

Altered expression of CD44 and DKK1 in the progression of Barrett's esophagus to esophageal adenocarcinoma

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Abstract Barrett's esophagus (BE) is an acquired condition in which the normal lining of the esophagus is replaced by intestinal metaplastic epithelium. BE can evolve to esophageal adenocarcinoma (EAC) through low-grade dysplasia (LGD) and high-grade dysplasia (HGD). The only generally accepted marker for increased risk of EAC is the presence of HGD, diagnosed on endoscopic biopsies. More specific markers for the prediction of EAC risk are needed. A tissue microarray was constructed comprising tissue samples from BE, LGD, HGD, and EAC. Marker expression was studied by immunohistochemistry using antibodies against CD44, DKK1, CDX2, COX2, SOX9, OCT1, E-cadherin, and β -catenin. Immunostaining was evaluated semi-quantitatively. CD44 expression decreased in HGD and EAC relative to BE and LGD. DKK1 expression increased in HGD and EAC relative to BE and LDG. CDX2 expression increased in HGD but decreased in EAC. COX2 expression decreased in EAC, and SOX9 expression increased only in the upper crypt epithelial cells in HGD. E-cadherin expression decreased in EAC. Nuclear β -catenin was not significantly different between BE, LGD, and HGD. Loss of CD44 and gain of DKK1 expression characterizes progression from BE and

LGD to HGD and EAC, and their altered expression might indicate an increased risk for developing an EAC. This observation warrants inclusion of these immunohistochemically detectable markers in a study with a long patient follow-up.

Keywords Barrett's esophagus ·
Esophageal adenocarcinoma · Dysplasia

Introduction

Barrett's esophagus (BE) is an acquired condition defined as the replacement of normal stratified squamous epithelium by metaplastic columnar epithelium in the distal esophagus [1–3]. BE is acquired due to prolonged gastro-esophageal reflux disease (GERD) which is increasing in incidence [4]. BE is a preneoplastic condition as it predisposes to the development of esophageal adenocarcinoma (EAC) via progression along low-grade dysplasia (LGD) and high-grade dysplasia (HGD). EAC is rapidly increasing in the western world; the incidence has increased over 70% in the last 20 years [5]. Almost without exception, EAC develops in BE [6] and, therefore, early recognition of this condition and endoscopic follow-up are potentially effective ways to prevent EAC.

BE evolves into EAC stepwise through increasing grades of dysplasia. As yet, the marker used for clinical decision making on eventual therapeutic intervention in a BE patient is the presence of HGD in endoscopic biopsies (during the follow-up of BE patients or a newly diagnosed GERD patient). In the presence of HGD, the patient runs a risk of over 40% to develop an EAC, and HGD is therefore an indication for an intervention, which used to be esophagectomy, but lately, more conservative approaches

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such as photodynamic therapy or mucosectomy have been developed [7, 8]. An important problem, however, is that of all patients with BE, only about 0.5% per year will eventually develop an adenocarcinoma [9]. A large number of patients are therefore kept under surveillance for the detection of a relatively small number of cancers. Consequently, markers are needed for the prediction of cancer risk in BE patients before HGD has developed.

Various attempts have been made to identify markers that can predict whether or not a BE patient will develop an EAC. Approaches chosen have included morphometry, cell adhesion molecule expression, DNA ploidy analysis, loss of heterozygosity at the chromosomal level, p53 mutations and immunohistochemical overexpression, proliferative activity (through immunohistochemical staining of the Ki-67 antigen), p16 anomalies (including promoter methylation, mutations, and loss of heterozygosity), activation of the Wnt pathway (through adenomatous polyposis coli mutations, β -catenin mutations, or one of the other players in Wnt signaling), analysis of patterns of promoter methylation, and more [10–16]. Although a voluminous literature exists on this subject, as yet, the only clinically used biomarker with a high predictive value is the presence of HGD [10].

In this study, we addressed this problem by examining the expression of genes involved in the Wnt pathway, Wnt downstream genes and key homeobox signaling pathway genes in the normal esophagus, BE with and without dysplasia, and EAC. The genes included in the study were selected based on their involvement in directing differentiation, including tissue architecture, in the gastrointestinal tract. The importance of these genes in gastrointestinal cancer has been repeatedly stipulated but almost never in BE or in EAC [13, 17–23]. Their expression was studied in retrospectively collected tissue samples selected from surgical resection specimens. The potentially predictive markers must subsequently be validated in a study on Barrett patients with long-term follow-up.

