

Sulphomucins favour adhesion of *Helicobacter pylori* to metaplastic gastric mucosa

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Abstract

Aims—To assess the influence of sulphomucin secretion on *Helicobacter pylori* colonisation and adhesion to metaplastic gastric cells.

Methods—Gastric biopsies from 230 *H pylori* positive patients with intestinal metaplasia were analysed. Sulphated mucins and *H pylori* were visualised using a new technique combining high iron diamine–alcian blue mucin stains with the Steiner silver stain for the bacteria.

Results—Sulphomucin secretion anywhere in the mucosa and a histological diagnosis of dysplasia increase the risk of *H pylori* adhesion to metaplastic cells (odds ratios 19.9 and 4.3, respectively). However, only 9.4% of cases showing sulphomucin secretion and 10.8% of cases with dysplasia had evidence of adhesion of *H pylori* bacteria to metaplastic cells.

Conclusions—The findings suggest that *H pylori* may play a role in the advanced stages of carcinogenesis. It will be of interest to investigate if the relative small proportion of type III metaplasias that actually progress to carcinoma show persistence of *H pylori*.

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Keywords: gastric intestinal metaplasia; sulphomucins; *Helicobacter pylori*; carcinogenesis

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Table 1 Numbers of cases with histological diagnosis based on haematoxylin and eosin staining according to the presence of sulphomucins in columnar and goblet cells

| Histological diagnosis | Sulphomucins | | | Total |
|----------------------------------|--------------|-----------|-----------|-------|
| | G-C- | G+C- | G+C+ | |
| Complete intestinal metaplasia | 92 (86.0) | 15 (14.0) | 0 (0) | 107 |
| Incomplete intestinal metaplasia | 0 (0) | 10 (25.0) | 30 (75.0) | 40 |
| Dysplasia | 0 (0) | 24 (28.9) | 59 (71.1) | 83 |
| Total | 92 (40.0) | 49 (21.3) | 89 (38.7) | 230 |

Percentages in parentheses.
C, columnar cell; G, goblet cell.
Overall p value = 0.0001, χ^2 test.

There is a general agreement that *Helicobacter pylori* increases gastric cancer risk¹⁻³ and that intestinal metaplasia of the gastric mucosa is a precursor of cancer.⁴ It is therefore somewhat paradoxical that colonisation by *H pylori* takes place where normal gastric surface epithelial (“foveolar”) cells are present, but not where they are replaced by metaplastic cells with intestinal phenotype.^{5,6} An exception to this rule has recently been reported by Genta *et al*,⁷ who documented adherence of *H pylori* to metaplastic cells with the “incomplete” phenotype, also known as “type III” or “colonic.” The latter type of metaplasia is associated with

secretion of sulphomucins.⁸ We examined the spacial relation between *H pylori*, metaplastic cells, and presence of sulphomucin secretion in biopsies from 230 patients from Nariño, Colombia.

Methods

Patients belong to a cohort with gastric cancer precursor lesions from the region of Nariño, Colombia, previously documented to have very high gastric cancer risk.⁹⁻¹¹ The study was approved by the Louisiana State University institutional review board. Four biopsies were evaluated for each patient: two from the antrum (lesser and greater curvatures), one from the incisura angularis, and one from the corpus (mid-portion of anterior wall). Subjects were selected because of a previous diagnosis of intestinal metaplasia with or without dysplasia, documented with haematoxylin–eosin, PAS–alcian blue, and high iron diamine–alcian blue stains.^{12,13} All patients had *H pylori* infection confirmed by the Steiner modification of the Warthin–Starry silver stain.¹⁴ Visual analogue scales¹⁵ were used to evaluate the density of *H pylori* colonisation. Published models were compared with the cases under study, which were graded as normal, mild, moderate, and marked. The Genta stain¹⁶ was performed to detect *H pylori* adherence to metaplastic cells expressing acid mucins. We developed a new technique using the high iron diamine–alcian blue method followed by the Steiner modification of the Warthin–Starry stain; this allows simultaneous visualisation of sulphated mucins, and *H pylori* and was used when adherence to acid mucin was previously detected with the Genta stain (see appendix). On haematoxylin–eosin stains the metaplasia was classified as “complete” type (type I or small intestinal) when in addition to the goblet cells, absorptive enterocytes with well developed brush border constituted the only intestinal phenotype observed. If irregular multivacuolated cells without brush border characterised portions of the metaplastic mucosa, such areas were classified as “incomplete” type (type III or colonic). Such islands of incomplete metaplasia were in most cases also accompanied by topographically independent foci of complete metaplasia. The presence of sulphomucins was evaluated independently in goblet cells and columnar cells. In all cases where sulphomucins were present in columnar cells they were also present in goblet cells. Therefore two positive patterns were recorded: goblet cells positive only (G+C-) or both goblet and columnar cells positive (G+C+).

