HHLA2 is a novel prognostic predictor and potential therapeutic target in malignant glioma

YANGZHI QI*, GANG DENG*, PENGFEI XU, HUIKAI ZHANG, FANEN YUAN, RONGXIN GENG, HONGXIANG JIANG, BAOHUI LIU and QIANXUE CHEN

Department of Neurosurgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. Glioma is the most common and aggressive tumor type of the central nervous system and is associated with poor prognosis. To date, novel emerging immunotherapies have significantly improved outcomes for patients with various cancer types. Human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2), a newly discovered immune checkpoint molecule, has demonstrated its potential as a novel therapeutic target. Therefore, the present study aimed to investigate the clinical prognostic value of HHLA2 in gliomas and its mechanistic role. A systematic review of datasets from The Cancer Genome Atlas was performed. The RNA-seq data of a total of 669 cases were analyzed and the biological function of HHLA2 was predicted by Gene Ontology (GO) and pathway enrichment analysis. Immunohistochemistry labelling images for HHLA2 was obtained from the Human Protein Atlas. xCell was used to comprehensively analyze the model of tumor-infiltrating immune cell in glioma. The Cox proportional hazards regression model was used to predict outcomes for glioma patients. The results revealed that the expression levels of HHLA2 were significantly lower in high-grade glioma, as well as glioma with wild-type isocitrate dehydrogenase, no deletion of 1p/19q and telomerase reverse transcriptase promoter mutation. Receiver operating characteristic analysis revealed that HHLA2 was a predictor of the neural subtype. The tumor-infiltrating immune cell model indicated that HHLA2 was negatively associated with tumor-associated macrophages. GO analysis and pathway enrichment analysis revealed that HHLA2-associated genes were functionally involved in inhibition of neoplasia-associated processes. HHLA2 was significantly negatively correlated with certain genes, including interleukin-10, transforming growth factor-β, vascular endothelial growth factor and δ-like canonical Notch ligand 4, and other immune checkpoint molecules, including programmed cell death 1, lymphocyte activating 3 and CD276. Survival analysis indicated that high expression of HHLA2 predicted a favorable prognosis. In conclusion, the present study revealed that upregulation of HHLA2 is significantly associated with a favorable outcome for patients with glioma. Targeting HHLA2 as an immune stimulator may become a valuable approach for the treatment of glioma in clinical practice.

Introduction

Glioma is the most common malignant tumor type of the central nervous system and the 5-year overall survival (OS) is <10%. According to the biological behavior and malignancy of the tumor, glioma may be divided into four grades from World Health Organization (WHO) grade I to IV. Low-grade glioma (LGG) includes WHO grade-I and -II tumors, while the other two grades III and IV are classified as high-grade glioma (HGG). Of note, glioblastoma multiforme (GBM), the most malignant glioma type with WHO grade IV, accounts for ~50% of glioma cases, and has a median survival time of 14.2 months and a 5-year survival rate of <5% (1). In the past decades, despite improvements in surgical, radio- and chemotherapies, the treatment of glioma has remained a tremendous challenge (2,3). However, with the development of novel emerging immunotherapies, which aim to reinvigorate antitumor immune responses, outcomes have been significantly improved in a variety of advanced hematologic and solid malignancies (4-8). This points out a new direction in terms of treatment strategies for glioma. Thus, novel therapeutic approaches targeting the interaction between the tumor microenvironment and immune response are urgently required in this field.

Various preclinical studies have demonstrated the success of immunotherapy-based approaches in animal models and numerous phase I and II clinical trials suggested immunotherapy to be safe and, in certain cases, improve progression-free survival (PFS) and OS (9-13). Preclinical studies using murine models with orthotopic-transplanted gliomas have provided a

Correspondence to: Professor Baohui Liu or Professor Qianxue Chen, Department of Neurosurgery, Renmin Hospital of Wuhan University, 9 Zhangzhidong Road and 238 Jiefang Road, Wuchang, Wuhan, Hubei 430060, P.R. China
E-mail: bliu666@whu.edu.cn
E-mail: chenqx666@whu.edu.cn

*Contributed equally

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marked benefit of checkpoint inhibitors used individually or in combination with other immunotherapeutic strategies (42). Numerous glioma-associated antigens, including interleukin (IL)-13 receptor subunit α2, human epidermal growth factor receptor 2, EPH receptor A2, gp100 and AIM-2 are being targeted in glioma (14-16). In addition, tumor-specific neoantigens, including epidermal growth factor receptor variant III, are being used to target tumor cells (16,17). The successful preclinical studies have prompted a number of clinical studies using dendritic cell vaccines (11). Furthermore, considerable progress has been achieved in immunotherapy with antibodies, adoptive T-cell transfer and chimeric antigen receptor T cells in their respective fields (18-20). Among the aforementioned therapeutic strategies, immune checkpoint blockade appears to be an exciting avenue that warrants further development based on the preclinical studies.

Immune checkpoint proteins are surface molecules on certain immune cell populations that activate or inhibit immune function when engaged to their ligands. Numerous studies have indicated an interaction between the expression of the co-inhibitory protein and tumor immune escape (21). Therefore, immunotherapy based on blocking the interaction between an immune checkpoint protein and its ligands has the potential to restore functional immune cells and inhibit tumor progression.

The B7 family, an important class of the immune checkpoint superfamily, has exhibited great potential for regulating T-cell function and participating in the immune response. The growing B7 family is now comprised of 10 members, including CD80 (B7-1), CD86 (B7-2), programmed cell death 1 ligand 1 (PD-L1 or B7-H1), PD-L2 (B7-DC), inducible T cell co-stimulator ligand (B7-H2), CD276 (B7-H3), B7-H4, V-set immunoregulatory receptor (B7-H5), B7-H6 and human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2 or B7-H7) (22). Among these ligands, PD-L1 and PD-L2 represent two ligands for the PD-1 receptor. Recent studies have indicated that upregulation of PD-1 and PD-L1 in tumor tissue was associated with poor prognosis in certain cancer types, demonstrating that PD-1 and PD-L1 may inhibit the function of T-cells and promote the immune escape of tumor cells (23-25). The clinical application of a specific antibody which inhibits the PD-1/PD-L1 pathway has achieved satisfactory curative effects (26,27). A recent study reported that upregulation of PD-1 in glioma predicted a poor prognosis (28), indicating the potential value of this immune checkpoint protein as a therapeutic target in glioma.

HHLA2 is the most recently discovered member of the B7 family. Transmembrane and immunoglobulin domain-containing 2 (TMIGD2, also known as IGPR-1 or CD28H) is the only known receptor identified for HHLA2 (29). While its exact function remains elusive, it has been reported to have co-stimulatory as well as co-inhibitory properties (30,31). Zhu *et al* (31) indicated that the interaction between CD28H and B7-H7 on antigen-presenting cells (APCs) co-stimulated human T-cell proliferation and cytokine production via a pathway involving AKT phosphorylation. By contrast, Zhao *et al* (30) proposed the opposite function for B7H7: In the presence of the T-cell antigen receptor (TCR) signaling pathway, B7-H7 inhibits the proliferation of $CD4^+$ and $CD8^+$ T cells. In addition, B7-H7 significantly reduces cytokine production by T cells, including interferon-γ, tumor necrosis factor-α, IL-5, IL-10, IL-13, IL-17α and IL-22. Thus, the ligation of B7-H7 to T cells suppresses T-cell responses. As with B7-H3, a T-cell co-inhibitory role and a co-stimulatory role have been reported for this ligand (22). One explanation is that HHLA2 has two ligands with opposite functions-TMIGD has a co-stimulatory role, while the other remains elusive. HHLA2 on APCs or tumor cells may interact with unknown ligands and exert a co-inhibitory function in the microenvironment of certain cancers. Furthermore, it may promote angiogenesis within the tumor microenvironment via its interaction with TMIGD2 expressed in the endothelium.

