Original Article

GGT5 as a promising prognostic biomarker and its effects on tumor cell progression in gastric cancer

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Background: Gastric cancer (GC) is a gastric malignant tumor with over 1 million new cases globally each year. There are many diagnostic methods for GC, but due to the hidden early symptoms of GC, early GC is easy to be missed and misdiagnosed, which affects the follow-up treatment of patients. The early and accurate diagnosis of GC is of great significance for the treatment and survival of GC patients. Our laboratory study found that gamma-glutamyl transferase (GGT) was highly expressed in GC patients, but the mechanism of GGT family genes in the occurrence and development of GC remained to be further studied. Therefore, this study aimed to explore the mechanism of GGT family functional gene GGT5 regulating the proliferation and migration of GC cells, and provide a possible new biomarker for the early diagnosis of GC.

Methods: The value of serum GGT in GC patients was first statistically analyzed. Then, The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets were used to analyze the mRNA expression of GGT5 in GC, and its clinical relationship and function. Furthermore, expression of GGT5 was reduced by lentivirus RNA interference and verified by polymerase chain reaction (PCR), Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) assays were used to detect cell proliferation after GGT5 knockdown. Scratch and Transwell assays were applied to observe cell migration after knockdown of GGT5. Finally, Western blot assays were observed to demonstrate PI3K/AKT-MAPK and MMPs expression levels after knockdown of GGT5.

Results: Serum GGT was expressed at a high level in GC patients. GGT5 was highly expressed in GC tissues, and was associated with poor prognosis and clinical stage of GC. GGT5 might be involved in the regulation of vascular development and angiogenesis, as well as in the mechanisms of cell motility and migration, and it was positively correlated with the PI3K/AKT pathway. The proliferation and migration capacity of GC cells was dampened by downregulation of GGT5. GGT5 mediated proliferation and migration of GC cells by directly targeting PI3K/AKT-MAPK-MMPs pathways.

Conclusions: Low expression of GGT5 reduced proliferation and migration in GC cells by modulating the PI3K/AKT-MAPK-MMPs pathway, and GGT5 might be a new target for GC.

Keywords: Gamma-glutamyl transferase 5 (GGT5); gastric cancer (GC); knockdown; prognostic markers; bioinformatics analysis

Submitted Dec 02, 2023. Accepted for publication Jun 10, 2024. Published online Aug 23, 2024.
doi: 10.21037/tcr-23-2222
View this article at: https://dx.doi.org/10.21037/tcr-23-2222

Introduction

Gastric cancer (GC) is a common cancer worldwide, ranking fifth in incidence and fourth in mortality. In 2020, there were more than 1 million new cases of GC and 769,000 deaths worldwide (1). At present, GC is difficult to diagnose in the early stage, and surgery, endoscopic resection, chemotherapy, biological agents and immunotherapy have been included in the treatment of GC, which reduces the mortality of GC to a certain extent, but the effect is not satisfactory. Therefore, new diagnosis or treatment methods are urgently needed to improve the clinical outcome of GC.

Gamma-glutamyl transferase (GGT) is a membrane-bound extracellular enzyme (2) that serves as a novel non-invasive marker that can be detected in the blood and used to indicate various diseases. Studies have shown that elevated serum GGT levels predict chronic diseases such as liver injury and insulin resistance (3,4). Apart from this, most studies have shown that GGT expression is significantly increased in human malignant tumors. For example, high GGT levels are associated with poor prognosis in cancers such as gallbladder cancer, pancreatic cancer, and epithelial ovarian cancer (5-7). GGT genes belong to a multigene family with at least seven genes to adapt to high physiological variations and expression in different physiological states and encode enzymes involved in glutathione (GSH) metabolism and amino acid transpeptidation which include GGT1, GGT2, GGT3P, GGT4P, GGT5, GGT6, GGT7 and GGT8P (8). GGT1 and GGT5 are the only two extracellular enzymes that cleave γ-glutamyl bonds (9,10).

Gamma-glutamyl transferase 5 (GGT5) belongs to the GGT family and has been shown to be the only GGT enzyme of having catalytic activity to date (10). GGT5 protein is expressed by local macrophages in multiple organs, including Kupffer cells in the liver and macrophages. GGT5 has been implicated in the control of re-oxidation (11), drug metabolism, immunological function (12), and other body functions, and there has been evidence that GGT5 may induce inflammation. Wei *et al.* had demonstrated that high levels of GGT5 in cancer-associated fibroblasts (CAFs) correlated with cancer cell survival and treatment resistance in lung adenocarcinoma (13). With the development of bioinformatics and the deepening of GC research (14-16), some papers have shown that GGT5 may play an important role in the pathogenesis and development of GC. However, the mechanism of GGT5 in GC remains unclear.

We hypothesized that GGT5 was related to tumor cell growth. In this study, bioinformatics analysis was used to explore the expression and function of GGT5 in GC. In addition, we conducted *in vitro* experiments to evaluate the impact of GGT5 knockdown on GC cell proliferation and migration. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2222/rc).

Highlight box

Key findings

- Knockdown of gamma-glutamyl transferase 5 (GGT5) reduced proliferation and migration in gastric cancer (GC) cells by modulating the PI3K/AKT-MAPK-MMPs pathway.

What is known and what is new?

- GGT5 is highly expressed in GC.
- GGT5 mediated proliferation and migration of GC cells by directly targeting PI3K/AKT-MAPK-MMPs pathways.

What is the implication, and what should change now?

- GGT5 might be a new target for GC and can be used for the diagnosis, prognosis and treatment of GC.

Methods

Bioinformatics analysis

A harmonized pan-cancer dataset was downloaded from the UCSC (http://xenabrowser.net/) datasets: TCGA Pan-Cancer, further extracted ENSG00000099998 (GGT5) gene expression data from each sample, log2(x+0.001) transformation was performed for each expression value. GGT5 expression in different tumors and the relationship between GGT5 and prognosis, immune infiltration, clinical stage were analyzed in Sangerbox (17) (http://www.sangerbox.com/tool). GSE27342, GSE29272 and GSE118916 expression profiles were obtained from the Gene Expression Omnibus (GEO) datasets (https://www.

ncbi.nlm.nih.gov/geo/). RNAseq data and corresponding clinical information for GC were obtained from the Cancer Genome Atlas (TCGA) datasets (http://portal.gdc.com), and differential mRNA expression was investigated using the R software Limma package. The LinkedOmic datasets (18) (http://www.linkedomics.org) and cBioPortal (19,20) (https://www.cbioportal.org/) were used to conduct a correlation study between GGT5 and other GC genes. In order to further determine the potential function of GGT5, ClusterProfiler (21) package in R software was used to analyze the GGT5 Gene Ontology (GO) functions and enrich Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, as well as to use LinkedOmic datasets for gene set enrichment analysis (GSEA) analysis.

