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G-protein Coupled Receptor 34 Promotes Gliomagenesis by Inducing Proliferation and Malignant Phenotype via TGF-Beta/Smad Signaling Pathway

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Abstract

Background: G-protein coupled receptor 34 (GPR34) is involved in cell motility, differentiation, and mitosis. GPR34 was reported to be highly expressed and play an oncogenic role in several solid tumors. Here, we investigated the mechanisms underlying how GPR34 promotes glioma progression. **Methods:** Bioinformatic analysis was performed on RNA-seq and clinical data from the gene expression omnibus (GEO), cancer genome atlas (TCGA), and Genotype-Tissue Expression (GTEx) databases. TIMER database and single-sample GSEA (ssGAEA) method were used to investigate the association between the GPR34 expression and immune infiltration level in glioma. Cox regression analysis was employed to ascertain whether the risk signature was an independent prognostic indicator for glioma. The viability and migratory/invasive potential of glioma cells were assessed using Cell Counting Kit-8, colony formation, wound healing, and Transwell assays. **Results:** We found that GPR34 expression was positively correlated with immune infiltration level and that high GPR34 level may be associated with poor prognosis in glioma. We further found that GPR34 may serve as an independent prognostic marker and prediction factor for the clinicopathological features of glioma. We showed that knocking down GPR34 attenuated the viability and migratory/invasive capacity of glioma cells (U251 and LN229), while GPR34 overexpression exerted the opposite effects. Additionally, core enrichment in the GSEA analysis indicated that GPR34-mediated gliomagenesis was associated with the cell cycle arrest, epithelial–mesenchymal transition (EMT), and activation of the TGF-β/Smad pathway; furthermore, inhibiting TGF-β/Smad signaling using LY2157299, a TGF-β inhibitor, reversed the oncogenic effects and malignant phenotype associated with GPR34 overexpression. **Conclusion:** GPR34 enhances the malignancy and carcinogenesis of glioma by promoting an EMT-like process, G1/S phase cell cycle transition, and TGF-β/Smad signaling. Accordingly, GPR34 likely functions as an oncogene in glioma and may represent a potential therapeutic target for this cancer.

Keywords

GPR34, glioma, EMT, cell cycle, TGF-beta/Smad signaling pathway, bioinformatic analysis

Abbreviations

CNS, central nerve system; DAB, 3-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, gene expression omnibus; GPR34, G-protein coupled receptor 34; GTEx, genotype-tissue expression; HGG, high-grade glioma; IHC, immunohistochemistry; LGG, low grade glioma; NC, negative control; OD, optical density; PVDF, polyvinylidene

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difluoride; qRT-PCR, quantitative real-time PCR; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TCGA, cancer genome atlas; WB, western blotting; WHO, World Health Organization.

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Introduction

Glioma is one of the most commonly diagnosed malignant tumors of the central nervous system (CNS), accounting for 80% of all CNS malignancies.[1] This tumor is characterized by extensive infiltrative growth in the brain parenchyma, but rarely metastasizes to distant organs. Despite the advances in multimodal therapeutic strategies, which combine microsurgery, chemotherapy, and radiotherapy, patients with glioma have a dismal prognosis, with a 5-year survival rate of <16 months.[2–4] Although several molecular targets and biomarkers have been established in glioma with the advent of biomedical technology,[5] most are of limited use in glioma diagnosis and treatment. This highlights the need for an in-depth investigation into the mechanisms involved in gliomagenesis and the identification of gene targets closely associated with prognosis to provide a theoretical basis for the early diagnosis of this tumor.

G-protein coupled receptor 34 (GPR34), a member of the G-protein coupled receptor (GPR) superfamily, is a seven-transmembrane protein encoded by a 1143-bp open reading frame. Multiple transcriptomic-based studies have reported that the expression of GPR34 was aberrantly elevated in astrocytes and, to a greater extent, also microglia, suggestive of a crucial role in microglial function.[6–9] Schoneberg et al reported that the number and morphology of microglia were altered in the CNS of GPR34-deficient mice. The authors further found that tumor necrosis factor-alpha (TNF-α) accumulation was increased and phagocytic activity was attenuated in microglia of these animals.[10] Although the GPR34 level was found to be upregulated in microglia following treatment with the demyelinating toxin cuprizone, GPR34 expression was downregulated in microglia of mice with kainic acid-induced epilepsy.[11,12] These observations suggest that GPR34 might play a crucial role in neuronal diseases *via* the regulation of microglial activity. In addition to its effect on neuroinflammation, GPR34 has also been reported to promote the malignant phenotype of many cancers, including cervical cancer, MALT lymphoma, and gastric adenocarcinoma,[13–15] as well as exert a wide range of cellular physiological functions, including in transcription and tumorigenesis, as well as in cell motility, differentiation, and growth.[16] However, whether GPR34 has prognostic value in glioma is unknown, as are the mechanisms underlying the effects of GPR34 in this tumor.

Here, we first identified a significant correlation between GPR34 overexpression and poor survival outcomes in patients with glioma. Subsequently, the oncogenic function of GPR34 and the associated mechanisms were explored in a series of *in vitro* experiments. Our results indicated that GPR34 enhances the malignancy and carcinogenesis of glioma by promoting G1/S phase cell cycle transition, epithelial–mesenchymal transition (EMT), and TGF-β/Smad signaling, and may represent a therapeutic target for the treatment of glioma.

Material and Methods

Patients and Tissue Sample Collection

Tissue samples were collected from 60 cases of glioma patients in Linyi People's Hospital. In general, the samples include 40 cases of diffuse gliomas and 20 cases of circumscribed glioma. According to the glioma classification (WHO CNS5 version),[4] the specimens were further reclassified as follows: (1) Adult-type diffuse glioma: 40 cases; (2) Pediatric-type diffuse high-grade glioma: 7 cases; (3) Pediatric-type diffuse high-grade glioma: 5 cases; (4) Circumscribed astrocytic glioma: 8 cases. For convenience, therapy and prognosis of tumor are reviewed in an order of various types of gliomas in a certain grade: WHOI:8 cases; WHOII:17 cases; WHOIII: 13 cases; WHOIV: 22 cases. CNS WHO grades 1-2 belong to low-grade glioma (LGG), while CNS WHO grades 3-4 belong to high-grade glioma (HGG). All normal brain tissue samples were obtained by decompression surgery for traumatic brain injury and glioma tissue specimens were obtained during surgical resection. All glioma samples were immediately snap-frozen at −80 °C. Glioma was confirmed by histopathological diagnosis and classified according to World Health Organization (WHO) criteria. Prior written informed consent was obtained from all patients and/or their legal guardians. This study protocol was also performed in accordance with the Declaration of Helsinki.

Cell Lines and Culture

The normal astrocyte cell line HA1800 and glioma cell line PT2 were donated by Central Laboratory of Linyi People's Hospital, the human glioma cell lines U251(CB55531061), TJ905(CB 45855702), A172(CB 65789047), SF295(CB 95777238), and LN229(CB 89733514) used in this study were purchased from the Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and incubated at 37 °C with 5% CO_2.

RNA Extraction and Real-Time qPCR Analysis

Total RNA was extracted from brain tissues and glioma cells using TRIzol reagent (Invitrogen, Waltham, MA, USA) and reverse transcribed into cDNA using Transcript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing,

China). qPCR was performed using Biosystem SYBR Green Master Mix (Toyobo, Japan). All specific primers in this research were designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The primer sequences for GPR34 amplification were 5′-CGACAACTTCAGTCAGCAGCTG-3′ (forward) and 5′-GGTTGGTCGCTATGATTGGTTA-3′ (reverse). GAPDH, 5′-CGGAGTCAACGGATTTGGTCGTATTGG-3′ (forward) and 5′-GCTCCTGGAAGATGGTGATGGGATTTCC-3′(reverse).
Each reaction was performed in triplicate. The mRNA level of GPR34 was normalized to that of GAPDH and calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.[17]

RNA Interference and Gene Overexpression

GPR expression was knocked down using small interfering RNA (siRNA). The siRNA targeting GPR34 (sequence #1: forward 5′-GAAAGAAAUUGUUCACAAATT-3′, reverse 3′-UUUGUGAACAAUUUCUUUCTT-5′; sequence #2: forward 5′-CGAAUAAUGUAUCAUAUUATT-3′, reverse 3′-UAAUAUGAUACAUUAUUCGTT-5′) and negative control siRNA were purchased from Genechem Co. Ltd. To overexpress GPR34, the coding sequence was obtained from NCBI and synthesized to facilitate colony. The plasmid for GPR34 overexpression (Genechem Co. Ltd) was transfected into glioma cells using Lipofectamine3000 (Invitrogen) according to the manufacturer's protocol.

Cell Counting Kit-8

A Cell Counting Kit-8 (CCK-8) assay was used to detect cell viability. Cells were seeded in 96-well plates (3×10^4 cells per well) in 200 μL of medium. CCK-8 solution (10 μL) and 100 μL of DMEM without FBS were added to each well. After 1 h of incubation at 37 °C, the absorbance (optical density) of each well was measured at 450 nm and cell viability was calculated. All assays were independently performed in triplicate.

