Decreased TFF2 Expression in the Gastric Antrum in Patients Infected with CagA-Positive *Helicobacter pylori*

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ABSTRACT: **Background:** The trefoils factor family is a relatively new family of peptides. Their abundant expression in the epithelial cells of the gastrointestinal tract in the normal physiological state and in various ulcerative conditions suggests an important role in mucosal defense and repair. Infection with *Helicobacter pylori* interferes with normal mucosal activity.

Objectives: To investigate whether *H. pylori* infection alters the expression of trefoils TFF1 and TFF2 in the gastric mucosa of patients with *H. pylori*-associated chronic active gastritis, positive or negative for the CagA strain.

Methods: During investigation for dyspepsia, gastric biopsies and blood samples were obtained from patients who underwent upper gastrointestinal endoscopy. Rapid urease testing, histology for determination of *H. pylori*-associated CAG and Western analysis for TFF1 and TFF2 expression with antisera were performed. CagA state was determined using a commercial kit.

Results: TFF2 expression was significantly reduced in both groups of patients with *H. pylori*-associated CAG compared to healthy patients without *H. pylori* infection, particularly in CagA-positive patients. TFF1 expression showed a tendency of reduction (not significant) in this group only.

Conclusions: These results suggest that *H. pylori*-associated CAG has a deleterious effect on the expression of TFF2 in the gastric antrum. This reduced expression may contribute to the damage induced to the gastric mucosa by *H. pylori*.

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KEY WORDS: *Helicobacter pylori*, chronic active gastritis (CAG), CagA, TFF1, TFF2

The trefoil factors are a newly recognized family of peptides that bear a three-loop trefoil domain. They are synthesized and secreted mainly by mucin-secreting epithelial cells lining the gastrointestinal tract. Three trefoil peptides are known in humans: TFF1 is expressed in the stomach TFF2 in both the stomach and duodenum, and TFF3 in the intestine [1]. Their expression in the normal physiological state and in various ulcerative conditions suggests an important role in mucosal defense and repair. The TFFs are expressed in a wide variety of ulcerative conditions of the gastrointestinal tract, including Barrett's esophagus [2], gastric and duodenal ulcers [3,4] as well as in the small and large intestine in Crohn's disease [5]. This prevalence emphasizes their importance as peptides involved in the repair of the gastrointestinal mucosa. Three different molecular forms of TFF1 were detected in normal stomachs: TFF1 monomer, dimer and a 25 kDa complex intimately associated with mucus [6,7]. It is now clear that all three mammalian trefoil factors are motogens, namely, able to promote cell migration without promoting cell division. They are all regulated at sites of mucosal injury and stimulate the repair process by stimulating the migration of surviving cells (epithelial restitution) [8,9]. It has been suggested that they participate, via mucin, in the correct organization of the mucous layer that protects the apical side of the mucosa from deleterious luminal agents [6,7]. Whether this occurs through direct stimulation of cellular differentiation or enhancement of mucosal protective properties (through an interaction with gastric mucins) remains to be elucidated. It has also been shown that oral and parenteral TFF2 accelerates the healing of gastric ulceration [10,11]. TFF2-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to injury from non-steroidal anti-inflammatory drugs [12].

The discovery of *Helicobacter pylori* and its relationship with peptic ulcer has been established [13,14]: *H. pylori* causes chronic active gastritis in virtually all infected individuals. Persistent inflammation of the gastric mucosa can ultimately lead to the loss of its normal architecture, with the gradual disappearance of gastric glands that contain specialized cells. In the resulting atrophic mucosa, inflammation and intestinal metaplasia may persist; thus *H. pylori* colonization density may decrease, and dysplasia, gastric cancer and lymphoma may eventually develop [15-17].

The aim of the study was to investigate whether *H. pylori* infection alters the expression of TFF1 and TFF2 in the gastric mucosa of patients with *H. pylori*-associated CAG, CagA positive and negative. This issue is relevant to the possible interference of *H. pylori* with mechanisms of mucosal repair.

