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ORIGINAL ARTICLE

PLEKHG5 is a novel prognostic biomarker in glioma patients

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Abstract

Background PLEKHG5, a Rho-specific guanine-nucleotide exchange factor, is involved in tumor cell migration, invasion and angiogenic potential. In this study, the expression pattern, prognostic value and function of PLEKHG5 in gliomas were investigated.

Methods Immunohistochemistry was used to determine the expression pattern of PLEKHG5 in 61 glioma patients after curative resection. Statistical analysis was performed to evaluate the diagnostic and prognostic significance of PLEKHG5. Gene ontology (GO) analysis, Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis and Gene set enrichment analysis (GSEA) were used to predict potential functions of PLEKHG5. Migration assay and western blot analysis determined PLEKHG5 function in glioma migration and invasion.

Results Increased PLEKHG5 expression levels were associated with higher glioma grades ($P < 0.05$). In addition, glioblastomas multiforme have higher ratio and stronger intensity of PLEKHG5 expression compared with low-grade gliomas. High expression level of PLEKHG5 indicated poorer prognosis and shorter survival time in all glioma patients ($P < 0.001$). GO analysis, KEGG pathway analysis and GSEA analysis suggested that PLEKHG5 was involved in glioma migration, invasion and epithelial–mesenchymal transition. Migration assay and western blot analysis revealed PLEKHG5 promoted glioma migration and invasion.

Conclusion Our results demonstrated PLEKHG5 could be used as a novel prognostic biomarker and anti-tumor target for glioma patients.

Keywords Glioma · PLEKHG5 · Novel prognostic biomarker · Tumor migration and invasion

Introduction

Glioma is the most common and malignant primary intracranial tumor [1]. Despite deeper understanding of molecular mechanisms in gliomas and advances in surgical techniques, radiotherapy and chemotherapy, the prognosis of glioma (especially glioblastoma multiforme (GBM), WHO grade IV glioma) patients remains poor. The GBM patients' median survival time is about 12–15 months [2]. The biological characteristics of gliomas include self-renewal, invasiveness, strong angiogenesis, high mortality and recurrence rates [3, 4]. Multiple researches have indicated that combined application of gene expression-based classification and histological classification may predict treatment effect and prognosis better than the histological classification alone [5]. In World Health Organization (WHO) 2016 classification of gliomas, isocitrate dehydrogenase (IDH) mutation and 1p/19q codeletion have been included as decisive markers for glioma

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classification. According to the WHO 2016 entities, astrocytoma, anaplastic astrocytoma and glioblastoma could be classified into IDH-mutant group and wild-type group. Moreover, 1p/19q codeletion become an important marker for oligodendroglioma, whose diagnosis must be based on IDH-mutant plus 1p/19q-codeleted [6]. Since gliomas have distinct molecular characteristics, it is necessary to identify novel biomarkers and molecular targets for glioma diagnosis, prognosis and therapy development.

Uncontrollable invasiveness, which limits the treatment effect and results in the tumor recurrence, is an important pathobiological characteristic of gliomas. Glioma migration and invasion can be regulated by many proteins and signaling pathways such as PI3K and MAPK pathways [7]. Among the genes and proteins which can exactly influence the glioma migration and invasion, the Rho family of GTPases, including RhoA, Rac1 and Cdc42, are key mediators of glioma migration and invasion [8]. They can regulate cell polarity and cell movement by regulating the organization of cytoskeleton [9]. Besides Rho GTPases, the Rho-specific guanine-nucleotide exchange factors (RhoGEFs) also play important roles, which can regulate GTPases activity by catalyzing the exchange of GDP for GTP [10].

Pleckstrin homology and RhoGEF domain containing G5 (PLEKHG5), which is mainly expressed in the nervous system, functions as a GEF to activate RhoA [11]. Many studies have demonstrated that PLEKHG5 is involved in endothelial cell migration [12], integrity of endothelial cell junction, function of cell barrier, vascular leakiness [13] and angiogenesis [14]. Furthermore, PLEKHG5 has also been reported to promote tumor cell migration and invasion, cancer stem cell spheroid formation and angiogenic potential [15, 16], suggesting PLEKHG5 may have an important role in tumor progression. However, the expression pattern, diagnostic and prognostic value of PLEKHG5 in human gliomas have not been determined.

Here, in this study we investigated the expression pattern of PLEKHG5 in human gliomas of different grades, its clinical significance and its potential functions. Our results illustrated that PLEKHG5 may be a novel prognostic biomarker and a potential anti-tumor target for human gliomas.

Materials and methods

Cell culture

The human GBM cell lines U87MG and U251 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). U87MG and U251 were cultured in DMEM (Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific). The cells were cultured at 37 °C in a humidified chamber containing 20% O_2 and 5% CO_2.