Colony Formation Assay

Transfected cells were seeded in six-well plate at a density of 500 cells per well and cultured in DMEM supplemented with 10% FBS for 2 weeks, fixed in 4% formaldehyde for 30 min, and stained with 1% crystal violet for 10 min. The colonies containing more than 60 cells were counted to exam clonogenicity. All assays were independently performed in triplicate.

Transwell Assay

The invasion assay was performed using a 24-well Transwell chamber (Corning, New York, USA) precoated with Matrigel (R&D System, USA). Approximately 5×10^4 cells in 100 μL of DMEM without FBS were placed in the upper Matrigel-coated chamber while 500 μL of DMEM containing 10% FBS was added to the lower chamber. After 24 h of incubation at 37 °C with 5% CO_2, the cells on the surface of the insert were removed using a cotton swab while the glioma cells that passed through the membrane (migrated cells) were stained with 1% crystal violet and counted under a microscope. Cell counts were expressed as the mean number of cells per field of view. All assays were independently performed in triplicate.

Wound Healing Assay

For the wound healing assay, transfected cells (5×10^5/mL) were seeded in a six-well plate and grown to 100% confluence. A pipette tip was used to make a scratch in the confluent cells. After washing twice with PBS, the plates were photographed under a microscope at 0, 24, and 48 h. The width of the scratch was used as a measure of cell migration.

Western Blot Assay

Equal amounts of protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electro-transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and incubated at 4 °C overnight with antibodies against GPR34 (1:1500), phosphorylated (p)-TGF-β (1:1000), p-Smad2 (1:1500), p-Smad3 (1:2500), Smad2/3 (1:1000 (all from Affinity), CDK2 (1:2500), CDK6 (1:2200), cyclin E (1:1500), MMP-2 (1:1500), MMP-7 (1:1000), ROCK1 (1:3000), E-cadherin (1:1500), N-cadherin (1:1500), Snail (1:1500), vimentin (1:2000), and GAPDH (1:2500 [all from Proteintech, Wuhan, China]). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2500, Affinity) at 37 °C for 3 h. Protein bands were visualized using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific). GAPDH served as a loading control.

Immunohistochemistry

Immunostaining was performed using an Ultra-Sensitive SP IHC Kit (Maxin Bio-technology, Beijing, China) according to the manufacturer's protocol. Sections were incubated with primary antibody targeting GPR34 (1:200, Proteintech) at 4 °C overnight, washed three times with PBS, and then incubated with HRP-conjugated polymers for 1 h. Following the addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin counterstaining, staining was analyzed under a bright field microscope (Nikon, Tokyo, Japan). Staining in the cytoplasm, membrane, and nucleus was considered positive and was scored according to the following scale, as previously described: 0, negative; 1, 0%-10%; 2, 10%-50%; 3, 50%-80%; 4, >80% positive cells.[15] Staining intensity was scored using the following scale: 0, negative; 1, low; 2, moderate; 3, strong. Scores ≤5 were defined as low GPR34 expression while scores >5 were defined as high GPR34 expression.

Immunofluorescence Staining

Glioma cells grown on coverslips (cultured to 70%-80% confluence) in six-well plates overnight were washed with PBS and

fixed in 4% paraformaldehyde for 15 min. The cells were then permeabilized using 0.1% Triton X-100 for 10 min, blocked with 5% BSA at 4 °C for 30 min, incubated with primary antibody, washed with PBS, and then incubated with secondary antibody. Nuclei were counterstained with DAPI at 4 °C for 10 min. Finally, the slides were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Flow Cytometry

Transfected cells (1×10^6 cells/mL) were harvested following trypsinization, resuspended in PBS, fixed in 70% cold ethanol at 4 °C overnight, and then incubated in PBS containing RNase A and propidium iodide (PI) (Beyotime) at 37 °C for 30 min. Cell cycle distribution was analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using Multi- Cycle software (version AV; Phoenix Flow Systems, San Diego, CA).

Bioinformatic Analysis

Glioma RNA-seq data from the Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases were downloaded from the University of California, Santa Cruz (UCSC) genome browser database (https://xenabrowser.net/). The raw data of five glioma microarray datasets were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The clinical data and related RNA-seq data from patients with glioma were obtained from CGGA (mRNA_array_325; mRNA_array_693 http://www.cgga.org.cn/). The Pearson's correlation coefficients of synergetic gene cluster analysis were calculated in R, the threshold values were set as adj.*P*.Value <.05 and $r > 0.7$. Gene set enrichment analysis (GSEA) was used to generate an ordered list of all the genes associated with the level of GPR34 and then to identify differentially enriched biological pathways between the high and low GPR34 expression groups. GPR34 expression was used as a phenotype label. Enriched biological pathways in each phenotype were ordered by normalized enrichment score (NES) and false discovery rate. Moreover, the R packages "ggplot2," "ggpubr," and "limma" "surfit" "ggsurvplot," among others, were utilized for visualizing the results of the bioinformatics analysis.

Statistical Analysis

All the experimental data were analyzed using Original 2018 and GraphPad Prism version 8 for Windows and presented as means ± SD. All experiments were performed in triplicate. The Student's *t*-test was used to analyze differences between two groups. Comparisons between three and more groups were determined using one-way ANOVA followed by Tukey's post-hoc test. Survival analysis was performed using the Kaplan–Meier (K–M) method. The χ^2 test was used to examine the relationship between GPR34 expression and clinicopathological characteristics. Univariate and multivariate Cox proportional hazards models were generated using R version 3.5.1. A *P*-value <.05 was considered statistically significant.

Results

GPR34 Expression was Upregulated in Glioma Specimens and Cell Lines

To preliminarily assess the GPR34 mRNA expression levels in several types of intracranial tumors, we first analyzed microarray GSE50161 from the GEO database. The results indicated that GPR34 mRNA expression was elevated in glioblastoma (GBM) and pilocytic astrocytoma tissues when compared with that in normal brain tissues and other primary brain tumor tissues (medulloblastoma and ependymoma) (Figure 1A). To further explore the expression patterns of GPR34 in normal brain and GBM tissues, we analyzed the GEO microarray data for glioma (GSE90598, GSE109857) and found that GPR34 expression was upregulated in GBM specimens (Figure 1B and C) ($P < .05$, $P < .001$, respectively). Next, to investigate GPR34 mRNA expression in different histological types of glioma tissues, we analyzed glioma datasets GSE4290 and GSE16011, and found that GPR34 expression increased with increasing glioma grade, and was highest in GBM (Figure 1D and E) ($P < .05$, $P < .01$, respectively). Glioma stem cells are a subpopulation of tumor cells with special ability to proliferation and differentiation in glioma, which are considered as the origin of glioma. Neural stem cells (NSCs) are capable of differentiating into astroglia, neuros, and oligodendrocytes, which are considered irreversible under normal condition and can potentially replace lose neural system after CNS injury and disease. Glioma cells and astrocyte were differentiated from GSCs and NSCs, respectively. In the GSE67089 microarray, CUX1mRNA was upregulated in GBM cells compared with astrocyte (NHA) ($P < .05$). Similarly, in terms of their progenitors, CUX1mRNA was aberrantly overexpressed in Mes-GSCs compared with NSCs ($P < .01$). Hence, we think CUX1 might exert a crucial role in gliomagenesis (Figure 1F). To further confirm the above results, we next evaluated GPR34 expression at both the mRNA and protein levels in non-tumor brain tissues ($n = 3$) and glioma tissues (LGG, $n = 3$; HGG, $n = 3$) obtained from Linyi People's Hospital using RT-qPCR and western blot assays, respectively. Consistent with that identified by bioinformatic analysis, the mRNA and proteinlevels of GPR34 were found to be aberrantly elevated in glioma tissues in tandem with increasing WHO grade (Figure 1G–I) ($P < .01$, $P < .001$, respectively). Finally, we examined the expression of GPR34 in astrocyte (HA1800) and glioma (A172, LN229, TJ905, PT2, U251, and SF295) cell lines, with the results revealing that the mRNA/protein levels of GPR34 were markedly higher in glioma cells (especially TJ905 cells) than in normal human astrocytes (Figure 1J–L) ($P < .01$, $P < .001$, respectively).

