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CDX2 and LI-Cadherin Expression in Esophageal Mucosa: Use of Both Markers Can Facilitate the Histologic Diagnosis of Barrett's Esophagus and Carcinoma

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Abstract

Background: Barrett's mucosa is a risk factor for esophageal adenocarcinoma and should be detected at an early stage. CDX2 and liver–intestine (LI)-cadherin are intestine-specific markers.Aberrant CDX2 expression has been demonstrated in Barrett's metaplasia, esophagitis, and intestinal metaplasia of the stomach. *Methods:* The relationship between CDX2 and LI-cadherin expression was investigated in normal gastroesophageal (n = 24) and in Barrett's (n = 20) mucosa, in low-grade (n = 15) and high-grade (n = 13) intraepithelial neoplasia (IEN) as well as in esophageal adenocarcinoma (n = 16), using immunohistochemistry. *Results:* Nuclear positivity for CDX2 coupled with membranous expression of LI-cadherin was observed in about 70% of the epithelial cells of Barrett's mucosa. The intensity of staining and the percentage of positive cells increased within the sequential steps of low-grade to high-grade IEN, whereas the normal cylindric epithelium lacked the expression of both. In adenocarcinoma, the expression of LI-cadherin and CDX2 was significantly weaker or absent. *Conclusions:* CDX2 and LI-cadherin are sensitive markers of intestinal metaplasia with or without dysplasia in the upper gastrointestinal tract. Both can be helpful for the early histologic diagnosis of Barrett's esophagus and its subsequent lesions; however, they do not significantly discern between different grades of dysplasia.

Keywords

Barrett's metaplasia, esophageal adenocarcinoma, CDX2, LI-cadherin, immunohistochemistry

Introduction

Reliable immunohistochemical methods to determine the exact dignity of an esophageal mucosa lesion are not yet available. Because of advances in minimally invasive treatment, such as endoscopic mucosal resection or laparoscopic surgery, it becomes more and more essential to evaluate reliable laboratory tests for this purpose. In this way it will be possible to minimize recurrent potential of individual tumors after surgery or even predict tumor aggressiveness or potential lymph node metastasis preoperatively. Furthermore, it might be possible to specify diagnosis, prognosis, and prediction of dysplastic lesions in the esophageal mucosa.

Barrett's esophagus, first described by the surgeon Norman Barrett in 1950[1] and refined in 1957,[2] occurs when the normal squamous epithelium of the distal esophagus is replaced by metaplastic columnar epithelium in response to chronic gastroesophageal reflux, which may progress to esophageal ulcer, stricture, dysplasia, or even adenocarcinoma. Although the specialized columnar epithelium is composed of both goblet and columnar nongoblet cells, only the former are considered as the hallmark of Barrett's esophagus.[3-6] The rapidly rising incidence of esophageal adenocarcinoma over the past 2 decades has driven efforts to identify patients with Barrett's esophagus.[7-9] The sensitivity and positive predictive values of standard upper endoscopy for diagnosing Barrett's esophagus have been

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reported as 82% and 34%, respectively.[10] This is secondary to the patchy and mosaic distribution of intestinal metaplasia within the columnar-lined esophagus. Thus, the diagnosis may be missed when goblet cells are inconspicuous or when small biopsies with crush artifacts are sent for examination. Accordingly, the availability of markers capable of detecting intestinal differentiation in the absence of goblet cells could be of clinical importance.

The caudal-type homeobox transcription factor (*cdx*) genes, gene products CDX1 and CDX2 are important in the early differentiation and maintenance of intestinal epithelium via regulation of intestine-specific gene transcription,[11-14] have attracted recent interest as early markers of Barrett's metaplasia. CDX2 expression has been demonstrated in Barrett's metaplasia, esophagitis, and intestinal metaplasia of the stomach in humans.[15-19] Evidence for a role of CDX2 in intestinal metaplasia also comes from animal studies; Mutoh et al[20] generated transgenic mice whose intestinal metaplasia was induced by expressing CDX2 in the stomach. The CDX2 gene is physiologically expressed throughout the small and large intestine, with the proximal limit occurring at the gastroduodenal junction. Therefore, it is considered that ectopic CDX2 expression may play a critical role in the development of intestinal metaplasia.