Patient samples

Normal brain tissues were collected from severe trauma patients who had decompression treatment. Glioma specimens of different grades were got from glioma patients ($n=61$) who had initial surgery in the Department of Neurosurgery, Qilu Hospital of Shandong University. This study got approved by the Ethics Committee of Qilu Hospital and the Institutional Review Board of Shandong University. We collected written informed consent from all the 61 glioma patients.

Immunohistochemistry staining

Human glioma samples were fixed with 4% paraformaldehyde. Paraffin-embedded glioma tissues were sectioned and antigen retrieval was performed using 1 mM EDTA (pH 8.0). Endogenous peroxidase activity was blocked by methanol containing 3% H_2O_2. The sections were firstly incubated with goat serum and then incubated with a PLEKHG5 antibody (Sigma-Aldrich, USA) at 4 °C overnight. The staining was visualized by DAB kit according to the instructions. Representative pictures were collected by an Olympus microscope.

Immunohistochemistry staining evaluation

PLEKHG5 expression levels were evaluated by the percentage of positive stained cells and the intensity of the staining. The percentage of immunoreactive cells was scored as follows: 0. 0%; 1. 1–10%; 2. 11–50%; 3. 51–75%; 4. over 75%. The intensity of staining was scored as follows: 0. negative; 1. weak staining; 2. moderate staining; 3. intense staining. The overall score of PLEKHG5 was the product of the percentage and the staining intensity. A total score of 0–12 was calculated and divided into 2 groups: PLEKHG5 low-expression group: the score was between 0 and 3; PLEKHG5 high-expression group: score was between 3 and 12. The PLEKHG5 high-expression group was further divided into different groups: PLEKHG5 (+) group: score was between 3 and 5; PLEKHG5 (++) group: score was between 5 and 7; PLEKHG5 (+++) group: score was between 7 and 10; PLEKHG5 (++++) group: score was between 10 and 12.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis

Using the Matlab software (https://cn.mathworks.com) and TCGA database, analysis of genes associated with PLEKHG5 was performed. By analyzing genes positively and

negatively associated with PLEKHG5 ($P < 0.05$) by utilizing DAVID web tool (https://david.abcc.ncifcrf.gov/home.jsp), the potential biological processes and signaling pathways which PLKEHG5 may be involved in were determined. Gene set enrichment analysis (GSEA) was performed using the specialized software (https://software.broadinstitute.org/) to analyze and determine the correlation between PLEKHG5 expression and genes from Molecular Signatures Database (MSigDB).

Gene knockdown by siRNA

U87MG and U251 were transfected with the PLEKHG5 siRNA (M-013873-01-0005) (Dharmacon, USA) using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. 48 h after transfection, cells were harvested for further investigation.

Migration assay

Migration assay was conducted with transwell chambers (Corning Costar, USA) according to the manufacturer's instructions. Treated glioma cells were seeded into the top chamber in DMEM and the bottom chamber was filled with DMEM containing 30% FBS. Glioma cells that migrated to the bottom of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet (Solarbio, China). Representative images were taken by an Olympus microscope.

Western blot

Glioma cells were lysed in RIPA buffer (Thermo Fisher Scientific) containing a protein inhibitor cocktail (Sigma-Aldrich). Protein concentrations were quantified using Pierce Protein Assay Kit (Pierce, USA). Proteins were run on SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane and detected by primary antibodies. The following primary antibodies were used: PLEKHG5 (Sigma-Aldrich), MMP2 (Abcam, UK, ab37150), MMP9 (Abcam, ab73734), mDia (Santa Cruz, USA, sc-373895), N-cadherin (Abcam, ab76011), Vimentin (Abcam, ab8069), GAPDH (Cell Signaling Technology, USA, 5174). Proteins were quantified using chemiluminescence (Bio-Rad, USA) according to the manufacturer's protocol.

Statistical analysis

Data analyses were performed using GraphPad Prism 6 and SPSS 20.0. Data are presented as the means ± SD. Kaplan–Meier analysis was used to estimate the survival curves. The correlations between PLEKHG5 expression and clinicopathological characteristics were determined by two-tailed χ^2 test. Comparisons between groups were analyzed using Student's test or ANOVA. $P < 0.05$ was regarded as statistically significant.

Results

The expression patterns of PLEKHG5 in diverse grades of glioma

To determine the potential function of PLEKHG5 in human gliomas, the expression levels of PLEKHG5 were investigated by immunohistochemistry (IHC) in normal brain tissues, low-grade gliomas (LGG) and GBMs. The IHC results of normal brain tissues and different grades of gliomas showed that compared to normal brain tissues and LGGs, GBMs had a relatively higher expression level of PLEKHG5 (Fig. 1a–f), which suggested that the expression level of PLEKHG5 might be associated with the grades of gliomas (Table 1). However, there was not a significant difference between the expression level of PLEKHG5 in LGGs and normal brain tissues (Fig. 1f). These results indicated that there was not a strong correlation between the grades of gliomas and the PLEKHG5 expression level in LGG. While the higher expression level of PLEKHG5 may indicate the higher grades of glioma tissues.