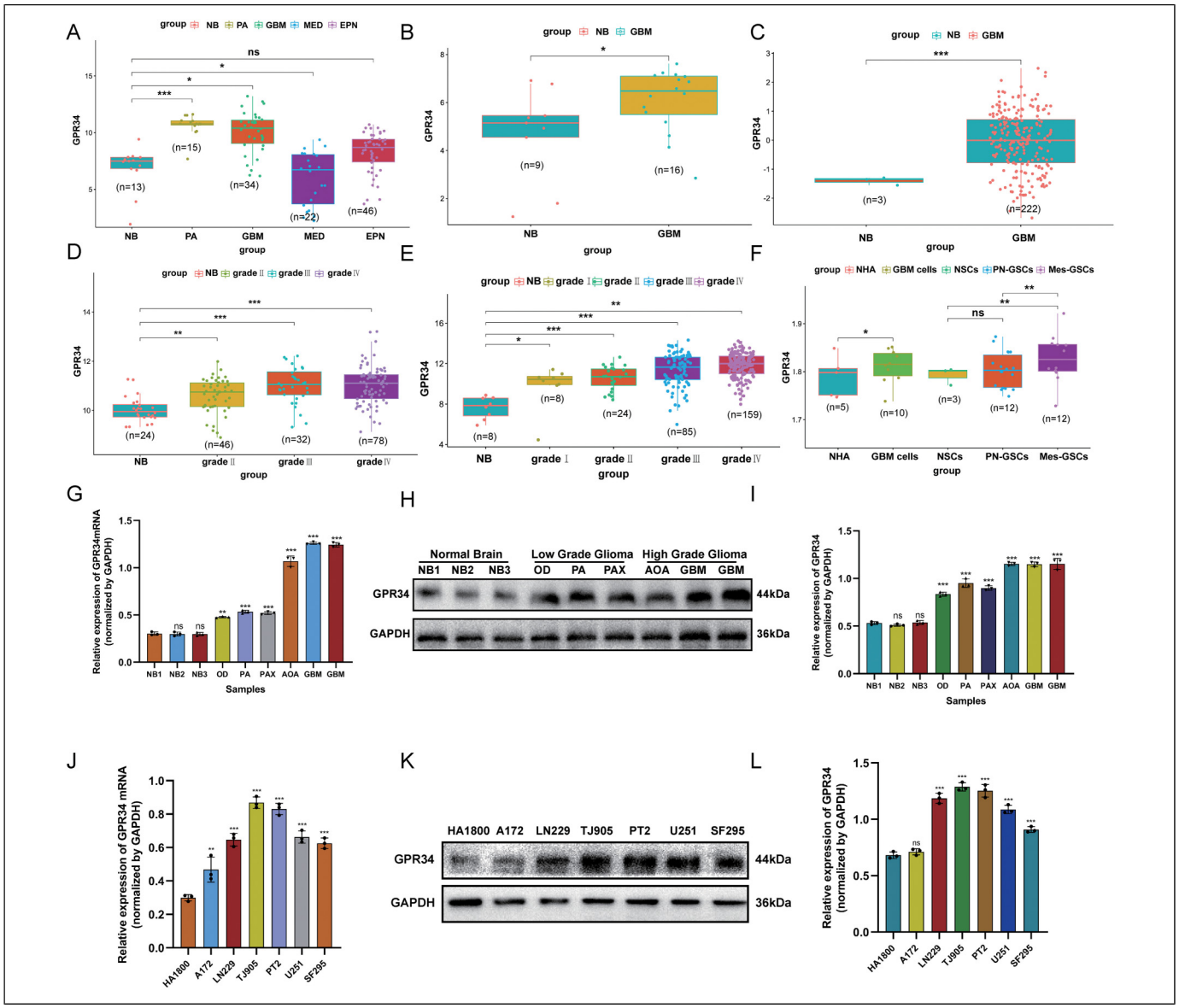

Figure 1. GPR34 was upregulated in glioma specimens and cell lines. (A) The expression level of GPR34 in normal brain (NB), glioblastoma (GBM), pilocytic astrocytoma (PA), medullablastoma (MED), and ependymoma (EPN). (B-E) Box plots implicating GPR34 expression levels in different pathological stage of glioma in GEO database. (F-H) qRT-PCR and western blot analysis of GPR34 in glioma tissues and non-tumor tissues collected from Linyi People's Hospital. (I-K) mRNA and protein level of GPR34 in human astrocyte cell lines and six glioma cells lines (A172, LN229, TJ905, PT2, U251, and SF295) measured by qRT-PCR and western blot. Data were presented as mean ± SD of three of separate experiments (*$P < .05$, **$P < .01$, ***$P < .001$, respectively).

Effects of GPR34 Expression on Immune Cell Infiltration

Infiltrating immune cells exerts an important role in inflammatory and immune responses against tumor cells, previous study has reported that GPR34 was mainly related to neutrophils,[18] hence, we hypothesized that GPR34 might affect the infiltration of immune cells in immune microenvironment of glioma. We determined Spearman correlation coefficients to access the potential association between various types of immune cells and GPR34 level via ssGSEA. In our results, GPR34 expression was positively associated with the abundances of macrophages ($r = 0.588$, $P < .001$), neutrophils ($r = 0.535$, $P < .001$), iDC ($r = 0.460$, $P < .001$), eosinophils ($r = 0.423$, $P < .001$), aDC ($r = 0.404$, $P < .001$), cytotoxic cells ($r = 0.331$, $P < .001$) as well as other cell types, however, GPR34 level was negatively associated with the abundance of pDC ($r = -0.206$, $P < .001$), Treg ($r = -0.217$, $P < .001$), and NK CD56 bright cells ($r = -0.399$, $P < .001$) (Figure 2B-J). We further examined the effect of GPR34 expression pattern on the immune infiltration, surprisingly, significant difference was detected in infiltration immune cells levels, mainly including macrophage, neutrophils, iDC, eosinophils, aDC, and cytotoxic cells ($P < .001$),

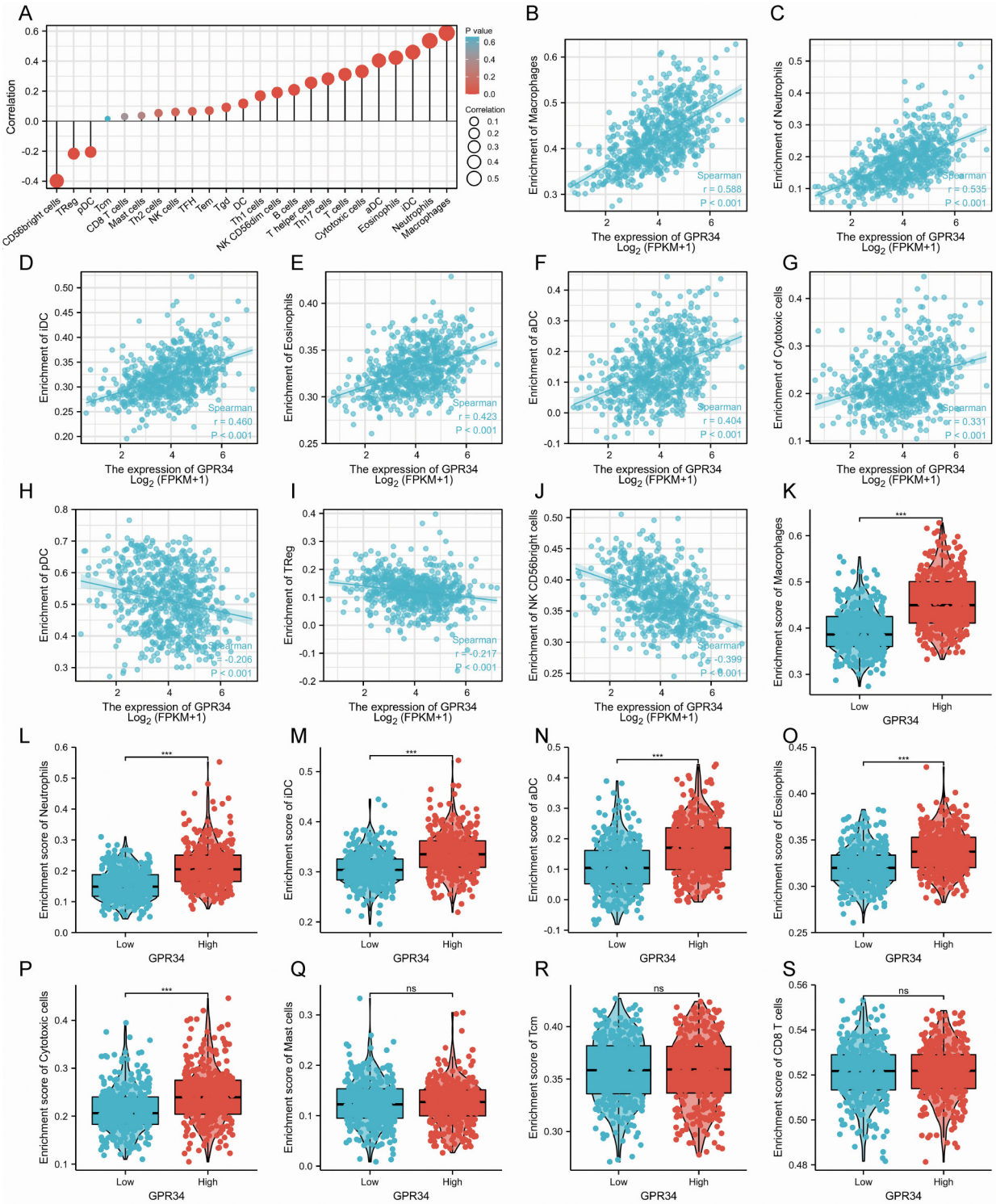

Figure 2. Correlation between immune cell infiltration and GPR34 levels. (A) Lollipop chart of GPR34 level in 24 types of immune cells. (B-J) Correlation between GPR34 expression and macrophage, neutrophils, iDC, eosinophils, aDC, cytotoxic cells, pDC, Treg as well as NK CD56 bright cells. (K-S) Violin plot showing the difference of macrophage, neutrophils, iDC, eosinophils, aDC, cytotoxic cells, mast cells, Tcm and CD8-T cells infiltration level between low- and high-GPR34 expression groups.

when GPR34 level was categorized into low and high groups (Figure 2K-S). While, we found no significant difference in Tcm cells, CD8-T cells, and mast cells. The results above indicated that glioma patients with GPR34 high expression were prone to present more immune cells (macrophage, neutrophils, iDC, aDC, etc) infiltrated than patients with GPR34-low.

