Trefoil factor 1 and gastrokine 2 inhibit *Helicobacter pylori*-induced proliferation and inflammation in gastric cardia and distal carcinogenesis

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Abstract. *Helicobacter pylori* (*H. pylori*) infection has been associated with non-cardia adenocarcinoma in the stomach, while its role in gastric cardia adenocarcinoma (GCA) remains controversial. In addition, the association between *H. pylori* and the protective factors trefoil factor 1 (TFF1) and gastrokine 2 (GKN2) in gastroesophageal adenocarcinomas has not been fully investigated. Therefore, the mRNA and protein expression levels of TFF1 and GKN2 in GCA and distal gastric adenocarcinoma (DGA) were analyzed using quantitative PCR (qPCR) and immunohistochemistry, and the association with *H. pylori* infection was investigated. In addition, the effects of TFF1 and GKN2 overexpression on *H. pylori*-induced cells were investigated using western blot and reverse transcription-qPCR analysis. The comparative analysis of 16S rRNA-positive mRNA expression between GCA and DGA showed no statistically significant difference. However, the rate of the *H. pylori* vacuolating toxin A (VacA) genotype was significantly higher in GCA (49.2%) compared with that in DGA (26.9%; $P<0.05$). *H. pylori* infection down-regulated the mRNA and protein expression levels of TFF1 and GKN2 in gastric tumor tissues, and the mRNA expression level of TFF1 and GKN2 was also markedly decreased *in vitro*. Furthermore, the cell proliferation varied in *H. pylori* total protein treatment group with the different doses. Notably, treatment with 20 μg/ml *H. pylori* total protein for 24 h resulted in the highest cellular proliferation rate. In addition, TFF1 and GKN2 overexpression inversely inhibited *H. pylori*-induced cell proliferation and upregulated NF-κB, tumor necrosis factor-α, IL-1β, IL-2, IL-4 and IL-6. The results of the present study indicate that *H. pylori*, particularly the VacA+ strain, plays an important role in GCA pathogenesis in high-risk areas of China, while TFF1/GKN2 inhibits *H. pylori*-induced cell proliferation and inflammation in GCA and DGA.

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Introduction

Globally, the incidence of gastroesophageal adenocarcinomas, primarily including gastric cardia adenocarcinoma (GCA) and esophageal adenocarcinoma (EA), has increased over the last 3 decades (1-4). In the Chinese population, EA is extremely rare (the age standardized rate: Approximately 0.4%) with a 77-fold lower incidence of esophageal squamous cell carcinoma in Chinese males, according to data from the GLOBOCAN 2012, and the majority of adenocarcinomas at the gastroesophageal junction are defined as GCA (5). GCA exhibits similar biological behavior to EA (6); however, there are significant differences with distal gastric adenocarcinoma (DGA). In high-risk areas of China, which had an annual gastric cancer mortality rate of 77.67/100,000/year in the 1990s, and were 3.29 and 3.43 times that of the national rate in 2004 and 2005, respectively (7,8), GCA shows different immunophenotypical patterns of cytokeratins, mucins and signaling molecular pathways (9-11). A genome-wide association study (GWAS) in Asia identified an association between single nucleotide polymorphisms (SNPs) in prostate stem cell antigen at rs2294693 and the risk of non-cardia cancer, but found no evidence for an association with cardia tumors (12). These findings indicated that GCA may be an independent entity of gastric cancer (GC).

It is well known that *Helicobacter pylori* (*H. pylori*) is associated with non-cardia adenocarcinomas (13,14), while gastroesophageal adenocarcinomas are primarily associated with gastroesophageal reflux disease (GERD), obesity and Caucasian ethnicity (15,16). However, the roles of *H. pylori* in gastroesophageal carcinogenesis remain controversial. Previous studies have demonstrated that *H. pylori* is a potential protective factor against gastroesophageal adenocarcinomas. In addition, it has also been shown that *H. pylori*-mediated chronic gastritis was inversely associated with GERD and

Barrett's esophagus (17). However, in Chinese areas with an increased incidence rate of upper gastrointestinal cancers and a high prevalence rate of *H. pylori* infection, for example the Chinese Cixian and Zanhuang counties, an increased proportion of GCA cases has been reported (1). In addition, a large cohort study in the Chaoshan region revealed that *H. pylori* infection was associated with an increased risk of GCA (18,19). Notably, a GWAS investigating genetic predisposition factors identified that SNP susceptibility loci and *H. pylori* infection were associated with an increased risk of GCA in a Chinese population (20). However, to the best of our knowledge, there is no evidence for a causal role of *H. pylori* in GCA.

Trefoil factor family (TFF) proteins comprise three small molecule polypeptides with a clover leaf-like disulfide structure (21). TFF1 is primarily secreted in gastric foveolar cells, while TFF2 is expressed in the neck cells and deep pyloric glands of the stomach, and TFF3 is highly expressed in intestinal goblet cells and gastrointestinal metaplasia (21). These peptides are physiologically resistant to proteolysis and the acidic environment, and develop to maintain mucosal integrity in stomach (21-23). In TFF1-knockout mice, the loss of TFF1 exhibit carcinogenic histological changes from gastritis to hyperplasia, ultimately leading to malignant adenocarcinoma in the gastric mucosa (22). Therefore, *H. pylori* has been widely considered to exhibit tumor suppressor effects in gastric carcinogenesis (23,24). It has been reported that TFF1 and gastrokine 2 (GKN2) are co-expressed by gastric mucus-secreting cells, and act by forming a heterodimer (25,26). These findings indicate that GKN2 may be involved in regulating the important anti-tumor effects of TFF1, but not TFF2 and TFF3, by forming a GKN2/TFF1 protein complex, (27). A recent study revealed that GKN2 exists in monomeric form in the corpus, and as a TFF1-GKN2 heterodimer in the antrum (28). However, comparative studies on both genes in different subtypes of GC are still at a preliminary stage. Therefore, the potential underlying mechanism of TFF1/GKN2 in *H. pylori*-mediated gastroesophageal adenocarcinomas remains elusive.

