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Does CDX2 expression predict Barrett's metaplasia in oesophageal columnar epithelium without goblet cells?

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SUMMARY

Background
Intestinal metaplasia (Barrett's oesophagus), but not cardiac-type mucosa in columnar-lined oesophagus, is regarded as premalignant. As intestinal metaplasia and cardiac-type mucosa are endoscopically indiscernible, it is difficult to take targeted samples from columnar-lined oesophagus with consequently a risk of having undetected intestinal metaplasia.

Aim
To investigate whether the intestinal markers CDX2, MUC2 and villin can predict the presence of undetected intestinal metaplasia in columnar-lined oesophagus.

Methods
Presence of intestinal metaplasia or cardiac-type mucosa was identified in 122 biopsy sets of columnar-lined oesophagus from 61 patients, collected at two subsequent follow-up upper endoscopies. CDX2, MUC2 and villin expression were determined by immunohistochemistry.

Results
All intestinal metaplasia samples (55) were positive for CDX2 and MUC2 and 32 of 55 for villin. CDX2 expression was detected in 23 of 67 (34%) samples with only cardiac-type mucosa. Detection of CDX2 in cardiac-type mucosa increased the likelihood of finding intestinal metaplasia in another biopsy set of columnar-lined oesophagus (odds ratio 3.5, 95% CI = 1.2–10, $P = 0.02$). MUC2 was positive in 13 of 23 (57%) of CDX2-positive cardiac-type mucosa samples, whereas villin was detected in seven of 23 (30%).

Conclusions
CDX2 expression in cardiac-type mucosa might be able to predict the presence of undetected intestinal metaplasia in columnar-lined oesophagus, and thus may be a putative marker for the presence of intestinal metaplasia in the absence of goblet cells.

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INTRODUCTION

Barrett's oesophagus (BO) is a premalignant condition caused by chronic gastro-oesophageal reflux,[1] which can progress from low-grade dysplasia to high-grade dysplasia, and subsequently to oesophageal adenocarcinoma.[2–5]

Barrett's oesophagus is characterized by the replacement of the squamous epithelium of the oesophagus by columnar epithelium with goblet cells [specialized intestinal metaplasia (IM)].[6] IM is associated with the expression of intestinal markers such as MUC2,[7] and villin.[8] Cardiac-type mucosa (CM) is also frequently observed in the columnar-lined oesophagus (CLO),[9] with the absence of goblet cells as the only histological difference compared with IM.[10] CM, in contrast to IM, is not regarded as a premalignant condition.[5, 11] Therefore, only patients with IM are currently advised to undergo periodic endoscopic surveillance to detect progression to dysplasia in an early, potentially curable stage.[12] Others have reported that patients with biopsies from CLO without IM were at an increased risk of having undetected IM. This was explained by either sampling error or developing IM over time. According to current guidelines, these patients would have been falsely excluded from a surveillance programme.[13]

The homeobox protein CDX2 is a transcription factor involved in the early intestinal differentiation of the epithelium of the intestines,[14–16] and its expression is also linked with BO,[17–19] suggesting that CDX2 is an early marker for the development of IM in the oesophagus as well. CDX2 regulates transcription of several intestinal genes, encoding proteins such as MUC2, alkaline phosphatase and sucrase-isomaltase.[20, 21] It has been reported that intestinal phenotypic modifications may also be detected in the absence of goblet cells by CDX2 expression in CLO.[22, 23] This epithelium has been regarded as being early-stage BO, but these studies were cross-sectional and therefore provided not enough evidence for this hypothesis.

The aim of this longitudinal study was to investigate whether intestinal markers for IM, i.e. CDX2 (early intestinal marker), MUC2 (goblet cell marker) and villin (late intestinal marker), were present in the columnar-lined segment of the oesophagus in the absence of a histological diagnosis of IM (defined by the presence of goblet cells). Furthermore, we investigated whether these markers were predictive for the presence of IM in CLO, not detected due to sampling error or to IM developing over time.

MATERIALS AND METHODS

Patients and materials

In this multicentre study, 108 patients were evaluated for this retrospective study for the presence of an endoscopic CLO of at least 2 cm, and at least two follow-up endoscopies with biopsies being performed. Based on these inclusion criteria, 47 patients were excluded, and consequently 61 patients could be included in this study. Biopsies were taken at different levels from the CLO and embedded together in one paraffin block. In this study, sections of these paraffin-embedded biopsy sets were used for evaluation. These slides were reviewed for the presence of IM by an expert gastrointestinal pathologist (HvD). Based on the presence of IM, patients were divided into three groups (Table 1): patients with IM in both biopsy sets (IM-group), patients with IM in one biopsy set and with only CM in the other biopsy set (discordant-group), and patients with only CM in both biopsy sets (CM-group). Patients with CM in the first endoscopy and IM in the second endoscopy, and visa versa, were taken together as the discordant-group.