Clinical information

A total of 124 primary GC cases (all patients underwent surgical resection by endoscope, laparotomy or laparoscopy, and had not received any treatment for GC before surgery. No epilepsy drugs. No other malignant tumor within 5 years) diagnosed and treated in The First Affiliated Hospital of Zhejiang Chinese Medical University in the past three years were selected. A total of 287 subjects (no abnormal blood lipids, normal tumor markers, and no other disease diagnosed within 1 year) who underwent a physical examination in the last year were selected as healthy controls. Biochemical indexes of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and GGT in GC patients prior to surgery and healthy controls (excluding abnormal liver function. ALT ranged from 5 to 40 U/L in males and 5 to 35 U/L in females, and AST 8 to 40 U/L.) were collected, and differences in GGT were analyzed. This research only collected the serum test data of the patients' clinical cases. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the Institutional Review Board (IRB) of The First Affiliated Hospital of Zhejiang Chinese Medical University (IRB: 2022-KL-150-01) and was exempted from informed consent by the IRB.

Cell culture

Human GC cell lines AGS, HGC-27 and MKN-45 were used in the experiments. HGC-27 was acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). AGS and MKN-45 were donated by the Key Laboratory of Digestive Pathophysiology of Zhejiang Province and the Department of Cell and Immunology of Zhejiang Chinese Medical University, respectively. HGC-27 and AGS were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), MKN-45 was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% FBS. All cells were maintained in 100 U/mL penicillin, and 100 μg/mL streptomycin at a humidified incubator (37 ℃, with 5% CO_2).

Cell transfections

The lentiviral vector has the characteristics of green fluorescent protein (GFP) gene and puromycin resistance, that is, the cells can express green fluorescence normally and are resistant to puromycin after successful transfection. According to the lentivirus transfection manual, 24 hours before transfection, target cells with good growth were selected, and 5×10^4 cells were added to each well of 6-well plates for culture under conventional culture conditions. When the cell confluence rate reached 70% after 24 h, the old medium was removed, and 1 mL of suspended diluted GGT5 knockdown lentivirus (sh-GGT5, Genechem, China) was added to each well (sh-GGT5 sequences were GCTCTTCTTCAACGGGACAGA). At the same time, the target cells were transfected with control negative lentivirus (sh-NC) to establish the control cell line (sh-NC sequences were TTCTCCGAACGTGTCACGT). Cells are routinely cultured in 37 ℃, 5% CO_2 incubator. Cell growth status was observed 12 h after transfection. If there was no obvious toxic effect, the conventional medium was replaced about 48 hours later. At 72 h after transfection, expression of GFP in cells could be observed under inverted phase contrast fluorescence microscopy. When the expression of green fluorescent protein was good and the growth was stable, puromycin (Beyotime, China, 2 μg/mL) was added to screen the cells with poor transfection. Polymerase chain reaction (PCR) was used to detect GGT5 expression in transfected cells. The cells with high fluorescence expression and stable growth could be used for subsequent cell experiments.

RNA isolation and PCR

Total RNA isolation was performed from cells using Trizol reagent (Glpbio, USA). After its concentration was determined, RNA was reversed-translated into the cDNA kit based on instructions from the BIO-

RAD script cDNA synthesis kit (Bio-Rad, USA). The primer sequences were as follows: forward primer 5'-GTCAGCCTAGTCCTGCTGG-3' and reverse primer 5'-GGATGGCTCGTCCAATATCCG-3' for GGT5; forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGT-3' for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was the reference gene for GGT5.The date were analyzed via using $2^{-\Delta\Delta Ct}$ method.

Cell Counting Kit-8 (CCK-8)

GC cell proliferation was assessed by CCK-8 assay (Beyotime, China). According to the manufacturer's instructions, 3000 cells were seeded into 96-well plates. The cells were cultured for 24, 48, and 72 h, CCK-8 reagent was added into each well, after incubation for another 2 h at 37 ℃, absorbance at 450 nm was measured using a multifunctional microplate reader.

5-ethynyl-2'-deoxyuridine (EdU) assays

The 5×10^5 cell suspension was injected into the 6-well plates. After the cells were cultured overnight and returned to normal, EdU labeling (Beyotime, China), fixation, washing and permeability were performed according to kit instructions. A volume of 500 μL of the Click reaction solution containing Azide 594 dye was added to each well for incubation, and then nuclear staining was conducted by using Hoechst dye, followed by fluorescence detection.

Transwell migration assays and wound healing assays

After the cells were digested by trypsin, 5×10^4 cell suspension was injected into 1% FBS medium and cultured in the 6-well plates starved for 24 h. Digestive cells again, 200 μL 2×10^4 cell suspension was inoculated in Transwell chambers (Corning, USA), placed in the 24-well plates, and cultured in the lower chamber of 10% FBS medium for 24 h. Then, after fixation with 70% methanol, crystal violet staining was carried out to stain the cells.

For wound healing assays, 5×10^5 cell suspension was seeded into 6-well plates. After 24 h, a scratch wound was made using a sterile 1,000 μL pipette tip in a monolayer of cells. The scratch wound was imaged at the 0, 24 and 48 h time points using microscope at the same location of 6-well plates. The wound healing rate was measured by the fracture area of the noncellular regions.

