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ORIGINAL ARTICLE

The homeodomain protein CDX2 is an early marker of Barrett's oesophagus

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Background: In Barrett's oesophagus (BO), squamous epithelium is replaced by specialised intestinal epithelium (SIE). Transcription factors associated with intestinal differentiation, such as CDX2, may be involved in BO development.

Aim: To investigate CDX2 expression in BO, squamous epithelium, and oesophageal adenocarcinoma (ADC).

Methods: CDX2 expression was assessed in 245 samples—167 biopsies of the columnar lined segment and 38 squamous epithelial biopsies of 39 patients with histologically confirmed BO (10 with ADC). Forty biopsies from 20 patients with reflux oesophagitis (RO) without BO were also evaluated. CDX2 protein was investigated immunohistochemically in 138 biopsies from 16 patients with BO, four with ADC, and 20 with RO. Cdx2 and Muc2 mRNA were detected semiquantitatively using 88 BO biopsies and squamous epithelium from 19 BO patients, and when present from ADC.

Results: SIE was present in 53/79 biopsies from the columnar lined segment; CDX2 protein was seen in all epithelial cells, but not in biopsies containing only gastric metaplastic epithelium (26/79), or in squamous epithelium (0/40) of patients with RO. Cdx2 mRNA was detected in all biopsies with goblet cell specific Muc2 transcription—indicative of SIE. Low Cdx2 mRNA expression was seen in 6/19 squamous epithelium samples taken 5 cm above the squamocolumnar junction of BO patients.

Conclusion: CDX2 protein/mRNA is strongly associated with oesophageal SIE. Cdx2 mRNA was present in the normal appearing squamous epithelium of one third of BO patients, and may precede morphological changes seen in BO. Therefore, pathways that induce Cdx2 transcription in squamous epithelial cells may be important in BO development.

Barrett's oesophagus (BO) is a complication of chronic gastro-oesophageal reflux disease (GORD). Although 20–30% of the Western population has regular GORD related complaints,¹ it is unclear why only 3–5% of these patients develop long segment BO.² BO has a prevalence of 0.03% in the Western population,³ and its development in patients with GORD is associated with obesity at a young age,⁴ increased duodeno-gastro-oesophageal reflux,^{5,6} complaints at an earlier age,⁵ a familiar predisposition,⁷ and a combination of obesity, presence of a hiatal hernia, and male sex.⁶ Patients with BO have an increased risk of developing adenocarcinoma of the oesophagus, with an annual incidence of 0.5% each year.⁸ Once these patients have developed adenocarcinoma, the prognosis is poor, with a five year survival rate of 5–20%.⁹

“Transcription factors that play an important role in normal intestinal differentiation may also play a role in the development of specialised intestinal epithelium in the oesophagus”

BO is characterised by the metaplastic replacement of squamous epithelial cells of the lower part of the oesophagus by specialised intestinal epithelium (SIE), which is associated with the presence of goblet cells and the expression of intestinal markers such as MUC2,¹⁰ alkaline phosphatase,¹¹ villin,¹² and sucrase isomaltase.¹³ The genetic events responsible for this process are largely unknown.

Transcription factors that play an important role in normal intestinal differentiation may also play a role in the development of SIE in the oesophagus. CDX2 is such a

transcription factor, and belongs to the caudal related homeobox gene family.¹⁴ CDX2 expression in the gastrointestinal tract is intestine specific, with a tightly regulated anterior boundary in the duodenum.¹⁵ CDX2 is involved in early differentiation and the maintenance of intestinal epithelial cells, characterised by the formation of multi-layered structures with microvilli.¹⁴ CDX2 also induces intestine specific transcription of the genes encoding MUC2, alkaline phosphatase, and sucrase isomaltase.^{16–18} Therefore, CDX2 is thought to be an important factor in the development and differentiation of intestinal epithelium.^{19,20}

Because BO is characterised by the development of SIE in the oesophagus, CDX2 may also play a role in the development of BO. To investigate whether CDX2 expression is associated with BO, and whether its expression may precede the morphological changes seen in BO, we determined its expression in the columnar epithelium of patients with BO, in squamous epithelium of patients with reflux oesophagitis only, and in oesophageal adenocarcinoma. Here, we show that CDX2 is expressed in SIE, but not in squamous epithelium of patients with reflux oesophagitis and in gastric metaplastic epithelium. Furthermore, Cdx2 mRNA was also detected in the squamous epithelium of one third of patients with BO, suggesting that CDX2 is indeed involved in the development of BO, and that its expression may precede the morphological changes seen in BO.