Table 2 Number of cases with adhesion of *H pylori* bacteria to metaplastic cells according to the presence of sulphomucins

| <i>H pylori</i> adherent to IM | Sulphomucins | | | Total |
|--------------------------------|--------------|-----------|-----------|-----------|
| | G-C- | G+C- | G+C+ | |
| Negative | 92 (42.4) | 47 (21.7) | 78 (35.9) | 217 (100) |
| Positive | 0 (0) | 2 (15.4) | 11 (84.6) | 13 (100) |
| Total | 92 (40.0) | 49 (21.3) | 89 (38.7) | 230 (100) |

Percentages in parentheses.

C, columnar cell; G, goblet cell; IM, intestinal metaplasia.

Overall p value = 0.001, χ^2 test.

STATISTICS

Statistical analysis was performed using the χ^2 method to compare frequencies. The Mantel-Haenzel test was used to analyse the relation between *H pylori* adherence and sulphomucins after adjusting for the gastric intestinal metaplasia extension and bacteria density. The interaction analysis was performed using logistic regression. A probability (p) value < 0.05 was considered statistically significant.

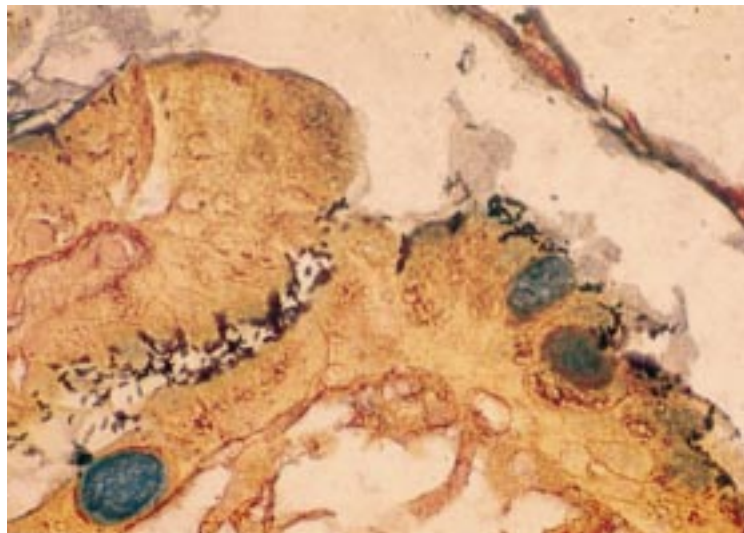


Figure 1 Photomicrograph of gastric biopsy showing colonies of *H pylori* on the gastric mucosa (long arrow) and as clusters adhering to sulphomucin secreting goblet cells (short arrows). (Modified high iron diamine-alcian blue-Steiner stains; magnification $\times 82$.)

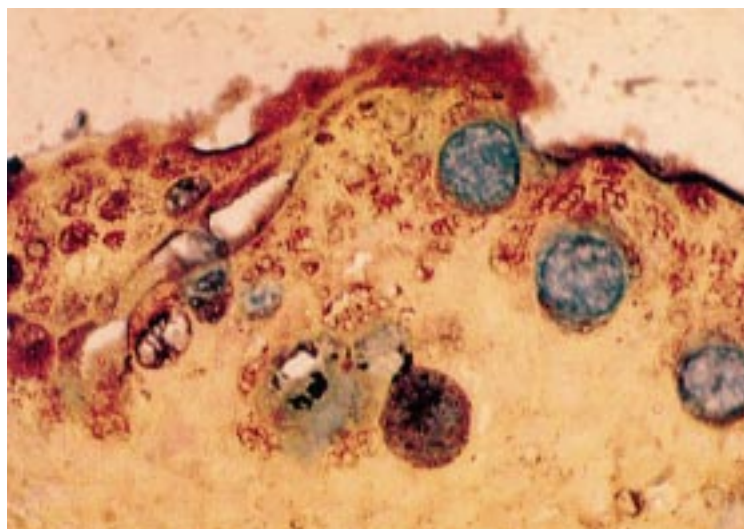


Figure 2 Photomicrograph of gastric biopsy showing adhesion of clusters of *H pylori* to the goblet cells expressing sulphomucins (Modified high iron diamine-alcian blue-Steiner stains; magnification $\times 82$.)