The present study aimed to determine the roles of *H. pylori* in the development of GCA and its effects on TFF1/GKN2 expression in high-risk areas in China. In addition, the effects of TFF1 and GKN2 overexpression on *H. pylori*-induced cell proliferation and inflammatory responses were also investigated *in vitro*.

Materials and methods

Patients. A total of 113 paraffin-embedded GC resection samples and non-cancerous adjacent tissues were obtained from the Department of Pathology, The Second Hospital of Hebei Medical University (Hebei, China), between January 2011 and December 2014. The center of the GCA was located 1 cm above and 2 cm below the anatomical location of the gastroesophageal junction, according to the Siewert classification system (29), while tumors located in the antrum and angle of the stomach were defined as DGA (9,10). The clinicopathological parameters are listed in Table SI. None of the patients received chemotherapy or radiotherapy prior to surgery.

The present study was approved by the local Ethics Committee, and written informed consent was obtained from all subjects prior to participation in the study.

Immunohistochemical (IHC) analysis. All the specimens are fixed with 4% formalin at room temperature for 4h, and embedded in paraffin. IHC analysis of *H. pylori*, TFF1 and GKN2 was conducted on the paraffin-embedded specimens (4-6 μm). Briefly, following deparaffinization with xylene and rehydration in a descending alcohol series at room temperature, high-pressure antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min, according to the retrieval protocol. Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase/methanol for 10 min. Subsequently, three serial sections were incubated overnight at 4˚C in a humidified chamber with antibodies against *H. pylori* (dilution, 1:100; cat. no. ab140128; Abcam), GKN2 (dilution, 1:100; cat. no. ARP65440-p050; Aviva Systems Biology Corp.) and TFF1 (dilution, 1:100; cat. no. EPR15377; Epitomics; Abcam). The assay was performed using a rabbit IHC kit (cat. no. FXP020; 4A Biotech Co., Ltd.) according to the manufacturer's recommendations. PBS was used as a negative control instead of the primary antibody. A total of 10 randomly selected high power fields (10X objective; x40 magnification) under light microscope were observed on each slide and samples with >5% positive cells were categorized as positive. All slides were judged by an experienced pathologist from the Department of Pathology, The Second Hospital of Hebei Medical University.

The odds ratio with 95% confidence intervals were used as measures of the association between *H. pylori* infection and the risk of GCA or DGA; and the data was statistically analyzed using the Mantel-Haenszel χ^2 test.

Cell culture and treatment. The normal gastric mucosa (NGM) epithelium cell line GES-1 was purchased from the Beijing Institute for Cancer Research. The SKGT-4 distal esophageal adenocarcinoma cell line (cat. no. CBP60462) was bought from Cobioer Biosciences Co., Ltd. The GES-1 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37˚C in a humidified atmosphere containing 5% CO_2. The SKGT-4 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) under the same conditions as GES-1 cells. The cells were then treated with different concentrations of *H. pylori* proteins (2.5, 5, 10, 20, 40, 80 and 160 μg/ml), and the solvent control group was treated with PBS.

MTT assay. The cells were treated with highly purified *H. pylori* protein (cat. no. 30-AH78; Fitzgerald Inc.). The GES-1 and SKGT-4 cells were cultured in the presence of increasing concentrations of *H. pylori* protein (2.5, 5, 10, 20, 40, 80 and 160 μg/ml) for 24 h, at 37˚C, following which the cells were incubated with MTT stock solution for 4 h. The inhibitory effect of *H. pylori* protein on cell proliferation was measured using a spectrophotometric microplate reader (BioTek Instruments Inc.) at 450 nm. Cell proliferation was determined and compared with the control group, using optical density (OD), and the following equation: Cell proliferation =

$$\frac{OD(experimental)-OD(blank)}{OD(control)-OD(blank)} \text{ x } 100\%.$$

Transfection. The recombinant plasmids pEZ-M02-GKN2 and pEZ-Lv105-TFF1, were synthesized by GeneCopoeia Inc.. A total of $1.0x10^5$ cells/ml were seeded into 6-well plates and then transfected using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's recommendations. Transfection efficiency was determined using western blot analysis, 48 h after cell transfection with the targeted expression plasmids of TFF1 and GKN2. Cells transfected with empty plasmids were regarded as the control group. Finally, the cells were treated with *H. pylori* protein (20 μg/ml) for 24 h and harvested for subsequent experimentation.

Reverse transcription-quantitative (RT-qPCR). Total RNA was extracted from cells following the aforementioned treatment using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Subsequently, cDNA was synthesized using GoScript™ Reverse Transcription System (Promega Corporation) according to the manufacturer instructions, and PCR amplification was performed with the appropriate set of primers (Invitrogen; Thermo Fisher Scientific, Inc.; Table SII) using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.) in an Mx3005p Real-Time PCR system (Agilent Technologies, Inc.). RT was performed under the following conditions: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 15 min. The thermocycling conditions of PCR amplification consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and elongation at 60˚C for 30 sec. The relative mRNA expression levels of the targeted genes were calculated according to the equation $2^{-\Delta\Delta Cq}$ (30), where ΔCq=Cq (targeted gene)-Ct (internal control gene; β-actin). All assays were performed in triplicate.

