GPR176 enhances the epithelial-mesenchymal transition in gastric cancer cells by activating the PI3K/AKT/mTOR signaling pathway

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Abstract. Gastric cancer is a malignancy with a high incidence and poor prognosis. The identification of novel molecular markers and elucidation of their underlying mechanisms may provide new avenues for improving therapeutic strategies. The present study analyzed the association between GPR176 expression and clinicopathological features using The Cancer Genome Atlas-Stomach Adenocarcinoma and GSE66254 datasets, and further validated the findings in patients from The First Affiliated Hospital of Guangxi Medical University (Nanning, China). The migratory and invasive abilities of gastric cancer cells were assessed using Transwell and wound-healing assays. Western blotting was carried out to evaluate the effects of GPR176 on the PI3K/AKT/mTOR signaling pathway. *In vivo* tumorigenesis assays in nude mice were carried out to confirm the role of GPR176 in tumor progression. Analysis revealed that GPR176 expression was significantly elevated in gastric cancer tissues and associated with unfavorable patient outcomes. Silencing GPR176 markedly suppressed the migration and invasion of gastric cancer cells, accompanied by inhibition of the PI3K/AKT/mTOR and EMT signaling pathways. These inhibitory effects were prevented by the overexpression of PIP5K1A. In line with the *in vitro* results, experiments with nude mice demonstrated that GPR176 knockdown impeded tumor growth, whereas its overexpression enhanced tumorigenicity. Furthermore, GPR176 suppression significantly attenuated EMT and PI3K/AKT/mTOR signaling *in vivo*, while GPR176 overexpression led to activation of these pathways. In summary, the present study identifies GPR176 as a novel prognostic biomarker in gastric cancer. Mechanistically, GPR176 promotes EMT and tumor progression, at least in part, through activation of the PI3K/AKT/mTOR signaling pathway.

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Abbreviations: GPR176, G protein-coupled receptor 176; PIP5K1A, phosphatidylinositol-4-phosphate 5-kinase type 1 α; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, gene ontology; RT-qPCR, reverse transcription-quantitative PCR; WB, western blotting; EMT, epithelial-mesenchymal transition

Key words: gastric cancer, GPR176, EMT, PI3K signaling pathway, PIP5K1A

Introduction

In 2020, the incidence of gastric cancer reached ~1.09 million cases, ranking 5th among all malignant tumors and accounting for 5.6% of all malignant diseases (1,2). The number of mortalities caused by gastric cancer totaled ~770,000, ranking fourth and accounting for 7.7% of overall malignant tumor-related fatalities (1,2). The high prevalence and poor prognosis of gastric cancer have markedly impacted the well-being of the population, especially in China (3). In 2022, China reported ~397,000 new cases of gastric cancer, accounting for 37% of the global total, with both incidence and mortality rates ranking third among malignant tumors in China (4,5). East Asia bears the brunt of the global burden, with ~60% of gastric cancer cases occurring in this region (6-8). Along with the health burden comes a pronounced economic loss to residents and the government due to the diagnosis and treatment of gastric cancer (9,10).

Early-stage gastric cancer often presents with inconspicuous symptoms, which leads to late detection, suboptimal treatment outcomes, high recurrence rates and low survival rates (6,10-12). The 5-year survival rate for advanced gastric cancer remains as low as 5% globally (13-15). Currently, the predominant treatment strategy for gastric cancer is comprehensive, with a primary emphasis on surgical intervention (16-18). Surgical procedures dominate the treatment landscape for early-stage gastric cancer, while chemotherapy improves survival and quality of life for locally advanced or metastatic cases (stage Ib to IIIb) (8,17,19,20). Despite the mature theory and practice of abdominal anatomy, coupled with inherent shortcomings of chemotherapy, advances in surgical and chemotherapeutic approaches to the treatment of gastric cancer have been limited (8). Overall, the efficacy of gastric cancer treatment remains unsatisfactory, with only modest improvements in prognosis (21).

New therapeutic approaches, such as targeted drugs and immunotherapy, offer hope for patients with gastric cancer (22,23). Targeting central mechanisms of tumor development, developing drugs that prevent uncontrolled cell proliferation or directly inducing apoptosis represents a promising path to a successful transformation and potentially a cure of advanced gastric cancer (24).

GPR176, a G protein-coupled receptor located on 15q14-q15.1, belongs to the G protein-coupled receptor family and functions as a cell surface receptor that responds to hormones, growth factors and neurotransmitters (25). GPR176 is primarily expressed in the brain, followed by the gallbladder and testis (25). While earlier studies focused primarily on the role and mechanisms of GPR176 in circadian rhythms (25,26,27), later research recognizes its potential in tumors (28-32). For example, Tang *et al* (28) revealed that GPR176 recruits GNAS, activates the cAMP/PKA/BNIP3L signaling pathway and inhibits mitochondrial autophagy, which promotes stem cell formation and proliferation of colorectal adenocarcinoma. Yun *et al* (29) demonstrated an association between the expression of GPR176 and the prognosis of breast adenocarcinoma. Interfering with GPR176 suppresses the PI3K/AKT/mTOR signaling pathway, glycolysis, epithelial-mesenchymal transition and proliferation of breast adenocarcinoma cells. The prognostic value of GPR176 in esophageal adenocarcinoma has also been established, indicating an association with prognosis and resistance of esophageal adenocarcinoma (30). Analyses of publicly available data have suggested an association between GPR176 and gastric cancer prognosis, warranting further exploration of underlying mechanisms (31,32).

PIP5K1A, which encodes the protein phosphatidylinositol-4-phosphate 5-kinase type 1 α, carries out a role in several processes, including activation of GTPase activity (33,34). It serves as an upstream regulator of the PI3K/AKT/mTOR signaling pathway and genetic or pharmacological inhibition of PIP5K1A markedly inhibits AKT phosphorylation (35,36).

Building upon previous research, the present study first evaluated the expression and prognostic significance of GPR176 in gastric cancer and further elucidated the mechanism by which GPR176 promotes cancer cell invasion and its functional interaction with PIP5K1A, using both *in vitro* and *in vivo* models.

Materials and methods

Data acquisition. RNA-sequencing (RNA-seq) data from 448 gastric cancer (STAD) samples, including 410 tumor and 38 normal samples, were collected, along with clinical data from 383 patients with STAD, sourced from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) (37). The Limma package in R 4.5.1 (https://bioconductor.org/packages/release/bioc/html/limma.html) facilitated the standardization of RNA-seq information (38). The transcriptomic details for all samples were preserved to investigate the nuanced differences between adenocarcinomatous and adjacent tissues. However, 17 samples with incomplete clinical information were excluded from subsequent survival analysis.

The RNA-seq data and associated clinical/prognostic information for 300 patients diagnosed with gastric cancer were methodically extracted from the GSE66254 dataset in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66254) (39). The original chip data from GSE66254 underwent a rigorous annotation and standardization process in R, utilizing the Limma package.

Patient samples. From the biobank of the Department of Gastrointestinal and Glandular Surgery at The First Affiliated Hospital of Guangxi Medical University (Nanning, China), tissue specimens (including gastric cancer tumor and adjacent non-tumorous tissues) were collected from 48 patients with gastric cancer who underwent surgery. Postoperative pathological reports indicated lymph node metastasis in 24 of these patients. These tissue samples were subsequently utilized to analyze the correlation between lymph node metastasis and the activation of the PI3K/AKT/mTOR and EMT signaling pathways, as well as the expression of GPR176.

Cell culture. The cell lines HGC-27 and NCI-N87 were procured from the American Type Culture Collection (The Global Bioresource Center; https://www.atcc.org/) cell repository. Culturing these cells involved a comprehensive medium consisting of DMEM (cat. no. 10566016; Gibco, Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (cat. no. 10099141C; Gibco; Thermo Fisher Scientific, Inc.), 1% streptomycin and 1% penicillin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.). The controlled environment for these cell cultures was maintained in incubators with 5% CO_2 and 37˚C.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). RNA extraction from each sample (gastric cancer tumor and adjacent non-tumorous tissues) was carried out using the TRI Reagent™, by following the manufacturer's protocol (cat. no. AM9738; Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, reverse transcription (RT) into cDNA was carried out according to the manufacturer's instructions for the PrimeScript RT Reagent Kit with gDNA Eraser (cat. no. RR047B; Takara Bio, Inc.). The primers were designed using Primer3 (https://primer3.ut.ee/) and are listed in Table SI. qPCR was performed using the FastStart Universal SYBR® Green Master Mix (cat. no. 06402712001; Roche Diagnostics GmbH) on an Applied Biosystems 7500 (Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (40).

Construction of lentivirus and stable cell lines. Design and packaging of overexpression (OE)/RNA interfering (RNAi) lentiviruses into cells was carried out by Shanghai GeneChem Co., Ltd. Lentiviruses for GPR176 overexpression, GPR176 knockdown (sh-GPR176), PIP5K1A overexpression and PIP5K1A knockdown (sh-PIP5K1A) were constructed using the wild-type sequences of GPR176 and PIP5K1A, respectively. Lentiviruses containing empty vectors were used as the control group for infection. The infection concentration of all lentiviruses in the present study was set to 5 multiplicity of infection (MOI), with an infection duration of 12 h in 37˚C cell

incubator. Puromycin was added at a concentration of 10 ng/ml to select cells with off-target effects and the selection period was 5 days in in 37˚C cell incubator. The GPR176 overexpression lentivirus contained the wild-type sequence of the GPR176 gene, while the PIP5K1A overexpression lentivirus contained the wild-type sequence of the PIP5K1A gene. The functional sequences of sh-GPR176 and sh-PIP5K1A are provided in Table SII. The efficacy of these lentiviruses was verified through a using RT-qPCR assay and western blotting (WB).