Table 1. Univariate Analysis and Multivariate Analysis of Overall Survival in the Primary Cohort (TCGA).

		Univariate analysis		Multivariate analysis	
Characteristics	Total (*N*)	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
WHO grade	634				
G2	223	Reference			
G3	243	2.999 (2.007-4.480)	**<.001***	2.076 (1.177-3.662)	**.012***
G4	168	18.615 (12.460-27.812)	**<.001***	4.875 (1.261-18.850)	**.022***
1p/19q codeletion	688				
codel	170	Reference			
non-codel	518	4.428 (2.885-6.799)	**<.001***	1.575 (0.741-3.346)	.238
Primary therapy outcome	314				
PR	64	Reference			
CR	138	0.767 (0.266-2.213)	.623	0.841 (0.248-2.856)	.781
PD	112	6.176 (2.684-14.210)	**<.001***	5.232 (1.873-14.618)	**.002***
Age	695				
<=60	552	Reference			
>60	143	4.668 (3.598-6.056)	**<.001***	4.301 (2.330-7.940)	**<.001***
IDH status	685				
WT	246	Reference			
Mut	439	0.117 (0.090-0.152)	**<.001***	0.551 (0.295-1.028)	.061
Gender	695				
Female	297	Reference			
Male	398	1.262 (0.988-1.610)	.062	1.999 (1.214-3.292)	.006
GPR34	695				
Low	347	Reference			
High	348	1.574 (1.234-2.006)	**<.001***	1.070 (0.642-1.783)	**.026***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, respectively.

GPR34 Overexpression was Correlated With Poor Prognosis in Patients With Glioma

A Cox proportional hazards model was applied to the primary cohort of TCGA to assess the ability of each variable to predict the prognosis of patients with glioma. Univariate and multivariate analyses indicated that factors such as the GPR34 expression level and WHO grade were significantly correlated with the prognosis of patients with glioma (Table 1). Next, we reviewed the clinical features of 60 glioma patients from Linyi People's Hospital (43 males, 17 females; mean age: 53 years [range 19-80]) and explored the association between the GPR34 expression level and clinicopathological characteristics using immunohistochemistry. We found moderate to strong cytoplasmic staining for GPR34 in LGG and HGG specimens, whereas normal brain tissues showed only negative/weak GPR34 staining in the cytoplasm (Figure 3A and B). We further found that GPR34 expression was correlated with tumor WHO grade and several parameters associated with malignant proliferation (Ki67, $P = .004$; mutated P53, $P = .022$) (Figure 3C, Table 2). To further address the potential effect of GPR34 in gliomagenesis, a K–M survival curve was generated to analyze the GPR34 expression profiles of glioma patients for whom overall survival (OS) data were available in the CGGA and TCGA databases. We found that patients with high GPR34 expression levels had shorter OS than those with low levels of GPR34 expression, irrespective of whether the glioma was primary or recurrent (Figure 3D and E) ($P < .05$). Similarly, follow-up data were collected for all 60 patients from Linyi People's Hospital, with K–M survival curve analysis indicating that high GPR34 expression was correlated with significantly shorter OS in patients with glioma, irrespective of glioma grade (all grade, $P = .032$, LGG, $P = .033$ or HGG, $P = .031$) (Figure 3F).

Knocking Down GPR34 Inhibited the Proliferation of Glioma Cells by Inducing G1/S Cell Cycle Arrest

To assess the function of GPR34 in glioma, pSin-GPR34-derived lentivirus and two different GPR34-siRNA vectors were used to respectively overexpress and knockdown GPR34 expression in the U251 and LN229 glioma cell lines (Figure 4A–D), following which the effects on cell viability were assessed using CCK-8 and colony formation assays. We found that U251 and LN229 cells overexpressing GPR34 exhibited a higher rate of proliferation relative to that in control cells, whereas the opposite effect was seen for cells transfected with GPR34-siRNA (Figure 4E–J) ($P < .05$). To further explore the biological function of GPR34 in gliomagenesis, synergetic gene cluster analysis was performed. Genes significantly associated with GPR34 were screened out using Pearson's correlation coefficient ($adj.P.Value < .05$, $r > 0.7$). The results of the cluster analysis indicated that GPR34-related genes might be involved in mitosis and the regulation of the G1/S phase cell cycle transition (Figure 5A). To determine the effect of GPR34 overexpression/knockdown on cell proliferation, a

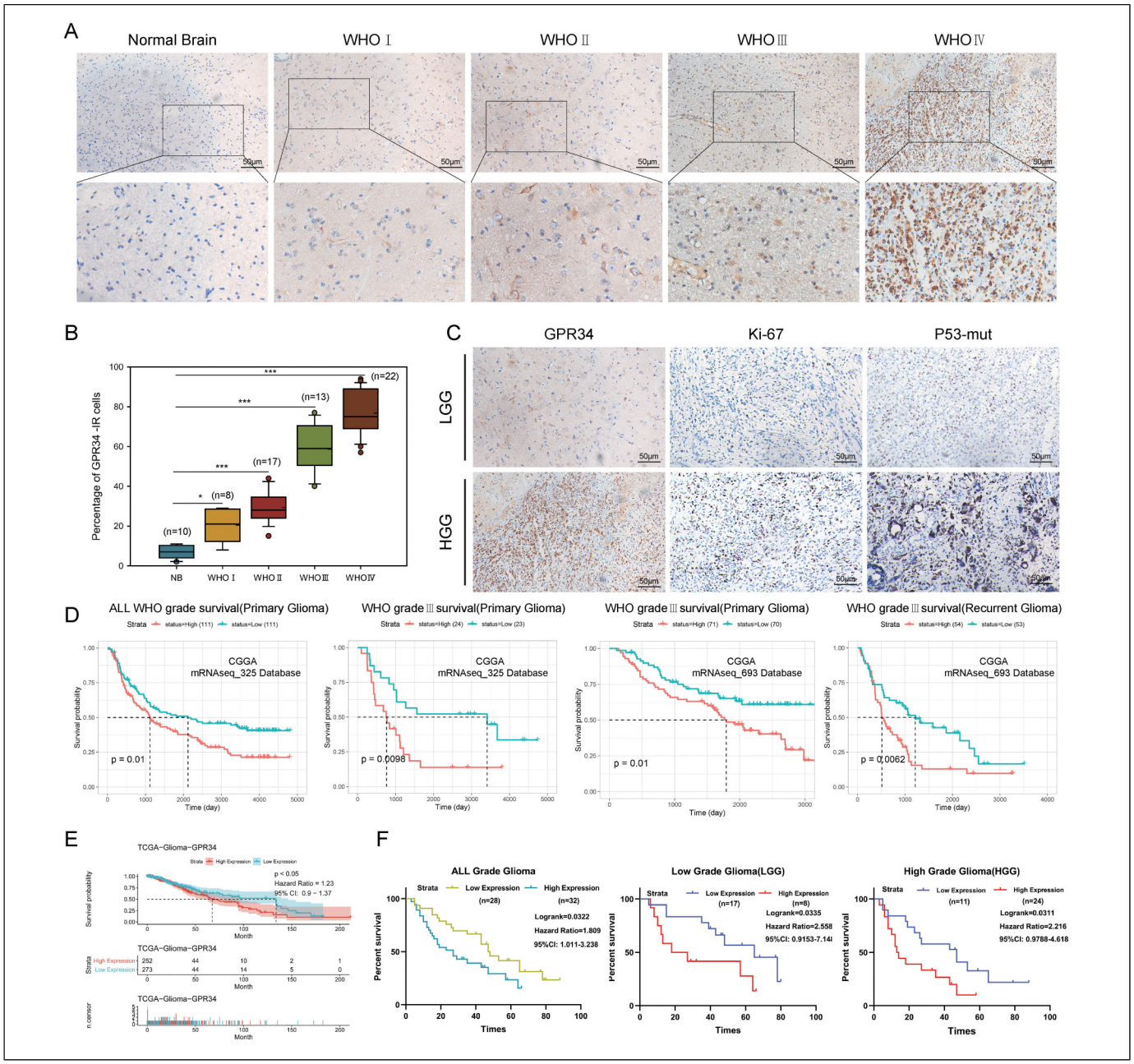

Figure 3. GPR34 overexpression was correlated with poor prognosis of glioma patients. (A and B) The expression of GPR34 in glioma tissues and non-tumor tissues (non-tumor, n = 10; WHOI, n = 8; WHOII, n = 17; WHOIII, n = 13; WHOIV, n = 22), measured by immunohistochemistry (IHC) assay. Scale bar, 50 μm. (C) IHC assay of GPR34 and malignant tumor biomarkers (Ki-67, P53mut) in patients with different grade of glioma. (D and E) Kaplan Meier (KM) curve comparing overall of primary and recurrent glioma patients according to expression of GPR34 in CGGA and TCGA database. (F) Kaplan-Meier analysis for overall survival (Linyi People's Hospital, n = 60) in glioma patients according to GPR34 expression level (Log-rank test, *$P < .05$, **$P < .01$, ***$P < .001$, respectively).

flow cytometric analysis was performed to detect the cell cycle distribution of glioma cells. The results showed that the knockdown of GPR34 led to a greater proportion of cells in the G1 phase (U251: From 32.97% to 51.02%; LN229: From 36.51% to 55.66%), while decreasing the percentage of cells in the S phase (U251: from 52.98% to 32.39%; LN229: from 49.36% to 27.56%) (Figure 5B and C) ($P < .05$). There was no statistical significance of change in the number of cells in G2M phase (U251: 14.05% vs 16.59%; LN229: 14.13% vs 16.78%). The expression of cell cycle-related proteins such as CDK2, CDK6, and cyclin E was markedly upregulated in GPR34siRNA-expressing U251 and LN229 cells compared with that in control cells ($P < .05$). The opposite trend was observed in glioma cells overexpressing GPR34 (Figure 5D–G) ($P < .05$).