Materials and methods

Selection of cases

The cases selected concerned a consecutive series of esophagectomy specimens received by the University Institute of Pathology in Lausanne, diagnosed as EAC between 1995 and 2007 (52 cases). Cases with insufficient tissue available (either having been used in previous studies or used in the diagnostic work-up) were excluded. In all, 44 cases were available for study. After re-assessment of the diagnosis by two experienced pathologists (WS and FB), 31 cases were retained based upon the following criteria:

presence of at least one or more areas of adenocarcinoma and/or HGD and/or LGD and/or intestinal metaplasia. The use of human tissues in this study was according to the criteria for the use of archival specimens as established by the local ethics review board.

Tissue microarray creation

Tissue samples to be included in the tissue microarray (TMA), notably with EAC, HGD, LGD, and BE, were identified on H&E stained tissue sections. From the 31 cases, 122 different tissue samples were included. Control tissue samples included in each TMA block comprised samples randomly selected from surgical resection specimens (normal ileum, normal duodenum, normal colon, normal esophagus, adenocarcinoma of the colon, and diffuse type and intestinal type adenocarcinoma of the stomach).

The TMAs were created in paraffin-embedded blocks of 2% agarose (Sigma A5093) mounted on a cassette using a manual TMA maker (Beecher Instruments Inc., Sun Prairie, WI, USA), as described previously [24]. Each TMA consisted of a 6×7 grid of 2 mm cores, allowing up to 41 tissue cores per TMA (one varying position was left empty for TMA recognition and proper orientation). Overall, four TMA blocks were constructed including 158 cores (64 EAC, 17 HGD, 19 LGD, and 22 BE samples and 36 control tissue samples). Sections (4 μ) were cut, stretched on a 56°C water bath, and mounted on SuperFrost Plus microscope slides (Menzel GmbH & Co KG, Braunschweig, Germany). On one H&E stained section, a final reference diagnosis was made for each tissue core (WS and FTB); this diagnosis was used in the study.

Selection of antibodies

Our selection of the target proteins was based upon the hypothesis that Wnt signaling and Wnt downstream genes play a key role in EAC development, in analogy to gastric and colorectal cancer. A first selection was made on the involvement of Wnt target genes in human gastrointestinal carcinogenesis as mentioned on the Wnt homepage (<http://www.stanford.edu/~rnusse/wntwindow.html>). A final selection was made based upon the published literature supporting potential involvement of the various proteins in gastrointestinal mucosa differentiation or in intestinal carcinogenesis [13, 18, 19, 25–31] and on the available antibodies applicable to routinely processed tissue specimens. As a readout protein for Wnt pathway activation, nuclear localization of β -catenin was used. As Wnt downstream proteins, we chose CD44, COX2, E-cadherin, CDX2, DKK1, and SOX9. Two homeobox proteins (CDX2 and OCT1) were included in the study, based on earlier reports indicating involvement of these proteins in intestinal metaplasia/dysplasia [21, 25, 30, 31].

Immunohistochemistry

Immunohistochemistry (IHC) was performed either manually or using an automated system (as indicated in Table 1). The choice of using one system or the other was based upon the quality of the staining obtained, some antibodies requiring a specially adapted protocol not easily adaptable to the staining machine.

Manual immunoperoxidase staining was performed, after dewaxing, quenching of endogenous peroxidase activity (by incubation with 3% hydrogen peroxide in demineralized water for 5 min), and antigen retrieval (2 min in a pressure cooker in ethylenediaminetetraacetic acid (EDTA) buffer, pH 9.0), using Envision+ (DAKO, Glostrup, Denmark) as detection system [24]. Exposure to primary antibodies, diluted as indicated in Table 1, was 2 h at room temperature. Visualization was performed with diaminobenzidine tetrachloride as chromogen. Sections were counterstained with hematoxylin.

Automated IHC was performed using the Bond IHC stainer (Vision BioSystems, Mount Waverley, Australia) with as secondary reagent, anti-goat Histofine max polymer (Nichirei Biosciences, Tokyo, Japan). Key program sequences were antigen retrieval (with EDTA buffer pH 9.0 for 20 min at 95°C or citrate buffer pH 9.0 for 20 min at 100°C), primary antibody incubation for 30 min, and incubation with the polymer kit for 15 min. In all experiments, negative control incubations (omission of the primary antibody) and positive control tissues were included.