WB. Extraction of proteins from cells (HGC-27 and NCI-N87) and tissues (gastric cancer and adjacent normal gastric) entailed a combination of RIPA reagent (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) and 1% PMSF (cat. no. P0100; Beijing Solarbio Science & Technology Co., Ltd.). Quantification of protein concentration was carried out using the BCA protein assay kit (cat. no. P0009; Beyotime Institute of Biotechnology). The proteins (2 μg) underwent separation using 10% SDS-PAGE electrophoresis and were then transferred to PVDF membranes. Following blocking with 5% skim milk at room temperature for 30 min, the PVDF membranes were incubated overnight at 4˚C with the diluent for the primary antibody. After two washes with PBS-Tween (1%), the membranes were incubated with secondary antibody diluent at 23˚C for 1 h. The visualization of protein bands was carried out using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). The information and usage concentrations of the corresponding primary antibodies are listed in Table SIII. HRP-conjugated Goat Anti-Mouse IgG (H+L) (1:100; cat. no. SA00001-1, Proteintech Group Inc.) and HRP-conjugated Goat Anti-Rabbit IgG (H+L) (1:100; cat. no. SA00001-2, Proteintech Group Inc.) were used as the secondary antibody.

Immunofluorescence (IF). IF staining was carried out following standard protocols (https://www.ptgcn.com/support/protocols/#if). Briefly, 1x10^5 HGC-27 and NCI-N87 cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.2% Triton X-100 (cat. no. 9002-93-1; Beijing Solarbio Science & Technology Co., Ltd.) and blocked with 5% normal goat serum (cat. no. SL038, Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h. Samples were then incubated with primary antibodies (Vimentin, 1:200; E-cadherin, 1:200) at 4˚C overnight, followed by Multi-rAb® CoraLite® Plus 488-Goat Anti-Rabbit Recombinant Secondary Antibody (H+L) (1:500; cat. no. RGAR002; Proteintech Group Inc.) and Multi-rAb® CoraLite® Plus 594-Goat Anti-Rabbit Recombinant Secondary Antibody (H+L) (1:500; cat. no. RGAR004; Proteintech Group Inc.) at room temperature for 1 h and DAPI (cat. no. 28718-90-3, Beijing Solarbio Science & Technology Co., Ltd.) nuclear counterstaining at room temperature for 15 min. Fluorescence images were captured with an Olympus inverted fluorescence microscope (Olympus IX73; Olympus Corporation) equipped with appropriate filter sets and a digital camera and analyzed using Olympus CellSens software 2.3.

Cell invasion assays. The cell suspension was obtained by digesting the cells with 1 ml trypsin (cat. no. T1321; Beijing Solarbio Science & Technology Co., Ltd.) for 2 min, adding 5 ml of complete medium, and then repeatedly pipetting the mixture. Before adding the cell suspension in 6.5 mm Transwell® with 8.0 μm Pore Polycarbonate Membrane Inserts (cat. no. 3422; Corning, Inc.), the Matrigel (cat. no. 356234; Beijing Solarbio Science & Technology Co., Ltd.) was prepared according to the manufacturer's instructions. Cells were plated in the upper chambers, and the culture medium was added to the lower chambers. Specifically, 200 μl of a serum-free cell suspension containing 100,000 cells was added to the upper Transwell inserts, while 600 μl of DMEM supplemented with 10% FBS was added to the lower chambers of a 24-well plate. The plate was then incubated for 48 h at 37˚C and 5% CO_2. After incubation, the insert was fixed with 4% paraformaldehyde at room temperature for 30 min and stained with a 1% crystal violet solution at room temperature for 20 min. Subsequent actions included the erasure of cells on the upper layer, which were then removed, washed with PBS, and the invaded cells were observed under an Olympus IX53.

Cell wound healing assays. Cells (HGC-27 cells and NCI-N87) were grouped according to the regulation of GPR176 and PIP5K1A, with the details stated in *Construction of lentivirus and stable cell lines*. To observe cell proliferation (HGC-27 and NCI-N87), a precise wound was induced by scratching the plate with a 200 μl suction tip when the cells covered 90-100% of the culture plate area. After washing twice with PBS, serum-free medium was added to the plate, and cells were cultured in incubators with 5% CO_2 and 37˚C. Subsequently, the width of the wound was observed and imaged consecutively under the Olympus IX53 at 0 and 48 h.

Nude-mouse transplanted tumor model construction. Male nude mice (n=40; 6-8 weeks old; 18-22 g) were housed under specific pathogen-free conditions with a controlled temperature of 22±2˚C, humidity of 50±10% and a 12-h light/dark cycle. All animals had ad libitum access to a standard laboratory diet and autoclaved water. The nude mice were randomly assigned into four groups (10 per group) and received subcutaneous injections of HGC-27 cells: sh-NC, sh-GPR-176, Lenti-null and Lenti-GPR-176, respectively. HGC-27 cells in a stable growth state were resuspended in pre-cooled PBS at a concentration of 1x10^7 cells/200 μl. This suspension was aseptically injected subcutaneously into the right flank of each mouse.

After transplantation, the general health status and body weight of the mice were monitored daily. Tumor volume was measured every two days using a digital caliper and calculated using the formula V=(length x width^2 x π)/6. To minimize observer bias, all measurements were conducted in a blinded fashion. Humane endpoints were strictly defined as follows: Tumor burden >1.5 cm in diameter, significant weight loss (>20% of initial body weight) or signs of severe distress. None of the animals reached these endpoints prior to the scheduled experimental conclusion. At the end of the experiment (day 28 post-inoculation), all mice were euthanized by CO_2 asphyxiation with a chamber displacement rate of 30-50% per min, followed by cervical dislocation to ensure mortality.

Statistical analysis. Each experiment was carried out to at least three independent replicates. Data analysis was conducted using IBM SPSS Statistics software (version 26.0;

IBM Corp.). Results are expressed as the mean ± standard error. Differences between two groups were assessed using an unpaired Student's t-test, while multiple comparisons were carried out by one-way or two-way ANOVA. Linear regression and correlation analysis were employed to assess the correlation between GPR176 and PIP5K1A expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GPR176 is associated with the prognosis and clinicopathologic factors of gastric adenocarcinomas. Expression matrices and corresponding clinical data were respectively obtained from TCGA-STAD dataset and GSE66254 dataset (37,39). The expression of GPR176, its prognostic significance and its association with clinicopathologic factors were evaluated. Analysis of the pan-cancer data from TCGA revealed significant differences in GPR176 expression between cancer and adjacent tissues in cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), stomach adenocarcinoma (STAD) and uterine corpus endometrial carcinoma (UCEC) (Fig. 1A). In the TCGA-STAD dataset, the expression of GPR176 was significantly higher in gastric cancer tissues when compared with adjacent tissues (Fig. 1B). Additionally, GPR176 expression levels were lower in patients with TNM stage I gastric adenocarcinoma when compared with patients with stage II/III/IV gastric adenocarcinoma (Fig. 1C), and GPR176 expression levels were significantly lower in patients with stage T1 when compared with patients with pathologic stage T2/T3/T4 (Fig. 1D).

Statistical analysis confirmed the association of GPR176 with tissue type and tumor stage, suggesting its importance in the occurrence and progression of gastric adenocarcinoma. Subsequently, in the TCGA-STAD and GSE66254 datasets, significant association was observed between GPR176 expression levels and overall survival/recurrence-free survival in gastric adenocarcinoma, with high GPR176 expression levels associated with worse prognosis (Fig. 1E-G). Using the median expression of GPR176 as a cut-off point, patients were categorized into GPR176-High and GPR176-Low groups. An increased proportion of patients at T3/T4 in the GPR176-High group was observed (Fig. 1H). The area under the receiver operating curve reached 0.8315, indicating GPR176 as a good biomarker to distinguish gastric adenocarcinoma from normal gastric tissue (Fig. 1I). Correlation analysis of GPR176 with PIP5K1A expression levels in gastric adenocarcinoma tissues using the TCGA-STAD dataset verified a linear positive association (Fig. 1J).

Nomogram construction. Nomogram were created based on GPR176 expression and clinicopathologic parameters. Univariate Cox regression analysis and multivariate Cox regression analysis were carried out using clinical case characteristics such as age, sex, TNM stage, T stage, N stage, M stage, histologic grade and GPR176 expression (Table SIV). The results of univariate Cox regression analysis results indicated that age, sex, TNM stage, T stage, N stage, M stage and histological grade were associated with the overall survival of patients with gastric adenocarcinoma. In Multivariate Cox regression analysis, only age, histologic grade and GPR176 expression were associated with overall survival of patients with gastric adenocarcinoma. The nomogram graph based on age, sex, TNM stage, T stage, N stage, M stage, histological grade and GPR176 expression were constructed to assess the risk of mortality for specific patients (Fig. 2A). The predictive power of the histogram was evaluated by comparing the grade between the training group and the validation group. The nomogram showed a high degree of overlap between the self-validation cohort and the training group in predicting the 1-, 3- or 5-year prognosis (Fig. 2B-D).