Table 2. Expression of GPR34 in Relation to Clinicopathological Features.

Clinicopathological features, Value	GPR34 expression			
	N	Low expression	High expression	*P* value
Gender				
Male	43(71.6)	21(48.8)	22(51.2)	
Female	17(28.4)	7(41.2)	10(58.8)	.592
Age				
<44	22(36.7)	12(54.5)	10(45.5)	
≥44	38(63.3)	16(42.1)	22(57.9)	.352
Tumor diameter				
<4 cm	19(31.7)	9(47.3)	10(52.7)	
≥4 cm	41(68.3)	19(46.3)	22(53.7)	.938
Tumor location				
Frontal	21(35.0)	12(57.2)	9(42.8)	
Temporal	15(25.0)	5(33.3)	10(66.7)	
Other	24(40.0)	11(45.8)	13(54.2)	.367
KPS				
<80	25(50.0)	11(44.0)	14 (56.0)	
≥80	35(50.0)	17(48.6)	18(51.4)	.727
WHO grade				
I-III	25(41.7)	17(68.0)	8(32.0)	**.005***
III-IV	35(58.3)	11(31.4)	24(68.6)	
P53mut expression				
Low	27(45.0)	17(63.0)	10(37.0)	**.022***
High	33(55.0)	11(33.3)	22(66.7)	
Ki-67 expression				
Low	19(31.7)	14(73.7)	5(26.3)	**.004***
High	41(68.3)	14(34.1)	27(65.9)	

*$P < 0.05$, **$P < 0.01$, respectively.

GPR34 Overexpression Promoted the Migration and Invasion of Glioma Cells

Next, the effect of GPR34 on the migratory and invasive potential of glioma cells was assessed by wound healing and Transwell assays using U251 or LN229 cells with knockdown/overexpression of GPR34. In Transwell assay, comparing with negative siRNA group, the number of glioma cells (per high power fields) transfected with GPR34-siRNA decreased 26.5% in U251 cell and 28.5% in LN229 cell. Whereas, the rate of migration of glioma cells transfected with the GPR34 overexpression vector was increased 42.3% (U251) and 40.08% (LN229) compared with empty vector-transfected cells (Figure 6A and B) ($P < .05$). The results of the wound healing assay demonstrated that glioma cells overexpressing GPR34 exhibited a noticeably enhanced rate of migration compared with that seen for empty vector-transfected controls (48 h, U251: 78.2% vs 62.2%; LN229: 81.5% vs 69.2%); in contrast, the migratory capacity of glioma cells (U251 and LN229) transfected with GPR34-siRNA was significantly reduced relative to that in the negative control group (48 h, U251: 65.0% vs 45.1%; LN229: 75.0% vs 58.5%) (Figure 6C and D) ($P < .05$). Subsequently, the impact of GPR34 on migration-associated proteins was detected by western blotting. The results revealed that the knockdown of GPR34 in U251 and LN229 glioma cells led to a notable downregulation of matrix metalloprotease-2 (MMP-2), MMP-7, and ROCK1 protein levels compared with that in the control group; the opposite results were obtained with GPR34 overexpression (Figure 6E–H) ($P < .05$).

GPR34 Promoted EMT and the TGF-B/Smad Signaling Pathway in Glioma Cells

To provide further insight into the molecular mechanism underlying the effects of GPR34 on gliomagenesis, GSEA of the GEO dataset was undertaken to identify the putative biological function of GPR34. GSEA led to the identification of 30 significantly enriched pathways related to GPR34 (adjusted *P*-value $< .05$), including 26 activated pathways (NES > 0) and four inactivated pathways (NES < 0) ($P < .05$) (Figure 7A and B). The results of the GSEA and co-expression analysis indicated that GPR34 may be involved in EMT ($P < .05$) (Figure 7C and D). EMT is associated with enhanced MMP activity, which induces glioma cell invasion *via* the degradation of extracellular matrix proteins. We have shown that GPR34 facilitates glioma malignancy. Consistent with this, we also found that the levels of several EMT markers, such as N-cadherin, Snail, and vimentin, were significantly decreased in GPR34-depleted U251 and LN229 cells; in contrast, the levels of E-cadherin were upregulated following GPR34 knockdown (Figure 7E–H) ($P < .05$). These effects on EMT markers were further confirmed *via* immunofluorescence staining, which showed that GPR34

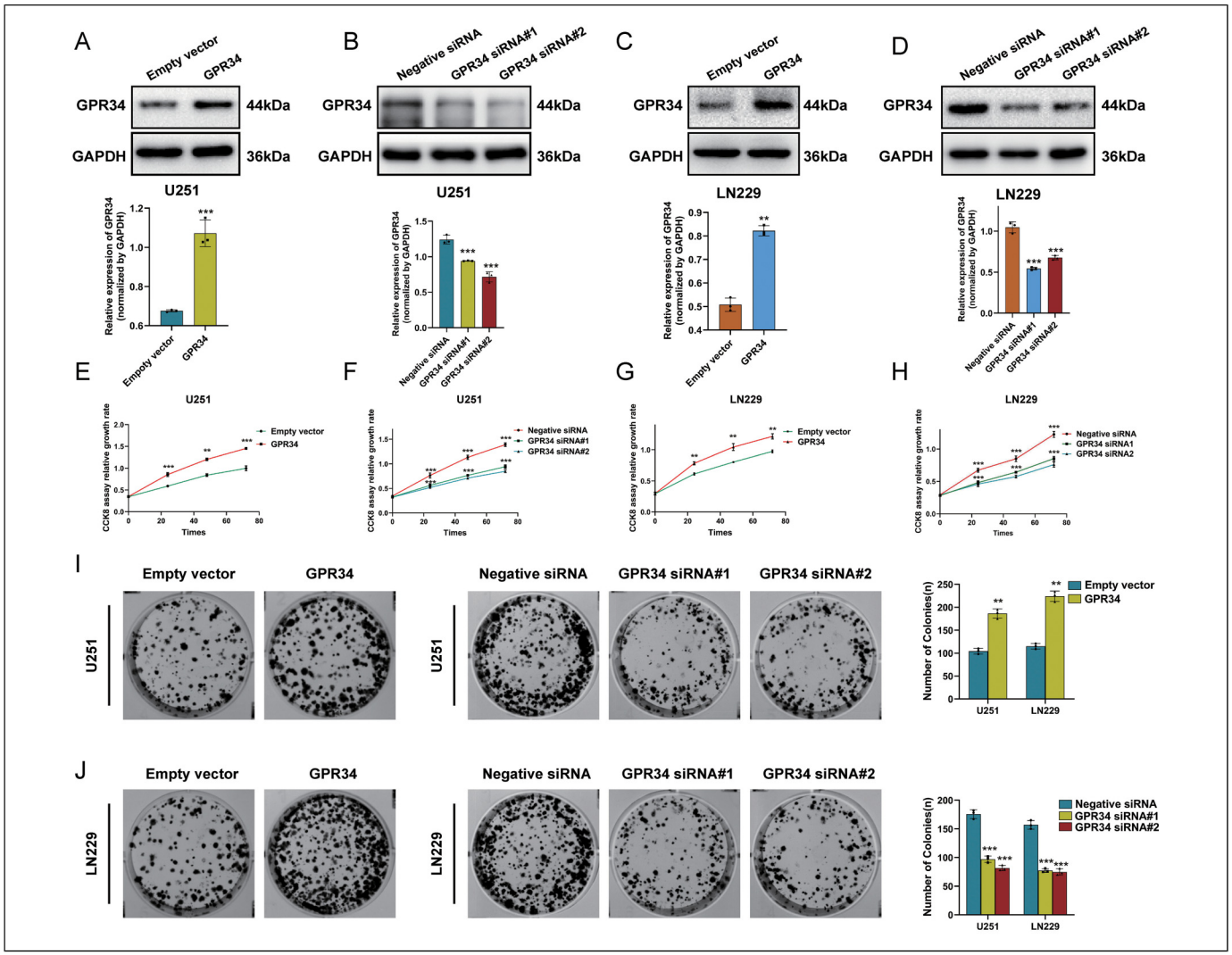

Figure 4. Effect of GPR34 expression on proliferation of glioma cells. (A-D) The interference effects of GPR34 siRNA and overexpression plasmid in U251 and LN229 cells. (E-H) Proliferation in U251 and LN229 cells was detected by CCK8 assay. (I-J) Colony formation assay of U251 and LN229 cells treated with GPR34 siRNA and overexpression plasmid after 14 days. Data were presented as mean ± SD of three of separate experiments (*$P < .05$, **$P < .01$, ***$P < .001$, respectively).

overexpression resulted in the upregulation of both vimentin and N-cadherin expression and a decrease in that of E-cadherin in U251 and LN229 cells (Figure 7I and J) ($P < .05$).