Abbreviations: BO, Barrett's oesophagus; GORD, gastro-oesophageal reflux disease; PCR, polymerase chain reaction; RT, reverse transcription; SIE, specialised intestinal epithelium

Table 1 Patient characteristics

Patient characteristics		No of patients	No of biopsies	Mean age (SD)	Male %
First group					
IHC	Adenocarcinoma	4	19	78.5 (2.7)	75%
	Barrett's oesophagus	16	79	70.8 (14)	66%
	Reflux oesophagitis	20	40	61.8 (11.6)	71%
Second group					
RT-PCR	Adenocarcinoma	6	12	68.9 (11.5)	57%
	Barrett's oesophagus	19	76	65.1(15.1)	55%

IHC, immunohistochemistry; RT-PCR, reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Patients and materials

CDX2 expression was analysed in 245 oesophageal samples. These consisted of 167 biopsies of the columnar lined segment and 38 squamous epithelial biopsies of 39 patients with histologically confirmed BO, of whom 10 also had an oesophageal adenocarcinoma, and 40 biopsies of 20 patients with reflux oesophagitis without BO.

CDX2 protein expression was analysed by immunohistochemistry (table 1) in 138 biopsies, consisting of 79 biopsies of the columnar lined segment, 19 oesophageal adenocarcinomas, and 40 squamous epithelium biopsies of the oesophagus. The four quadrant biopsies taken at 2 cm intervals from the columnar lined segment were pooled, formalin fixed, and paraffin wax embedded. Biopsies of the colon were used as a positive control.

Biopsies from a second group of patients, not related to the first group, were used for mRNA analysis, because this analysis could not be performed on the formalin fixed, paraffin wax embedded samples. One hundred and seven oesophageal biopsies were collected at endoscopy from 19 patients with BO (table 1), six of whom also had oesophageal adenocarcinoma. Biopsy specimens were obtained from the columnar mucosa of the oesophagus (n = 38), the adenocarcinoma if present (n = 12), and the squamous epithelium—5 cm above the neosquamous-columnar junction (n = 38). For each of these locations, the biopsies from each location (two of the BO segment, two of the squamous epithelium, and when present two of the oesophageal adenocarcinoma) of individual patients were pooled, snap frozen, and used for RNA extraction (see below). An additional biopsy was taken next to the previous biopsies from the BO segment, and was used for the histological evaluation of the presence of SIE (n = 19). All columnar segments lining the oesophagus at endoscopy of both groups of patients were at least 3 cm in length or more. Biopsies of the colon were used as positive controls for Cdx2 mRNA analysis. Our study was approved by the central committee on research involving human subjects in The Netherlands in 2002.

Histological analyses

Sections from the biopsies and part of the biopsies taken for RNA analysis were stained with haematoxylin and eosin and

evaluated for the presence of SIE and/or adenocarcinoma. Staining with Alcian blue at pH 2.5 was performed to facilitate the detection of mucin producing goblet cells.²¹ The inflammatory response in biopsies of patients with reflux oesophagitis and BO, which were used for immunohistochemistry, was graded by the Ismail-Beigi classification²² for squamous epithelium and by the updated Sydney system^{23–24} for columnar epithelium.