Histology and immunohistochemistry

Six consecutive sections of 4 μm each from every biopsy set were mounted on adhesive slides, dried overnight at 37 °C, and deparaffinized with xylene. The first of these serially sectioned slides was stained with haematoxylin and eosin (H&E) to determine the type of columnar epithelium (CM or IM). Alcian Blue and periodic acid-Schiff (PAS) stainings in consecutive

Table 1. Classification of patients in groups, based on histology results from two subsequent endoscopies

	IM-group	Discordant-group		CM-group
First endoscopy	IM	IM	CM	CM
Second endoscopy	IM	CM	IM	CM
No. of patients	15	16	9	21

IM, intestinal metaplasia; CM, cardiac-type mucosa.

slides were performed to facilitate the detection of mucin producing goblet cells. The next three slides were used for immunohistochemistry.

For immunohistochemistry, antigen retrieval was performed by boiling the deparaffinized samples in 10 mM monocitric acid buffer (pH 6.0) for 15 min, and slowly cooling down to room temperature (RT). Prior to immune staining, endogenous peroxidase activity was blocked by incubating the slides in a 0.5% solution of H_2O_2 in phosphate-buffered citric acid for 15 min at RT. Samples were washed for 5 min with TRIS-buffered saline (TBS) (pH 7.4). This was repeated two times. The samples were incubated in TBS buffer containing 10% rabbit non-immune serum (Dako, Glostrup, Denmark) and 10% normal human plasma (Dako) for 20 min. Sections were incubated for 16 h at 4 °C with respectively primary antibody anti-CDX2 (clone 392M; Biogenex, San Ramon CA, USA) in a 1:100 dilution, anti-MUC2 (clone Ccp58; Novocastra, Newcastle upon Tyne, UK) in a 1:100 dilution or anti-villin (clone CWWB1; Lab Vision, Fremont CA, USA) in a 1:2000 dilution. Samples were again washed three times for 5 min with TBS (pH 7.4). Subsequently, biotin-labelled rabbit-anti-mouse antibody (Dako) was used as second antibody, followed by the addition of a streptavidin–horseradish peroxidase complex (Dako) using 3-amino-9-ethylcarbazole as substrate. Slides were analysed for nuclear CDX2 staining, cytoplasmic MUC2 staining and brush border villin staining by two independent investigators (MK, DAB) who were blinded for the presence or absence of IM. CDX2 expression was considered positive if a clear red staining of at least five adjacent nuclei in the same gland was seen, to exclude incidental false positive nuclei. MUC2 expression was present if a red staining in the cytoplasm of (goblet) cells was observed. Villin expression was visualized as a red staining near the apical border of cells.

Statistical analysis

The chi-squared test, Mann–Whitney test and Kruskal–Wallis test were used to compare the patient characteristics and the immunohistochemical stainings between the three patient groups. A P-value <0.05 was considered significant. Odds ratios (ORs) with a 95% confidence interval were used as an estimate of the relative risk for the presence of IM. Calculations were initially done with upper endoscopies as the unit of analysis, ignoring the statistical dependency of endoscopies within the same patients. Subsequently, analyses were repeated with the consideration of only one endoscopy per patient. Statistic analyses were conducted using SPSS software (SPSS version 11.0; SPSS, Chicago, IL, USA).

RESULTS

The presence of IM

Intestinal metaplasia was defined as the presence of goblet cell containing glands. In addition to goblet cells, non-goblet cells can also stain positive with alcian blue. Therefore, the presence of IM was evaluated by light-microscopic examination of H&E stained slides. Consecutive alcian blue and PAS stained slides were only used to confirm a diagnosis of IM.

Intestinal metaplasia was observed in 55 of 122 (45%) biopsy sets. In 67 of 122 (55%) biopsy sets, only CM was present. The mean number of biopsies taken was five in the IM-group and four in the discordant- and CM-group (similar at the two endoscopies in each group), which was not significantly different (Table 2). When correcting for the length of the columnar segment, the mean number of biopsies taken per centimetre was not different in IM and CM biopsy sets (respectively 1.5/cm (range 0.1–4.3) and 1.5/cm (range 0.3–4.0), $P = 0.68$). Based on the presence of IM, the IM-group consisted of 15 patients, the discordant-group of 25 patients, and the CM-group of 21 patients. Of all patient characteristics, only the length of the CLO differed significantly between the three groups ($P = 0.016$), with CLO being longer in the IM-group, compared with the discordant- and the CM-group (Table 2).