GBMs have higher ratio and stronger intensity of PLEKHG5 expression

Next, we investigated the ratios of glioma tissues which expressed PLEKHG5. We found that although some of Grade III and IV glioma tissues had at most very modest expression of PLEKHG5 (Fig. 2a), GBMs had a higher ratio of PLEKHG5 expression when compared with LGGs (Fig. 2b). The ratio of PLEKHG5 expression increased with increasing glioma grade (Fig. 2b), suggesting there was a correlation between glioma grades and the ratio of PLEKHG5 expression. Furthermore, we found GBMs had a stronger intensity of PLEKHG5 expression compared with LGG (Fig. 2c). More than half of GBMs had a relatively strong intensity (+ + + ~ + + + +) of PLEKHG5 expression (Fig. 2c). While compared to GBMs, most LGG had a weaker intensity (+ ~ + +) of PLEKHG5 expression (Fig. 2c). The intensity of PLEKHG5 expression also increased with increasing glioma grade (Fig. 2c), suggesting the relation between glioma grades and the intensity of PLEKHG5 expression. Collectively, these results suggested that there were correlations between the grades of gliomas and

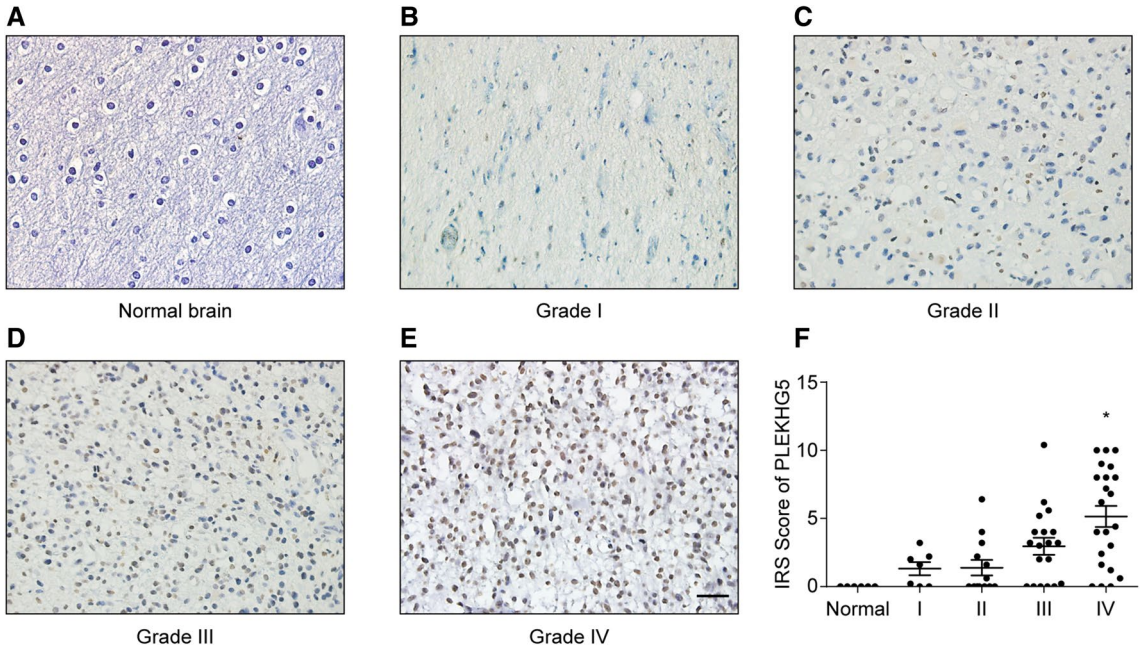

Fig. 1 The expression pattern of PLEKHG5 in diverse tumor grades of gliomas. **a** Representative image of IHC staining for PLEKHG5 in sections from normal tissue. **b** Representative image of IHC staining for PLEKHG5 in sections from grade I gliomas. **c** Representative image of IHC staining for PLEKHG5 in sections from grade II gliomas. **d** Representative image of IHC staining for PLEKHG5 in sections from grade III gliomas. **e** Representative image of IHC staining for PLEKHG5 in sections from grade IV gliomas. **f** Quantification of IHC staining for PLEKHG5 in sections from normal tissue and different grades of gliomas. Scale bar 50 μm

Table 1 Correlation between PLEKHG5 expression and clinicopathological characteristics in glioma

Characteristics	Cases	PLEKHG5 expression		*P* value
		Low[a]	High[b]	
Age (year)				
< 45	27	22	5	0.222
≥ 45	34	23	11	
Gender				
Male	41	22	19	0.640
Female	20	12	8	
WHO grade				
I–II	20	16	4	**0.010**
III–IV	41	17	24	
Radiotherapy				
Yes	44	25	19	0.695
No	10	5	5	
Chemotherapy				
Yes	40	21	19	0.445
No	14	9	5	

Bold numbers indicate a statistically significant difference in this analysis

[a]IRS of PLEKHG5 expression less than 3 (including 3)

[b]IRS of PLEKHG5 expression more than 3

the ratios of PLEKHG5 expression as well as the intensity of PLEKHG5 expression.