GSEA of the GEO microarray dataset also revealed that genes associated with the TGF-β/Smad signaling pathway were enriched in glioma tissues exhibiting high GPR34 expression, suggesting that the effects of GPR34 on gliomagenesis may be mediated through the TGF-β/Smad pathway ($P < .05$) (Figure 7A–C). Consistent with the western blot results, the levels of several canonical TGF-β/Smad signaling targets, including p-TGF-β-R1, p-Smad2, and p-Smad3, were significantly decreased or elevated under GPR34 inhibition or overexpression (Figure 8A–D) ($P < .05$). To further validate that the malignant phenotype induced by GPR34 was mediated *via* the activation of TGF-β/Smad signaling, we blocked the TGF-β/Smad pathway in GPR34-overexpressing cells using the TGF-β inhibitor LY2157299. As shown in Figure 8, treatment with LY2157299 significantly attenuated the GPR34 overexpression-induced proliferative ($P < .05$) (Figure 8E–G) and invasive ($P < .05$) abilities of glioma cells (Figure 8H and I), as well as EMT-like processes ($P < .05$) (Figure 8J and K). Combined, these findings indicated that GPR34 induced glioma malignancy and tumorigenesis *via* activating the TGF-β/Smad signaling pathway, at least in part.

Discussion

GPR34 is a seven-transmembrane receptor reported to be preferentially expressed in microglia; however, it is also known to play crucial roles in mast cell degranulation and to be aberrantly elevated in neuroinflammation induced by cuprizone, a demyelinating toxin.[11,19,20] Growing evidence has indicated that GPRs are involved in tumorigenesis as

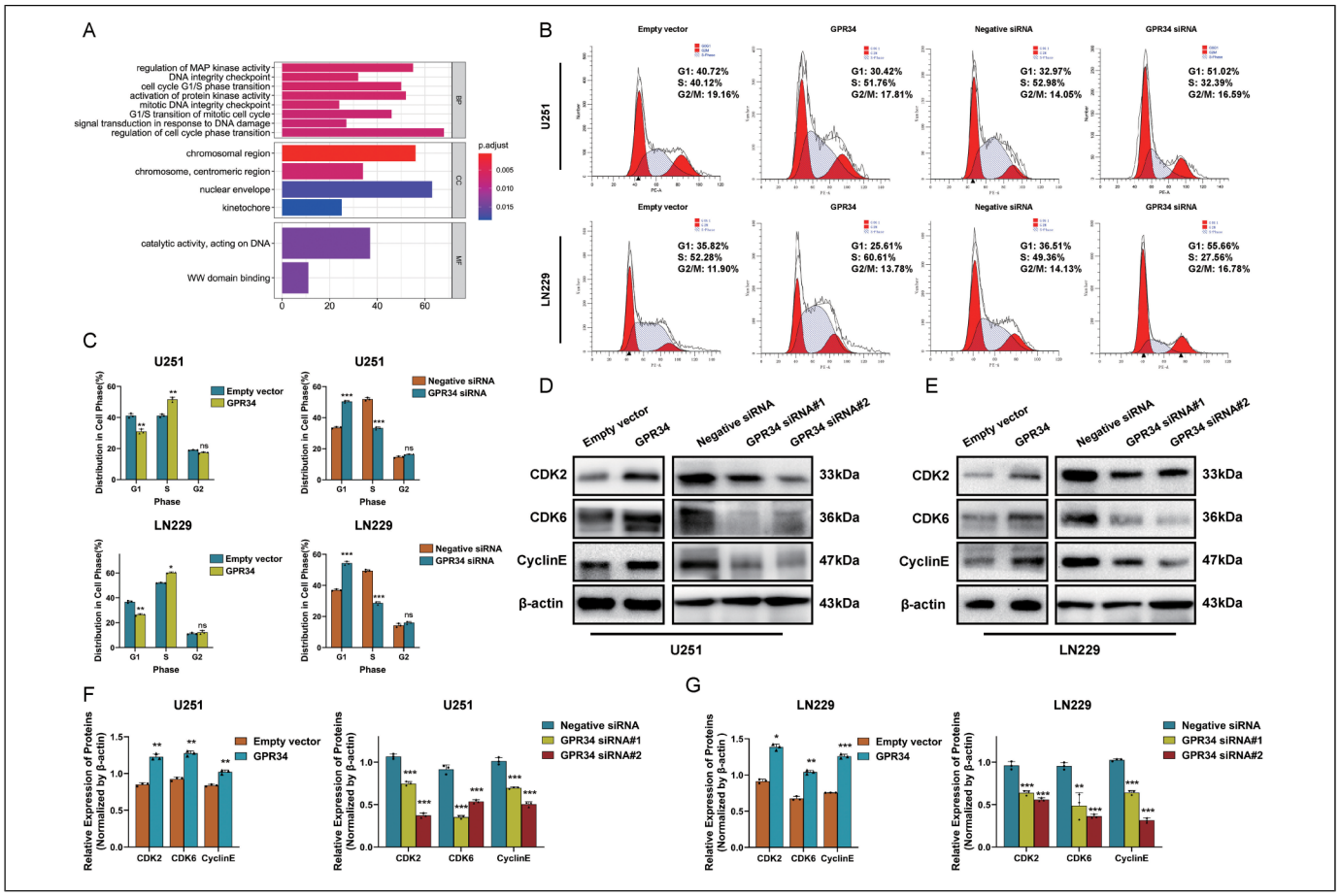

Figure 5. Knocking down GPR34 induced G1/S cell cycle arrest in glioma cells. (A) Biological process (BP), cell components (CC), and molecular function (MF)-related synergetic genes cluster analysis of GPR34. (B-C) Flow cytometry data represented more cells were arrested in S phase of cell cycle in the GPR34 group compared with empty vector group, while, GPR34 knockdown induced G1 phase arrest of glioma cells. (D-G) Western blot showed the expression of relevant cell cycle proteins in U251 and LN229 cells. Data were presented as mean ± SD of three of separate experiments (*$P < .05$, **$P < .01$, ***$P < .001$, respectively).

well as the regulation of angiogenesis and tumor cell survival.[21,22] Recent studies have implicated aberrant GPR34 expression in the development of several malignant solid tumors, including melanoma, MALT lymphoma, leukemia, cervical cancer, and gastric cancer.[13,15,16,23,24] GPR34 expression was reported to be dysregulated in MALT lymphoma; GPR34 overexpression was found to induce CRE/AP1-mediated transcription and activation of the PKC/MAPK/NF-κB signaling pathways, which was proposed as a novel mechanism underlying MALT lymphoma development and progression.[14] We have previously shown that, in $P185^{YR}$-transformed Ba/F3 cells, GPR34 participated in BCR/ABL-induced leukemogenesis and imatinib resistance.[24] Tan et al found that GPR34 expression was markedly upregulated in cervical cancer cell lines and that the transcriptional repression of miRNA-381 resulted in the activation of GPR34, suggesting that the miRNA-381/GPR34 axis may serve as a therapeutic target for the treatment of cervical cancer.[13] Additionally, Yu et al reported that the knockdown of GPR34 negatively affected the tumorigenesis, infiltration depth, and lymph node metastasis of human gastric cancer.[15]

In the present study, we confirmed that the GPR34 level increased with increasing glioma grade and identified GPR34 as a novel factor involved in glioma malignancy. Based on these observations, we further explored the biological functions and the molecular mechanisms underlying the effects of GPR34 in glioma and found that GPR34 could induce gliomagenesis (promoted cell proliferation, invasion, and migration). The stage of cell cycle arrest can vary under different intracellular stresses. Cyclin D1 is a critical regulator of the G1/S cell cycle checkpoint, combined with CDK4/6 to form complex triggering uncontrolled cell proliferation. Besides, the cyclin E/CDK2 complex also controls the G1/S cell cycle transition.[25–27] To the best of our knowledge, this is the first study to report that the knockdown of GPR34 induces G1/S phase transition to inhibit the proliferation of glioma cells, thereby playing a critical role in gliomagenesis.