Assessment of immunoreactivity

The immunoreactivity was microscopically evaluated independently by two observers (WS and PY) in terms of the percentage of cells stained (in deciles). Given the zonal architecture of BE mucosa, the percentage of positive cells was noted separately for the upper crypt half and the lower crypt half in BE, LGD, and HGD but only as a percentage

of all cells in EAC. No major discrepancies occurred between the two assessors; the mean of the two assessments was used for statistical calculations.

Data was entered in SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA), and descriptive analysis was undertaken, together with a one-way ANOVA test to assess statistical significance between the values obtained for localization and diagnostic category. For comparison between BE, LGD, and HGD, upper and lower crypt half were considered separately. For comparisons with EAC, the highest (either upper or lower) crypt value was used. Differences between diagnostic categories were analyzed using Tukey's honestly significant difference post hoc test, graphically visualized by box-plot graphs. In the graphs, for the sake of clarity, outliers have been indicated separately (with the symbols *white circle* or *filled star* in the figures, the numbers referring to the outlying sample number), but the data points were included in the statistical calculations. The number of samples available precluded the use of validated statistical approaches (receiver operating characteristic (ROC) analysis) to determine eventual cut-points. To explore potential diagnostic use of these markers, we arbitrarily chose 30% loss of CD44 (absence of membranous immunostaining in at least 30% of the cells) and 30% gain of DKK1 (cytoplasmic staining of at least 30% of cells) as cut-points.

Results

The results obtained by scoring of the immunostained TMAs are illustrated in the figures and summarized in Table 2. We will briefly describe immunoreactivity patterns per studied antigen.

CD44 and DKK1

In general, CD44 immunoreactivity was found on the plasma membrane of epithelial cells. A minority of the

Table 1 Antibodies used with their dilution, antigen retrieval method, and immunohistochemistry method

Antigen	Source	Clone	Species	Concentration	IHC method
β-catenin	Novocastra	17C2	Mouse	1:100	A
CD44	CHUV	Wild-type PCA	Mouse	1:50	A
COX2	Cayman	synth pept	Mouse	1:1,000	B
DKK1	Lifespan	PCA	Rabbit	1:100	B
E-cadherin	DAKO	NCH-3.8	Mouse	1:40	A
CDX2	Novocastra	AMT28	Mouse	1:50	A
OCT1	LabVision	PCA	Rabbit	1:50	A
SOX9	Chemicon	PCA	Rabbit	1:400	A

A: manual Envision system (DAKO, Glostrup, Denmark), heat-induced epitope retrieval: pressure cooker 2 min EDTA pH 9.0

B: bond automated IHC system (Vision BioSystems, Mount Waverley, Australia), heat-induced epitope retrieval in EDTA pH 9.0
PCA polyclonal antibody

Table 2 Semiquantitative results of immunohistochemical staining

Antibody		BE		LGD		HGD		EAC		<i>p</i> value	Significant between diagnoses
		Mean	SD	Mean	SD	Mean	SD	Mean	SD		
CD44	Crypt	86	18	85	13	53	30			<0.001	BE and LGD vs HGD
	Upper	44	33	46	36	40	36			0.843	
	Total	86	18	85	13	47	36	48	37	<0.001	
DKK1	Crypt	19	12	21	20	49	28			0.001	BE and LGD vs HGD
	Upper	11	13	19	26	29	33			0.084	
	Total	16	15	21	25	35	37	51	35	<0.001	
CDX2	Crypt	50	27	59	23	61	26			0.264	BE vs HGD HGD vs EAC
	Upper	32	25	43	33	63	23			0.005	
	Total	48	28	48	33	66	23	33	34	0.002	
OCT1	Crypt	33	26	42	28	47	27			0.320	
	Upper	20	23	26	28	32	30			0.349	
	Total	28	29	35	31	41	32	29	30	0.446	
SOX9	Crypt	95	4	94	2	95	5			0.720	BE and LGD vs HGD
	Upper	81	11	79	21	93	5			0.010	
	Total	95	4	89	22	96	4	89	15	0.100	
COX2	Crypt	93	15	93	21	94	12			0.982	BE, LGD, and HGD vs EAC
	Upper	91	14	90	23	96	10			0.582	
	Total	95	8	93	21	96	10	73	24	<0.001	
E-cadherin	Crypt	100	1	100	0	100	0			0.480	BE, LGD, and HGD vs EAC
	Upper	100	1	100	0	100	0			0.490	
	Total	100	1	100	0	100	0	79	31	<0.001	
β-catenin	Cytoplasm	97	11	96	18	72	33	63	34	<0.001	BE and LGD vs EAC
	Nuclear	0	0	1	3	1	3	6	14	0.150	

