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ORIGINAL ARTICLE

Aberrant epithelial expression of trefoil family factor 2 and mucin 6 in *Helicobacter pylori* infected gastric antrum, incisura, and body and its association with antralisation

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Aims: To determine gastric expression of trefoil family factor 2 (TFF2) and MUC6 in *Helicobacter pylori* positive and negative subjects, and its association with antralisation at the gastric incisura.**Methods:** Gastric biopsies from the antrum, incisura, and body of 76 dyspeptic patients without ulcers were used for the determination of *H pylori* infection, histological changes, and epithelial TFF2 and MUC6 expression.**Results:** In the foveola, the rates of TFF2 and MUC6 immunostaining were greater in *H pylori* infected (n = 27) than in uninfected patients (n = 49) at the antrum (59.3% v 4.1% for TFF2 and 63.0% v 4.1% for MUC6; both p < 0.001) and incisura (44.4% v 2.0% for TFF2 and 48.1% v 0% for MUC6; both p < 0.001). In the deeper glands, the rates were also greater in *H pylori* infected than in uninfected patients at the incisura (85.2% v 22.4% for both TFF2 and MUC6; p < 0.001). Antral-type mucosa was present at the incisura in 28 of the 76 patients. TFF2 and MUC6 expression in the foveola and deeper glands was significantly associated with antral-type mucosa, independent of *H pylori* status.**Conclusions:** *Helicobacter pylori* infection increases the expression of TFF2 and MUC6 in the gastric epithelium. Aberrant TFF2 and MUC6 expression is associated with antralisation of gastric incisura.

Mucins are high molecular weight glycoproteins that are the major components of the mucous viscous gel covering the surface of epithelial tissues. To date, 11 distinct human epithelial mucins have been identified.¹ Mucins are expressed in a site specific fashion. The gastric mucosa normally expresses mucins MUC1, MUC5AC, and MUC6.^{1–5} These mucins are believed to play an important role in the protection of the gastric mucosa from extremes of pH, proteases, toxins, mechanical irritation, and pathogenic organisms.^{1–5}

“Whether or not trefoil family factor 2 and mucins are involved in antralisation is unknown”

Trefoil family factor (TFF) peptides are a relatively newly discovered family of peptides comprising small secretory peptides bearing one or more trefoil domain. In humans, three TFF peptides have been identified, namely: TFF1/pS2 (or breast cancer oestrogen inducible peptide), TFF2/hSP (or spasmodic polypeptide), and TFF3/ITF (or intestinal trefoil factor).^{6–8} TFFs are mainly synthesised and secreted by mucin secreting epithelial cells lining the gastrointestinal tract and are thus closely associated with mucins.^{1–9} In normal gastric mucosa, TFF1 co-localises with MUC5AC in the surface/foveolar epithelium, whereas TFF2 is expressed together with MUC6 in the mucous cells of the regenerative zone of the body mucosa and in the antral glands.^{10–11} The role of TFFs in mucosal defence and healing is also becoming increasingly clear, although the mechanism needs to be further elucidated.^{8–12} More recently, it has been found that the expression pattern of mucins and TFF peptides alters in gastric precancerous lesions and neoplasia, and these altered expression patterns may be used as prognostic indicators, implying involvement of these factors in tumour progression.^{3–7, 8, 11–18}

Helicobacter pylori is recognised as a major cause of chronic gastritis and peptic ulcer disease, and is a definite pathogen

for the development of gastric cancer.^{19–21} However, the exact mechanisms by which infection with the organism leads to gastric cancer have not been fully elucidated. Previous studies have shown that *H pylori* infection is associated with changes in the expression of mucins in the gastric epithelium.^{3–5} However, these studies only investigated gastric biopsies taken from the gastric antrum, so that global changes in the expression of these factors in the stomach could not be revealed. Moreover, the possible changes in gastric epithelial expression of TFF2 in the presence of *H pylori* infection have rarely been reported.²² In addition, our previous studies have shown that *H pylori* infection is associated with antralisation—that is, the presence of antral-type mucosa in the gastric incisura, body, and/or fundus or gastric mucosal transformation from transitional or body type to antral type, which is strongly associated with gastric atrophy and intestinal metaplasia.²³ Moreover, cell proliferation and apoptosis and their related proteins such as Bcl-2 and Bax alter in the antralised gastric incisura.²⁴ These observations suggest that antralisation is primarily the result of *H pylori* infection in most cases, and may be an important step (or histological marker) in the process of gastritis progressing to precancerous lesions. However, whether or not TFF2 and mucins are involved in antralisation is unknown. Therefore, the aim of our study was to determine the expression of TFF2 and MUC6 in normal and *H pylori* infected gastric epithelium at the gastric antrum, incisura, and body, and to explore the association between the aberrant expression of TFF2 and MUC6 and antralisation at the incisura.