Furthermore, genomic DNA used for qPCR was directly extracted from the paraffin-embedded tissues, including GC and non-cancerous mucosa of adjacent tissues, using a Genomic DNA kit (Tiangen Biotech Co., Ltd.), to detect *H. pylori* 16S rRNA, cytotoxin-associated gene A (CagA) and vacuolating toxin A (VacA). The optimal cut-off points of *H. pylori* 16S rRNA, CagA and VacA detection levels were calculated by accepting Ct ≤35 and ΔCt ≤15 as a positive result. It is reported that *H. pylori* 26695 protein could affect cell proliferation and apoptosis, and then was involved in GC. Therefore, the primers were designed based on the complete genome sequence of *H. pylori* 26695 (GenBank, AE000511.1).

Western blot analysis. Total protein was extracted from cells using lysis buffer (1% Triton X-100; 150 mM NaCl; 2 mM EDTA; 50 mM Tris-HCl) supplemented with a phosphatase inhibitor cocktail. Following protein quantification by BCA Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.) using Gen5 1.0 software (BioTek Instruments, Inc.) on a spectrophotometer microplate reader (BioTek Instruments, Inc.), a total of 20-50 μg protein extract was analyzed using SDS-PAGE (10-15% gels) and then electrophoretically transferred onto a polyvinylidene fluoride membrane. Non-specific sites on the membranes were blocked at room temperature for 60 min with 5% skimmed milk in TTBS, supplemented with Tween-20. Following incubation with the primary antibodies overnight at 4˚C, the membranes were then probed with a HRP-conjugated secondary antibody (dilution, 1:5,000) at room temperature for 2 h. Finally, the immunoreactive bands were visualized using Pierce™ Electrochemiluminescent Western Blotting Substrate (cat. no. UC280185; Thermo Fisher Scientific, Inc.) on ImageQuant™ LAS 4,000 (serial no. 2639042; GE Healthcare Life Sciences) and their densities was quantified and normalized to β-actin.

The primary antibodies used in the present study included: Rabbit anti-human β-actin (1:10,000; cat. no. AC026), a monoclonal antibody purchased from ABclonal Biotech Co., Ltd.. Rabbit anti-human cyclin dependent kinase 4 (CDK4; 1:5,000; cat. no. ab108357), cyclin B1 (1:10,000; cat. no. ab32053), cyclin D (1:200; cat. no. ab16663), proliferating cell nuclear antigen (PCNA; 1:2,000; cat. no. ab92552), NF-κB (1:1,000; cat. no. ab207297) and phosphorylated (p)-NF-κB (1:1,000; cat. no. ab239882) purchased from Abcam. Goat anti-mouse IgG (H&L), HRP-conjugated secondary antibody (1:5,000; cat. no. S0002) and goat anti-rabbit IgG (H&L), HRP-conjugated secondary antibody (1:5,000; cat. no. S0001) obtained from Affinity Biosciences.

Statistical analysis. SPSS v21.0 software (IBM Corp.) for Windows was used for statistical analysis, and clinical variables were analyzed using χ^2 or Fisher's exact test, where appropriate. The qPCR data on *H. pylori* between GCA and DGA was performed using χ^2 or Fisher's exact test. Quantitative data are presented as the mean ± SD, and the data were analyzed using an unpaired t-test. Differences among >2 groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. All the statistical tests were two-tailed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H. pylori infection in patients with GCA and DGA. The expression of 16S rRNA, CagA and VacA was quantified using qPCR. Among 113 patients with GC, 75.2, 30.1 and 38.9% were positive for *H. pylori* 16S rRNA, CagA and VacA expression, respectively.

The comparative analysis of 16S rRNA expression level revealed no statistically significant difference between patients with GCA and DGA (73.8 vs. 76.9%; $P>0.05$; Table I). However, the mRNA expression level of VacA was significantly higher in patients with GCA (49.2%) compared with that in patients with DGA (26.9%; $P<0.05$). Notably, in samples from male patients >60 years of age, with lymph node metastasis or stage III GCA, increased VacA levels were detected compared with those in patients with DGA and matched pathological features ($P<0.05$; Table II). In DGA, but not in GCA ($P>0.05$), a significant association between CagA and age ($P<0.05$), and VacA and lymph node metastasis ($P<0.05$) was observed. These findings suggest that *H. pylori* may serve an important role in GCA and DGA pathogenesis. Furthermore, subjects with VacA-positive *H. pylori* infection may exhibit an increased risk of GCA in high incidence areas of China.

TFF1 and GKN2 expression levels in GCA and DGA. It has been reported that TFF peptides are primarily expressed in gastric mucous cells and are resistant to proteolysis and acidic environments under physiological conditions; thus, resulting

Table I. H. pylori infection in patients with GCA or DGA.

Group	Cases, n	Positive, n (%)	Negative, n (%)	OR (95% CI)[a]	P-value
H. pylori 16S rRNA					
GCA	61	45 (73.8)	16 (26.2)		
DGA	52	40 (76.9)	12 (23.1)	0.844 (0.357-1.996)	0.699
H. pylori CagA					
GCA	61	19 (31.1)	42 (68.9)		
DGA	52	15 (28.8)	37 (71.2)	1.116 (0.497-2.504)	0.790
H. pylori VacA					
GCA	61	30 (49.2)	31 (50.8)		
DGA	52	14 (26.9)	38 (73.1)	2.627 (1.190-5.800)	0.016

[a]Obtained using Mantel-Haenszel χ^2 test. GCA, gastric cardia adenocarcinoma; DGA, distal gastric adenocarcinoma; *H. pylori*, *Helicobacter pylori*; OR, odds ratio.

Table II. Expression of TFF1 and GKN2 in GCA and DGA.