Exploration of the mechanisms of GPR176 action using bioinformatics tools. Based on the expression levels of GPR176, patients in the TCGA-STAD dataset were categorized into high and low expression groups. Differential expression analysis was carried out using the Limma package for RNA-seq and a volcano plot was generated (Fig. 3A). The corresponding pie charts showed that 326 genes were upregulated, and 581 genes were downregulated. Functional enrichment analysis of differentially expressed genes associated with GPR176 revealed enrichment in signaling pathways such as 'PI3K-Akt signaling pathway'; Fig. 3B), and cellular functions such as 'Cell adhesion' and 'G-protein-coupled receptor signaling' (Fig. 3C). Gene Set Enrichment Analysis indicated associations between GPR176 and tumor-related signaling pathways such as 'cell adhesion molecules CAMs', 'Hedgehog signaling pathway', 'Jak-stat signaling pathway' and 'MAPK signaling pathway' (Fig. 3D). CDK2, FOXO1, FOXO3 and VEGFA are downstream target genes of the PI3K/AKT/mTOR signaling pathway, reflecting the activation/inhibition status of the pathway (41). Linear regression analysis using RNA-seq data from gastric adenocarcinoma tissues in TCGA-STAD revealed an association between GPR176 and CDK2, FOXO1, FOXO3 and VEGFA (Fig. 3E).

GPR176 enhances the migration and invasion capabilities of gastric cancer cells and induces EMT. To confirm the effect of GPR176 on the biological behavior of gastric cancer cells and explore the corresponding mechanisms, lentiviral vectors were used to overexpress and knockdown GPR176 expression levels. RT-qPCR and WB confirmed the satisfactory efficiency of OE lentivirus and RNAi lentivirus in HGC-27 and NCI-N87 cells (Fig. S1A). Subsequently, scratch-wound healing and Transwell assays were carried out to evaluate the effects of modulating GPR176 expression on cell migration and invasion. After upregulation of GPR176, the migration and invasion ability of cells increased significantly, while downregulation of GPR176 resulted in a significant decrease in this ability (Fig. 4A and B). Subsequent RT-qPCR analysis revealed a significant increase in the mRNA levels of EMT-activating genes, such as CDH2, VIM and SNAI1, following upregulation of GPR176 (Fig. 4C). By contrast, the expression of the gene CDH1 which encodes E-cadherin, associated with the inhibition of the EMT pathway (42), significantly decreased (Fig. 4C). Conversely, downregulation of GPR176 led to a significant decrease in the mRNA levels ofCDH2, VIM and SNAI1, accompanied by a significant

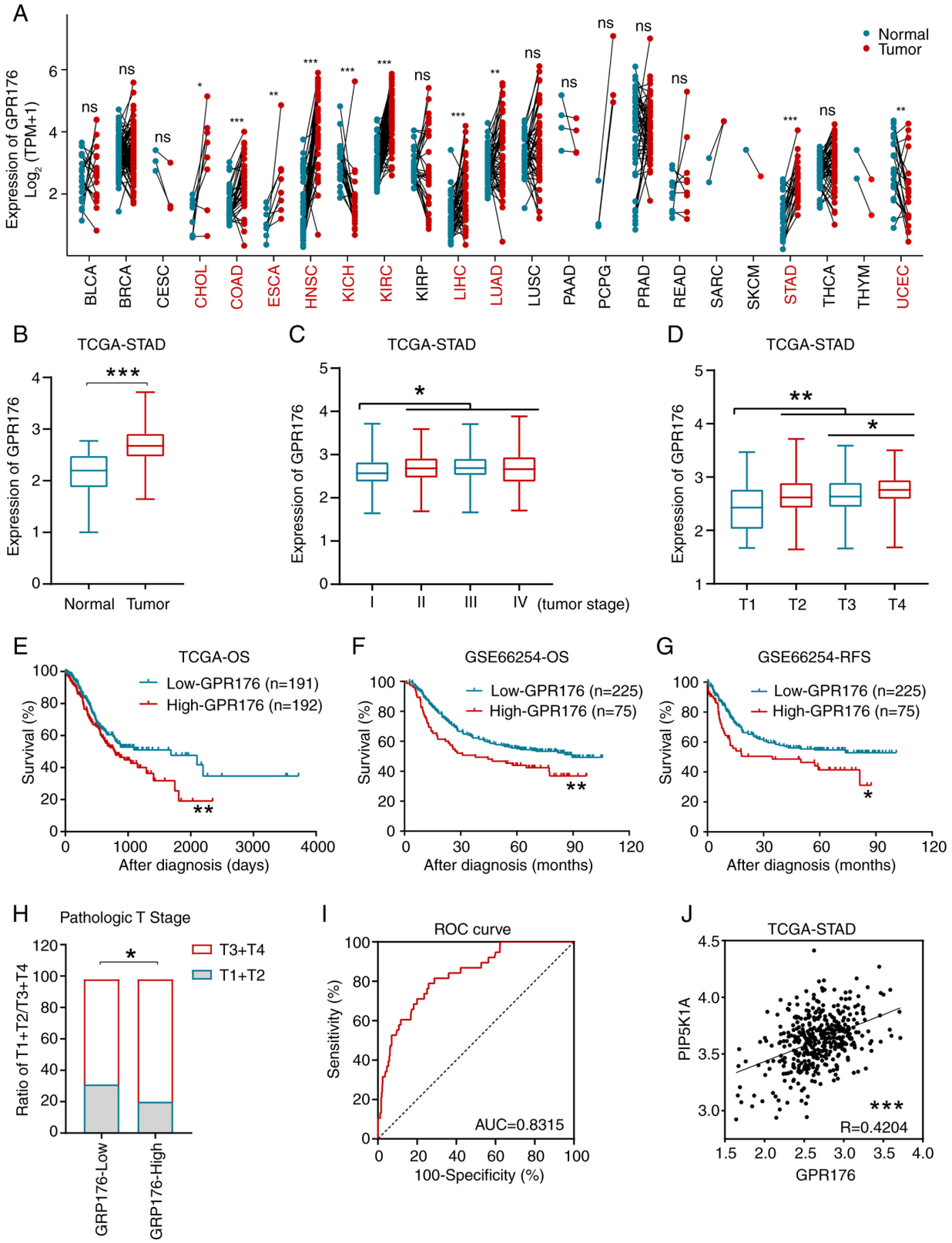

Figure 1. GPR176 expression levels were associated with clinicopathological characteristics and prognosis in STAD patients. (A) GPR176 expression levels in common malignant tumors and adjacent tissues. (B) Comparison of GPR176 expression levels in STAD tumor tissues and adjacent normal stomach tissues. (C) Expression levels of GPR176 mRNA in STAD tumors after TNM staging (I, II, III and IV). (D) Expression levels of GPR176 mRNA in STAD tumors at pathologic T1, T2, T3 and T4 stages. (E) Tn the TCGA-STAD dataset, OS of patients with STAD with GPR176 high and low expression groups using the median value as a cut-off. (F) In the GSE66254 dataset, OS of patients with STAD with GPR176 high and low expression groups using the 75th percentile value as a cut-off. (G) In the GSE66254 dataset, RFS of patients with STAD with GPR176 high and low expression groups using the 75th percentile as a cut-off. (H) Association of GPR176 expression with pathologic T staging in the TCGA-STAD dataset. (I) The ROC curve based on GPR176 plotted for distinguishing between gastric cancer and adjacent tissues. (J) Significant positive correlation between GPR176 and PIP5K1A expression in the gastric cancer samples in the TCGA-STAD dataset. ROC, receiver operating characteristic; TCGA, The Cancer Genome Atlas; STAD, stomach adenocarcinoma; OS, overall survival; RFS, Recurrence-free survival. *P<0.05, **P<0.01, ***P<0.001. Unpaired Student's t-test was used in Fig. 1A-D, Survival analysis with Kaplan-Meier method was used in Fig. 1E-G; Chi-squared test was used in Fig. 1F; Receiver Operating Characteristic (ROC) was used in Fig. 1G; Linear regression was used in Fig. 1H.

