vs. GBM). Intriguingly, compared with GBM, astrocytoma (ASTR, HR=0.401, 95%CI: 0.265-0.607, $P < 0.001$), oligodendroglioma (ODG, HR=0.332,

95%CI: 0.204-0.541, $P<0.001$) and mixed glioma (HR=0.393, 95%CI: 0.233-0.661, $P<0.001$) were pertinent to better prognosis of glioma patients. Remarkably, age (HR=2.137, 95%CI: 1.511-3.021, $P<0.001$), tumor grade (HR=2.461, 95%CI: 1.632-3.713, $P<0.001$) and *MGMT* status (HR=1.680, 95%CI: 1.248-2.261, $P<0.001$) were significantly associated with gliomas' prognosis, with a C-index of 0.854 (95%CI: 0.830-0.878). Table 3 showed the results from the univariate and multivariate Cox regression analyses. A nomogram integrating the prognostic model and the above mentioned clinical characteristics was developed for predicting the 1-OS, 3-OS, and 5-OS of glioma patients (Figure 6F). Additionally, calibration curves (Figure 6G) were adopted to evaluate the consistency between the predictive values from the nomogram and observed values in 1-OS, 3-OS and 5-OS of glioma patients, showing that the nomogram had superior predictive capacity.

Validation of the Prognostic Model

We selected a total of 588 glioma patients from the CGGA database as an external validation cohort. Based on equation 1, the risk score of each patient was calculated. Then, KM survival curve revealed that the high risk glioma population had worse OS than low risk patients, grouped by a optimal cut-off value of 0.2926944, and these results were consistent with the TCGA training set (Figure 7A). And the 5-year survival rates of high and low risk patients were 28.50% and 66.30%, respectively. The risk distribution, patients survival status as well as the heatmap of the four gene-based signature were also depicted (Figure 7B). Then, the AUC of the ROC curves for 1-year, 3-year and 5-year OS were 0.758, 0.763 and 0.738, respectively (Figure 7C). Also, multivariate Cox proportional hazard regression analysis for the risk score and clinical characteristics (such as age, glioma grade, *MGMT* status and histological subtypes) was performed, indicating that the risk score (HR=1.423, 95%CI: 1.104-1.834, $P=0.0065$) was an independent prognostic indicator (Figure 7D), with a C-index of being 0.783 (95%CI: 0.759-0.807). Additionally, we constructed a nomogram via multivariate Cox regression analysis for the risk score and clinical traits (Figure 7E). We found that ASTR and GBM had higher scores than oligoastrocytoma (OAST) and ODG patients in the nomogram, which might be related to the presence of anaplastic astrocytoma (AASTR) in the ASTR patients. Ultimately, the calibration curves (Figure 7F) discovered that the nomogram had good predictive performance for the 1-OS, 3-OS, and 5-OS of glioma patients.

Comparison of the Four Gene Expression Levels using the CGGA Cohort

We used the CGGA dataset to compare the expression of the four gene based on *IDH* status, denoting *IDH* mutant glioma with higher *LCAT* (Figure 8A, $P<0.0001$), *UST* (Figure 8B, $P<0.0001$), *GALNT13* (Figure 8C, $P<0.0001$) and *SMPD3* (Figure 8D, $P<0.0001$) levels. Also, we performed survival analyses to determine the impact of the four core genes on glioma survival. The results indicated that these core genes with high expression were involved in favorable prognosis of the glioma (Figure 8E-H, $P<0.001$).

Immunohistochemistry

Due to the absence of immunohistochemical results of the *LCAT* protein in normal brain and glioma tissues in the HPA database, the comparison between the two groups were unavailable. Compared with

normal cerebellum tissues, the *UST*, *GALNT13* and *SMPD3* brown protein granule in LGG and HGG tissues were decreased (Figure 9), indicating that the consistent results of gene and protein expression in three core genes.

