GNG5 is a novel oncogene associated with cell migration, proliferation, and poor prognosis in glioma

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Abstract

**Background:** Although many biomarkers have been reported for detecting glioma, the prognosis for the disease remains poor, and therefore, new biomarkers need to be identified. **GNG5**, which is part of the G-protein family, has been associated with different malignant tumors, though the role of **GNG5** in glioma has not been studied. Therefore, we aimed to identify the relationship between **GNG5** and glioma prognosis and identify a new biomarker for the diagnosis and treatment of gliomas.

**Methods:** We used data on more than a thousand gliomas from multiple databases and clinical data to determine the expression of **GNG5** in glioma. Based on clinical data and CGGA database, we identified the correlation between **GNG5** and multiple molecular and clinical features and prognosis using various analytical methods. Co-expression analysis and GSEA were performed to detect **GNG5**-related genes in glioma and possible signaling pathways involved. ESTIMATE, ssGSEA, and TIMER were used to detect the relationship between **GNG5** and the immune microenvironment. Functional experiments were performed to explore the function of **GNG5** in glioma cells.

**Results:** **GNG5** is highly expressed in gliomas, and its expression level is positively correlated with pathological grade, histological type, age, and tumor recurrence and negatively correlated with isocitrate dehydrogenase mutation, 1p/19 co-deletion, and chemotherapy. Moreover, **GNG5** as an independent risk factor was negatively correlated with the overall survival time. GSEA revealed the potential signaling pathways involved in **GNG5** function in gliomas, including cell adhesion molecules signaling pathway. The ssGSEA, ESTIMATE, and TIMER based analysis indicated a correlation between **GNG5** expression and various immune cells in glioma. In vivo and in vitro experiments showed that **GNG5** could participate in glioma cell proliferation and migration.

**Conclusions:** Based on the large data platform and the use of different databases to corroborate results obtained using various datasets, as well as in vitro and in vivo experiments, our study reveals for the first time that **GNG5**, as an oncogene, is overexpressed in gliomas and can inhibit the proliferation and migration of glioma cells and lead to poor prognosis of patients. Thus, **GNG5** is a potential novel biomarker for the clinical diagnosis and treatment of gliomas.

**Background**

Glioma is the most common malignant tumor of the central nervous system (CNS), showing high recurrence and a high mortality rate [1]. Although extensive research has been performed on the etiology, diagnosis, and treatment of gliomas, the prognosis is still unsatisfactory despite active comprehensive treatment being adopted for glioma patients, including maximum surgical resection, postoperative radiotherapy, and chemotherapy [2]. To improve the diagnostic accuracy of glioma and predict the prognosis of glioma patients more accurately, The World Health Organization (WHO) in 2016 defined the subclassification of glioma using molecular parameters including the co-deletion of chromosomal 1p and 19q(chr1p/19q), and the mutational status of isocitrate dehydrogenase (IDH) [1]. However, as
malignant tumors are highly heterogeneous, using these widely used biological markers is not sufficient for the precise diagnosis and treatment of glioma. Therefore, there is an urgent need for novel molecular markers with high specificity and sensitivity to improve the molecular diagnosis and treatment of glioma.

G-proteins are a family of proteins that participate in multiple cellular functions such as cell division, differentiation, and metastasis during embryonic development through direct interaction with G protein-coupled receptors [3, 4]. The formation of gliomas is related to embryonic development; for example, the epithelial-mesenchymal transition (EMT) is a process vital for embryonic development, and it has been shown to regulate the progression and invasion of glioma [5, 6]. Multiple studies have confirmed the significance of G-protein family members in the pathological progression of cancer. For example, *GNG7* is an epigenetic silencing gene involved in the malignant progression of renal clear cell carcinoma and esophageal cancer [7, 8]; *GNG4* function shows a high degree of correlation with liver cancer and colorectal cancer and is used in their diagnosis and prognosis [9, 10]; and *GNG11* promotes the adhesion, migration, and invasion of gastric cancer cells [11]. Thus, the G-protein family is associated with tumor progression and may be used as potential biomarkers for the diagnosis and treatment of tumors.

We focused on the *GNG5* protein of the G-protein family, and few reports describe the role of *GNG5* in tumors. *GNG5*, as a gamma subunit of G-protein, localized to the human chromosome 1p22, and is composed of 3 exons and 2 introns, and regulates the occurrence and development of many diseases [12]. Additionally, it acts as a regulator of islet function regulating the secretion of insulin, and is also used as a marker of melanosis coli to regulate cellular apoptosis [13, 14]. *GNG5* has been shown to play an important role in various cancers such as endometrial carcinoma [15] where it is highly expressed, and in invasive ductal carcinoma of the breast [16] where it regulates the secretion of E-cadherin through the Wnt signaling pathway. However, the role of *GNG5* in contributing to the pathological mechanism in glioma has not been reported.

We focused on exploring the expression pattern of *GNG5* in glioma from gene level to protein level, examining the underlying relationship between *GNG5* and the clinical and molecular characteristics of glioma in patients, and revealing the potential biological function of *GNG5* in the pathological progression of glioma. The results obtained could enhance our understanding of the complex molecular mechanisms of glioma and provide a promising novel target for combating the disease.