Western blotting

The proteins in cells were extracted by using the Radio Immunoprecipitation Assay (RIPA) lysis buffer containing a mixture of protease and phosphatase inhibitors (Beyotime, China), and were mixed with 5× loading buffer (Beyotime, China) at a ratio of 4:1, boiled for denaturation. The proteins were prepared with sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to the polyvinylidene fluoride membrane. Then the membranes were blocked with 5% skimmed milk powder and incubated at 4 ℃ overnight with primary antibodies (*Table 1*). After recovery of the primary antibody, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (HRP-labeled goat anti-mouse IgG, Sangon Biotech Cat# D110087, RRID: AB_2940948; HRP-labeled goat anti-rabbit IgG, Sangon Biotech Cat# D110058, RRID: AB_2940954) for 2 h at 37 ℃. The Enhanced Chemiluminescence (ECL) chemiluminescence reagent (Biosharp, China) was then used to develop imaging. The relative expression of the target protein was calculated using grey value analysis software.

Statistical analysis

Each experiment was repeated three times independently. Statistical analysis and drawing were performed using IBM SPSS Statistics 25 software (IBM Corp., Chicago, IL, USA), GraphPad Prism 8 (GraphPad Software, CA, USA). Variance analysis, independent *t*-tests and Chi-squared tests were performed to assess differences between groups. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 were considered to indicate statistical significance.

Results

Upregulation of GGT5 expression in GC and its association with clinicopathological parameters

First, serum GGT expression levels of GC patients and healthy controls were analyzed, and the results showed that GGT levels were highly expressed in GC (P<0.05) (*Figure 1A*, *Table 2*). TCGA and GEO datasets were used to further confirm our results. We detected high expression of GGT5 in GC tissues and found that patients with high expression of GGT5 had a significantly poor prognosis (*Figure 1B-1D*). In addition, the association between GGT5 expression and clinicopathological parameters of GC was investigated. GGT5 expression was significantly increased

Table 1 Primary antibodies

Primary antibodies	Host	Dilution ratio	Brand	Catalogue number	RRID
GAPDH	Rabbit	1:10,000	Affinity, USA	AF7021	AB_2839421
AKT	Mouse	1:2,000	Proteintech, USA	60203-2-Ig	AB_10912803
P-AKT	Mouse	1:2,000	Proteintech, USA	66444-1-Ig	AB_2782958
PI3KP85	Rabbit	1:2,000	Beyotime, China	AF7742	AB_2844106
ERK	Rabbit	1:2,000	Beyotime, China	AF1051	AB_2923146
P-ERK	Rabbit	1:2,000	Beyotime, China	AF1891	AB_2923145
P38	Rabbit	1:2,000	Bioss, China	bs-0637R	AB_10856281
P-P38	Rabbit	1:2,000	Bioss, China	bs-0636R	AB_10856595
STAT5b	Rabbit	1:5,000	Abcam, UK	ab178941	AB_2885102
MMP2	Rabbit	1:5,000	Abcam, UK	ab92536	AB_10561597
MMP9	Rabbit	1:5,000	Abcam, UK	ab76003	AB_1310463
EGFR	Rabbit	1:5,000	Abcam, UK	ab52894	AB_869579

RRID, Research Resource Identifier.

in advanced tumor specimens, and was positively correlated with immune infiltration of GC and correlated with the T stage, grade and stage of GC (*Figure 1E,1F*). Meanwhile, the analysis of the datasets showed that the occurrence of GC was not related to sex and age (*Figure 1G,1H*).

Through differential expression analysis, 806 differentially expressed genes were finally obtained, of which 802 were upregulated and 4 were downregulated in GC. Fold change and corrected P values were used for volcano plots, with red dots indicating significantly up-regulated genes, blue dots representing the genes with significant difference down, and gray dots indicating insignificant genes (*Figure 2A*). As the *Figure 2B-2J* shows, EMILIN1, HIC1, C1R and TMEM119 etc. were positively correlated genes with GGT5, LRPPRC, MELK, MAD2L1 and UCHL5 etc. were negatively correlated genes with GGT5 (*Figure 2B-2J*). Functional enrichment analysis of differential genes showed that the molecular functions of differential gene products were mainly related to vascular smooth muscle contraction, regulation of vascular development and angiogenesis, regulation of actin cytoskeleton, cell adhesion molecules, regulation of cell motility and migration, and extracellular matrix (ECM) organization (*Figure 2K-2L*). At the same time, the expression of GGT5 was found to be slightly positively correlated with the PI3K/AKT pathway (*Figure 2M*).

Low expression of GGT5 reduced GC cell proliferation

To identify the role of GGT5 on GC, first, we detected transfection efficiency after transfection of sh-NC and sh-GGT5 into GC cells. The fluorescence efficiency of transfection was observed by fluorescence microscopy to reach 75%, and then unstable cells were screened by puromycin. Subsequently, PCR revealed that GGT5 expression was significantly reduced by transfection with sh-GGT5 in GC cells (*Figure 3A,3B*). Next, to determine whether GGT5 could affect GC cell proliferation, EdU and CCK8 assays were performed. The results showed that the proliferation capacity was inhibited in sh-GGT5 GC cells (*Figure 3C,3D*).

Low expression of GGT5 inhibited GC cell proliferation by downregulating the PI3K/AKT-MAPK signaling pathway

To further elucidate the detailed mechanisms of sh-GGT5 reduced proliferation in GC cells, PI3K/AKT-MAPK pathways were investigated. As expected, treatment with sh-GGT5 significantly decreased phosphorylation of P38 and ERK compared to the control group (P<0.001). At the same time, western blotting results showed that the expression of AKT, P-AKT and PI3KP85 protein was reduced (P<0.001) in GC cells when transfected with sh-GGT5. Furthermore,