TABLE 2 Univariate and Multivariate Cox regression analysis of the core genes

Genes	Univariate analysis			Multivariate analysis			
	HR	95%CI	P-value	HR	95%CI	P-value	Coef.
<i>LCAT</i>	0.937	0.920-0.954	<0.0001	0.972	0.955-0.989	0.00124	-0.028787
<i>UST</i>	0.94	0.926-0.954	<0.0001	0.975	0.958-0.993	0.00767	-0.024918
<i>IPMK</i>	0.466	0.392-0.553	<0.0001				
<i>NEU4</i>	0.937	0.921-0.953	<0.0001				
<i>GALNT13</i>	0.938	0.928-0.949	<0.0001	0.969	0.954-0.984	<0.0001	-0.031937
<i>PDE6B</i>	1.257	1.187-1.33	<0.0001				
<i>SMPD3</i>	0.686	0.630-0.747	<0.0001	0.766	0.707-0.831	<0.0001	-0.26647

HR, hazard ratio; Coef, coefficients; CI, confidence interval; *LCAT*, Lecithin-Cholesterol Acyltransferase; *UST*, Uronyl 2-Sulfotransferase; *IPMK*, Inositol Polyphosphate Multikinase; *NEU4*, Neuraminidase 4; *GALNT13*, Polypeptide N-Acetylgalactosaminyltransferase 13; *PDE6B*, Phosphodiesterase 6B; *SMPD3*, Sphingomyelin Phosphodiesterase 3; **Bold** text shows the *P*-value less than 0.05.

TABLE 3 The univariate and multivariate Cox regression analyses for clinical characteristics

Traits	Univariate			Multivariate		
	HR	95%CI	<i>P</i> -value	HR	95%CI	<i>P</i> -value
Age	5.688	4.262-7.591	<2e-16 ***	2.137	1.511-3.021	1.75e-05 ***
Gender	1.113	0.849-1.458	0.439	-	-	-
Grade	5.327	3.699-7.673	<2e-16 ***	2.461	1.632-3.713	1.76e-05 ***
Risk_Score	7.315	5.424-9.865	<2e-16 ***	2.840	1.961-4.113	3.28e-08 ***
MGMT_status	3.326	2.516-4.397	<2e-16 ***	1.680	1.248-2.261	0.000622 ***
Histological subtypes (Ref.=GBM)						
ASTR	0.126	0.088-0.180	<2e-16 ***	0.401	0.265-0.607	1.56e-05 ***
ODG	0.068	0.044-0.103	<2e-16 ***	0.332	0.204-0.541	9.64e-06 ***
Mixed_glioma	0.084	0.054-0.132	<2e-16 ***	0.393	0.233-0.661	0.000440 ***

MGMT, O-(6)-methylguanine-DNA methyl transferase; GBM, glioblastoma; ASTR, astrocytoma; ODG, oligodendroglioma; HR, hazard ratio; **Bold** text shows the *P*-value less than 0.05; ***, *P*<0.0001.

Discussion

Glioma is one of the most common malignant primary brain tumors. Generally, *IDH* mutation is frequently observed in grades II-III, with a frequency of 80% (45). *IDH* mutation confers a survival advantage for glioma patients compared to patients with the *IDH* wild-type (7), serving as an independent predictive indicators. Several pieces of evidence suggest that *IDH1/2* mutations are gain-of-function mutations, making synthetic metabolites shift from α-KG to 2-HG, which correlates with hypermethylated phenotype of glioma and propel its progression (15, 46). This process is opposite to *IDH* wild-type glioma, with catalysis of NADP⁺-dependent isocitrate oxidative decarboxylation to α-KG (47). However, Waitkus et al. demonstrated that an *IDH1* mutation (R132H, a commonly mutated site) reduced metabolites in the tricarboxylic acid (TCA) cycle and diminishes the growth of glioma cells (48).

As the importance of cancer stem cell indices is gradually highlighted, many studies have employed the WGCNA method to investigate the influence of mRNAsi on tumors, such as breast cancer (49), bladder cancer (50), lung squamous cell carcinoma (22), lung adenocarcinoma (51), endometrial carcinoma (52), hepatocellular carcinoma (33) and glioma (53). Nevertheless, the presence of *IDH* mutations in glioma makes it different from other tumors. Malta et al. demonstrated that *IDH* mutations in the glioma population negatively correlated with mRNAsi values (24). At the same time, metabolic alterations caused by *IDH* mutations also in turn give rise to the dedifferentiation of glioma cells. Due to these dedifferentiation and metabolic changes conferring *IDH*-mutant glioma patients a better prognosis than *IDH* wild-type glioma patients. However, pathogenesis of gliomas remains unclear. Accordingly, in the

present study, we used GBM and LGG samples from the TCGA database, with corresponding mRNAsi values of each sample, to explore the mRNAsi relationship to clinical characteristics, especially in terms of *IDH* status. Next, WGCNA was employed to identify the genes in each module associated with the mRNAsi trait, and cancer stem cell-related metabolic genes were determined after identification of metabolism-related genes from 70 KEGG pathways. Ultimately, a four-gene signature was constructed via univariate and multivariate Cox regression analyses, and this signature was verified using internal (TCGA cohort) and external datasets (CGGA validation set). Our results showed that the four-gene signature (*LCAT*, *UST*, *GALNT13* and *SMPD3* genes) was independent indicator for the prediction of glioma survival. We also found that the four genes were protective factors for glioma patients, with high expression in *IDH* mutant glioma samples.