Group	N	TFF1 (%)	GKN2 (%)
NGM	23	20 (87.0)	18 (77.3)
GCA	61	26 (42.6)[a]	18 (29.5)[a]
DGA	52	22 (42.3)[a]	15 (28.8)[a]

[a]P<0.05 vs. NGM. TFF1, trefoil factor family 1; GKN2, gastrokine 2; GCA, gastric cardia adenocarcinoma; DGA, distal gastric adenocarcinoma; NGM, normal gastric mucosa.

in maintenance of mucosal integrity in the stomach (21-23). In addition, GKN2, which acts by interacting with TFF1 to form a heterodimer, also serves an important role in maintaining mucosa integrity (25,26). Therefore, TFF1 and GKN2 protein expression levels were detected in GC tissues using IHC staining, to further investigate their association with *H. pylori* infection. The positive staining of TFF1 and GKN2 in IHC analysis was indicated by brown granules in the cytoplasm (Fig. 1A). The positive rates of TFF1 and GKN2 in 113 GC samples were 42.5 and 29.2%, respectively, and were significantly lower compared with those in NGM (P<0.05; Table II).

No statistically significant differences were detected in TFF1 and GKN2 expression levels between GCA and DGA. However, the expression levels of both proteins were significantly lower in both types of gastric adenocarcinoma compared with NGM (P<0.05; Table II). Furthermore, TFF1 and GKN2 expression was associated with lymph node metastasis and invasion depth in DGA (P<0.05; Table III).

H. pylori downregulates TFF1 and GKN2 expression in vivo and in vitro. The incidence of positive TFF1 and GKN2 expression was significantly decreased in *H. pylori*-positive GCA (45 cases) and DGA (40 cases) compared with that in GCA and DGA *H. pylori*-negative samples (P<0.05; Fig. 1B and Table IV). Furthermore, to verify the effects of *H. pylori* on TFF1 and GKN2 expression, GES-1 and SKGT-4 cells were treated with different concentrations of *H. pylori* protein for 24 h and the results showed that *H. pylori* treatment significantly reduced TFF1 and GKN2 mRNA and protein expression levels (P<0.05; Fig. 1C-E). Taken together, the results demonstrate that *H. pylori* infection decreases the expression of both protective factors *in vitro* and *in vivo*.

TFF1/GKN2 expression inhibits H. pylori-induced cell proliferation. It has been reported that *H. pylori* protein induces cell proliferation and apoptosis in human gastric epithelial cell lines (31-33). To further investigate the tumorigenic potential of *H. pylori*, its effects on GES-1 and SKGT-4 cell proliferation were determined. The results showed that cell survival rate in the *H. pylori*-treated groups varied with the different doses (Fig. 2A). Notably, treatment with 20 μg/ml *H. pylori* total proteins for 24 h resulted in the highest cell proliferation rate (P<0.05; Fig. 2A). Therefore, 20 μg/ml *H. pylori* total proteins were used for the following experiment. *H. pylori* treatment significantly increased the mRNA and protein expression levels of CDK4, PCNA, cyclin B1 and cyclin D (P<0.05; Fig. 2B-D).

To investigate whether TFF1/GKN2 regulated *H. pylori*-induced proliferation, cells were transfected with TFF1 and GKN2 overexpression plasmids and their protein expression levels were determined. Western blot analysis demonstrated that both targeted genes were successfully overexpressed (Fig. 2E). In addition, the overexpression of TFF1 and GKN2 significantly decreased cell proliferation, compared with that in the *H. pylori*-treatment group (P<0.05; Fig. 2F).

TFF1/GKN2 expression decreases the H. pylori-induced inflammatory response. To investigate the role of TFF1 and GKN2 in the regulation of the *H. pylori*-induced inflammatory response, the activation status of NF-κB was evaluated using western blot analysis. The analysis showed that NF-κB p65 were significantly increased following treatment with *H. pylori* proteins compared with those in the control group (P<0.05; Fig. 3A and B). Although there were not significant differences of the phosphor/total NF-κB p65 ratio between before and after treatment (Fig. 3C), overexpression of TFF1 and GKN2 inhibited *H. pylori*-mediated upregulation of NF-κB p65, compared with *H. pylori*-treated group (P<0.05; Fig. 3A and B).

