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Original Article

Identification of three glioblastoma subtypes and a six-gene prognostic risk index based on the expression of growth factors and cytokines

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Abstract: Glioblastoma multiforme (GBM) is the most common and invasive tumor of the central nervous system. Growth factors and cytokines (GFCKs) play a crucial role in tumor invasion. In the present study, GFCK expression profiles from GBM patients in the Chinese Glioma Genome Atlas were used to perform sample clustering with non-negative matrix factorization. Three GBM subtypes were identified based on differences in GFCK expression, and the subtypes differed in characteristics and prognosis. A prognostic risk index (RI) comprising six GFCKs (BMP2, CCN3, GKN1, LIF, MDK, and SEMA3G) was defined using univariate Cox hazard analysis and multivariate stepwise Cox regression. The RI was validated in two independent data sets and may be independent of some known prognostic factors. Our results suggest that GBM occurs as different subtypes expressing different patterns of GFCKs and that these expression patterns can be captured in an RI that can predict prognosis.

Keywords: Glioblastoma multiforme, growth factor, cytokine, molecular subtyping, prognosis

Introduction

Glioblastoma (GBM) accounts for 16% of all primary brain tumors, making it the most common primary malignant brain tumor [1]. Risk factors for GBM remain poorly understood; the only well-known cause is ionizing radiation [2]. Similarly, little progress has been made in prolonging survival of patients with GBM. The disease remains one of the deadliest types of tumor worldwide; the rate of recurrence is high and prognosis is dismal, with a 5-year survival rate of approximately 5% [3]. Standard management for patients with GBM is surgical resection followed by radio- and chemotherapy [4, 5]. Decades of research into new therapies have largely failed because GBM is so heterogeneous and invasive [6-9].

More effective treatment of GBM may depend on defining tumor subtypes that may be particularly susceptible to certain therapies [10-13]. We wondered whether it would be possible to define GBM subtypes based on expression of growth factors and cytokines (GFCKs), since these molecules drive tumor cell invasion [17]. Such invasion makes it difficult to achieve curative effects even with extensive surgical resection [14, 15]: cancer cells may already have infiltrated unresected areas of the brain, where they may form new tumors after surgery [16]. Inhibiting GFCK receptors can transiently slow progression of lung cancer [18], colorectal cancer [19] and hepatocellular carcinoma [20]. Already one anti-GFCK therapy against GBM has been approved by the US Food and Drug Administration: the therapeutic antibody bevacizumab targets the vascular endothelial growth factor (VEGF) receptor [21].

We hypothesized that analyzing the expression pattern of GFCKs in GBM might allow us to dif-

ferentiate subtypes, which may allow stratification of patients by prognosis and response to therapies.

Materials and methods

Data processing

The glioma data sets mRNAseq_693 and mRNAseq_325 (last update: November 28, 2019) containing mRNA sequencing (mRNA-seq) data and clinical information were downloaded from the Chinese Glioma Genome Atlas (http://cgga.org.cn/index.jsp). A third GBM data set in The Cancer Genome Atlas (TCGA, https://www.cancer.gov/) was downloaded from UCSC Xena (http://xena.ucsc.edu/public); this data set contained clinical information and gene expression profiles based on the Affymetrix Human Genome U133a array platform.

Low-grade glioma samples were removed from the mRNAseq_693 and mRNAseq_325 data sets. Samples without survival time were not included in the survival analysis. The mRNAseq_693 data set was used for molecular subtyping and creating a risk index (RI) for predicting prognosis. The mRNAseq_325 and TCGA_ array data sets were used for validating the prognostic value of the RI. GFCKs in each sample were determined from the Molecular Signature database (version 7, https://www.gsea-msigdb.org). The workflow of the present study is shown in **Figure 1**.

Nonnegative matrix factorization (NMF) and identification of marker genes

Nonnegative matrix factorization (NMF), similar to principal component analysis or independent component analysis, aims to explain the observed data using a limited number of basic components. NMF has been used to computational biology [22], predict cis-regulatory elements [23], characterize gene function [24], predict phenotype from data across different microarray platforms [25], and define cancer subtypes based on gene expression profiles [26]. NMF can be performed faster than principal component or independent component analyses, and it can generate more stable clusters [27]. Thus, NMF was applied in the present study.

GFCKs were ranked by variance based in the mRNAseq_693 data set, and the top 20% of GFCKs showing highest variance were subjected to NMF using the *NMF* package in R (https://cran.r-project.org/package=NMF). The *extractFeatures* function in R was used to define marker genes for the resulting GBM subtypes.

Gene set enrichment analysis (GSEA)

GSEA [28, 29] was applied to explore the potential biological characteristics of each GBM subtype. The GSEA Java software (version 4.0.3) was used, and the reference gene set was the hallmark gene set *h.all.v7.0.symbols.gmt* [30]. Gene sets with a false discovery rate < 0.25 after 1,000 permutations were considered to be significantly enriched.

GFCK expression-based prognostic RI for GBM

Univariate Cox hazard analysis was applied to the mRNAseq_693 data set in order to identify individual GFCKs that might affect overall survival. Then multivariate stepwise Cox regression was performed to establish a GFCK expression-based prognostic RI for each GBM as follows: $RI = Expression_{gene1} \times \beta_{gene1} + Expression_{gene2} \times \beta_{gene2} + Expression_{gene3} \times \beta_{gene3}...$, where β is the estimated regression coefficient of a certain GFCK and is derived from the multivariable Cox regression. Patients with GBM were divided into low- or high-risk groups according to median RI. Time-dependent receiver operating characteristic analysis was used to assess the prognostic value of the RI using the *survivalROC* package in R [31]. We also benchmarked the RI's prognostic value against that of routine clinicopathologic characteristics, including age [32], chemotherapy [4], radiotherapy [33], mutation in the isocitrate dehydrogenase (IDH) gene [32], and 1q19p co-deletion [34].

Statistical analysis

All analyses were performed using R version 3.6.1 (https://www.r-project.org/). Gene expression differences between groups were assessed for significance using the unpaired Student's *t* test. Univariate and multivariate Cox proportional hazard modeling was used to identify prognosis-related variables. Survival was compared between groups of patients using Kaplan-Meier analysis and the log-rank method. Differences associated with $P < 0.05$ were considered significant.