The expression of HHLA2 has been reported in a large proportion of tumor specimens, including breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney and esophageal, but not in endometrial, gallbladder, laryngeal, stomach and uterine cancer or in lymphoma (29). To date, no systematic study on the expression status and biological function of HHLA2 in patients with glioma has been performed, to the best of our knowledge. The present study aimed to examine the expression of HHLA2 in normal brain specimens and tumor specimens obtained from patients with glioma. Furthermore, the potential mechanistic role of HHLA2 in glioma and the association between HHLA2 expression and tumor behavior were investigated, and its clinical utility as a prognostic predictor was assessed.

Table I. Information of patients with glioma.

TCGA database variable	No. of cases (N=669)
Information of TCGA patients	
Age (years)	
<48	312
≥47	297
Missing data	60
Sex	
Male	355
Female	254
Missing data	60
IDH	
Mutant	429
Wild-type	232
Missing data	8
OS (months)	
<26	442
≥26	225
Missing data	2
Status	
Survival	428
Dead	239
Missing data	2

TCGA, The Cancer Genome Atlas; OS, overall survival.

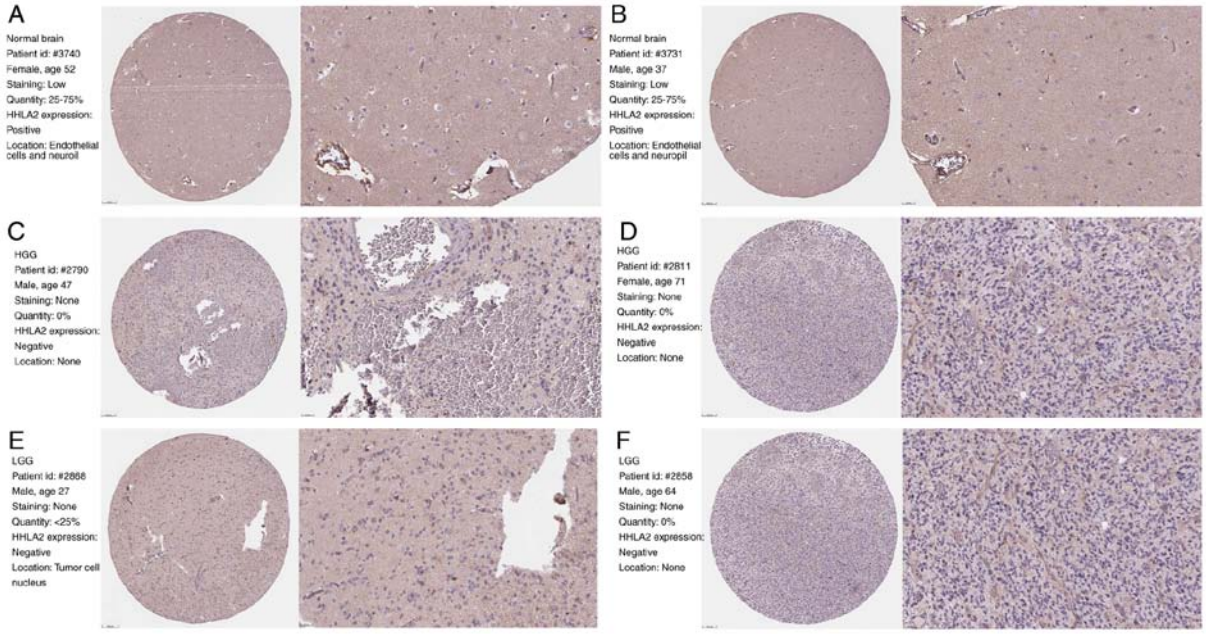

Figure 1. Representative specimens exhibiting HHLA2 IHC labeling pattern in normal brain, low-grade glioma, and high-grade glioma. HHLA2 IHC are presented. (A and B) Normal brain. (C and D) Low-grade glioma. (E and F) High-grade glioma. Scale bar, 100 and 25 μm, respectively.

Materials and methods

Sample and data collection. RNA sequencing data from human glioma samples were obtained from The Cancer Genome Atlas (TCGA) database (http://www.tcga.org/) and downloaded from the GlioVis database (http://gliovis.bioinfo.cnio.es/). The dataset contained 515 LGG samples, 152 GBM samples and 2 undefined samples (Table I). The characteristics of the patients are listed in Table I. Furthermore, data regarding IDH mutation, 1p/19q co-deletion and telomerase reverse transcriptase (TERT) mutation for the TCGA cohort were obtained by whole-exon sequencing or pyrosequencing.

Immunohistochemistry (IHC). The IHC labelling images of normal brain tissue and tumor tissue were obtained from The Human Protein Atlas (http://www.proteinatlas.org/). The Human Protein Atlas used anti-HHLA2 (cat. no. HPA055478; Sigma-Aldrich; Merck KGaA) as a primary antibody and the tumor tissues were obtained from TCGA database.

Model of tumor-infiltrating immune cells (TIICs). xCell (http://xcell.ucsf.edu/) was used to obtain the expression level of 64 types of immune cells in the tumor microenvironment of patients with glioma. Then 20 types of immune cells with significant difference in infiltration ratio were screened out using the R package 'limma' (http://bioconductor.riken.jp/packages/3.0/bioc/html/limma.html).

Functional enrichment analysis. Gene ontology (GO) and pathway enrichment analysis [Kyoto Encyclopedia of Genes and Genomes (KEGG)] were performed to analyze the genes associated with HHLA2 by using Metascape (http://metascape.org). Enriched ontological terms and pathways with P<0.05 were selected and presented in a heatmap using the R package 'ComplexHeatmap' (http://www.bioconductor.org/packages/stats/bioc/ComplexHeatmap/).

Cox proportional hazards regression model. The prognostic value of each factor was first assessed by univariate Cox proportional hazards regression. Subsequently, statistically significant genes were used to construct the multivariate Cox regression model. Glioma samples were divided into high-expression and low-expression groups based on the median level of HHLA2 expression. Kaplan-Meier survival curves were generated to assess the prognostic value of the model using the R package 'survival' (https://CRAN.R-project.org/package=survival). A receiver operating characteristic (ROC) curve was generated to assess the accuracy of the model with the R package 'survivalROC' (https://CRAN.R-project.org/package=survivalROC).

Statistical analysis. Statistical analysis was mainly performed with R (https://www.r-project.org/) with several publicly available packages. P<0.05 was considered to indicate statistical significance. (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, respectively, as indicated in the figures and legends).

Results

HHLA2 expression is absent in GBM. To evaluate the expression level of HHLA2 in glioma, the IHC staining data of glioma and normal brain tissue were obtained from the Human Protein Atlas dataset and analyzed individually. Overall, positive staining for HHLA2 was observed in endothelial cells and neuropils of normal brain tissue (Fig. 1A and B), while staining was negative in glial cells and neurons. Furthermore, no tumoral HHLA2 expression was detected in HGG (Fig. 1C and D). Of note, only a small percentage of LGG samples were positive for HHLA2 and IHC labeling was

