Effects of *Helicobacter pylori* on the expression of the FTO gene and its biological role in gastric cancer

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Abstract. *Helicobacter pylori* (*Hp*) is a primary risk factor for gastric cancer. The fat mass and obesity-associated (FTO) gene is associated with the development and progression of various cancer types such as glioma, leukemia, breast cancer and colorectal cancer. The aim of the present study was to investigate the effect of *Hp* infection on the expression of FTO and its roles in gastric cancer. It was found that the expression levels of both FTO mRNA and protein were significantly increased in *Hp*-infected human gastric mucosal epithelial cells and Mongolian gerbil gastric tissues. The expression of FTO in gastric cancer tissues was higher than that in para-cancer tissues. Data from The Cancer Genome Atlas demonstrated that FTO expression in gastric cancer tissues was significantly higher than that in normal tissues. Patient survival rate was significantly decreased in patients with high expression levels of FTO. It was also demonstrated that FTO expression was associated with several pathological parameters, such as tumor stage, metastasis stage and the American Joint Committee on Cancer stage. The FTO gene was positively correlated with 16,601 genes in gastric cancer and negatively correlated with 3,623 genes. Gene Ontology enrichment analysis demonstrated that FTO was significantly enriched in the regulation of gene expression and oxidative RNA demethylase activity, and it was associated with components such as the RNA N6-methyladenosine methyltransferase complex and nuclear speckle. In addition, knockdown of the FTO gene inhibited the migration and invasion of *Hp*-infected cells. In conclusion, the data suggests that *Hp* infection leads to upregulation of the FTO gene, which may be related to patient survival rate, tumor staging and other pathological parameters of patients with gastric cancer. It also suggests that FTO promotes proliferation and migration of gastric cancer cells, which may be involved in the pathogenesis of *Hp*-induced gastric cancer.

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Introduction

Gastric cancer is one of the most common malignancies worldwide and the fourth leading cause of cancer-related death worldwide (1). In China, gastric cancer is the fourth and third most common cancer in terms of incidence and mortality, respectively (2). Although the morbidity and mortality rates resulting from gastric cancer are exhibiting a significant downward trend, the 5-year overall survival rate of patients with advanced distant metastasis is still <5% (3). Therefore, it is urgent to elucidate the molecular mechanism underlying the occurrence of gastric cancer and reduce its incidence (4). Gastric cancer development results from a combination of factors, of which *Helicobacter pylori* (*Hp*) infection is the most important risk factor (5). Our research group previously found that *Hp* infection causes upregulation of the fat mass and obesity-associated (FTO) gene. The FTO gene, also known as ALKBH9, is located on chromosome 16q12.2 and was the first N6-methyladenosine (m6A) demethylase to be identified (6). Recent research suggests that variants of the FTO gene are not only associated with obesity and metabolic disorders in humans, but also with cancer (7). Abnormal expression of FTO promotes tumorigenesis, progression and chemotherapy resistance in several cancer types, including breast (8), ovarian (9) and colorectal cancer (10). Previous research also suggests that FTO may be a potential marker of gastric cancer (11-13). The increased expression of FTO in gastric cancer promotes cell proliferation, migration and invasion (14) and is associated with tumor stage and poor prognosis (15).

To the best of our knowledge, there are currently no published data determining whether *Hp* infection affects

FTO expression in gastric cancer. Therefore, the present study aimed to investigate this question by using *Hp*-infected cells and a gerbil animal model to analyze FTO expression in gastric cancer and adjacent tissues, and to examine the effect of FTO gene knockdown on cell migration and invasion in the context of *Hp* infection.

Materials and methods

Human cell lines and cell culture. Human gastric mucosal epithelial cell line GES-1 and gastric adenocarcinoma cell line MKN45 were obtained from the Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The human gastric epithelial adenocarcinoma cell line AGS and the human kidney 293T cell line were purchased from The American Type Culture Collection. All cell lines were cultured in RPM1640 medium with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific) and 100 U/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific) at 37˚C, and in a humidified incubator at 10% CO_2.

Hp culture and infection. The *Hp* GZ7 strain (GenBank accession ID, KR154737.1) was determined to be a typical East Asian strain ($CagA^+$) by sequencing in our previous study (16), where it was isolated from clinical gastric cancer tissues with informed consent from the patients and the approval of The Ethics Committee of Guizhou Medical University [Guiyang, China; approval no. 2017(43)]. *Hp* GZ7 was cultured for 48-72 h at 37˚C in a microaerophilic atmosphere on a Columbia agar plate (Thermo Fisher Scientific) containing 10% sheep's blood. Gastric mucosal GES-1, AGS and MKN45 cells were cultured for 24 h in 6-well plates containing antibiotic-free medium and subsequently infected with *Hp* at a multiplicity of infection (MOI) of 40. The cells were infected for 24 h before they were harvested for western blotting and reverse transcription-quantitative PCR (RT-qPCR). The uninfected cells served as a negative control. All cells were incubated at 37˚C and in a humidified incubator at 10% CO_2.

Plasmid construction and lentiviral coating. The sequence of short hairpin FTO (shFTO) was 5'-CACCAAGGAGAC TGCTATTTA-3' and that of nonsense shRNA (sh-NC) was 5'-GCUUCGCGCCGUAGUCUUA-3'. In the design of shFTO and sh-NC, restriction sites (*Bam*HI and *Eco*RI) and a loop at both the 5' and 3' ends and in the middle of the sequence were added. For plasmid construction, the shRNA was added to a 0.2 ml centrifuge tube, placed in a PCR machine at 95˚C for 5 min, and then allowed to stand at room temperature for 20 min to form a double-stranded oligo fragment. The psi-LVRU6GP vector (Guangzhou Fulengen Co., Ltd.) was digested with *Bam*HI and *Eco*RI at 37˚C for 1 h, fragments were separated by 0.8% agarose gel electrophoresis, and were then purified using a gel recovery kit (Tiangen Biotech Co.). The double-stranded oligo fragment was mixed with the digested vector, and the ligase reaction was carried out at 16˚C overnight with T4 DNA ligase. The ligated product was transformed into competent *Escherichia coli* DH5α cells and positive clones were selected for using ampicillin. Plasmid DNA was extracted by DNA plasmid extraction kit (Tiangen Biotech Co.) for sequencing and identification. 293T cells were cultured until 80% confluency and then transfected with 2 μg shFTO plasmid or empty vector plasmid and lentivirus package plasmids psPASX2 (1.5 μg) and PMD2G (0.5 μg) at the ratio of 4:3:1 in the presence of 5 μl H4000 (Engreen Biosystem Ltd.). After 48 h of transfection, the virus supernatant was extracted and used to infect AGS and MKN45 cells at an MOI of 40. When the AGS and MKN45 cells reached 90% confluency, puromycin (final concentration, 1 μg/ml) was added and stable cell lines with knocked down FTO expression were isolated after 1 week.