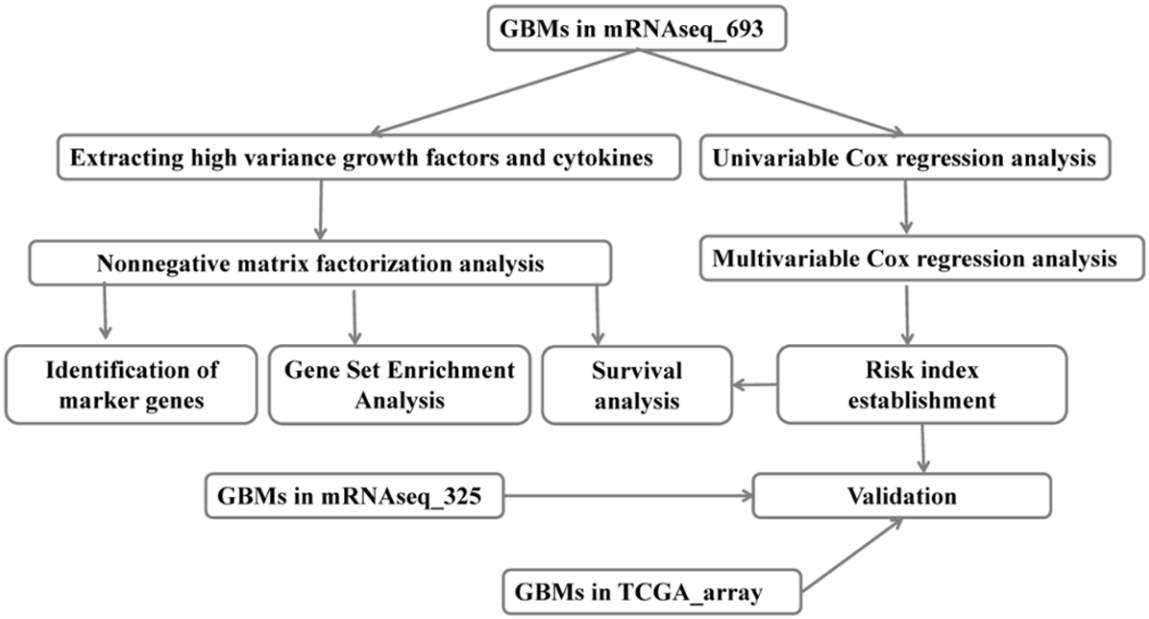


Figure 1. Workflow of the present study. GBM, glioblastoma; TCGA, The Cancer Genome Atlas.

Results

Heterogeneity in GFCK expression may be a basis for defining GBM subtypes

A total of 453 GFCKs were collected from the Molecular Signature database, while 340 GFCKs were detected in the mRNAseq_693 data set. The expression of 68 GFCKs with highest variance were analyzed using NMF (**Figure 2A**). The consensus matrix showed good classification (**Figure 2B**): 237 patients with GBM were divided into three subtypes (**Figure 2C**). Type II GBM was associated with higher frequency of IDH mutations and X1q19p co-deletion than types I or III (**Figure 2D**).

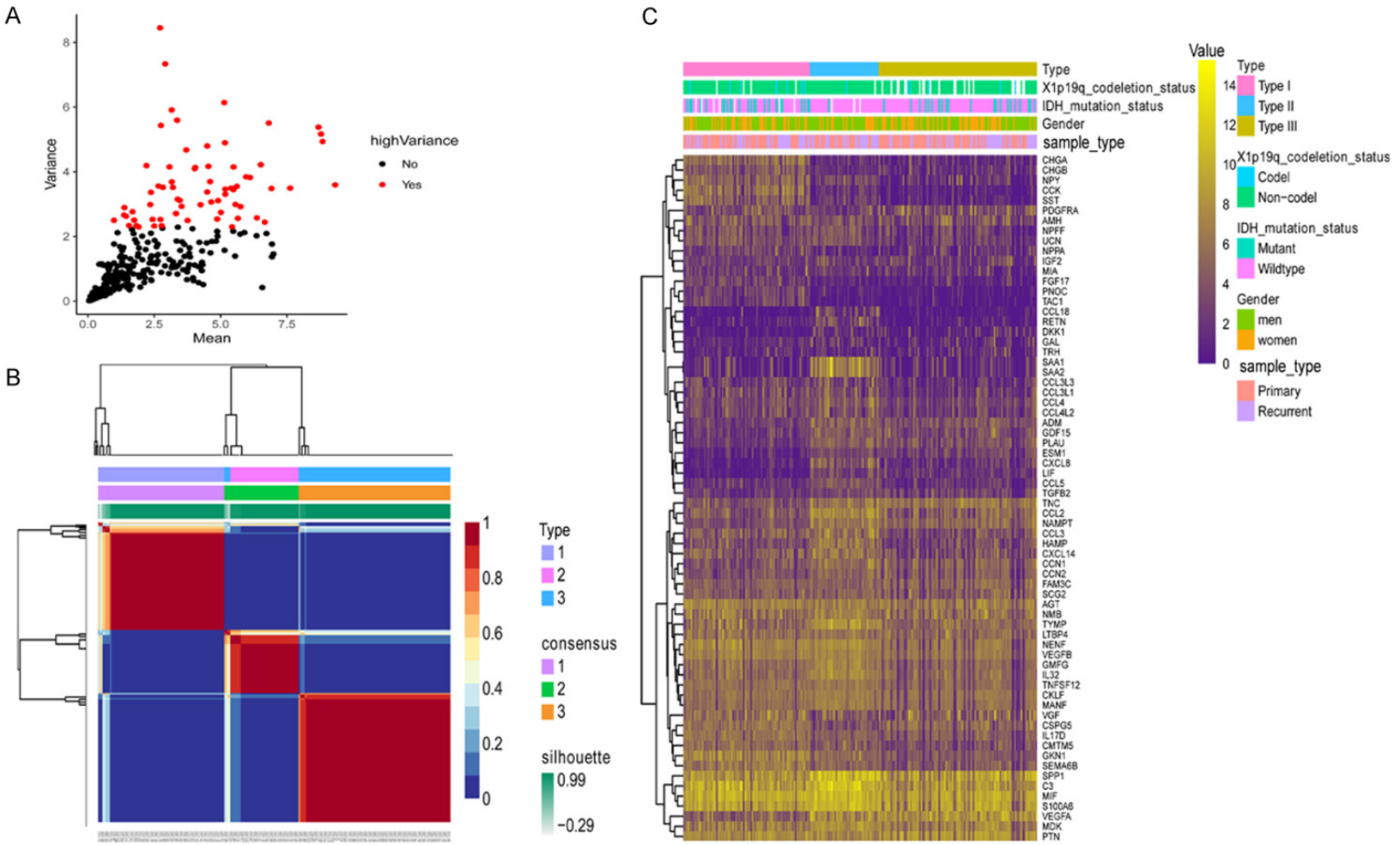
GFCK-based GBM subtypes present different phenotypes