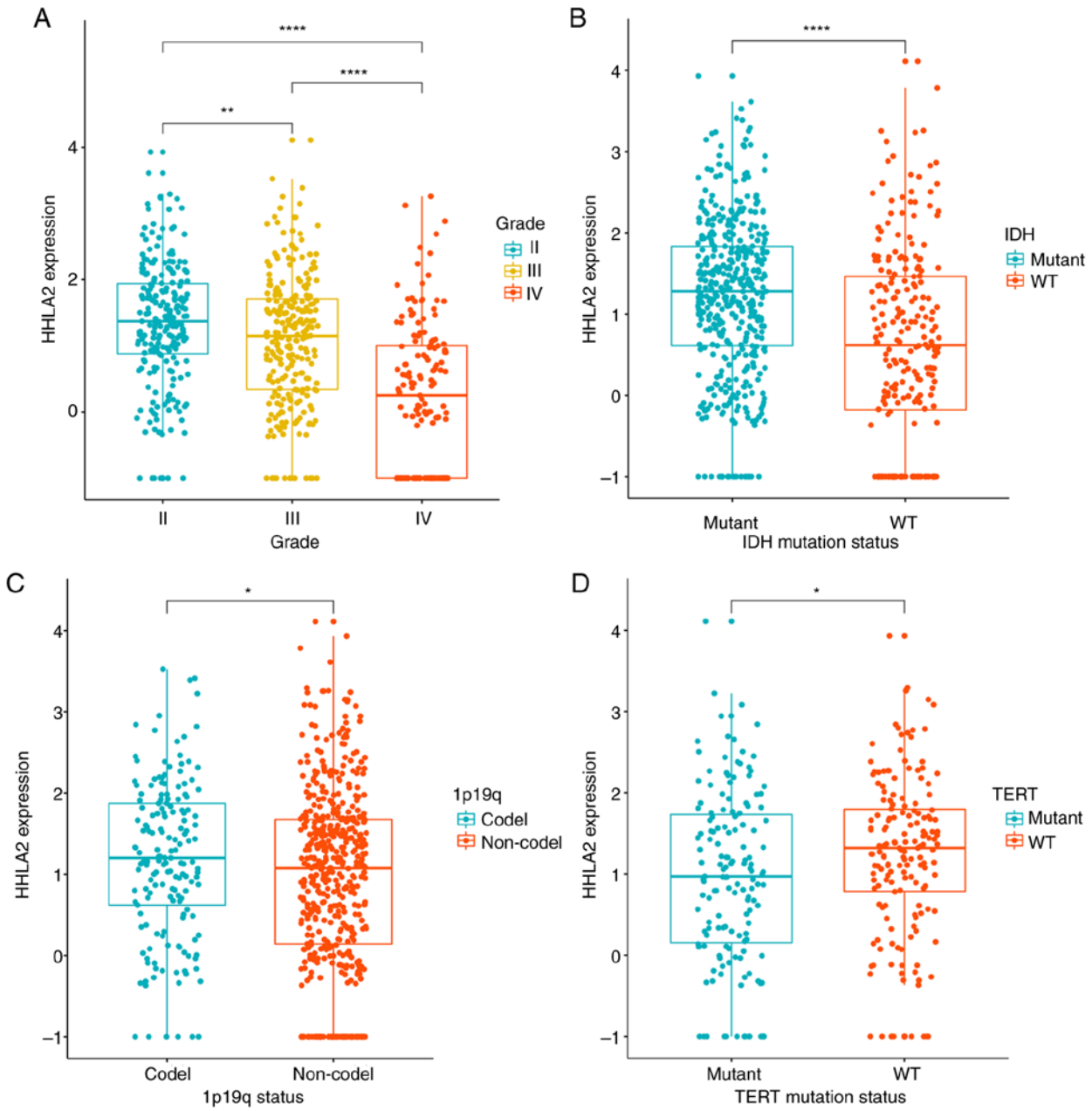

Figure 2. HHLA2 expression status in malignant glioma. (A) HHLA2 expression was significantly decreased in WHO III grade glioma than WHO II grade glioma (WHO III 224 vs. WHO II 226, **P<0.01), and was also significantly decreased in WHO IV grade glioma than WHO II grade glioma (WHO IV 150 vs. WHO II 226, ****P<0.0001) and WHO III grade glioma (WHO IV 150 vs. WHO III 224, ****P<0.0001). (B) HHLA2 is expressed at a higher level in IDH-mutant glioma (mutant 429 vs. wild-type 233, ****P<0.0001). (C) The 1p/19q co-deletion group had a higher expression of HHLA2 (co-deletion 169 vs. no co-deletion 494, *P<0.05). (D) The TERT wild-type group had a higher expression of HHLA2 (mutant 155 vs. wild-type 166, *P<0.05). HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2; WT, wild-type.

observed in tumor cell nuclei rather than endothelial cells, while other samples with LGG were negative for HHLA2 (Fig. 1E and F). This result indicated that with the increasing degree of tumor malignancy, HHLA2 expression in glioma was gradually reduced until it was absent.

Downregulated HHLA2 predicts poor prognosis in glioma. To explore the prognostic role of HHLA2 in glioma, the association between HHLA2 and several prognostic factors was analyzed. The results indicated that the mRNA expression levels of HHLA2 were significantly decreased with the increase in the grade of glioma and that the expression level was lowest in GBM (P<0.0001; Fig. 2A), indicating a strong correlation between HHLA2 expression and malignancy of glioma. IDH-mutant and 1p/19q co-deletion types were associated with a better outcome in glioma. When taking into account the IDH mutation status, it was indicated that HHLA2 expression was significantly higher in the IDH mutant group than in the IDH wild-type group (P<0.0001; Fig. 2B). Furthermore, compared with the 1p/19q no-deletion group, the 1p/19q co-deletion group had a higher expression of HHLA2 (P<0.05; Fig. 2C). TERT promoter mutations are usually considered to be associated with poor outcome (32), and the present results revealed that in the TERT wild-type group, the

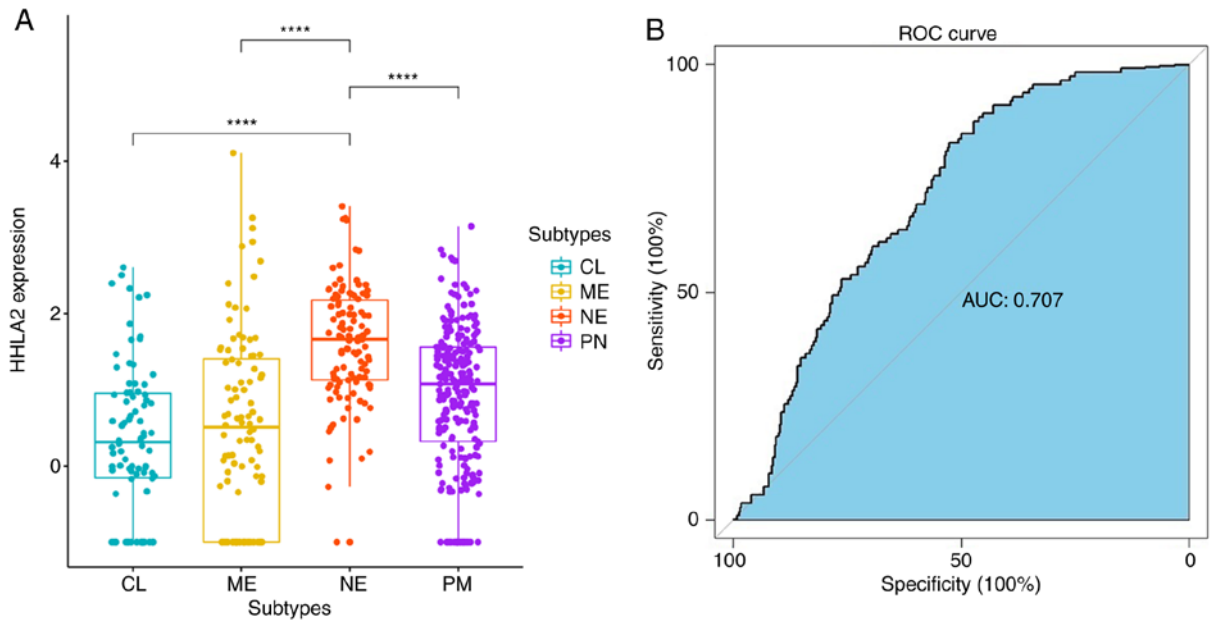

Figure 3. High expression of HHLA2 is a good predictor for the NE subtype. (A) HHLA2 was significantly increased in the NE subtype (n=110) than other subtypes (n=418, ****P<0.0001). (B) ROC curve analysis revealed that HHLA2 had a high sensitivity and specificity to predict the NE subtype. The AUC was 0.707. HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2; NE, neural; ROC, receiver operating characteristic; AUC, area under curve; CL, classical; ME, mesenchymal; PN, proneural.

expression of HHLA2 was significantly increased (P<0.05; Fig. 2D). These results indicated HHLA2 expression was more prevalent in glioma with lower malignancy.