**Methods**

*Data collection*

A comparison of the transcript levels of *GNG5* in different tumors was performed using Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn). To identify differences in expression for *GNG5* between gliomas and the control group, RNAseq data from 698 gliomas and 5 normal brain tissues were downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), and microarray data of the GSE131273 dataset from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. RNA sequencing data from 1018 gliomas and their
corresponding clinical information was downloaded from the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/help.jsp) database. The relationship between the expression level of GNG5 and clinicopathological features was further analyzed using the data obtained from CGGA. The patients were divided into older and younger groups according to the median age of the patients, and high and low expression groups according to the average expression level of GNG5 in the samples. Additionally, we obtained the overall survival (OS) information for glioma patients from the GSE53733 dataset, including 23 patients who showed long-term survival (> 36 months), 16 patients that showed short-term survival (< 12 months), and 31 patients who displayed intermediate survival. We analyzed the difference in expression of GNG5 among the three groups of patients with different survival phenotypes. The Human Protein Atlas (HPA, https://www.proteinatlas.org/) was used to validate GNG5 expression at the protein level.

**Gene set enrichment analysis (GSEA) and immune correlation analysis**

GSEA is a tool developed by research teams at MIT and Harvard University's Broad Institute to analyze genome-wide expression profiling from microarray data [17], and we used it for analyzing GNG5 related pathways and molecular mechanisms in glioma. We used 1000 gene set permutations for each analysis using the expression level of GNG5 as a reference phenotype tag. The resulting enriched pathways were analyzed based on nominal (NOM) P-values and normalized enrichment scores (NES). We examined the relationship between the expression of GNG5 in glioma tissues and the immune microenvironment using the single-sample gene set enrichment analysis (ssGSEA) to calculate the enrichment of 29 immune cell geneset signatures in each glioma sample based on data downloaded from the CGGA database [18-20]. Further, a cluster analysis of glioma samples was performed based on the quantitative results obtained from the ssGSEA analysis. The ESTIMATE method (estimate R package) was used to evaluate the immune scores, tumor purity, and the stromal score for each glioma sample [21]. Tumor IMMune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) is an ideal public database for studying the infiltration abundance of tumor-infiltrating immune cells. The database pre-evaluated the immune infiltration levels of six immune infiltrating cells (including B cells, CD4 + T cells, CD8 + T cells, neutrophils, macrophages and dendritic cells) in 10897 cancer samples by complex statistical methods. We examined the relationship between the expression of GNG5 and the infiltration abundance of the above six immune cells in glioma using TIMER [22].

**Patients and tissue preparation**

Forty primary glioma samples and five non-glioma samples were selected from the Department of Neurosurgery of the First Affiliated Hospital of Harbin Medical University. The tissues were cut into tissue blocks (5 mm thick). One block was fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin, and the others were frozen in liquid nitrogen.

**Cell lines and cell culture**
The human astrocyte (HA) cell line and glioma cell lines LN229 and T98 were donated by the Microbiology Laboratory of Henan Provincial People's Hospital. The glioma cell lines U251 and A172 were purchased from Procell Life Science & Technology Co. Ltd (Wuhan, China). Cells were cultured in DMEM high-sugar medium (Procell, PM150210) containing 10% fetal bovine serum (FBS, Gibco, lot: 10099-141c) and 1% penicillin-streptomycin mixture in a 37 °C humidified incubator with 5% carbon dioxide.

**Cell transfection**

For transient transfection, cells were seeded in 6-well plates, 100 pmol siRNA was transfected into each well using siRNA-Mate (GenePharma, Shanghai, China), and the transfection efficiency was detected by real-time quantitative polymerase chain reaction (RT-qPCR) at 36h after transfection. Lentivirus targeting GNG5 (shGNG5) was constructed according to the siRNA sequences with the highest knockdown efficiency (Table S1, Figure 7B), while empty sequence lentivirus was constructed as a control (shNC). For stable transfection, serum-free medium containing viral uid was added when the density after glioma cell seed plate reached 50%, according to the instructions, and replaced with complete medium 24h later. For stable transfection, when the cell density reached 50%, serum-free medium containing lentivirus was added according to the instructions and replaced with complete medium 24 h later. Medium containing 2 μg/mL puromycin was used to select stably transfected cells.

**RNA isolation and RT-qPCR**

Total RNA was collected using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and RNA was reverse transcribed using the transcriptor first-strand cDNA synthesis kit (Hoffmann-La Roche, Basel, Switzerland), according to the manufacturer's instructions as described previously [23]. RT-qPCR was conducted with SYBR Green on a 7500HT Fast Realtime System (Applied Biosystems, Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for the normalization of GNG5. The relative fold-change in the expression of GNG5 was determined by using the relative quantification method. The specific primer sequences used in the reverse transcription experiments are shown in Table S2 and were purchased from Ribo Bio-Technology (Guangzhou, China).

**MTT assay**

Cell proliferation was detected by MTT assay. The transfected cells were seeded into 96 well plates (1000 cells/well). 100 μL serum-free medium containing 10 μL MTT (Solarbio, China) was added to each well and incubated at 37 °C for 4h. Then the supernatant was removed, and 110 μL DMSO was added. After shaking for 10 minutes in the dark at room temperature, the absorbance of the solution was measured at 490 nm with a spectrophotometer.

