SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Data acquisition and processing

Gene expression profiles and paired clinical information were downloaded from The Cancer Genome Atlas (https://portal.gdc.cancer.gov) to verify the expression and prognostic impact of FLG in GC based on the GPL570 platform tested by Affymetrix Human Genome U133 Plus 2.0 Array.

FLG expression analysis

We utilized Tumor Immune Estimation Resource version 2 (TIMER2) to analyze FLG expression levels between tumors and paired normal tissues. In tumor types with no or limited normal tissues, we investigated FLG expression by using 'Gene Expression Profile' section of Gene Expression Profiling Interactive Analysis (version 2) (GEPIA2) to combine data from normal tissues in the Genotype-Tissue Expression (GTEx) database, under parameters of log2FC (fold change) cutoff = 1 and *p*-value cutoff = 0.01.

UALCAN tool was utilized to perform protein expression analysis. Protein expression data were from Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset. Besides, we downloaded and analyzed immunohistochemical images of FLG protein expression in human normal tissues and tumor tissues from the Human Protein Atlas (HPA).

Survival prognosis analysis and ROC curve analysis

We obtained overall survival (OS) and disease-free survival (DFS) significance

maps and Kaplan–Meier (K-M) survival plots of FLG by utilized ‘Survival Analysis’ section of GEPIA2. We utilized R package p ROC (version 1.17.0.1) to analyze data and R package ggplot2 (version: 3.3.3) to visualize the results.

The PrognoScan database complies accessible tumor microarray datasets, facilitating the correlation of gene expression and survival including OS and disease-free survival (DFS). We evaluated the relationship between FLG expression and prognosis in diverse tumor types using the PrognoScan database (http://www.abren.net/PrognoScan/) with a threshold P-value of 0.05.

Genetic alteration analysis

The cBioPortal tool and GSCALite were employed to analyze FLG genetic alterations. Alteration frequency and type and mutated site for FLG from TCGA were obtained and analyzed. We input ‘FLG’ to ‘Quick Search’ module, and genetic alterations and mutated site data can be obtained from ‘Cancer Types Summary’ and ‘Mutations’ modules.

Immune infiltration analysis

MCPCOUNTER, TIDE and EPIC algorithms were employed to investigate the correlation between FLG expression and infiltration of CAFs in the ‘Immune Association’ section of TIMER2.

We obtained the tumor dataset from the UCSC database. Immediately, the FLG expression data in each sample were extracted. Besides, tumor gene expression profiles were extracted. Subsequently, we mapped them to GeneSymbol. Finally, ESTIMATE was used to calculate stromal, immune, and estimate scores, and the corr.

test was utilized to calculate Pearson's correlation between FLG expression and immune infiltration scores.

Protein network and FLG-related gene enrichment analysis

GeneMANIA website was utilized to find functionally similar genes using a large body of genomics and proteomics data, and we used it to search for genes that are functionally similar to FLG. Also, a human FLG-binding protein co-expression network can be obtained by STRING tool. All gene symbols were used as input gene symbols for Gene Ontology pathway enrichment analysis.

Western blotting

After quantification and denaturation, protein was subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated separately with primary antibodies against anti-human FLG antibody (1:500 dilution), against anti-human N-cadherin antibody (1:1000 dilution), against anti-human E-cadherin antibody (1:1000 dilution), against anti-human Vimentin antibody (1:1000 dilution), against anti-human ZEB1 antibody (1:500 dilution) and a mouse anti-human β-actin (1:500 dilution). The secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibody was used as a 1:5000 dilution. The film is scanned and the optical density value of the target strip is analyzed with the Gel image processing system.

CCK8 detection

GC cells transfected with si-FLG and si-NC in logarithmic growth phase were seed in 96-well plates at 5×10^3 cells/well density, and 5 multiple wells were designed for each group. After incubation for 0h, 24h, 48h, 72h and 96h, the supernatant was discarded and 100 μl complete medium was added to each well, then a volume of 10

μl CCK8 solution was added to each well and incubated in an incubator for 1 h. The wavelength at OD450 was measured in a multiplate reader.