The expression patterns of PLEKHG5 are related to the glioma patients' survival

Next, we intended to identify the correlation between the glioma patients' survival and the expression patterns of PLEKHG5. To confirm the quality of the clinical data we got, we analyzed the association between the glioma grades and the patients' survival time. We found the higher grade of the gliomas always indicated a poorer prognosis and shorter survival time in our clinical data (Fig. 3a), which suggested that the clinical data was of good quality. Then we determined the relation between patient survival and expression level of PLEKHG5. We found that patients with PLEKHG5 expressed gliomas always had a poorer survival time (Fig. 3b). We also confirmed these findings in LGG, high-grade (grade III–IV) gliomas, anaplastic gliomas and GBMs (Fig. 3c–f). In accordance with our previous findings, PLEKHG5 expressed gliomas usually meant poorer prognosis. Then, we investigated the correlation between patient survival and different expression levels of PLEKHG5 in gliomas. Higher expression levels of PLEKHG5 in gliomas referred to a poorer patient survival (Fig. 3g), and similar result could be observed in high-grade (grade III–IV) gliomas and GBMs (Fig. 3h, i). At last, we determined the

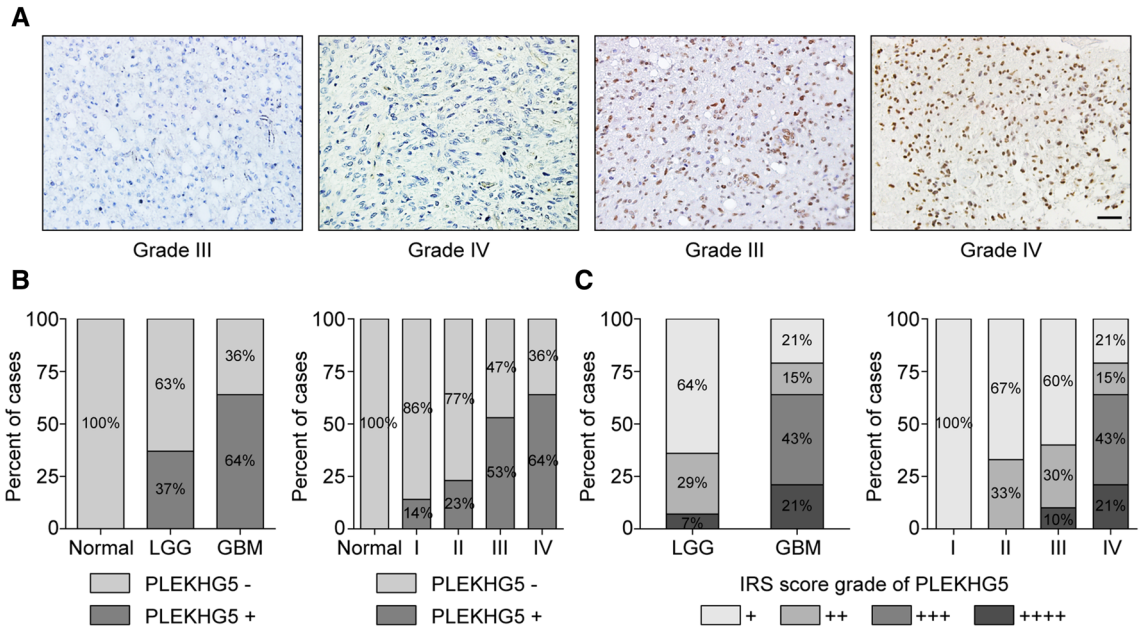

Fig. 2 GBMs have higher ratios and stronger intensity of PLEKHG5 expression. **a** Representative images of IHC staining negative and positive for PLEKHG5 in sections from grade III and grade IV gliomas. **b** Quantification of ratio of IHC staining positive for PLEKHG5 in sections from gliomas of diverse grades. **c** Quantification of IHC staining grade for PLEKHG5 in sections from gliomas of diverse grades. Scale bar 50 μm

prognostic value of PLEKHG5 in TCGA database. Consistent with the above findings, high PLEKHG5 expression had significantly worse prognosis than low PLEKHG5 expression in GBM patients in TCGA database (Fig. 3j). All these results demonstrated that the expression patterns of PLEKHG5 were related to the glioma patients' survival.