To further elucidate the association between HHLA2 expression and molecular subtypes, patients were divided into four groups according to subtypes defined by TCGA. Upregulated HHLA2 expression was observed in the neural (NE) subtype rather than in proneural (PN), classical (CL), and mesenchymal (ME) subtypes (P<0.0001; Fig. 3A). In addition, ROC curves were used to evaluate the specificity and sensitivity of our previous findings, indicating that the expression status of HHLA2 may serve as a good predictor for the neural subtype of gliomas [area under curve (AUC)=0.707; Fig. 3B].

Model of tumor-infiltrating immune cells (TIICs) and tumor-associated macrophages (TAMs) in glioma. To date, tumor immunotherapy has yielded significantly improved outcomes in a variety of advanced hematologic and solid malignancies, including glioma. Hence, to further understand the function of immune cells in the tumor microenvironment, the expression model of TIICs in patients with glioma was explored using xCell (http://xcell.ucsf.edu/) and several immune cells with significant difference in infiltration ratio were screened out (Table II). It was revealed that macrophages were markedly increased in GBM (Fig. 4A). TAMs, developed from monocytes, have been confirmed to be the most important type of immune cell in the stroma of tumors, accounting for 50% of the total number of immune cells, and to have an important role in neoplasia, metastasis, immune escape and tumor angiogenesis (33,34). Previous studies have also indicated that HHLA2 is constitutively expressed on human monocytes and takes part in angiogenesis (21). In the present study, it was observed that TAMs were significantly higher in the HHLA2 low-expression group (Fig. 4B) and predicted a worse prognosis (Fig. 4C). The aforementioned results indicated that HHLA2 may have an important role in the tumor immune microenvironment, tumor angiogenesis and the process of monocytes developing into TAMs. Thus, the association between HHLA2 and TAMs may provide a novel therapeutic method.

Correlation of HHLA2 and associated immune molecules. To further explore the function of HHLA2 in the immune microenvironment, the correlation between HHLA2 and several immune-associated molecules was analyzed at the mRNA level. Pearson correlation analysis indicated that HHLA2 was revealed to be negatively correlated with co-inhibitory immune checkpoint molecules, including programmed cell death 1 (PD-1; r=-0.172), lymphocyte activating 3 (LAG3; r=-0.171), cytotoxic T-lymphocyte associated protein 4 (CTLA4; r=-0.045) and CD276 (r=-0.434; Fig. 5A). However, HHLA2 was positively correlated with CD160 (r=0.261), which is commonly known as a stimulatory molecule (21,29,30). In addition, common immune inhibitors, including IL-10 and transforming growth factor (TGF)-β, were significantly higher in the HHLA2 low-expression group (Fig. 5C and D). These results indicated that HHLA2 may co-stimulate the immune response and have a positive role in tumor immune microenvironment.

Mechanism of HHLA2 acting on TAMs. It has been reported that cytokines, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), have important roles in the formation of TAMs and tumor angiogenesis (33,34). To further assess the mechanisms by which HHLA2 acts on TAMs in malignant glioma, five common molecules associated with angiogenesis were selected and analyzed individually (35,36). In the TCGA dataset, it was observed that HHLA2 was significantly negatively correlated with molecules including

Table II. Differentially expressed immune cells.

Immune cells	Log FD	Adjusted P-value
Upregulated		
Smooth muscle	-0.162091191	3.79E-46
Macrophages M1	-0.035218908	3.26E-26
DC	-0.047622184	4.18E-26
CLP	-0.064684851	4.44E-26
Th2 cells	-0.056711574	8.06E-25
Macrophages	-0.05311427	1.73E-22
Macrophages M2	-0.022494652	2.36E-19
Mesangial cells	-0.013731035	4.96E-19
Astrocytes	-0.034756525	7.22E-17
Epithelial cells	-0.008775589	1.14E-16
Downregulated		
$CD4^+$ Tcm	0.041603702	1.88E-76
Eosinophils	0.012507035	6.73E-69
Tregs	0.018249872	9.22E-44
Neurons	0.085024979	1.87E-40
Platelets	0.007315915	2.78E-38
Hepatocytes	0.001416468	1.99E-37
Basophils	0.025025816	5.25E-29
Pericytes	0.040365872	8.10E-23
Class-switched memory B-cells	0.010062028	2.55E-16
Myocytes	0.00361773	5.36E-12

FD, fold-change; DC, dendritic cell; CLP, common lymphoid progenitor; Th2 cells, helper T cells 2; Tcm, central memory T cells; Treg, regulatory T cells.

VEGF (r=-0.400), δ-like canonical Notch ligand 4 (DDL4; r=-0.358), PDGFA (r=-0.228), fibroblast growth factor receptor 1 (FGFR1; r=-0.164) and hepatocyte growth factor (HGF; r=-0.137; Fig. 5B). These results demonstrated that overexpression of HHLA2 may have a potential application in anti-tumor angiogenesis treatment and inhibiting the formation of TAMs by decreasing VEGF and PDGF.

Enrichment analysis of HHLA2-associated genes. To further explore the biological function of HHLA2 in glioma, an enrichment analysis with Metascape (http://metascape.org) was also performed. Genes significantly associated with HHLA2 expression were screened out by Pearson correlation analysis (Pearson |R|>0.4, P<0.05). Sequentially, 234 positively correlated genes and 211 negatively correlated genes were analyzed individually. It was revealed that positively correlated genes were involved in membrane trafficking, the glutamate receptor signaling pathway, regulation of neuronal death, response to toxic substances, regulation of protein ubiquitination and negative regulation of protein modification process (Fig. 6A), while negatively correlated genes were involved in processes that promote abnormal proliferation, including cell division, DNA replication, DNA repair, activation of E2F transcription factor 1 (E2F1) target genes at the G1/S checkpoint, the FOXM1 pathway and the ATR pathway (Fig. 6B). These results indicated that HHLA2 may have an important role in preventing normal neurons from damage, promoting the immune response and inhibiting neoplastic cell proliferation.

Patients with increased HHLA2 have a favorable survival prognosis. As HHLA2 expression was correlated with favorable prognostic factors, the prognostic value of HHLA2 expression in glioma as well as in GBM was then explored. Patients were divided into a high-expression group and a low-expression group based on the median HHLA2 level. Kaplan-Meier analysis demonstrated that higher HHLA2 expression was associated with a better outcome in patients with glioma of all grades (P<0.0001) as well as in GBM patients (P=0.021; Fig. 7A and B).

To further comprehend this model, a survival analysis was performed in four different subtypes of glioma defined by TCGA (Fig. 8A-D). The results demonstrated that no statistical significance was detected in the CL, NE and PN subtypes (Fig. 8A, C and D). However, higher HHLA2 expression was significantly associated with a better prognosis in the ME subtype (P=0.013; Fig. 8B). In addition, compared with the CL and ME subtypes, patients with the NE and PN subtypes had a significantly better outcome (P<0.0001; Fig. 8E).

In order to take into account key clinical and molecular factors, the Cox proportional hazards model was further applied. Univariate analysis indicated that age, IDH status, grade and HHLA2 expression were significantly associated with OS (P<0.0001; Table III). Furthermore, multivariate analysis indicated that age, IDH status and grade were independent prognostic factors (P<0.0001; Table III). However, HHLA2 expression was not an independent prognostic factor according to the multivariate analysis (P=0.257).