CDX2 expression

To investigate the expression of CDX2 protein in IM and CM, CDX2 staining was evaluated. CDX2 expression was observed in all IM-positive biopsy sets (Table 3; Figure 1a,b), i.e. in 30 of the IM-group and in 25 of the discordant-group. In addition, CDX2 expression was also observed in 23 of 67 (34%, 95% CI: 23–47) biopsy sets without IM (Table 3; Figure 1c,d).

CDX2 was more frequently observed in IM-negative biopsy sets of patients of the discordant-group, in which the other biopsy set was positive for IM (13/25; 52%), than in patients of the CM-group, in which IM was absent in both biopsy sets (10/42; 24%) ($P = 0.019$). The presence of CDX2 in CM therefore

Table 2. Patients characteristics

	IM-group	Discordant-group	CM-group	*P*-value
No. of patients	15	25	21	
Mean age at first endoscopy in years (range)	59 (28–82)	58 (39–78)	52 (27–74)	0.30
Mean length of the CLO in cm (range)	4 (2–8)*	3 (2–7)	3 (2–5)	0.016
Mean number of biopsies (range)	5 (1–17)	4 (1–7)	4 (1–8)	0.27
Interval between subsequent endoscopies in months (range)	42 (12–158)	31 (4–112)	30 (4–117)	0.54
Proton-pump inhibitor use (%)	11/13 (85%)	20/22 (91%)	12/19 (63%)	0.18

IM, intestinal metaplasia; CM, cardiac-type mucosa; CLO, columnar-lined oesophagus.
* Significantly different.

Table 3. Results of immunohistochemical stainings of all biopsy sets

	IM			CM		
Group (no. pts)	IM (30)	Discordant (25)	Total (55)	Discordant (25)	CM (42)	Total (67)
CDX2-positive	30 (100%)	25 (100%)	55 (100%)	13 (52%)**	10 (24%)	23 (34%)
MUC2-positive	30 (100%)	25 (100%)	55 (100%)	11 (44%)	5 (12%)	16 (24%)
Villin-positive	22 (73%)	10 (40%)	32 (58%)	4 (17%)*	3 (7%)	7 (10%)

IM, intestinal metaplasia; CM, cardiac-type mucosa.
* One sample could not be evaluated as no enough tissue was available.
** $P = 0.019$ (compared with CDX2 expression in biopsy sets with CM of the CM-group).

significantly increased the likelihood of observing IM in another biopsy set of the CLO (OR 3.5, 95% CI: 1.2–10, $P = 0.021$), regardless if CM in the discordant-group was present in biopsies from the first or second upper endoscopy. When we calculated the predictive value of CDX2 expression in biopsies with CM taken during the first endoscopy, for the presence of IM in biopsies of the next endoscopy, and visa versa, the ORs were similar (respectively 4.0, 95% CI: 0.8–21, $P = 0.10$ and 3.2, 95% CI: 0.8–13, $P = 0.10$). In one patient of group 3, both IM-negative biopsies were positive for CDX2.

A longer segment of CLO was not associated with a higher change of CDX2 being present in CM ($P = 0.135$). There was no correlation between the use of proton-pump inhibitors and the presence of CDX2 in CM ($P = 0.42$).

MUC2 expression

Mucins are large glycoproteins forming the main components of the gel-like mucous layer on the surface of the intestine, protecting the mucosa against damaging luminal contents, such the gastro-oesophageal refluxate.[24] MUC2 is a mucin specific for IM.[25–27] As CDX2 regulates the transcription of MUC2,[21] we evaluated the expression of MUC2 in IM and CM. MUC2 staining in goblet cells was found in all biopsy sets with IM (Table 3, Figure 2a), and was mainly localized in the cytoplasm alongside the membrane. Moreover, MUC2 was also expressed in CM in 16 of 67 (24%) samples without IM. In CM, MUC2 was expressed in the entire cytoplasm of non-goblet columnar cells that did not stain positive with alcian blue (Figure 2b). Thirteen of 16 (81%) MUC2-positive CM samples were also positive for CDX2 in the same region.