Type I GBM was associated with the marker genes TAC1, CCK, CHGA, PNOC, SST, FGF17, CHGB, and NPY (**Figure 3A**). Type II was associated with the marker genes SAA1/2, RETN, and CXCL8 (**Figure 3B**), and Type III was associated with PDGFRA, ESM1, and CSPG5 (**Figure 3C**). The marker genes identified for Types I and II were expressed at significantly higher levels in those subtypes than in Type III, but the same was not true for marker genes of Type III. Thus, the marker genes for Type III should be considered tentative.

The potential biological characteristics of each GBM subtype were investigated using GSEA. Type I GBM showed significant enrichment in the KRAS signaling down (DN) hallmark gene set (**Figure 4A**), while Type II was enriched in several hallmark gene sets: the epithelial-mesenchymal transition, KRAS signaling up, mTORC1 signaling, P53 pathway, and PI3K/AKT/mTOR signaling (**Figure 4B**; Table S1). Type III GBM showed enrichment in the hallmark gene sets of E2F targets and G2M checkpoint (**Figure 4C**). These differences among the three subtypes suggest different phenotypes. Indeed, we found that prognosis was poorer for Type III patients than for patients with other subtypes (**Figure 4D**).

GFCK-based prognostic RI for GBM

Based on the mRNAseq_693 data set, univariate Cox hazard analysis identified 29 GFCKs as associated with survival (Table S2), and multivariate stepwise Cox regression identified six GFCKs (BMP2, CCN3, GKN1, LIF, MDK, and SEMA3G) as independent prognostic factors. This led us to define a prognostic RI as RI = $\text{Expression}_{\text{BMP2}} \times (-0.3286) + \text{Expression}_{\text{CCN3}} \times 0.15059 + \text{Expression}_{\text{GKN1}} \times (-0.19827) + \text{Expression}_{\text{LIF}} \times (-0.13721) + \text{Expression}_{\text{MDK}} \times 0.1625 + \text{Expression}_{\text{SEMA3G}} \times (-0.36129)$.


A
Variance
Mean
highVariance
No
Yes
B
Type
1
2
3
consensus
1
2
3
silhouette
0.99
−0.29
C
Type
X1p19q_codeletion_status
IDH_mutation_status
Gender
sample_type
Value
Type
Type I
Type II
Type III
X1p19q_codeletion_status
Codel
Non−codel
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Mutant
Wildtype
Gender
men
women
sample_type
Primary
Recurrent

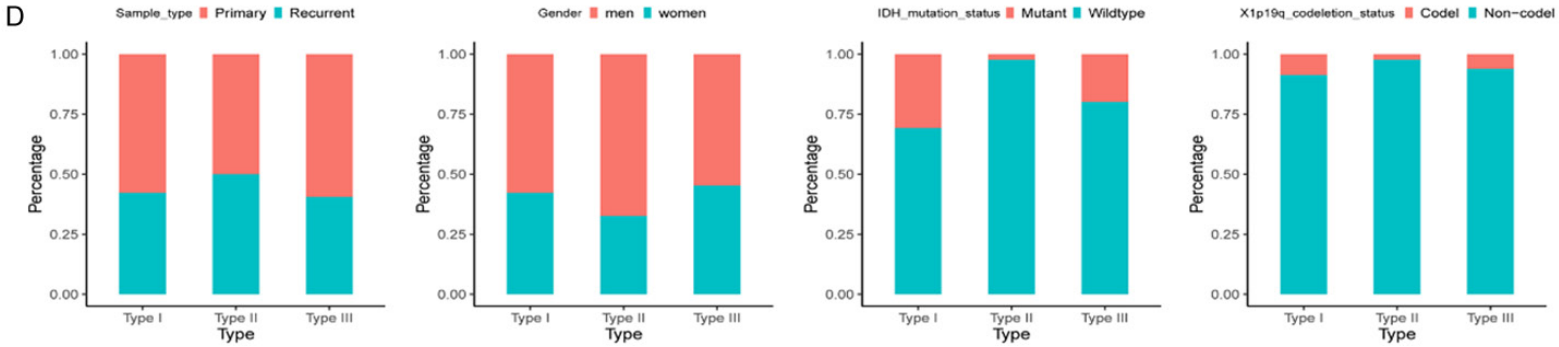


Figure 2. Sample clustering using nonnegative matrix factorization. A. Selection of highly variant genes. B. Consensus clustering matrix. C. Expression heatmap of highly variant genes in the three subtypes of glioblastoma multiforme. D. Proportions of tumor sample types, sex, mutation in isocitrate dehydrogenase (IDH), or 1p19q co-deletion in subtypes I-III.