**Scratch wound healing assay**

Cells stably transfected with shGNG5 and shNC were cultured in 6-well plates, respectively. Three replicate wells per cell were performed for parallel experiments. When cells grew to 90% confluence, each
well was scratched with a 200 µl sterile pipette tip and then washed 3 times with 1× PBS to remove detached cells. Each well was added with an appropriate amount of fresh serum-free medium and placed in a 37 °C incubator for 24 h. Photographs of the same area of the wound were taken under 40× magnification conditions using a phase-contrast microscope.

Transwell migration assay

200µL serum-free medium containing 2´10^4 transfected cells were seeded into 24 well transwell upper chambers (Corning, USA), respectively. 600µL of complete medium with 10% FBS was added to the lower chamber. After incubation for 24h, the non migrated cells on the surface of the upper chamber were erased, then the upper chamber was fixed with 4% paraformaldehyde for 30 minutes at room temperature and stained with 1% crystal violet for 5 minutes. The staining results were observed under a microscope and five fields were randomly selected to count the cells. All assays were independently performed in triplicate.

Colony-forming assay

Transfected cells were resuspended and seeded in 6-well plates (200 cells/well) and cultured in complete medium for 10 days. Cell colonies were fixed using 4% paraformaldehyde for 30 minutes at room temperature and stained using 1% crystal violet for 5 minutes. Then, cell colonies were observed under a microscope and imaged, and the number of cell colonies was counted using ImageJ software (Version 1.52).

Determination of cell cycle distribution

The transfected cells were collected and fixed using ice-cold 70% ethanol for 24h. According to the cell cycle detection kit instructions (SevenSea Pharmatech, China), cells were then centrifuged and washed with sterile PBS, incubated with 500 µL of staining solution containing 12.5 propidium iodide and 10 µL RNase A for 30 min at 37 °C in the dark. Then, cell cycle distribution was analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Western blot

Total protein was prepared from transfected cells using RIPA buffer with a proteinase inhibitor (EpiZyme, China). The lysates were incubated on ice for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4 °C, and the protein concentration was measured by a BCA kit (Boster, Wuhan, China). Equal quantities of protein were electrophoresed through a 12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked and then probed with the appropriate primary antibody, such as VCAM1 (1:250, Proteintech, USA), ICAM1 (1:1000, Cell Signaling Technology, USA), CDH2 (1:800, Cell Signaling Technology, USA), SDC2 (1:800, Proteintech, USA), and GAPDH (1:5000, Bioworld Technology, USA), overnight at 4°C. Subsequently, the membranes were incubated with the goat anti-Rabbit IgG secondary antibody (EarthOx Biotechnology) at room
temperature for one hour. The protein blots were developed using a chemiluminescence reagent (ECL) kit (Beyotime Biotechnology).

**Immunochemical staining**

For immunohistochemical (IHC) staining, tissue sections of 4 μm thickness were placed in xylene and graded alcohols for deparaffinization and hydration. Heat-induced antigen retrieval was performed in EDTA (PH 8.0) buffer using a microwave oven for 15 minutes. Blocking was done with 10% goat serum to reduce nonspecific staining. The appropriate amount of primary antibody working solution was then dropped onto the sections and incubated overnight at 4°C. Primary antibodies included *GNG5* (1:100, Novus Biologicals, USA) and *IDH1 R132H* (Zhongshan Jinqiao, China). The staining results were observed under a light microscope, and ten 40× visual fields were randomly selected and photographed. The IHC results were analyzed using ImageProPlus software (version 6.0). For cellular immunofluorescence staining, transfected cells were resuspended and seeded in 24 well plates placed with round coverslips (Solarbio, China). Cells were fixed using 4% paraformaldehyde after 24h and subsequently permeabilized using 0.5% Triton X-100. Primary antibody Ki67 (1:100, abbkine, China) was added onto the slides and incubated overnight at 4 °C. DyLight 594 IgG (1:100, abbkine, China) was added onto the slides as a fluorescent secondary antibody for 2h at room temperature. DAPI was then used for nucleus staining, and the staining results were observed and imaged under a fluorescence microscope.

**In vivo tumor formation assay**

Four week old male nude mice were purchased from the Charles River Animal Experimental Center (ZheJiang, China), and U251 cells stably transfected with shGNG5 and shNC were collected, respectively. The cell concentration was resuspended to 2 x 10^7 / mL and then 100 μL of cell suspension was subcutaneously injected into the root of the right thigh of each nude mouse. The tumor sizes were measured every five days starting the second week. Tumor volume was calculated by the formula: volume = (longest diameter × shortest diameter^2) / 2 [24]. The mice were sacrificed after 5 weeks, and the tumors were removed and weighed. The protocol was in accordance with the regulations of the animal ethics committee of Harbin Medical University.