Go and KEGG pathway analysis of PLEKHG5 and related genes

To further determine PLEKHG5's potential functions in glioma progression, we performed correlation analysis of PLEKHG5 expression in TCGA database and subsequently got 8646 PLEKHG5-associated genes. 4368 genes of PLEKHG5-associated genes are positive-related and the other 4278 genes are negative-related (Supplementary Table 1). Using the PLEKHG5 associated genes, GO analysis suggested that PLEKHG5 positively related genes were mainly involved in cell migration, cell adhesion, angiogenesis, cell proliferation and extracellular matrix disassembly (Fig. 4a). While PLEKHG5 negatively related genes were likely to mainly participate in brain development and nervous system development, including synapse organization and chemical synaptic transmission (Fig. 4a). KEGG pathway analysis showed that PLEKHG5 positive-related genes were enriched in regulation of actin cytoskeleton, cell adhesion and signaling pathways associated with tumor progression (PI3K-Akt signaling pathway, chemokine signaling pathway and JAK-STAT signaling pathway) (Fig. 4b). PLEKHG5 negative-related genes were associated with neural activity and neural signaling (glutamatergic synapse, GABAergic synapse, axon guidance, Hippo signaling pathway and Wnt signaling pathway) (Fig. 4b). Next, using TCGA database, patients were divided into PLEKHG5-high and PLEKHG5-low groups. The association between PLEKHG5 expression and epithelial–mesenchymal transition (EMT), actin-based cell movement, extracellular matrix disassembly and regulation of actin cytoskeleton was analyzed by Gene set enrichment analysis (GSEA). The genes signatures of EMT, actin-based cell movement, extracellular matrix disassembly and regulation of actin cytoskeleton were all highly enriched in patients with PLEKHG5 high expression (Fig. 4c), suggesting PLEKHG5 was involved in these biological processes. To sum up, these results suggested that PLEKHG5 may participate in the regulation of actin cytoskeleton, extracellular matrix disassembly and EMT to regulate the glioma cell migration and invasion.

PLEKHG5 promoted glioma cell migration and invasion

To validate the results of GO, KEGG and GSEA analysis, we performed migration assay to determine PLEKHG5 effect on glioma motility. Migration assay revealed that knockdown of PLEKHG5 significantly suppressed U87MG and U251 glioma cells migration (Fig. 5a, b).