PATIENTS AND METHODS

Patients and gastric biopsies

Seventy six patients (M/F, 35/41; mean age, 42; SD, 13 years) referred for upper endoscopy at Queen Mary Hospital, Hong

Abbreviations: CI, confidence interval; OR, odds ratio; SPEM, spasmodic polypeptide expressing metaplasia; TFF, trefoil family factor

Kong because of dyspeptic and reflux symptoms were selected for our study. At endoscopy, four biopsies were obtained from each patient: two from the gastric antrum, one from the body, and one from the incisura. One antral biopsy was used for an in house rapid urease test. The other biopsies were fixed in formalin and embedded in paraffin wax for histological examination and immunohistochemistry. The following groups of patients were excluded from our study: (1) those who had been taking aspirin or non-steroidal anti-inflammatory drugs over the past six months; (2) those who had been taking antibiotics, H₂ receptor blockers, bismuth, or proton pump inhibitors in the preceding four weeks; and (3) those who had undergone previous gastric surgery, those with endoscopic evidence of gastroduodenal ulceration, or those with histological evidence of gastric precancerous lesions (atrophy, intestinal metaplasia, and dysplasia), gastric carcinoma, or lymphoma.

All patients gave informed written consent, and this project was approved by the ethics committee of The University of Hong Kong.

Detection of *H pylori* infection and histological examination

Sections (4 µm thick) of the paraffin wax embedded tissue were stained with haematoxylin and eosin. Histological changes and helicobacter-like organisms were examined by experienced pathologists who were blinded to all clinical and endoscopic information. The in house rapid urease test used in our study has been shown to have a sensitivity of 99% and specificity of 100% for the detection of *H pylori*.²⁵ Patients were defined as *H pylori* positive if positive by both the rapid urease test and histology. Patients were defined as *H pylori* negative if all tests were negative. Patients positive by the rapid urease test only or histology only were not included in our study.

The updated Sydney system was used to assess the severity and activity of gastric inflammation.²⁶ Chronic gastritis was said to be definitely present when the severity of inflammation at any site of the stomach was classified as grade 2 or 3. The mucosa of gastric biopsies taken from different sites was classified as antral (mucous secreting or pyloric) type, body (acid secreting or oxyntic) type, or transitional (junctional) type according to the definitions set out in the updated Sydney system.²⁶ The characteristic feature of antral-type mucosa is the presence of coiled and branching antral glands, which are lined by mucous cells that are interspersed with endocrine cells (chiefly G and D types), and a few parietal cells. The glands in body-type mucosa are straight tubes that constitute acid producing parietal cells, along with scattered mucous cells in their upper portion and mainly chief cells in their lower portion, with scattered argyrophilic endocrine cells. Transitional-type mucosa is a mixture of the architectural features and cell types found in the antral and body types.²⁶

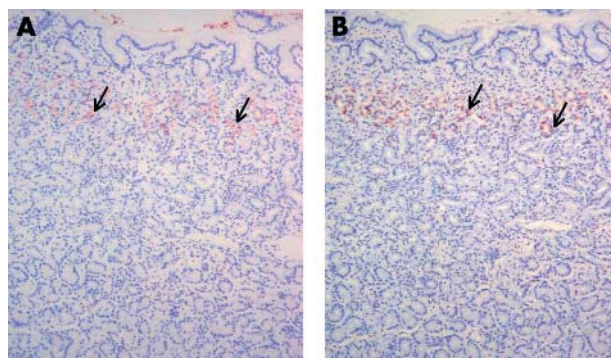


Figure 2 The expression of trefoil family factor 2 (TFF2) and MUC6 in normal body mucosa. The expression of (A) TFF2 and (B) MUC6 is limited to the mucous neck cells in the regenerative zone (arrows). Original magnification, ×100.