PATIENTS AND METHODS

Informed consent was obtained from all patients, and the Helsinki Committee of our facility approved the protocol.

TFF = trefoils factor family
CAG = chronic active gastritis

Thirty-two patients (age 50.4 ± 17 years) referred for evaluation of dyspepsia underwent upper GI endoscopy. Exclusion criteria were gastric ulcer, erosions or malignancy diagnosed during the procedure, alcohol abuse, severe concomitant diseases, previous gastric surgery, use of proton pump inhibitors, GI bleeding preceding the endoscopy, and treatment with NSAIDs, corticosteroids and prostaglandin analogues. All patients had macroscopically normal stomach at gastroscopy (findings were recorded). A blood sample and seven antral biopsies were taken with standard endoscopic biopsy forceps from within 2 cm of the pylorus from each patient. Patients were divided into three groups: group 1 – patients with *H. pylori*-associated CAG, CagA-positive serum (7 males, 2 females); group 2 – patients with *H. pylori*-associated CAG, CagA-negative serum (5 males, 7 females); and group 3 (control group) – patients with no evidence for *H. pylori* infection or inflammation (6 males, 5 females).

RAPID UREASE TESTS

One biopsy from each patient was used for rapid urease test immediately after removal, using H. pylori ONE kit (GI Supply Camp Hill, PA, USA).

HISTOLOGY

As *H. pylori* infection tends to be patchy, its status was also assessed by histology. Two biopsies (from each subject) were fixed in buffered formalin solution and stained with hematoxylin-eosin and Giemsa. Sidney classification was used to classify *H. pylori*-associated CAG [18].

WESTERN ANALYSIS

Four antral biopsies from each subject were used for peptide extraction according to Newton et al. [6]. Briefly, the biopsy specimens from each subject were homogenized in 67 mM sodium phosphate buffer pH 6.5 containing 1 mM iodoacetamide, 4 mM PMSF, 5 mM benzamidine HCl, 10 mM EDTA, 100 mM aminocaproic acid and 10 mM N-ethyl maleimide. The homogenates were centrifuged at 100,000 g for one hour at 4°C. The supernatants were immediately frozen in liquid nitrogen and stored at -70°C. Protein concentrations were measured in the samples using the Bradford protein assay (BIORAD) and 20 µg from each sample were used for Western analysis. For analysis of TFF2 by Western blotting, samples were denatured by boiling for 10 minutes in the presence of 2.5% β-mercaptoethanol and immediately electrophoresed on sodium dodecyl sulphate polyacrylamide gels containing 20% acrylamide in the separating gel and 10% acrylamide in the stacking gel. Proteins were then transferred from the gels to 0.2 µm nitrocellulose membrane (Schleicher and Schuell, Germany) using a semi-dry transfer apparatus (Biometra, Germany) for 15 min at 5 mA/cm^2. The next day membranes were blocked with 4% (wt/vol) skim milk powder in Tris buffered saline (20 mM Tris, 140 mM NaCl, 0.05% Tween-20, pH 7.4) for 1 hour at room temperature, and then incubated with a 1/1000 dilution of anti-TFF2 antiserum in blocking solution for 2 hours at room temperature. Goat anti-rabbit-HRP conjugate (Sigma, Israel) was used as the secondary antibody and the signal was detected on X-ray films using the chemiluminescence reagents of the EZ-ECL kit (Biological Industries, Beit HaEmek, Israel). For Western analysis of TFF1, samples (without denaturation) were electrophoresed in gels containing 20% acrylamide in the separating gel and 4% acrylamide in the stacking gel. The conditions for protein transfer and membrane blotting with anti-TFF1 were as described above for anti-TFF2.

The anti-human TFF1 and anti-human TFF2 sera were kindly provided by Dr. C.L. Tomasetto (Strasbourg, France) and Dr. A.S. Giraud (Melbourne, Australia) respectively. These sera specificities were determined previously [19,20].

DENSITOMETRY ANALYSIS

The densities of the bands from the films of the Western blots were analyzed using the BioCapt and Bio-Profil (Bio-1D) softwares.