Discussion

The present study first focused on detecting the expression level of HHLA2 in normal brain tissue and tumor tissue obtained from patients with glioma by IHC labeling in the Human Protein Atlas dataset. By individually analyzing the normal brain tissue and tumor tissue, it was revealed that HHLA2 was absent in normal brain cells, including glial cells and neurons, but abundant in endothelial cells. Furthermore, it was observed that tumoral HHLA2 expression was absent in HGG, particularly in GBM. Of note, in contrast to the aforementioned, weak expression of HHLA2 in the nuclei of tumor cells was observed in part of the patients with LGG. This result suggested a downward trend in HHLA2 expression with the increase in the degree of malignancy of the tumor, which is opposite to previous results according to which HHLA2 expression was not detected in most organs, but was widely expressed in human cancers of the breast, lung, thyroid, skin, pancreas, ovary, liver, bladder, colon, prostate, kidney and esophagus (29). However, Yan *et al* (37) also reported that HHLA2 was widely overexpressed in early pancreatic precancerous lesions compared with pancreatic cancer, although it was not expressed in normal acinar, islet and ductal cells. Furthermore, overexpression of HHLA2 was indicated to be significantly associated with a better outcome. In addition, Zhu *et al* (31) indicated that HHLA2

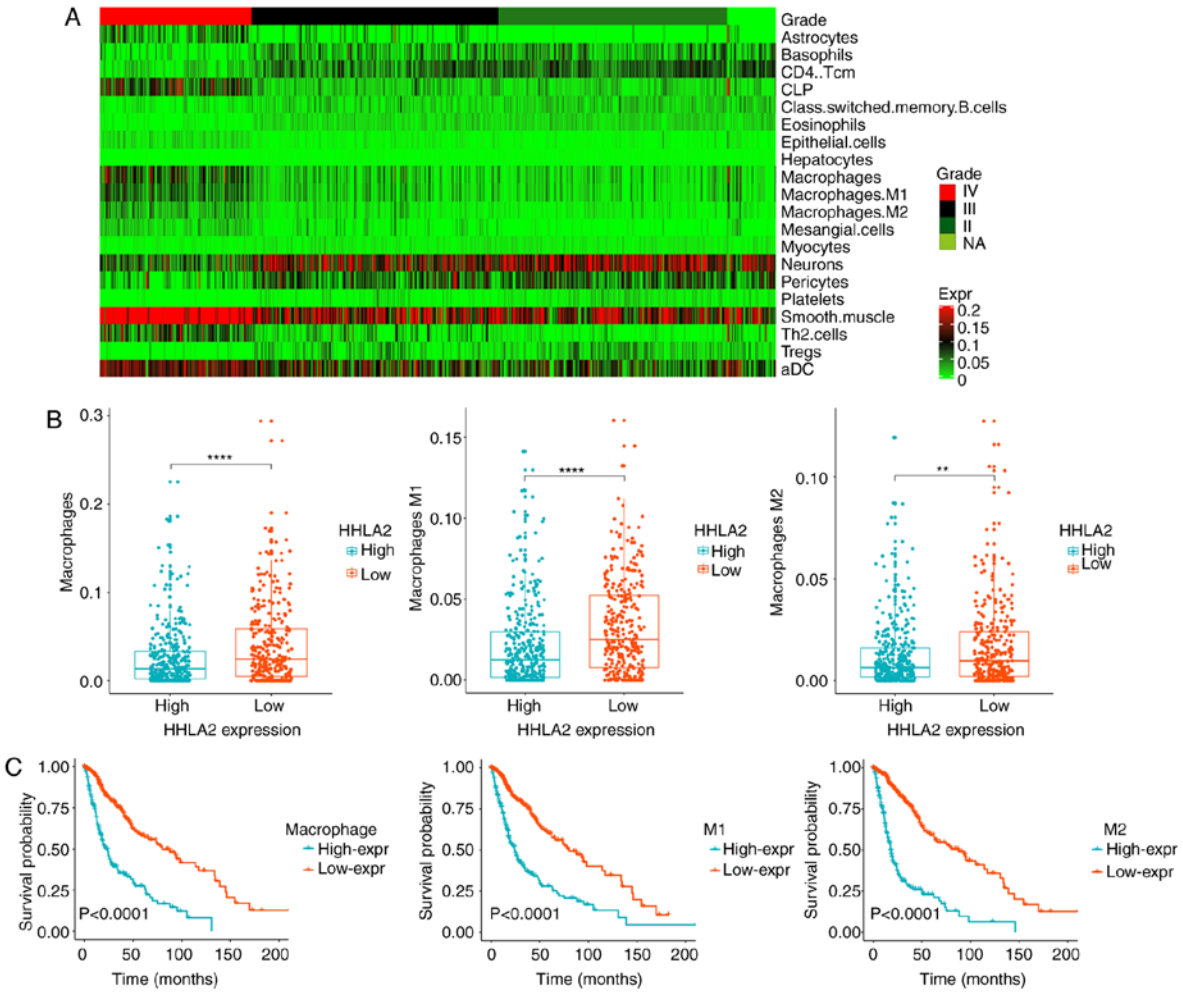

Figure 4. TIIC model and TAM expression in glioma. (A) Immune cell expression heatmap in glioma (top 20 increased and decreased cells). (B) TAMs were significantly higher in the HHLA2 low-expression group (****P<0.0001, ****P<0.0001, and **P<0.01, respectively). (C) Low expression of TAMs predicted better outcome (P<0.0001). TIIC, tumor-infiltrating immune cell; TAM, tumor-associated macrophage; HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2.

engaged with CD28H to co-stimulate human T-cell proliferation and cytokine production via a pathway involving AKT phosphorylation. Based on the aforementioned results, it is reasonable to assume that higher HHLA2 expression in the early stage of glioma co-stimulated the immune response, while the expression decreased with the increasing malignancy of the tumor. It may be speculated that the expression model of HHLA2 in glioma is similar to that in the pancreas. However, most patients with glioma only present at the hospital after evident clinical symptoms have occurred. On this account, it is difficult to obtain tumor tissue in the early stage of glioma for IHC labeling and detection of HHLA2 expression. Furthermore, the present cancer model was validated in the TCGA dataset at the mRNA level at the same time.

Through analysis of TCGA, the largest cancer dataset, HHLA2 expression in malignant glioma was assessed at the transcriptional level. It was revealed that HHLA2 expression in GBM was significantly lower than that in glioma of other grades. According to a study by Eckel-Passow *et al* (32), a single TERT mutation predicted poor prognosis in glioma, while IDH mutation and 1p/19q co-deletion predicted a favorable outcome. In the present study, tumors with IDH mutation, 1p/19q co-deletion and wild-type TERT expressed higher levels of HHLA2, which was in line with our previous assumption. Furthermore, the expression levels of HHLA2 were detected in four different molecular subtypes of glioma defined by TCGA: PN, NE, CL and ME (38,39). Studies have indicated that PN and NE subtypes mostly occurred among LGG and were associated with a favorable prognosis, while the CL and ME subtypes were associated with a worse outcome (38,40). Of note, in the NE subtype, a significantly higher expression of HHLA2 compared with that in the other subtypes was observed, and the HHLA2 expression status was a good predictor for NE-subtype glioma. These results were in line with a previous study and indicated that higher expression of HHLA2 may predict a favorable outcome (37).

The present study further explored the expression model of TIICs in glioma, and it was indicated that macrophages were markedly increased in GBM vs. LGG. TAMs, developed from monocytes, have been confirmed to be the most important type of immune cell in the stroma of tumors, accounting for 50% of all immune cells, and to have an important role in neoplasia, metastasis, immune escape and tumoral angiogenesis (33,34). Immature monocytes migrate to tumor tissues and develop