Results

For descriptive purposes, the relation between the presence of *H pylori* in gastric tissue and sulphomucin distributions was studied in those biopsies with a histological diagnosis of gastric intestinal metaplasia.

Based on the analysis of all biopsies for 230 patients, table 1 shows the association between the type of metaplasia, as evaluated in haematoxylin-eosin stains, and the presence of sulphomucins in columnar and goblet cells. All cases of dysplasia had adjacent areas of incomplete intestinal metaplasia as well as sulphomucins. Fourteen per cent of cases with exclusively complete intestinal phenotype had some sulphomucins in goblet but not in columnar cells.

Table 2 shows a positive association between adhesion of *H pylori* bacteria to metaplastic cells and the secretion of sulphomucins. Even though the specific site of sulphomucin secretion may not coincide exactly with the site of *H pylori* presence, all specimens showing *H pylori* adherence to metaplastic cells had sulphomucins somewhere in the section (figs 1 and 2). The odds ratio for bacterial adhesions given the presence of sulphomucins anywhere in the specimen was 19.9 (p = 0.002), indicating that sulphomucin secreting metaplasias are more likely to allow bacterial adhesions to metaplastic cells than those secreting only sialic mucins. If only columnar cells secreting sulphomucins are counted, the odds ratio was 9.8 (p = 0.0001). In no case were bacterial adhesions to mucin goblets visualised in the absence of sulphomucin secretion somewhere in the specimen. Despite the strong association of sulphomucin secretion with bacterial adhesions, the effect was observed in only 9.4% of the patients (13/138).

Table 3 shows the association between bacterial adhesion and histological diagnosis based on haematoxylin-eosin stains. The odds ratio of bacterial adhesion, given the presence of incomplete intestinal metaplasia phenotype, was 26.3 (p = 0.001). A histological diagnosis of dysplasia increased the risk of bacterial adhesion by a factor of 4.3 (p = 0.010). All cases of dysplasia with bacterial adhesion were mild. Tables 2 and 3 reflect the close correlation between the histological classification of metaplasia by haematoxylin-eosin and by mucin histochemistry stains.

Table 4 shows a positive association between bacterial adhesion to metaplastic cells, the density of bacterial colonisation in the non-metaplastic mucosa, and the extension of the gastric intestinal metaplasia. The phenomenon of *H pylori* adherence and sulphomucins was correlated with extensive intestinal metaplasia (p = 0.032) and a severe degree of bacterial colonisation (p = 0.007). Even though 5.6% of the biopsies had *H pylori* adherence to metaplastic cells, abundant colonisation by itself does not explain the adherence. A multivariate model (logistic regression) showed that, even after adjusting for the density of *H pylori*, adherence was significantly more common in patients with extensive intestinal metaplasia.

Table 3 Numbers of cases with bacterial adhesion according to histological diagnosis based on haematoxylin–eosin staining

| <i>H pylori</i> adherent to IM | Histological diagnosis | | | Total |
|--------------------------------|------------------------|-----------|-----------|-----------|
| | CIM | IIM | Dysplasia | |
| Negative | 107 (49.3) | 36 (16.6) | 74 (34.1) | 217 (100) |
| Positive | 0 (0) | 4 (30.8) | 9 (69.2) | 13 (100) |
| Total | 107 (46.5) | 40 (17.4) | 83 (36.1) | 230 (100) |

Percentages in parentheses.

CIM, complete intestinal metaplasia; IIM, incomplete intestinal metaplasia; IM, intestinal metaplasia.

Overall p value = 0.002 by χ^2 test.

Table 4 Extension of gastric intestinal metaplasia and density of *H pylori* colonisation according to *H pylori* adherent status

| <i>H pylori</i> density | <i>H pylori</i> adherent to IM | | Total |
|-------------------------|--------------------------------|-----------|-----------|
| | Negative | Positive | |
| Scarce | 144 (98) | 3 (2) | 147 (100) |
| Abundant | 73 (88) | 10 (12) | 83 (100) |
| IM extension | | | |
| <60% | 115 (99.1) | 1 (0.9) | 116 (100) |
| ≥60% | 102 (89.5) | 12 (10.5) | 114 (100) |

Percentages in parentheses.

IM, intestinal metaplasia.

Overall p value = 0.002 by χ^2 test.