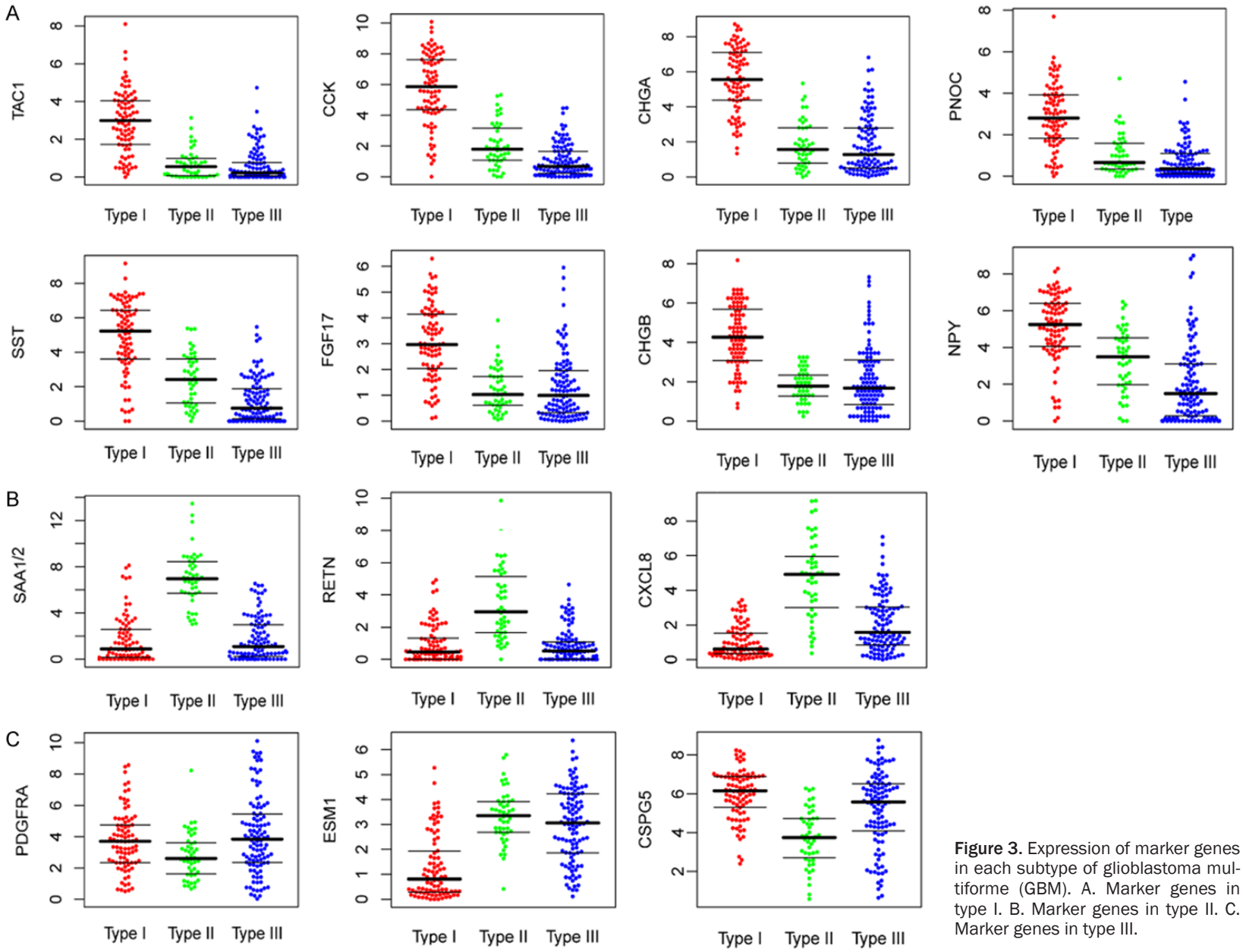


Figure 3. Expression of marker genes in each subtype of glioblastoma multiforme (GBM). A. Marker genes in type I. B. Marker genes in type II. C. Marker genes in type III.