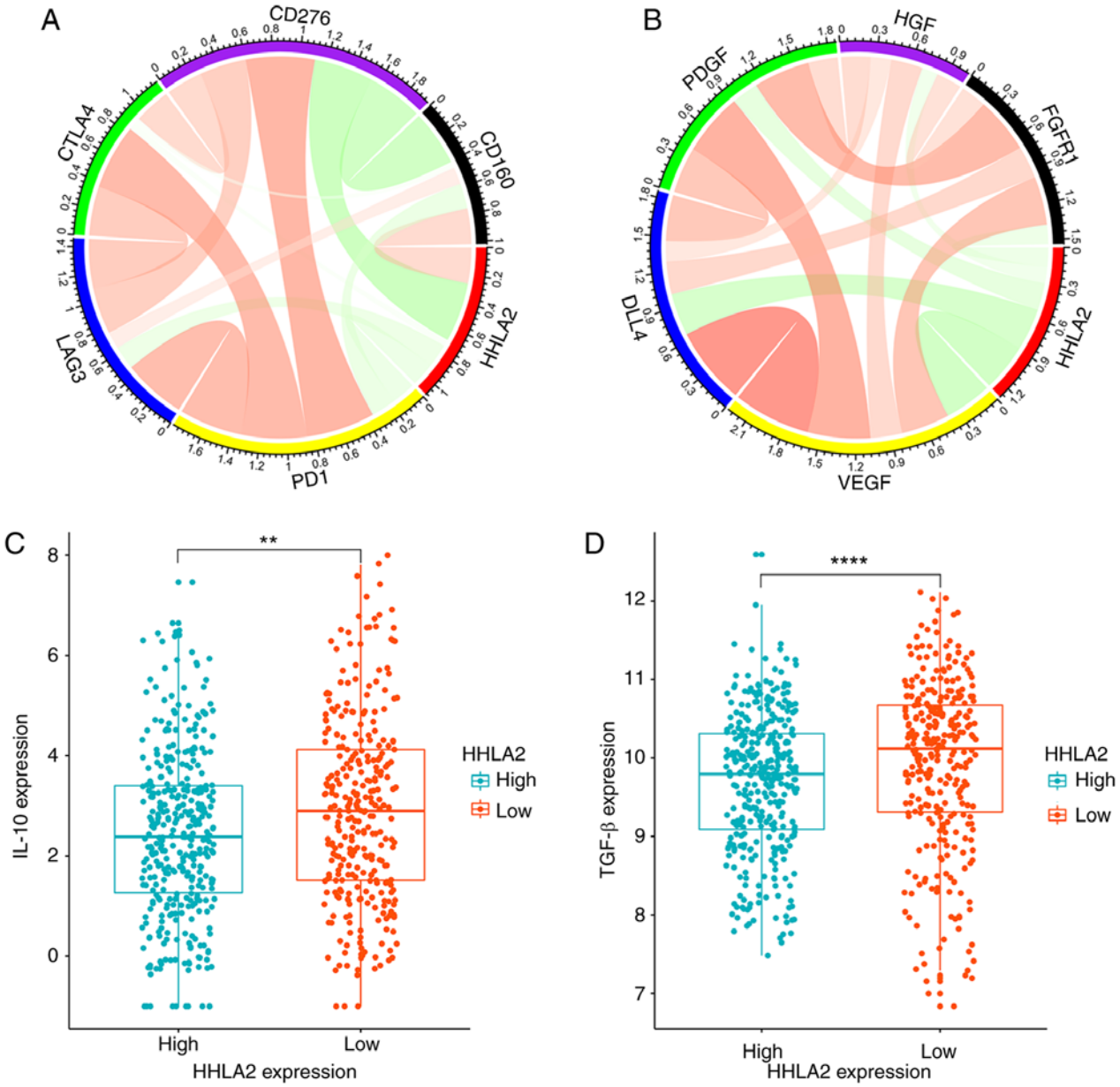

Figure 5. Correlation of HHLA2 and related molecules. (A) HHLA2 was positively correlated with CD160 and negatively correlated with PD-L1, LAG3, CTLA4, and CD276. (B) HHLA2 was negatively correlated with angiogenesis molecules, including VEGF, DLL4, PDGF, FGFR1 and HGF. Color intensity and the size of the circle are proportional to the correlation coefficients. (C and D) IL-10 and TGF-β, representative immune inhibitors, were significantly higher in the low-HHLA2 expression group (**P<0.01 and ****P<0.0001, respectively). HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2; PD-L1, programmed cell death 1 ligand 1; LAG3, lymphocyte activating 3; CTLA4, cytotoxic T-lymphocyte associated protein 4; DLL4, δ-like canonical Notch ligand 4; FGFR1, fibroblast growth factor receptor 1; HGF, hepatocyte growth factor.

into TAMs through several cytokines, including VEGF, PDGF, colony-stimulating factor-1 (CSF-1) and C-C motif chemokine ligand 2. Notably, a previous study confirmed that HHLA2 was constitutively expressed on human monocytes (22). Thus, the association between TAMs and the expression levels of HHLA2 was explored in the present study. Lower TAMs were observed in the HHLA2 high-expression group and predicted a better outcome. To further explore the mechanism of HHLA2 acting on TAMs, several cytokines linked to angiogenesis and processes that develop monocytes into TAMs were selected for assessment, revealing that HHLA2 was negatively correlated with VEGF and PDGF. These results demonstrated that HHLA2 may inhibit TAM development and tumor angiogenesis via anti-VEGF and anti-PDGF processes. Kumar *et al* (41) reported that anti-CSF1 receptor, specifically targeting TAMs, plus anti-PD1 treatment significantly improved therapies, compared with anti-PD1 treatment alone. HHLA2, which may not only inhibit TAM development but also co-stimulate immune function, has promising potential in immune therapy for patients with glioma.

The function of HHLA2 in the immune microenvironment of glioma was then assessed. Taking its role as an immune stimulator into account, its correlation with several common immune inhibitors was explored. According to Kamran *et al* (42), TGF-β and IL-10 are central to maintaining the immunosuppressive microenvironment of glioma. Thus, TGF-β and IL-10 were selected as representative immune inhibitors and included in the analysis. It was observed that IL-10 was significantly increased in the low HHLA2 expres-