Immunohistochemistry

Biopsy samples were serially sectioned at 4 µm, mounted on adhesive slides, dried overnight at 37°C, and dewaxed with xylene. Antigen retrieval was performed in 10mM monocitric acid (pH 6.0) at 100°C for 15 minutes. After cooling, the samples were blocked with non-immune serum for 30 minutes. The sections were stained using the primary antibody against CDX2 (1/100 dilution; Biogenex, San Ramon, California, USA), followed by the addition of a biotinylated rabbit secondary antibody (Dako, Glostrup, Denmark) and streptavidin-alkaline phosphatase complex (Dako). A red colour was developed using new fuchsin substrate.

Semiquantitative RT-PCR

Total RNA was isolated using TRIzol-reagent (Invitrogen, Groningen, The Netherlands), and the remaining chromosomal DNA was subsequently removed with the DNA free RNA kit (Zymo, Orange, California, USA). Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using intron spanning primers for Cdx2 (5'-CCCAGCGGCCAGCGCGAAACCTGT/5'-TATTGTCTTTTGTCTGGTTTTCA) and Muc2 (5'-CAGGATGGCGCCTTCTGCTA/5'-ATGCTGCTCCAAGCTGAGGT). Amounts of Cdx2 and Muc2 mRNA were standardised to those of β actin using the β actin primers 5'-CAAGGCCAACC GCGAGAAG and 5'-CAGGGTACATGGTGTTGCC. cDNA was synthesised with the use of avian myeloma virus reverse transcriptase (Promega, Madison, Wisconsin, USA). Primers were annealed by cooling down from 70°C to room temperature, followed by cDNA synthesis by incubation for 30 minutes at 42°C. PCRs (total volume of 25 µl) contained 1 µl of the cDNA solution, 1 × PCR core buffer (Promega), 2mM MgCl₂, 0.4µM forward and reverse primer, 200µM of each nucleotide (Promega), and 0.02 U/µl Taq polymerase (Promega).

Table 2 Histological classification of inflammation

Reflux oesophagitis		Barrett's oesophagus			
Histological classification Ismail-Beigi ²²		Histological classification Updated Sydney ²⁴	Acute		Chronic
1	7/20 (35%)	1	4/16 (25%)	5/16 (31%)	
2	7/20 (35%)	2	7/16 (44%)	7/16 (44%)	
3	6/20 (30%)	3	5/16 (31%)	4/16 (25%)	
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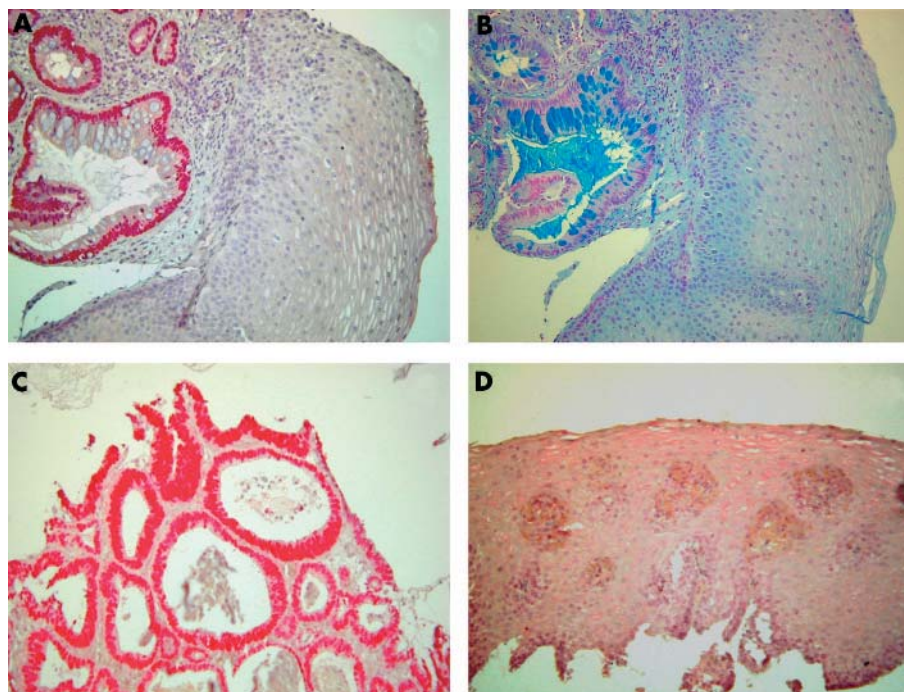