Cadherins are transmembrane glycoproteins responsible for Ca^{2+}-dependent cell–cell adhesion.[21] Furthermore, they are involved in the maintenance of tissue structure and morphogenesis.[21,22] Today there is increasing evidence that cadherin-mediated cell adhesion additionally plays a crucial role in carcinoma cell behavior.[23,24] Liver–intestine (LI)-cadherin is a structurally unique member of the cadherin superfamily as it contains 7 instead of 5 molecular domains as a hallmark.[25,26] LI-cadherin is one of the transcriptional targets of CDX2.[25-27] It is selectively expressed on the basolateral surface of enterocytes and goblet cells in the small and large intestine but not in the upper gastric tract. In contrast to rat, LI-Cadherin is not expressed in the liver.[28] Aberrant LI-cadherin expression has been shown to be a sensitive marker for early detection of gastric intestinal metaplasia and well-differentiated adenocarcinomas[28] as well as a marker of hepatocellular carcinomas[29] and pancreatic ductal adenocarcinomas.[30] Ko et al[19] detected overexpression and colocalization of CDX2 and LI-cadherin in gastric intestinal metaplasia and adenocarcinoma and presumed that aberrant upregulation of CDX2 and consequently the activation of intestinal genes may be one of the possible mechanisms linked to the induction of intestinal metaplasia.

However, LI-cadherin expression has yet not been studied comparatively with that of CDX2 in esophageal disorders. Accordingly, we focused on specialized columnar epithelium in Barrett's mucosa without dysplasia, with low-grade and high-grade intraepithelial neoplasia (IEN) as well as on invasive adenocarcinoma to gain insight into the role of the homeotic gene cdx2 and its target LI-cadherin within the metaplasia–dysplasia–adenocarcinoma sequence.

Materials and Methods

Patients and Samples

Participants of the study were 70 patients whose samples had been examined at the Institute of Pathology at the University Hospital Charité, Berlin, in the years 2004 (n = 4), 2005 (n = 16), and 2006 (n = 50). The mean patient age was 64 years (minimum 32 years, maximum 97 years, standard deviation 12.5 years), 19 were women and 51 were men. The samples were obtained as biopsies (n = 50) or mucosectomy specimens (n = 6) during esophagogastroscopy or as esophageus resections by open surgery (n = 14). Formalin-fixed, paraffin-embedded tissue samples were selected from the archive, and the hematoxylin and eosin (H&E)-stained slides were reviewed to verify the initial diagnoses and to select suitable areas for immunohistochemical stainings. When available, adjacent nonneoplastic epithelium was evaluated in samples from intraepithelial or invasive neoplasia, so the total number of evaluated tissues was n = 88. These covered the whole spectrum from normal gastroesophageal mucosa (n = 24) through Barrett's mucosa without dysplasia (n = 20), with low-grade (LG-IEN, n = 15) and high-grade intraepithelial neoplasia (HG-IEN, n = 13) to invasive adenocarcinoma (n = 16). The diagnoses were made according to the criteria of the WHO classification of tumors, 2000.[31] All patients had given informed consent to the use of the tissue samples in this study, in accordance with the Helsinki Declaration of the World Medical Association.

Immunohistochemistry

Consecutive microsections of 2-µm thickness were deparaffinized with xylene, and antigen demaskation was performed in boiling sodium citrate buffer (0.01 mM; pH 6.0). Endogenous peroxidase was quenched by incubation with peroxidase block (DAKO, Glostrup, Denmark) for 10 minutes. The antibodies against LI-cadherin (anti-LI-cadherin goat polyclonal LI-cadherin primary antibody; Santa Cruz Biotechnology, Santa Cruz, CA) and CDX2 (Biogenetics, Padua, Italy) were used at 1:1000 and 1:100 dilutions, respectively. For antibody detection the LSAB plus HRP kit (DAKO) was used according to the manufacturer's protocol. DAB was used as chromogen, and the sections were then counterstained with haematoxylin for 2 minutes.