Immunohistochemistry for TFF2 and MUC6 in gastric epithelium

Biopsy specimens taken from the antral, body, and incisura of all patients were subjected to immunohistochemistry for the assessment of TFF2 and MUC6 expression in the gastric epithelium. Briefly, tissues embedded in paraffin wax were cut into sections 4 µm thick, dewaxed, and dehydrated for 10 minutes. The sections were treated in 3% hydrogen peroxide for five minutes, and then incubated with monoclonal antibody against TFF2 (undiluted), MUC6 (diluted 1/100; both from Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) overnight at 4°C, followed by the application of biotinylated link antibody and streptavidin–horseradish peroxidase (LSAB kit; Dako Corporation, Carpinteria, California, USA). Finally, the sections were developed with diaminobenzidine–hydrogen peroxidase substrate, and lightly counterstained with haematoxylin.

The intensity of cytoplasmic immunostaining for TFF2 and MUC6 was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. Cells with moderate or strong immunostaining were classed as positively stained. In addition, the percentage of positively stained cells over the total number of cells counted was calculated. TFF2 and MUC6 expression in the gastric foveola was defined as present when the percentage of positively stained cells over the total number of foveolar cells counted (> 300) was ≥ 5%. Because it has been found that TFF2 and MUC6 are expressed ubiquitously in the regenerative zone of the gastric mucosa, the expression of TFF2 and MUC6 only in the deeper gastric glands outside the regenerative zone was measured in our study, and was defined as present if the percentage of positively stained cells over the total number of cells counted (> 300) within these glands was ≥ 5%.

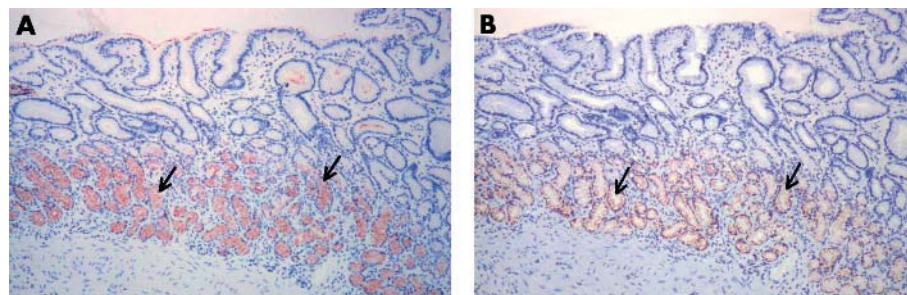


Figure 1 The expression of trefoil family factor 2 (TFF2) and MUC6 in normal antral mucosa. There is uniform staining of (A) TFF2 and (B) MUC6 in the regenerative zone and the deeper mucous glands (arrows). Original magnification, ×100.

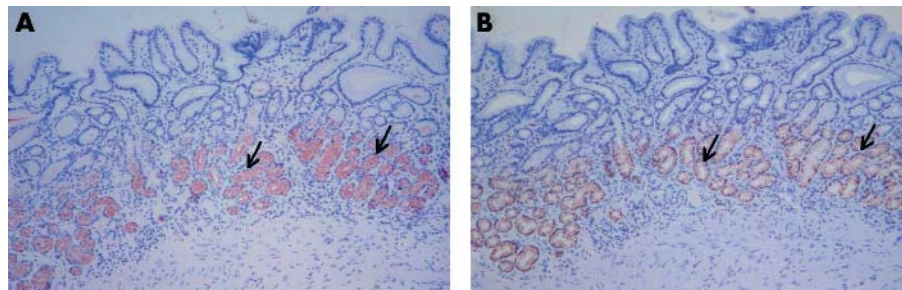


Figure 3 The expression of trefoil family factor 2 (TFF2) and MUC6 in normal incisura mucosa. The mucosa exhibits transitional or body-type histology, and the expression of (A) TFF2 and (B) MUC6 is limited to the mucous neck cells (arrows) in most cases. Original magnification, $\times 100$.