Figure 1 CDX2 protein in Barrett's oesophagus, oesophageal adenocarcinoma, and reflux oesophagitis. (A) Positive nuclear staining (red) for CDX2 was seen in 16 of 16 samples of Barrett's oesophagus. A representative slide from patient 10 is shown. (B) The presence of CDX2 was associated with goblet cells, which are characteristic of Barrett's oesophagus, as was shown using Alcian blue (pH 2.5) staining of a serially sectioned slide of the same patient. (C) CDX2 was also present in four of four adenocarcinomas; a representative slide from patient 3 is shown. (D) CDX2 was absent in the squamous epithelium of all patients with reflux oesophagitis (0 of 20).

PCR conditions were 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute. PCR products were size separated on a 2% agarose gel and stained with ethidium bromide. Band size and intensity were determined with the Kodak 1D software version 3.5 (Kodak, Rochester, New York, USA). Bands were standardised against the β actin house-keeping gene, as described previously.²⁵

Statistical analyses

All continuous variables were expressed as mean (SEM). Statistical analyses were done by means of the Fisher's exact test for immunohistochemistry and the Mann Whitney U test for the semiquantitative RT-PCR data. A two sided p value < 0.05 was considered significant.

RESULTS

Histology

SIE was seen in 53 of 79 (67%) biopsies from the columnar lined segment and was absent in 26 (33%) biopsies. The biopsies without SIE comprised gastric type (cardiac or fundic type) epithelium. SIE was also absent in all 40 reflux oesophagitis biopsies, which contained only squamous epithelium in all biopsies.

The inflammatory response in BO was graded according to the updated Sydney system, in which the inflammation is divided into four categories based on an acute component (numbers of neutrophils and eosinophils) and a chronic component (mononuclear cell count) in the epithelium.²⁴ This system was originally developed for glandular epithelium of the stomach, but has also been shown to be useful in the inflammatory classification of BO.²⁴ The acute component of inflammation in BO samples ranged from mild to severe in most biopsies, with four patients with BO having grade 1, seven grade 2, and five grade 3 inflammation, according to the updated Sydney classification (table 2). The chronicity of the inflammation ranged from mild to severe, with five patients with BO having grade 1, seven having grade 2, and four having grade 3. The inflammation in the 40 squamous epithelium biopsies of the 20 patients with reflux oesophagitis was graded as grade 1 in seven patients, grade 2 in

seven, and grade 3 in six, according to the Ismail-Beigi classification (table 2). For this reason, we assumed that the biopsies were representative of the whole spectrum of inflammation in both BO and reflux oesophagitis.

CDX2 expression

All oesophageal biopsies (53 SIE, 26 gastric type, 19 oesophageal adenocarcinoma, and 40 inflamed squamous epithelium) were analysed for the presence of the CDX2 protein by immunohistochemistry. All 53 biopsies with SIE had positive nuclear staining for CDX2 in the epithelium (fig 1A). This staining was associated ($p < 0.001$) with the presence of goblet cells, as detected in serially sectioned slides by staining with Alcian blue at pH 2.5 (fig 1B). Nuclear CDX2 expression was also seen in all 19 oesophageal adenocarcinoma samples (fig 1C). Cytoplasmic staining for CDX2 was not seen. Nuclear or cytoplasmic staining for the CDX2 protein was absent in the 26 gastric type epithelium biopsies and in the 40 squamous epithelium biopsies (fig 1D).

Molecular analysis

The presence of Cdx2 mRNA was evaluated in the second group of 19 patients with BO by RT-PCR, and this was normalised for β actin transcription (fig 2A). For all 19 patients, the presence of SIE was confirmed in the biopsies taken adjacent to those used in the transcriptional analysis. Cdx2 mRNA was detected in 13 of 19 BO segments and in four of six oesophageal adenocarcinomas (fig 2B). The amounts of Cdx2 mRNA did not differ significantly between BO and oesophageal adenocarcinoma ($p = 0.9$) (fig 2B).