LCAT (Lecithin-Cholesterol Acyltransferase) encodes an extracellular cholesterol esterifying enzyme, a lecithin-cholesterol acyltransferase. The esterification of cholesterol plays a pivotal functional role in transporting cholesterol (54). Given that lipid metabolism is also involved in metabolic reprogramming, *LCAT* may serve as an important prognostic biomarker. A previous study, Ouyang et al., constructed a twelve-gene signature, including the *SPP1*, *KIF20A*, *HMMR*, *TPX2*, *LAPTM4B*, *TTK*, *MAGEA6*, *ANX10*, *LECT2*, *CYP2C9*, *RDH16* and *LCAT* genes, for predicting the OS in hepatocellular carcinoma (HCC). That study also indicated that *LCAT* was expressed at a low level in HCC patients (55). Additionally, *UST* (Uronyl 2-Sulfotransferase), located on chromosome 6q25.1, encodes a protein that transfers sulfate to the 2 site of uronyl residues, including iduronyl residues and glucuronyl residues (56). Nikolovska et al. recently demonstrated that *UST* participates in melanoma cell migration, and knock-down of the *UST* gene suppressed cell motility and adhesion (57), which may relate to glycosaminoglycan metabolism, but the specific mechanism is unclear. The absence of studies about the association between the *UST* gene and glioma to date encourages us to investigate this question in the future. The *GALNT13* (Polypeptide N-Acetylgalactosaminyltransferase 13) gene encodes the GALNT13 protein, which catalyzes the initial reaction in O-linked oligosaccharide biosynthesis, holding the possibility of transferring an N-acetyl-D-galactosamine residue to a serine or threonine residue on the protein receptor, and is the pivotal enzyme involved in the synthesis of O-glycans, especially Tn epitopes in neurons (58). Ducray et al. used real-time RT PCR to detect 22 DEGs between 1p19q co-deleted and epidermal growth factor receptor (*EGFR*) amplified gliomas and found that *GALNT13* was down-regulated in glioma patients with *EGFR* amplification compared with normal brain samples (59). Additionally, the findings in our study denote that a low expression level of *GALNT13* is correlated with unfavorable outcome in glioma patients. Meanwhile, the results of Nogimori et al. also demonstrated that *GALNT13* was a the prognostic factor for lung cancer (60). The protein encoded by the *SMPD3* (Sphingomyelin Phosphodiesterase 3) gene, mainly expressed in neurons of the central nervous system (CNS), catalyzes the hydrolysis process of sphingomyelin to generate phosphocholine and ceramide (61), which mediates a wide range of cellular functions and is able to independently modulate cell growth, differentiation, cell cycle and its apoptosis (62). The occurrence of mutations in *SMPD3* may be conducive to leukemia formation (63). *SMPD3*, functions as a tumor suppressor gene in HCC via integrative genomic analysis and affects tumor invasion. Decreased *SMPD3* is an independent predictive factor influencing the early recurrence of HCC

(64). The above mentioned evidence suggests that these four genes may be the prognostic indicators of tumors, but the specific mechanism needs to be explored.

The present study provides insights into the impact of cancer stem cell (CSC)-associated metabolic genes on glioma survival. To our knowledge, metabolic reprogramming of CSC may be characterized by carcinogenic activity (65-67). A number of previous studies have shown metabolic alterations in *IDH* mutant glioma. Accordingly, we have identified stemness-related metabolic genes and established a four gene-based prognostic model, which raises hope for clinical use.

However, some limitations also need to be mentioned. First, we used data from public databases to perform analysis, and lack experimental verification of gene expression and exploration of the specific mechanisms and pathways. Furthermore, our laboratory also lacked the experimental condition for inhibiting the genes or knocking down them to confirm whether these four hub genes can be mechanistically targeted to reduce glioma malignancy. Thus, the lack of experimental verification is our study's major limitations. Besides, the expression of *LCAT* protein between normal brain and glioma tissues was inaccessible in the HPA database. However, the tissues of glioma patients also hardly to collect in clinic for verifying its expressions, which needs the cooperation of neurosurgeons in the near future. Also, other molecular alterations, such as *TERT* promoter mutations, *ATRX* mutations or loss and co-deletion of chromosome 1p/19q may exert impact on gliomas' prognosis. Due to lack of sufficient information on the molecular characteristics in the assessed glioma samples, we did not take these factors into consideration. Ultimately, multi-center prospective studies will be required for evaluation the clinical application of the four gene prognostic signature.