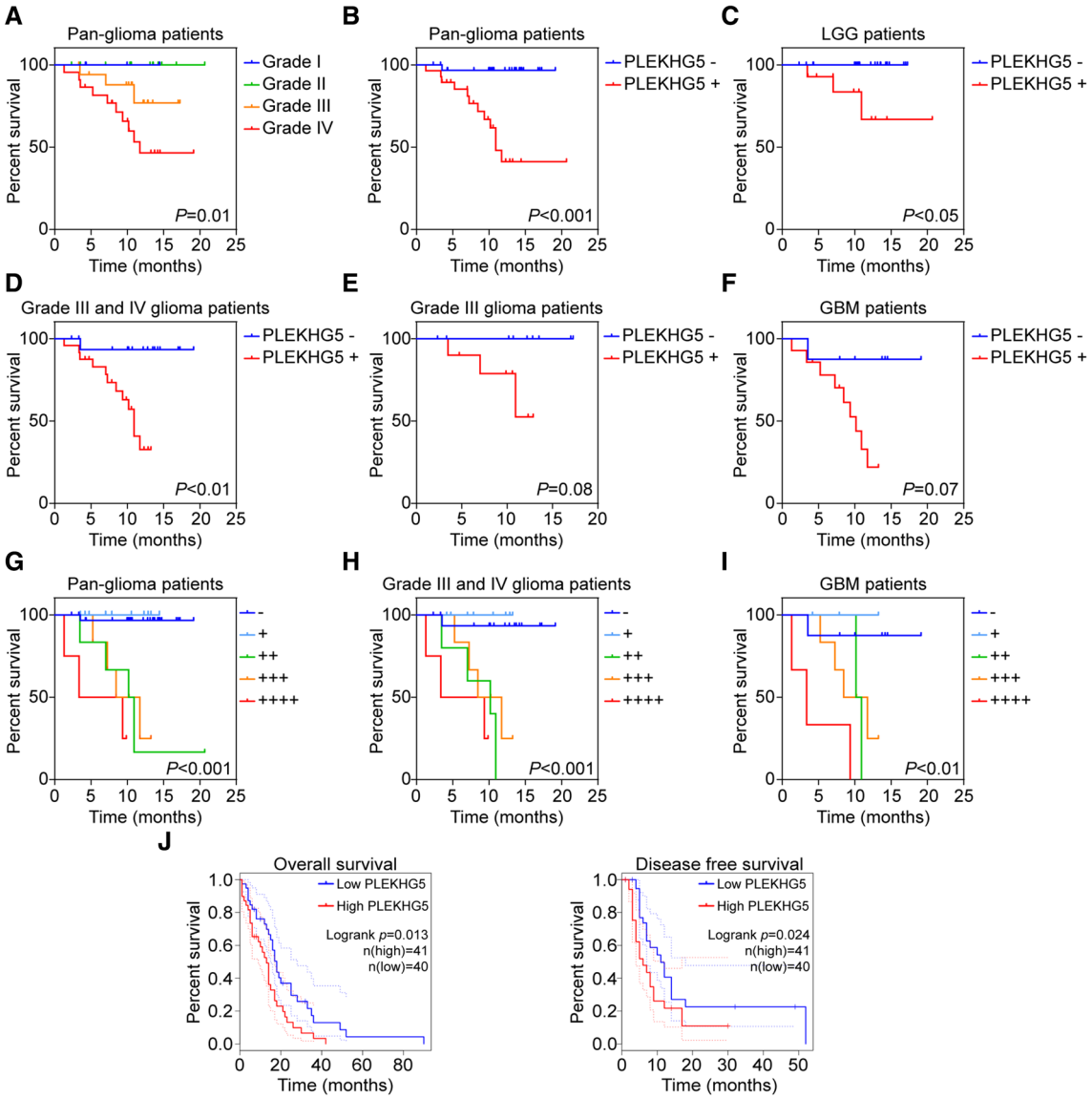

Fig. 3 Prognostic effects of PLEKHG5 expression level on glioma patients. **a** Kaplan–Meier survival curves of glioma patients according to the glioma grades. **b** Kaplan–Meier survival curves of glioma patients according to positive (+) or negative (−) expression of PLEKHG5. **c** Kaplan–Meier survival curves of patients with LGG according to positive (+) or negative (−) expression of PLEKHG5. **d** Kaplan–Meier survival curves of patients with high-grade gliomas (WHO III and IV) according to positive (+) or negative (−) expression of PLEKHG5. **e** Kaplan–Meier survival curves of patients with grade III gliomas according to positive (+) or negative (−) expression of PLEKHG5. **f** Kaplan–Meier survival curves of patients with GBMs according to positive (+) or negative (−) expression of PLEKHG5. **g** Kaplan–Meier survival curves of glioma patients according to the PLEKHG5 IRS grades. **h** Kaplan–Meier survival curves of patients with high grades gliomas (WHO III and IV) according to the PLEKHG5 IRS grades. **i** Kaplan–Meier survival curves of patients with GBMs according to the PLEKHG5 IRS grades. **j** Kaplan–Meier survival curves of glioma patients' overall survival (OS) and disease-free survival (DRS) according to the expression level of PLEKHG5. The cutoff level was set at the quartile value of the PLEKHG5 levels

Western blot analysis showed that PLEKHG5 knockdown inhibited MMP2 and MMP9 expression, both of which were important matrix metalloproteinases for glioma migration and invasion, suggesting PLKEHG5 promoted glioma migration at least partly by regulating extracellular matrix (ECM) degradation (Fig. 5c). Moreover, PLEKHG5 knockdown downregulated the expression of mDia, which acted as RhoA effector to regulate the F-actin cytoskeleton to support tumor cell motility [17], implying PLEKHG5 was involved in glioma cell cytoskeleton organization (Fig. 5c). While PLEKHG5-siRNA had no effect on mesenchymal factors N-cadherin and Vimentin,