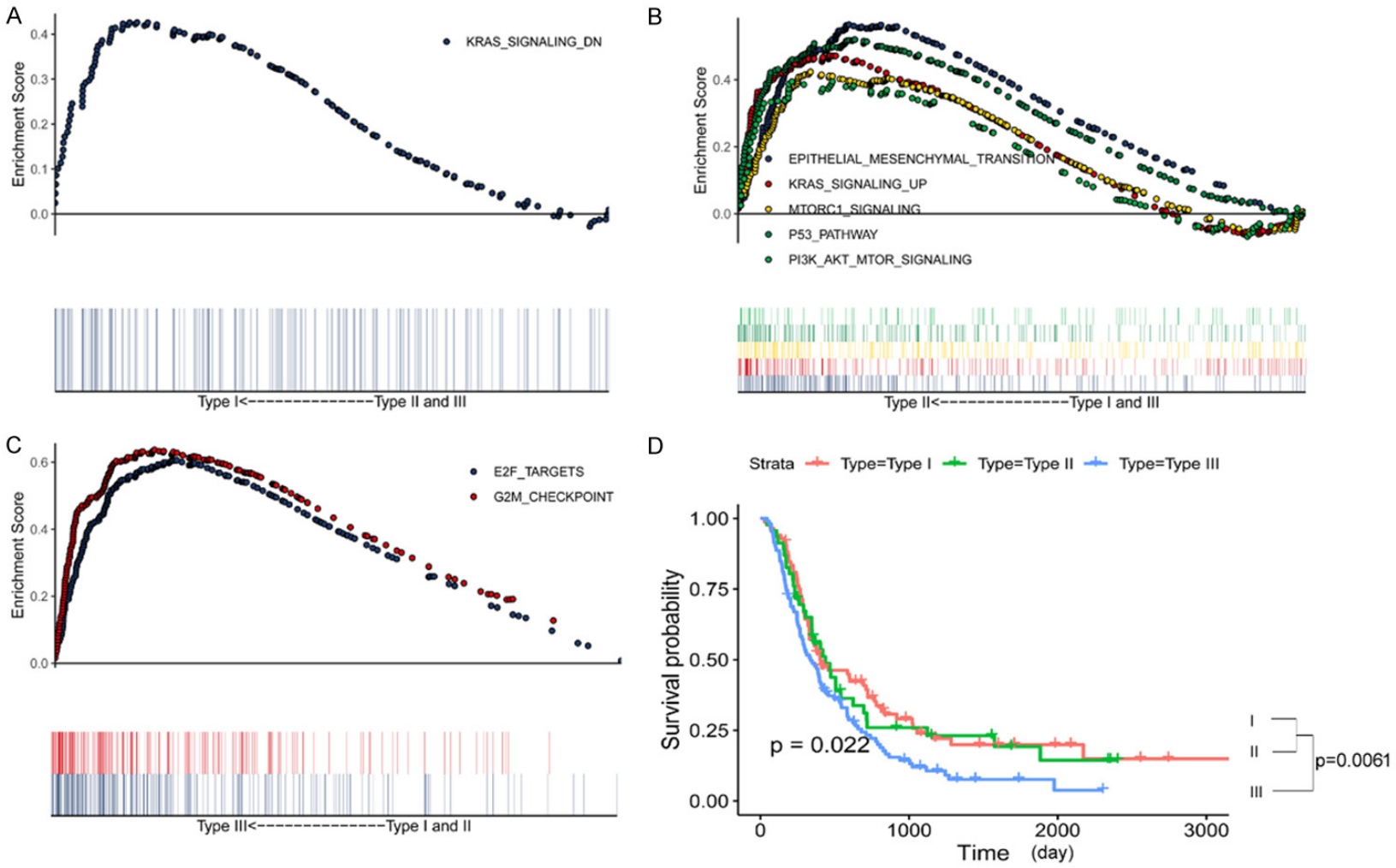


Figure 4. These three glioblastoma multiforme (GBM) subtypes vary in biology and prognosis. A. The KRAS signaling down (DN) hallmark gene set was significantly enriched in type I GBM. B. Multiple hallmark gene sets were significantly enriched in type II GBM. C. The hallmark gene sets E2F targets and G2M checkpoint were significantly enriched in type III GBM. D. Prognosis was worse for Type III than for Type I and II GBM.

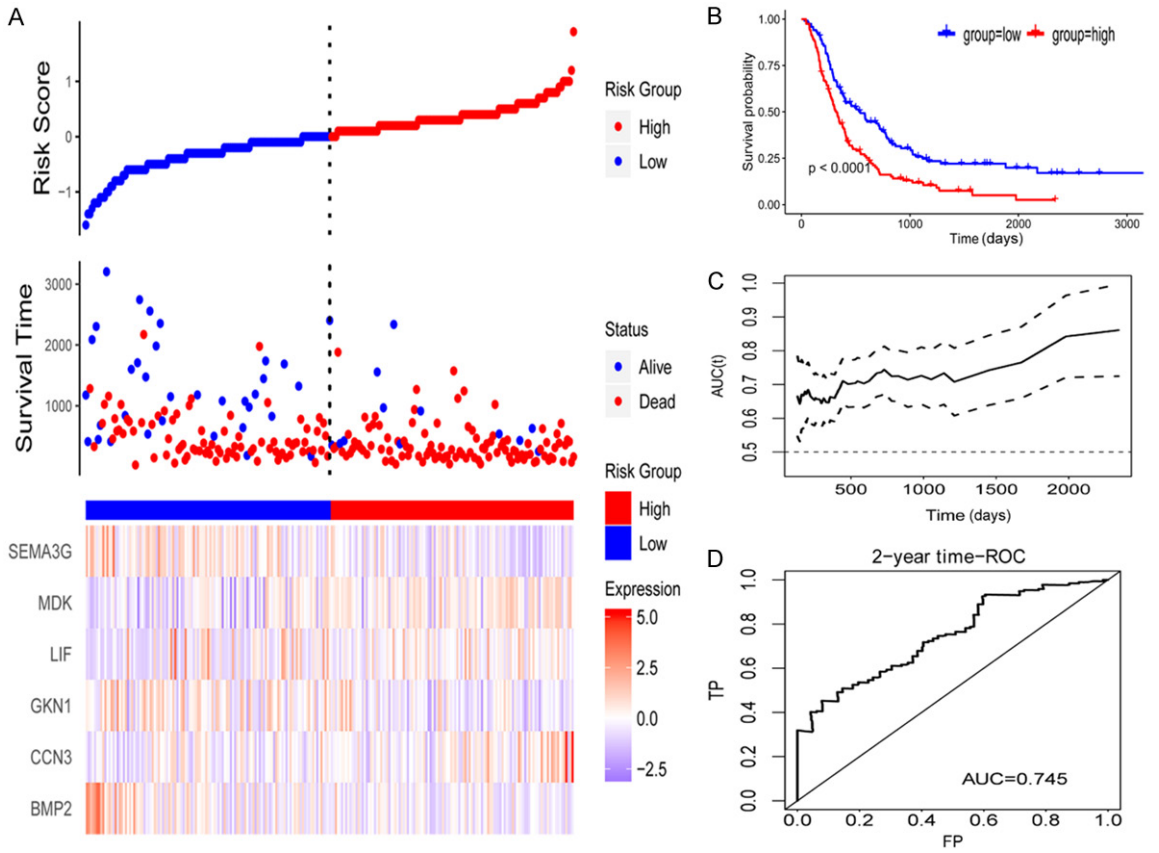


Figure 5. Establishment of a GFCK expression-based prognostic risk index. A. Distribution of prognostic risk index values, survival time, survival status and expression heatmap of the six selected GFCKs in glioblastoma multiforme (GBM) tissues in the CCGA mRNAseq_693 data set. B. Kaplan-Meier curves of patients stratified as low- or high-risk based on the median value of the risk index. C. Area under the receiver operating characteristic curve (AUC) to assess the ability of the risk index to predict survival. D. Time-dependent receiver operating characteristic curve for predicting 2-year survival. TP, true positive; FP, false positive.

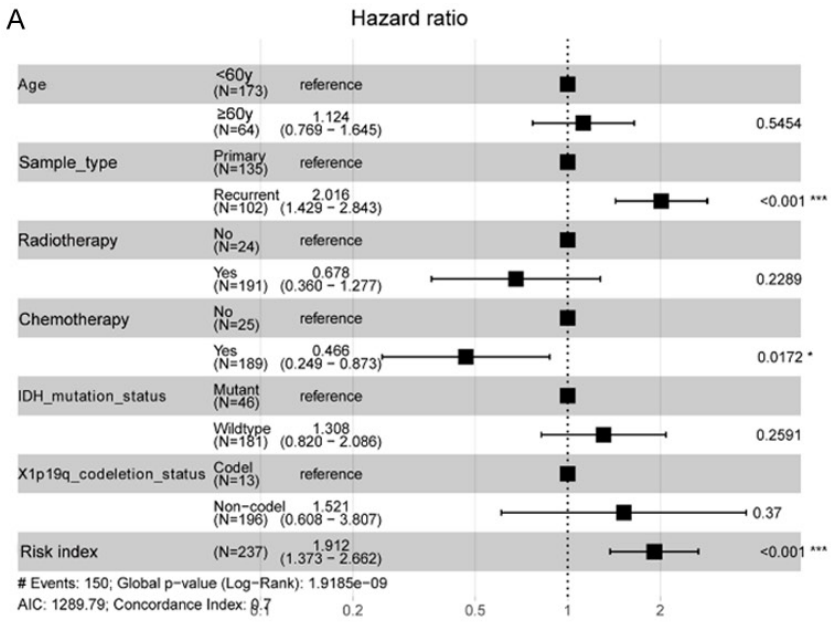
Univariate Cox hazard analysis showed that the RI was significantly associated with survival (hazard ratio 2.277, P = 1.38e-09), and the median RI was used to stratify patients into a low-risk group (< median RI) or high-risk group (≥ median RI) (**Figure 5A**). Overall survival was significantly shorter in the high-risk group (log-rank P < 0.001, **Figure 5B**). Time-dependent ROC curves suggested that the RI predicted survival more accurately over longer than shorter periods (**Figure 5C**). The area under the ROC curve for predicting 2-year survival was 0.745 (**Figure 5D**). The RI predicted prognosis independently of the following known prognostic factors: age, chemotherapy, radiotherapy, IDH mutation, and 1q19p co-deletion (**Figure 6A**).