Statistical analysis

Data were expressed as the percentage of positive cases over the total number of cases. Statistical analysis was performed using SPSS system (version 10.0; SPSS Inc, Chicago, Illinois, USA). The association between the expression of TFF2 and MUC6 and *H pylori* infection at different gastric sites was assessed using the χ^2 test, with Yates’s correction if required, or Fisher’s exact test; odds ratios (OR), 95% confidence intervals (CI), and two tailed p values were calculated. The α level of significance was set at $p < 0.05$.

RESULTS

Expression of TFF2 and MUC6 in normal gastric epithelium at different gastric sites

In the normal stomach, staining for TFF2 and MUC6 in the gastric foveola was very weak or not discernable. In the gastric antrum, there was uniform staining of TFF2 and MUC6 in the regenerative zone and the deeper mucous glands (fig 1). In gastric body mucosa, TFF2 and MUC6 were expressed together only in the regenerative zone, where mucous neck cells were present (fig 2). In gastric incisura mucosa, the expression of TFF2 and MUC6 was limited to the regenerative zone in most cases (fig 3; table 1).

Expression of TFF2 and MUC6 in *H pylori* infected gastric epithelium at different gastric sites

Of the 76 patients, 27 were positive for *H pylori* infection. Chronic gastritis (grade 2 or 3) was present in 25 *H pylori* positive patients and four *H pylori* negative patients.

In general, *H pylori* infection was associated with increased expression of TFF2 and MUC6 in both the gastric foveola and the deeper gastric glands at the antrum (fig 4), body (fig 5), and incisura (fig 6). Foveolar staining for TFF2 and MUC6 appeared to be limited to the bottom of the pits.

In *H pylori* negative patients, the positive rate for TFF2 expression in the foveola at the antrum, incisura and body was 4.1%, 2.0%, and 4.1%, respectively. However, in *H pylori* infected patients, the rates increased to 59.3%, 44.4%, and

14.8%, respectively. TFF2 expression in the foveola was seen more often in patients with *H pylori* infection than in those without *H pylori* infection at the gastric antrum (OR, 34.2; 95% CI, 6.83 to 171.0; $\chi^2 = 29.30$; $p < 0.001$) and incisura (OR, 38.4; 95% CI, 4.61 to 320.2; $\chi^2 = 19.19$; $p < 0.001$; table 1). In *H pylori* positive patients, TFF2 expression in the deeper glands was present in 100%, 85.2%, and 14.8% of cases at the antrum, incisura, and body, respectively, whereas the rates were 100%, 22.4%, and 2.0%, respectively, in those without *H pylori* infection. The positive rate for TFF2 expression in the deeper glands at the incisura was significantly higher in *H pylori* infected patients than in uninfected patients (OR, 19.9; 95% CI, 5.66 to 69.8; $\chi^2 = 27.71$; $p < 0.001$; table 1).

MUC6 expression was similar to TFF2 expression (table 1). In the foveola, MUC6 expression was significantly higher in *H pylori* positive patients than in *H pylori* negative patients at the antrum (63.0% v 4.1%; OR, 40.0; 95% CI, 7.94 to 201.1; $\chi^2 = 32.19$; $p < 0.001$) and incisura (48.1% v 0%; $\chi^2 = 25.17$; $p < 0.001$). In the deeper glands, MUC6 expression was present more frequently in *H pylori* positive patients than in *H pylori* negative patients at the incisura (85.2% v 22.4%; OR, 19.9; 95% CI, 5.66 to 69.8; $\chi^2 = 27.71$; $p < 0.001$; table 1).

Association between the expression of TFF2 and MUC6 and antralisation of gastric incisura

Antral-type mucosa was present at the incisura in 28 of 76 of patients—20 of the 27 *H pylori* positive and eight of the 49 *H pylori* negative patients (table 1). Seventy two patients had antral-type mucosa and four patients had transitional-type mucosa in the antrum. At the gastric body, antral-type mucosa was seen in only one *H pylori* positive patient and the transitional type in six (three *H pylori* positive and three *H pylori* negative) patients (table 1).