Although the precise molecular regulatory network involved in GPR34-mediated tumorigenesis remains to be elucidated, several studies have reported that GPR34 can activate the PI3K/PDK1/AKT signaling pathway.[15] In our study, we

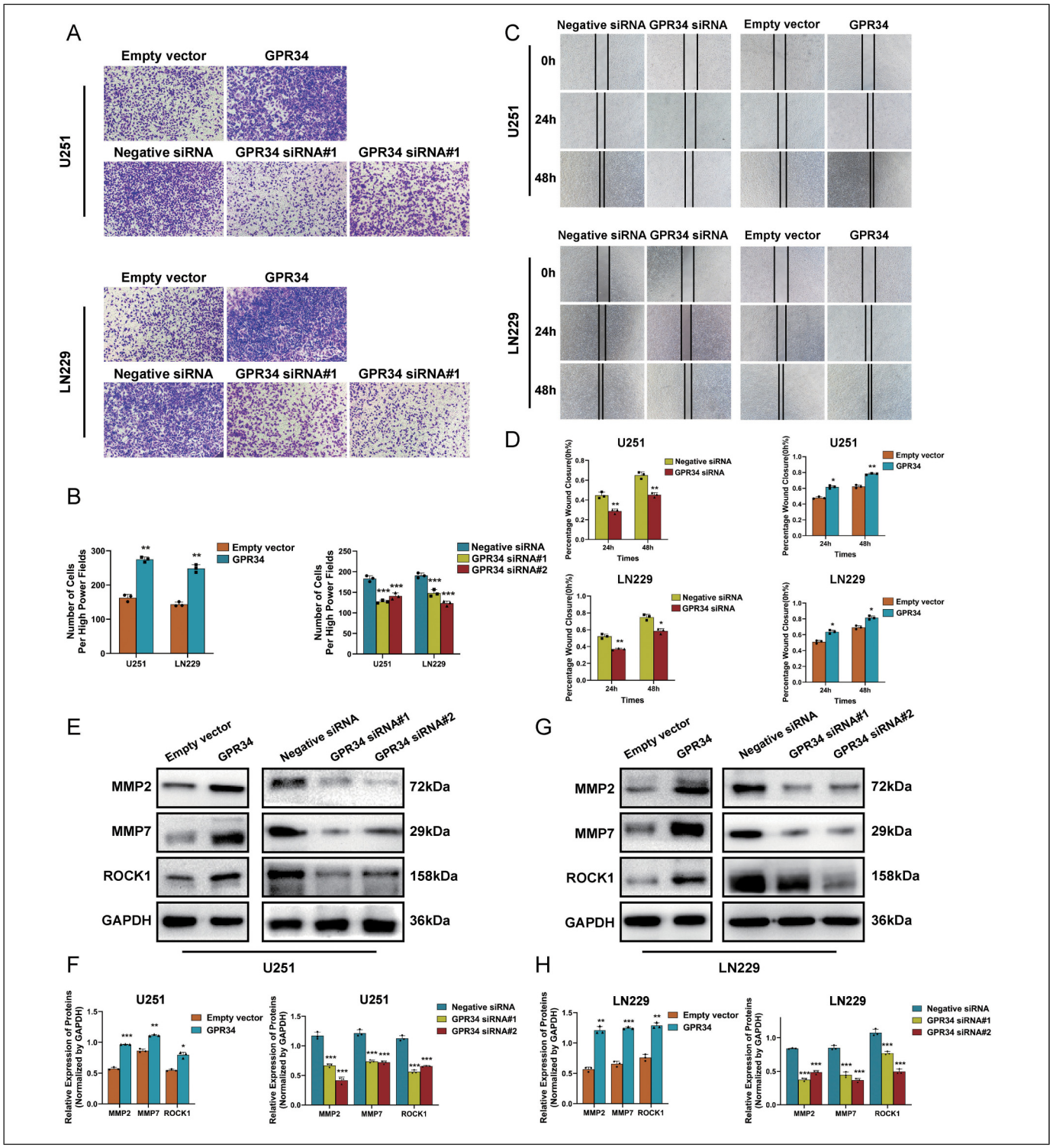

Figure 6. GPR34 overexpression promoted migration and invasion of glioma cells. (A and B) Transwell assay to access the effects of GPR34 cell migration and invasion in U251 and LN229 cells. (C and D) Wound healing assay was used to detect the cell migration ability after overexpression or knockdown of GPR34 after 24 and 48 h in U251 and LN229 cells (magnification ×200). (E-H) Western blot assay to determine the expression changes of invasion markers in GPR34-silencing/overexpressing U251 and LN229 cells. Quantification graphs were shown in F-H (*$P < .05$, **$P < .01$, ***$P < .001$, respectively).

provide preliminary evidence to indicate that GPR34 may also be involved in the regulation of EMT and the TGF-β/Smad signaling pathway. Although some behavioral characteristics were observed to differ between EMT-related processes in traditional epithelial tumors and those in neuroepithelial tumors, several EMT biomarkers were reported to play a crucial role in glioma

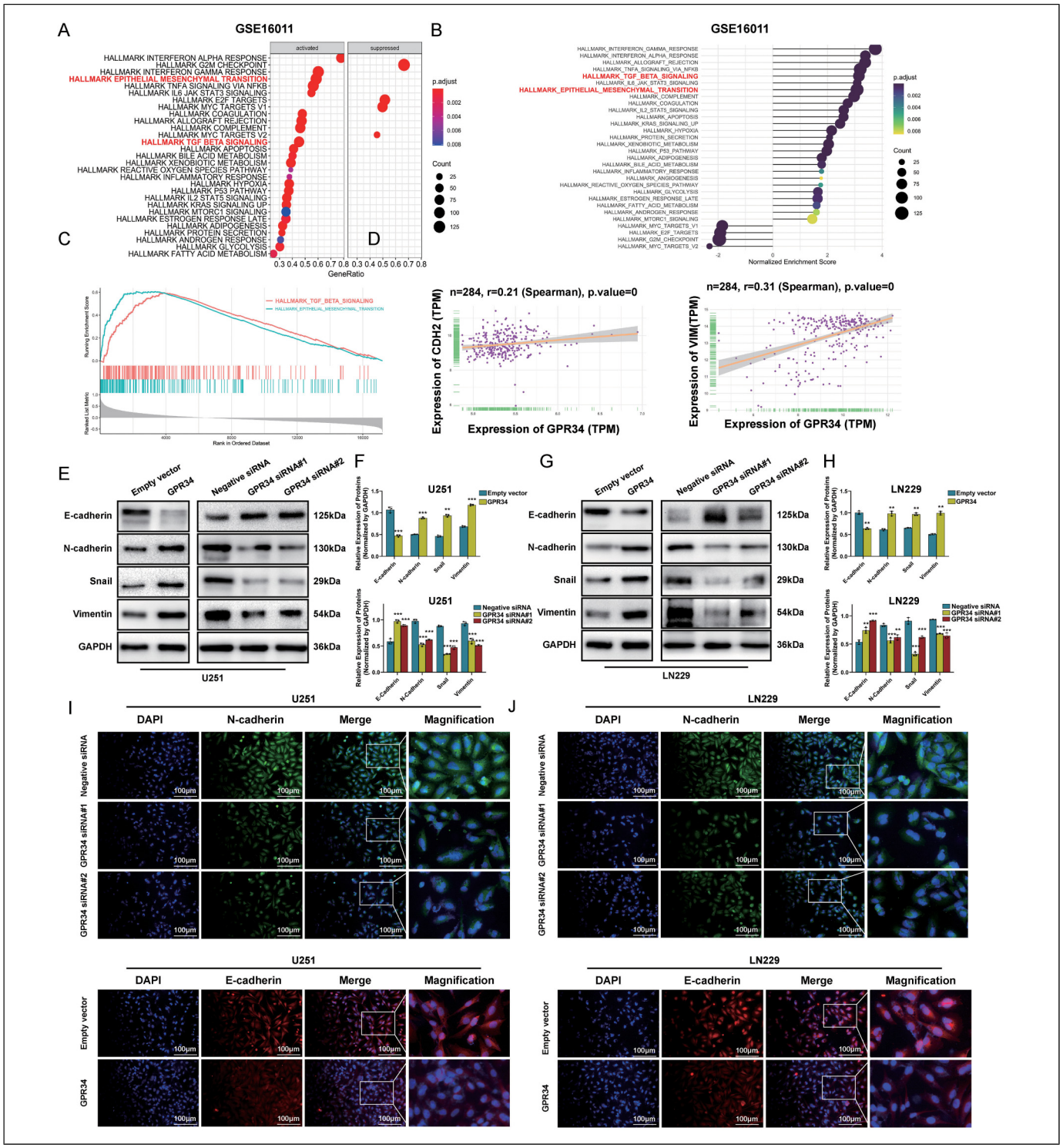

Figure 7. The overexpression of GPR34 induced the epithelial-mesenchymal transition (EMT) in glioma cells. (A and B) Gene set enrichment analysis (GSEA) pathways enriched by GPR34. The dotplot showed the 26 up-regulated (NES>0) and 4 down-regulated (NES<0) pathways. (C) GSEA enrichment analysis represented that enrichment of epithelial-mesenchymal transition (EMT) and TGFβ/Smad pathways. (D) Correlation of GPR34 with several key regulatory factors (N-cadherin [CDH2] and VIM) of epithelial-mesenchymal transition (EMT) in glioma samples based on the data from GEO database. (E-H) Western blotting showing the decreased protein levels of mesenchymal marker (N-cadherin, vimentin, and snail) and the increased protein levels of epithelial marker (E-cadherin) after knockdown of GPR34 in U251 and LN229 cells, while conversed phenomenon was presented following GPR34 overexpression treatment. (I-J) Immunofluorescence staining showing the same outcomes as for GPR34 in U251 and LN229 cells after siRNA treatment and overexpression plasmid treatment. The scale bar corresponds to 100 μm (* $P<.05$, ** $P<.01$, *** $P<.001$, respectively).