Stomach tissue sections of Mongolian gerbils infected with Hp. In our previous study (17), *Hp* NCTC 11637 (ATCC 43504, CagA-positive) was used to intragastrically infect Mongolian gerbils for 24 months to establish *Hp*-related gastric disease models successfully. In these gerbils, chronic superficial gastritis, erosive gastritis, atrophic gastritis, intestinal metaplasia and well-differentiated gastric cancer were pathologically observed at 3, 6, 12 and 24 months after *Hp* infection. At different time points, the gerbils (3 gerbils per time point) were sacrificed after anesthesia, and their stomach tissues were removed, fixed and embedded in paraffin (17). In the present study, the paraffin-embedded gerbil stomach tissues of 3 months after *Hp* infection were cut into 5-μm sections for IHC staining. The animal study was approved by the Animal Care Welfare Committee of Guizhou Medical University (Guiyang, China; approval no. 1900801).

Human gastric tissue. A total of 20 cases of surgically resected gastric cancer and corresponding para-cancerous tissue specimens were collected from patients with gastric adenocarcinoma (13 male and 7 female patients; age, 51.2±9.6 years; range, 40-73 years), who were admitted to the Affiliated Hospital of Guizhou Medical University from March, 2021, to December, 2021. Informed and signed consent was obtained before surgery, and the research protocol was approved by The Ethics Committee of Guizhou Medical University [Guiyang, China; approval no: 2019(19)].

Immunohistochemistry. Paraffin-embedded tissue sections were deparaffinized in xylene for three 10 min intervals, incubated in 100% ethanol for three 10 min intervals, and then in a series of graded ethanol solutions (90, 70, 50 and 30% ethanol) for 3 min each. The tissue sections were placed in a boiling pressure cooker for 2.5 min for antigenic repair. After cooling for 10 min in 3% H_2O_2, sections were washed in PBS for 10 min and then incubated with the FTO antibody (1:3,000; cat. no. 27226-1-AP; Proteintech Group, Inc.; Wuhan Sanying) for 2 h at room temperature. Sections were then washed three times with PBS for 10 min intervals and incubated with the goat anti-rabbit IgG antibody labeled with peroxidase HRP (1:10,000; cat. no. SA00001-2; Proteintech Group, Inc.; Wuhan Sanying) at room temperature for 2 h. Sections were then washed three times with PBS for 10 min intervals. A total of 1 ml reagent A and 50 μl reagent B from the DAB Detection Kit (cat. no. ZLI-9018; OriGene Technologies, Inc.) was mixed, two drops were added to the tissue sections for 1-3 min, and then sections were incubated in PBS to stop the reaction. Slides were stained in hematoxylin for 3 min at room temperature, washed in distilled water, stained in ammonia reverse blue

solution for 1-2 min, washed in distilled water, dehydrated in a graded ethanol series (50, 70, 90% and absolute ethanol), incubated three times in xylene solution for 10 min intervals, and then mounted. The FTO expression level of each sample was judged according to the number of stained cells and the depth of staining using an optical microscope. Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used to calculate the mean integrated optical density of active areas in each group, and the average optical density was taken for analysis.

RT-qPCR. The total RNA of GES-1 and AGS cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific). A Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Ltd.) was used for the reverse transcription of RNA according to the manufacturer's instructions. qPCR was next performed using a 2X SYBR Green Master Mix (Roche Diagnostics, Ltd.) in an Illumina Eco™ Real-Time PCR system (Gene Company, Ltd.). The amplification program was as follows: 95˚C for 10 min then 38 cycles of 95˚C for 12 sec, 60˚C for 18 sec and 72˚C for 30 sec. GAPDH served as a normalization control, and the $2^{-\Delta\Delta Cq}$ approach (18) was employed to calculate relative gene expression. Primer 5.0 (PREMIER Biosoft) was used to design primers for qPCR analysis. The FTO primer sequence was 5'-AACACCAGG CTCTTTACGGTC-3', which was synthesized by Sangon Biotech Co., Ltd.

Western blotting. Total protein of GES-1 and AGS cells was extracted according to the instructions of the high potency RIPA lysate (tissue/cell) research reagent kit (Beijing Solarbio Science & Technology Co., Ltd.). Protein was quantified using a BCA protein quantification kit (Beijing Solarbio Science & Technology Co., Ltd.). For denaturation, 5X loading buffer was added to the protein sample at a 4:1 ratio and boiled for 10 min. A total of 20 μg protein was run on a 10% gel using SDS-PAGE at 80 V for 2 h, proteins were transferred to a polyvinylidene fluoride membrane for another 2 h and the membrane was then blocked with a solution of 20 ml PBS containing 0.05% Tween20 (1X PBST) with 1 g non-fat milk powder at room temperature for 2 h. Incubation with the FTO primary antibody (1:3,000; cat. no. 27226-1-AP; Proteintech Group, Inc.; Wuhan Sanying) was conducted at 4˚C overnight. The membrane was washed three times with 1X TBST solution and incubated with the goat anti-rabbit IgG antibody labeled with peroxidase HRP (1:10,000; cat. no. SA00001-2: Proteintech Group, Inc.; Wuhan Sanying) at room temperature for 2 h. Signals were visualized using chemiluminescence ECL (Thermo Fisher Scientific) and imaged using a chemiluminescence imager (Amersham; Cytivia). ImageJ software (version 1.41; National Institutes of Health) was used to quantify the gray value of the image, and the relative protein expression using the ratio of the FTO to HRP-conjugated mouse anti-ACTB monoclonal antibody (1:10,000; cat. no. HRP-60008: Proteintech Group, Inc.; Wuhan Sanying) or HRP-conjugated mouse anti-GAPDH monoclonal antibody (1:10,000; cat. no. HRP-60004: Proteintech Group, Inc.; Wuhan Sanying) was calculated.