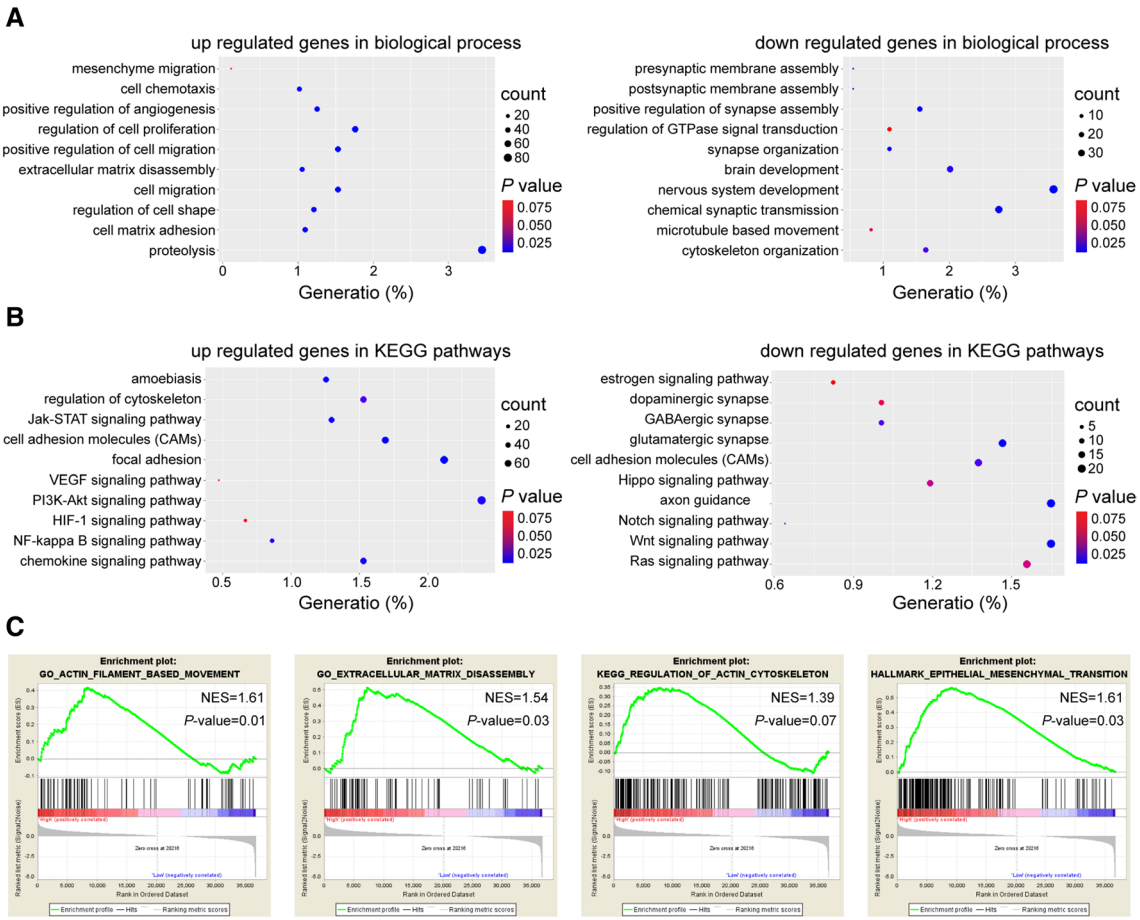

Fig. 4 Go and KEGG pathway analysis of PLEKHG5 and related genes. **a** Biological processes analysis of the positively and negatively correlated genes. **b** KEGG analysis of the positively and negatively associated genes. **c** GSEA suggesting positive association of PLEKHG5 with actin filament-based movement, extracellular matrix disassembly, regulation of actin cytoskeleton and epithelial mesenchymal transition. *NES* normalized enrichment score

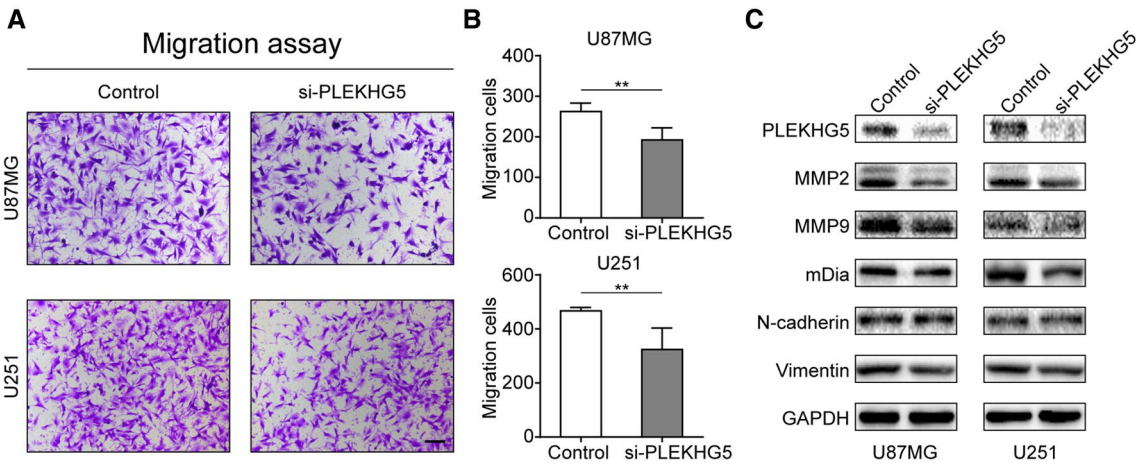

Fig. 5 PLEKHG5 promoted glioma cell migration and invasion. **a** Analysis of glioma cells migration by transwell assay. Indicate treated glioma cells that migrated to the bottom surface were stained with crystal violet and observed by light microscopy. Scale bar 100 μm. **b** Quantification of migrated glioma cells with indicate treatment. **c** Western blot analysis of PLEKHG5, MMP2, MMP9, mDia, N-cadherin and Vimentin in indicate treated U87MG and U251 glioma cells

indicating PLEKHG5 may not participate in the regulation of EMT (Fig. 5c).