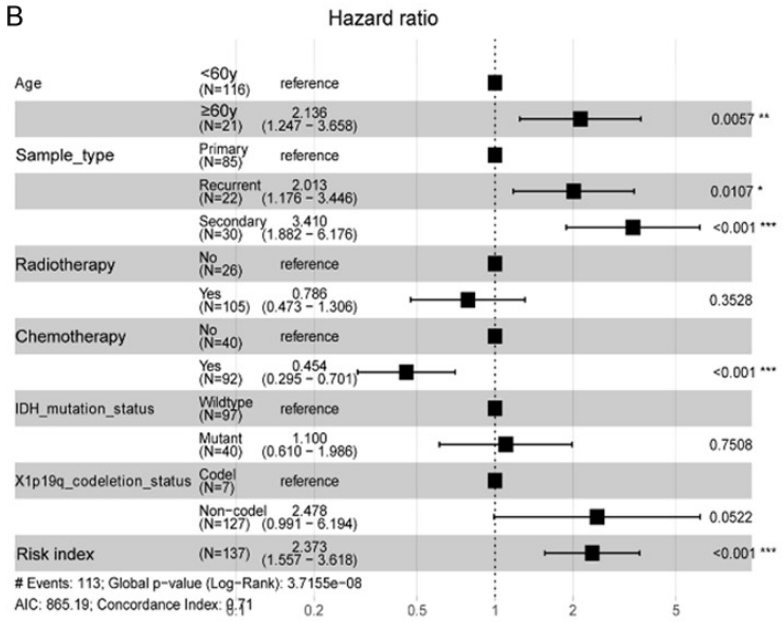
Validation of the GFCK-based prognostic RI

The prognostic RI was validated using the mRNAseq_325 and TCGA_array data sets. In both data sets, univariate Cox hazard analysis showed that the RI was significantly associated with survival (mRNAseq_325, hazard ratio 1.790, P < 0.001; TCGA_array, hazard ratio 1.287, P = 0.004), and it predicted prognosis independently of previously published clinicopathological factors (**Figure 6B**, **6C**). In both data sets, overall survival was shorter for the high-risk than for the low-risk group, although this difference achieved statistical significance only in the mRNAseq_325 data set (**Figure 6D**, **6E**).

Discussion

Personalized medicine plays an increasingly important role in cancer treatment. It is critical to identify "at-risk" patients and help them make decisions according to individual risk levels or subtypes. For GBMs, IDH mutation and O6-methylguanine-methyltransferase (MGMT)


A
Hazard ratio
Age
<60y (N=173) reference
≥60y (N=64) 1.124 (0.769 − 1.645) 0.5454
Sample_type
Primary (N=135) reference
Recurrent (N=102) 2.016 (1.429 − 2.843) <0.001 ***
Radiotherapy
No (N=24) reference
Yes (N=191) 0.678 (0.360 − 1.277) 0.2289
Chemotherapy
No (N=25) reference
Yes (N=189) 0.466 (0.249 − 0.873) 0.0172 *
IDH_mutation_status
Mutant (N=46) reference
Wildtype (N=181) 1.308 (0.820 − 2.086) 0.2591
X1p19q_codeletion_status
Codel (N=13) reference
Non−codel (N=196) 1.521 (0.608 − 3.807) 0.37
Risk index (N=237) 1.912 (1.373 − 2.662) <0.001 ***
Events: 150; Global p−value (Log−Rank): 1.9185e−09
AIC: 1289.79; Concordance Index: 0.7
0.1
0.2
0.5
1
2


B
Hazard ratio
Age
<60y (N=116) reference
≥60y (N=21) 2.136 (1.247 − 3.658) 0.0057 **
Sample_type
Primary (N=85) reference
Recurrent (N=22) 2.013 (1.176 − 3.446) 0.0107 *
Secondary (N=30) 3.410 (1.882 − 6.176) <0.001 ***
Radiotherapy
No (N=26) reference
Yes (N=105) 0.786 (0.473 − 1.306) 0.3528
Chemotherapy
No (N=40) reference
Yes (N=92) 0.454 (0.295 − 0.701) <0.001 ***
IDH_mutation_status
Wildtype (N=97) reference
Mutant (N=40) 1.100 (0.610 − 1.986) 0.7508
X1p19q_codeletion_status
Codel (N=7) reference
Non−codel (N=127) 2.478 (0.991 − 6.194) 0.0522
Risk index (N=137) 2.373 (1.557 − 3.618) <0.001 ***
Events: 113; Global p−value (Log−Rank): 3.7155e−08
AIC: 865.19; Concordance Index: 0.71
0.1
0.2
0.5
1
2
5