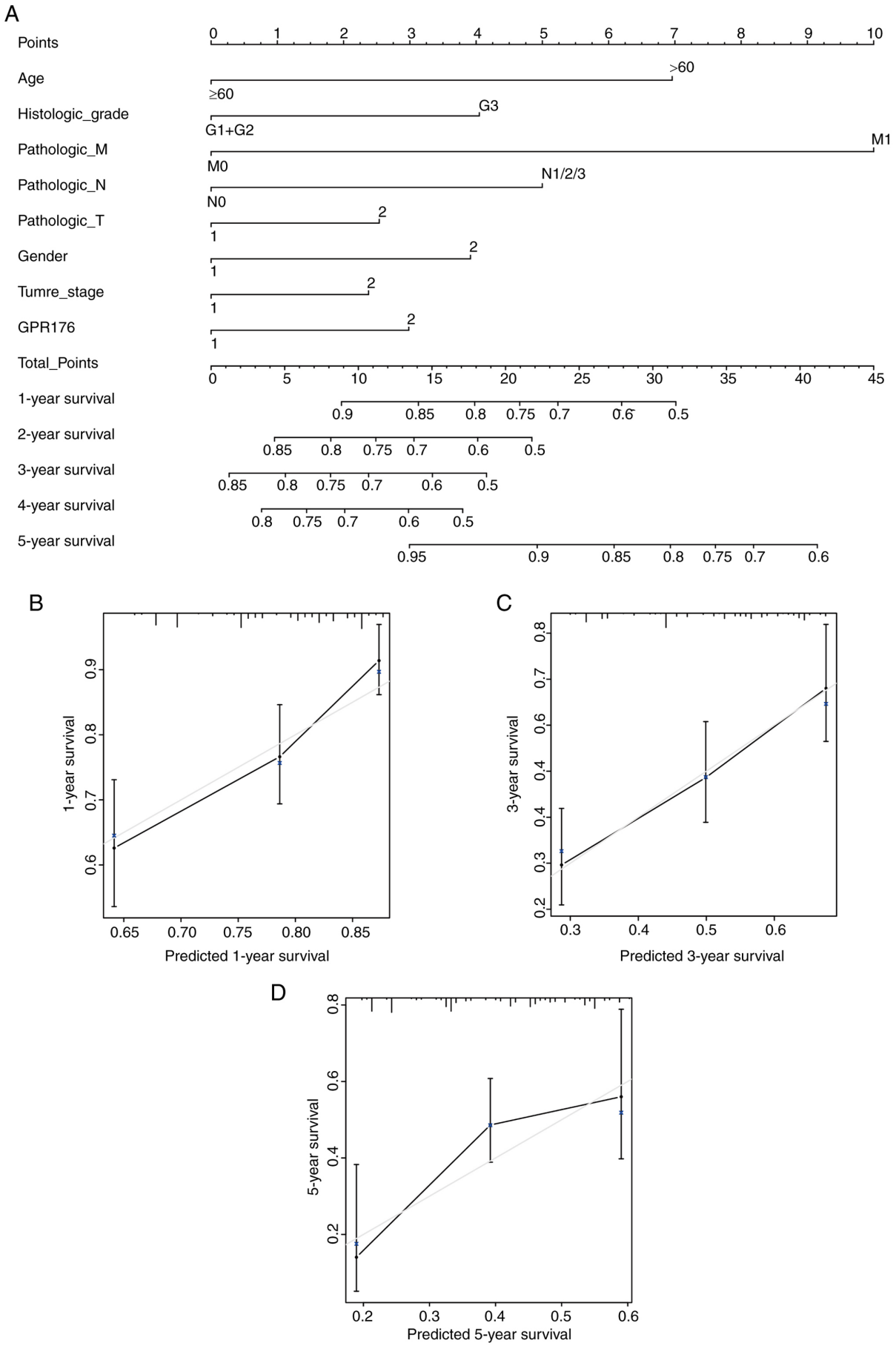

Figure 2. The Nomogram was constructed based on GPR176 and clinical-pathological features in the TCGA-STAD dataset. (A) Nomogram; (B) fitted curve evaluating the accuracy of on1-year survival prediction; (C) fitted curve evaluating the accuracy of 3-year survival prediction; (D) fitted curve evaluating the accuracy of 5-year survival prediction.

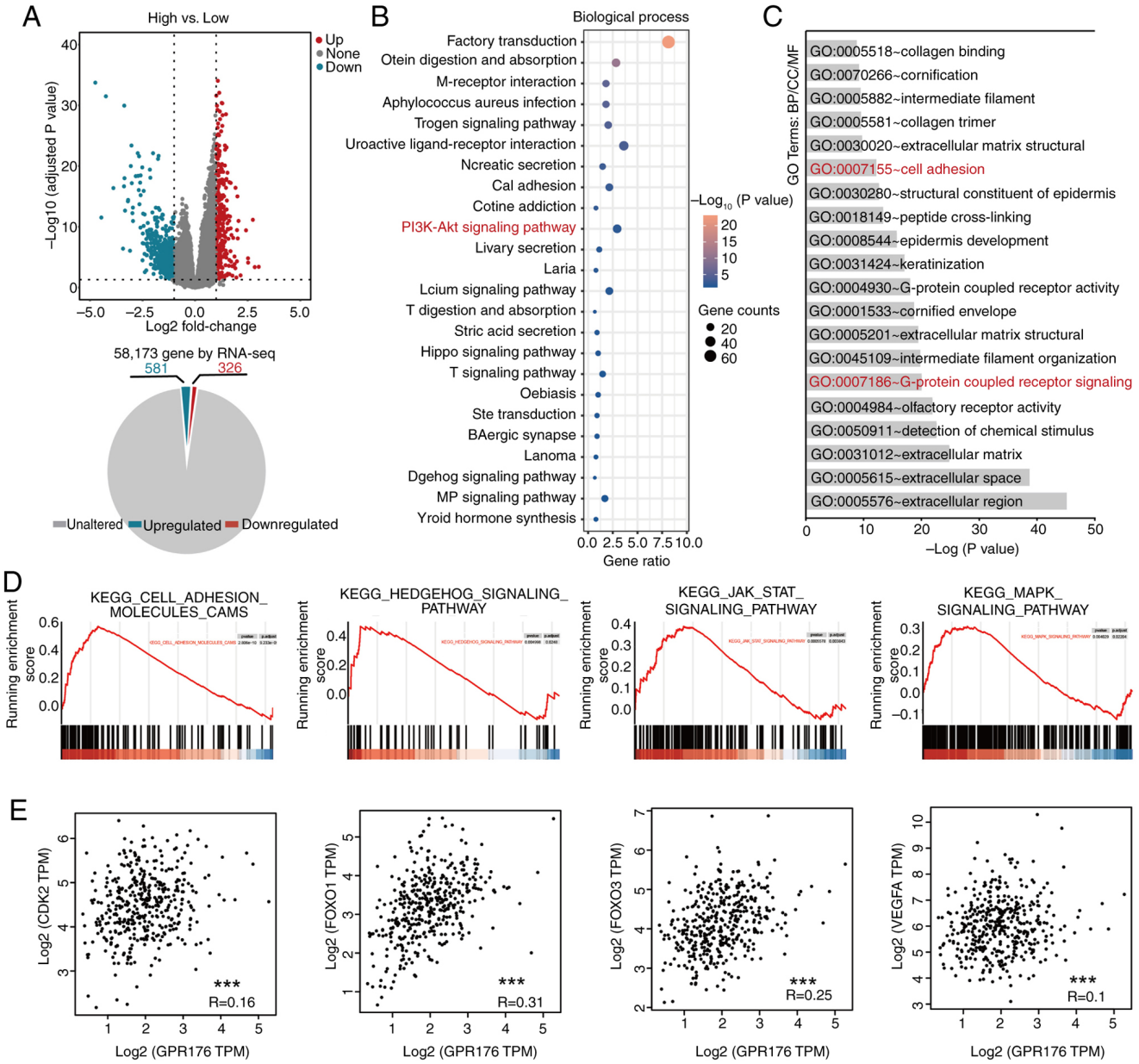

Figure 3. Bioinformatics analysis revealed potential mechanisms through which GPR176 may exert its functions. (A) Volcano plot of differentially expressed genes between GPR176 high and low expression groups in the TCGA-STAD dataset. (B) KEGG signaling pathways enriched based on differentially expressed genes. (C) GO terms enriched based on differentially expressed genes. (D) Gene Set Enrichment Analysis results based on differentially expressed genes. (E) Correlation between GPR176 expression and the expression of target genes of PI3K/AKT/mTOR signaling pathway, including CDK2, FOXO1, FOXO3 and VEGFA. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes. ***P<0.001.

increase in the mRNA levels of CDH1, associated with the inhibition of the EMT pathway (Fig. 4C). WB results corroborated the RT-qPCR results and revealed an increase in the protein concentration of N-cadherin, vimentin and Snai1, and a decrease in the protein concentration of E-cadherin after upregulation of GPR176 (Fig. 4D), with the corresponding bar chart shown in Fig. S2A and B. IF showed that GPR176 upregulation increased vimentin and reduced E-cadherin, whereas GPR176 knockdown had the opposite effect (Fig. S1E). The results from RT-qPCR, WB, and IF experiments showed that GPR176 expression was associated with the activation of the EMT signaling pathway and increased migration and invasion of gastric adenocarcinoma cells.

Downregulation of PIP5K1A prevents the effects of GPR176 on cell migration, invasion, EMT and on the PI3K/AKT/mTOR pathway. Previous studies have confirmed that PIP5K1A is an upstream molecule in the PI3K/AKT/mTOR signaling pathway (30-33). Analysis of RNA-seq data from the TCGA-STAD dataset in the present study revealed a significant positive association between GPR176 and PIP5K1A expression (Fig. 1J). Therefore, we hypothesized that GPR176 may activate the PI3K/AKT/mTOR signaling pathway by inducing upregulation of PIP5K1A expression. Interference and overexpression experiments of PIP5K1A were performed based on the overexpression of GPR176 and the repression of GPR176. Analysis revealed that interference with PIP5K1A expression