Results of descriptive and statistical analysis for obtaining *p* values using ANOVA test. A Tukey's honestly significant difference post hoc test was performed for assessing the significance of the differences between diagnoses. As statistically significant, we considered $p < 0.05$. Mean and SD values are noted in percentages

epithelial cells expressed CD44 circumferentially; the vast majority showed focal membrane expression. Stromal cells also stained strongly but were not included in the cell count. Significant loss of CD44 staining of crypt epithelial cells was found in HGD in comparison with BE and LGD (Fig. 1a–c). This was also found for CD44 staining in HGD and EAC vs BE and LGD, as graphically illustrated in Fig. 1d.

DKK1 stained the cytoplasm with appreciable differences in intensity between samples. EAC showed more intense immunoreactivity (Fig. 2a–c). More lower crypt cells expressed DKK1 in HGD than in BE and LGD. In EAC, strong diffuse cytoplasmic immunoreactivity was noted. Overall, DKK1 expression was significantly higher in HGD and EAC than in BE and LGD (Fig. 2d; Table 2).

Given the consistent differences in CD44 and DKK1 expression notably between LGD and HGD, we chose as arbitrary cut-points 30% loss of CD44 and 30% gain of DKK1 expression. With these parameters, the combination of loss of CD44 and gain of DKK1 was only found in HGD samples but with low (29%) sensitivity for HGD (Table 3).

Altered expression of at least one marker attained high specificity and sensitivity (94% and 80%, respectively).

CDX2

CDX2 was only expressed in the nuclei of epithelial cells. EAC often stained diffusely. CDX2 expression was significantly higher in upper crypt epithelium in HGD than in upper crypt epithelium in BE (Fig. 3a–c; Table 2). Overall, CDX2 expression was significantly lower in EAC than in HGD (Fig. 3d; Table 2).

SOX9

SOX9 showed a nuclear staining pattern with limited variation between tissue samples. In some HGD cases, strong SOX9 staining was noted in upper crypt epithelium (Fig. 4a, b). SOX9 expression was not strikingly increased in each HGD case but, overall, significantly higher in HGD than in BE or LGD (Fig. 4c).

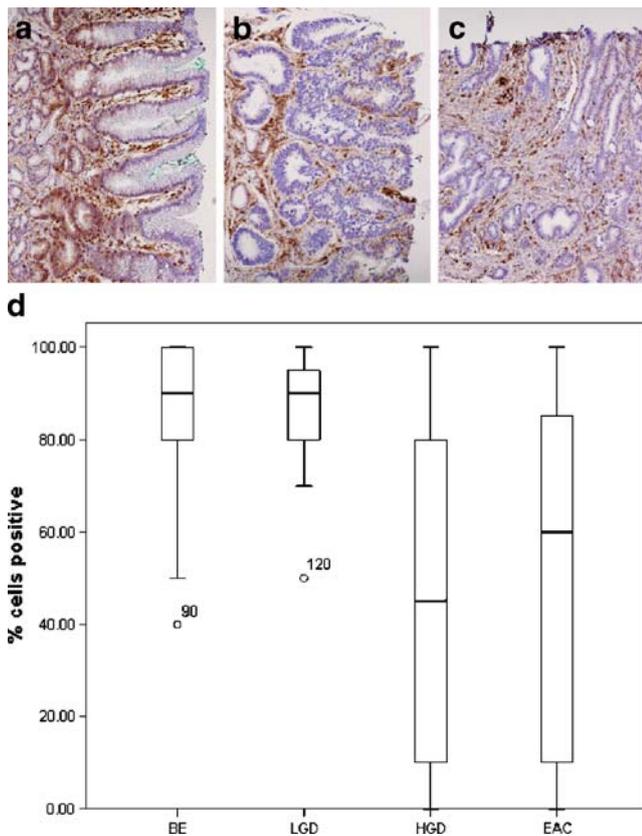


Fig. 1 CD44 staining in BE (a), HGD (b), and EAC (c). Crypt cells in BE show strong membranous staining, which is lost in HGD and EAC. Note that stromal cells also show CD44 immunoreactivity. **d** Box plot of the percentage of CD44 positive cells per diagnostic category (BE and LGD vs HGD and EAC, $p < 0.001$)

COX2

COX2 showed strong immunoreactivity in the cytoplasm of epithelial cells (Fig. 5a, b). In EAC, expression was lower (Fig. 5c). In BE, LGD, and HGD, COX2 expression was significantly higher than in EAC (Fig. 5d). COX2 was also expressed in inflammatory cells, notably macrophages, but this was not taken into account in scoring the results.