Discussion

The reason for the preferential colonisation of *H pylori* in gastric epithelial foveolar cells but not in metaplastic cells is unknown. Since the former secrete neutral mucins and the latter secrete acid mucins, it appears reasonable to suggest that mucin secretions determine colonisation in the gastric microenvironment. Neutral mucins may be a necessary nutrient, or acid mucins may be toxic for the bacteria. Our findings that bacterial colonisation occurred in metaplastic cells only if sulphomucins were secreted may indicate that some acid mucins are not toxic—that is, those containing sulphur atoms may allow bacterial growth in their immediate vicinity. By inference, it could be proposed that sulphomucins may provide some nutrient for helicobacter bacteria.

H pylori has been classified as a human carcinogen.² The mechanism of cancer induction and the stage at which it takes place are unknown. Since *H pylori* induces inflammation decades before an eventual neoplastic outcome, it would be logical to assume that the effect takes place early in the process of carcinogenesis. However, it has been suggested that oxygen radicals produced during the inflammatory process may induce DNA damage and eventually cause neoplastic transformation.¹⁷ Such radicals are present as long as there is inflammation and may therefore damage DNA at all stages of the carcinogenic process. That *H pylori* may play a role in advanced stages of the carcinogenic process has been suggested recently by Uemura *et al* in a non-randomised clinical trial.¹⁸ Their preliminary study reports that successful treatment of *H pylori* infection reduces the risk of metachronous cancer after endoscopic resection of gastric carcinoma limited to the mucosa. Although their report needs replication, it provides an interesting suggestion for further research.

If *H pylori* plays a role in the late stages of gastric carcinogenesis, the presence in sulphomucins secreting metaplastic cells may be involved mechanistically. *H pylori* has been shown to be capable of catalysing nitrosation reactions¹⁹ and participating in N-acetyltransferase activities.²⁰ It is therefore possible that the presence of the bacteria in the vicinity of dysplastic cells contributes to the progression of the precancerous process. As mentioned above, oxygen radicals induced by bacteria may produce DNA damage. If the target is a dysplastic cell, further mutations may result in invasive neoplastic clones.

Our findings confirm those of Genta *et al* and Steadman *et al*.^{7, 21} Further elucidation of these observations may help understand the microenvironmental requirements of *H pylori* colonisation in the human gastric mucosa as well as the mechanism by which *H pylori* infection increases the risk of gastric cancer.

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Appendix

Staining procedure for high iron diamine–alcian blue–Steiner method

- 1 Pre-clean glass slides and glassware using the following steps:
 - 10% nitric acid, 1 h
 - Running tap water, 15 min
 - Deionised water, 4 times
 - Dry up at 60°C or at room temperature
- 2 Cut 5 μ m tissue sections. Heat fix them to the glass slides for 1 h or overnight at 60°C
- 3 Deparaffinise and hydrate to distilled water: Three changes of xylene, 5 min each
Two changes of absolute ethanol and two changes of 95% ethanol, 3 min each
Wash thoroughly in running tap water for at least 2 min
- 4 Stain for 18 to 24 h in freshly prepared high iron diamine solution; keep in the dark at room temperature
- 5 Rinse “in and out” with distilled water
- 6 Alcian blue solution, 30 min (filter before use)
- 7 Rinse “in and out” with distilled water
- 8 Rinse with deionised water, 4 times
- 9 Microwave in 1% uranyl nitrate (100% power), 2 min
- 10 Let stand at room temperature, 5 min
- 11 Rinse with deionised water, 4 times
- 12 Microwave in 1% silver nitrate (100% power), 2 min
- 13 Let stand at room temperature, 10 min
- 14 Rinse with deionised water, 4 times
- 15 Five dips in 95% ethyl alcohol and five dips in absolute alcohol
- 16 2.5% gum mastic, 5 min
- 17 Air dry the sections, 1 min
- 18 Microwave the reducing solution (100% power), 1.5 min
- 19 Add the 0.04% silver nitrate and immerse the slides
- 20 Let stand at room temperature, 2 min
- 21 Microwave the reducing solution (30% power), 2 min
- 22 Let stand at room temperature, 3 min
- 23 Take out the positive control, rinse it in deionised water five dips and in 95% alcohol five dips. Check under the microscope, it should show black spirochetes in a light yellow background; if is not good return it to the stain container and microwave (30% power) 2 min plus 2 min at room temperature and check it again.
- 24 Rinse with deionised water to stop reduction, 4 times
- 25 Dehydrate through graded alcohols and clear in xylene
- 26 Mount with synthetic resin.

Results

Most sulphomucins stain dark brown to black, sialomucins stain blue. *Helicobacter pylori* stains black. Background yellow.

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