**Statistical analysis**

We used R (v3.5.1) for statistical analysis. The Wilcoxon rank-sum or Kruskal test was used to analyze the correlation between the expression level of *GNG5* and clinical data of glioma patients. A Cox's regression model and the Kaplan-Meier method were used to analyze the relationship between the expression level of GNG5 and the patients' OS. COX test was used for univariate and multivariate analysis to reveal the risk factors affecting glioma prognosis. A Mann-Whitney test, Chi-square or Fisher's exact test was performed to analyze the different expression of *GNG5* between two groups using the Graphpad Prism 8.0 software.
Results

Clinical characteristics of the patients involved in the study

We collected 40 glioma samples, including 19 males and 21 females, and 8 cases with WHO grade II, for a total of 10 and 22 patients with WHO grade III and IV, respectively (Table 1). Furthermore, we used data from the CGGA database that contains a large number of glioma gene expression profiles and a vast amount of clinical information data to further analyze the relationship between \textit{GNG5} expression and the clinical features of glioma. RNA sequencing data of 749 patients, including 442 males and 307 females, with complete clinical information, were screened from 1018 samples based on the CGGA database. In total, we analyzed 502, 222, and 25 cases of primary, recurrent, and secondary gliomas, respectively. An analysis of the pathological results showed that there were 218, 240, and 291 cases with WHO grade II, grade III, and grade IV gliomas, respectively. In addition, 40.28\% of the patients (n=410) showed mutations in \textit{IDH}, and 33.30\% patients (n=339) were \textit{IDH} wildtype. Only 15.23\% of patients (n=155) showed a 1p19q co-deletion, whereas 58.35\% (n=594) did not have this genotype. These results are summarized in Table S3.

Table 1 Clinical information of 40 glioma samples
| Gender      | Group | N (%) | Group      | N (%)    | P value |
|-------------|-------|-------|------------|----------|---------|
| Female      | High  | 21 (52.5%) | Low GNG5  | 13       | 0.2049  |
| Male        |       | 19 (47.5%) |            |          |         |
| Clinical stage |     |             |            |          | <0.001* |
| WHO II      | High  | 8 (20%)    | Low GNG5  | 0        |         |
| WHO III     |       | 8 (25%)    |            | 0        |         |
| WHO IV      |       | 22 (55%)   |            | 20       |         |
| Age (years) | <=47.15 | 21 (52.5%) |   >47.15  | 19 (47.5%) | 0.5273  |
| Staining of IDH1 R132H |   |             | Positive   | 18 (45%) | 0.0309* |
|             |        |            | Negative   | 22 (55%) |         |

High or low is divided by the median value. Chi-square or Fisher’s exact test. *P < 0.05 was considered statistically significant.

GNG5 is highly expressed in gliomas

We analyzed GNG5 expression in different tumors and control groups using GEPIA, including 9,663 samples from 33 tumors and 5,540 corresponding control samples. GNG5 expression was elevated in tumor tissues including glioblastoma (GBM), lower-grade glioma (LGG), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), skin cutaneous melanoma (SKCM), and uterine carcinosarcoma (UCS), and showed significantly lower expression in acute myeloid leukemia (LAML) relative to the control group (Figure 1A). These findings suggest that GNG5 is highly expressed in a variety of tumors. To further reveal the expression level of GNG5 in glioma and normal brain tissues based on transcriptome data in TCGA database and chip data in GSE131273 dataset, respectively. And the results showed that GNG5 expression was significantly higher in gliomas relative to normal brain tissue (Figure 1B, C, P < 0.001), which was consistent with the results of RT-qPCR (Figure 1D, P < 0.05). Furthermore, IHC staining results based on clinical samples showed that the expression level of GNG5 in gliomas was significantly higher than that in control groups and higher in high-grade gliomas than in low-grade gliomas (Figure 1E), and this result was also confirmed in the HPA database (Figure S1A). The above results were validated by different databases and clinical samples from transcriptome and protein levels, revealing that GNG5 is significantly highly expressed in gliomas.

High expression of GNG5 is a predictor of poor prognosis

Analysis of follow-up results of 40 glioma patients showed that patients with high expression of GNG5 had shorter OS (Figure 1F, P < 0.05), and this result was confirmed in multiple datasets containing large samples. The average follow-up time for the 749 patients whose data for survival analysis is included in this study was 3.22 years. The results showed that high expression of GNG5 is significantly correlated with reduced survival of glioma patients (Figure 2A, P < 0.001); these results were verified based on TCGA database and GSE53733 dataset (Figure S1B, C). Moreover, a time-dependent receiver operated characteristic (ROC) analysis showed that the values for area under the ROC curve (AUC) were 0.714, 0.792, and 0.821 for one, three, and five-year OS, respectively (Figure 2B). Therefore, our results indicate that GNG5 may serve as a biomarker for glioma patients, especially for the five-year OS group.

The results of a univariate analysis suggest that the high expression level of GNG5 in glioma patients is associated with reduced OS, primary recurrence or secondary (PRS) type, histological type, pathological grade, age, chemotherapy status, presence of mutations in the IDH gene, and a 1p/19q co-deletion (Figure 2C). Additionally, the multivariate analysis results indicate that the expression of GNG5, PRS type, pathological grade, age, chemotherapy status, presence of mutations in the IDH gene, and 1p/9q co-deletion were independently correlated with OS (Figure 2D). The above data indicate GNG5 may serve as a prognostic factor and increased GNG5 expression is associated with poor OS.