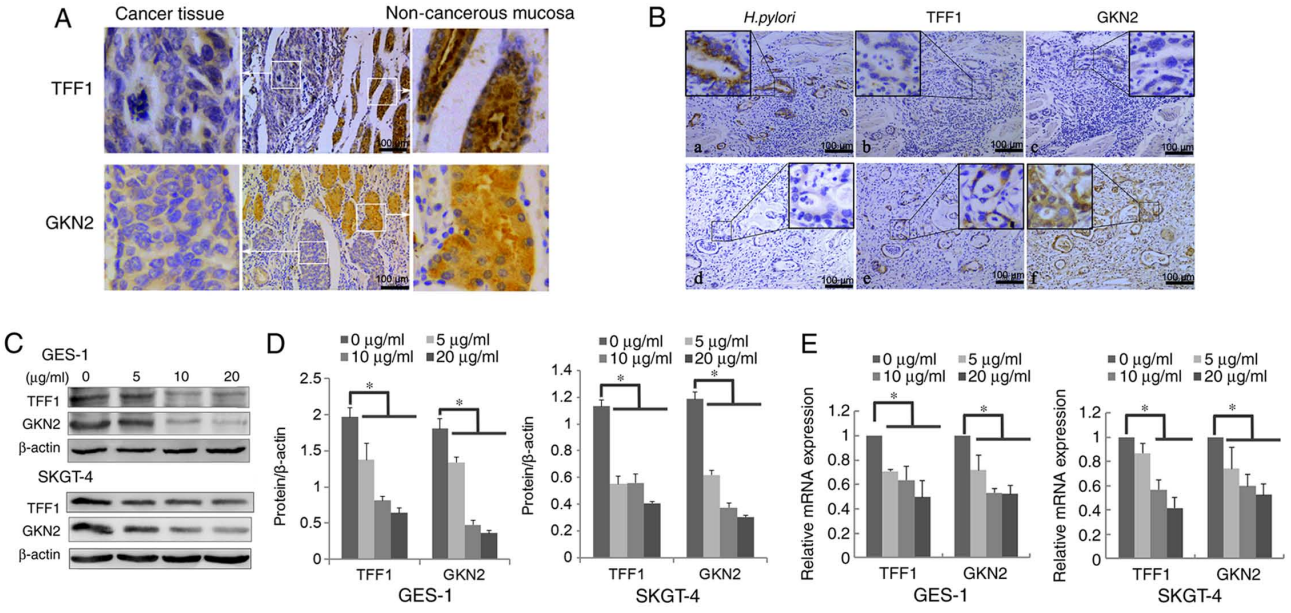

Figure 1. *H. pylori* downregulates TFF1 and GKN2 mRNA and protein expression *in vivo* and *in vitro*. (A) Protein expression level of TFF1 and GKN2 in non-cancerous mucosa (right) and cancer tissue (left) using IHC. (B) Protein expression of (a) *H. pylori*+, (b) TFF1-, (c) GKN2-, (d) *H. pylori*-, (e) TFF1+ and (f) GKN2+ in continuous slices of GCA and DGA using IHC. (C-E) *H. pylori* treatment for 24 h significantly downregulated the (C and D) protein and (E) mRNA expression level of TFF1 and GKN2 in GES-1 and SKGT-4 cells (*P<0.05). Data are presented as the mean ± SD of triplicate experiments, and the data were analyzed using ANOVA and Tukey's post hoc test. IHC, immunohistochemistry; GCA, gastric cardia adenocarcinoma; DGA, distal gastric adenocarcinoma; *H. pylori*/Hp, *Helicobacter pylori*; NGM, normal gastric mucosa; -, negative; +, positive.

It has been reported that bacterial-induced activation of NF-κB induces the secretion of proinflammatory cytokines, which in turn leads to inflammation (34-36). Therefore, the mRNA expression levels of the inflammatory cytokines IL-6, IL-2, IL-4, IL-1β and tumor necrosis factor α (TNF-α), were evaluated. RT-qPCR analysis demonstrated that the mRNA expression levels of all the inflammatory cytokines tested were significantly increased in the *H. pylori*-treated group, compared with the control group (P<0.05; Fig. 3D). However, overexpression of TFF1 or GKN2 in GES-1 and SKGT-4 cells significantly downregulated the expression of the aforementioned cytokines, compared with *H. pylori*-treated group (P<0.05; Fig. 3D). These results indicate that to some extent, TFF1 and GKN2 overexpression suppresses the *H. pylori*-induced inflammatory response.

Discussion

The present study aimed to investigate the association between *H. pylori* infection and the expression of TFF1 and GKN2 in GCA and DGA. The results demonstrated that there was no statistically significant difference in *H. pylori* infection rate between GCA and DGA. Furthermore, *H. pylori* infection significantly decreased TFF1 and GKN2 *in vitro*, and *H. pylori* protein treatment also inhibited the mRNA and protein expression level of both genes *in vivo*. These findings indicate that *H. pylori*-mediated loss of TFF1 and GKN2 expression may contribute to the development of GCA and DGA.

Unlike the clear association between *H. pylori* and DGA pathogenesis, its association with GCA remains controversial. Several studies have reported that there was no association between *H. pylori* infection and gastroesophageal cancers (37,38), while others have demonstrated a positive association (18,39). It is reported that gastroesophageal cancers are inversely associated with *H. pylori* infection, particularly the CagA strain (40). In addition, declining prevalence of *H. pylori* infection has been reported to coincide with an increased incidence rate of GCA (14,41). These findings support a potential protective effect of *H. pylori* infection against gastroesophageal cancers. However, a recent meta-analysis revealed an association between *H. pylori* infection, particularly CagA-positive strains, and the relative risk of Barrett's esophagus (42). In regions, for example Cixian County and Zanhuang County with a high incidence rate of gastroesophageal cancers and an increased prevalence of *H. pylori* infection in China, GCA accounts for a large proportion of cases (1). Notably, in a Chinese population, the studies showed that *H. pylori* infection was associated with an increased risk of GCA (18,20). In the present study, no statistically significant difference was observed in 16S rRNA positive rate between GCA and DGA. Notably, GCA samples displayed significantly higher VacA rates compared with DGA samples. Research in the last few decades has revealed that VacA is a key toxin for *H. pylori* pathogenesis and has a variety of effects on gastric epithelial cells. All identified *H. pylori* strains possess the VacA gene; however, there was significant sequence diversity in VacA genes among numerous isolated strains (43,44). In the present study, the *H. pylori* 26695 strain (GenBank, AE000511.1), including 16S rRNA, CagA and VacA was detected. Our results showed that there are no differences in *H. pylori* infection between GCA and DGA. This was consistent with several other studies in the Chinese population (18,20), and further confirms that *H. pylori* may be an important factor in GCA pathogenesis and patients who are VacA-positive may have a high-risk to develop GCA, in high incidence regions in China.

Table III. Association between *Helicobacter pylori*, TFF1 and GKN2 and the pathological characteristics in patients with GCA (n=61) and DGA (n=52).