Immunofluorescence. The cell culture medium in the confocal dish was discarded and the GES-1 and AGS cells were washed with PBS three times for 5 min intervals. To fix the cells, 1 ml 4% paraformaldehyde was added and incubated for 30 min at room temperature, before washing with PBS three times for 5 min intervals. Cells were permeated with 0.3% Triton X-100 for 50 min at room temperature, before washing with PBS three times for 5 min intervals. The cells were blocked with 5% Bovine Serum Albumin BSA-V (cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. The cells were incubated with FTO primary antibody (1:50; cat. no. 27226-1-AP; Proteintech Group, Inc.; Wuhan Sanying) overnight at 4˚C and then washed with PBS three times for 5 min intervals. CoraLite594-conjugated goat anti-rabbit IgG(H+L) (1:200; cat. no. SA00013-4; Proteintech Group, Inc.; Wuhan Sanying) was added to the cells and incubated for 1 h at room temperature. Then, 100 μl of Antifade Mounting Medium with DAPI (cat. no. P0131-25 ml; Beyotime Institute of Biotechnology) was added to each well in turn. The cells were carefully covered with a slide and imaged by a laser confocal microscope. Image fluorescence intensity was quantified using ImageJ software (version 1.41; National Institutes of Health).

Bioinformatics analysis-acquisition and preprocessing of gastric cancer data in the Cancer Genome Atlas (TCGA) database. FTO RNA-seq V2 mRNA expression data from specimens in the gastric cancer dataset (including 408 gastric cancer specimens and adjacent tissues) were downloaded from the TCGA database (https://tcga-data.nci.nih.gov/tcga/) and preprocessed. From these, 211 specimens were screened for sex, age, tumor size (T1, T2, T3 and T4), lymph node metastasis (N0, N1 and Nx), distant metastasis (M0, M1 and Mx) and American Joint Committee on Cancer (AJCC) pathological feature stage (AJCC stage I + II, III + IV) (19). A total of 378 cases from TCGA database were selected as the gastric cancer group. In addition, according to the level of FTO mRNA expression, cases in the gastric cancer group were divided into the high expression group and the low expression group according to the median value, with 180 cases in each group.

Bioinformatics analysis-FTO-related gene screening and gene function enrichment analysis. To analyze the genes that were positively and negatively correlated with FTO expression the LinkedOmics database (http://linkedomics.org/login.php) was used. The CANCER COHORT was selected as 'TCGA_STAD', the SEARCH DATASET as 'HiSeq RNA', the SEARCH DATASET ATTRIBUTE as 'FTO', the TARGET DATASET as 'HiSeq RNA' and the STATISTICAL METHOD as 'Pearson Correlation test'. Gene function enrichment analysis was performed using the STRING website (https://cn.string-db.org/). The website operation steps were followed to carry out Gene Ontology (GO) Enrichment analysis for 'Molecular Function', 'Biological Process' and 'Cell Component'.

Wound healing assay. A total of $1x10^6$ AGS and MKN45 cells were cultured in a 6-well plate overnight. When the degree of confluence reached ~90%, a horizontal cell scraping strip of equal width (~500 μm) was drawn through the center of the well, and the wounded cells were washed with PBS and incubated for a further 48 h in the medium with 4% FBS.

Imaging was conducted using an optical microscope at 0 and 48 h to measure the cell damage. The migration ability of cells was evaluated by comparing the relative wound width at 48 h with the wound width at 0 h. The relative scratch healing rate was calculated as follows: Percentage relative scratch healing rate=[gap distance (T0-T48)/gap distance at T0] x100%, with T0 and T48 corresponding to the width of the wound at 0 and 48 h, respectively.

Invasion assay. Invasion assays were performed in Transwell plates (Costar; Corning, Inc.) precoated with Matrigel (BD Bio sciences). The chamber coating was performed at 37˚C for 1 h. A total of $2x10^4$ of each cell line (AGS and MKN45) diluted in serum-free DMEM medium was added to the upper chamber and 500 μl DMEM containing 10% FBS was added to the lower chamber as a nutritional attractant. After incubating for 48 h at 37˚C, the cells inside the upper chamber were removed and the cells invading through the membrane were fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were then stained with 0.1% crystal violet for 30 min at room temperature and counted under an optical microscope.

Statistical analysis. The SPSS 18.0 software (SPSS, Inc.) was used for data analysis. Unpaired t-test was used to compare the two independent *Hp* infected and uninfected groups. To analyze the differences in FTO expression between gastric cancer tissues and paired adjacent tissue, paired t-test was used. One-way ANOVA followed by Dunnett's post hoc tests was used when multiple groups were compared respectively with control groups. One-way ANOVA followed by Tukey's post hoc test was used to compare all pairs of columns containing multiple groups. The following settings for GEPIA were used as: 'Expression on Box Plots', 'Gene as FTO', '|log2FC| Cutoff as 1', 'P-value Cutoff as 0.01', 'Datasets as STAD', 'Log Scale as log2(TPM + 1)', 'Jitter Size as 0.4' and 'Matched normal date' as 'TCGA normal and GTEx data'. Survival curve analysis was performed using the Kaplan-Meier method and the log-rank test using GEPIA data. To analyze the relationship between FTO expression and clinicopathological parameters, χ^2 test was used. Spearman's correlation test was used for analysis of FTO-related gene screening and Fisher's exact test was used for gene function enrichment analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hp infection upregulates FTO expression. Of the cell lines used, the cell lines with lowest FTO expression were the AGS and GES-1 cell lines. Therefore, these two cell lines were selected for follow-up experiments (Fig. 1A). Following GES-1 and AGS infection with the *Hp* GZ7 strain at MOI 40:1 for 24 h, FTO mRNA and protein levels were detected by RT-qPCR and western blotting, respectively, and both the mRNA and protein levels in infected cells were significantly higher than those in uninfected cells (Fig. 1B and C). Immunofluorescence demonstrated that FTO was mainly expressed in the cytoplasm of gastric epithelial cells, and the total fluorescence intensity of FTO in *Hp* infected cells increased (Fig. 1D). The expression level of FTO protein in the gastric tissues of Mongolian gerbils infected with *Hp* was also significantly higher than that of the control group (Fig. 1E).