Relationship between GNG5 and the underlying molecular and clinical characteristics in glioma patients

To clarify the correlation between highly expressed GNG5 in gliomas and clinicopathological features, we first analyzed the clinicopathological information of 40 glioma patients and found that the expression level of GNG5 was significantly correlated with the pathological grade of gliomas (Table 1). To explore whether the expression level of GNG5 is also associated with more clinicopathological features in a large dataset, we further analyzed the relationship between GNG5 expression level in the 749 samples from the CGGA database and the various tumor subtypes, their pathological classification, molecular
classification, tumor treatment, and the age of the patients. The results indicated that the GNG5 expression level in gliomas was significantly correlated with the pathological grade and the age of the patients (Figure 3A, C, P<0.001). The average level of GNG5 expression in tissue from patients with recurrent gliomas was higher than in patients with primary and secondary tumors (Figure 3B, P<0.001). Similar results were found based on correlation analysis between GNG5 expression level in gliomas and different pathological subtypes, such as the expression levels of GNG5 in recurrent astrocytoma, recurrent anaplastic astrocytoma, and recurrent anaplastic oligodendroglioma were higher than that in corresponding primary pathological subtypes, as shown in Figure 3G.

Reports from research conducted previously show that patients with IDH mutations or a 1p/19q co-deletion had longer survival and better prognosis [25]. Interestingly, we found that the expression level of GNG5 in patients with mutations in the IDH gene was lower than in patients with wildtype IDH (Figure 3D). Similarly, patients with a 1p/19q co-deletion had significantly lower expression of GNG5 than in patients without the co-deletion (Figure 3E). Furthermore, to visualize the association between GNG5 expression and IDH mutation in clinical samples, we performed IHC staining on clinical samples, which showed a significant correlation between IDH mutation and GNG5 expression levels in gliomas (Table1, Figure S2). Together, these results further indirectly reveal that the expression of GNG5 correlates with the prognosis of glioma patients. When a grouping of patients based on whether they received chemotherapy or not was performed, we found that patients who received chemotherapy showed an upregulation of GNG5 expression relative to those who did not receive chemotherapy (Figure 3F). However, radiotherapy did not affect GNG5 expression (data was not shown). One explanation for this observation may be that the surgical resection of the tumor and subsequent chemotherapy. Taken together, these results indicate that a correlation exists between GNG5 expression and the molecular and clinical characteristics of glioma.

**Co-expression analysis of GNG5**

To further explore the function of GNG5, the limma package (Bioconductor) was used in the R statistical software to analyze the coexpression genes with GNG5. We found 4517 genes associated with GNG5 (correlation coefficient (Cor) > 0.5, P < 0.001), of which 29 genes were negatively correlated with GNG5 and a total of 4488 showed a positive correlation. A heatmap of the top 20 genes associated with GNG5 is shown in Figure 4A and the five genes showing significant positive or negative correlation with GNG5 is shown in Figure 4B. The five genes that showed positive correlation with GNG5 expression were RPF1 (Cor = 0.895, P = 0.000), AK2 (Cor = 0.893, P = 0.000), TMSB4X (Cor = 0.875, P = 4.249 x 10^{-322}), POP4 (Cor = 0.874, P = 4.247 x 10^{-320}) and RER1 (Cor = 0.866, P = 1.400 x 10^{-307}) (Figure 4C-E and S3A, B), while the top five genes that were negatively correlated with GNG5 expression were RIMS1 (Cor = -0.665, P = 4.201 x 10^{-131}), CDYL2 (Cor = -0.61, P = 6.459 x 10^{-105}), RN7SL4P (Cor = -0.605, P = 1.370 x 10^{-102}), ATRNL1 (Cor = -0.595, P = 1.953 x 10^{-98}) and TUB (Cor = -0.589, P = 3.049 x 10^{-96}) (Figure 4F-H and S3C, D). Thus, GNG5 expression is correlated with various genes in gliomas.

**GNG5 regulates signaling pathways, including cell adhesion molecules in glioma**

GSEA was used to identify GNG5 related signaling pathways involved in gliomas. A P<0.05 and a false discovery rate (FDR) <0.25 represented a significant enrichment in the results (in the enrichment of MSigDB Collection). Five pathways, including those involving the ECM-receptor interaction, focal adhesion, cell adhesion molecules, toll-like receptor signaling pathway, and nod-like receptor signaling pathway, showed significantly differential enrichment in samples from patients showing the GNG5 high expression phenotype based on NES, NOM P-values, and FDR values (Figure 5A, S3E-H; Table 2), indicating a potential role for GNG5 in the development of glioma. Since cell adhesion molecules have an essential role in the proliferation and migration of tumors, to clarify whether glioma cells are involved in cell adhesion molecule regulation, we performed in vitro experiments. We found that decreasing the expression level of GNG5 in glioma cells could significantly affect the expression of VCAM1, ICAMI, CDH2, and SDC2, which are key molecules of cell adhesion molecules (Figure 5B, C). Therefore we are more confident that GNG5 can exert its biological effects by regulating different signaling pathways including cell adhesion molecules in glioma.

**Table 2** Signaling pathways enriched in high expression of GNG5.
| Gene set name                                      | NES | NOM  | P-value | FDR  |
|----------------------------------------------------|-----|------|---------|------|
| KEGG_ECM_RECEPTOR_INTERACTION                      | 1.85| 0.008| 0.1     |      |
| KEGG_FOCAL_ADHESION                                | 1.81| 0.004| 0.076   |      |
| KEGG_CELL_ADHESION_MOLECULES_CAMS                 | 1.77| 0.026| 0.096   |      |
| KEGG_TOLL LIKE_RECEPTOR_SIGNALING_PATHWAY          | 1.76| 0.002| 0.092   |      |
| KEGG_NOD LIKE_RECEPTOR_SIGNALING_PATHWAY           | 1.75| 0.016| 0.093   |      |

**GNG5 is related to the immune microenvironment**

Next, we explored whether there is a correlation between GNG5 expression and the tumor immune microenvironment. We analyzed 29 immune-system related gene sets characterizing different types and functions of immune cells (Table S4). Based on data from the CGGA database, ssGSEA was used to quantify and hierarchically cluster immune cells in tumor samples into three groups. According to the clustering heat map of immune cell gene sets in the three groups, a high immunity group (high-immune), medium immunity group (mid-immune), and low immune activity group (low-immune) were defined (Figure 6A). Moreover, the high-immune group had a significantly higher immune score than that of the low-immune group, though the tumor purity showed the opposite characteristics (Figure 6B, C).