Flow cytometry assay

GC cells transfected with si-FLG and si-NC in logarithmic growth phase were cultured in 6-well plates with 5×10^5 cells/well. The cells of each group were centrifuged at 150g for 5min and then collected, the supernatant was carefully removed. The cells were washed with PBS twice and resuspend with 500 μl binding Buffer. According to manufacturer's instructions, samples were stained with Annexin V-FITC and propidium iodide. Then, incubation at room temperature for 15min away from light and an Aria flow cytometer was utilized to display the apoptotic cell population. Finally, FlowJo software was used to analyze data.

Transwell assay

Single cell suspensions (2.5×10^4 cells/well) were added to the upper chambers and allowed to invade for 24 h at 37°C in a CO_2 incubator. Cells remaining attached to the upper surface of the filters were carefully removed with cotton swabs. The membranes were fixed with 4% paraformaldehyde for 20 min, and stained with 0.1% crystal violet for 20 min at room temperature and examined by light microscopy.

Cell invasion was assayed in BD Bio-coat Matrigel invasion chambers with an 8-μm pore size polycarbonate filter coated with Matrigel. DMEM (500 μl) containing 10% FBS was added to the lower chambers. Data are presented as mean ±SD of three independent experiments.

Wound healing assay

According to the experimental plan, cells of each group were cultured until the density was fused. Before the experiment, the medium was changed to serum-free medium and 10μg/ml of mitomycin C was added. Cells in each group were scratched

with 200 ul pipette tip, and the surface was cleaned with serum-free medium once to remove the cell debris, and then cultured with serum-free medium. Cells were evaluated at 0, 24, 48, 72 and 96 h using an inverted phase-contrast microscope

Statistical analysis

Results from the Oncomine database included fold change, *p*-value, and gene rank. The PrognoScan and GEPIA databases produced HR and *p*-values or Cox P-values according to a log rank test. The correlation between FLG expression and other related immune genes was investigated using the Spearman method and the correlation strength was categorized according to R values. R values of 0.80-1.00 were deemed very strong, R values of 0.60-0.79 were strong, R values of 0.40-0.59 were moderate, R values of 0.20-0.39 were weak, and R values of 0.00- 0.19 were categorized as very weak.

Data were analyzed using GraphPad Prism (version 6.00) and SPSS (version 20.0). We employed One-way ANOVA to compare multiple groups, and Student's t-test to compare two groups. Data were reported as means ± SD. $P \leq 0.05$ was considered statistically significant.

Detailed Information of Reagents

Dulbecco's modified Eagle's medium (DMEM; Servicebio, China);

RPMI-1640 medium (1640; Servicebio, China);

10% fetal bovine serum (FBS; PAN, Germany);

0.25% Trypsin-EDTA (Sigma,USA)

Phosphate Buffered Saline (PBS, Sangon Biotech, China)

Trizol (Invitrogen, USA);

Lipofectamine 3000 (Invitrogen, USA);

Puromycin (Invitrogen, USA);

Lipofectamine 3000 (Invitrogen, USA);

Reverse transcription kit (Transgene, China);

SYBR Green PCR Master Mix (Transgene, China);

Anti-human FLG antibody (Affinity, China);

Anti-human E-cadherin antibody (Santa Cruz Biotechnology, CA, USA);

Anti-human N-cadherin antibody Santa Cruz Biotechnology, CA, USA);

Anti-human vimentin antibody (Santa Cruz Biotechnology, CA, USA);

Anti-human ZEB1 antibody (Santa Cruz Biotechnology, CA, USA);

Mouse anti-human β-actin (Wanleibio, China);

Peroxidase -conjugated goat anti-rabbit or anti-mouse antibody (Wanleibio, China);

Immobilon Western Chemiluminescent HRP substrate (Millipore, USA);

4% Paraformaldehyde (Aladdin, China);

BD Bio-coat Matrigel invasion chambers (BD Biosciences, USA);

Crystal violet (Amresco , USA);