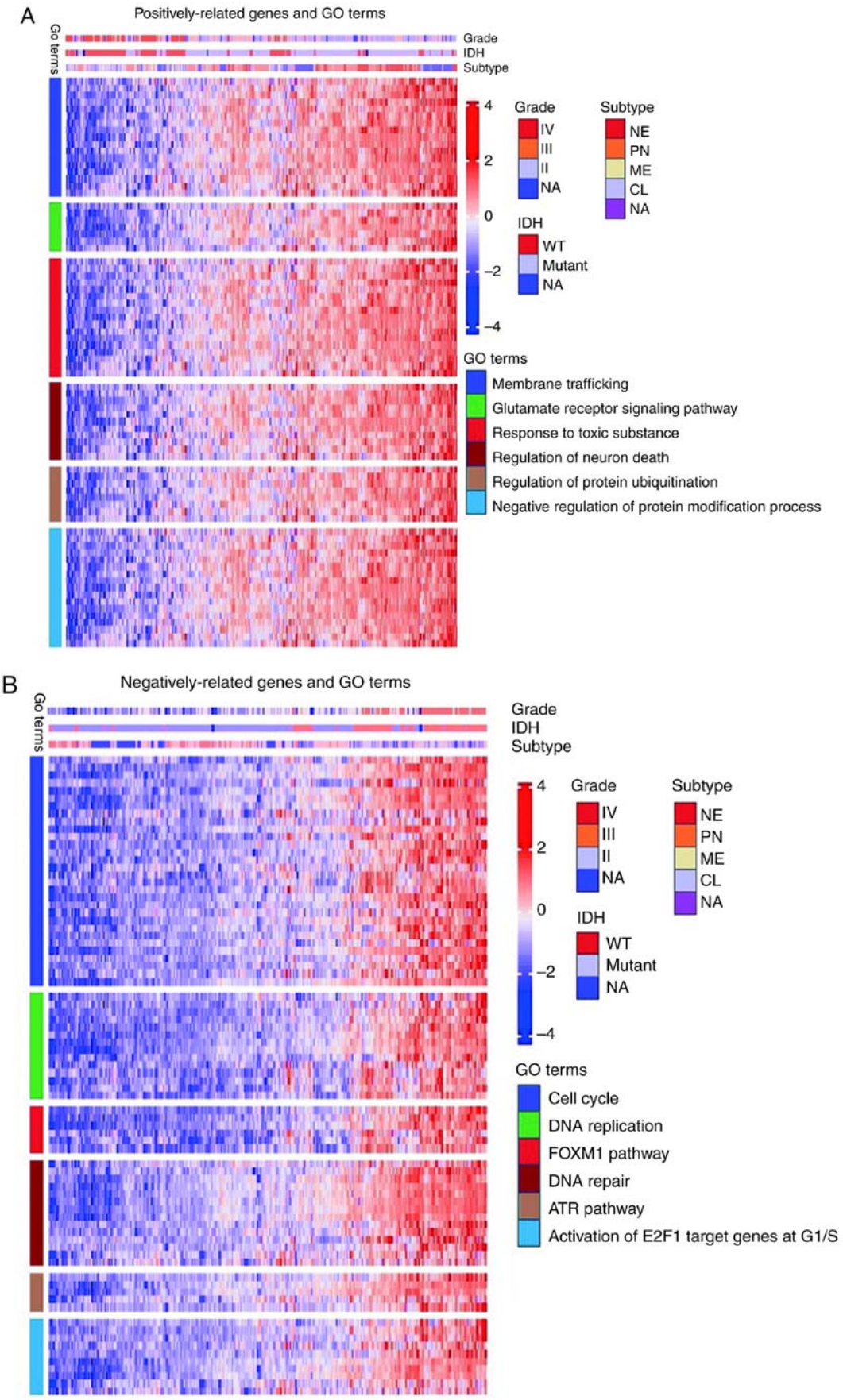

Figure 6. Enrichment analysis of HHLA2-related genes. (A) The positively-related genes were involved in biological process including membrane trafficking, the glutamate receptor signaling pathway, regulation of neuron death, response to toxic substance, regulation of protein ubiquitination, and negative regulation of protein modification process. (B) Negatively-related genes were involved in biological processes including cell division, DNA replication, DNA repair, activation of E2F1 target genes at G1/S, the FOXM1 pathway, and the ATR pathway. HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2.

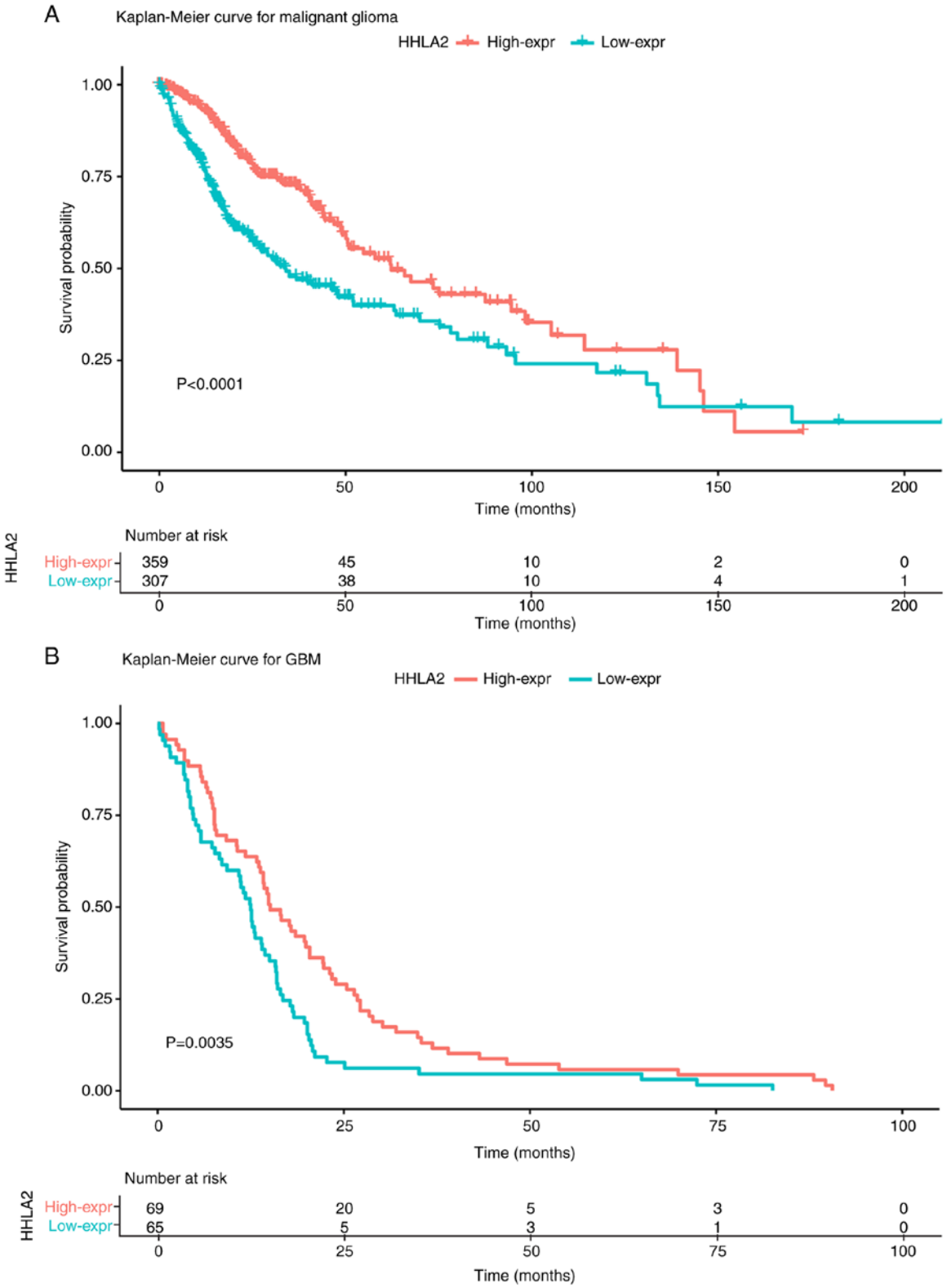

Figure 7. Overexpression of HHLA2 is associated with better prognosis in glioma and GBM. (A and B) Kaplan-Meier survival analysis revealed that high expression of HHLA2 conferred a significantly better prognosis in patients with malignant glioma (hig 359 vs. low 307, P<0.0001) and GBM (high 69 vs. low 65, P<0.01), respectively. HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2.

sion group, which was also the case for TGF-β. The function of HHLA2 was then explored in-depth via GO and KEGG enrichment analysis. The results demonstrated that HHLA2 was positively associated with response to toxic substances, regulation of neuronal death, the glutamate receptor signaling pathway and regulation of protein processes. This indicated that HHLA2 is able to prevent normal neurons from damage by negatively regulating the glutamate receptor signaling pathway. Furthermore, genes which were negatively correlated with HHLA2 were enriched in cell cycle, DNA