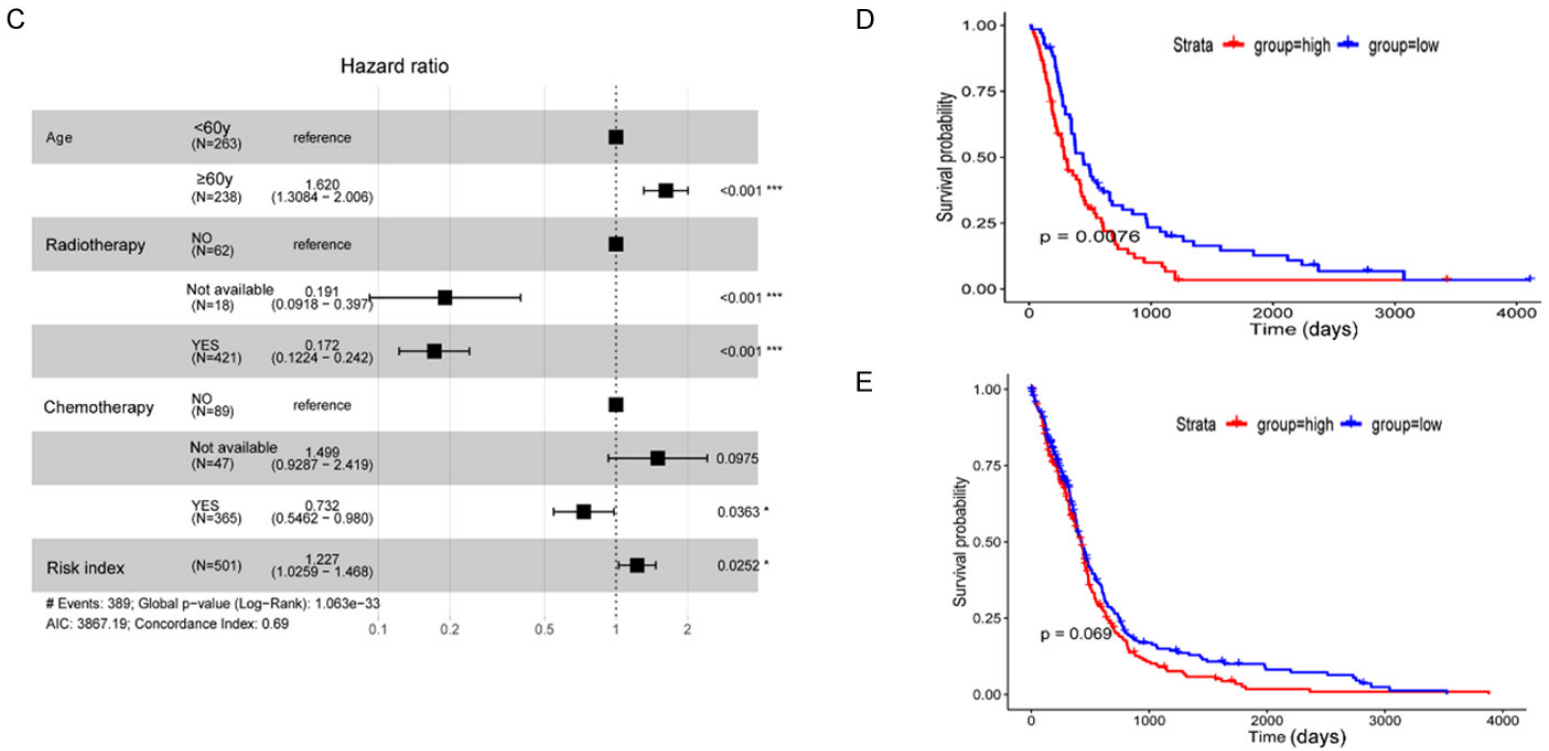


Figure 6. Validation of the GFCK expression-based prognostic risk index (RI). (A-C) Multivariate analyses of clinicopathological features and the RI in glioblastoma multiforme (GBM) tissues (A) in the CCGA mRNAseq_693 data set, (B) CCGA mRNAseq_325 data set, or (C) TCGA_array data set. (D, E) Kaplan-Meier curves of patients stratified as low- or high-risk based on median RI value in the (D) CCGA mRNAseq_325 data set or (E) TCGA_array data set. AIC, Akaike Information Criterion.

promoter methylation have proven to be reliable prognostic biomarkers [9]. However, IDH mutations are found in fewer than 5% of patients with primary GBM [35]. A methylated MGMT promoter is associated with longer survival of GBM patients, in part because of increased sensitivity to temozolomide [36]. The TCGA classification system has defined four subtypes of GBM (classical, mesenchymal, proneural and neural) based on comprehensive analysis of genetic alterations and expression [12]. These subtypes differ in prognosis [37] and response to chemoradiotherapy [12]. However, the classification system has not found widespread use in the clinic because it requires complex data that are costly to collect.

As a potentially more straightforward, accessible alternative, we have developed a system for classifying GBM subtypes based on expression of 68 GFCKs. Three subtypes were identified, each with a unique hallmark gene set and different clinical characteristics and prognosis. Type I GBM is associated with high expression of TAC1, CCK, CHGA, PNOC, SST, FGF17, CHGB, and NPY, and it is enriched for the hallmark gene set down-regulated by KRAS signaling. Type II GBM is associated with high expression of SAA1/2, RETN, and CXCL8, and it is enriched in several typical cancer-related hallmark gene sets: the epithelial-mesenchymal transition, KRAS signaling up, mTORC1 signaling, P53 pathway, and PI3K/AKT/mTOR signaling. Type III GBM, while clearly different from the other subtypes in clinical profile and prognosis, appears more difficult to define genetically: no genes were identified as uniquely up-regulated in tumors of this subtype. Nevertheless, we did find the subtype to be associated with enrichment in two cell cycle-related hallmark gene sets: E2F targets and G2M checkpoint. Further work is needed to understand the genetic basis for this subtype. It may also be that this subtype is defined more by environment-gene interactions than by genomic alterations.

Our GFCK-based classification may help clarify some of the difficulties in treating GBM. For example, treatments targeting the PI3K/AKT/mTOR signaling pathway have proven ineffective in the longer term [38], and our results suggest that this may be, in part, because only one of the three GBM subtypes is likely to respond to it. Our classification suggests the usefulness of targeting mTORC1, consistent with an analysis confirming mTOR as an important therapeutic target [39]. Our results suggest that GBM of Type III involve perturbations of the cell cycle, consistent with the observation that approximately 80% of GBM cases feature dysregulation of the CDK4/6-Rb-E2F axis in the cell cycle [35, 40, 41]. In these ways, our classification scheme may help guide improvements in GBM management.