At the gastric incisura, the expression of TFF2 and MUC6 was significantly associated with the presence of antral-type mucosa. The rates of TFF2 and MUC6 expression in the

Table 1 Expression of trefoil family factor 2 (TFF2) and MUC6 in *Helicobacter pylori* positive and negative patients at different gastric sites in relation to the mucosal types

	Percentage of cases with positive TFF2/MUC6 staining								
	Antrum			Incisura			Body		
	N	Foveola	Gland	N	Foveola	Gland	N	Foveola	Gland
<i>H pylori</i> negative	49	4.1/4.1	100/100	49	2.0/0	22.4/22.4	49	4.1/2.0	2.0/2.0
Antral type	45	4.4/4.4	100/100	8	0/0	75.0†/75.0†	0	NA	NA
Transitional type	4	0	100/100	28	3.6/0	17.9/17.9	3	0/0	0/0
Body type	0	NA	NA	13	0/0	0/0	46	4.3/2.2	2.2/2.2
<i>H pylori</i> positive	27	59.3*/63.0*	100/100	27	44.4*/48.1*	85.2*/85.2*	27	14.8/14.8	14.8/11.1
Antral type	27	59.3/63.0	100/100	20	55.0/60.0	100†/100†	1	100/100	100/100
Transitional type	0	NA	NA	6	16.7/16.7	50.0/50.0	3	33.3/33.3	66.7/66.7
Body type	0	NA	NA	1	0/0	0/0	23	8.7/8.7	4.3/0

* $p < 0.001$, compared with *H pylori* negative cases; † $p < 0.001$, compared among antral type, transitional type, and body type, χ^2 test, $df = 2$. df , degrees of freedom; NA, not available.

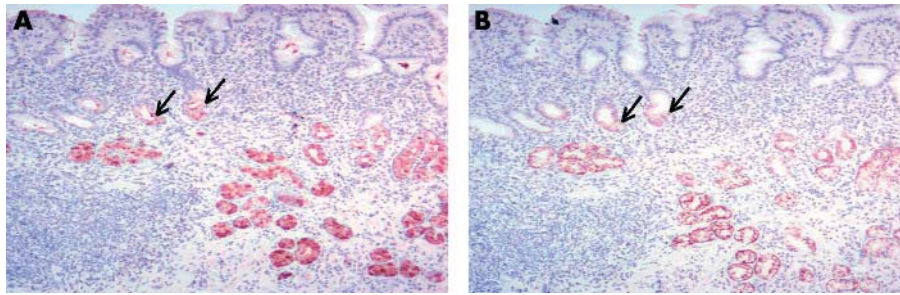


Figure 4 The expression of trefoil family factor 2 (TFF2) and MUC6 in antral mucosa with *Helicobacter pylori* infection. In addition to the regenerative zone and the deeper mucous glands, the expression of (A) TFF2 and (B) MUC6 is also seen in the foveola (arrows). Original magnification, $\times 100$.

foveola were 39.3%, 5.9%, and 0%, respectively, in antral-type, transitional-type, and body-type mucosa (degrees of freedom = 2; $\chi^2 = 43.74$; $p < 0.001$). In the deeper glands, the rates of TFF2 and MUC6 expression were 92.9%, 23.5%, and 0%, respectively, in antral-type, transitional-type, and body-type mucosa (degrees of freedom = 2; $\chi^2 = 15.62$; $p < 0.001$). When patients with and those without *H pylori* infection were analysed separately, there was an association between the presence of antral-type mucosa and the expression of TFF2 and MUC6 in the deeper glands, but not in the foveola (table 1).

DISCUSSION

Our present study showed that *H pylori* infection was associated with significant changes in the expression of TFF2 and MUC6 at the gastric antrum, incisura, and body. In the normal gastric antrum, there was no expression of TFF2 and MUC6 in the foveolar epithelium. Both TFF2 and MUC6 were expressed in the regenerative zone and the deeper portion of the antral glands. These findings are in agreement with previous studies using antral biopsies.³⁻⁵ Moreover, we found that TFF2 and MUC6 were expressed only in the regenerative zone of the body mucosa, and, in most cases, of the incisura mucosa. However, in the presence of *H pylori* infection, the distribution of TFF2 and MUC6 expression was altered: these molecules were frequently expressed in the foveola of the antrum and incisura. This finding supports the previous observation by Byrd *et al*, who only used antral biopsies, that MUC6 expression was limited to the mucous glands of *H pylori* negative patients, but that 72% of *H pylori* positive patients expressed MUC6 in the surface mucous cells.³ Moreover, our present study showed that both TFF2 and MUC6 were also expressed in the deeper glands of the body, especially the incisura mucosa, in the presence of *H pylori* infection.