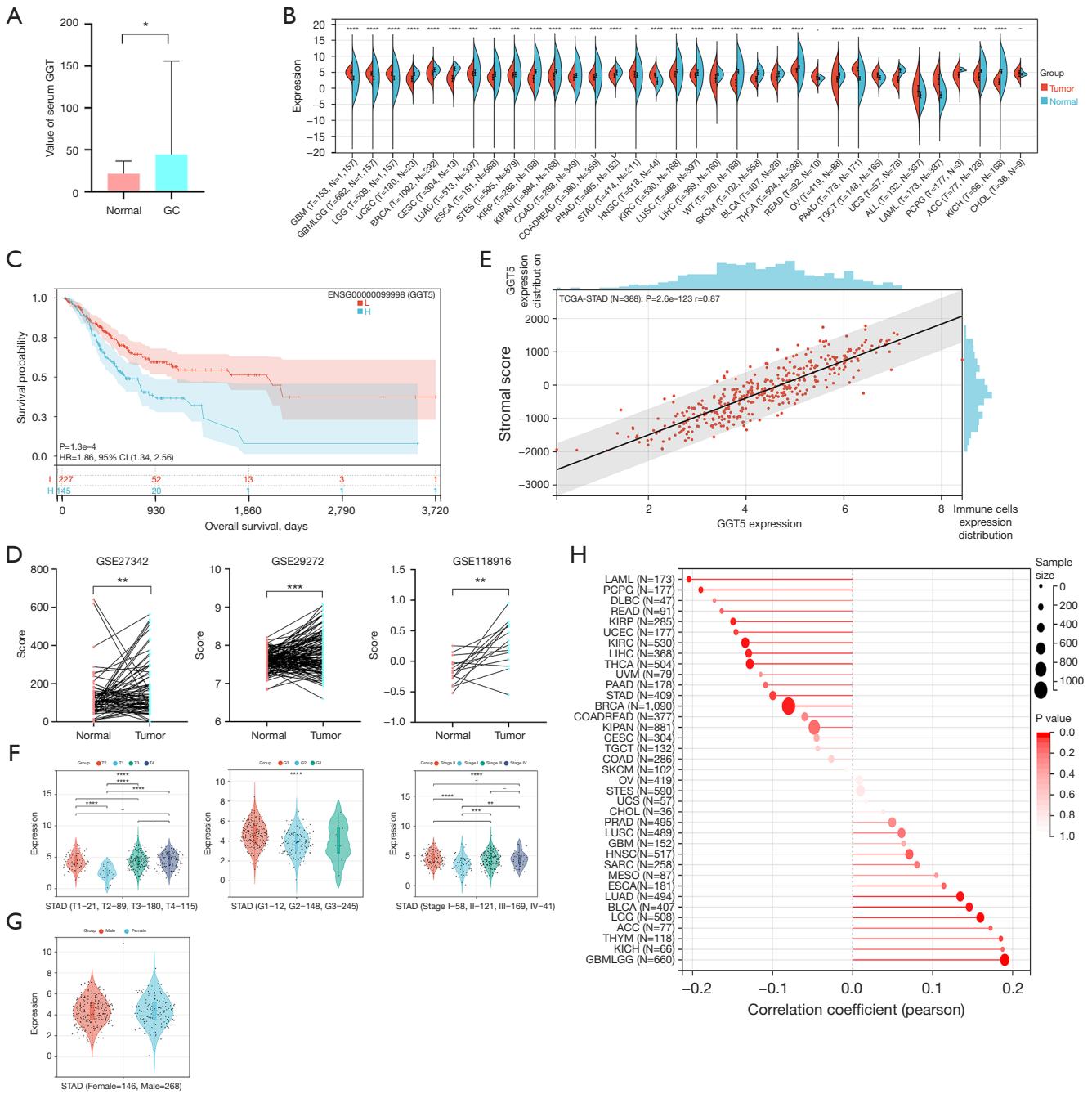

Figure 1 Upregulation of GGT5 expression in GC and its association with clinicopathological parameters. (A) Serum GGT levels in clinical GC patients. (B) Expression of GGT5 in GC tissues in TCGA datasets. (C) Relationship between GGT5 expression and prognosis. (D) Expression of GGT5 in GC tissues in GEO datasets. (E) Connection of GGT5 with immune infiltration in GC. (F) Relationship between GGT5 expression and clinical staging. (G) Gender analysis of GC tissue. (H) Age analysis of GC tissue. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. GC, gastric cancer; GGT, gamma-glutamyl transferase; GGT5, gamma-glutamyl transferase 5; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus.

Table 2 Statistical analysis of clinical data from GC patients and healthy controls

Variable	GC patients (n=124)	Healthy controls (n=287)	P value
GGT (U/L)	44.48±111.48	21.62±14.9	0.02*
Age (years)	60.17±11.21	57.80±10.08	0.50
Gender (cases)			
Male	76	156	
Female	48	131	0.19

GGT and age were expressed as mean ± standard deviation, gender was expressed as number of cases. And n represented the number of cases in the GC patients or healthy controls. GGT and age were analyzed by using independent *t*-tests to assess differences between groups. Gender was analyzed by using Chi-squared tests to assess differences between groups. *, P<0.05. GC, gastric cancer; GGT, gamma-glutamyl transferase.

expression of the nuclear transcription factor STAT5b (P<0.001) was inhibited after GGT5 knockdown (*Figure 4*). The results evidently demonstrated low expression of GGT5 could affect the GC progression by regulating the PI3K/AKT-MAPK signaling pathway.

GGT5 knockdown inhibited GC cell migration

Next, the functionalities of GGT5 on GC cell migration were investigated. As expected, scratch test results showed that GGT5 knockdown abrogated the migration capacity of GC cells (P<0.01). In addition, the Transwell assay was used to measure cell migration again. The results provided evidence that was consistent with the provided scratch test results (P<0.001) (*Figure 5A*,*5B*). To confirm the influence of GGT5 on MMPs signaling in GC cells, western blotting showed that expression of MMP2 (P<0.001), MMP9 (P<0.001) and EGFR (P<0.01) decreased when GGT5 was knocked down (*Figure 5C*). These results suggested that GGT5 promoted GC migration by activating the MMPs signaling pathway.

Discussion

GC is the fourth most common cancer in men and the seventh most common cancer in women. Early GC can be treated with endoscopic mucosal resection or endoscopic submucosal dissection for a good long-term prognosis (22,23). Improvements in hygiene and the elimination of H. Pylori have significantly lowered global statistics of GC, but there is still a long way to improve survival rates for advanced and metastatic GC (24). Carcinoembryonic antigen (CEA) and carbohydrate antigen 199 (CA199) are frequently used in GC diagnosis and prognosis. However, existing circulating biomarkers have showed low sensitivity and specificity (25). Therefore, it is important to find an effective therapeutic target and new biomarkers.