	CGA, n (%)						DGA, n (%)					
Pathological characteristics	Total, n	16S rRNA	CagA	VacA	TFF1	GKN2	Total, n	16S rRNA	CagA	VacA	TFF1	GKN2
Sex												
Male	54	39 (72.2)	16 (29.6)	27 (50.0)	24 (44.4)	17 (31.5)	42	34 (81.0)	12 (28.6)	11 (26.2)[a]	18 (42.9)	12 (28.6)
Female	7	6 (85.7)	3 (42.9)	3 (42.9)	2 (28.6)	1 (14.3)	10	6 (60.0)	3 (30.0)	3 (30.0)	4 (40.0)	3 (30.0)
Age, years												
≤60	20	17 (85.0)	10 (50.0)	12 (60.0)	5 (25.0)	4 (20.0)	27	23 (85.2)	11 (40.7)	9 (33.3)	11 (40.7)	7 (25.9)
>60	41	28 (68.3)	9 (22.0)[b]	18 (43.9)	21 (51.2)	14 (34.1)	25	17 (68.0)	4 (16.0)[b]	5 (20.0)[a]	11 (44.0)	8 (32.0)
Lymph node metastasis												
Positive	34	25 (73.5)	10 (29.4)	16 (47.1)	12 (35.3)	8 (23.5)	30	21 (70.0)	7 (23.3)	3 (10.0)[a]	8 (26.7)	4 (13.3)
Negative	27	21 (77.8)	9 (33.3)	14 (51.9)	14 (51.9)	10 (37.0)	22	19 (86.4)	8 (36.4)	11 (50.0)[c]	14 (63.6)[c]	11 (50.0)[c]
Invasion depth												
T1+T2	10	8 (80.0)	4 (40.0)	5 (50.0)	5 (50.0)	4 (40.0)	13	9 (69.2)	3 (23.1)	4 (30.8)	10 (76.9)	6 (46.2)
T3+T4	51	37 (72.5)	15 (29.4)	25 (49.0)	21 (41.2)	14 (27.5)	39	31 (79.5)	12 (30.8)	10 (25.6)	12 (30.8)[d]	9 (23.1)
Stage												
I	7	5 (71.4)	2 (28.6)	2 (28.6)	3 (42.9)	3 (42.9)	6	5 (83.3)	0	1 (16.7)	5 (83.3)	3 (50.0)
II	20	15 (75.0)	7 (35.0)	12 (60.0)	12 (60.0)	6 (30.0)	19	15 (78.9)	8 (42.1)	10 (52.6)	11 (57.9)	9 (47.4)
III	31	23 (74.2)	10 (32.3)	16 (51.6)	9 (29.0)	7 (22.6)	24	19 (79.2)	7 (29.2)	3 (12.5)[a]	6 (25.0)	3 (12.5)
IV	3	2 (66.7)	0	0	2 (66.7)	2 (66.7)	3	1 (33.3)	0	0	0	0

[a]P<0.05, GCA vs. DGA; [b]P<0.05, ≤60 vs. >60 in GCA or DGA; [c]P<0.05, positive lymph node metastasis vs. negative lymph node metastasis in DGA; [d]P<0.05, T1/T2 vs. T3/T4 in DGA. GCA, gastric cardia adenocarcinoma; DGA, distal gastric adenocarcinoma.

Table IV. *Helicobacter pylori* infection decreased the expression of TFF1 and GKN2 in GCA and DGA.

	GCA		DGA	
Groups	n (%)	P-value	n (%)	P-value
TFF1+/*H. pylori*+	15/45 (33.3)		13/40 (32.5)	
TFF1-/*H. pylori*+	30/45 (66.7)		27/40 (67.5)	
TFF1+/*H. pylori*-	11/16 (68.8)		9/12 (75.0)	
TFF1-/*H. pylori*-	5/16 (31.3)	0.014[a]	3/12 (25.0)	0.009[a]
GKN2+/*H. pylori*+	10/45 (22.3)		8/40 (20.0)	
GKN2-/*H. pylori*+	35/45 (77.8)		32/40 (80.0)	
GKN2+/*H. pylori*-	8/16 (50.0)		7/12 (58.3)	
GKN2-/*H. pylori*-	8/16 (50.0)	0.036[a]	5/12 (41.7)	0.010[a]

[a]P<0.05, *H.* cases vs. *H. pylori*-cases. TFF1, trefoil factor family 1; GKN2, gastrokine 2; GCA, gastric cardia adenocarcinoma; DGA, distal gastric adenocarcinoma; *H. pylori*, *Helicobacter pylori*.