FTO expression in gastric cancer is associated with patient prognosis. A total of 20 gastric cancer specimens and 20 corresponding adjacent tissue sections were examined by immunohistochemistry to determine the differences in FTO expression levels. Positive staining of FTO protein was visualized as brown-yellow granules, mostly located in the cytoplasm and nucleus (Fig. 2A). The protein level of FTO in gastric cancer tissues was also significantly higher than that in the adjacent tissues (Fig. 2A). FTO mRNA expression data based on a total of 408 gastric cancer cases and 211 normal tissues were available from the TCGA database. Bioinformatics analysis indicated that the mRNA expression of FTO in gastric cancer was significantly higher than that in normal tissues (Fig. 2B). Kaplan-Meier survival curves were used to explore the prognostic value of FTO expression on the overall survival rate of patients with gastric cancer. The overall survival rate of patients with high FTO expression was significantly lower than that of patients with low FTO expression (Fig. 2C).

FTO expression is associated with some clinicopathological parameters in patients with gastric cancer. The gastric cancer group included 242 males and 136 females; 118 cases aged ≤60 years and 260 aged >60 years; 102 cases of T1+T2 stage and 276 of T3+T4 stage; 121 cases of N0 stage, 99 of N1 stage, 81 of N2 stage, 72 of N3 stage and 5 of Nx stage; 334 cases in M0 stage, 26 in M1 stage and 18 in Mx stage; 182 cases in Stage I+II and 196 in Stage III+IV. The χ^2 test was used to examine the relationship between FTO mRNA expression and the clinicopathological characteristics of patients with gastric cancer in the TCGA database. The results demonstrated a positive association between the level of FTO mRNA expression and clinicopathological parameters in patients with gastric cancer, such as T stage, M stage and AJCC stage, and a high expression level of FTO mRNA was associated with higher T stage, M stage and AJCC stage. There was no association between FTO mRNA expression level and sex, age or N stage of patients (Table I).

FTO gene in gastric cancer is related to genes associated with RNA stability and RNA metabolism. The LinkedOmics database was used to analyze genes related to FTO in patients with gastric cancer, based on Spearman's correlation test. The FTO gene in gastric cancer was positively correlated with 16,601 genes, including ZFP90, chromodomain helicase DNA binding protein 9 (CHD9) and protocadherin γ subfamily B7 (PCDHGB7) (Fig. 3A), and was negatively correlated with 3,623 genes, including halo acid dehalogenase like hydrolase domain containing 3 (HDHD3) and estrogen related receptor α (ESRRA) (Fig. 3B). GO enrichment demonstrated that RNA stability and RNA metabolism were the biological processes in which FTO related genes were most significantly enriched during gene expression regulation (Fig. 3D). The molecular functions of FTO-related genes that were most significantly enriched were oxidative RNA demethylase activity and mRNA binding. The cellular components most significantly enriched were the RNA m6a methyltransferase complex and nuclear speckles. The enrichment results of FTO-related genes

