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Original Research

PLEKHA4 is Associated with Tumour Microenvironment, Stemness, Proliferation and Poor Prognosis of Gliomas

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Abstract

Background: Glioma is the most common intracranial malignancy. Immune-infiltration and tumour stemness are associated with the prognosis of glioma. Although pleckstrin homology containing family A, number 4 (*PLEKHA4*) is widely expressed in various human cancers, its role in glioma remains unclear. **Methods**: We examined the features and clinical significance of *PLEKHA4* in gliomas by analysing relevant data from the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases. Gene set enrichment analysis (GSEA) was performed to determine the possible functions and pathways involving *PLEKHA4* in glioma. The relationship between *PLEKHA4* expression and the degree of oncogenic dedifferentiation was analysed using stemness scores (ss) calculated from epigenetic and transcriptomic features. We also explored the relationship between *PLEKHA4* expression and immune cell infiltration in gliomas using the CIBERSORT databases. Furthermore, drug sensitivity analysis was performed using datasets from the GDSC and GTRP databases. In addition, we performed relevant *in vitro* experimental studies. **Results**: *PLEKHA4* DNA hypomethylation status was associated with its high expression in glioma tissues as well as poor prognoses. Univariate and multivariate Cox analyses indicated that *PLEKHA4* expression may be considered as an independent prognostic factor in patients with glioma. GSEA indicated that high *PLEKHA4* expression was associated with Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Wingless-Type MMTV Integration Site Family (Wnt), JUN N-terminal kinase (JNK) signalling pathways and involved in apoptotic, cytoskeletal, and cell adhesion biological processes (BPs). In addition, increased *PLEKHA4* expression was associated with higher glioma stemness scores than lower *PLEKHA4* expression levels. Furthermore, the expression of *PLEKHA4* was shown to be associated with glioma infiltration by $CD4^+$ T cells, B cells, neutrophils, macrophages, and dendritic cells. Drug sensitivity analysis also showed that *PLEKHA4* expression was negatively correlated with the sensitivity of several small molecule kinase inhibitors. Furthermore, *in vitro* experiments confirmed that *PLEKHA4* knockdown inhibited the proliferation of glioma cells. **Conclusions**: *PLEKHA4* is highly expressed in glioma tissues and correlated with tumour stemness, immune cell infiltration and proliferation, suggesting its potential as a novel prognostic biomarker and therapeutic target in glioma.

Keywords: glioma; *PLEKHA4*; prognostic biomarkers; methylation; immune cell infiltration; tumour stemness; drug sensitivity

1. Introduction

Neuro-oncological classificational guidelines of the World Health Organization (WHO) categorizes gliomas into four grades, with grades III and IV being associated with worse clinical outcomes [1]. Despite the availability of various treatment options, such as surgery, postoperative adjuvant radio-chemotherapy, electric field therapy, and immunotherapy, the prognoses for gliomas remain unsatisfactory, the 5-year survival rate for glioblastoma, which has the worst prognosis, is only 6.8% [2,3]. Therefore, finding novel prognostic biomarkers and effective therapeutic targets which may lead to enhanced glioma treatment and disease outcomes should be considered as important.

Targeting the Wnt signalling pathway as a therapeutic option against gliomas is increasingly attracting attention [4,5]. Aberrant activation of canonical Wnt signalling components has been commonly found in malignant gliomas. Furthermore, these alterations mediate a variety of biological processes (BPs) leading to malignant tumour behaviours, such as the maintenance of glioblastoma (GBM) stem cells, enhancement of migration and invasion, and induction of drug resistance [6–10]. However, the non-canonical Wnt pathway, which is known to participate in the invasiveness and mesenchymal transition of glioma cells, has also been found to promote malignancy [11,12].

Phosphoinositides, which are membrane lipids, act as key signalling molecules that regulate the functioning of proteins by recruiting them to the plasma membrane and changing their conformation [13,14]. The pleckstrin homology (PH) domain, one of the most widely distributed structural domains in the human proteome, is found in over

250 human proteins. Furthermore, the PH domain binds to phosphatidylinositol (PIP), a specific lipid component, to localize to the cell membrane [15]. PH containing family A, number 4 (*PLEKHA4*), is a PH domain-containing protein, the function of which remains largely uncharacterized. Recent studies have shown that *PLEKHA4* influences the intracellular ubiquitination level of dishevelled segment polarity protein (DVL) ubiquitination, which is mediated via sequestering a key E3 ligase adaptor within phosphatidylinositol-4,5-bisphosphate 4-phosphatase 2 (PI [4,5] P2)-rich plasma membrane clusters, thereby regulating the Wnt/PCP signalling pathway [16]. *PLEKHA4* knockdown was also found to attenuate Wnt/β-catenin signalling and inhibit melanomas growth [17]. Although this evidence implies a relationship between *PLEKHA4* and Wnt signalling, the role of *PLEKHA4* in human disease remains largely unknown.

In view of the dearth of previous research studies that investigated the potential role of *PLEKHA4* in glioma, we examined the prognostic significance of *PLEKHA4* in glioma by analysing the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases [18,19]. Gene set enrichment analysis (GSEA) was performed to analyse the possible functions and pathways of *PLEKHA4* in glioma. The relationship between PLEKHA4 expression and the degree of oncogenic dedifferentiation was analysed using stemness scores calculated from epigenetic and transcriptomic features. The association between *PLEKHA4* expression and immune cell infiltration in gliomas was assessed using the CIBERSORT databases. Furthermore, drug sensitivity analysis was performed using datasets from the Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Treatment Response Portal (GTRP). It is surmised that our findings would enhance the diagnosis and treatment of gliomas.

2. Materials and Methods

2.1 Analysis of PLEKHA4 Expression

We downloaded RNA sequencing (RNA-seq, Illumina Inc, San Diego, CA, USA) data of 701 patients, including 532 cases of low-grade glioma (LGG) and 169 cases of GBM, from the UCSC Xena database for further analysis [20]. Downloading from the CGGA website, enabled a dataset comprising 1013 cases of glioma patients to be obtained (625 cases of LGG and 388 cases of GBM). Information from the Gene Expression Profiling Interactive Analysis (GEPIA) database was used to analyse the of *PLEKHA4* in various cancers [21]. The Gliovis database [22] was used to assess the correlation between *PLEKHA4* expression and various clinical features including WHO tumour grade, sex, methylation status of the O(6)-methylguanine-DNA methyltransferase (*MGMT*) gene promoter, sequence deletions on chromosomes (Chr) 1p/19q, mutation status of the gene encoding isocitrate dehydrogenase (*IDH*), and different glioma molecular subtypes (mesenchymal, classical, and proneural). We used representative immunohistochemical (IHC) staining images of *PLEKHA4* in gliomas obtained from The Human Protein Atlas (HPA) online database (http://www.proteinatlas.org).

2.2 Survival Analysis

Kaplan–Meier survival analysis was used to determine the relationship between the expression levels of *PLEKHA4* and overall survival (OS) in patients with glioma. The R package rms was used to build a nomogram based on the Cox method and the prognostic values of the features of interest was assessed. Risk scores were obtained from the analysis and receiver operating characteristic (ROC) curves were plotted to show their predictive values for TCGA and CGGA glioma.

2.3 Co-Expression Analysis and GSEA

PLEKHA4 gene co-expression data were obtained from the LinkedOmics database (http://linkedomics.org/). The AmiGO 2 database (http://amigo.geneontology.org/) was searched for both non-canonical and canonical Wnt signalling pathway information. Enrichment analysis was performed to assess the functional enrichment of 100 genes that were positively associated with *PLEKHA4* in gliomas. GSEA was conducted using GSEA software (version 3.0, Broad Institute Inc, Cambridge, MA, USA) [23].

2.4 Pan-Cancer Tumour Stemness Correlation Analysis

We downloaded the TCGA pan-cancer (N = 10,535, G = 60,499) database, which consists of a uniformly normalized dataset, and extracted expression data for the *PLEKHA4* (ENSG00000105559) gene from each sample. We obtained RNAss (RNA stemness scores) and DNAss (DNA stemness scores) by calculating epigenetic and transcriptomic features for each tumour, respectively, as previously reported by Malta *et al*. [24]. Finally, we integrated the stemness scores and gene expression data of the samples to perform correlation analyses using the Sangerbox tool (http://sangerbox.com/).

2.5 Immune Microenvironment Analysis

We used the R package ESTIMATE to calculate the stromal, immune, and ESTIMATE (version 1.0.13, https://bioinformatics.mdanderson.org/estimate/) scores of each patient. CIBERSORT (https://cibersort.stanford.edu/) was used to assess the immune infiltration scores [25].

2.6 Drug Sensitivity Analysis

PLEKHA4 expression and drug sensitivity were investigated using the GDSC and Clinical Trial Reporting Program (CTRP) databases [26,27].

2.7 Cell Culture and PLEKHA4-Targeted Short Interfering RNA (siRNA) Transfection

Human glioma (LN-229 and U-118MG) cell lines were obtained from Meison Cell Corporation (Jinhua, Zhejiang, China). All cells were tested for STR analysis and mycoplasma contamination. When the LN-229 and U-118MG cells, cultures in six-well plates, reached 70–80% confluency, they were respectively transfected with siRNA (32330, Genepharma Inc, Suzhou, Jiangsu, China), using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). The siRNA sequences are shown (**Supplementary Table 1**).