OCT1

OCT1 was expressed in nuclei of epithelial and stromal cells. In BE, crypt cell nuclei were most intensely stained. Significant differences were not noted between LGD and HGD or EAC (data not shown).

E-cadherin

E-cadherin was strongly immunostained with a membranous pattern in all samples with the exception of EAC. In EAC, staining was decreased and very heterogeneous (data

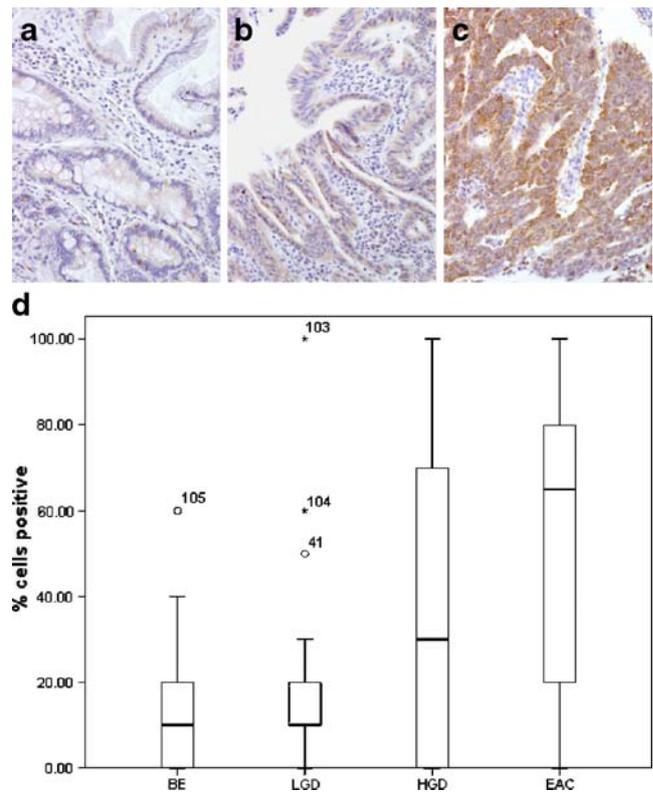


Fig. 2 DKK1 expression in BE (a), HGD (b), and EAC (c). Cytoplasmic DKK1 expression was focal and weak in BE, more widespread in HGD, and strong in EAC. **d** Box plot of percentage of cells expressing DKK1 per diagnostic category (BE and LGD vs EAC, $p < 0.001$)

not shown), some areas retaining E-cadherin expression and others with complete loss. In the invasion front in EAC, individual invasive cells did not show membranous staining but occasionally cytoplasmic staining.

Table 3 Sensitivity and specificity of CD44 loss and DKK1 gain for the differentiation between LGD and HGD

Marker expression	Histology		
	HGD	LGD	Total
CD44L/DKK1G	5	0	5
CD44L/DKK1N or CD44N/DKK1G	11	4	15
CD44N/DKK1N	1	15	16
Total	17	19	36

CD44L=CD44 loss (more than 30% of cells)

CD44N=normal pattern of CD44 expression

DKK1G=gain in DKK1 expression (more than 30% of the cells)