Interestingly, we found that the expression of GNG5 was significantly increased in the high-immune group, and decreased in the low-immune group (P<0.001, Figure 6D). Further, we used TIMER to analyze the relationship between GNG5 expression and infiltration abundance of six immune cells (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) after purity correction in glioma [22, 26]. We found that GNG5 expression was positively correlated with the infiltrating abundance of CD8+T cell (Cor = 0.154, P = 7.29 x 10^{-4}), B Cell (Cor = 0.453, P = 1.40 x 10^{-25}), macrophages (Cor = 0.544, P = 9.10 x 10^{-38}), CD4+T cells (Cor = 0.549, P = 6.68 x 10^{-39}), neutrophils (Cor = 0.554, P = 1.25 x 10^{-39}), dendritic cell (Cor = 0.582, P = 2.06 x 10^{-44}) in LGG. B Cell (Cor = 0.112, P=0.022), macrophages (Cor = 0.179, P=0.000), and dendritic cells (Cor=0.241, P=5.88 x 10^{-7}) were corrected with GNG5 expression in GBM (Figure 6E). These results suggest that GNG5 may be a potential factor influencing the immune microenvironment in glioma.

**Downregulated GNG5 could inhibit glioma cell proliferation and migration**

RT-qPCR results showed that the expression level of GNG5 in many kinds of glioma cells was significantly higher than that in HA cells (Figure 7A). To explore the role of GNG5 in gliomagenesis, we constructed and screened siRNA sequences targeting GNG5 (Figure 7B, Table S1). To observe the effect of downregulated GNG5 on migration and proliferation of tumor cells, we established a U251 glioma cell line with stable knockdown of GNG5. MTT assay indicated that the cell proliferation ability decreased obviously after reducing the expression of GNG5 (Figure 7C), which was similarly confirmed by cell clonogenic assay (Figure 7D-E) and Ki67 IF staining (Figure 7F-G). In addition, we also found that the migration ability of tumor cells was reduced after the reduction of GNG5 expression and was significantly different from that of the control group (Figure 7H-I, P< 0.001). Compared with the control group, the number of glioma cells migrated significantly decreased in shGNG5 group (Figure 8A-B). Further, we
found that knockdown of GNG5 could arrest the cells in the G1 phase (Figure 8C-D), suggesting that GNG5 may play a biological function by participating in the cell cycle process. Moreover, subcutaneous tumorigenesis experiments showed that downregulation of GNG5 could significantly inhibit the growth of glioma tissues (Figure 9, \( P < 0.05 \)). The results of in vivo and in vitro experiments indicated that GNG5 could participate in the malignant progression of gliomas by participating in the proliferation and migration of gliomas.

**Discussion**

G-proteins consisting of \( \alpha \), \( \beta \) and \( \gamma \) subunits are also known as GTP binding proteins, which mediate the transduction of a variety of hormones and neurotransmitters across the membrane and subsequently trigger a series of physiological and biochemical reactions in cells [27, 28]. G-proteins regulate basic life processes such as cell metabolism, secretion, growth, proliferation, differentiation, distortion, pathological changes, and cell death [4]. GNG5, a subunit of G-protein, has been reported to promote the proliferation and migration of tumor cells [29]. However, to date, few studies have reported the role and mechanism of action for GNG5 in tumors.

To explore the expression patterns of GNG5 in various tumors, we searched for GNG5 in the GEPIA database. We found that GNG5 was significantly highly expressed in a variety of cancers, including gliomas. (Figure 1A). To further understand the role of GNG5 in tumors, we focused on its role in glioma. We obtained GNG5 expression data from TCGA and GEO databases and clinical samples. Our results showed that GNG5 expression was increased in glioma based on data from sequencing, microarrays, and qPCR data (Figure 1B-D). Previous studies have suggested that GNG5 shows elevated expression in endometrial cancer and in infiltrating ductal carcinoma of the breast [15, 16]. Furthermore, POP4, a GNG5 coexpressing promoter gene, is highly expressed in prostate cancer cells, while RIMS1, a GNG5 coexpressing suppressor gene, is lowly expressed in craniopharyngioma [30, 31]. Moreover, we validated the expression pattern of GNG5 protein in glioma samples obtained from clinic and HPA databases, and a high level of GNG5 was observed in glioma (Figure 1E, S1A). Moreover, our follow-up results suggested that GNG5 highly expressed in gliomas could significantly decrease patients' OS. Therefore, we hypothesized that GNG5 might be an oncogene in glioma.