2.8 RNA Extraction and Reverse Transcription Quantitative Polymerase Chain Reaction (RT–qPCR)

Total RNA was extracted from cell samples using Trizol (T9424, Sigma-Aldrich Inc, Springfield, MO, USA). Next, the concentration of RNA was detected using a NanoDropOne spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Furthermore, the RNA was reverse transcribed using a cDNA synthesis kit (RR036A, Takara Bio Inc, Yamanashi, Japan). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using TBGreen (RR430A, Takara Bio Inc, Yamanashi, Japan) with a LightCycler96 system (version 1.1, Roche Diagnostics Inc, Basel, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize *PLEKHA4* levels; primer sequences for the RT-qPCR are shown (**Supplementary Table 1**).

2.9 Cell Proliferation Assay

Approximately 4000 LN-229 and U-118MG cells were first inoculated on 96-well plates, and then transfected with *PLEKHA4*-siRNA the next day. To assess the effect of *PLEKHA4* on the malignant biological behaviour of glioma cells, the effect of *PLEKHA4* knockdown was examined by continuously monitoring cell numbers using the IncuCyte system (20× objective), during which period images were acquired every 3 h for 96 h, following which they were analysed.

2.10 Statistical Analysis

R software (v.4.0.5, RStudio Inc., Boston, MA, USA) was used to statistically analyse the datasets from TCGA and CGGA databases. Wilcoxon rank sum and chi-square tests for continuous and categorical variables, respectively. The correlation between clinical characteristics and OS was assessed using multivariate and univariate Cox regression analyses. Correlations between *PLEKHA4* expression and immune infiltration and tumour stemness scores (DNAss and RNAss) were calculated and assessed using Spearman's correlation coefficient. Results with a false discovery rate (FDR) <0.25 and $p < 0.05$ were considered statistically significant.

3. Results

3.1 PLEKHA4 Expression is Elevated in Gliomas and Correlated with Malignant Phenotype

The results of the pan-cancer analysis revealed that *PLEKHA4* expression levels in different tumours of most cancer types varied compared to levels in normal tissues (Fig. 1A). The transcript levels of *PLEKHA4* in LGG and GBM were significantly higher than those in normal brain tissues (Fig. 1B).

An assessment of the correlation between *PLEKHA4* expression and molecular characteristics of patients with glioma showed higher *PLEKHA4* expression levels in gliomas without Chr 1p/19q co-deletions, mesenchymal subtypes, *IDH* wild-type (WT) gliomas, and *MGMT* non-methylated gliomas (Fig. 1C). Furthermore, analysis of the in-situ expression of *PLEKHA4* using the HPA database, showed higher *PLEKHA4* levels in both GBM and LGG (Fig. 1D).

3.2 Prognostic Significance of PLEKHA4 Methylation in Glioma

DNA methylation can regulate gene expression without affecting the sequence of genes. Therefore, identifying the methylation status of *PLEKHA4* can help elucidate the phenomenon of increased *PLEKHA4* mRNA expression in gliomas. The *PLEKHA4* gene has seven methylation sites, and in LGGs, six of them (cg01093065, cg06339706, cg06705122, cg15549821, cg18689573, and cg23002721) were negatively correlated with *PLEKHA4* mRNA expression levels, whereas one (cg25261547) was positively correlated (Fig. 2A). The six negatively correlated methylation sites had a lower methylation level in GBM than they did in LGG (Fig. 2B,C), whereas the *PLEKHA4* mRNA expression level was higher in GBM than it was in LGG (Fig. 1B). In addition, hypermethylation at cg06339706 and cg06705122 sites significantly affected the prognosis of patients with LGG, who showed a longer survival time than patients without hypermethylation (Fig. 2D,E).

3.3 Association between PLEKHA4 Expression and Prognosis and Prognostic Predictor Construction

Dichotomous grouping of patients from the TCGA and CGGA databases showed that high PLEKHA4 expression was mostly associated with a worse OS (Fig. 3A,B). Univariate and Cox multifactor regression analysis showed that *PLEKHA4* expression with tumour grade (LGG vs GBM), *MGMT* promoter status (methylated vs unmethylated), Chr7 gain and Chr10 loss status (yes vs no), Chr 1p/19q co-deletion (yes vs no), *IDH* status (Mut vs WT), and age were independent prognostic factors (Tables 1,2). The results suggest that gliomas expressing high levels of *PLEKHA4* may have a worse prognosis compared to low levels of *PLEKHA4* expression.

Furthermore, we integrated data on survival status, survival time, and seven characteristics in a sample of 621

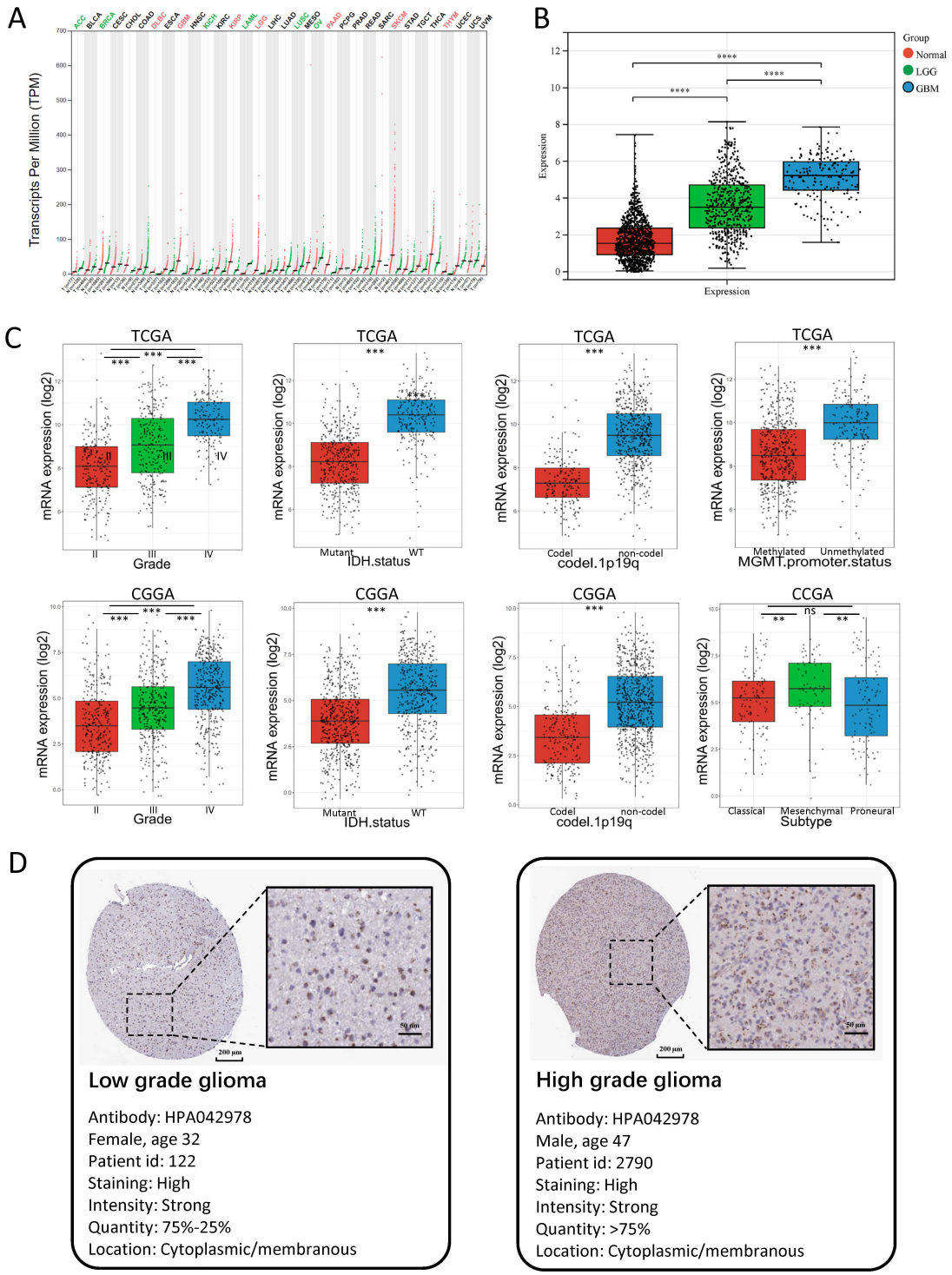

Fig. 1. Pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression is increased in gliomas. (A) Expression pattern of *PLEKHA4* mRNA in The Cancer Genome Atlas (TCGA) tumours, with normal tissues matched to the Genotype–Tissue Expression (GTEx) database. (B) *PLEKHA4* expression levels in TCGA gliomas and normal tissues, with normal tissues matched to those in the GTEx database. (C) Correlation of *PLEKHA4* expression with various clinical features, including World Health Organization (WHO) tumour grade, isocitrate dehydrogenase (*IDH*) mutation status, 1p/19q co-deletion status, methylation status of O(6)-methylguanine-DNA methyltransferase (*MGMT*) gene promoter and different glioma molecular subtypes. (D) PLEKHA4 protein staining in different grades of glioma tissues from Human Protein Atlas. The scale bar represents 200μm and 50μm. (ns, no significance, $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). LGG, low-grade glioma; GBM, glioblastoma; CGGA, Chinese Glioma Genome Atlas; TCGA, The Cancer Genome Atlas.