Conclusions

Taken together, a prognostic four gene signature was constructed via integrative bioinformatics analysis combining the TCGA and CGGA cohorts. This prognostic model effectively stratified glioma patients and was found to be an independent factor for predicting gliomas' OS. Our findings may provide insight into individualized tumor therapies in the context of clinical settings. Thus, we recommended that this classifier should be employed to assess the prognostic risk of glioma patients. And a multitude of prospective studies will be needed to verify our findings.

Declarations

AUTHOR CONTRIBUTIONS

YT, YJ and YG contributed to the planning of the study. YT and YJ drafted the manuscript. HT and YG revised the manuscript. DZ, JF, ZZ and YL downloaded the data. YT, YJ and YG established the four gene signature. YT, YJ, JY, ZZ and HT made contributions to validation of the prognostic model. YT and YJ prepared all tables and figures. YT, DZ, JF, JY, HT and YG preformed the interpretation of all results. All the authors reviewed and approved the final manuscript:

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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DATA AVAILABILITY STATEMENT

The TCGA-GBM, TCGA-LGG (<https://portal.gdc.cancer.gov>); mRNAsi and EREG-mRNAsi (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5902191/>); clinical information of TCGA samples (www.cbioportal.org); mRNA Sequencing data of CGGA_mRNAseq_693 and CGGA_mRNAseq_325 (www.cgga.org.cn/); metabolic genes lists (<https://figshare.com/s/d2a968666f6f4c9ea224>); The human protein atlas database (HPA, <https://www.proteinatlas.org/>).

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Figures

Figure 1

Flow chart of the present study. TCGA, The Cancer Genome Atlas; mRNAsi, gene expression-based stemness indices; IDH, isocitrate dehydrogenase; WGCNA, weighted gene correlation network analysis; IHC, immunochemistry; CGGA, the Chinese Glioma Genome Atlas; C-index, concordance index; ROC, receiver operating characteristics.

Figure 2

mRNAi in relation to clinical traits of glioma patients. (A) Boxplot of mRNAi in glioma samples, stratified by age; (B) boxplot of mRNAi in glioma patients, grouped by gender; (C) boxplot of mRNAi in glioma patients based on IDH status; (D) boxplot of mRNAi in glioma patients on the basis of tumor grade; (E) KM survival curves displaying the overall survival of glioma patients divided into high or low mRNAi groups based on the optimal cut-off values calculated by X-tile software; (F) KM survival curves showed the overall survival of glioma patients with high or low-ERGE-mRNAi values. mRNAi, gene expression-based stemness indices; IDH, isocitrate dehydrogenase; IDH_mut, IDH mutation; IDH_wt, IDH wild type; ERGE-mRNAi, epigenetically regulated-mRNAi; LGG, low-grade glioma; HGG, high-grade glioma; KM, Kaplan-Meier.

Figure 3

WGCNA and identification of core genes. (A) The network topology of a variety of soft threshold powers (β); The left panel presents the scale-free fit index, with the y-axis showing R^2 and the x-axis showing the soft threshold powers, where six was selected as the appropriate power value for next analysis. The right panel denotes that mean connectivity (y-axis) gradually decreases as the β values (x-axis) increase; (B) Cluster dendrogram of glioma patients. Each branch is representative of a gene and the each color is a co-expression module; (C) Correlation between the modules and clinical traits (mRNA_{si} and EREG-mRNA_{si}). The correlation coefficient of each cell refers to the correlation of gene modules in relation to clinical characteristics, with its the size increasing from blue to red; (D) Scatter plot for module membership (MM) vs. gene significance (GS) for mRNA_{si} in the blue module; (E) Identification of seven core genes via a Venn plot. WGCNA, weighted gene correlation network analysis; mRNA_{si}, gene expression-based stemness indices; EREG-mRNA_{si}, epigenetically regulated-mRNA_{si}; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 4

Functional enrichment analyses in the blue module. GO enrichment analysis in the (A) BP category, (B) CC category, (C) MF category; (D) KEGG pathway enrichment analysis. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 5