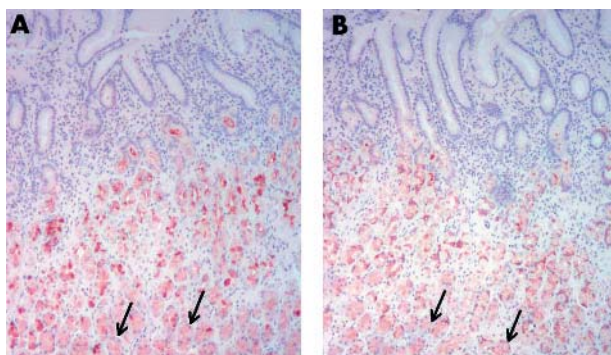


Figure 5 The expression of trefoil family factor 2 (TFF2) and MUC6 in body mucosa with *Helicobacter pylori* infection. The expression of (A) TFF2 and (B) MUC6 is seen in the regenerative zone and in the deeper glands (arrows). Original magnification, $\times 100$.

TFF2 is upregulated in diverse pathological conditions, and its expression profile broadens to include the regenerative epithelia of the entire gastrointestinal tract.²⁷ For example, expression of TFF2 and other trefoil peptides is increased at sites of gastric ulceration, duodenal ulceration, and Crohn's disease.²⁸⁻³⁰ Thus, increased expression of TFF2 may be a potentially important mucosal defence mechanism. Previous studies have shown that there is an association between TFFs and mucins.⁹⁻¹¹ TFFs interact with mucous, and stabilise the mucous gel by binding the long mucous molecules together.¹⁷ It has been suggested that TFF2 may function with MUC6 to strengthen the mucous barrier.¹⁷ Moreover, Hoosain *et al* showed that a highly purified preparation of porcine TFF2 was able to stimulate [3H] thymidine incorporation in HCT116 colon carcinoma cells, suggesting a role in cell proliferation.³¹ Recently, Farrell *et al* also showed that TFF2 deficient mice have a 35% reduction in the fundic proliferation rate compared with wild-type mice.¹⁸ Those results suggest a physiological role for TFF2 in promoting mucosal healing through the stimulation of cell proliferation.

"Our present study showed that both trefoil family factor 2 and MUC6 were also expressed in the deeper glands of the body, especially the incisura mucosa, in the presence of Helicobacter pylori infection"

Helicobacter pylori infection is associated with antralisation (or so called pseudopyloric metaplasia) at the gastric incisura, body, and fundus.^{23 24 32} It has been established that *H pylori* infection induces the apoptosis of gastric epithelial cells, and subsequently stimulates cell proliferation in the gastric mucosa.³³ Indeed, Wong *et al* found that resected specimens taken from the gastric body or fundus, most infected with *H pylori*, showed the presence of antral-type glands entirely surrounded by acid secreting mucosa.³⁴ Moreover, they reported that there was a pronounced increase of mucous neck cells in fundus glands adjacent to areas of pseudopyloric metaplasia, and that the secretory phenotype of pseudopyloric metaplasia in the fundus (typically, expression of TFF2) resembled that of the mucous neck cells.³⁴ These findings indicate that pseudopyloric metaplasia occurs in the body glands as a result of hyperplasia of mucous neck cells, and represents a mucosal response to damage associated with *H pylori* infection.^{23 34 35} Furthermore, Schmidt *et al* reported that the spasmolytic polypeptide (TFF2) expressing metaplastic (SPEM) lineage was closely associated with fundic *H pylori* infection.³⁶ Thus, we propose that the hyperplastic mucous neck cells move both upwards and particularly downwards in the oxyntic tubule, replacing the specialised parietal and chief cells, to create a mucous cell lineage. This process can occur focally, occupying a single oxyntic tubule, groups of tubules, or on a fairly massive scale with many tubules involved.³⁷ Eventually a mucous gland, which resembles a pyloric gland, is formed, and thus antralisation of the proximal gastric mucosa, particularly the incisura,