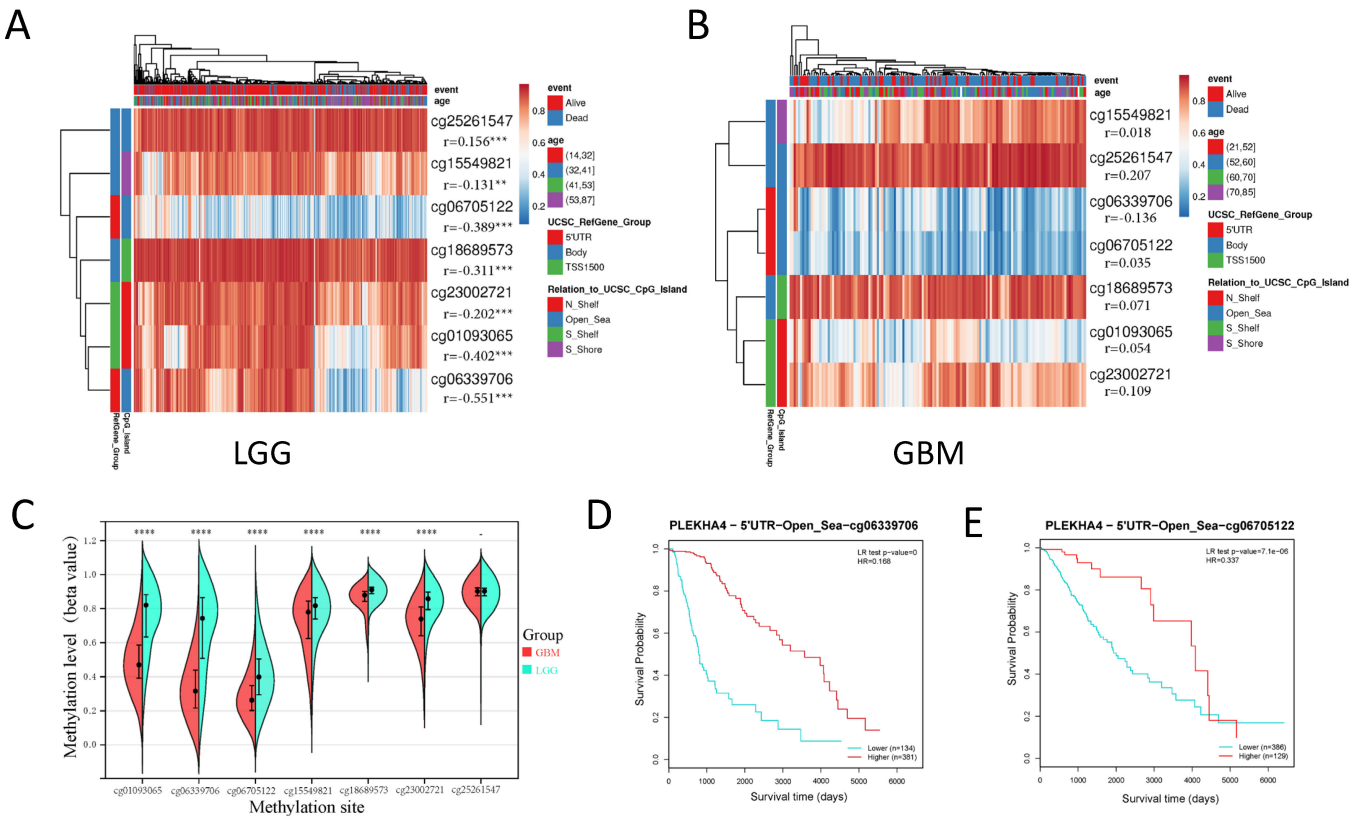

Fig. 2. Methylation analysis and its prognostic significance in glioma. (A,B) Heat map showing methylation sites in LGG and GBM. (C) Comparison of the methylation degree of pleckstrin homology containing family A, number 4 (*PLEKHA4*) between LGG and GBM. (D,E) Kaplan–Meier curves show that hypermethylation of CpG sites (cg06339706 and cg06705122) corresponds to longer survival in patients with LGG (**$p < 0.01$, ***$p < 0.001$ and **** $p < 0.0001$). LGG, low-grade glioma; GBM, glioblastoma.

Table 1. Univariate regression and multivariate survival model of prognostic covariates in patient data from The Cancer Genome Atlas (TCGA) glioma dataset.

Characteristics	Univariate Cox Regression		Multivariate Cox Regression	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
PLEKHA4				
Increasing Expression	1.690 (1.550–1.843)	<0.001	1.172 (1.030–1.332)	0.016
Grade				
LGG vs GBM	8.967 (6.795–11.832)	<0.001	2.139 (1.455–3.145)	<0.001
Sex				
Female vs Male	1.166 (0.899–1.512)	0.246		
Age				
Increasing years	1.069 (1.058–1.079)	<0.001	1.043 (1.030–1.056)	<0.001
MGMT promoter status				
Methylated vs unmethylated	3.301 (2.492–4.373)	<0.001	1.335 (0.953–1.870)	0.092
Chr7 gain and chr10 loss				
Chr7 gain and chr10 loss vs others	8.133 (6.092–10.857)	<0.001	1.457 (0.989–2.145)	0.057
IDH status				
WT vs Mut	9.995 (7.478–13.359)	<0.001	1.940 (1.112–3.386)	0.020
1p/19q Codeletion				
codel vs non-codel	4.598 (2.906–7.276)	<0.001	1.700 (0.974–2.953)	0.062

HR, hazard ratio; 95% CI, 95% confidence interval; GBM, glioblastoma; MGMT, O(6)-methylguanine-DNA methyltransferase; IDH, isocitrate dehydrogenase; WT, wild-type; Mut, mutant.

Table 2. Univariate regression and multivariate survival model of prognostic covariates in patient data from Chinese Glioma Genome Atlas (CGGA) glioma dataset.

Characteristics	Univariate Cox Regression		Multivariate Cox Regression	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
PLEKHA4				
Increasing Expression	1.269 (1.269–1.219)	<0.001	1.107 (1.050–1.168)	<0.001
Grade				
G2 vs G3 vs G4	2.813 (2.503–3.162)	<0.001	2.278 (1.956–2.654)	<0.001
Sex				
Female vs Male	0.985 (0.836–1.159)	0.852		
Age				
Increasing years	1.029 (1.021–1.036)	<0.001	1.009 (1.002–1.016)	0.001
IDH status				
WT vs Mut	3.093 (2.611–3.664)	<0.001	1.19 (0.954–1.476)	0.125
1p/19q codeletion				
codel vs non-codel	4.508 (3.412–5.956)	<0.001	2.389 (1.732–3.295)	<0.001
Radiotherapy status				
Yes vs no	1.021 (0.811–1.285)	0.860	0.736 (0.567–0.956)	0.022
Chemotherapy status				
Yes vs no	1.524 (1.255–1.849)	<0.001	0.807 (0.641–1.016)	0.068

HR, hazard ratio; 95% CI, 95% confidence interval; WT, wild-type; Mut, mutant.