The GGT gene belongs to a multi-gene family and is involved in the occurrence and development of various malignant tumors (6,9,10). GGT1 and GGT5 are the only two extracellular enzymes in the GGT family that can cleave the γ-glutamyl bond. They produce precursor amino acids such as cysteine and cystine by hydrolyzing γ-glutamyl substrates, which are used to maintain the γ-glutamyl cycle in tumor cells and thereby promote the occurrence and development of tumors (26). Song *et al.* found that GGT5 may affect the trait of GGT (27). This study found that the preoperative serum GGT value of GC patients was higher than that of the healthy control group (*Figure 1A*). Analysis in the TCGA datasets revealed that GGT5 was highly expressed in GC tissues (*Figure 1B*). Therefore, this study focused on the functional study of GGT5 in GC.

GGT5 has been proved to be a catalytically active GGT enzyme, and is involved in regulating the body's functions, including reoxidation, drug metabolism and immune functions (11,12). When there is unregulated uptake of amino acid metabolic enzymes and changes in metabolite-driven genes, it can affect the occurrence and development of tumors (28,29). GGT5 is mainly involved in the extracellular catabolism of GSH, hydrolyzing it to glutamic acid, cysteine and glycine (30). Tumor cells cannot directly absorb GSH, but rely on high concentrations of cysteine and cystine in serum and interstitial fluid to maintain intracellular GSH levels. Therefore, the cysteine generated by GGT5 decomposing GSH becomes an important raw material required for tumor cells to synthesize GSH (26). Meanwhile, GSH also plays a role in cell cycle regulation and cell signal transduction (31,32). Studies have shown that GSH has an inhibitory effect on PI3K/AKT pathway (33), and its expression level may decrease with the high expression of GGT5 in GC, while the decrease of GSH level will cause the activation of p38MAPK and AKT (34,35). Gene enrichment analysis showed that GGT5 overexpression may be closely related to MAPK pathway and PI3K/AKT pathway (36,37). In addition, GGT5 can convert LeukotrienceC4 into active leukotrienceD4, thus playing an important role in tumor

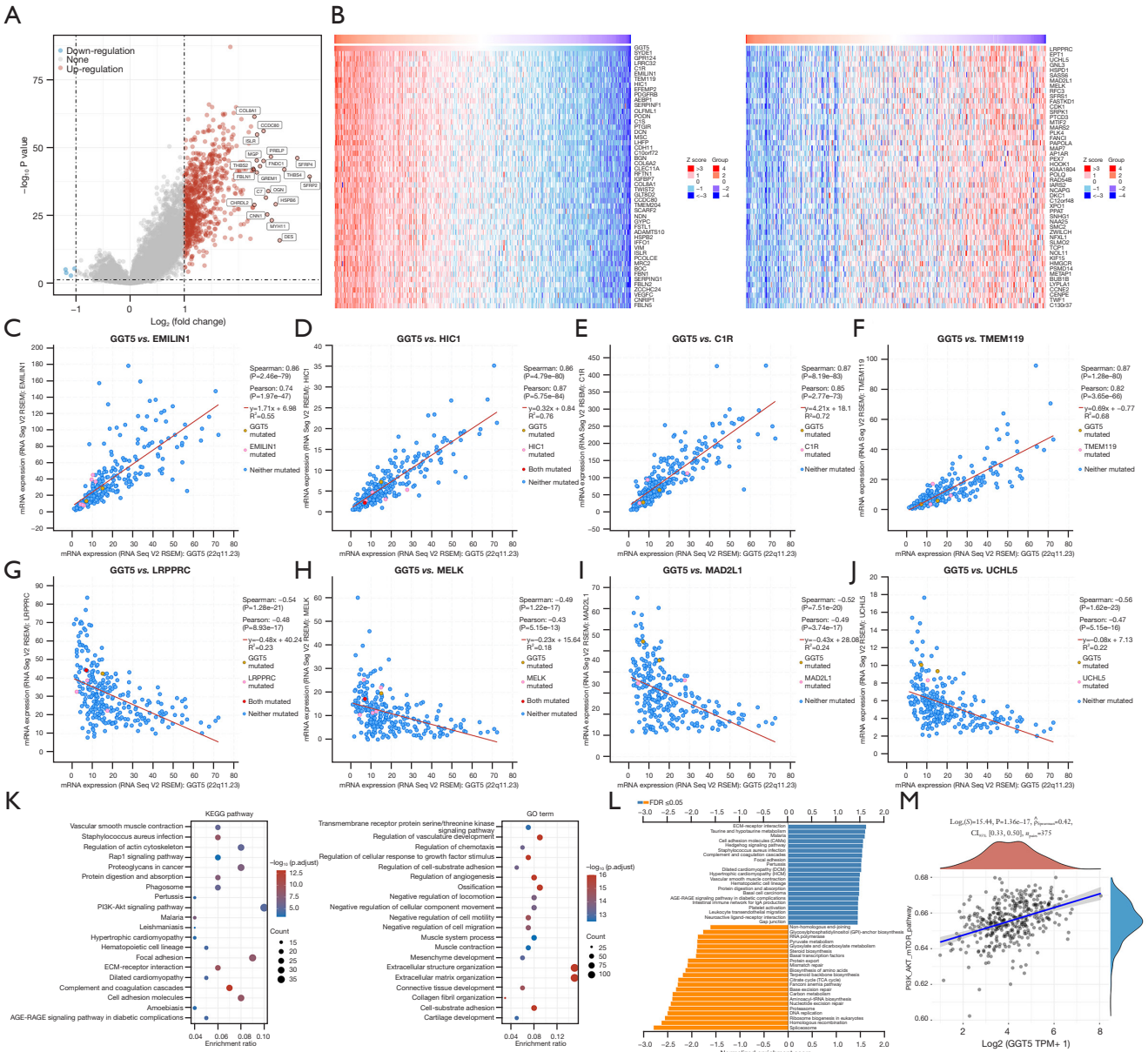

Figure 2 Potential functions of GGT5. (A) Volcanic plots for differentially expressed gene. (B-J) Significant positively/negatively correlated genes associated with GGT5. (K) KEGG pathway analysis and GO analysis for biological processes. (L) GESA was performed for functional enrichment analysis. (M) Relationship between expression of GGT5 and PI3K/AKT pathway. GGT5, gamma-glutamyl transferase 5; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, gene ontology; GSEA, gene set enrichment analysis.