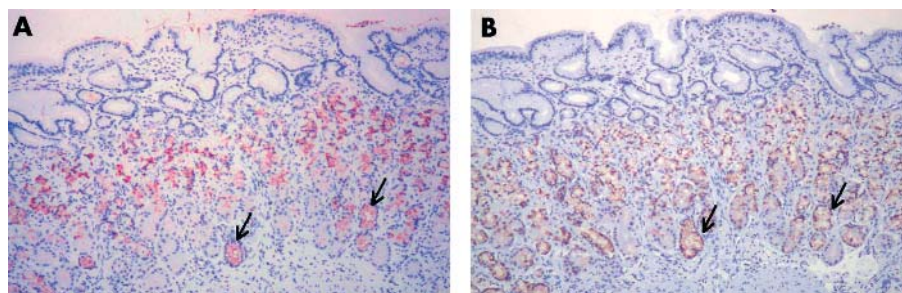


Figure 6 The expression of trefoil family factor 2 (TFF2) and MUC6 in the incisura mucosa with *Helicobacter pylori* infection. The mucosa exhibits antral-type histology, and the expression of (A) TFF2 and (B) MUC6 is seen in the deeper glands (arrows), in addition to the regenerative zone. Original magnification, $\times 100$.

follows. It is most likely that the weakened antralised mucosa in the proximal stomach is prone to further damage by *H pylori*, resulting in ulceration even in the presence of subnormal acid production.^{32 38}

More importantly, the expression pattern of mucins and TFFs alters in gastric precancerous lesions and neoplasia, and thus the altered expression pattern of these factors may be involved in the development of gastric cancer.^{2 3 6-8 11-18 39} It has been shown that mice lacking TFF1 develop antral adenoma or carcinoma, and TFF1 expression is lost in 50% of human gastric cancers, suggesting that TFF1 may be a tumour suppressor gene.^{8 17} More recently, studies have shown that SPEM is associated with the development of gastric cancer, suggesting that SPEM is a potential precursor lesion of gastric cancer.^{40 41} In our present study, we found that alteration of TFF2 and MUC6 expression was associated with antralisation of gastric incisura, indicating that antralisation is histogenetically identical to SPEM. Because there was a close topographical association between the expression of TFF2 and MUC6 in the deeper glands and antralisation of gastric incisura was present in both *H pylori* positive and negative patients, we suggest that the expression of TFF2 and MUC6 in the deeper glands may be a useful biological marker for antralisation. Furthermore, based on the findings of previous and present studies, we hypothesise that the increased expression of TFF2 and MUC6 in *H pylori* infection is a host response to damage induced by the infection. These factors are associated with the regulation of cell proliferation; thus, an increase in these factors, along with other growth factors, will be followed by increased cell proliferation.^{17 30} However, *H pylori* induced mucosal damage (including epithelial apoptosis) that is accompanied by an overmatched cell proliferation may result in hyperplasia of the gastric mucosa and transformation of the gastric mucosa into antral-type mucosa (antralisation), which is believed to increase the risk of the development of gastric neoplasia.^{23 24 32 33} Further studies are required to verify this hypothesis.

In conclusion, *H pylori* infection increases the expression of TFF2 and MUC6 in the gastric epithelium, which may be a protective response of the host to the mucosal damage induced by *H pylori* infection. Aberrant expression of TFF2 and MUC6 is associated with antralisation of the gastric

incisura, and thus may be involved in and used as a biological marker for the process.

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Take home messages

- Helicobacter pylori* infection increases the expression of TFF2 and MUC6 in the gastric epithelium, and this may be a protective response of the host to the mucosal damage induced by *H pylori* infection
- Aberrant TFF2 and MUC6 expression is associated with antralisation of gastric incisura
- This increased expression of TFF2 and MUC6 might be a useful biological marker for antralisation

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