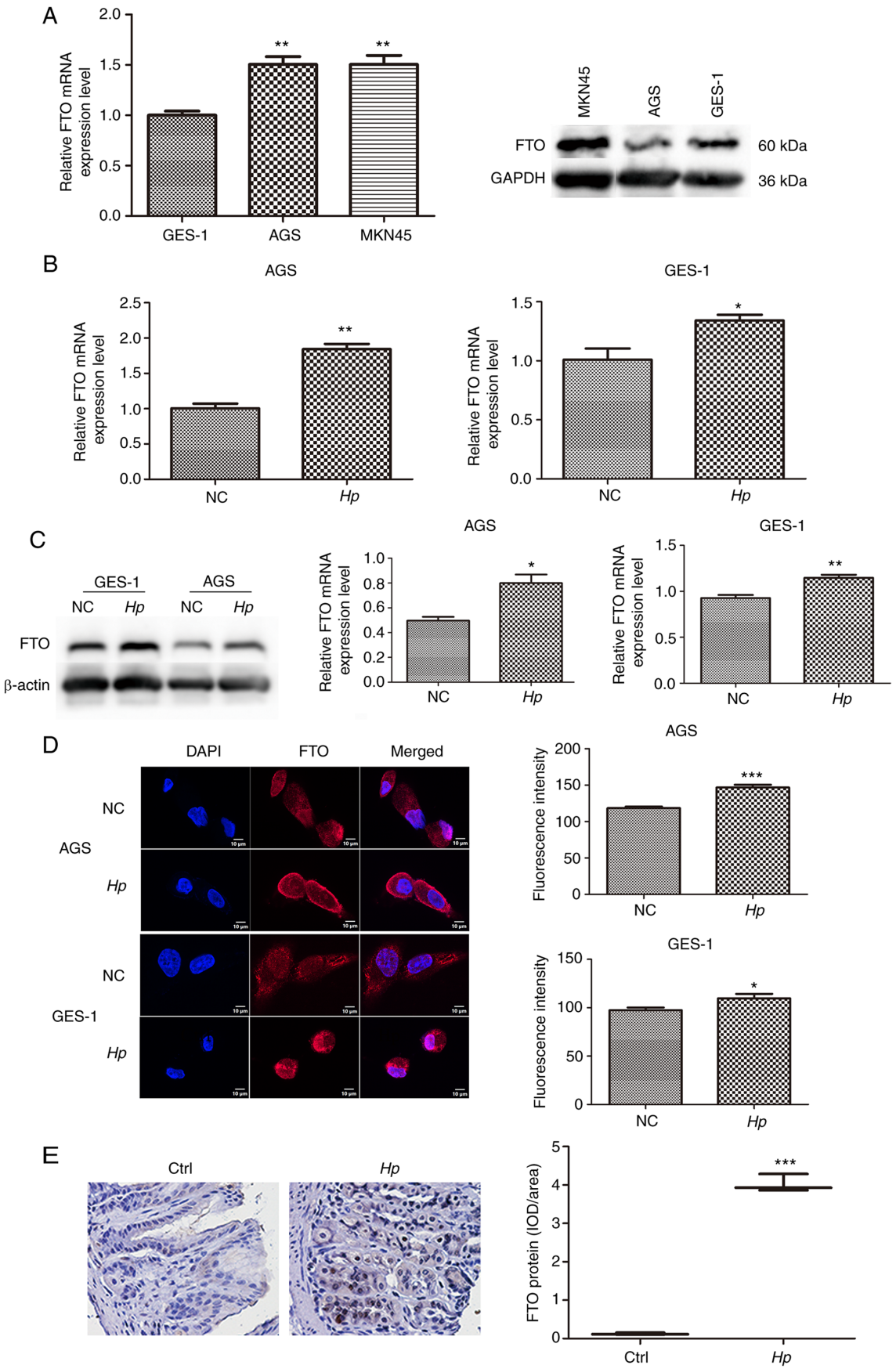

Figure 1. Effects of *Hp* infection on the expression of the FTO gene. (A) FTO mRNA and protein in GES-1 cells and various gastric cancer cell lines. Data were analyzed using one-way ANOVA followed by Dunnett's post hoc tests (**P<0.01 vs. GES-1). (B) FTO mRNA levels in AGS and GES-1 cells infected with *Hp* (*P<0.05 and **P<0.01 vs. NC). (C) FTO protein expression levels after *Hp* infection of AGS and GES-1 cells *Hp* (*P<0.05 and **P<0.01 vs. NC). (D) FTO expression and localization in *Hp* infected AGS and GES-1 cells *Hp* (*P<0.05 and ***P<0.001 vs. NC). (E) FTO protein expression in the gastric tissues of Mongolian gerbils 3 months after *Hp* administration (***P<0.001 vs. Ctrl). Data are presented as mean ± SD. Error bars represent SD. Ctrl, control; FTO, fat mass and obesity-associated; *Hp*, *Helicobacter pylori*; NC, negative control.

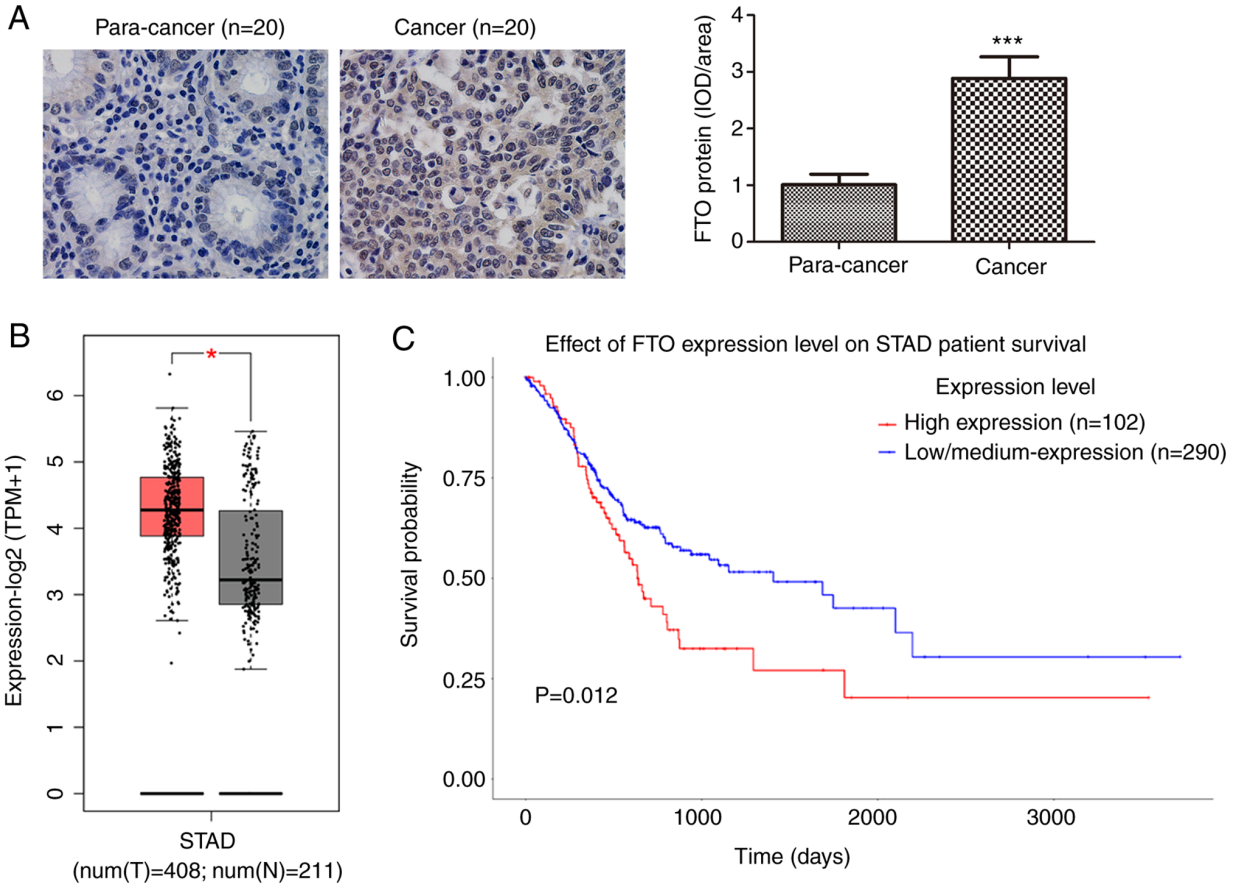

Figure 2. The expression of FTO in GC tissues and its correlation with the prognosis of patients with GC. (A) FTO protein expression levels in 20 human GC tissues and their corresponding adjacent tissues (x400 magnification) (***P<0.001 vs. para-cancer). (B) A boxplot showing FTO RNA expression in 408 GC tissues and 211 normal gastric tissues [data from The Cancer Genome Atlas and GTEx]. (C) Kaplan-Meier analysis of relationship between FTO expression and prognosis of gastric cancer. *P<0.05. GC, gastric cancer; FTO, fat mass and obesity-associated; STAD, stomach adenocarcinoma; TPM, transcripts per million.

suggest a possible function for FTO in cell proliferation and metabolism and a role in the tumorigenesis and progression of gastric cancer.