Kaplan-Meier survival curves of seven core genes. Survival curve analysis of (A) LCAT expression (8.0); (B) UST expression (10.6); (C) IPMK expression (1.4); (D) NEU4 expression (5.1); (E) GALNT13 expression (6.3); (F) SMPD3 expression (1.3); (G) PDE6B expression (2.3) in relation to glioma patient OS. The values in brackets are their optimal cut-off values calculated by X-tile software. LCAT, Lecithin-Cholesterol Acyltransferase; UST, Uronyl 2-Sulfotransferase; IPMK, Inositol Polyphosphate Multikinase; NEU4,

Neuraminidase 4; GALNT13, Polypeptide N-Acetylgalactosaminyltransferase 13; PDE6B, Phosphodiesterase 6B; SMPD3, Sphingomyelin Phosphodiesterase 3

Figure 6

Construction and evaluation of a prognostic model in the TCGA training cohort. (A) The patient distribution, survival status and a heatmap of high/low risk score calculated by the prognostic model composed of four core genes; (B) Kaplan-Meier survival curve analysis of OS in patients with glioma on the basis of the four gene signature in the training cohort. The risk table shows the total number of

patients at risk in the high/low risk group at the corresponding time; (C) GO Chord plot of four core gene enrichment analysis; (D) The time-dependent receiver operating characteristics (ROC) curves of the four gene signature for predicting 1-, 3-, and 5-year OS of glioma patients; (E) Multivariate cox proportional hazard regression analysis for the prognostic model and significant clinical traits; (F) Development of a nomogram to predict the 1-year OS, 3-year OS, and 5-year OS of glioma patients. (G) Calibration curves for evaluating the consistency between predicted probability and the actual probability for 1-year OS, 3-year OS, and 5-year OS from nomogram. TCGA, The Cancer Genome Atlas; GO, gene ontology; BP, biological process; MF, molecular function; OS, overall survival; AIC, Akaike information criterion; AUC, area under the curve; LGG, low grade glioma; HGG, high grade glioma; ASTR, astrocytoma; ODG, oligodendroglioma; GBM, glioblastoma

Figure 7

Validation of the four gene signature for the prediction of glioma survival in the CGGA validation cohort. (A) Survival curve showing the impact of risk score on glioma survival. (B) Risk plot, survival status of glioma patients, and heatmap of the four gene signature expression profile. (C) ROC curves of the four gene signature reveal its predictive performance for glioma survival at 1-year, 3-year and 5-year time points. (D) A forest plot demonstrating the prognostic model as an independent prognostic indicator of glioma patients. (E) A nomogram containing the risk score and essential clinical characteristics for

prediction of the 1-year OS, 3-year OS, and 5-year OS of glioma patients. (F) Calibration plots assess the predictive capacity of the nomogram used to predict 1-year OS, 3-year OS, and 5-year OS of glioma. CGGA, the Chinese Glioma Genome Atlas; OS, overall survival; AIC, Akaike information criterion; ROC, receiver operating characteristics; AUC, area under the curve; LGG, low grade glioma; HGG, high grade glioma; ASTR, astrocytoma; ODG, oligodendroglioma; OAST, oligoastrocytoma; GBM, glioblastoma

Figure 8

Assessment of the gene expression of four genes in different IDH status and their impact on glioma survival. Boxplot of (A) LCAT, (B) UST, (C) GALNT13 and (D) SMPD3 gene expression in IDH mutant and IDH wild-type glioma patients from CGGA cohort. Additionally, survival curves separately present the relationships between (E) LCAT, (F) UST, (G) GALNT13 and (H) SMPD3 gene expression and the OS of glioma patients derived from the CGGA database. IDH, isocitrate dehydrogenase; IDH_mut, IDH mutation; IDH_wt, IDH wild type; LCAT, Lecithin-Cholesterol Acyltransferase; UST, Uronyl 2-Sulfotransferase; GALNT13, Polypeptide N-Acetylgalactosaminyltransferase 13; SMPD3, Sphingomyelin Phosphodiesterase 3; OS, overall survival; CGGA, the Chinese Glioma Genome Atlas.

Figure 9

The results of IHC staining for protein expression of core genes in the HPA database. The brown granule is representative of protein expression. IHC, immunochemistry; HPA, the human protein atlas; LGG, low grade glioma; HGG, high grade glioma; UST, Uronyl 2-Sulfotransferase; GALNT13, Polypeptide N-Acetylgalactosaminyltransferase 13; SMPD3, Sphingomyelin Phosphodiesterase 3.

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