CagA STATE

Level of anti-CagA antibodies was determined in the patients' sera using a commercial kit (Genesis Diagnostics, Cambridgeshire, UK).

STATISTICAL ANALYSIS

Wilcoxon rank sum test was used for analysis of the densitometry data. *P* values < 0.05 were considered significant.

RESULTS

HISTOLOGY OF GASTRIC TISSUES

Histopathology of gastric tissues [Figure 1] showed normal appearance in group 3 (control group) and marked chronic inflammation, lymphoid follicles formation and prominent germinal centers in groups 1 (patients with *H. pylori*-associated CAG, CagA positive) and 2 (*H. pylori*-associated CAG, CagA negative). As shown in Figure 1A and B, the histopathological findings in groups 1 and 2 were similar.

Examination of gastric glands revealed infiltration by polymorphonuclear cells with no differences between patients in groups 1 and 2 [Figure 2A and B]. *H. pylori* bacilli on the surface of the foveolar epithelium and lumen in these groups are shown [Figure 2C and D].

EXPRESSION OF TFF2 IN THE ANTRUM

Western analysis using anti-TFF2 serum detected only one band with an estimated molecular mass of ~21 kD, corre-

sponding to the glycosylated form of TFF2 [21]. The expression of TFF2 was decreased in patients with *H. pylori*-associated CAG compared to patients without *H. pylori* infection [Figure 3A]. The decrease in TFF2 expression was especially prominent in CagA-positive patients [Figure 3A].

Quantification of the Western signals by densitometry analysis showed that the differences in TFF2 expression among the three groups were significant: *H. pylori*-associated CAG patients with CagA-negative serum or CagA-positive serum had significantly lower TFF2 signals than patients without *H. pylori* infection ($P = 0.039$ and $P = 0.001$ respectively) [Figure 3B]. A significant difference was also found between the two groups of patients with *H. pylori*-associated CAG ($P = 0.034$) [Figure 3B]. The non-specific vague signal at ~60 kD [Figure 3A] was not quantified by densitometry.

EXPRESSION OF TFF1 IN THE ANTRUM

Western analysis using anti-TFF1 serum detected one band with molecular mass of 6.5 kD [Figure 4], corresponding to the monomeric form of TFF1 [6]. A great variability in the expression of TFF1 was observed among patients within the same group, mainly in the control group and in the CAG patients negative to CagA [Figure 4]. Statistical analysis did not show significant differences among the various groups of patients. However a tendency of reduction in TFF1 expression was found in the CAG group with positive CagA reactivity ($P = 0.097$).

DISCUSSION

The purpose of the present study was to evaluate the possible effect of *H. pylori* infection on the expression of TFF1 and TFF2 in non-ulcerated antral mucosa with no evidence of gastric cancer. We speculate that *H. pylori* infection may have a modulator effect on the expression of trefoils. Our results show significantly reduced expression of TFF2 in patients with *H. pylori*-associated CAG. This decrease was more pronounced in patients infected with the CagA strain. These findings may partially explain the correlation between the increased severity of gastritis and the infection with *H. pylori* CagA strain. Our results are supported by a recent study that examined the immunohistochemistry expression of TFF2 and *H. pylori* infection in carcinogenesis of the gastric mucosa, and concluded that the effect of the pathogen on the expression of TFF2 depends on the status of gastric mucosa [22].

A similar tendency was found for TFF1, but with no statistical significance, concurring with a previous report that analyzed TFF1 gene expression in non-ulcerated mucosa and showed that induction of TFF1 mRNA was not related to *H. pylori* status or to the severity of gastritis [3]. Still, our

Figure 1. Histopathology of gastric mucosa showing marked chronic inflammation with lymphoid follicles formation and prominent germinal centers. **[A]** Patient with CAG, *H. pylori* positive, CagA positive. **[B]** Patient with CAG, *H. pylori* positive, CagA negative. **[C]** Control of normal gastric mucosa (*H. pylori* negative). Germinal centers are indicated by arrows. (Hematoxylin-eosin staining, **[A]** and **[B]** low power magnification and **[C]** intermediate power magnification)