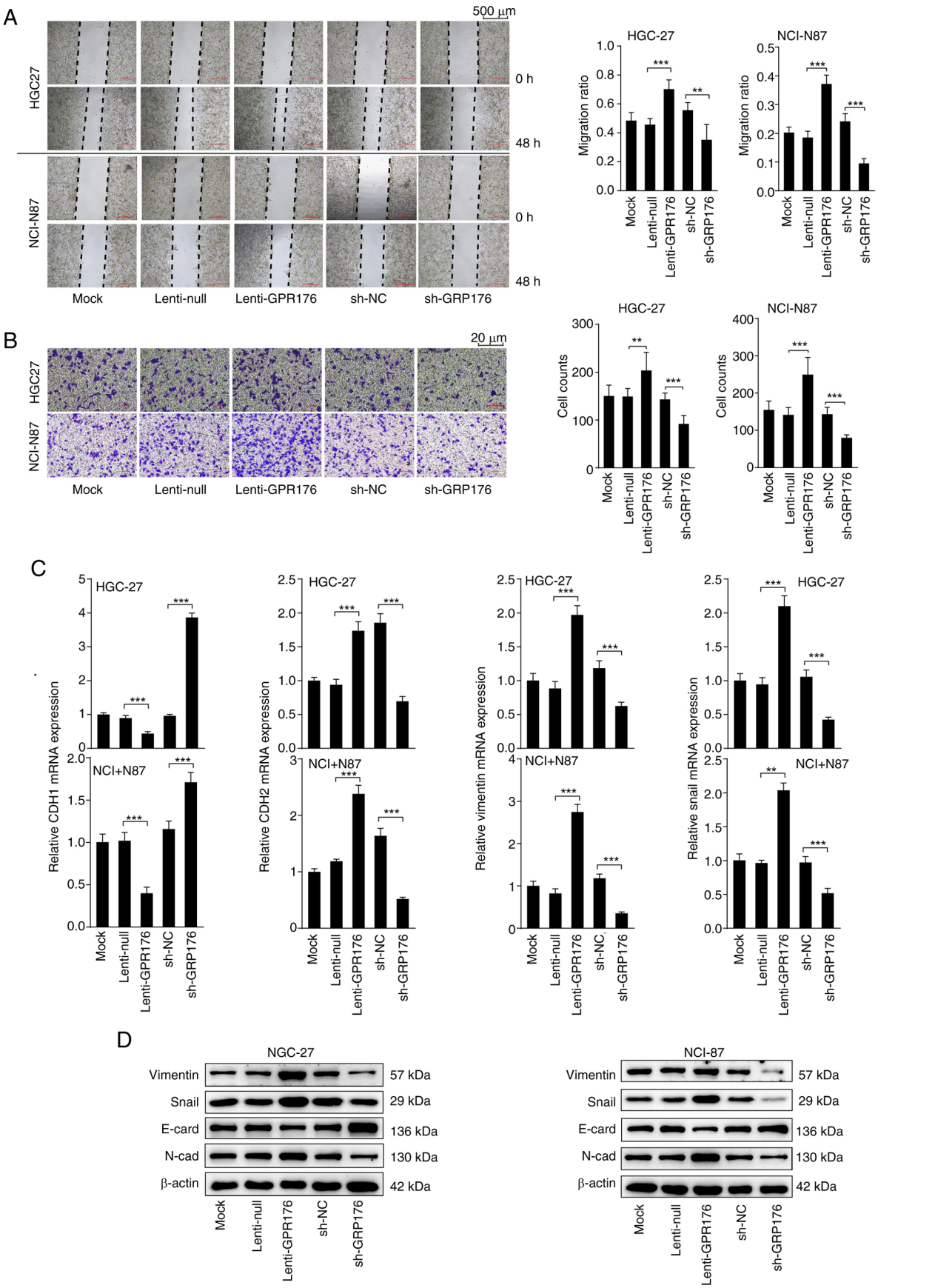

Figure 4. Genetic regulation of GPR176 influences cell invasion/migration and EMT signaling pathways in gastric cancer cells. (A) Upregulation and downregulation of GPR176 promote and inhibit migration of gastric cancer cells, respectively. (B) Upregulation and downregulation of GPR176 promote and inhibit invasion of gastric cancer cells, respectively. (C) Effects of upregulation and downregulation of GPR176 on mRNA expression of EMT pathway genes in HGC-27 and NCI-N87 cells. (D) Effects of upregulation and downregulation of GPR176 on protein expression in the EMT pathway in HGC-27 and NCI-N87 cells, with the corresponding bar chart shown in Fig. S2A and B. EMT, epithelial-mesenchymal transition; sh-NC, short hairpin negative control; cad, cadherin. Statistical analysis was carried out using one-way ANOVA. ***P<0.001, **P<0.01.

significantly prevented the migration and invasion induced by GPR176 overexpression (Fig. 5A and B). Conversely, downregulation of PIP5K1A also significantly reversed the slowed migration and invasion of gastric cancer cells caused by GPR176 knockdown (Fig. 5A and B). Following this, WB was carried out to evaluate the phosphorylation levels of molecules within the PI3K/AKT/mTOR pathway and the EMT signaling pathway, along with assessing the expression levels of PIP5K1A. The activation of the PI3K/AKT/mTOR pathway and EMT signaling pathway after GPR176 upregulation was attenuated by the downregulation of PIP5K1A. Conversely, the inhibition of the PI3K/AKT/mTOR pathway and EMT signaling pathway after GPR176 downregulation was counteracted by the OE of PIP5K1A (Fig. 6A and B), with the corresponding bar chart shown in Fig. S3A and B. The results of the RT-qPCR assay were in concordance with the aforementioned experiments, indicating a preventing in changes to the mRNA expression levels of molecules in the EMT signaling pathway after GPR176 upregulation, which was suppressed by the downregulation of PIP5K1A in HGC-27 and NCI-N87 cells; similarly, in HGC-27 and NCI-N87 cells, the downregulation of GPR176 resulted in prevention of the mRNA expression levels of molecules in the EMT signaling pathway, under the upregulating influence of PIP5K1A (Fig. 7A-L).

The status of EMT and PI3K/AKT/mTOR between para-cancer and cancer tissues, as well as the expression of PIP5K1A and GPR176. Firstly, patients with gastric cancer were categorized into two groups based on the presence or absence of lymph node metastasis: Those with lymph node metastasis and those without. Further classification was made based on histological type, distinguishing between the para-cancer group and the cancer group. Initially, WB was employed to assess the protein levels of E-cadherin, N-cadherin, PIP5K1A, GPR176, AKT, p-AKT, mTOR and p-mTOR in each group. The findings revealed that irrespective of lymph node metastasis, the expression of E-cadherin in the cancer group was significantly reduced compared with the para-cancer group. Conversely, levels of N-cad, PIP5K1A, GPR176, p-AKT, p-mTOR, p-AKT/AKT and p-mTOR/mTOR were increased in the cancer group compared with the para-cancer group (Fig. 8A-F). The representative blots of WB experiments are referenced in Fig. 8G.

Subsequently, validation of the expression levels of key genes in the EMT pathway and PI3K/AKT/mTOR signaling pathway, as well as PIP5K1A and GPR186, was conducted using RT-qPCR. Among patients without lymph node metastasis, no significant differences were observed in GPR186 and CDH2 (N-cadherin) expression levels between the para-cancer and cancer groups (Fig. 8I and K). However, CDH1 (E-cadherin) expression was significantly reduced in the cancer group compared with the para-cancer group (Fig. 8H). Conversely, PIP5K1A expression was markedly higher in the cancer group compared with the para-cancer group (Fig. 8J). In patients with lymph node metastasis, there were no significant differences in CDH1 expression between the para-cancer and cancer groups (Fig. 8L). Nevertheless, GPR186, CDH2 and PIP5K1A expression levels were significantly increased in the cancer group compared with the para-cancer group (Fig. 8M-O). In summary, the expression levels of PIP5K1A and GPR176 were increased in the cancer group compared with the para-cancer group. Additionally, activation of the EMT pathway and PI3K/AKT/mTOR pathway was evident in the cancer group.

GPR176 promotes the growth of transplanted tumors by activating the PI3K/AKT/mTOR pathway. *In vitro* experiments provided evidence that GPR176 could activate the PI3K/AKT/mTOR pathway, facilitating EMT and promoting cell proliferation by inducing the overexpression of PIP5K1A. To further validate the role of GPR176 in gastric cancer, a nude mouse subcutaneous tumor experiment was designed in the present study to assess the influence of GPR176 expression on the PI3K/AKT/mTOR pathway and EMT. In comparison with the Lenti-NC group, the GPR176 upregulation group exhibited significantly larger tumor volume and weight. Conversely, relative to the sh-NC group, the sh-GPR176 group displayed a substantial reduction in tumor volume and weight (Fig. 9A). Subsequent examination of the mRNA expression levels of EMT pathway molecules, consistent with the *in vitro* results, revealed activation of the EMT pathway following GPR176 upregulation, while the EMT signaling pathway was inhibited after GPR176 downregulation (Fig. 9B). Western blot analysis suggested the activation of the EMT and PI3K/AKT/mTOR pathways following GPR176 upregulation, as indicated by increased levels of key pathway markers. Conversely, GPR176 downregulation resulted in changes indicative of pathway inhibition. (Fig. 9C), with the corresponding bar chart shown in Fig. S4A-G.

Discussion

Gastric cancer is a highly malignant tumor characterized by the absence of typical early symptoms, often leading to late-stage diagnosis and missing of the optimal treatment window (4,5). This poses several challenges for the treatment of gastric cancer (43). In the advanced stages, gastric cancer has usually spread to lymph nodes or other organs, making treatment considerably more difficult (44-46). Due to the different subtypes of gastric cancer, each with different biological behaviors and treatment responses, the development of universal treatment plans is complex and requires more individualized and precise treatment strategies (47,48). Some patients develop resistance to conventional chemotherapeutic agents, leading to a decrease in treatment efficacy and requiring constant adjustment of drug combinations during treatment to overcome the adaptability of the tumor (49,50). Given these challenges, there is an urgent need for further research in the field of gastric cancer treatment to develop more effective and personalized treatment strategies.

GPR176 is generally considered to be associated with circadian rhythms (26). However, some researchers have begun to recognize its role in tumors. For example, Tang *et al* (28) found that GPR176 interacts with the G protein GNAS to inhibit mitochondrial autophagy in colorectal cancer cells, thereby promoting cancer progression. In the present study, compared with normal gastric tissue, upregulation of GPR176 expression in gastric cancer was observed, with significant differences in expression between different TNM stages and tissue types. This heterogeneous expression suggests that GPR176 may play different biological roles in different subtypes of gastric cancer.