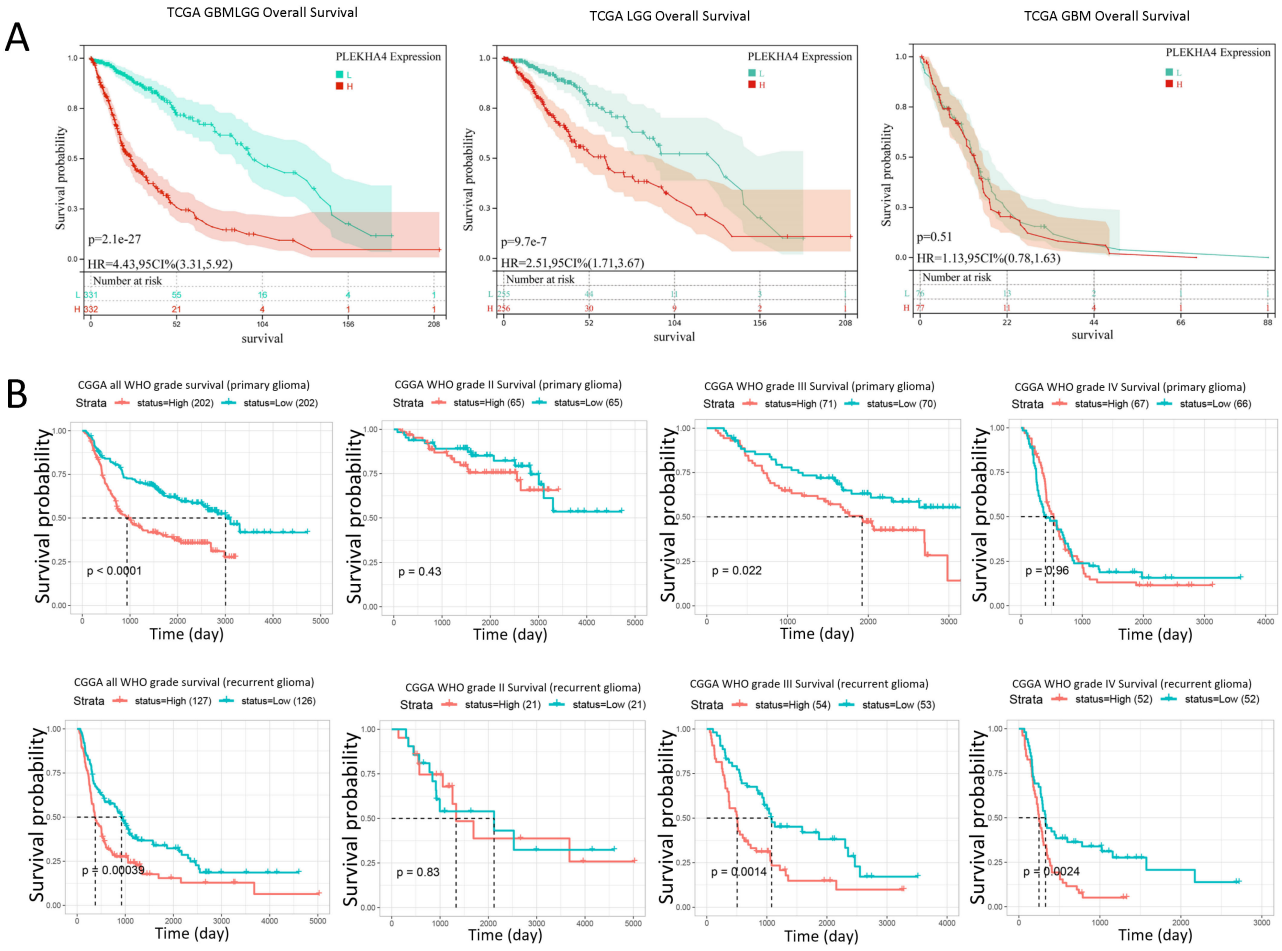

Fig. 3. Prognostic value analysis of pleckstrin homology containing family A, number 4 (*PLEKHA4*) in glioma. Prognostic value of *PLEKHA4* in glioma examined using (A) The Cancer Genome Atlas (TCGA) and (B) Chinese Glioma Genome Atlas (CGGA) database.

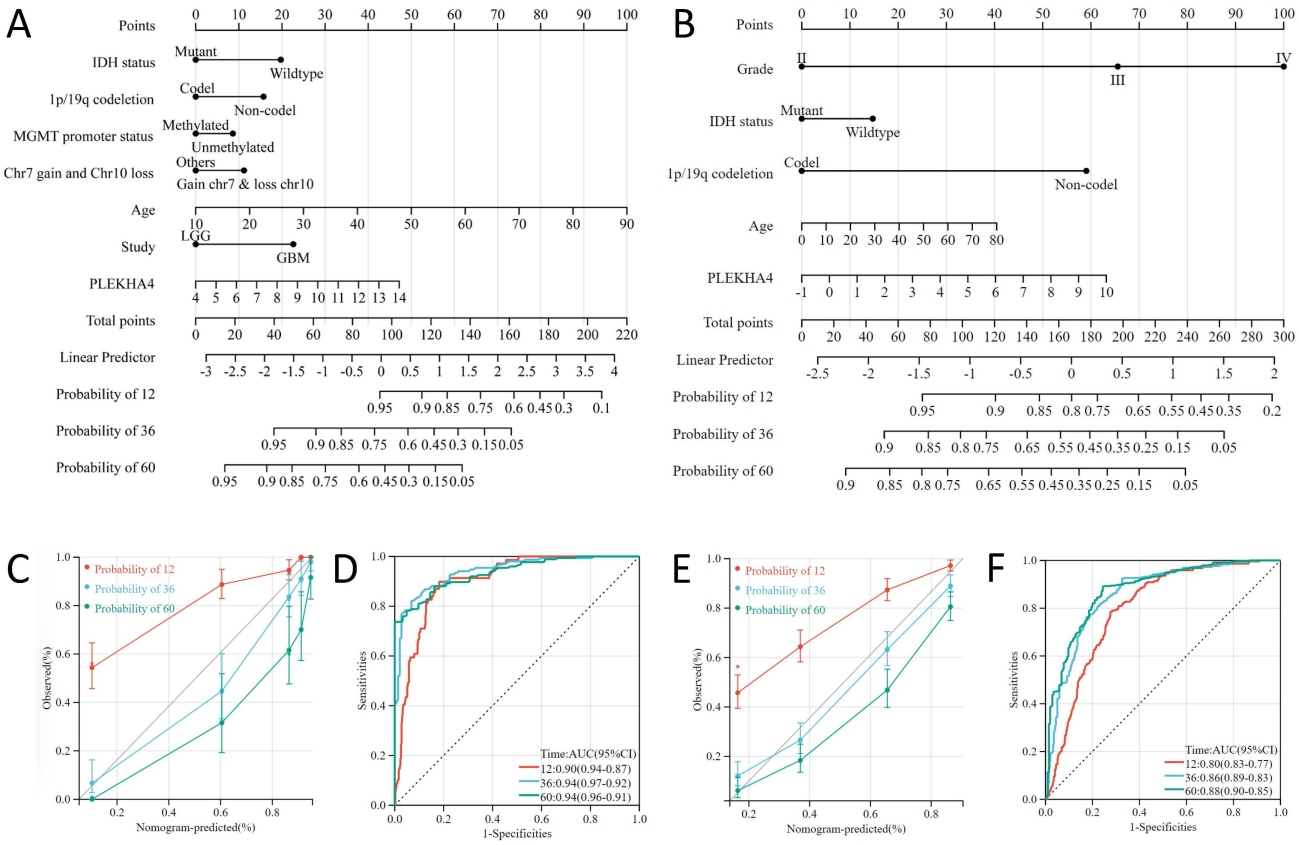

Fig. 4. Prognostic prediction of glioma in patients using pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression. Construction of nomogram to predict the overall survival (OS) for glioma patient data, (A) The Cancer Genome Atlas (TCGA) and (B) the Chinese Glioma Genome Atlas (CGGA). Calibration curves show the OS of (C) TCGA and (E) CGGA glioma cohorts. Risk scores were obtained from analysis and receiver operating characteristic (ROC) curves show predictive value with (D) TCGA and (F) CGGA glioma. IDH, isocitrate dehydrogenase; MGMT, O(6)-methylguanine DNA methyltransferase

TGCA gliomas to create a nomogram to assess the prognostic significance of these characteristics (Fig. 4A). The overall C-index of the model for the TCGA cohort was 0.865 (95% confidence interval [CI], 0.844–0.887; $p = 4.096 \times 10^{-243}$). We integrated data on survival time, survival status and five characteristics in a sample of 983 CGGA gliomas to create a nomogram to assess the prognostic significance of these characteristics (Fig. 4B). The overall C-index of the model for the CGGA cohort was 0.759 (95% CI, 0.740–0.778; $p = 8.297 \times 10^{-158}$) . The line graph predicted the 1-, 3-, and 5-year OS of patients with glioma (Fig. 4C,E). In addition, the risk score was calculated based on the results of the Cox regression analysis and the plotted ROC curves showed area under the curve (AUC) values of 0.90, 0.94, and 0.94 at 1, 3, and 5 years, respectively in TCGA glioma (Fig. 4D). In addition, AUC values for the CGGA glioma cohort were 0.80, 0.86, and 0.88 at 1, 3, and 5 years, respectively (Fig. 4F).

3.4 PLEKHA4 Co-Expression and Gene Enrichment Analysis

The analysis of co-expressed genes showed that 10,510 and 9605 genes were positively and negatively correlated with *PLEKHA4* expression, respectively. The top 50 genes positively associated with *PLEKHA4* were selected based on correlation, following which heat maps were constructed (Fig. 5A). In addition, enrichment analysis of the top 100 genes that showed a positive correlation with *PLEKHA4* in gliomas was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases. The results were visualized using bubble plots.

KEGG pathway enrichment revealed that the co-expressed genes were enriched in pathways and processes such as tuberculosis, human immunodeficiency virus 1 infection, phagocytosis, NOD-like receptor signalling, influenza A, leukocyte trans-endothelial migration, and antigen processing and presentation (Fig. 5B). The GO analysis of BPs suggested that the co-expressed genes were involved in processes, such as response to biotic stimulus, defence response, immune effector process, defence response to other

organism, innate immune response, response to cytokines, cell activation, response to interferon gamma, and cytokine-mediated signalling pathway (Fig. 5C). GO cellular component (CC) analysis indicated that the co-expressed genes were involved in components, such as the Golgi apparatus; anchoring junction; cell substrate junction; phagocytic vesicle; and peptidase inhibitor complex (Fig. 5D). Furthermore, the GO molecular function (MF) analysis suggested that the co-expressed genes were involved in the functions including identical protein binding; cysteine type endopeptidase activity involved in the apoptotic process; phospholipase a2 inhibitor; deoxycytidine deaminase; cytidine deaminase; and phospholipase inhibitor activities; S100 protein binding; and card domain binding (Fig. 5E).

3.5 GSEA of PLEKHA4

Signalling pathway analysis using a GSEA tool based on the KEGG database revealed that high *PLEKHA4* expression was associated with janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling, regulation of actin cytoskeleton, apoptosis, chemokine signalling, and cancer (Fig. 6A). Annotation of GO BP terms suggested that high *PLEKHA4* expression may be associated with regulation of T cell activation, and the JUN N-terminal kinase (JNK) cascade, as well as the positive regulation of canonical Wnt signalling pathway (Fig. 6B). Annotation of GO CC terms suggested that high *PLEKHA4* expression may be associated with protein complexes involved in cell adhesion, actin filament, actin filament bundle, and actin cytoskeleton (Fig. 6C). Annotation of GO MF terms suggested that high *PLEKHA4* expression levels may be associated with cysteine type endopeptidase regulator activity involved in the apoptotic process, integrin binding, and laminin binding (Fig. 6D, Table 3).