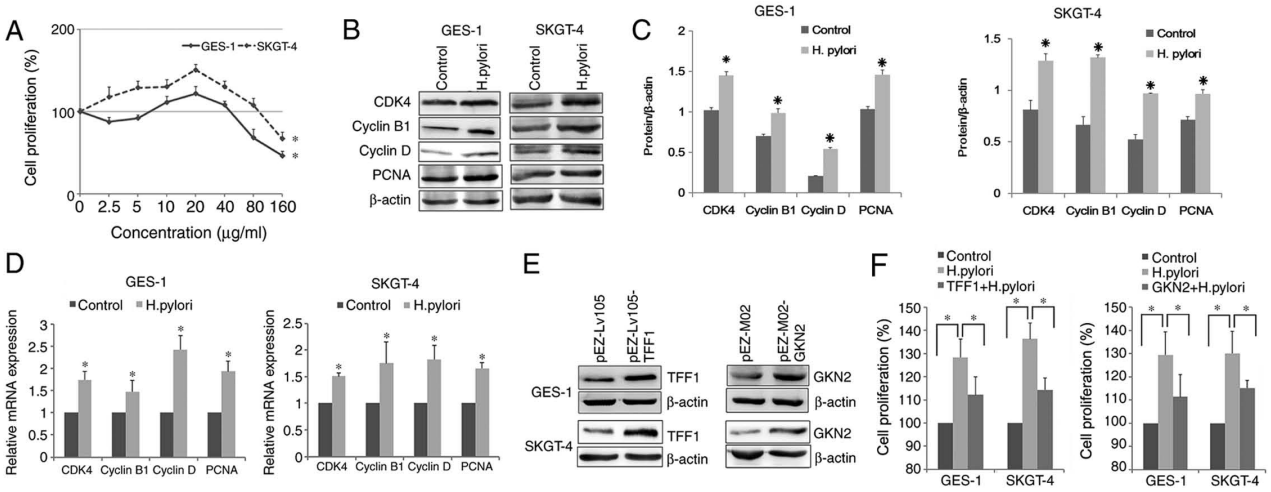

Figure 2. *H. pylori* affects the proliferation of host cells. (A) *H. pylori* total protein affected cell proliferation of GES-1 and SKGT-4 with their respective controls (*P<0.05). Treatment of cells with 20 μg/ml *H. pylori* total protein for 24 h resulted in the highest cell proliferation rate (*P<0.05). Percentage of cells proliferation was determined using an MTT assay. Data are presented as the mean ± SD of triplicate experiments. (B-D) *H. pylori* increased the (B and C) protein and (D) mRNA expression levels of CDK4, cyclin B1, cyclin D and PCNA compared with that in the solvent control group (*P<0.05), and the data were analyzed using an unpaired t-test. (E) Successful overexpression of TFF1 (pEZ-Lv105-TFF1) and GKN2 (pEZ-M02-GKN2) was confirmed in the GES-1 and SKGT-4 cells using western blot analysis. (F) Overexpression of TFF1 and GKN2 inhibited *H. pylori*-induced cell proliferation (*P<0.05). Cell proliferation was detected using an MTT assay. Experiments were repeated 3 times and the data are presented as the mean ± SD. *H. pylori*, *Helicobacter pylori*; PCNA, proliferating cell nuclear antigen; CDK4, cyclin dependent kinase 4; TFF1, trefoil factor 1; GKN2, gastrokine 2.

To further investigate the effects of *H. pylori* on host cell self-protection mechanisms, the protein expression levels of two protective molecules were detected in GC and normal gastric epithelial tissue specimens. Both GCA and DGA exhibited significantly lower expression of TFF1 and GKN2 compared with that in NGM. Notably, *H. pylori*-positive GCA and DGA tissues displayed significantly decreased TFF1 and GKN2 expression compared with that in their respective *H. pylori*-negative tumor tissues. It has been reported that TFF1 and GKN2 expression is downregulated in GC (45,46). The protein and mRNA expression of GKN1, the other member of GKN family, was decreased in the mucosa of patients with GC and with *H. pylori*-positive chronic gastritis (34), and exosomal GKN1 protein could inhibit gastric carcinogenesis (35). In particular, decreased expression of GKN2 has been associated with shorter overall survival in the intestinal subtype of GC (36). A previous study demonstrated that TFF1 acts by interacting with GKN2 to form a heterodimer, which is stabilized with an intermolecular disulfide bond (25). GKN2 and TFF1 co-expression induces G_1/S cell cycle arrest in MKN1, MKN28 and MKN45 cells, thus inhibiting the proliferation of gastric cancer cells (47). Therefore, TFF1 and GKN2 are tumor suppressive factors and their absence may contribute to carcinogenesis in GC (22). In the present study, *H. pylori* significantly downregulated the mRNA and protein expression levels of these aforementioned protective factors, in two different cell lines. These findings suggest that *H. pylori*-mediated TFF1 or GKN2 downregulation may be associated with the development of GCA and DGA.