DKK1N=less than 30% DKK1 expression

Specificity of CD44L/DKK1G for HGD 5/5=100%

Sensitivity of CD44L/DKK1G for HGD 5/17=29%

Specificity of at least one aberrant marker for HGD 16/17=94%

Sensitivity of at least one aberrant marker for HGD 16/20=80%

Specificity of CD44N/DKK1N for LGD 15/19=79%

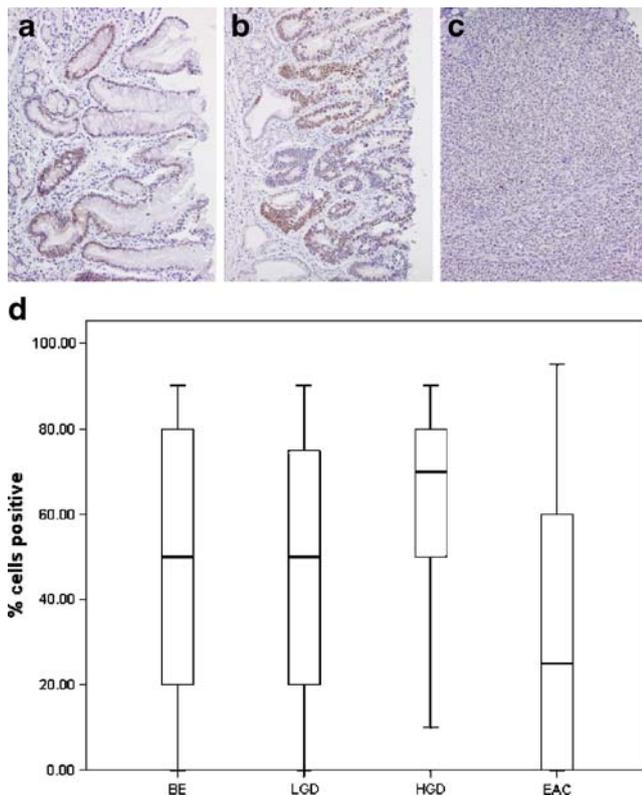


Fig. 3 CDX2 expression in BE (a), HGD (b), and EAC (c). Nuclear expression was observed in BE in the crypt epithelium, extending to upper crypt epithelium all the way to the surface in HGD. CDX2 expression was decreased in EAC. **d** Box plots of the percentage of CDX2 positive cells per diagnostic category (BE vs HGD, $p=0.005$; HGD vs EAC, $p=0.002$)

β -catenin

In BE and LGD, strong membranous staining was found. BE and LGD showed a higher percentage of β -catenin positive cells than EAC. Nuclear staining was found almost exclusively in EAC (Fig. 6). Statistical analysis indicated a significant decrease in membranous β -catenin expression in EAC (Table 2).

Discussion

The aim of this study was to identify proteins that show altered expression during the progression of BE to EAC, with as final goal the definition of markers that will allow the identification of BE patients with a high risk for EAC development. Gene expression profiling studies with this aim have been published [32] but, as yet, have not resulted in significant progress.

In the design of the study, several elements merit brief consideration. Firstly, the choice of the material studied: we conducted the study on a limited number of tissue samples (122) from 31 patients. We do not consider the small

number of patients an important limitation in our study, as the intention was to identify promising markers rather than validating them in a long-term follow-up case control approach. Therefore, the endpoint we used was not patient outcome but histological diagnosis per sample. Further studies would have to include BE and LGD samples from patients who did not develop HGD or EAC.

A second consideration is our choice to score immunoreactivity in BE, LGD, and HGD separately in the lower and the upper crypt. This division is somewhat arbitrary and could be subject to inter-observer variation. We attempted to improve the reproducibility of the scoring results through implication of two independent observers. These obtained very similar results, confirming the reliability of our observations. A further consideration is the way we compared BE, LGD, and HGD with EAC. As in EAC, mucosal architecture is completely lost, rendering lower and upper crypt scores without meaning; we compared scores in EAC with the highest score (either upper crypt or lower crypt) in LGD and HGD for our statistical evaluations. We