Histopathological Evaluation

All sections were evaluated on a LaborLux (Leitz, Bensheim, Germany) light microscope at 25×, 100×, 200×, and 400× magnification and images were captured by a 3-CCD camera, model KY-F75U (JVC Professional Products Company, Wayne, NJ).

The staining intensity was graded semiquantitatively from 0 (=*absent*) through 1 (=*slight*), 2 (=*moderate*) to 3 (=*strong*). Also, the relative number of positive stained cells compared with all epithelial cells of that lesion was estimated in measures of 10%. The accuracy of this percentage is slightly limited because on immunohistochemically stained slides the histomorphology is more ambiguous when compared with H&E morphology and therefore the borders of the lesion, especially in cases of IEN, cannot be determined exactly. Another limitation to the preciseness is the variability of the immunohistochemical expression within one lesion. Most tumors showed areas of no, moderate, and strong expression side by side, which was combined to a single score.

In analogy to Remmele and Stegner,[32] an immunoreactivity score (IRS) was calculated as the product of the value for staining intensity (from 0 to 3) and a value for the percentage of positive cells, defined as follows: 0, no positive cells; 1, 10% positive; 2, 20% to 50% positive; 3, 60% to 80% positive; and 4, 90% to 100% positive; the total IRS ranging from 0 to 12.

Statistical Analysis

For descriptive statistics, arithmetic mean and median were acquired. The correlation between CDX2 and LI-cadherin staining was examined using Pearson's correlation coefficient. To analyze significances between the different kinds of leasons, the Mann–Whitney test was used, and significance was assumed when $P < .05$. All calculations were performed using SPSS 14.0 software (SPSS Inc, Chicago, IL).

Results

Using the described staining techniques, CDX2 showed a brown nuclear reaction and LI-cadherin a brown membranous staining. In all of the examined lesions the expression patterns of both LI-cadherin and CDX2 were heterogeneous. Whereas only the normal cylindric epithelium was almost entirely negative for both antibodies, all other lesions had areas of no, weak, moderate, and strong staining side by side. LI-cadherin was accentuated at the luminal side of the epithelium, whereas CDX2 expression was found in cells at the luminal surface and in the foveolae (Figure 1). The immunohistochemical evaluation is summarized in Table 1. In the normal epithelium, almost no staining was observed. In Barrett's mucosa, there was a moderate staining in about 70% of the epithelial cells, which was very similar in LG-IEN. The strongest expression was found in HG-IEN, whereas the staining for both CDX2 and LI-cadherin was weaker in invasive adenocarcinoma.

Pearson's correlation coefficient revealed a significant ($P < .001$) correlation between CDX2 and LI-cadherin staining, with regard to the staining intensity as well as the percentage of positive cells and the IRS (Table 2).

Statistical evaluation revealed that both CDX2 and LI-cadherin expressions differed significantly between normal epithelium and all other examined lesions. No significant differences could be found between Barrett's mucosa and IEN. The expression of CDX2 and LI-cadherin was significantly weaker in invasive carcinoma than in HG-IEN. Also, the staining of LI-cadherin, but not of CDX2, was significantly weaker in invasive carcinoma than in Barrett's mucosa and in LG-IEN. The *P* values are summarized in Table 3.