Villin expression

Villin is an actin-binding cytoskeletal protein essential for brush border formation (microvilli) in normal end-differentiated epithelial cells of the intestine.[28]

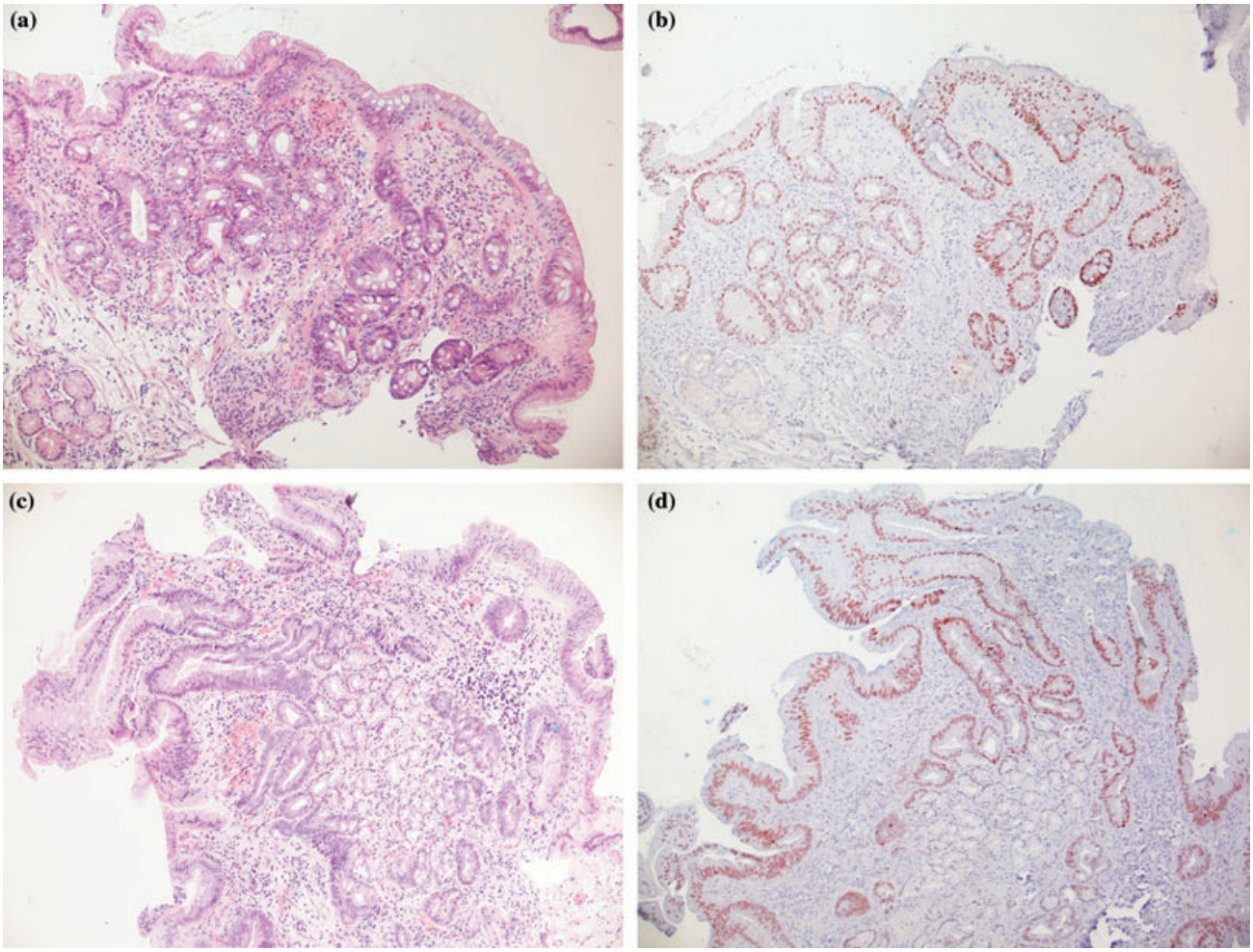

Figure 1. CDX2 expression in columnar epithelium of the oesophagus. (a) Intestinal-type columnar epithelium with goblet cells (haematoxylin-eosin). (b) Nuclear staining (red) for CDX2 in intestinal-type columnar epithelium in a serial section of the same patient as in (a). (c) Cardiac-type columnar epithelium without goblet cells (haematoxylin-eosin). (d) CDX2 expression in cardiac-type columnar epithelium in a serial section of the same patient as in (c). Original magnifications ×100.