In addition, we created a prognostic RI based on the expression of six GFCKs (BMP2, CCN3, GKN1, LIF, MDK, and SEMA3G). We validated the RI using two independent data sets from different platforms, suggesting that it may be a reliable prognostic marker. Indeed, it appears to predict prognosis independently of several known prognostic factors, and it can stratify patients into those showing shorter or longer survival, similar to previous multiple studies [42, 43]. However, while the prognostic difference was not significant in the data set from TCGA. This may result from too much censored data in TCGA or racial effects [44].

Unsurprisingly, several studies pointed out that the six GFCKs were associated with GBM. BMP2 may sensitize GBM stem-like cells to temozolomide by affecting HIF-1α stability and MGMT expression [45]. Ablating LIF may potently attenuate GBM growth enhancement [46]. and MDK expression correlates with GBM progression [47]. We also found the expression of CCN3, GKN1 and SEMA3G were associated with prognosis of patients with GBM. These roles of three GFCKs in GBM were needed further investigation.

Although our study provides new insights into GBM, it has several limitations. First, no suitable tool is currently available to predict new data using an NMF model, so our GBM classification system requires validation and perhaps improvement based on another independent data set based on the same platforms. Second, we were limited to publicly available data, so we were unable to examine whether our GBM subtypes also differ in metastatic behavior, response to chemotherapy, or tumor recurrence. Thirdly, it is not clear whether these six GFCKs are causal or merely markers for the prognosis of patients with GBM before furthermore molecular experiment.

Despite these limitations, we were able to define GBM subtypes based on GFCK expression patterns, from which we also derived a prognostic RI. If confirmed in prospective studies, our findings may aid in clinical decision-making to improve GBM management, and they may help guide further research into GBM onset and progression.

Acknowledgements

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Disclosure of conflict of interest

None.

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Table S1. Gene sets enriched in type II glioblastoma multiforme

Name	Size	Enrichment score	Adjusted P*
HALLMARK_ALLOGRAFT_REJECTION	186	0.699	0.000
HALLMARK_COMPLEMENT	192	0.591	0.000
HALLMARK_IL2_STAT5_SIGNALING	197	0.582	0.001
HALLMARK_COAGULATION	119	0.573	0.001
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	48	0.682	0.001
HALLMARK_INTERFERON_GAMMA_RESPONSE	193	0.690	0.001
HALLMARK_INFLAMMATORY_RESPONSE	193	0.646	0.002
HALLMARK_IL6_JAK_STAT3_SIGNALING	80	0.697	0.002
HALLMARK_APOPTOSIS	159	0.548	0.002
HALLMARK_TNFA_SIGNALING_VIA_NFKB	196	0.667	0.003
HALLMARK_P53_PATHWAY	195	0.520	0.006
HALLMARK_XENOBIOTIC_METABOLISM	184	0.470	0.009
HALLMARK_HYPOXIA	191	0.548	0.009
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	198	0.565	0.009
HALLMARK_INTERFERON_ALPHA_RESPONSE	95	0.680	0.009
HALLMARK_KRAS_SIGNALING_UP	191	0.473	0.012
HALLMARK_GLYCOLYSIS	189	0.482	0.022
HALLMARK_UV_RESPONSE_UP	156	0.381	0.119
HALLMARK_ESTROGEN_RESPONSE_LATE	194	0.338	0.121
HALLMARK_ADIPOGENESIS	194	0.399	0.136
HALLMARK_FATTY_ACID_METABOLISM	150	0.400	0.137
HALLMARK_PI3K_AKT_MTOR_SIGNALING	102	0.395	0.146
HALLMARK_MTORC1_SIGNALING	197	0.424	0.150
HALLMARK_ANGIOGENESIS	34	0.449	0.159
HALLMARK_MYOGENESIS	192	0.322	0.161
HALLMARK_APICAL_JUNCTION	195	0.323	0.174
HALLMARK_CHOLESTEROL_HOMEOSTASIS	71	0.370	0.174
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	108	0.393	0.196
HALLMARK_PEROXISOME	101	0.337	0.221

*Adjusted by the false discovery rate.

Table S2. Genes associated with survival of glioblastoma multiforme patients in univariate Cox hazard analysis

Gene	β	HR (95% CI)	*P* value
APLN	0.123	1.13 (1.02-1.25)	0.018
BMP2	-0.171	0.843 (0.747-0.951)	0.005
CCK	-0.0564	0.945 (0.894-1)	0.048
CCL19	-0.151	0.86 (0.748-0.989)	0.035
CCN3	0.169	1.18 (1.05-1.34)	0.007
CHGA	-0.066	0.936 (0.881-0.995)	0.033
CHGB	-0.0832	0.92 (0.85-0.996)	0.039
CRH	-0.343	0.709 (0.538-0.935)	0.015
CTF1	0.182	1.2 (1.02-1.41)	0.024
EDN3	-0.166	0.847 (0.722-0.995)	0.044
EGF	0.275	1.32 (1.02-1.7)	0.037
GDF10	-0.247	0.782 (0.649-0.94)	0.009
GKN1	-0.0786	0.924 (0.86-0.994)	0.034
LIF	0.0894	1.09 (1-1.2)	0.049
MDK	0.119	1.13 (1.04-1.22)	0.003
NRG3	-0.144	0.866 (0.755-0.992)	0.039
PDGFA	0.143	1.15 (1.02-1.3)	0.019
PLAU	0.0941	1.1 (1.02-1.19)	0.018
PRLHR	-0.532	0.588 (0.433-0.797)	0.001
SEMA3G	-0.292	0.747 (0.604-0.922)	0.007
SEMA4C	0.256	1.29 (1.04-1.6)	0.020
SPP1	0.0642	1.07 (1-1.13)	0.037
STC1	0.147	1.16 (1.02-1.31)	0.019
TAC1	-0.0944	0.91 (0.829-0.999)	0.047
TGFB2	0.128	1.14 (1.03-1.25)	0.008
TNC	0.154	1.17 (1.08-1.26)	0.000
TNFRSF11B	0.229	1.26 (1.09-1.46)	0.002
TNFSF14	0.246	1.28 (1.1-1.49)	0.001
VEGFA	0.0925	1.1 (1.03-1.17)	0.003

HR, hazard ratio; CI, Confidence Interval.