immunity (38). Overexpression of GGT5 in tumor tissues is positively correlated with PD-L1 expression and CD8 T cell infiltration (39), and overexpression of GGT5 in follicular dendritic cells can impair the ability of P2RY8 to enhance B cell restriction of germinal center (40). Moreover, the balance between GGT5 and oxidative stress cascade regulation plays a key role in steroid production (11). In summary, GGT5 can regulate the occurrence and development of tumors through pathways such as the metabolism of GSH, immune infiltration, and signal pathways. GGT5, as an oncogene, was activated in breast cancer (41), lung cancer (13,42,43) and liver cancer (2,44),

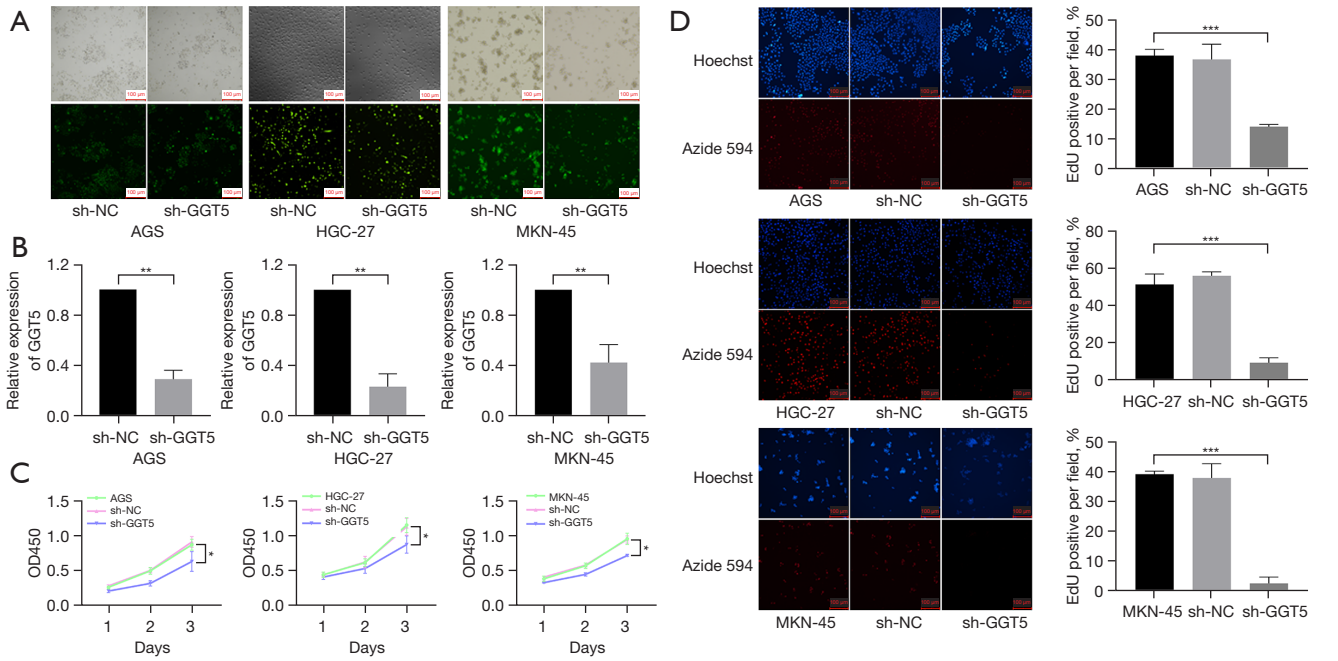

Figure 3 Low expression of GGT5 reduced GC cell proliferation. (A) Transfection efficiency with GFP by fluorescence microscopy. (B) The transfection efficiency was tested by PCR. (C) Proliferation was detected by CCK-8. (D) Cell multiplication was measured by EdU. Use the Azide 594 dye to detect proliferative cells, and the Hoechst dye to stain the cell nucleus. *, P<0.05; **, P<0.01; ***, P<0.001. GC, gastric cancer; GGT5, gamma-glutamyl transferase 5; GFP, green fluorescent protein; PCR, polymerase chain reaction; CCK-8, cell counting kit-8; EdU, 5-ethynyl-2'-deoxyuridine.

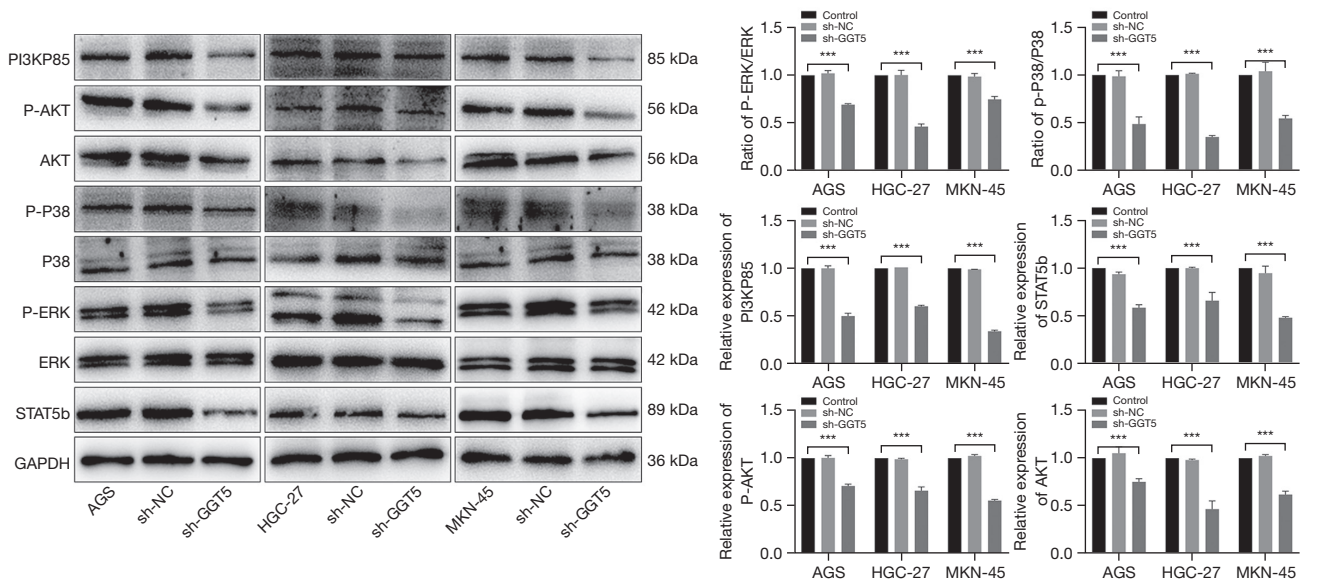

Figure 4 Low expression of GGT5 inhibited GC cell proliferation by downregulating the PI3K/AKT-MAPK signaling pathway. ***, P<0.001. GC, gastric cancer; GGT5, gamma-glutamyl transferase 5.