To determine whether the expression of Cdx2 precedes the morphological changes seen in BO, the amounts of Cdx2 mRNA were also determined in squamous epithelium biopsies obtained 5 cm above the neosquamous-columnar junction of patients with BO (fig 3). Low amounts of Cdx2 mRNA were present in six of 19 samples of squamous epithelium (fig 3). The relative amounts of Cdx2 mRNA in the squamous epithelium were significantly lower ($p < 0.01$) than those seen in BO tissue (fig 2B). The presence of goblet cells, characteristic of BO, was evaluated by the detection of goblet cell specific Muc2 mRNA. Cdx2 mRNA was present in

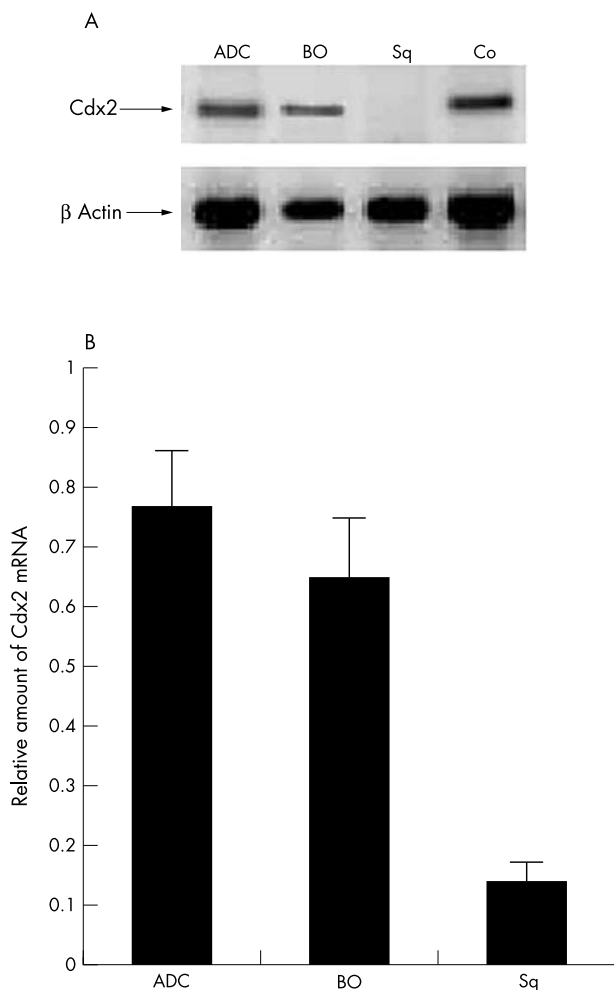


Figure 2 Cdx2 mRNA in Barrett's oesophagus, squamous epithelium, and oesophageal adenocarcinoma. (A) Cdx2 mRNA was detected by reverse transcription polymerase chain reaction (95 bp fragment) in four of six oesophageal adenocarcinoma (ADC) samples, in 13 of 19 Barrett's oesophagus (BO) samples, in six of 19 squamous epithelium (Sq) samples, and in colon control tissue (Co). (B) Relative amounts of Cdx2 mRNA in squamous epithelium, BO, and ADC. Amounts of Cdx2 mRNA were normalised against β actin. The mean Cdx2 mRNA values in squamous epithelium ($n = 6$) were significantly lower than those seen in BO ($n = 13$) ($p < 0.01$). The relative amounts of Cdx2 mRNA did not differ significantly between BO ($n = 13$) and ADC ($n = 4$) (not significant; $p < 0.1$).

all Muc2 positive samples. Furthermore, samples without the presence of Cdx2 mRNA did not contain Muc2 transcripts. Muc2 mRNA was absent in only three BO samples with Cdx2 transcription (fig 3). In addition, Muc2 transcripts were not detected in the squamous epithelium samples.