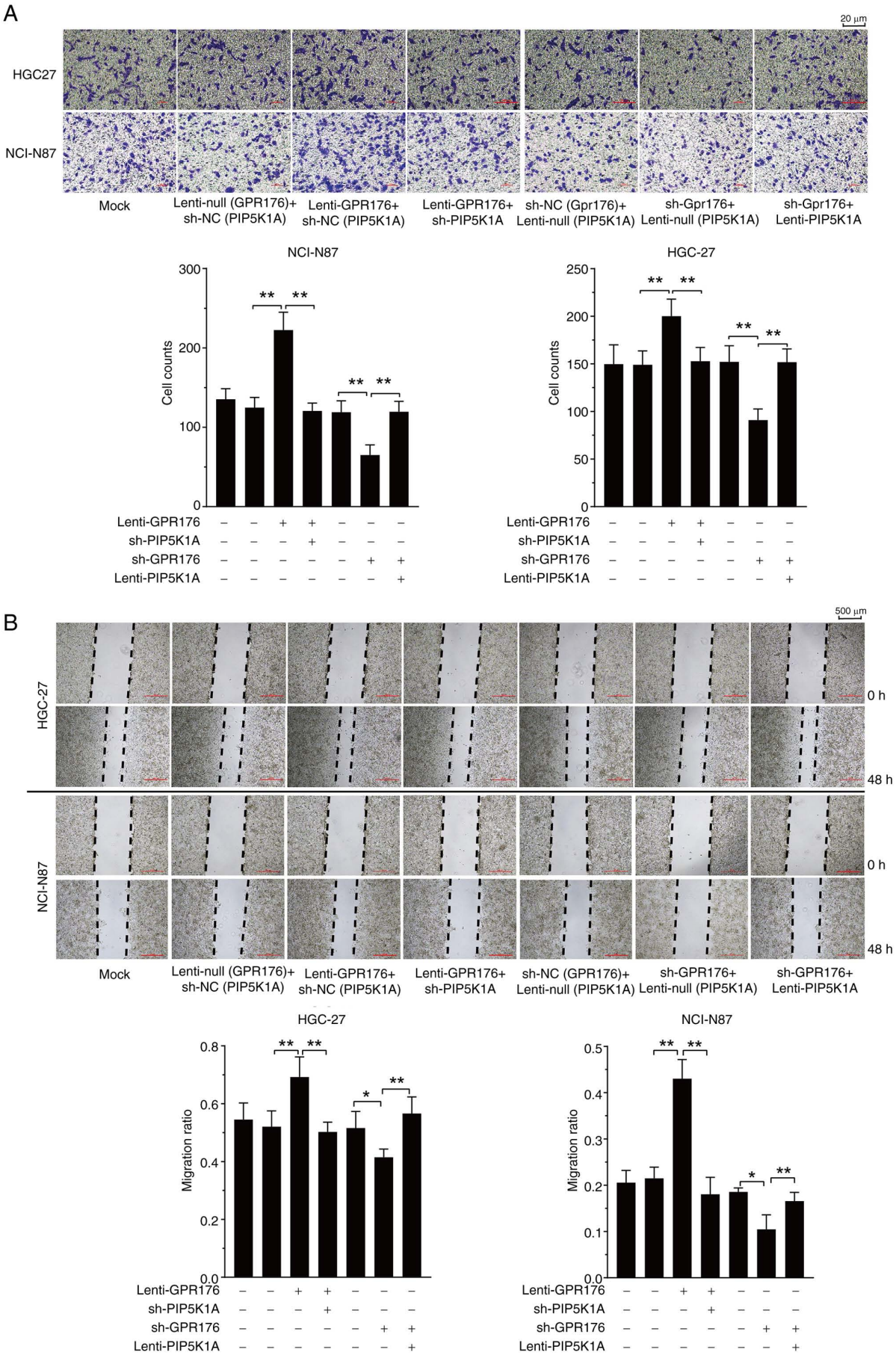

Figure 5. Genetic regulation of PIP5K1A counteracts the effects of GPR176 on invasion, migration. (A) Combined genetic regulation of GPR176 and PIP5K1A on cell invasion. (B) Combined genetic regulation of GPR176 and PIP5K1A on cell migration. sh-NC, short hairpin-negative control. Statistical analysis was carried out using two-way ANOVA. *P<0.05, **P<0.01.

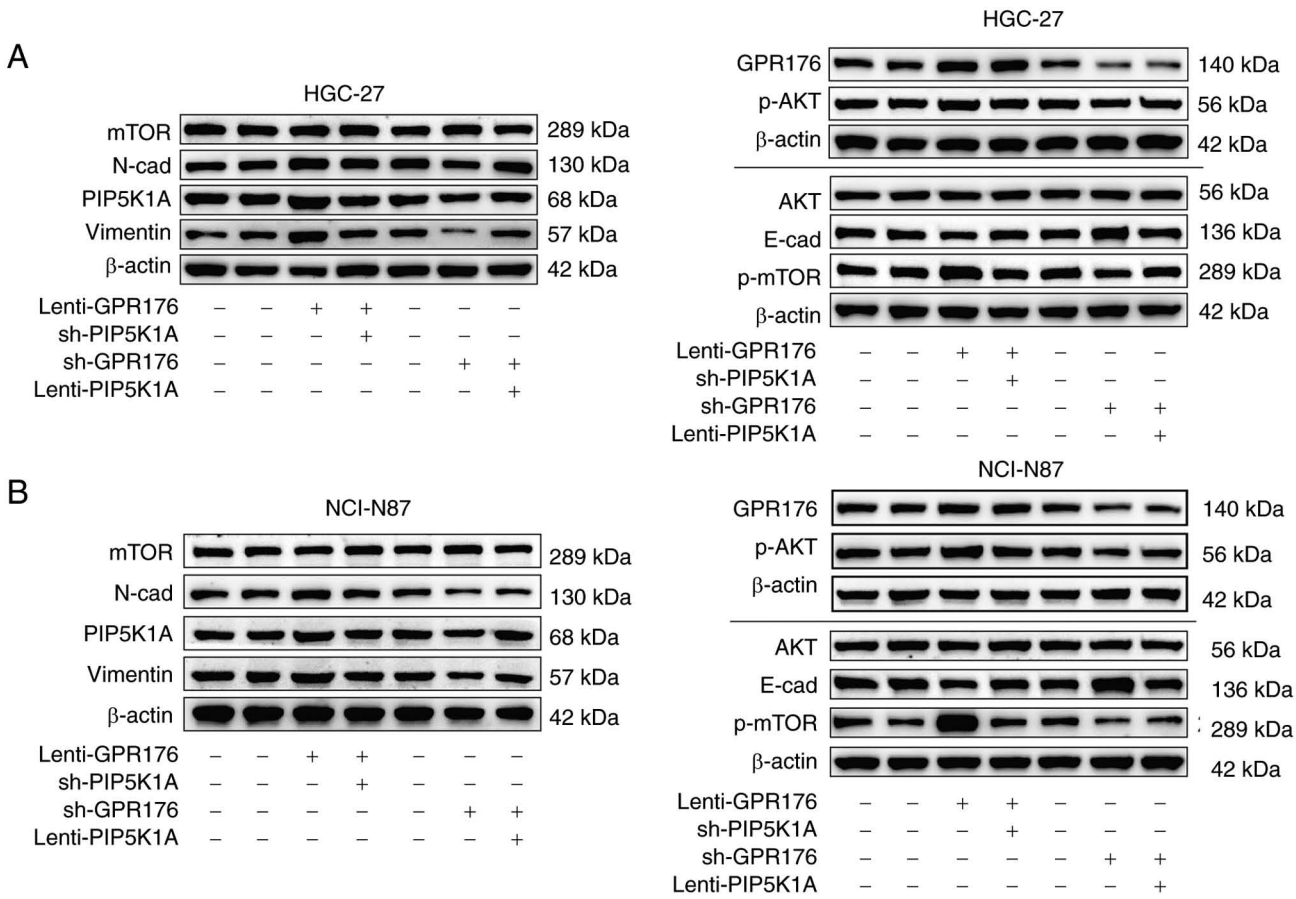

Figure 6. Genetic regulation of PIP5K1A counteracts the effects of GPR176 on EMT pathway and PI3K/AKT/mTOR pathway. (A) Combined genetic regulation of GPR176 and PIP5K1A on protein expression and phosphorylation in the EMT pathway and PI3K/AKT/mTOR pathway in HGC-27 cells, with the corresponding bar chart shown in Fig. S3A. (B) Combined genetic regulation of GPR176 and PIP5K1A on protein expression and phosphorylation in the EMT pathway and PI3K/AKT/mTOR pathway in HGC-27 cells, with the corresponding bar chart shown in Fig. S3B. EMT, epithelial-mesenchymal transition; sh, short hairpin-; Lenti, Lentivirus; cad, cadherin.

Through integrated analysis of data from TCGA and GEO databases, it was clarified that high GPR176 expression is significantly associated with shorter overall survival and disease-free survival in patients with gastric cancer. This suggests that GPR176 could serve as an independent prognostic marker, providing a new molecular standard for the assessment of patient survival. Similarly, researchers such as Yun *et al* (29) have previously suggested that GPR176 may also serve as a prognostic biomarker in breast cancer. Bioinformatic analysis revealed that the gene expression profile regulated by the accumulation of GPR176 is associated with signaling pathways such as PI3K/AKT/mTOR. This suggests that GPR176 may be involved in the development of gastric cancer via this signaling pathway. Further experimental verification demonstrated an association between GPR176 and the PI3K/AKT/mTOR signaling pathway and provided an experimental basis for understanding the specific role of GPR176 in cell proliferation, survival and metastasis.

The PI3K signaling pathway carries out a key role in the proliferation and progression of various cancer cells, including gastric cancer (51,52). Numerous previous studies have indicated that the PI3K signaling pathway promotes the progression of gastric cancer through various mechanisms, including inhibiting apoptosis, inducing drug resistance, facilitating metastasis and promoting angiogenesis (53-55).

After activation by PI3K and PIP2, AKT kinase relocates downstream to the cell membrane, triggering its conformational activation (56). AKT carries out a key role in activating the PI3K axis and elevated expression of AKT and p-AKT has been detected in >74% of gastric cancer cases (57). Aberrant expression of p-AKT is associated with the overexpression of PI3K and HER2, and high levels of p-AKT are regarded as indicators of tumor progression, metastasis, and poor prognosis in gastric cancer (58).