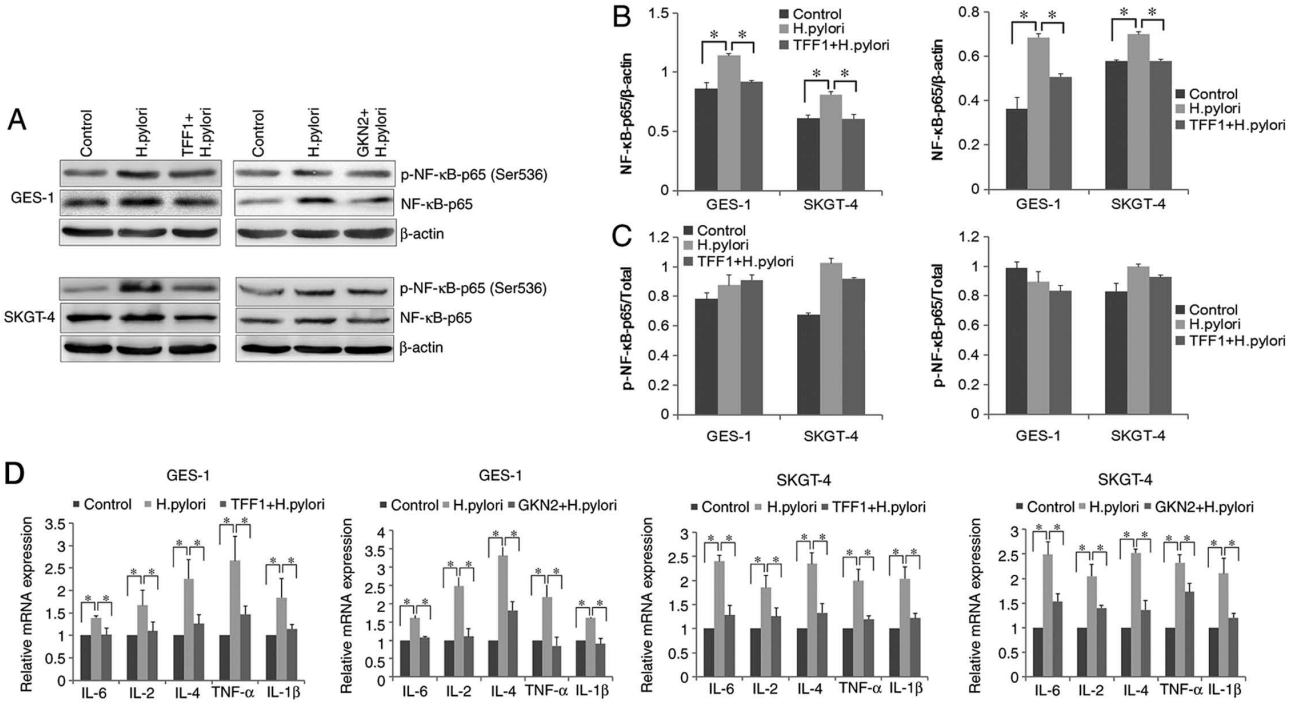

Figure 3. Overexpression of TFF1 and GKN2 inhibits the *H. pylori*-induced inflammatory response. (A) NF-κB-p65 and p-NF-κB-p65 (Ser536) were detected using western blot analysis. (B and C) NF-κB-p65 and its phosphorylated form (Ser536) were significantly increased following treatment with *H. pylori* proteins, and overexpression of TFF1 and GKN2 significantly inhibited *H. pylori*-mediated regulation of NF-κB p65 (*P<0.05). (D) H. pylori (20 μg/ml) significantly increased the mRNA expression level of IL-6, IL-2, IL-4, IL-1β and TNF-α (*P<0.05), whereas the overexpression of TFF1 and GKN2 decreased these inflammatory cytokines (*P<0.05). Data are presented as the mean ± SD of triplicate experiments. *H. pylori*, *Helicobacter pylori*; TFF1, trefoil factor 1; GKN2, gastrokine 2; TNF, tumor necrosis factor; p, phosphorylated.

It is widely recognized that *H. pylori* induces chronic gastritis, which is considered to be the first step in gastric carcinogenesis (48-50). In addition, *H. pylori* serves an important role in the activation of NF-κB (48). Therefore, the bacterial-mediated activation of NF-κB induces the secretion of proinflammatory cytokines such as TNF-α and IL-1β, which in turn leads to inflammation (48-50). Furthermore, JHP0290, a functional protein from *H. pylori* was found to bind to several cell types, including gastric epithelial cell lines, macrophage and neutrophils and induce TNF-α release, which was partly dependent on the activation of NF-κB (31,32). HP1286, a conserved secreted protein from strain 26695, could induce apoptosis in adenocarcinoma gastric cells (33). Previous research in a TFF1 knock-out mouse model showed that the majority of inflammation-related genes, such as NF-κB, TNF-α and IL-1β and the downregulation of TFF1 were involved in the induction of inflammation, which in turn resulted in gastric tumorigenesis (49,50). With respect to GKN2, it was found to recruit neutrophils and induce the release of inflammatory factors, for example NF-κB and IL-1β, contributing to inflammation in stress-induced gastric lesions (51). To investigate *H. pylori* infection in host cell self-protection mechanisms in the present study, *H. pylori* total protein, primarily containing urease, CagA and cytotoxin A antigens, was selected to treat GES-1 and SKGT-4 cells *in vitro*. NF-κB p65 was significantly increased in both cells following *H. pylori* protein treatment. Notably, overexpression of TFF1 and GKN2 inhibited *H. pylori*-induced increase of NF-κB p65 and reversed the mRNA expression levels of inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-4, and IL-6) following *H. pylori*-mediated upregulation. The aforementioned results indicate that TFF1 and GKN2 may protect gastric epithelial cells from damage mediated by inflammatory cytokines.

It is well known that inflamed tissues express several growth factors, including epidermal growth factor and platelet-derived growth factor, which directly promote cell proliferation (52). The results of the current study further demonstrate that *H. pylori* regulates cell proliferation differently for distinct concentrations. In particular, treatment of cells with 20 μg/ml *H. pylori* total protein resulted in the highest proliferation rate. Furthermore, TFF1 and GKN2 overexpression inhibited *H. pylori*-mediated cell proliferation. It has been reported that the proliferation rate of gastric epithelial cells in patients with CagA-positive *H. pylori* was significantly higher compared with that in patients who were CagA-negative (53). However, *in vitro*, CagA acts as a potent inhibitor of cell proliferation, which is inconsistent with its oncogenic role (54). Therefore, CagA may be involved in a mechanism that converts the response of the host cells between growth inhibition and stimulation, depending on varying infective dosage (53,54). In addition, several *H. pylori* virulence factors, for example JHP0290 and SlyD have been identified to regulate the proliferation of gastric epithelial cells (32,55). The aforementioned results indicated that *H. pylori*, to some extent, induces cell proliferation and the inflammatory response in host cells. Furthermore, overexpression of TFF1 and GKN2 may reduce this response.

In conclusion, the present study demonstrated that there was no statistically significant difference in *H. pylori* infection rate between GCA and DGA. This finding indicates that *H. pylori*,

particularly the VacA+ strain, may play an important role in GCA, in high risk-areas of China. The results indicated that *H. pylori* decreased the mRNA and protein expression levels of TFF1 and GKN2 in GCA and DGA, while TFF1/GKN2 overexpression significantly reduced *H. pylori*-induced cell proliferation and inflammation in GCA and DGA; however there was a lack of a suitable negative control in *H. pylori* protein treatment. In addition, the mechanism between *H. pylori* infection and self-protection of the host cell remains unclear, and further investigation is required in the future. Notably, it might be important to select an appropriate negative control, for example a denatured version of the *H. pylori* proteins or proteins derived from harmless gut flora, for the *H. pylori* protein experiments.

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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

WL, DZ, BC, XZ and XW performed the experiments and prepared the figures. JL and LX prepared the figures and analyzed the data. WL, JL and LX performed critical revision of the manuscript and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the local Ethics Committee of Hebei Medical University and written informed consent was provided by all the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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