DISCUSSION

We have shown that CDX2 protein is present in BO containing SIE, as was recently reported by others.^{26, 27} CDX2 expression was also detected in oesophageal adenocarcinoma. In contrast, no CDX2 protein was seen in biopsies containing gastric type epithelium of the distal oesophagus taken from patients with BO, or in the squamous epithelium of patients with reflux oesophagitis without BO. However, low amounts of Cdx2 mRNA were detected in approximately one third of the squamous epithelium samples of patients with BO. The presence of Cdx2 mRNA also correlated with goblet cell specific Muc2 mRNA in BO samples (fig 3).

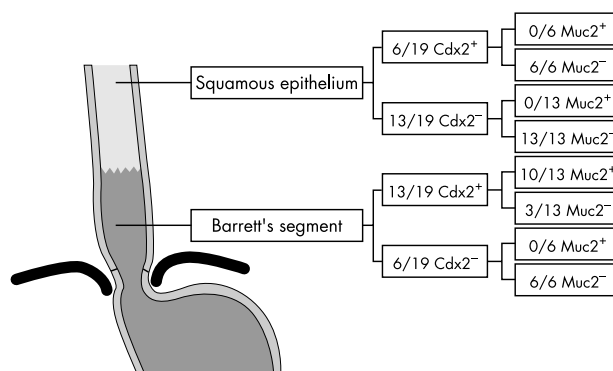


Figure 3 Schematic overview of the distal oesophagus, summarising the transcription of Cdx2 mRNA and goblet cell specific Muc2 mRNA in biopsies taken from the Barrett's segment and the squamous epithelium of patients with Barrett's oesophagus.

The homeobox protein CDX2 is involved in the differentiation and maintenance of intestinal epithelium.¹⁴ Expression of CDX2 is detected at the time of morphogenesis in the visceral endoderm of mouse intestine,²⁸ and continues to be present throughout adulthood, but then is normally restricted to the intestine.²⁹ It is detectable in the crypts of the intestine and in the villi,¹⁵ and is thought to be a key regulator of intestinal differentiation.¹⁹ Exogenous expression of CDX2 in IEC6 cells, an undifferentiated rat intestinal cell line that does not express CDX2, causes differentiation of IEC6 cells into goblet cells and absorptive enterocytes.¹⁴ Similar observations have been made in an animal model, in which ectopic expression of CDX2 induced the development of metaplastic changes of the gastric antrum, and in *Helicobacter pylori* related intestinal metaplasia of the human stomach.^{30, 31} These metaplastic changes of the mouse gastric antrum were also characterised by the development of goblet cells and absorptive enterocytes, and the expression of intestine specific proteins such as MUC2, alkaline phosphatase, villin, guanylyl cyclase C, and trefoil factor 3.²¹ In contrast, heterozygous CDX2 knockout mice developed polyp-like lesions in their colon during the first 3 months of life, which lacked CDX2 expression.³² These lesions were composed of heterotopic, well differentiated, stratified squamous epithelium, stomach, and small intestinal mucosa.³³ It was concluded that CDX2 directs epithelial differentiation towards a caudal phenotype. For these reasons, CDX2 expression is thought to be an early marker of intestinal differentiation, and may therefore play a role in the development of SIE in the lower part of the oesophagus, as seen in BO.

"We hypothesise that inflammation in the oesophagus caused by duodeno-gastro-oesophageal reflux induces CDX2 expression in a subset of patients"

Although all additional biopsies taken from the BO segment for histological evaluation in the group of patients in whom Cdx2 mRNA was determined showed SIE, Cdx2 mRNA was not detected in six of 19 (32%) BO segments. In the biopsies taken from these segments, Muc2 transcription was absent, which suggests that goblet cells were not present in these samples. Because goblet cells are a hallmark of BO, these biopsies may have contained another type of columnar epithelium, probably gastric type epithelium, as was detected in 26 of 79 biopsies of the BO segment in our study. This is in agreement with findings in another study, which reported that goblet cells were only found in 51% of patients with