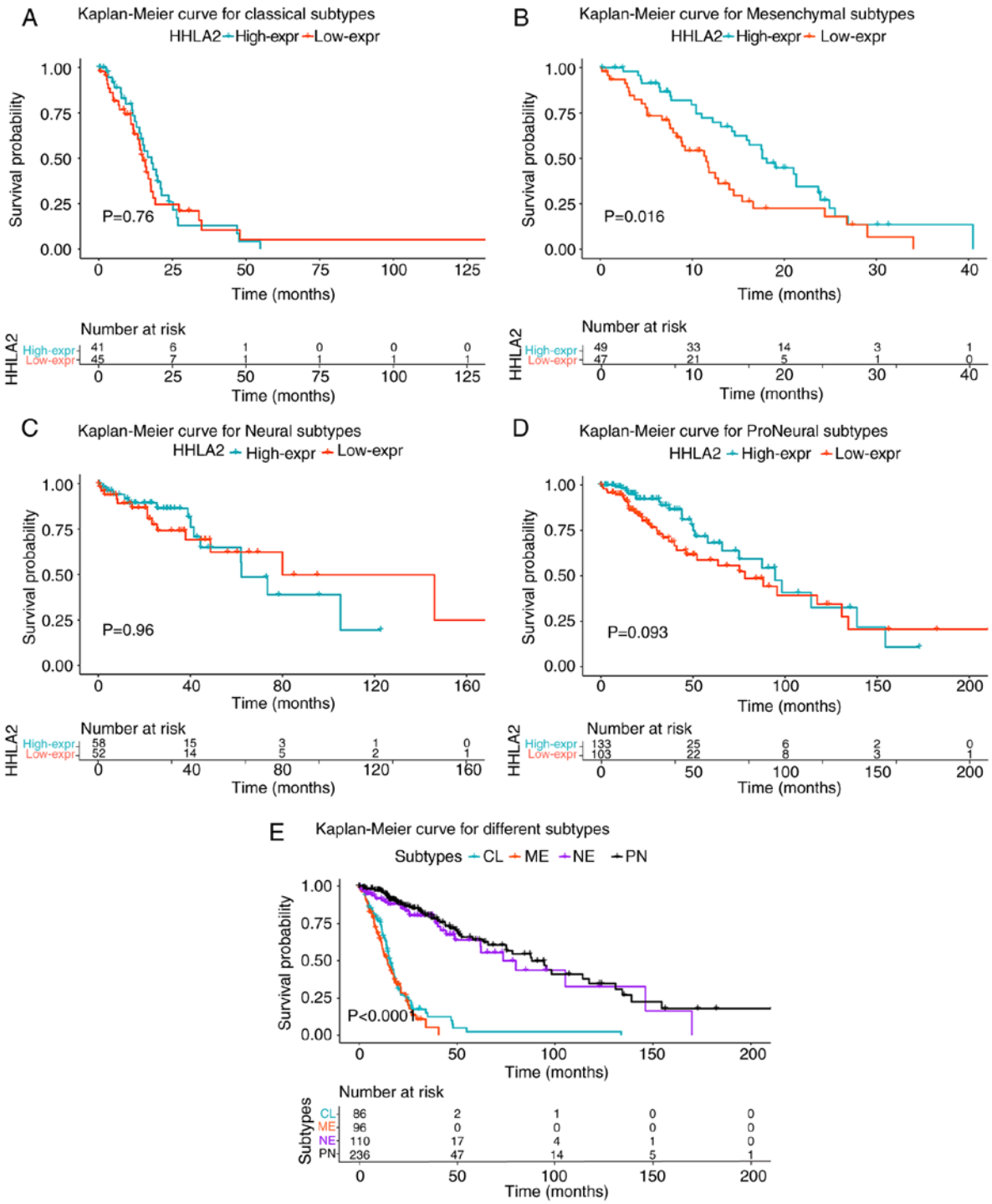

Figure 8. Overexpression of HHLA2 predicts better outcomes in ME subtypes. (A-D) Kaplan-Meier survival analysis revealed that overexpression of HHLA2 conferred a significantly better prognosis in the ME subtype (high 49 vs. low 47, P<0.05). (E) PN and NE subtypes displayed a better outcome than other subtypes (P<0.0001). HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2; ME, mesenchymal; PN, proneural; NE, neural; CL, classical.

replication, DNA repair, activation of E2F1 target genes at G1/S, the FOXM1 pathway and the ATR pathway. Activation of E2F1 target genes at G1/S, the FOXM1 pathway and the ATR pathway have been previously confirmed to promote neoplastic proliferation and tumor progression (43-45). These results indicated that HHLA2 inhibits tumor progression in the early stage of glioma by participating in early immune response and inhibiting neoplastic proliferation and angiogenesis. Hence, exploring the mechanism of HHLA2 acting on tumor cells may facilitate the discovery of a cure for this disease.

Notably, the present study was the first to report on the prognostic significance of HHLA2 in glioma. Kaplan-Meier survival analysis revealed that higher HHLA2 expression was consistently and significantly associated with better survival in glioma and GBM. Furthermore, a subsequent Kaplan-Meier survival curve analysis for each subtype yielded a similar result for the ME subtype of glioma. Lin *et al* (40) reported

Table III. Univariate and multivariate Cox analysis for OS.

Characteristic	P-value	HR	95% CI
Univariate			
Age	<0.001	4.819	3.505-6.625
Grade	<0.001	0.099	0.074-0.133
IDH Wild-type	<0.001	8.848	6.707-11.670
HHLA2	<0.001	0.526	0.406-0.681
Multivariate			
Age	<0.001	1.963	1.339-2.880
Grade	<0.001	0.193	0.133-0.279
IDH Wild-type	<0.001	2.373	1.655-3.404
HHLA2	0.257	0.854	0.650-1.122

OS, overall survical; CI, confidence interval; HR, hazard ratio; HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2.

that the CL subtype had the worst outcome and accounted for a large part of GBM, while the NE and PN subtypes had a high proportion of LGG with a better outcome. These results may explain for the absence of a significant correlation between higher HHLA2 expression and better prognosis in the CL, NE and PN subtypes of glioma. Furthermore, the univariate analysis indicated that HHLA2 expression was a significant prognostic factor, while multivariate analysis demonstrated that the expression of HHLA2 was not an independent prognostic factor. Regarding the limitations of the present study, part of the patients included were lost to follow-up. Thus, the potential of HHLA2 to be an independent prognostic predictor for glioma should be further investigated in a larger and more comprehensive dataset.

However, in other types of human cancer, HHLA2 expression is not always a favourable predictor for patient survival. The prognostic significance of HHLA2 expression in osteosarcoma and colorectal carcinoma was identified to be correlated with metastasis and poor survival (46,47). Similarly, in triple-negative breast cancer, overexpression of HHLA2 was associated with lymph node positivity and advanced stage of the disease at the time of diagnosis, and also with an increased risk of recurrence (29). HHLA2 has also been reported to predict a favorable outcome in pancreatic ductal adenocarcinoma and gastric cancer (37,48). One explanation is that HHLA2 has two opposite ligands (22), including TMIGD that has a co-stimulatory role, while the other ligand remains elusive. TMIGD was indicated to be the major receptor in certain cancer types due to the tumoral immune microenvironment. It is not uncommon for members of the B7 family to have a dual function depending on the immune environment, tumor microenvironment or interaction with different receptors (22). HHLA2 belongs to group III of the B7 family, which also includes B7-H3 (also known as CD276) and B7x. As with HHLA2, the prognostic significance of B7-H3 and B7x also remains to be further delineated (49,50). Wang *et al* (51) reported that B7-H3 was positively associated with the Toll-like receptor signaling pathway and predicted poor survival for glioma patients. Zhou *et al* (52) also identified that overexpression of B7-H3 was associated with the malignancy grade of brainstem gliomas. However, results demonstrating B7-H3 as a co-stimulatory molecule associated with prolonged survival in pancreatic cancer (53) and gastric cancer (54) have also been reported. Therefore, the association between HHLA2 and other immune checkpoint molecules was then analyzed, revealing a negative correlation with checkpoint inhibitors, including PD-1, LAG3 and B7-H3. These results indicated that anti-PD-1 plus anti-B7-H3 treatment may be a strategy for glioma treatment, as it may lead to upregulation of HHLA2. Notably, the present study was the first to indicate that HHLA2 may act as a co-stimulatory molecule in glioma, in contrast to other B7 family members, which are commonly characterized as immune checkpoint inhibitors.

To date, observable progress has been made in the area of immunotherapy. However, blocking the PD-1/PD-L1 pathway was only effective in a small number of cases and most patients with glioma still suffered from disease. Thus, novel therapeutic strategies targeting other immune checkpoint molecules are in urgent demand. HHLA2, acting as an immune stimulator and inhibiting the formation of TAMs, may be a potential therapeutic target.

To the best of our knowledge, the present study was the first to explore the biological function and clinical roles of HHLA2 in glioma. The results indicated that HHLA2 acts as an immune stimulator and inhibits the formation of TAMs. Furthermore, HHLA2 expression was significantly correlated with a favorable outcome. The present study indicated its potential as a prognostic predictor and novel therapeutic target.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YQ, GD and QC designed this study. YQ, GD, PX, HZ, FY, RG, HJ and BL performed the data collection and collation. All the authors were involved in the analysis and interpretation of data. YQ wrote the paper, with the help of the co-authors. GD, BL and QC reviewed and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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