FTO gene knockdown decreases Hp-induced migration and invasion of gastric cancer cells. Lentiviral expression vectors were used to establish stable AGS and MKN45 cell lines containing a knocked down FTO gene. Western blot analysis demonstrated that FTO was successfully knocked down in AGS and MKN45 cells, which was rescued by *Hp* infection (Fig. 4A). *Hp* infection of cell lines with or without a knocked down FTO gene were used to investigate the role of FTO on *Hp*-induced cell migration and invasion abilities by wound healing and Transwell assays. The results demonstrated that *Hp* enhanced cell migration and invasion abilities in AGS and MKN45 cells (Fig. 4B and C). However, the migration and invasion abilities of cells were reduced in cell lines where the FTO gene had been knocked down. Altogether, these findings suggested that knocking down the FTO gene may inhibit the promoting effect of *Hp* on the migration and invasion ability of gastric cancer cells.

Discussion

The occurrence and development of gastric cancer is a complex process, involving a variety of factors and molecular pathways (20,21), most of which remain to be elucidated (22). *Hp* is a gram-negative bacterium that can selectively colonize the gastric mucosa. A study showed that *Hp* infection is one of the most important risk factors for gastric cancer (23). In total, ~90% of non-cardia cancers are associated with *Hp* infection (24). *Hp* infection can lead to chronic immune pathological damage to the gastric mucosa, and to the secretion of a variety of cytokines and chemokines, such as interleukin (IL)-8 (25) and IL-6 (26), which cause chronic inflammation (27). There is a stepwise process from early phenotype to the development of gastric cancer: Normal gastric mucosa to chronic atrophic gastritis to intestinal metaplasia to gastric mucosal epithelium atypical hyperplasia to gastric cancer (28,29). Although *Hp* has been listed as a class I carcinogen since 1994 (30), the specific mechanisms underlying carcinogenesis remain unclear.

Due to the lack of effective biomarkers, the prognosis of patients with early gastric cancer is still poor (31). In the absence of effective therapies, the search for new biomarkers is critical for the optimization of targeted therapies. The FTO gene encodes an α-ketoglutarate-dependent hydroxylase-related non-heme iron nucleoprotein, originally identified as a regulator of body weight and obesity (32). Studies have shown that FTO can act as a demethylase of m6A to participate in the development and progression of various cancer types such as breast cancer (8,33,34). FTO can demethylate m6A to increase the mRNA stability in tumor pathways and promote

Table I. Relationship between the expression level of FTO mRNA and the clinicopathological parameters of patients with gastric cancer.

Clinicopathological feature	FTO mRNA		χ^2	P-value
	Low	High		
Sex, n			0.46	0.83
Male	122	120		
Female	67	69		
Age, n			3.154	0.076
>60 years	138	122		
≤60 years	51	67		
T stage, n			6.499	0.011
T1+T2	62	40		
T3+T4	127	149		
N stage, n			5.417	0.2471
N0	67	54		
N1	52	47		
N2	40	41		
N3	28	44		
NX	2	3		
M stage, n			6.758	0.0341
MO	174	160		
M1	11	15		
MX	4	14		
AJCC stage, n			5.129	0.024
I+II	102	80		
III+IV	87	109		

AJCC, American Joint Committee on Cancer; FTO, fat mass and obesity-associated (mRNA); M, metastasis (stage); N, node (stage); T, tumor (stage).

tumor progression (35). Zhang *et al* (36) demonstrated that FTO removes the m6A modification from homeobox B13 (HOXB13) mRNA, abolishes YTH domain-containing family protein 2-mediated HOXB13 degradation, promotes HOXB13 protein expression, activates the WNT signaling pathway and promotes endometrial cancer invasion and metastasis. FTO has also been demonstrated to promote breast cancer cell invasion and migration through the FTO/micro-RNA -181b-3p/ADP ribosylation factor-like GTPase 5B signaling pathway (8). The pro-apoptotic gene, BCL2 interacting protein 3 (BNIP3), is a downstream target of the FTO-mediated m6A modification (15). FTO can demethylate BNIP3 m6A mRNA and induce its degradation, thereby promoting proliferation of breast cancer cells and inhibiting apoptosis. Tang *et al* (37) demonstrated that knockdown of FTO reduces the proliferation of pancreatic cancer cells, and identified c-Myc as the main target of FTO, suggesting that FTO may be necessary for pancreatic cancer progression. Although it is widely appreciated that FTO is involved in multiple pathways involved in the development and progression of various cancer types, the exact physiological function of this gene remains unclear.

In previous research by our group, *Hp*-CagA transfected gastric cancer cells were used for transcriptome sequencing and it was found that the expression of FTO mRNA was increased (38). It was speculated that *Hp* may play a role in the development and progression of gastric cancer by increasing FTO expression. In the present study, gastric epithelial cells were infected with *Hp* and an increase in FTO mRNA and protein levels were observed, compared with uninfected cells. Using immunofluorescence, it was also observed that *Hp* infection increased the level of FTO protein. These findings were further confirmed using a gerbil model. These results add to the hypothesis that FTO may play an important role in *Hp*-related gastric cancer.