Take home messages

- The expression of CDX2 protein and mRNA is strongly associated with the presence of specialised intestinal epithelium in the oesophagus
- Cdx2 mRNA was also present in endoscopically normal appearing squamous epithelium of one third of patients with Barrett's oesophagus (BO) who have, and may precede the morphological changes seen in BO
- Pathways that induce Cdx2 transcription in squamous epithelial cells may be important in the development of BO

3–4 cm columnar-like epithelium of the oesophagus on a first endoscopy.³⁴ This increased to 88.9% after three endoscopies.³⁴ This suggests that the absence of SIE in the biopsies taken from the columnar lined segment might be the result of sampling error. Because in our study only one of the six patients negative for Cdx2 mRNA had an oesophageal adenocarcinoma, and no dysplasia was seen in the adjacent biopsies taken for routine screening in the other five patients, it is unlikely that a neoplasm that is associated with a decreased number of goblet cells was present in these biopsies.

To assess whether CDX2 is an early marker for metaplastic replacement in the oesophagus, Cdx2 mRNA expression was also determined in reflux exposed squamous epithelium of patients with BO. Low amounts of Cdx2 mRNA were indeed seen in six of the 19 squamous epithelium samples tested (fig 3). In addition, transcription of Muc2 was not detected in these samples, which excludes the possibility that SIE was covered by a stratified epithelial layer. This indicates that healthy appearing squamous epithelium 5 cm above the squamo-columnar junction of the oesophagus in a subset of patients with columnar metaplasia of the distal oesophagus may already have undergone molecular changes, which may make them prone to the development of SIE, although this needs to be determined in a longitudinal follow up study of patients with reflux oesophagitis without BO. Patient to patient variation in the extent of reflux, the severity of inflammation, and the effect of the medication used, may explain why not all squamous epithelium samples of patients with BO contained detectable amounts of Cdx2 mRNA.

The development of BO is associated with the pathological reflux of acid³⁵ and/or bile.³⁶ Taken together with recent reports that CDX2 expression can be induced in keratinocytes by prolonged exposure to acid,³⁷ Cdx2 transcription may be an early step in the metaplastic replacement of oesophageal squamous epithelium by SIE. We hypothesise that inflammation in the oesophagus caused by duodeno-gastro-oesophageal reflux induces CDX2 expression in a subset of patients. Pathways involved in de novo CDX2 expression in oesophageal squamous epithelium may be important for the development of BO. Elucidating these pathways may result in a greater understanding of why only a subset of patients with GORD develop BO.

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ECHO

H pylori strains from gastric MZBL have common genetic marker



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Researchers may have discovered a key determinant of a rare complication of gastric lymphoma—a virulence sequence of *Helicobacter pylori*—in a comparative molecular study.

They looked for genetic markers in *H pylori* strains isolated from patients with gastric extranodal marginal zone B cell lymphoma (MZBL) of the mucosa-associated lymphoid tissue (MALT)-type and strains from age matched patients with gastritis only. They used subtractive hybridisation, which allows genes or sequences from isolated strains to be compared with a chosen control strain. The two strains used were a “tester” gastric MZBL strain and a “driver” gastritis strain chosen as the control, which had six known *H pylori* virulence genes in common.

Two open reading frames (ORFs) from the hybridisation were significantly linked with gastric MZBL over gastritis strains: JHP950 (74% v 49%) and JHP1462 (26% v 3%). JHP950 proved specific for gastric MZBL when tested against a group of strains from patients with duodenal ulcer and patients with adenocarcinoma, with significant prevalences (49% and 39%, respectively), and is therefore the candidate marker for gastric MZBL. Both ORFs coded for unknown products.

The researchers tested 43 strains from patients with gastric MZBL, 39 from patients with gastritis only, 41 from duodenal ulcer, and 28 from gastric adenocarcinoma. All patients were age matched.

H pylori causes gastric adenoma and MALT lymphoma and also 80% of gastric MZBL. Bacterial virulence factors may be responsible, but the researchers had previously ruled out all six *H pylori* virulence factors and instead investigated genetic variation specific to MZBL strains.

▲ Lehours P, et al. *Gut* 2004;**53**:931–937.