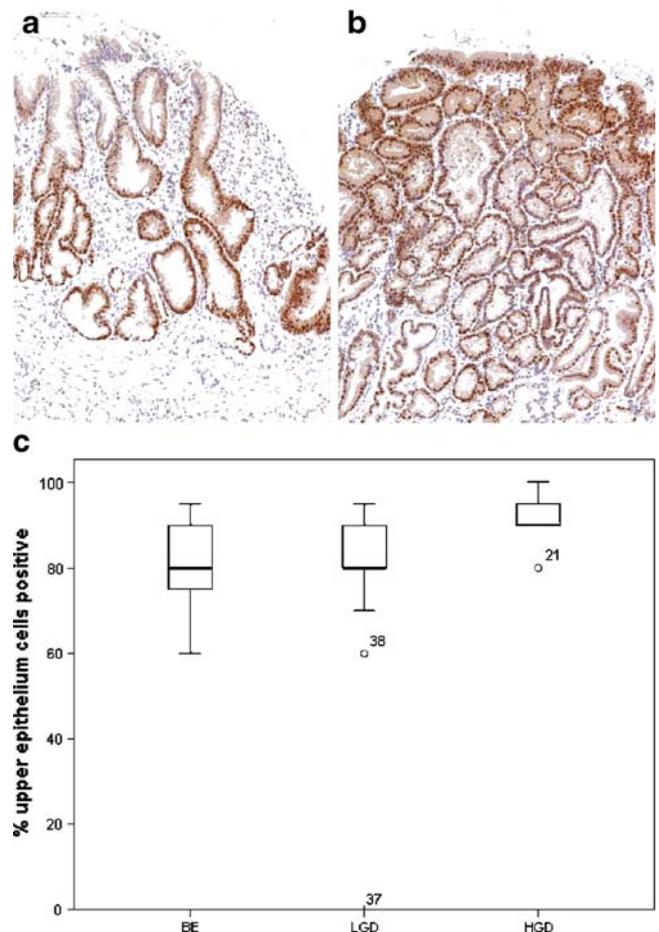


Fig. 4 Nuclear SOX9 expression was observed in BE mainly in the lower crypt region (a) but in HGD, extended all the way to the surface (b). **c** Box plot of the percentage SOX9 positive cells per diagnostic category (BE and LGD vs HGD and EAC, $p=0.01$)

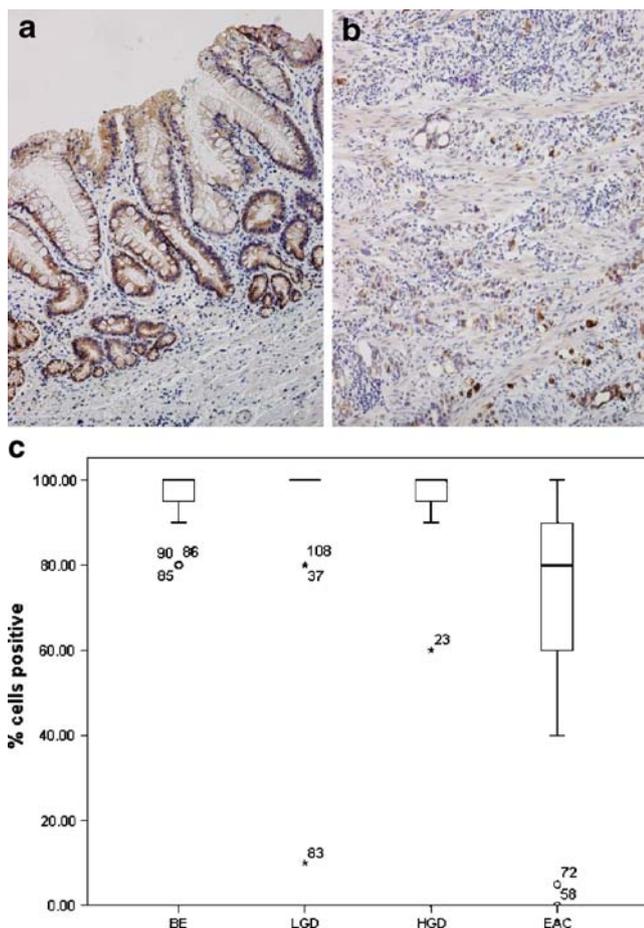


Fig. 5 Diffuse cytoplasmic COX2 expression was found in BE (a), LGD, and HGD. The percentage of COX2 positive cells was decreased in EAC (b). **c** Box plot of the percentage of COX2 positive cells per diagnostic category (BE, LGD and HGD vs EAC, $p < 0.001$)

deem this justified as no important differences were noted when the mean of the two values was used.

Finally, we scored immunoreactivity as a continuous variable rather than in terms of positive or negative, as cut-points could not be established using validated statistical approaches (e.g., ROC analysis). Using this experimental design, we found the expression of several of the studied markers to differ significantly between BE, LGD, HGD, and EAC. Taking as an arbitrary cut-point 30% loss of CD44 and 30% gain of DKK1 expression, the combination of these two markers seems promising.

CD44 and DKK1

CD44 has been extensively studied in colorectal cancer [33] and, also, in BE where decreased expression of wild-type CD44 in glandular epithelium was reported [34, 35] with a shift from focal in BE to more diffuse in BE with dysplasia. We found wild-type CD44 expression to have significantly decreased in the crypt base epithelium only in HGD, which

is in contrast to the findings of Menges et al. [35, 36]. Also, in EAC, we noted a decreased level.

Dickkopf-1 (DKK1) is a Wnt antagonist protein that specifically inhibits Wnt canonical signaling by interacting with the co-receptor Lrp-5/6 and, thus, prevents Wnt and Frizzled from forming a ternary complex with Lrp-5/6 [37]. Expression of members of the DKK1 family has been studied before in colorectal cancer, showing a differential expression pattern between normal colon epithelium and colorectal cancer [29]. However, epigenetic silencing of DKK1 has been reported in colorectal cancer [38, 39]. DKK1 has been studied before in reflux esophagitis and in BE but not in EAC [40]. We found DKK1 expression to have increased significantly in HGD and EAC in comparison with BE and LGD. This result is counterintuitive, as silencing of its repressor conceptually should go along with activated Wnt signaling. Overexpression of a repressor could, however, also signify activation downstream of its site of action, sustained expression being the result of a defective feedback loop. It has in addition been reported that DKK1 may inhibit tumorigenesis through Wnt pathway independent mechanisms.