Discussion

This study analyzed the complex expression patterns of CDX2 and LI-cadherin during the development of Barrett's epithelium to adenocarcinoma according to the metaplasia–dysplasia–adenocarcinoma sequence. Barrett's esophagus is a complication of longstanding gastroesophageal reflux, which remains asymptomatic in most cases. It is considered a risk factor for esophageal adenocarcinoma, which has a poor prognosis unless detected at an early stage.[33-36] Therefore, the presence of Barrett's mucosa is used as a marker for the identification of those patients who are in need of endoscopic surveillance. At present the best predictor of the future development of a carcinoma in a given patient with Barrett's esophagus is still the histologic identification of dysplasia,[37] whereby the HG-IEN carries the highest relative risk (33%)[38] of malignant progression in Barrett's esophagus with a 5-year incidence for adenocarcinoma of 59%.[39] Several studies have been done based on the hope that, in future, specific molecular changes might serve as reliable markers for the early identification of patients who are likely to develop carcinoma. Numerous studies examined the immunohistochemical expression of p53 and ki-67 as biomarkers of malignant transformation, and they found concordantly, that both markers independently are able to predict progression to high-grade lesions and carcinoma, irrespective of the histopathologic diagnosis.[40,41] On the other hand, the histopathologic diagnosis is the best validated biomarker of malignant progression, because it has been examined in prospective interventional studies. Results on that level of evidence are still pending