In the present study, using immunohistochemistry on 20 gastric cancer and adjacent tissue sections, it was observed that the expression level of FTO in human gastric cancer tissue was higher than that in adjacent normal tissues. The analysis using the TCGA database also demonstrated that patients with gastric cancer had a higher expression level of FTO than the controls. Survival curve analysis showed an association between a high expression level of FTO and poor survival rate of patients with gastric cancer. Altogether, these data suggest a role of FTO in the occurrence of gastric cancer. A comparison of clinicopathological parameters in patients with gastric cancer with high and low FTO expression levels indicated a statistically significant association between FTO expression and T stage, M stage and AJCC stage, but no association with age, sex or N stage. These results were consistent with the research of Xu *et al* (14).

Using the LinkedOmics database, genes closely related to FTO were examined and it was determined that the FTO gene in gastric cancer was positively correlated with 16,601 genes, including ZFP90, CHD9 and PCDHGB7. A study demonstrated that PCDHGB7 induces apoptosis of breast cancer cells and is involved in cancer development and progression (39). However, it was also found that FTO was negatively correlated with 3,623 genes, including HDHD3 and ESRRA. Chen *et al* (40) demonstrated that ESRRA enhanced the metabolism and proliferation abilities of cancer cells. Wang *et al* (41) also demonstrated that ESRRA may be mainly involved in cellular metabolism processes, and demonstrated a role for ESRRA in the tumorigenesis and progression of uteri corpus endometrial carcinoma. In the present study, the GO enrichment results demonstrated that the biological processes of FTO-related genes were associated with gene expression regulation, RNA stability and RNA metabolism, and the molecular functions of FTO-related genes were associated with oxidative RNA demethylase activity and mRNA binding. Regarding the cellular components, enrichment to the RNA m6a methyltransferase complex and nuclear speckles was observed. These results suggest that FTO may have a role in cell proliferation and metabolism and possibly in the tumorigenesis and progression of gastric cancer. The *in vitro* experiments confirmed that FTO knockdown reduced the invasion and migration abilities of AGS and MKMN45 cells induced by *Hp* infection. *Hp* may affect the stability of downstream gene transcription levels by upregulating the expression of the FTO gene (from the transcription and translation level), causing changes in the m6A level of downstream target genes and therefore a biological behavioral change of gastric cancer

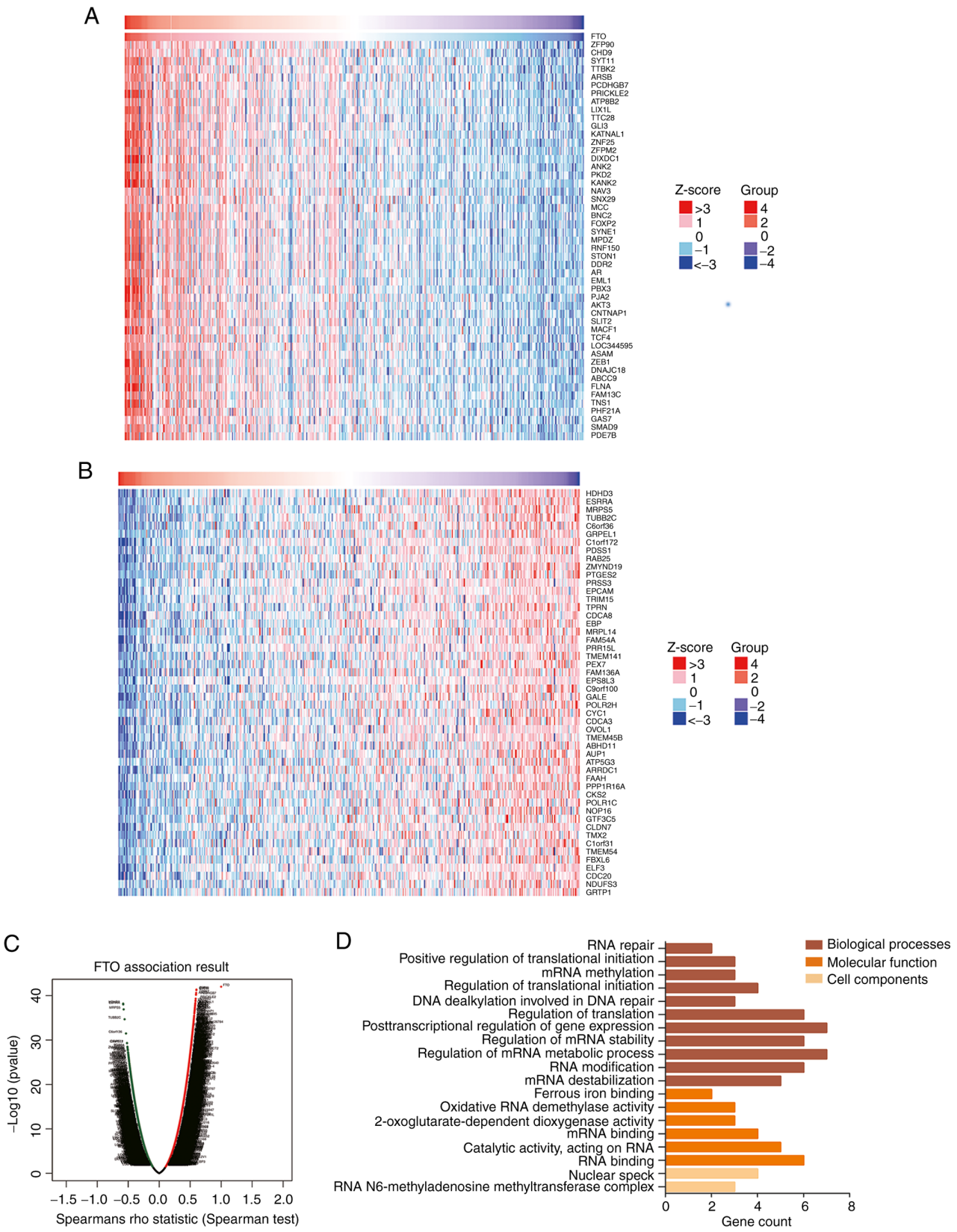

Figure 3. FTO-related genes and functional enrichment analysis in gastric cancer. (A) Genes positively correlated with FTO. (B) Genes negatively correlated with FTO. (C) FTO-related gene analysis in gastric cancer. (D) Gene Ontology enrichment analysis. FTO, fat mass and obesity-associated.

cells. Therefore, high FTO expression induced by *Hp* infection may be related to the progression of gastric cancer tumors, suggesting that FTO may be an important biomarker in the occurrence, development and prognosis of gastric cancer.