Analysis of the TCGA molecular subtypes reveals that the majority of gastric cancer cases studied display varying degrees of PIK3CA gene mutations, along with amplifications of RTK genes such as EGFR and HER2 (48). Genomic amplifications markedly contribute to tumor progression. Amplification of PIK3CA is associated with tumor progression, prognosis and the development of gastric cancer resistance (59). The substantial involvement of the PI3K/AKT/mTOR signaling pathway in gastric cancer progression suggests that targeting this signaling axis holds potential for cancer therapy (60).

The present study identified the upregulation of GPR176 as a promoter of EMT in gastric cancer cells, thereby revealing a mechanism that drives tumor invasion and metastasis. *In vitro* assays confirmed that GPR176 overexpression enhances cell migration and invasion, whereas its knockdown produces the opposite effect. These findings highlight the pivotal role of

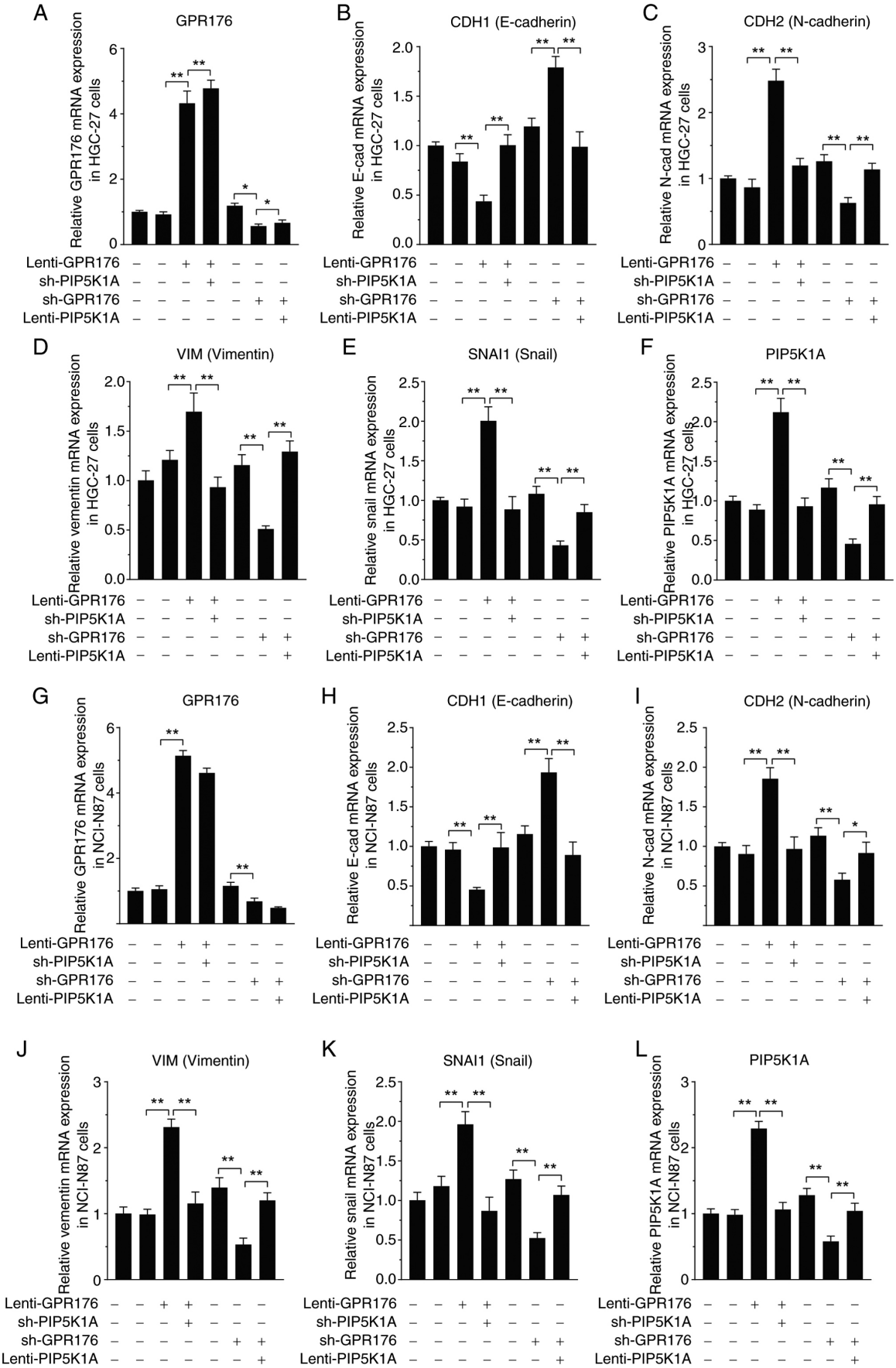

Figure 7. Genetic regulation of PIP5K1A counteracts the effects of GPR176 on the expression of EMT pathway genes at the mRNA level. Combined genetic regulation of GPR176 and PIP5K1A on the expression of (A) GPR176 (B) CHD1 (E-cadherin), (C) CHD2 (N-cadherin) (D) VIM (Vimentin), (E) SNAI1 (Snail), (F) PIP5K1A in in HGC-27 cells. Combined genetic regulation of GPR176 and PIP5K1A on the expression of (G) GPR176, (H) CHD1 (E-cadherin), (I) CHD2 (N-cadherin), (J) VIM (Vimentin), (K) SNAI1 (Snail), (L) PIP5K1A in NCI-N87 cells. EMT, epithelial-mesenchymal transition; sh-, short hairpin-; cad, cadherin. Statistical analysis was performed using two-way ANOVA. $^{**}P<0.01$, $^{*}P<0.05$.

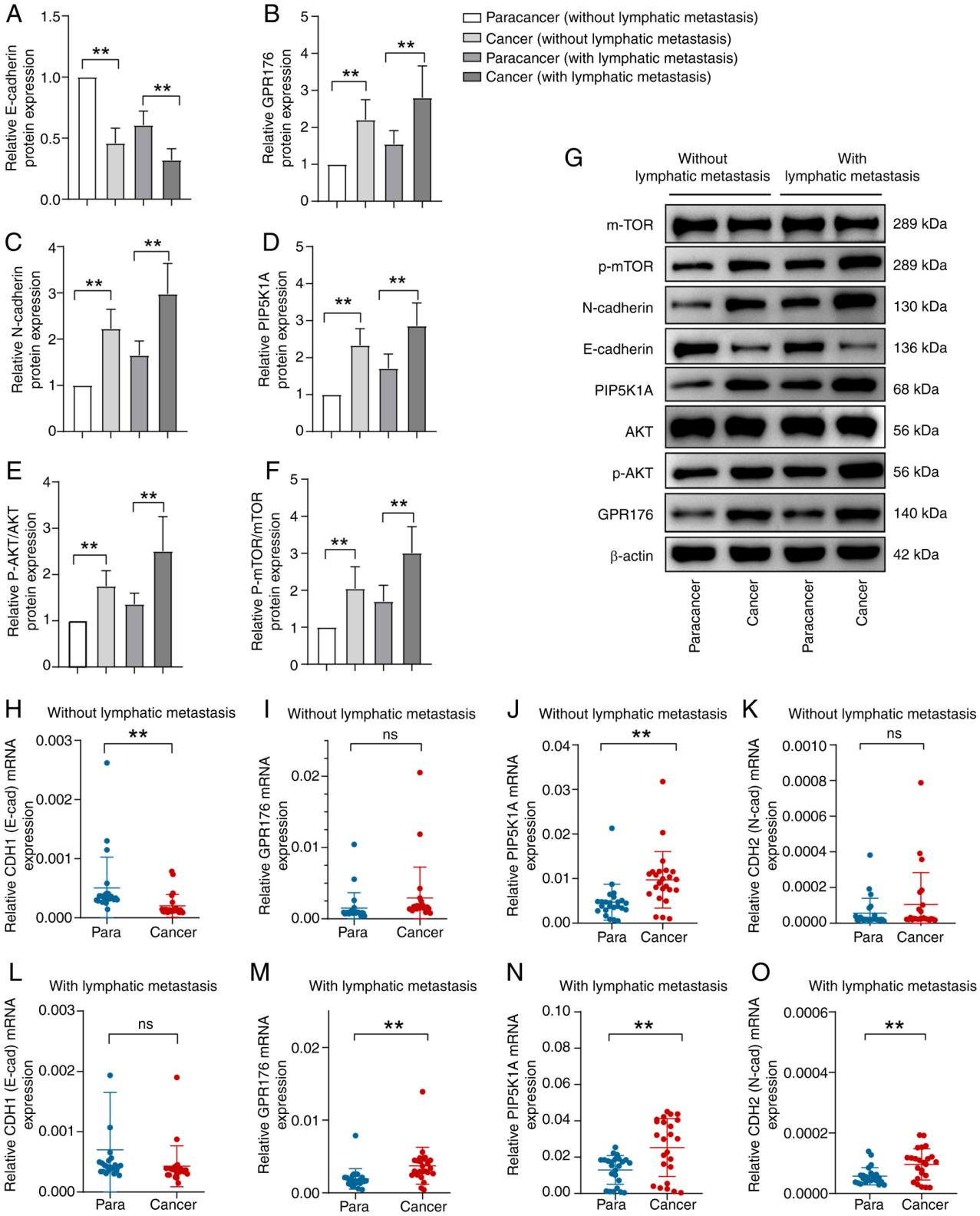

Figure 8. A significant difference was observed in the expression of key molecules in the EMT and PI3K/AKT/mTOR pathways, as well as in GPR176 and PIP5K1A levels, between metastatic and non-metastatic tumor tissues. (A) The relative expression of E-cadherin at the protein level in the specific group. (B) Relative expression of GPR176 at the protein level in the specific group. (C) Relative expression of N-cadherin at the protein level in the specific group. (D) Relative expression of PIP5K1A at the protein level in the specific group. (E) Relative expression of p-AKT/AKT at the protein level in the specific group. (F) Relative expression of p-mTOR/mTOR at the protein level in the specific group. (G) The representative blots of key molecules of the EMT signaling pathway and PI3K/AKT/mTOR pathway at the protein level in the specific group, as well as the expression of GPR176 and PIP5K1A. (H) Relative expression of CDH1 (E-cadherin) at the mRNA level in the specific group. (I) Relative expression of GPR176 at the mRNA level in the specific group. (J) Relative expression of PIK5K1A at the mRNA level in the specific group. (K) Relative expression of CDH2 (N-cadherin) at the mRNA level in the specific group. (L) Relative expression of CDH1 (E-cadherin) at the mRNA level in the specific group. (M) Relative expression of GPR176 at the mRNA level in the specific group. (N) Relative expression of PIK5K1A at the mRNA level in the specific group. (O) Relative expression of CDH2 (N-cadherin) at the mRNA level in the specific group. Para, para-cancer. For experiments in Fig. 8A-J, statistical analysis was performed using two-way ANOVA. For the experiments presented in Fig. 8K-R, an unpaired Student's t-test was used to assess the statistical significance of differences between the two groups. **P<0.01.