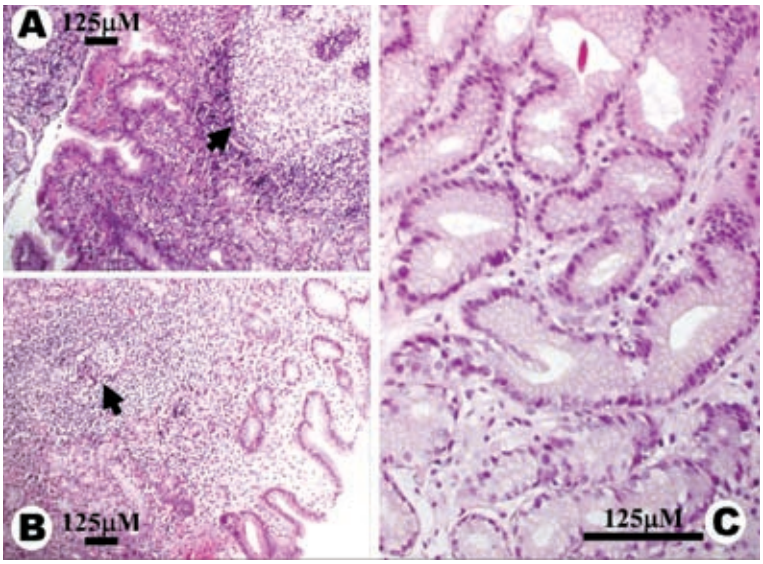


Figure 2. Histopathology of gastric mucosa showing gastric glands infiltrated by polymorphonuclear cells. **[A]** and **[C]** positive for *H. pylori* and CagA. **[B]** and **[D]** positive for *H. pylori* and negative for CagA. These photomicrographs (high power magnification, hematoxylin-eosin staining) show infiltration by polymorphonuclear cells, indicated by arrows in **[A]** and **[B]** and presence of abundant *H. pylori* bacilli, indicated by arrows in **[C]** and **[D]** on the surface of the foveolar epithelium and lumen.

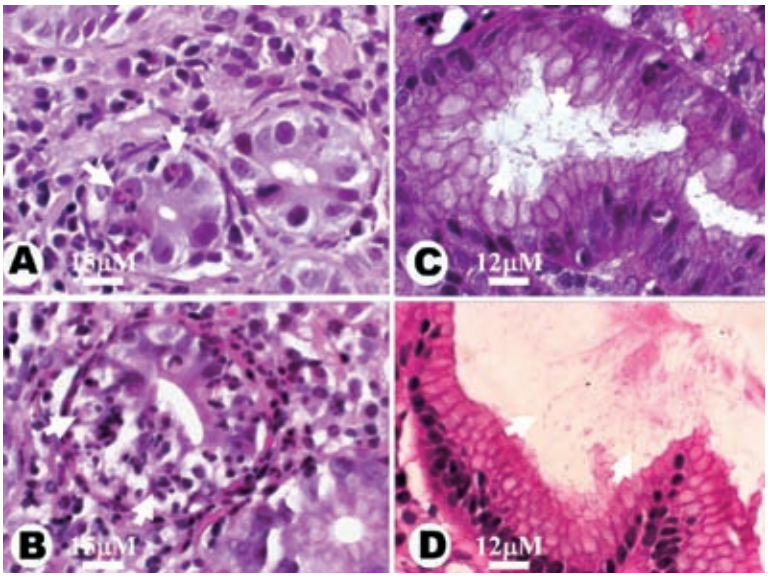


Figure 3. Expression of TFF2. Proteins from antral biopsies of patients with or without H. pylori-associated CAG were separated by gel electrophoresis and studied by Western analysis with anti-TFF2 **[A]**. Twenty µg from each sample were used for the analysis. The representative results of two patients with no evidence for *H. pylori* (lanes 5,6), two patients with *H. pylori*-associated CAG Cag-negative serum (lanes 2 and 3) and two patients with *H. pylori*-associated-CAG CagA-positive serum (lanes 1 and 4) are shown. Molecular size markers are indicated on the left. TFF2 levels were quantified by densitometry analysis of the bands obtained in the Western blot films **[B]**. TFF2 levels in the three groups of patients were expressed in arbitrary densitometry units and statistical analysis was performed using Wilcoxon rank sum test. The mean and distribution are presented.