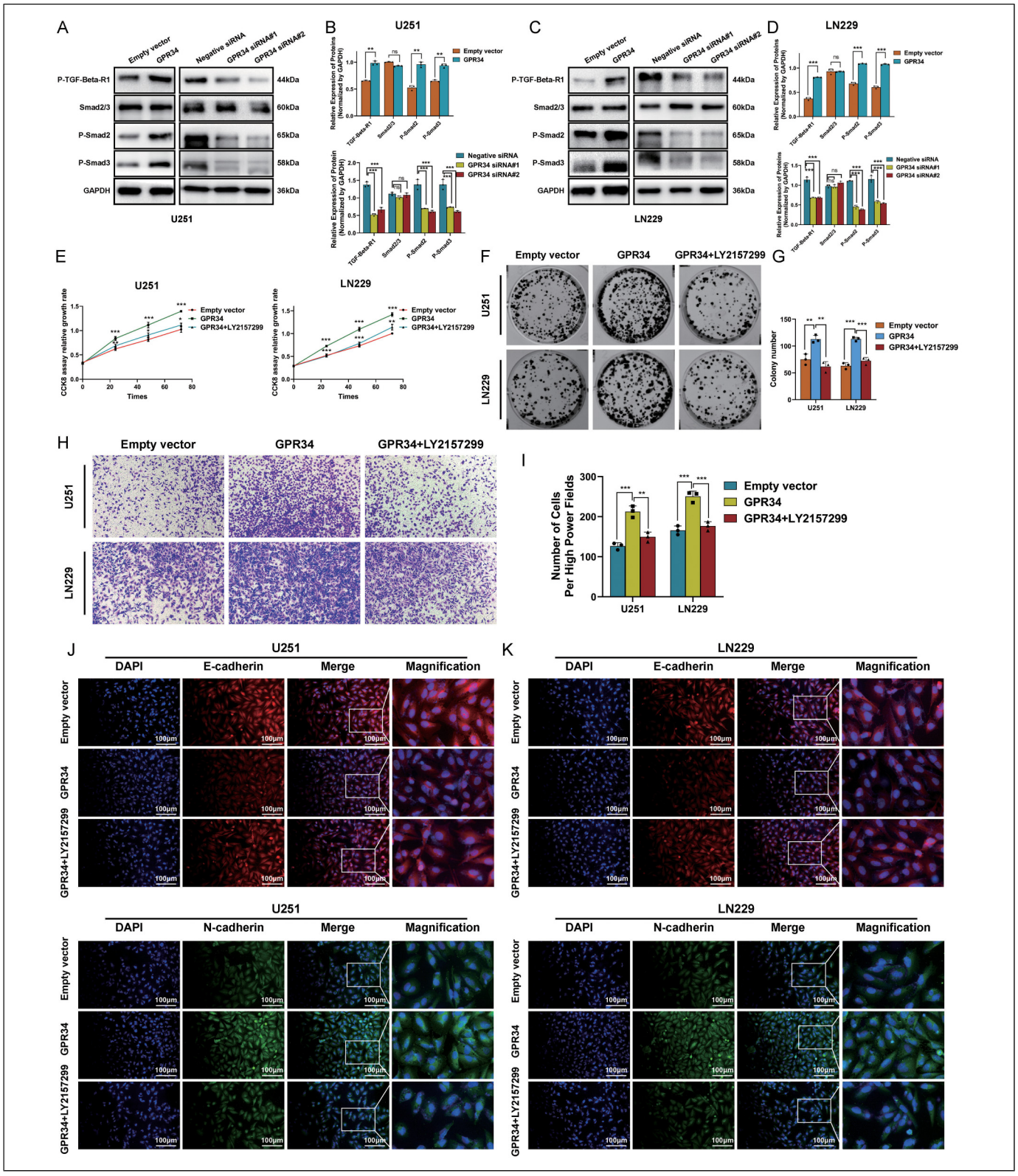

Figure 8. GPR34 promoted the malignant phenotype by activating TGFβ/Smad signaling. (A-D) Western blot detection of P-TGFβR1, smad2/3, P-samd2, P-smad3 in U251 and LN229 cells GPR34-siRNA or overexpression plasmid treatment. (E-G) After being treated with TGFβ inhibitor LY2157299, representative images of U251-GPR34-plasmid and LN229-GPR34-plasmid cell proliferation and invasion in the CCK8 and Transwell assay. (H-I) Representative micrographs of violet-stained U251-GPR34 and LN229-GPR34 cell colonies treated with LY2157299. (J-K) Immunofluorescence staining analysis showed the protein level of EMT-related markers (E-cadherin and N-cadherin,) in LN229-GPR34 and U251-GPR34 after inhibiting TGFβ/Smad pathway (* $P < .05$, ** $P < .01$, *** $P < .001$, respectively).

malignancy.[28] For instance, Slug was shown to facilitate the proliferation and angiogenesis of GBM *in vivo*[29] as well as enhance the migratory and invasive capacity of glioma cells *in vitro*. Moreover, the EMT-related biomarker Snail was reported to promote gliomagenesis *via* the induction of an EMT-like process *in vitro*,[30] while Twist1 was found to accelerate GBM tumorigenesis by activating the EMT pathway.[31] Hence, the term "EMT-like process" or "glial-to-mesenchymal transition" has frequently been proposed as an alternative to EMT in gliomas.[32]

In our study, we confirmed that GPR34 knockdown could alter the levels of EMT-related markers (Snail, vimentin, N-cadherin, and E-cadherin), which suggested that GPR34-induced gliomagenesis may involve the regulation of an EMT-like process. In addition, the TGF-β/Smad signaling pathway, as a fundamental growth control signaling played a pivotal role in the varieties of tumorigenesis, leading to a defined cellular response *via* the activation of p-TGF-β/p-Smad2/p-Smad3 target genes. One study showed that a relative change rather than an absolute change in TGF-β expression is crucial for the activation of downstream target genes.[33] Importantly, the expression of several canonical TGF-β/Smad signaling targets, including p-TGF-β-R1, p-Smad2, and p-Smad3, were significantly decreased or elevated under GPR34 inhibition or overexpression in our study. Furthermore, the inhibition of the TGF-β/Smad pathway using LY2157299 significantly attenuated the enhanced proliferative and invasive capabilities of glioma cells as well as an EMT-like process induced by GPR34 overexpression.

Interferons are pleiotropic cytokines expressed in several types of cancer cells and tumor microenvironment, exerting a crucial pro-tumoral role in tumorigenesis.[34,35] Recently, many studies into the inflammation microenvironment of tumor tissues have proved the hypothesis that a close association exerts between tumor metastasis progression and inflammation response. EMT was initially found in the process of embryonic development. However, it also played an important role in tumor metastasis.[36,37] Lv et al has reported that interferon-γ consensus sequence-binding protein induced proliferation, invasion, and EMT-like phenomena via TGF-β pathway in human osteosarcoma U2OS cells.[38] In addition, the TNFα/NFκB signaling has been considered as the "major culprit" contributing to the inflammatory response in glioma and other cancer.[39–41] Furthermore, previous study has reported that multiple loss-function mutations in genes affected by NFκB were involved in many types of abnormalities, such as defects of CNS and impaired innate/acquired immune response.[42,43] Many evidence to support the fact that AKT could phosphorylate IκBα and IκB kinase after the activation of NFκB. Then, IκBα was degraded followed by nuclear translocation of P65(RelA) (one of the NFκB family members), which has been identified to promote EMT of glioma cells.[44–46] Wang et al demonstrated that miR-19a/b could induce malignant phenotype and EMT-like progress via AKT- NF-κB pathway in glioma cell.[39] In our study, the results of GPR34-related GSEA analysis indicated that interferon and TNFα/NFκB pathway might participate in the GPR34-induced gliomagenesis. Importantly, we also found that glioma patients with GPR34 high expression were prone to present more immune cells infiltrated than patients with GPR34-low level. Taken together, the effect of interferon and TNFα/NFκB signaling pathways on GPR34-induced malignant phenotype in glioma needed to be further explored in the future. There are some limitations in our study: firstly, the number of glioma patients incorporated in multivariate/univariate cox analysis was reduced as many patients with glioma had missing integrated data on all variables from TCGA public database. Secondly, the number of healthy samples (as controls) was small, so additional researches are needed to balance sample size. Lastly, this study mainly focused on the prognosis value of GPR34 in glioma. As the TGF-β/Smad signaling pathway is involved in several human diseases, including vascular diseases and cancer, further work is needed to determine whether GPR34 may also play a role in glioma stemness.[47]

Conclusion

We found that GPR34 expression was positively correlated with the malignant phenotype and immune cell infiltration of glioma and may serve as a novel biomarker of poor prognosis for patients with this disease. We further found that GPR34 facilitated the proliferation, migration, and invasion of glioma cells. Importantly, we showed for the first time that GPR34 can induce G1/S cell cycle transition and an EMT-like process in glioma. Finally, our data indicated that the effects of GPR34 in glioma may be mediated through the regulation of the TGF-β/Smad signaling pathway. Combined, these observations highlight the potential of GPR34 as a promising therapeutic target for the treatment of glioma.

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Authors' Note

FF, XH designed the study, YC researched the literature. FF and YC wrote the manuscript and performed bioinformatics analysis. XH, FF contributed to figures. YC and FF performed the invitro experiments and data analysis. FF, XH supervised the study. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The study protocol was approved by the Ethics Committees of Linyi People's Hospital (No. 10023).

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