We further loaded 151 Wnt signalling pathway-related genes (**Supplementary Table 2**), which were identified by searching the AmiGO 2 database. The heat map displayed the top 60 genes associated with *PLEKHA4* expression among these genes, indicating a potential regulatory role for *PLEKHA4* in the activities of the Wnt signalling pathway in glioma (Fig. 6E).

3.6 PLEKHA4 Expression and Tumour Stemness Scores

RNAss and DNAss were calculated based on transcriptomic and epigenetic feature sets, respectively, and an analysis of their correlation with *PLEKHA4* expression in pan-cancer showed relatively higher correlation with glioma than with other cancers (Fig. 7A,C). Meanwhile, *PLEKHA4* expression was significantly correlated with glioma cell stemness (Fig. 7B,D).

3.7 PLEKHA4 Expression and Tumour Microenvironment in Patients with Glioma

The correlation between high *PLEKHA4* expression and T cell activation prompted us to further investigate the relationship between *PLEKHA4* expression and the immune microenvironment in gliomas. The results indicated that increased *PLEKHA4* expression was associated with higher immune, stromal, and ESTIMATE scores (Fig. 8A–C). CIBERSORT analysis indicated that regulatory T cells (Treg), resting memory $CD4^+$ T cells, M2 and M1 macrophages, naive B cells, $\gamma\delta$T cells, activated dendritic cells, and neutrophils were enriched in the group showing high *PLEKHA4* expression (Fig. 8D). Correlation analysis also confirmed that *PLEKHA4* expression was significantly associated with increases in resting memory $CD4^+$ T cells, Treg, M2 and M1 macrophages, naive B cells, gamma delta T cells, activated dendritic cells, and neutrophil immune cells (Fig. 8E).

3.8 PLEKHA4 Expression and GDSC and CTRP Drug Sensitivity in Pan-Cancer

The results of the drug sensitivity analysis showed that *PLEKHA4* expression was possibly negatively correlated with the sensitivity of various small molecule kinase inhibitors, such as selumetinib, trametinib, and dabrafenib, in the GDSC database (Fig. 9A). The CTRP drug sensitivity analysis also showed a correlation with small molecule kinase inhibitors, while *PLEKHA4* expression was negatively correlated with virofenib drug sensitivity (Fig. 9B).

3.9 PLEKHA4 Knockdown Inhibited in Vitro GBM Cells Proliferation

The results of analyses utilized to investigate the potential role played by *PLEKHA4* in glioma progression were validated vis conventional experimental methods. We transfected LN-229 and U-118MG cells with siRNA and verified *PLEKHA4* knockdown efficiency using RT-PCR assay (Fig. 10A,B). The association between *PLEKHA4* and the malignant biological behaviour of glioma cells was assessed via image analysis, which indicated that knockdown of *PLEKHA4* expression can inhibit glioma cell proliferation (Fig. 10C–F).

4. Discussion

Glioma, which is recognized as the most common primary malignancy of the central nervous system in adults [28]. The primary treatment protocol for glioma involves a combination of therapies, including surgery, radiotherapy, chemotherapy, and electric field therapy [29]. Despite tremendous advances in the diagnosis and treatment of gliomas, the prognoses remain unsatisfactory, especially in GBM, and the OS rates are low.

In recent years, with the advancement of molecular biology technology, considerable research results have been made in molecular markers and pathways that drive the malignant phenotype of glioma, and precision medicine driven by molecular pathways has targeted the treatment of glioblastoma, starting from the pathogenic mechanism, providing a very attractive and promising solution for tu-

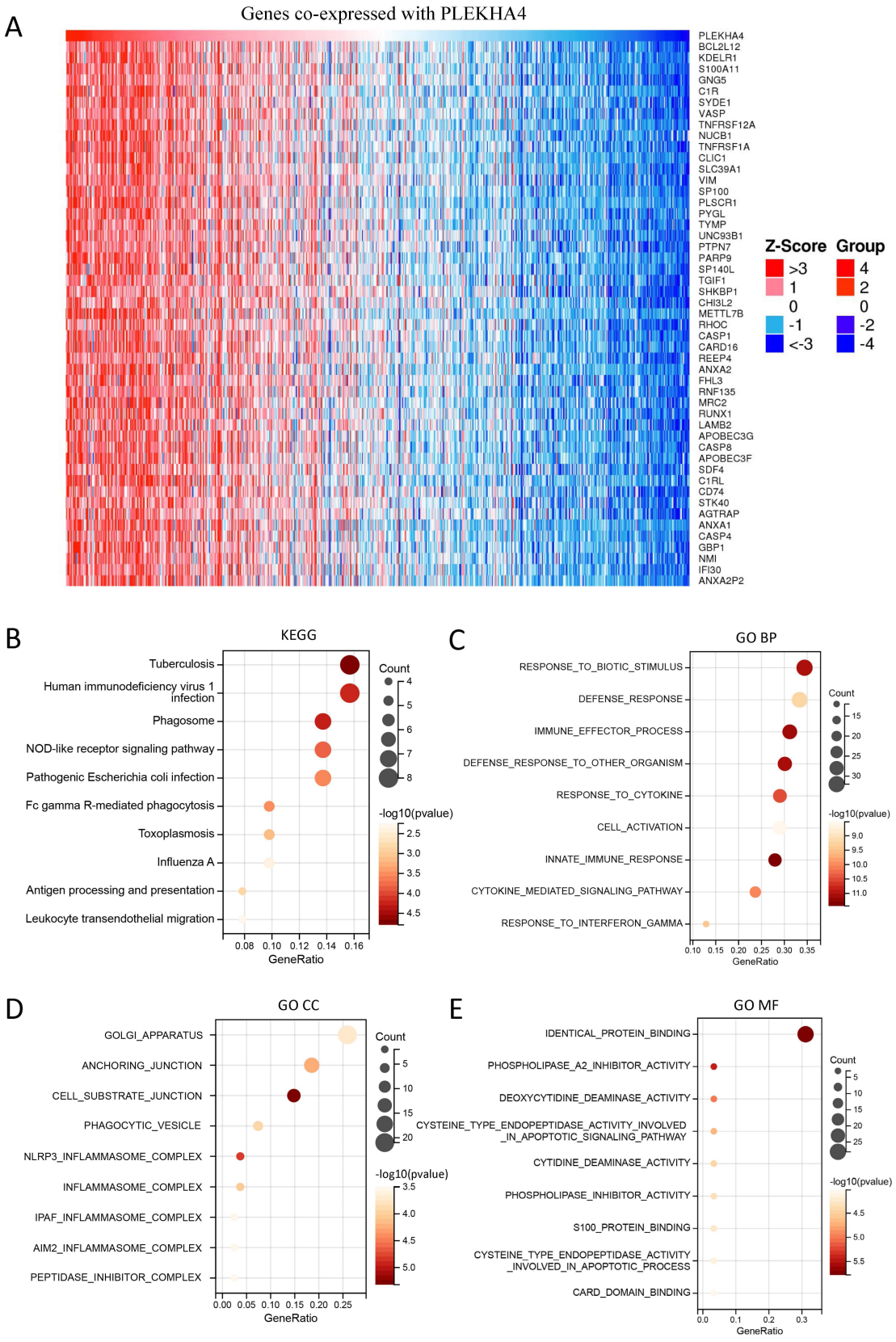

Fig. 5. Functional analysis of pleckstrin homology containing family A, number 4 (*PLEKHA4*) in gliomas. (A) LinkedOmics database exploration of genes co-expressed with *PLEKHA4* in gliomas. (B–E) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of genes co-expressed with *PLEKHA4* in gliomas.