Discussion

In recent years, gene expression-based classification, diagnosis, prognosis prediction and treatment have been of great interest for anti-tumor research. More and more evidence has demonstrated the potentials of the genes differentially expressed in normal tissue and tumors as diagnostic, prognostic indicators and therapeutic targets. Here, in this study, for the first time, we determined PLEKHG5, as a Rho-specific guanine-nucleotide exchange factor, to be a novel prognostic biomarker in glioma patients. The higher expression level of PLEKHG5 suggested a higher grade of glioma tissues. What's more, the ratio and the intensity of PLEKHG5 expression were both associated with the grades of gliomas. GBMs always had higher ratio and stronger intensity of PLEKHG5 expression compared with LGGs. And the expression patterns of PLEKHG5 were also related with glioma patient survival time. Higher expression of PLEKHG5 usually indicated a poor prognosis and short survival time of glioma patients.

Metastasis is the main reason that results in the tumor recurrence. To invade into the surrounding tissue, the glioma cells have to degrade the extracellular matrix (ECM). Among the main steps in which tumor cells invade, ECM disassembly is an important process. Matrix metalloproteinases (MMPs) are the main proteins which can degrade various components of the ECM. MMP-mediated ECM degradation promotes tumor invasion and metastasis. Among all the MMPs, MMP2 and MMP9 have been paid more attention since elevated expression of MMP2 and MMP9 have been reported in gliomas, breast cancers and lung cancers [18]. MMP2 and MMP9 are considered critical and essential in brain tumor migration and invasion [19]. By GO, KEGG and GSEA analysis, we found PLEKHG5 was involved in ECM disassembly. Further migration assay and western blot analysis demonstrated that the knockdown of PLEKHG5 expression significantly inhibited glioma migration and the expression of MMP2 and MMP9. All these results suggested that PLEKHG5 participated in tumor cell migration and invasion by regulating MMPs which can degrade ECM.

Besides secreting proteolytic enzymes to degrade the ECM, actin cytoskeleton-based tumor cell movement is also important. It helps the tumor cells move to the area where the ECM has been degraded by proteolysis, further intravasate into lymphatic or blood vessels and eventually spread to the distant organs [20]. The Rho family of GTPases are the key proteins which can not only contribute to organization of the actin and microtubule cytoskeletons but also regulate cell morphogenesis, cell polarity, cell migration and cell invasion [9]. Among all the members of Rho family of GTPases, RhoA, Rac1 and Cdc42 are well-characterized and regarded as key regulators of cell cytoskeleton, cell migration and invasion [21]. RhoA is described to regulate the assembly of stress fibers. Rac1 is important in the formation of lamellipodia and Cdc42 is believed to influence the assembly of filopodia [21]. In addition, as two major effectors of Rho, Rho-associated coiled-coil-containing protein kinase (ROCK) and mammalian homologue of Drosophila diaphanous (mDia) are also important in regulation of actin cytoskeleton. Some researches demonstrated that Rho/ROCK signaling could maintain the actomyosin contractility and subsequently make cells with amoeboid invasiveness [22]. While mDia also participated in the cell adhesion and cell migration and some studies indicated that mDia and ROCK could antagonize with each other to influence Rho-induced cell migration [23]. By performing GO and KEGG pathway analysis, we also found PLEKHG5 had close relation with actin-based cell movement and regulation of actin cytoskeleton. The migration assay and western blot analysis demonstrated that the knockdown of PLEKHG5 expression significantly inhibited glioma migration and the expression of mDia. These results suggested that PLEKHG5 was also involved in tumor cell migration and invasion by regulating assembly of actin cytoskeleton.

In conclusion, we determined PLEKHG5 as a novel prognostic biomarker which were associated with the glioma grades and glioma patients' survival time. We also identified PLEKHG5 was associated with many key steps in tumor metastasis. The PLEKHG5 may regulate secretion of proteolytic enzymes and assembly of actin cytoskeleton to influence glioma cell migration and invasion. The exact functions of PLEKHG5 on glioma motility and the underlying mechanisms required further and more investigations. Our results were based on a single hospital-based retrospective study. It should be noted that there might be unmeasured differences which might influence the results. A multicenter prospective study is required to confirm PLEKHG5 effect in glioma prognosis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Research involving human participants and/or animal Ethical approval for using human samples in this study was obtained from the local ethics committee.

Informed consent Patients gave consent for the use of their tumor tissues for future investigations, which had been performed for many years at time of the initial diagnosis.

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