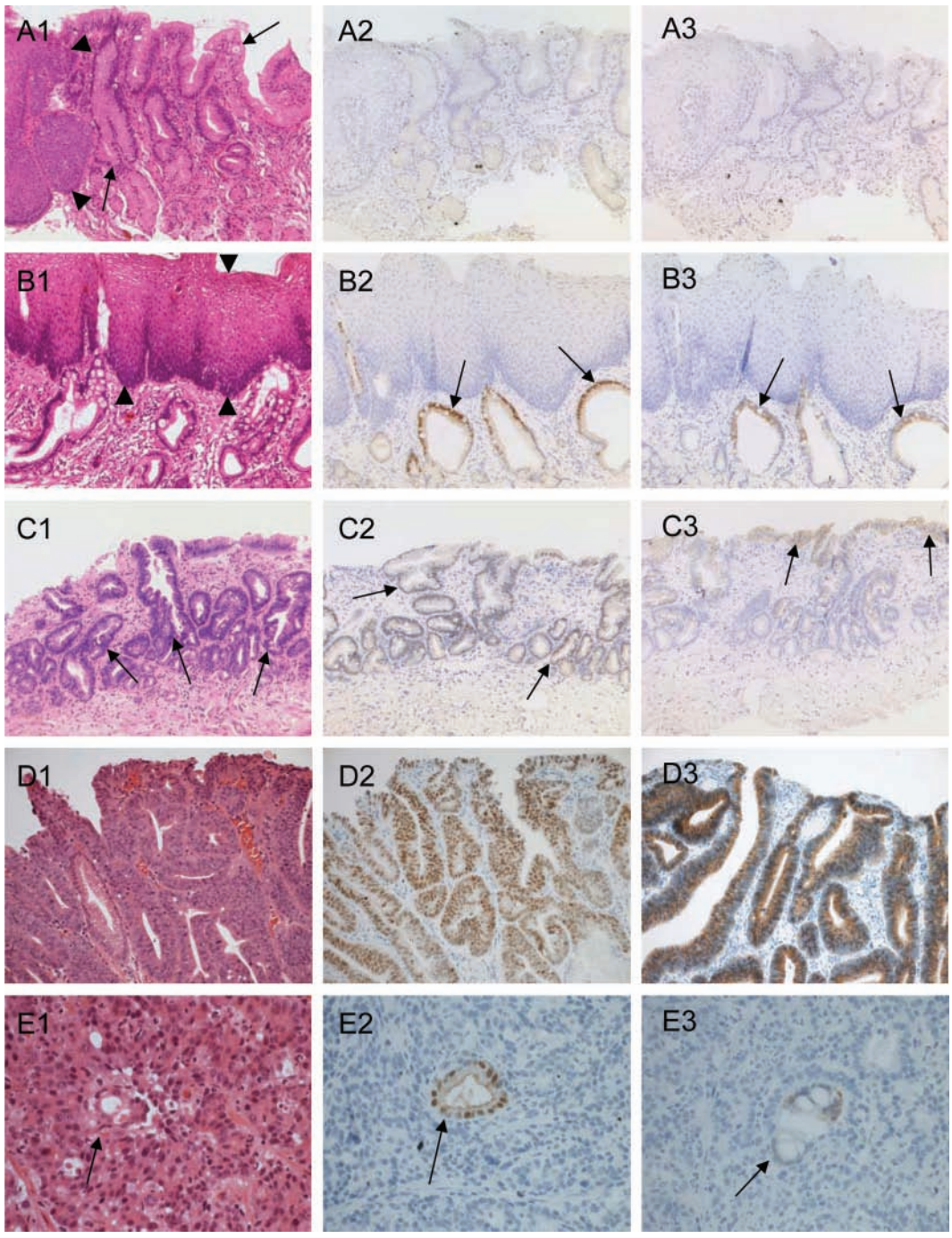

Figure 1. Hematoxylin and eosin histology (column 1), CDX2 immunohistochemistry (column 2) and Li-cadherin immunohistochemistry (column 3)

Row A, In the normal epithelium (arrows, glandular epithelium, arrowheads, squamous epithelium), no staining reaction is visible. Row B, In Barrett's mucosa, which is in this case lying under intact squamous epithelium (arrowheads), the expression of CDX2 and LI-cadherin is almost concordant (arrows). Row C, The low-grade intraepithelial neoplasia (LG-IEN) is characterized by irregularly shaped glands and enlarged but still rather monomorph nuclei (arrows). CDX2 positive cells can be found at the surface and in the deeper glands (arrows), whereas the LI-cadherin staining is strongest at the surface (arrows; rows A, B, and C original magnification 100×). Row D, This case of high-grade intraepithelial neoplasia (HG-IEN) shows distorted, unorganized glands, and the nuclear atypia is higher than in LG-IEN (column 1). The staining pattern is similar to Barrett's mucosa and LG-IEN; this case exhibits a particularly strong and continuous staining (original magnification 200×). Row E, This invasive adenocarcinoma shows small, heavily distorted glandular structures, mitoses, and atypical nuclei with prominent nucleoli; superposed by a neutrophilic inflammatory infiltrate. The tumor is negative for both CDX2 and LI-cadherin, whereas an entrapped remaining gland (arrow) is positive for both markers (original magnification 400×).

Table 1. Arithmetic Mean (Median) of Staining Intensity, Percentage of Positive Cells, and IRS (Columns) for Each Type of Lesion Examined (Rows)

	CDX2			LI-Cadherin		
Diagnosis	Intensity	Percentage	IRS	Intensity	Percentage	IRS
Normal, n = 24	0.4 (0.0)	2.5 (0.0)	0.4 (0.0)	0.1 (0.0)	0.4 (0.0)	0.1 (0.0)
Barrett, n = 20	1.9 (2.0)	65.0 (75.0)	5.9 (6.0)	2.0 (2.0)	71.5 (80.0)	6.2 (6.0)
Low-grade IEN, n = 15	2.0 (2.0)	61.4 (65.0)	5.6 (6.0)	2.2 (2.0)	59.3 (65.0)	6.3 (6.0)
High-grade IEN, n = 13	2.5 (3.0)	73.1 (80.0)	7.9 (8.0)	2.5 (3.0)	62.3 (70.0)	7.3 (9.0)
Carcinoma, n = 16	2.1 (2.0)	47.5 (45.0)	5.1 (4.0)	1.8 (2.0)	31.9 (20.0)	3.9 (3.5)

Abbreviations: LI-cadherin, liver–intestine cadherin; IRS, immunoreactivity score; IEN, intraepithelial neoplasia.