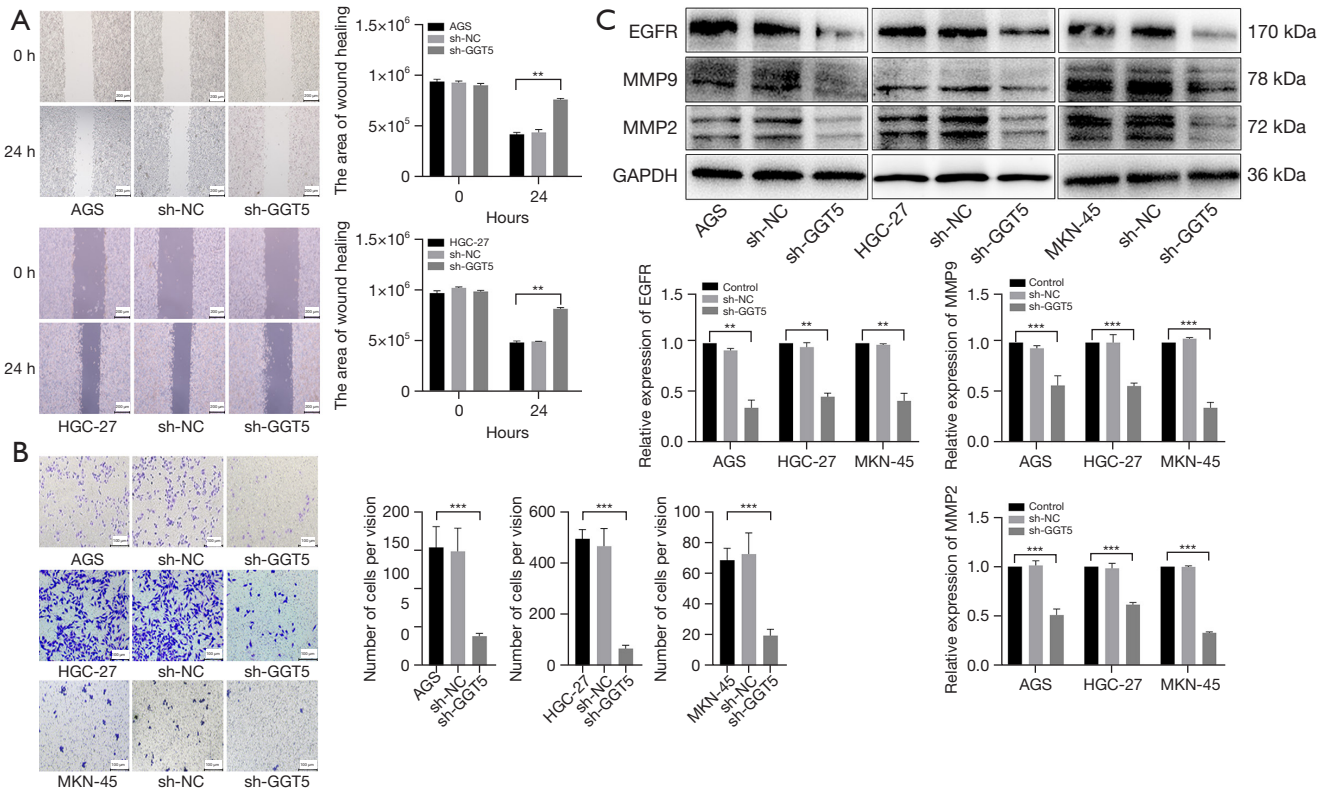

Figure 5 GGT5 knockdown inhibited GC cell migration. (A) Migratory capacity was detected by wound healing assays. (B) The migration ability of GC cells was verified by Transwell staining with crystal violet. (C) The MMPs pathway was examined by western blotting. **, P<0.01; ***, P<0.001. GC, gastric cancer; GGT5, gamma-glutamyl transferase 5; MMPs, matrix metalloproteinases.

causing the enhancement of tumor cell proliferation and invasion. However, the mechanism of GGT5 in GC was still unclear. Therefore, this study mainly explored the mechanism of GGT5 in GC, and provided a basis for GGT5 to be used as a diagnostic target for GC.

In our work, we further found that GGT5 was indeed highly expressed in GC through GEO datasets. In addition, bioinformatics analysis showed that increased GGT5 expression levels would lead to poorer outcomes in GC patients and was positively correlated with immune infiltration of GC (*Figure 1C-1E*). The study also investigated the relationship between GGT5 expression and clinicopathological parameters of GC, and the results showed that GGT5 expression increased significantly in advanced GC tumor specimens, which was correlated with the T stage, grade and stage of GC, and was independent of age and sex (*Figure 1F-1H*). Our work explored the mechanism of GGT5 in GC cells. We found that low expression of GGT5 could inhibit the growth and migration of GC cells and alter the biological characteristics of GC cells by CCK-8, EdU, scratch and Transwell experiments (*Figures 3C,3D,5A,5B*). PI3K/AKT signaling may promote cell survival, which have been identified as drivers of oncogenesis and cancer progression, and MAPKs are also important pathways in regulating tumor development (45-47). The results were shown in *Figure 4*, GGT5 knockdown significantly decreased AKT, P-AKT, PI3KP85 levels as previously described (*Figure 2E*) and reduced phosphorylation of P38 and ERK compared to the control group.