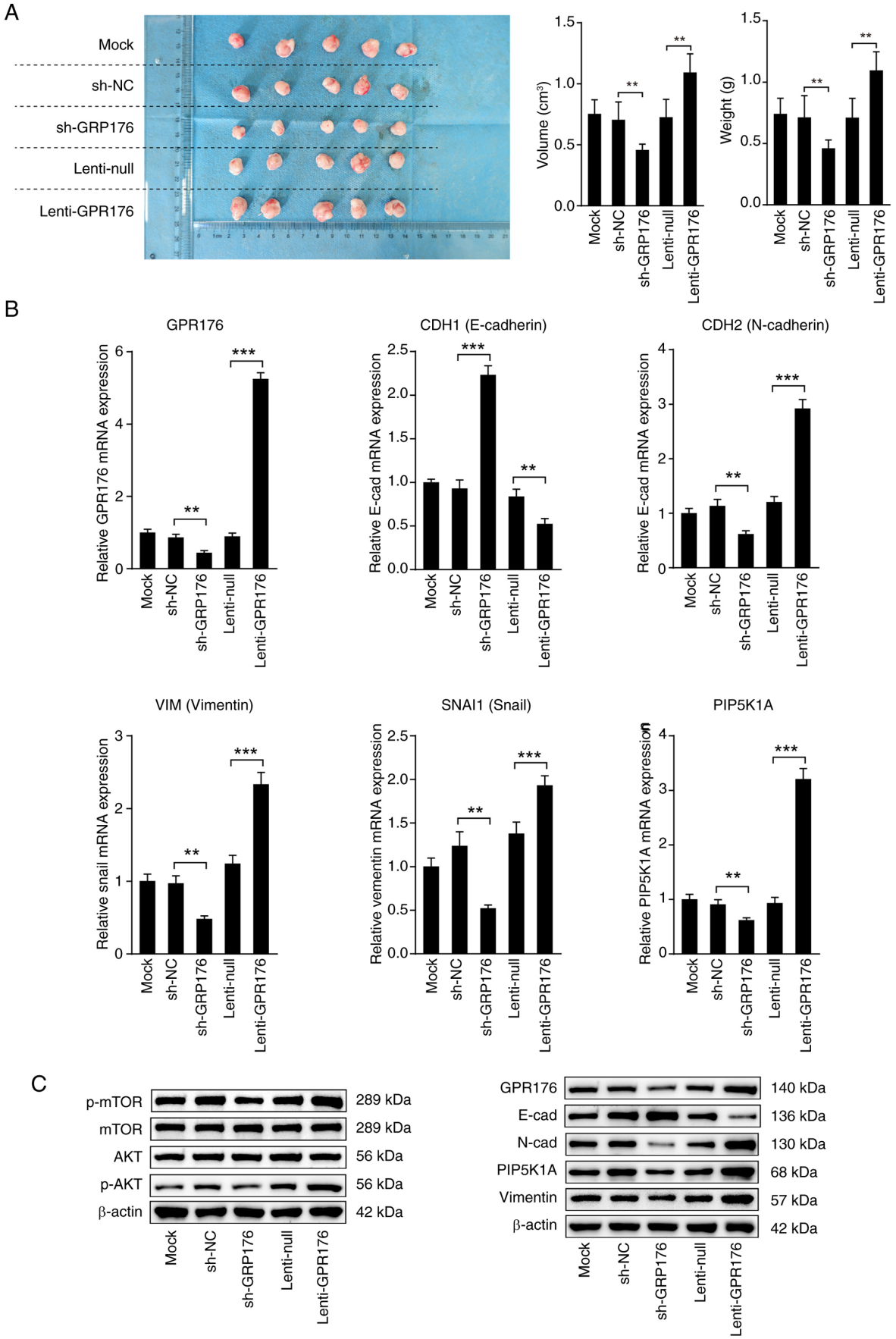

Figure 9. Genetic regulation of GPR176 expression affects mouse subcutaneous graft tumor growth, PI3K/AKT/mTOR pathway and the EMT pathway. (A) Effects of upregulation and knockdown of GPR176 expression on tumor growth. (B) Effects of GPR176 expression regulation on the expression of PIP5K1A and EMT signaling pathway genes. (C) Effects of GPR176 expression regulation on the EMT signaling pathway and PI3K/AKT/mTOR pathway. SH-, short hairpin-; NC, negative control; EMT, epithelial-mesenchymal transition; cad, cadherin. Statistical analysis was performed using an unpaired Student's t-test. **P<0.01, ***P<0.001.

GPR176 in regulating EMT and suggest that it may serve as a novel modulator of gastric cancer progression. Future studies should further dissect the upstream regulators and downstream effectors of GPR176 in EMT, as well as its interactions with other EMT-related transcription factors and regulatory networks.

The present study also identified an association between GPR176 and PIP5K1A, and that downregulation of PIP5K1A prevented the effects of GPR176 on migration, invasion, EMT and activation of the PI3K/AKT/mTOR pathway. This indicates that GPR176 may exert its tumor-promoting function, at least in part, through synergistic regulation with PIP5K1A. Additional studies employing gene knock-in models, CRISPR/Cas9-mediated editing or co-immunoprecipitation assays are warranted to validate the direct interaction between GPR176 and PIP5K1A and to elucidate their precise molecular mechanisms. Although the present study highlighted the involvement of GPR176 in the PI3K/AKT/mTOR signaling pathway, it is important to note that this pathway represents a common oncogenic mechanism (61-63). Future research should therefore investigate the potential upstream regulators that modulate GPR176 expression, such as transcription factors, epigenetic modifications or non-coding RNAs, which may contribute to its dysregulation in gastric cancer. In addition, downstream interactions of GPR176 beyond PI3K/AKT/mTOR, including crosstalk with MAPK/ERK or Wnt/β-catenin pathways, may further shape tumor cell proliferation, survival and metastatic behavior. Exploring these upstream and downstream aspects will broaden the understanding of GPR176-mediated oncogenic signaling and may uncover novel therapeutic opportunities.

As a member of the G protein-coupled receptor family, GPR176 remains relatively underexplored in both physiological and pathological contexts. Findings of the present study expand the current understanding of its role in gastric adenocarcinoma, particularly in relation to cell signaling, proliferation, invasion and metastasis. Given the high heterogeneity of gastric cancer and the variable therapeutic responses among patients, studying GPR176 expression and function across tumor subtypes could contribute to the development of more individualized treatment strategies. Furthermore, the observed association between GPR176 expression and patient survival underscores its potential as a prognostic biomarker and as a candidate for risk stratification in clinical practice.

Furthermore, the findings of the present study indicate that high GPR176 expression in gastric adenocarcinoma is associated with tumor invasion, metastasis and poor prognosis, highlighting its potential translational significance. GPR176 may serve not only as a prognostic biomarker for assessing patient survival and recurrence risk but also as a promising therapeutic target due to its key role in tumor cell proliferation, migration and activation of the PI3K/AKT/mTOR pathway. Future studies should further validate the predictive value of GPR176 in independent patient cohorts and explore the feasibility of targeting GPR176 through small-molecule inhibitors, antibodies or gene-editing strategies, providing new avenues for personalized therapy and precision oncology.

Despite these insights, several limitations should be acknowledged. First, the bioinformatics analyses were based on retrospective datasets (TCGA-STAD and GSE66254) with limited sample sizes, which may introduce selection bias. Second, although the prognostic value and functional role of GPR176 were validated through *in vitro* and *in vivo* experiments, the models may not fully capture the complexity of the human tumor microenvironment. Third, the mechanistic investigations mainly focused on the PI3K/AKT/mTOR signaling pathway and EMT, while other potential pathways or crosstalk mechanisms remain unexplored. Finally, the translational relevance of GPR176 as a biomarker or therapeutic target requires further validation in large-scale, prospective and independent clinical cohorts. Addressing these limitations will be key for advancing the clinical application of GPR176 in gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GM conceived and designed the research program; GM and KL contributed to acquisition of data; CH, HR, DL, GZ and JC performed data analysis. GM, ZH, HR and KL performed the experiments. KL wrote the manuscript. GM guided and supervised the manuscript. All authors read and approved the final manuscript. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The investigation had been approved by the ethics committee of Guangxi Medical University the first affiliated hospital, Nanning, China (approval no. 2023-S243-01)]. All methods in this research were carried out in accordance with Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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