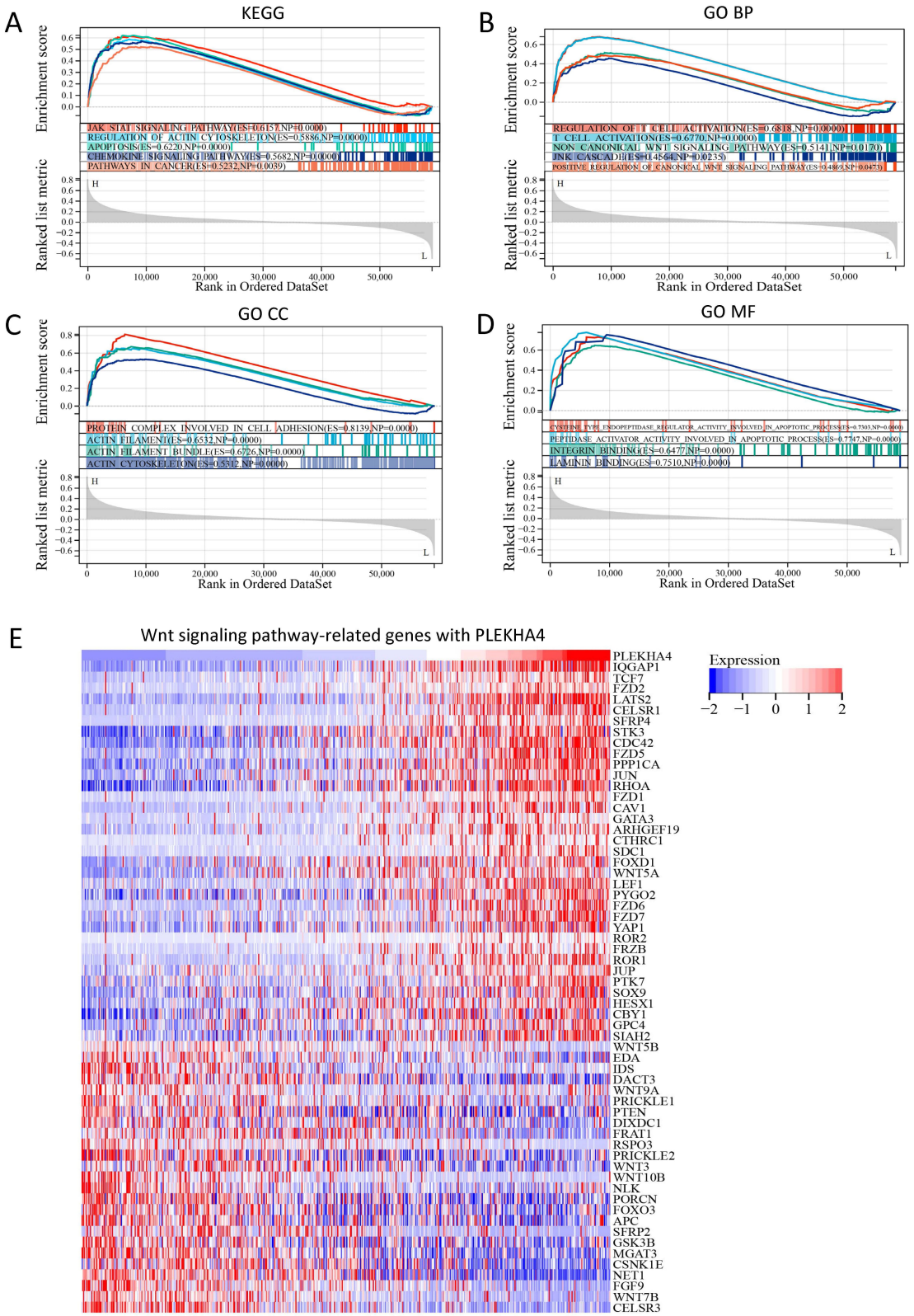

Fig. 6. Gene set enrichment analysis (GSEA) of pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression levels. (A–D) Kyoto Encyclopedia of Genes and Genomes (KEGG); Gene Ontology (GO) biological process (BP); GO cellular component (CC); and GO molecular function (MF) analyses were performed using the GSEA tool. (E) *PLEKHA4* and top 60 Wnt pathway-related genes are shown in the heat map.

Table 3. Gene set enrichment results.

Term	ES	NES	*p*-value	FDR
KEGG				
JAK_STAT_SIGNALING_PATHWAY	0.6157	2.079	0.0003	0.001
REGULATION_OF_ACTIN_CYTOSKELETON	0.5886	2.0027	0.0009	0.008
APOPTOSIS	0.622	1.9287	0.0045	0.038
CHEMOKINE_SIGNALING_PATHWAY	0.5682	1.8992	0.0051	0.053
PATHWAYS_IN_CANCER	0.5232	1.8015	0.0039	0.0144
GO BP				
REGULATION_OF_T_CELL_ACTIVATION	0.6818	2.1428	0.0003	0.009
T_CELL_ACTIVATION	0.677	2.157	0.0003	0.005
NON_CANONICAL_WNT_SIGNALING_PATHWAY	0.5141	1.6453	0.017	0.0462
JNK_CASCADE	0.4564	1.6178	0.0235	0.0549
POSITIVE_REGULATION_OF_CANONICAL_WNT_SIGNALING_PATHWAY	0.4869	1.5319	0.0473	0.0864
GO CC				
PROTEIN_COMPLEX_INVOLVED_IN_CELL_ADHESION	0.8139	2.1833	0	0
ACTIN_FILAMENT	0.6532	2.1771	0.0006	0.003
ACTIN_FILAMENT_BUNDLE	0.6726	2.1257	0.0009	0.005
ACTIN_CYTOSKELETON	0.5312	1.9945	0.0042	0.034
GO MF				
CYSTEINE_TYPE_ENDOPEPTIDASE_REGULATOR_ACTIVITY_INVOLVED_IN_APOPTOTIC_PROCESS	0.7303	2.1321	0.0003	0.001
PEPTIDASE_ACTIVATOR_ACTIVITY_INVOLVED_IN APOPTOTIC_PROCESS	0.7747	2.1056	0.0014	0.005
INTEGRIN_BINDING	0.6477	2.0892	0.0018	0.009
LAMININ_BINDING	0.751	2.0147	0.0047	0.04

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; BP, biological function; CC, cellular components; MF, molecular function; ES, Enrichment score; NES, normalized enrichment score; FDR: false discovery rate. Gene sets with $p < 0.05$ and FDR <0.05 are considered significant.

mour treatment [30]. However, at present, the exploration of targeted drugs based on clinical practice has not achieved satisfactory results [31]. Therefore, the identification of relevant molecular markers driving tumour malignancy and the screening of highly effective and specific drugs remain the biggest research challenges at present.

In the present study, we sought to clarify the mechanisms underlying characteristic malignant glioma phenotypes and to identify potential prognostic markers [30]. The findings of our study confirmed that *PLEKHA4* expression is regulated by DNA methylation and that *PLEKHA4* expression is associated with poor prognosis. Moreover, it was found that *PLEKHA4* expression showed potential as an independent prognostic factor, because it accurately predicted disease prognosis along with other features. Finally, our *in vitro* experiments also confirmed that knocking down *PLEKHA4* expression significantly reduced the proliferative capacity of glioma cells.

The results of our investigation in to the functioning of *PLEKHA4* in gliomas, indicated that high *PLEKHA4* expression levels may be associated with multiple signalling pathways, including JAK/STAT, Wnt, and JNK. In addition to our findings, several other studies have also demonstrated that *PLEKHA4* may influence the intracellular levels of DVL and further regulate the Wnt pathway in that manner [16,17]. Therefore, we suggest that *PLEKHA4* may play a regulatory role in the Wnt pathway in glioma. Particularly in the non-canonical Wnt/PCP pathway, *PLEKHA4* has been shown to be associated with the cytoskeleton and JNK pathway. Furthermore, our GSEA indicated that *PLEKHA4* may be involved in the regulation of these BPs and signalling pathways, thus substantiating our findings pertaining to its function. GO terms such as cell adhesion and actin filament, which are closely related to glioma tumour invasiveness [32,33], were observed to be enriched by this study. These findings indicate that *PLEKHA4* may be involved in regulating canonical and non-canonical Wnt pathways in gliomas, thereby influencing their malignancy. Notably, JAK/STAT as well as canonical and non-canonical Wnt signalling pathways all play important roles in mesenchymal transition [34–36]. Additionally, we found that high *PLEKHA4* expression may be associated with cellular components, such as protein complexes involved in cell adhesion, actin filaments, actin bundles, and actin cytoskeleton. Furthermore, our study revealed that *PLEKHA4* is highly expressed in mesenchymal subtypes. Considered together, these findings indicate that *PLEKHA4* may be involved in mesenchymal transition.

Glioma stem cells are closely linked to tumorigenesis and tumour maintenance, and act as key factors in the