In conclusion, the present study demonstrated that *Hp* infection can lead to an increase in the expression level of FTO in gastric cancer cells, with a high expression of FTO being associated with poor prognosis of patients with gastric

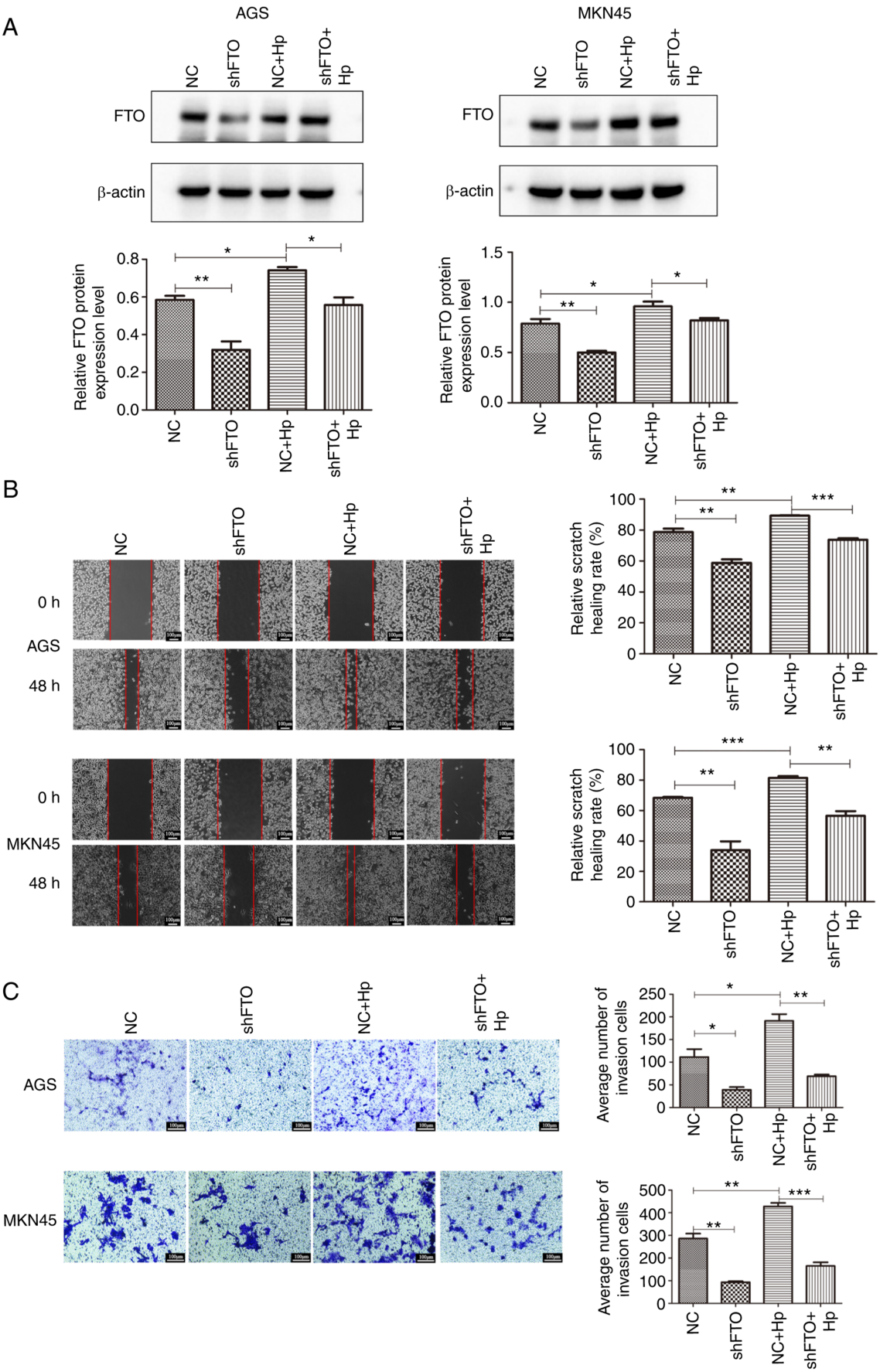

Figure 4. Effects of knocking down FTO expression on the migration and invasion abilities of *Hp*-infected AGS and MKN45 cells. (A) Western blot detection of FTO protein expression in AGS and MKN45 cells. (B) The cell migration ability of *Hp*-infected AGS and MKN-45 cells after knockdown of the FTO gene. (C) The invasion ability of *Hp*-infected AGS and MKN-45 cells after knockdown of the FTO gene. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as mean ± SD. Error bars represent the SD. *P<0.05, **P<0.01, ***P<0.001. FTO, fat mass and obesity-associated; *Hp*, *Helicobacter pylori*; NC, negative control; sh, short hairpin.

cancer. High FTO expression levels induced by *Hp* might play an important role in regulating the proliferation and migration of gastric cancer cells. These results suggest a novel molecular mechanism underlying gastric cancer induced by *Hp* infection that may be of therapeutic interest.

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Availability of data and materials

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

Authors' contributions

YX and JZ conceived and designed the experiments. SG, QW and LB conducted most of the experiments and analyses. XH, ZW, LL, LW and YZ confirm the authenticity of all the raw data. YX and SG drafted the manuscript. XH, ZW, LL, LW and YZ were responsible for overseeing the manuscript. YX, SG, QW, LB, XH, ZW, LL, LW, YZ and JZ were responsible for writing and revising the manuscript, and contributed significantly to the study design, data collection, data analysis and interpretation of data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental processes with human specimens were checked and authorized by The Ethics Committee of Guizhou Medical University [Guiyang, China; approval no, 2019(19)]. All subjects gave their informed consent for inclusion before participating in the study. The study was conducted following the Declaration of Helsinki. The animal study was approved by the Animal Care Welfare Committee of Guizhou Medical University (Guiyang, China; approval no. 1900801).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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