Table 2. Pearson's Correlation Coefficients for the CDX2 and LI-Cadherin Stainings Regarding Staining Intensity, Percentage of Positive cells, and IRS[a]

	CDX2			LI-Cadherin		
	Intensity	Percentage	IRS	Intensity	Percentage	IRS
CDX2						
Intensity	1.000	0.747	0.889	0.808	0.628	0.710
Percentage	0.747	1.000	0.897	0.816	0.817	0.851
IRS	0.889	0.897	1.000	0.806	0.740	0.826
LI-cadherin						
Intensity	0.808	0.816	0.806	1.000	0.795	0.898
Percentage	0.628	0.817	0.740	0.795	1.000	0.934
IRS	0.710	0.851	0.826	0.898	0.934	1.000

Abbreviations: LI-cadherin, liver–intestine cadherin; IRS, immunoreactivity score.
[a]All correlations are significant at the .01 level.

Table 3. *P* Values Assessed by Mann–Whitney Test for Significant Differences Between the Examined Lesions (Rows) Regarding the Percentage of Positive cells and the IRS of the CDX2 and LI-Cadherin Immunohistochemistry (Columns)[a]

	CDX2		LI-Cadherin	
Compared Lesions	Percentage	IRS	Percentage	IRS
Normal vs all other	*0.000*	*0.000*	*0.000*	*0.000*
Barrett vs LG-IEN	0.459	0.866	0.092	0.899
Barrett vs HG-IEN	0.526	0.089	0.323	0.191
LG-IEN vs HG-IEN	0.111	0.094	0.561	0.331
Carcinoma vs Barrett	0.078	0.510	*0.001*	*0.022*
Carcinoma vs LG-IEN	0.135	0.346	*0.033*	*0.037*
Carcinoma vs HG-IEN	*0.018*	*0.028*	*0.024*	*0.013*

Abbreviations: LI-cadherin, liver–intestine cadherin; IRS, immunoreactivity score; LG-IEN, low-grade intraepithelial neoplasia; HG-IEN, high-grade intraepithelial neoplasia.
[a]*P* Values <.05 are given in italics.

for p53 and ki-67.[37] But both markers have been shown to reduce the interobserver variability of pathologists when assessing esophageal biopsies. In one study, the κ values between 3 observers increased from 0.240 in H&E assessment to 0.520 in the ki-67 and to 0.715 in the p53 assessment, showing the usefulness of these markers in routine diagnostics.[42] In reverse transcription-polymerase chain reaction (RT-PCR) analysis, Eda et al[16] demonstrated that CDX2 emerged already at the stage of esophagitis in inflammatory squamous mucosa. In immunohistochemistry, a granular cytoplasmatic reactivity could be observed within the squamous epithelium, and there was 100% concordance between the CDX2 expression determined by RT-PCR and by immunohistochemistry.

Eda et al[15] also demonstrated the expression of CDX2 in chronic gastritis and concluded that inflammation may play an important role in the induction of CDX2 expression. According to their finding, CDX2 expression is not the result of, but the trigger of the development of intestinal metaplasia. Functional studies have also shown CDX2 to regulate intestine-specific gene transcription in vivo, as evidenced by binding to several intestine-specific promoters and activating the transcription, as that of LI-cadherin, an intestine-specific adhesion molecule with aberrant expression in intestinal metaplasia and well-differentiated adenocarcinomas of the stomach.[27,28] Another study compared the expression of LI-cadherin in human gastric carcinoma with clinicopathological parameters, prognosis, and CDX2 expression and observed an association with aggressiveness of gastric carcinoma so that LI-cadherin was considered as a good marker for high-grade malignancy.[43]