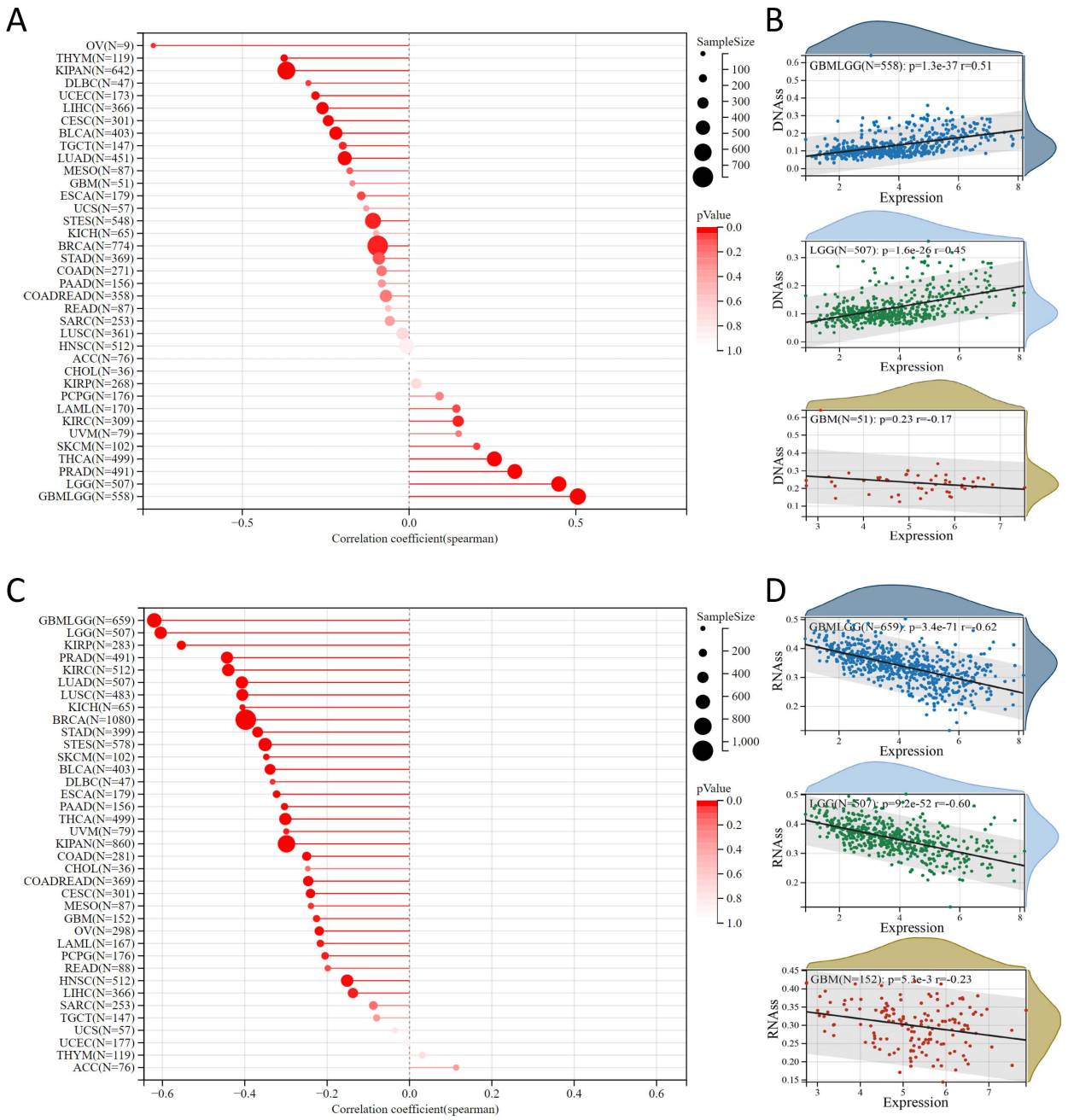

Fig. 7. Analysis of the correlation between pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression and tumour stemness. Correlation between *PLEKHA4* expression and DNA and RNA stemness scores (DNAss and RNAss, respectively) in (A–D) TCGA gliomas.

resistance shown by gliomas to standard therapies and recurrence [37,38]. Molecular markers linked to glioma stem cells have demonstrated a significant correlation with prognosis [39]. The unique role plaed by the Wnt pathway in maintaining the stemness of glioma cells may qualify it as a potential anti-cancer treatment strategy [10,40]. Because *PLEKHA4* has been shown to promote the malignant phenotype of melanocytic tumours via the Wnt pathway, we performed a pan-cancer analysis of *PLEKHA4* expression and tumour stemness, which demonstrated a significant correlation in gliomas. These results suggest that *PLEKHA4* may influence glioma cell stemness; however, more studies may be needed to confirm these findings.

Immunotherapy, which has emerged as a promising treatment strategy against glioma, warrants exploration. The immune microenvironment evidently exerts a significantly negative effect on glioma therapy, by promoting malignant growth and treatment resistance [41–43]. Many previous studies have also confirmed the association between immune cells and tumour prognosis [44,45]. In this study, our result show that high *PLEKHA4* expression levels may be associated with the regulation of T cell activation. Fur-

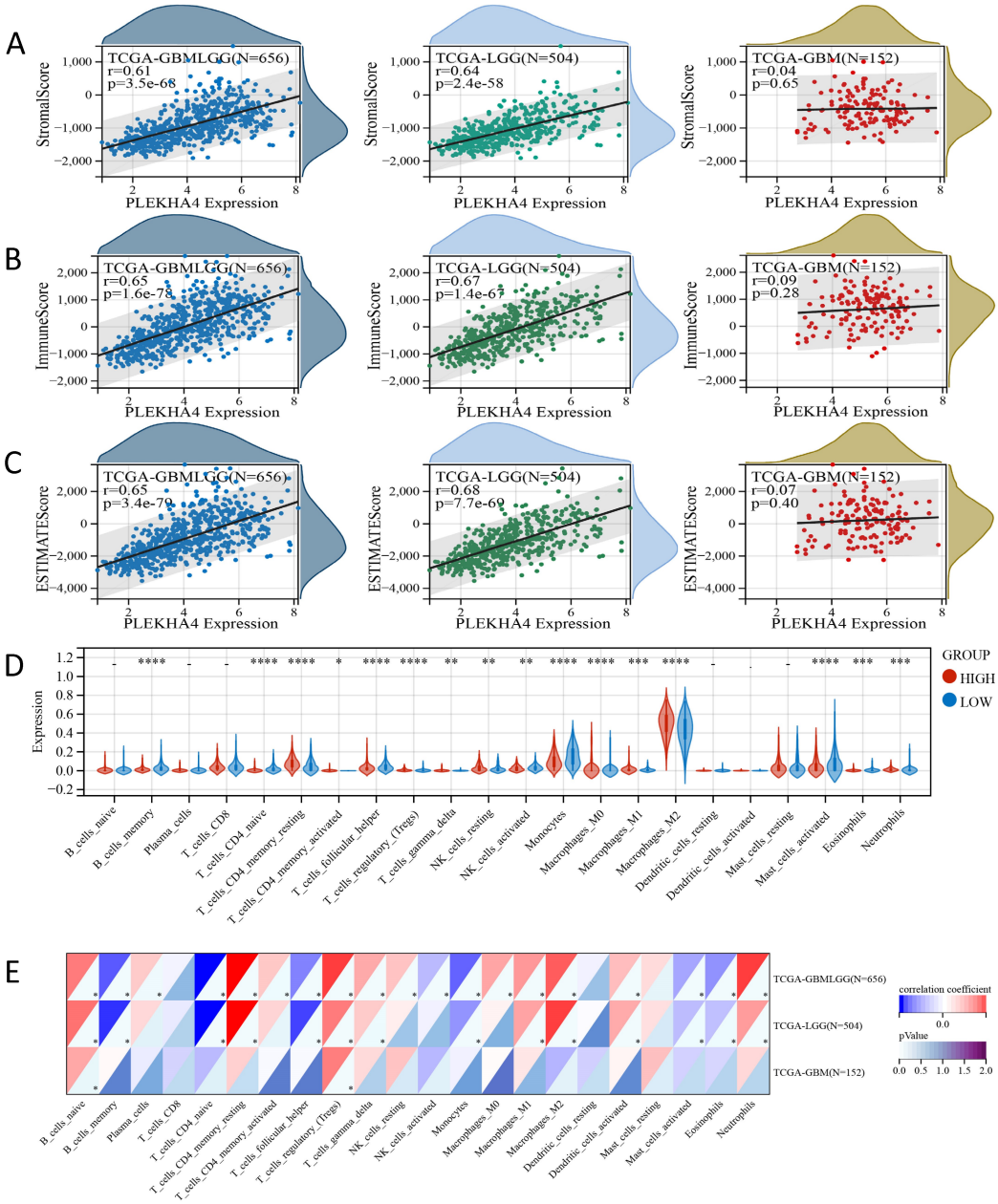

Fig. 8. The expression levels of Pleckstrin homology containing family A, number 4 (*PLEKHA4*) were correlated with the immune microenvironments of gliomas. (A–C) Correlation between *PLEKHA4* expression and immune, stromal, and ESTIMATE scores. (D) Violin plot depicting CIBERSORT scores of 22 types of immune cells in high versus low expression groups. (E) Correlation analysis of *PLEKHA4* expression levels using CIBERSORT score of 22 types of immune cells in The Cancer Genome Atlas (TCGA) glioma. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

ther analyses revealed that high *PLEKHA4* expression was associated with higher stromal, immune and ESTIMATE scores. Results of the CIBERSORT database analyses revealed that *PLEKHA4* was correlated with a variety of immune cells, especially T cell immune infiltration. In Zhang *et al.*'s [46] study, it was also shown to be associated with T cell infiltration. These results suggest that *PLEKHA4* may influence immune cell infiltration and thus facilitate prediction of the unique landscape of glioma immune infiltration.

We also explored the correlation between *PLEKHA4* expression and tumour drug sensitivity in pan-cancer, and found a negative correlation with several small molecule kinase inhibitors, including the mitogen-activated protein kinase kinase (MEK) inhibitors, selumetinib and trametinib, and the V-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors dabrafenib and vemurafenib. Small molecule inhibitors are inceasingly gaining attention as powerful tools for tumour-targeted therapy [47].