Therefore, the presence of a brush border of the oesophageal columnar epithelium can be demonstrated by villin expression. We investigated whether villin protein was also expressed in CM in addition to the intestinal markers CDX2 and MUC2. One CM sample could not be evaluated, as there was not enough tissue available for staining. Villin expression was observed in 32 of 55 (58%) of IM-positive biopsy sets (Figure 3a). In seven of 66 (11%) CM samples, villin expression was found (Figure 3b), of which five were also CDX2 positive. Four CM samples (6%) were positive for CDX2 and MUC2, as well as for villin.

DISCUSSION

Patients with CM in CLO are currently excluded from surveillance endoscopy, as they are regarded as IM negative and thus as not having a premalignant condition.[13] This study shows a significant relationship between the intestinal marker CDX2 in CM and the presence of IM in biopsies taken at another time point, as CDX2 stained positive in 52% of CM biopsy sets of the discordant-group (with an OR of 3.5), in which the biopsy set of the other endoscopy was positive for IM (Table 2). In our opinion it is unlikely that, despite the two-dimensional analysis of the biopsies, goblet cells

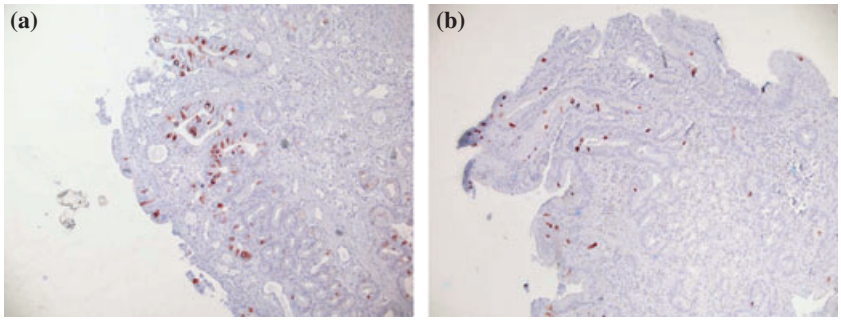

Figure 2. MUC2 expression in columnar epithelium of the oesophagus. (a) MUC2 staining in goblet cells (red) in intestinal-type columnar epithelium. (b) MUC2 expression in cardiac-type columnar epithelium without goblet cells in a serial section of the same patient as in Figure 1c. Note that the MUC2 expression is not associated with goblet cells. Original magnifications ×100.

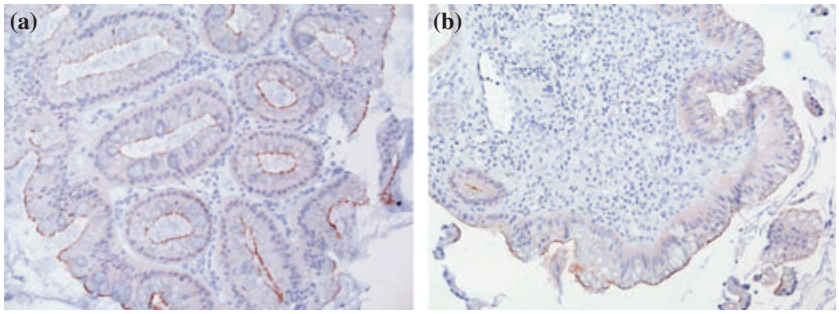

Figure 3. Villin expression in columnar epithelium of the oesophagus. (a) Villin staining of the brush border (red) in intestinal-type columnar epithelium. (b) Villin expression in cardiac-type columnar epithelium without goblet cells. Note that the villin expression is not associated with goblet cells. Original magnifications ×200.

have been missed in these CM biopsy sets, as the CDX2 expression was often observed in large areas without goblet cells (Figure 1c,d), and, in addition, in the six consecutive slides also no goblet cells were observed. Therefore, CDX2 staining may represent a useful histological marker for the presence of IM in CLO despite the absence of goblet cells suggestive for IM.

CDX2 expression in CM as an indicator for the presence of IM has been reported in previous studies.[19, 22] These studies were however cross-sectional, which means that biopsies were only evaluated at one time-point. In contrast, this study was a longitudinal study, in which biopsy sets of two subsequent endoscopies were compared.