To elucidate in large sample data that GNG5 is an oncogene and can lead to a poorer prognosis for patients, we obtained clinical information on more than a thousand glioma samples from TCGA, CGGA, and GEO databases. The results show that increased expression of GNG5 could indeed cause a poor prognosis of glioma patients. Moreover, as shown in figure 2B, GNG5 has a high diagnostic value and can be used as a biomarker to predict glioma prognosis. Additionally, genes coexpressed with GNG5, such as AK2, are highly expressed in lung cancer and can lead to poor prognosis in patients [32]; besides, RER1 as a coexpressed gene with GNG5, could promote the progression of pancreatic cancer and reduce the survival rate of patients [33]. Although the high expression of GNG5 in glioma could reduce patients' OS rate, whether the expression of GNG5 is necessarily correlated with such a prognosis for glioma patients was further examined. Results from univariate and multivariate analyses showed that increased
expression of \( GNG5 \) is an independent risk factor for glioma patients. The above results indicate that \( GNG5 \) is indeed an oncogene in gliomas and is predictive of a worse prognosis for patients. Furthermore, we found that \( GNG5 \) expression levels are closely related to the clinical features associated with glioma prognosis, especially with the pathological grade and primary recurrence status of glioma (Figure 3, Table 1). However, the results based on clinical data were not as significant in the correlation analysis between the expression level of \( GNG5 \) and age in the CGGA database, possibly due to the small sample size. Some studies have reported that glioma patients with a mutation in the \( IDH \) gene and a 1p/19q co-deletion have a better prognosis [34, 35]. As we predicted, we found that the average expression level of \( GNG5 \) was lower in samples from patients with mutations in the \( IDH \) gene and those with a 1p/19q co-deletion. Moreover, since approximately 90% of \( IDH \) mutations occur in the \( IDH1 \) gene and more than 85% of mutations occur at R132H [25, 36], through IHC analysis, we found that \( GNG5 \) was significantly associated with IDH mutation at the protein level. The above results reveal \( GNG5 \) as an oncogene in gliomas from different perspectives and portend a worse prognosis for patients. However, the mechanism explaining how \( GNG5 \) leads to a poor prognosis for glioma patients is yet to be elucidated.

We next undertook a GSEA to understand the functioning of \( GNG5 \) in glioma. We found that high expression of \( GNG5 \) promotes the activation of a series of cancer-related signaling pathways (Figure 6). Similar interactions have been described previously; for example, the ECM-receptor interaction is involved in the proliferation and invasion of cancer cells [37, 38], and focal adhesion is involved in the metastasis and invasion of cancer cells [39]. Additionally, the toll-like receptor promotes the immune evasion of glioma by down-regulating the MHC II molecules in microglia [40], and cell adhesion molecules and the nod-like receptor play a significant role in the proliferation, invasion, and metastasis of glioma cells [41-44]. A particular gene may play a role in a variety of signaling pathways in the pathological process of diseases. Used as a tool to reveal the molecular mechanisms underlying diseases, GSEA is widely used and has high reliability. Compared with traditional methods of analysis, GSEA has the advantage of using large sample sizes and can avoid the biases inherent in experimental results caused by artificial threshold setting [17, 45]. Furthermore, based on the results of GSEA, we found that decreasing the expression levels of \( GNG5 \) could significantly affect the expression of key molecules of cell adhesion molecules signaling pathway, such as \( VCAM1, ICAM1, CDH2, \) and \( SDC2 \) [47], from the mRNA and protein levels. It is well known that cell adhesion molecules play an essential role in tumors' malignant progression [46]. Moreover, previous studies have shown that downregulation of \( VCAM1, ICAM1, \) and \( CDH2 \) can inhibit cancer cell proliferation and migration [46, 48-50]. In our study, we found that the expression of \( VCAM1, ICAM1, CDH2 \) was down-regulated, while the expression of \( SDC2 \) was up-regulated after knocking down the expression of \( GNG5 \) in glioma cells. The expression level of \( SDC2 \) was found to be up-regulated after knocking down the expression of \( GNG5 \) in the present study, possibly related to reducing its methylation level. Previous studies have shown that \( SDC2 \) has a higher methylation level in cancer tissues [51, 52], which indirectly supports our hypothesis. These results further illustrate that downregulation of \( GNG5 \) can inhibit glioma cell proliferation and migration through the cell adhesion molecules pathway. Together, we believe that the signaling pathways enriched with \( GNG5 \) in glioma, as revealed in our study,
could provide the basis for further investigations into the specific mechanisms underlying \textit{GNG5} functioning in glioma.