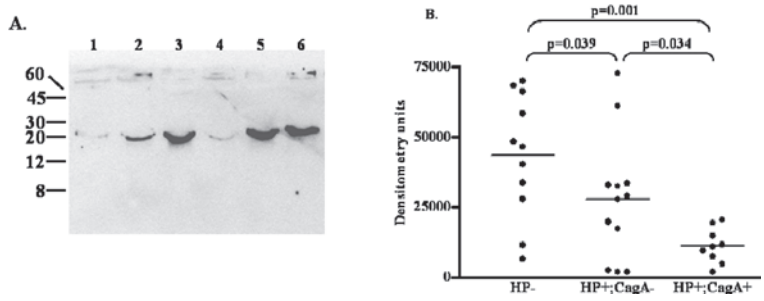
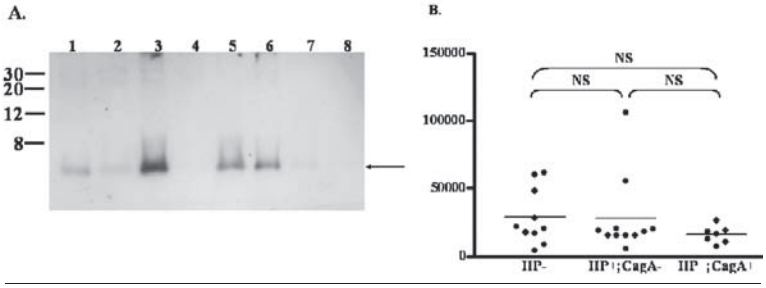


Figure 4. Expression of TFF1. Proteins from antral biopsies of patients with or without H. pylori-associated CAG were studied by gel electrophoresis and Western analysis with anti-TFF1 **[A]**. Twenty µg from each sample were used for the analysis. The representative results of three patients with no evidence for *H. pylori* (lanes 6–8), three patients with *H. pylori*-associated CAG CagA-negative serum (lanes 3–5) and two patients with *H. pylori*-associated CAG CagA-positive serum (lanes 1 and 2) are shown. Molecular size markers are indicated on the left. Arrow indicates TFF1 signal. TFF1 signals were quantified by densitometry analysis of the Western blot films and expressed in arbitrary densitometry units **[B]**. The mean and distribution are presented.



results show a tendency of reduction in TFF1 expression in the CAG group positive to CagA, in agreement with a recent report that showed decreased expression of both TFF1 and TFF2 in patients infected with *H. pylori* [23], and in contrast to another study that showed an increased TFF2 expression in *H. pylori* infection [24]. Both studies [23,24] used immunohistochemistry to quantify trefoil expression.

Our results suggest that *H. pylori*-associated gastritis has a deleterious effect on the expression of TFF2 in the gastric antrum. This reduced expression may contribute to the damage induced to the gastric mucosa by *H. pylori*. Two possible mechanisms can be suggested: TFFs participate, via mucin binding, in the correct organization of the mucous layer that protects the apical side of the mucosa from deleterious luminal agents [6,7] including proton permeation [25]. The reduction in TFF2 expression may affect the protective effects of the mucous layers, exposing the cells to these damaging agents. A second possible mechanism that should be considered is an inhibitory effect in the motogen properties of TFFs (promotion of cell migration), decreasing the migration of surrounding cells and affecting the epithelial restitution [8,9].

CagA-positive *H. pylori* strain exerts a more diminishing effect on the expression of TFF2. These findings may partially explain the correlation between the increased severity of gastritis and gastric ulcers and the infection with CagA strain. Our results add new light on the relationship between *H. pylori* gastritis and the development of gastric mucosal damage. These relations between *H. pylori* and the expression of various trefoil peptides deserve further investigation.

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