Previously, it has been suggested that there are two possible reasons for not detecting IM.[13] First, several authors have proposed that IM may develop over time in a two-step process. It has been suggested that multilayered epithelium, with morphological and immunohistochemical characteristics of both squamous and columnar epithelium, may represent a transitional stage in the development of BO.[29] Others have suggested that IM develops from previously induced CM in the oesophagus under influence of chronic inflammation.[13, 30–32] According to this theory, the finding of CDX2 expression in CM, and in a subset also expression of MUC2 and villin, could indicate early intestinal differentiation prior to morphologic changes such as goblet cells,[19, 33] and in this way being an intermediate stage in the differential shift of CM towards IM.[28, 30]

The second possibility for not detecting IM is sampling error. Although IM is predominantly present in the proximal end of the CLO,[34] IM and CM may have a patchy distribution. As IM and CM are endoscopically indiscernible from each other, and the presence of IM can be very focal,[35] sampling error for the detection

of IM may occur.[13] Sixteen of the 25 patients of the discordant-group had IM in their first, and CM in their second biopsy set (Table 1). It seems likely that in these cases the finding of no IM can be contributed to sampling error. The likelihood of detecting IM increased with the number of biopsies taken, and therefore taking not enough biopsies could be a reasonable explanation for missing IM in this group. As in this study the mean number of biopsies taken per cm was similar in the IM samples and the CM samples, the possibility of sampling error seems to be ruled out. However, as IM has a patchy appearance in the CLO but is predominantly located at the proximal end of the CLO,[12, 34] it is possible that despite taking the same numbers of biopsies, IM could be missed due to taking proportionally less biopsies of the proximal part of the CLO. A similar explanation can be given for the other nine patients of the discordant-group who had CM detected at their first endoscopy, whereas IM was found in biopsies from the second endoscopy. As the mean interval between two subsequent endoscopies in the discordant-group was with 30 months relatively long, and the development of IM is thought to be a slow process, it is also possible that IM in this subgroup has developed over time.

Although a final conclusion on the cause of not detecting IM in one set of biopsies cannot be given, the ORs for the predicting value of CDX2 in CM in the different subgroups were similar, and thus it is reasonable to assume that CDX2 expression in CM represents a reliable marker for the detection of the premalignant IM in CLO at another time point. In line with this assumption, it is likely that the 24% with CDX2 expression in CM biopsy sets in whom IM was not detected in both biopsy sets taken at different time points, will show IM in biopsies taken at a next endoscopy. Unfortunately, because of exclusion from the surveillance programme, these patients have currently not undergone another follow-up upper endoscopy to evaluate this.

CDX2 is a transcription factor for MUC2, which is a mucin specific for IM.[25–27] In our study, as expected, all IM biopsies stained positive for MUC2. In 13 of 23 (57%) of the CDX2-positive CM biopsies, MUC2 staining was also positive. Villin expression was observed in 58% of the IM-positive samples. This lower result of villin expression in IM compared with CDX2 expression and MUC2 expression has been suggested to be caused by the fact that the quantity of villin protein needs to have a sufficient level to result in a mature brush border.[36, 37] In addition to villin expression in IM, five of 23 (22%) of the CDX2-positive CM samples also showed villin expression, suggesting the presence of end-differentiated intestinal characteristics in CM. Although less frequent, the presences of MUC2 and villin expression in CM are supportive for the value of CDX2 as indicator of IM in CLO.

A possible limitation of this study is the use of one single technique to detect CDX2 in the biopsies. The major reason that we only used immunohistochemistry was that additional techniques such RT-PCR,[18] could not be performed on our paraffin-embedded tissue, but only on fresh snap frozen biopsies, which were not available in this retrospective study. However, as we performed the CDX2 immunohistochemical stainings with a commonly used dilution,[17, 18] which showed only very specific nuclear staining without background staining in the cytoplasm of cells, it is unlikely that the immunohistochemistry may have resulted in false positive results.

In conclusion, this study shows that the presence of CDX2 in CM might be able to predict the presence of IM in CLO, which was otherwise not detected because of sampling error or developing of IM over time. This suggests that CDX2 staining could be used as an additional marker for the presence of IM in CLO in the absence of goblet cells. A prospective follow-up study on patients with CM in their biopsies should be performed to confirm the predictive value of CDX2. Nonetheless, as the presence of IM is still the gold standard for the presence of premalignant BO, we suggest an additional endoscopy in patients in whom CDX2 expression in CM is demonstrated. This should include the taking of extensive biopsies for the detection of IM (especially near the squamo–columnar junction) to evaluate if endoscopic surveillance is indeed indicated in these patients.

CONFLICTS OF INTEREST

We declare that we have no conflict of interest.

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APPENDIX

Flow cytometry for the detection of Barrett's patients at risk for developing adenocarcinoma (CYBAR) study group

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