In our study, we observed nuclear positivity for CDX2 coupled with membranous expression of LI-cadherin in about 70% of the epithelial cells of the Barrett mucosa, and the intensity of staining and the distribution of expression increased within the sequential steps of low-grade to high-grade dysplasia, whereas only the normal cylindric epithelium lacked expression of both. Then in adenocarcinoma the expression of LI-cadherin and CDX2 was significantly weaker. In all cases, the expression of LI-cadherin was tightly coupled with that of CDX2, in accordance with previous studies. Also remarkable in some cases was the heterogeneity of staining intensity, with intensely stained next to weakly stained cells. Because of this heterogeneity, the use of CDX2 and LI-cadherin immunohistochemistry as an additional diagnostic tool is limited. However, a strong staining reaction may confirm a diagnosis of dysplasia, and an abrupt loss of immunoreactivity points at areas suspicious of beginning invasion. Given the molecular evidence suggesting that CDX2 is one of the master regulatory genes in intestinal differentiation, the finding of CDX2 and its gene product LI-cadherin in esophageal biopsies would assist in confirming the diagnosis of intestinal metaplasia and identify those in need of endoscopic surveillance. Because CDX2 expression is an early event in intestinal differentiation, its presence and that of LI-cadherin indicates that the molecular machinery for intestinal differentiation is in place and therefore the expression of these 2 proteins might actually be more sensitive for the diagnosis of Barrett's esophagus and its upstream lesions than routine histologic evaluation for the presence of goblet cells.

However, this is not a prospective study, so it still has to be examined if this speculation proves true. A further limitation of our data is that the expression of both markers is no reliable indicator for different degrees of dysplasia. Although LG-IEN and HG-IEN showed a slightly stronger expression of both markers than nondysplastic Barrett mucosa, there was no significant difference. Therefore, in routine histopathologic diagnostics, both markers will not greatly facilitate the often difficult decision between Barrett's esophagus with or without dysplasia. They might be useful again to decide between HG-IEN and invasive carcinoma, as the expression of both was significantly reduced in the invasive lesions. The aforementioned markers p53 and ki-67 do not perform much better in that respect: Most studies used them as predictive markers for the progression to high-grade lesions[40,41] and therefore cannot be compared with our study, which does not aim at making a prediction. Both markers do not facilitate the differentiation between Barrett esophagus without and with low-grade dysplasia, but are expressed in significantly more cells in high-grade dysplasia and in carcinoma according to Lörinc et al,[42] whereas according to Feith et al[44] only ki-67 expression differs between Barrett esophagus with and without dysplasia, whereas p53 expression does not.[44] In both studies, even among high-grade lesions there are cases with low or no expression of p53 and ki-67. In accordance with Phillips et al[18] we observed LI-cadherin and CDX2 expression also in nongoblet columnar epithelial cells (Figures 1B to 1E), supporting the hypothesis of CDX2 driving the process of intestinalization with more and less activity remarkable in the heterogenous expression patterns. Concordant with the observations in adenocarcinomas of the stomach[19,28] and those of the esophagus,[18] the intensity of the CDX2 and LI-cadherin staining varied with the degree of differentiation: poorly differentiated and invasive esophageal adenocarcinoma exhibited weaker, patchy, or no staining. A possible explanation for this phenomenon may be that cancer cells become less differentiated during progression, resulting in a downregulation of CDX2 and LI-cadherin. In tumors, oncogenes are activated and the CDX2 gene has been found to be downregulated by the oncogenic RAS in the human colonic cell lines Caco-2 and HT-29 via activation of the PKC pathway.[45] But in contrast to Phillips et al,[18] we did not observe decreased CDX2 staining in high-grade dysplasia, but rather increase of expression when compared with nondysplastic Barrett's epithelium.

In summary, this study confirms that both CDX2 and, as never described before, LI-cadherin are sensitive markers of intestinal differentiation in the upper gastrointestinal tract and may be useful in the diagnosis of Barrett's esophagus. Additionally, we have demonstrated the presence of LI-cadherin besides CDX2 protein in cases of low-grade and high-grade dysplasia and adenocarcinoma, the latter with loss or decrease of detectable proteins. Whether both molecules may serve as positive predictors for the

development of esophageal adenocarcinoma because of their strongest expression in high-grade IEN requires further, preferably prospective studies.

Declaration of Conflicting Interests

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