Recently, immunotherapy has been shown to be a new treatment strategy for glioma, and genes related to glioma immunity have been reported frequently [53, 54]. However, the association of \textit{GNG5} with glioma immunity has not been reported. Our results suggest that \textit{GNG5} may participate in the malignant progression of glioma through ECM-receptor interaction, toll-like receptor pathway, and the nod-like receptor signaling pathways, which are reported to be related to the immune response process [55, 56]. Therefore, we hypothesized that \textit{GNG5} might be involved in the immune response in glioma. To verify this, we analyzed 29 immune-associated gene sets in glioma samples using ssGSEA and ESTIMATE. The results showed that the expression of \textit{GNG5} in glioma was correlated with immune activity (Figure 7D). Previous studies have reported a correlation between programmed death-1/ligand-1 (PD-1/PD-L1) and immune activity in tumors in triple-negative breast cancer [19]; Moreover, a large number of studies have shown that ssGSEA and ESTIMATE are highly reliable in the evaluation of tumor immune stratification and immune invasion analysis [21, 57-60]. Furthermore, we analyzed the correlation between the expression of \textit{GNG5} and infiltration level of tumor-infiltrating immune cells using TIMER. The results suggest that \textit{GNG5} is correlated with the infiltration abundance of various immune cells in glioma, especially macrophage, dendritic cells, and neutrophils in low-grade glioma (Figure 7E). Though there are currently few reports describing the relationship between the G-protein family and cancer immunity, correlations between single genes and cancer immunity have been observed. For example, CD70, which is highly expressed in glioma, can promote macrophage infiltration into a tumor [53], and JAK1 was shown to be positively correlated with the infiltration of various immune cells in breast cancer, such as CD8+ T cells and dendritic cells, etc. [61]. Additionally, LAYN expression was positively correlated with the infiltration of CD4+T and CD8+T cells, macrophages, neutrophils, and dendritic cells in colon and gastric adenocarcinomas [62]. These results indicate that \textit{GNG5} is a highly expressed gene in glioma, is associated with immune activity, and future research could help establish the immune response related to \textit{GNG5} function in glioma.

In order to study the function of \textit{GNG5} in glioma cells \textit{in vitro} and \textit{in vivo}, we knocked down the expression level of \textit{GNG5} in glioma cells by cell transfection technology. We found that knockdown of the expression level of \textit{GNG5} significantly reduced the metastatic and proliferative abilities of glioma cells. This effect may be related to the involvement of \textit{GNG5} in the cell cycle, as downregulated \textit{GNG5} can significantly arrest glioma cells in the G1 phase. \textit{In vivo} experiments further confirmed that the growth tendency of tumor tissue could be obviously inhibited by downregulated \textit{GNG5}.

Although we used bioinformatics analysis and large datasets to uncover the role of \textit{GNG5} in glioma, our study has a few limitations. First, as extensive clinical sample information in the public databases is not always available, it is difficult to comprehensively evaluate the correlation between \textit{GNG5} and the clinical features of glioma patients. However, it is difficult to avoid the problem of the lack of clinical sample information in public databases, as this data is collated from different research centers. Second, through comprehensive biological analysis, we found that GNG5 may be involved in a variety of biological
processes to affect the prognosis of gliomas, although the in vitro and in vivo experiments involved in this study are only preliminary verification. However, our results confirm the reliability of biological analysis results, which lays a foundation for further study of various mechanisms and elucidating the mechanism of disease in the future.

Conclusion

Our study shows that \textit{GNG5} is highly expressed in gliomas, and this expression is correlated with various molecular and clinical features of glioma patients. High expression levels of \textit{GNG5} predict a poor prognosis in glioma patients. Decreasing the expression of \textit{GNG5} can inhibit glioma cell proliferation and migration. Additionally, \textit{GNG5} may participate in glioma's pathological progress through the signaling pathways related to cancer, such as cell adhesion molecules signaling pathway. These results indicate that \textit{GNG5} is a novel oncogene in glioma and could provide a potential biomarker for diagnosing and treating gliomas.

Abbreviations

AUC: area under the receiver operated characteristic curve; CGGA: Chinese Glioma Genome Atlas; CNS: central nervous system; Cor: correlation coefficient; EMT: epithelial-mesenchymal transition; FDR: false discovery rate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GBM: glioblastoma; GEO: Gene Expression Omnibus; GEPIA: Gene Expression Profiling Interactive Analysis; HPA: Human Protein Atlas; IDH: isocitrate dehydrogenase; IHC: immunohistochemical; LAML: acute myeloid leukemia; LGG: lower-grade glioma; NES: normalized enrichment scores; NOM: nominal; OS: overall survival; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PD-1/PD-L1: programmed death-1/ligand-1; PRS: primary recurrence or secondary; ROC: receiver operated characteristic; SKCM: skin cutaneous melanoma; ssGSEA: single-sample gene set enrichment analysis; TCGA: The Cancer Genome Atlas; TIMER: Tumor IMmune Estimation Resource; UCS: and uterine carcinosarcoma; WHO: World Health Organization.

Declarations

\textit{Ethics approval and consent to participate}

All protocols of this study were approved by the Ethics Committee of the First Clinical College of Harbin Medical University. All clinical samples involved in this study have been informed consent of the patients. The public data involved in this study were all downloaded from public databases, therefore there was no new informed consent required.

\textit{Consent for publication}

Not applicable.
Availability of data and materials

The datasets generated and/or analysed during the current study are available in the CGGA database (http://www.cgga.org.cn/), TCGA database (https://portal.gdc.cancer.gov/), and datasets from GEO (https://www.ncbi.nlm.nih.gov/geo/). The data of RT-qPCR and cell function experiments during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' Contributions

Hong Shen, Zhenfeng Jiang, Yanzheng Gao, and Zhiguo Lin conceived and designed the experiment. Binchao Liu, Binfeng Liu, and Zhishuai Ren analyzed and checked the data. RNA extraction, RT-qPCR and follow-up of glioma patients were carried out by Han Xian, Yanbiao Wang and Yan Shi. Wang Zhang, Miaomiao Jiang and Zhendong Liu performed cell function experiments, immunohistochemical staining and subcutaneous tumorigenesis in nude mice. Wang zhang and Meng Na wrote the manuscript. All authors read and approved the final manuscript.

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