The tumor microenvironment (TME) of GC is composed of ECM, fibroblasts, endothelial cells, mesenchymal stem cells, macrophages, lymphocytes, neutrophils and other cell components (48). The stomach has a strong acidic environment and a unique endocrine system, which also makes the TME of GC distinctive (49). In GC, the characteristics of stromal cells are associated with the deterioration of patient survival rate and treatment

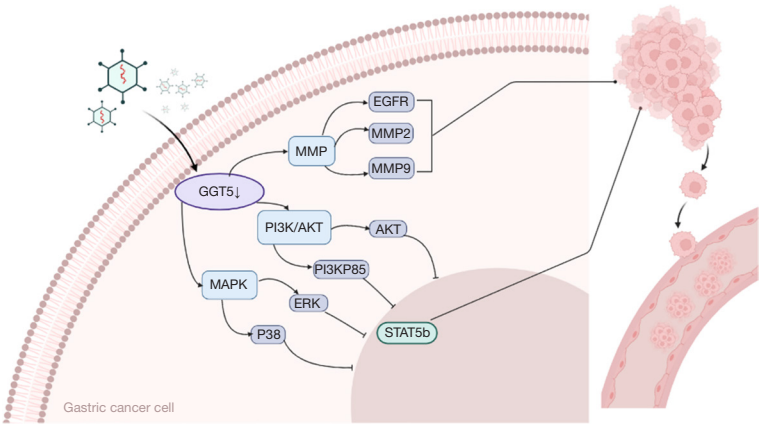

Figure 6 Potential mechanisms for knockdown of GGT5 prevent GC progression. GGT5 knockdown might inhibit PI3K/AKT-MAPK-MMPs pathways, thus preventing GC cell proliferation and migration. GC, gastric cancer; GGT5, gamma-glutamyl transferase 5. Created with BioRender.com.

resistance, and promote tumor invasion through activating stromal remodeling, immune crosstalk, metabolic effects, and soluble secreted factors (50,51). ECM and matrix together constitute the first barrier in the process of tumor metastasis, and its degradation is a key link in tumor invasion and metastasis. Studies have shown that the matrix metalloproteinases (MMPs), as important enzymes that can degrade ECM, play an important role in mediating tumor angiogenesis, metastasis and invasion (52,53). Western blotting showed that MMP2, MMP9, EGFR were inhibited by knockdown of GGT5 (*Figure 5C*). Therefore, GGT5 knockdown could regulate MMPs protein expression and inhibit GC cell metastasis through the ECM of the TME.

By differential expression gene analysis, we concluded that the GGT5 gene might act as a prognostic biomarker in GC (*Figure 2A*). Co-expressed EMILIN1, HIC1, C1R, and TMEM119 genes were positively correlated with GGT5 and had been associated with tumor growth and poor cancer prognosis (54-57). Co-expressed genes LRPPRC, UCHL5, MAD2L1, and MELK negatively correlated with GGT5 might be novel therapeutic targets for GC in the future (58-62) (*Figure 2B-2J*). Angiogenesis is required for aggressive tumor growth and metastasis (63), impairment of PI3K and MAPK signaling pathways may affect hematological parameters (64), MMPs may also contribute to the formation of the premetastatic microenvironment by secreting proangiogenic factors (65). To investigate the probable biological processes of GGT5, we performed GO and KEGG analyses on the GGT5 gene. Enrichment analysis of the GGT5 gene showed that the regulation of angiogenesis, cell motility and migration, cell adhesion molecules, ECM, and other related processes were significantly enriched, indicating that GGT5 was associated with metastasis and invasion of GC (*Figure 2C,2D*).

The study found that high levels of GGT5 in CAFs of lung adenocarcinoma contribute to cancer cell survival and drug resistance, indicating that GGT5 may be a promising therapeutic target for lung adenocarcinoma (13). Nevertheless, the role of GGT5 in the treatment resistance of GC still needs to be further investigation, with the aim of providing a theoretical basis for the development of GGT5 as a therapeutic target for GC. It has been reported that cell surface receptors transduced signals from ECM into cells, which may govern a variety of cellular processes including survival, growth, migration, and differentiation (66). A recent review showed that the ECM may be involved in deregulation of pre-malignant lesions and GC (67). Therefore, the role of GGT5 in EMT needs further study. In addition, the competing endogenous RNA network has been extensively studied in recent years (68,69), so we will further investigate the mechanism of GGT5 in GC to further elucidate the role of GGT5 in GC.

In summary, the potential function of GGT5 in GC was explored in our study. We concluded that GGT5 knockdown might inhibit GC progression by targeting PI3K/AKT-MAPK-MMPs pathways (*Figure 6*), and proposed that GGT5 might be a novel target for GC therapy.

Conclusions

In this study, we investigated the expression and function of GGT5 in GC and the underlying mechanism of GGT5 in tumor progression. Knockdown of GGT5 expression might inhibit GC cell proliferation and migration by inhibiting PI3K/AKT-MAPK-MMP pathways. GGT5 might be a potential therapeutic target for GC.

Acknowledgments

We acknowledge the Key Laboratory of Digestive Pathophysiology of Zhejiang Province and the Department of Cell and Immunology for providing GC cell lines.
Funding: This work was supported by Natural Science Foundation of Zhejiang Province (No. LQ19H160044 to W.C./No. LGF21H160016 to Jian Xu), and Zhejiang Province Traditional Chinese Medicine Science and Technology Project (No. 2023ZL056 to Z.Z.).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2222/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2222/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2222/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2222/coif). W.C. reports receiving funding from Natural Science Foundation of Zhejiang Province (No. LQ19H160044). Z.Z. reports receiving funding from Zhejiang Province Traditional Chinese Medicine Science and Technology Project (No. 2023ZL056). Jian Xu reports receiving funding from Natural Science Foundation of Zhejiang Province (No. LGF21H160016). The other authors have no conflicts of interests to disclose.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board (IRB) of The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China (IRB: 2022-KL-150-01) and was exempted from informed consent by the IRB.

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Cite this article as: Chen W, Yang F, Shen H, Xu J, Chen J, Zhang Z, Xu J, Xu B. GGT5 as a promising prognostic biomarker and its effects on tumor cell progression in gastric cancer. Transl Cancer Res 2024;13(8):4459-4473. doi: 10.21037/tcr-23-2222