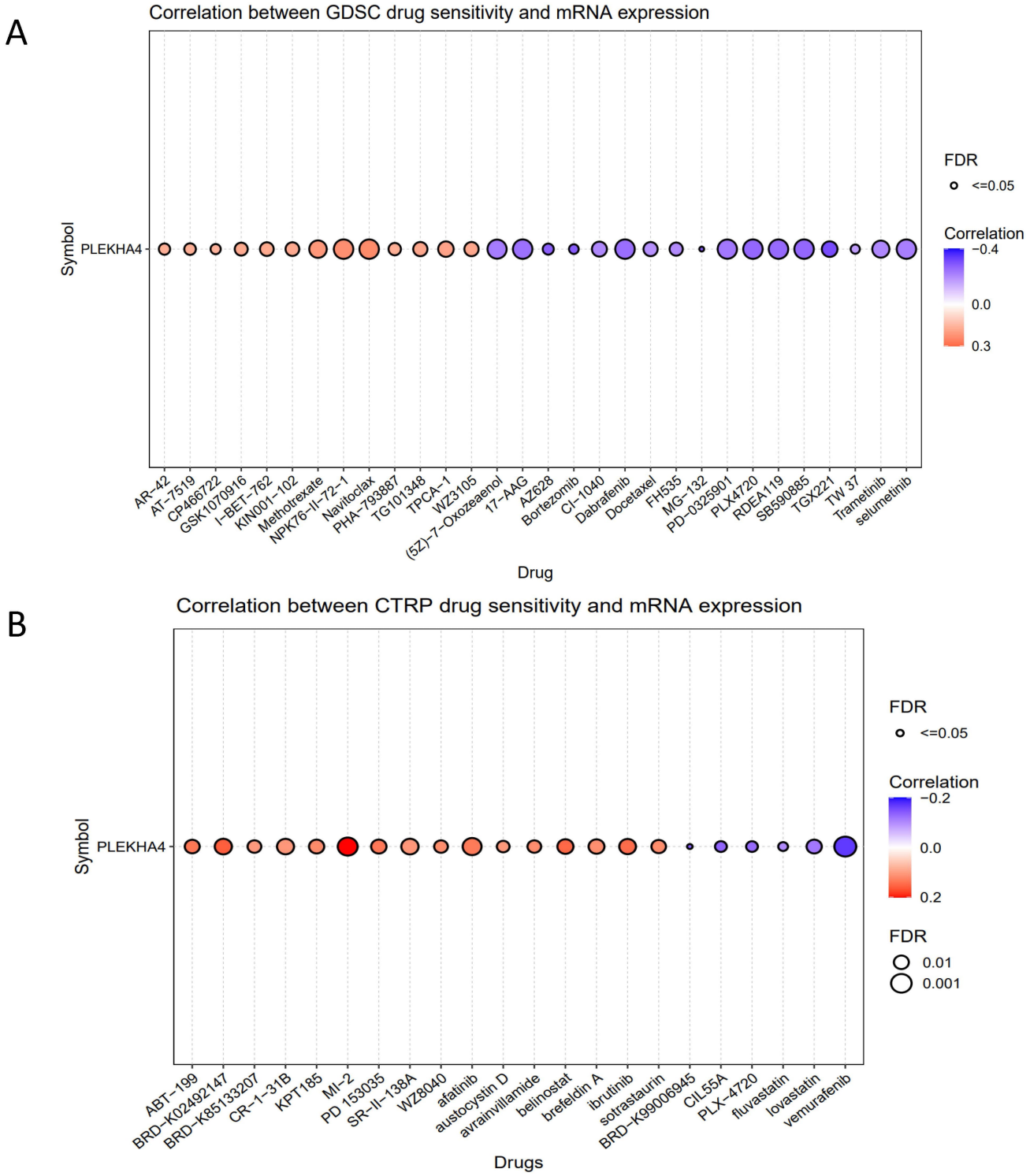

Fig. 9. Drug sensitivity analysis. Correlation between pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression and (A) Genomics of Drug Sensitivity in Cancer (GDSC) and (B) Cancer Treatment Response Portal (GTRP) drug sensitivity in pan-cancer. FDR, false discovery rate.

PLEKHA4 knockdown exhibited additive effects with the BRAF inhibitor, encofenib, which attenuates the growth of melanoma xenografts *in vivo* [17]. Our current results may thus contribute to the providision of new concepts that may help enhance drug treatment aimed at gliomas.

This study was affected by several limitations. First, the sample size was restricted, and thus further validation of these findings using a wider range of patients may be warranted. Moreover, *in vitro* experiments targeting drug sensitivity were not performed, and animal experiments were

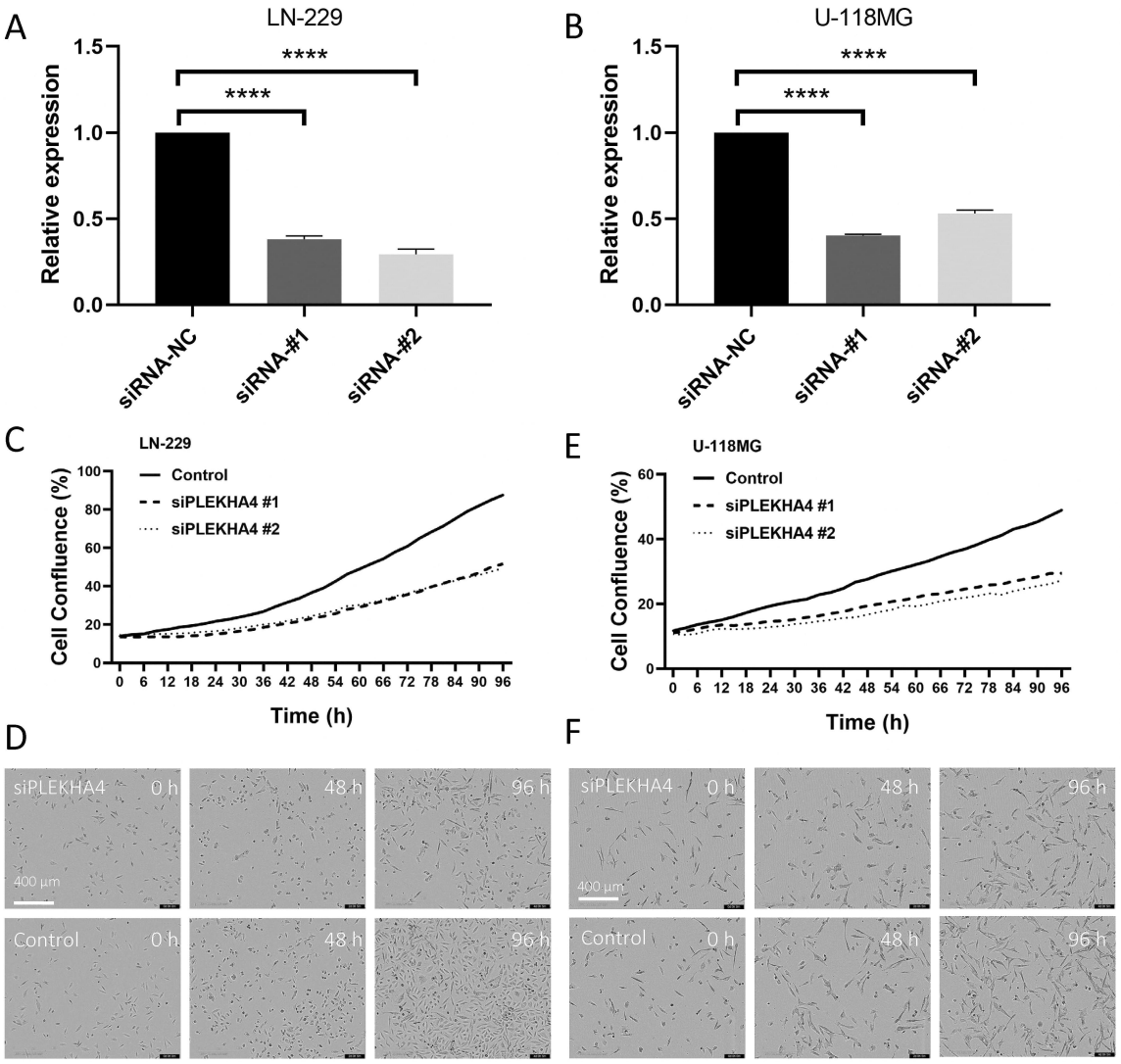

Fig. 10. The effect of pleckstrin homology containing family A, number 4 (*PLEKHA4*) expression on glioma cell proliferation. (A,B) Comparison of the efficiency of *PLEKHA4* knockdown using small interfering RNA (siRNA) sequences in LN-229 and U-118MG cell lines. IncuCyte automated bright-field imaging of cell proliferation in (C,D) LN-299 and (E,F) U-118MG glioma cells treated with siRNAs targeting different regions of *PLEKHA4* (si*PLEKHA4* #1, #2) or negative control siRNAs (****$p < 0.0001$).

not conducted. Furthermore, the study did not investigate the specific mechanisms linking *PLEKHA4* with immune infiltration, which may require further exploration.

5. Conclusions

The findings of this study strongly suggests that the DNA hypomethylation of *PLEKHA4* significantly increases its expression in gliomas. Therefore, *PLEKHA4* shows potential as a novel prognostic biomarker and a treatment target for patients with glioma. Our multidimensional analysis suggested that the biofunctions of *PLEKHA4* may be involved in regulating Wnt signalling, apoptosis, immune cell regulation, cell adhesion, and stemness maintenance in glioma. Moreover, the immune microenvironment analysis indicated that *PLEKHA4* expression may be associated with higher ESTIMATE scores and immune cell infiltration in gliomas. Furthermore, these findings suggested that *PLEKHA4* expression may be involved in the functional regulation of $CD4^+$ T immune cells, suggesting that it may contribute to the altered immune status. Drug sensitivity analysis also revealed a negative correlation between *PLEKHA4* expression and several small molecule kinase inhibitors. Finally, *in vitro* experiments confirmed that knockdown of *PLEKHA4* expression inhibited the proliferation of glioma cells. Therefore, our findings strongly suggest that *PLEKHA4* may play a key role in the progression of gliomas and thus provide a new target in the search for effective treatments for glioma.

Availability of Data and Materials

The original datasets presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author Contributions

XG, YL, BG and XM conceived and designed the study; XG and SH performed the experiments; HY and SH participated in data analysis and model construction; XG and YL wrote this paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable. Molecular and clinical data used in the study was collected from public depository and ready for academic research use.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2205130.

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