In all, loss of CD44 and gain of DKK1 expression might be taken as indicators of the progression from LGD to HGD. This is a novel finding and warrants detailed study, including confirmation by western blotting and reverse transcription polymerase chain reaction, as a marker for progression of BE to EAC.

CDX2, OCT1, SOX9, and COX2

The caudal-related homeobox gene CDX2 encodes an intestine-specific transcription factor crucial for the regulation of differentiation of intestinal cells [21]. Immunostaining of CDX2 has been proposed as a useful marker for the identification of intestinal metaplasia in BE [15, 25, 31, 41]. Overall, we observed increased CDX2 expression in

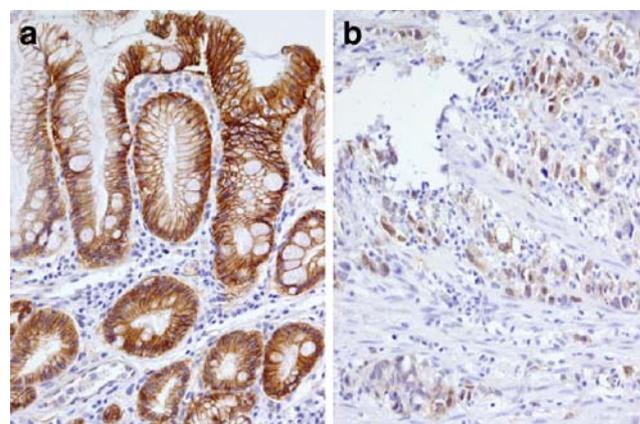


Fig. 6 β -catenin expression shows a strong membranous immunostaining pattern in BE (a) but a nuclear staining pattern in EAC (b)

HGD relative to BE and LGD, which then decreases in EAC. The widely varying values in individual cases preclude the use of CDX2 for the diagnosis of HGD. Our observations match those of Villanacci et al. [25]. It has been reported that CDX2 expression is modulated by OCT1, but we did not find OCT1 expression to differ significantly between the diagnostic categories, suggesting that the changes in CDX2 expression we observed are not due to concomitant changes in OCT1 expression, as previous research suggests [21]. How CDX2 and OCT1 interact remains largely unclear, and more research on this subject has to be conducted.

SOX genes constitute a family of transcription factors, belonging to a super-family known as the high mobility group box and play an important role in, among others, the development of the intestinal tract [42]. SOX9 has been reported to be regulated by the Wnt pathway, and it represses CDX2 expression in intestinal crypts [43].

We found SOX9 expression to have slightly but significantly increased in upper crypt epithelium in HGD. For SOX9 and CDX2 to both show increased expression in the upper crypt epithelium in HGD was an unexpected result, given the repressive influence of SOX9 on CDX2 expression [43]. In the regulation of CDX2 expression, evidently, other factors than only SOX9 are involved. The wide variation in expression of SOX9 in individual cases precludes its use for diagnostic purposes.

COX2 was shown to be functionally active in BE since treatment with COX2 inhibitors reduced proliferative activity of BE cells in culture as well as of EAC cells [2]. COX2 polymorphisms have been reported to be associated with increased risk for EAC [44]. An effect of the polymorphism studied (the 8473 C allele) on the immunohistochemical expression level was, however, not reported. We found the percentage of COX2 expressing cells were significantly reduced in EAC, relative to the other disease categories. This matches the results obtained by Villanacci et al. [25], who attributed the lower expression of COX2 to the poor differentiation grade of the EAC in their series. In our group of EAC, the number of poorly differentiated EACs was also high. Our data, notably the absence of any difference in COX2 expression between BE, LGD, and HGD, imply that COX2 is not a potential marker for progression of BE towards EAC.

E-cadherin and β -catenin

E-Cadherin belongs to the family of the cadherin transmembrane proteins, which play an important role in cell adhesion notably in the formation of adherence junctions through homotypic interactions. Earlier studies on the involvement of E-cadherin in the development of EAC suggest that there is decreased expression with progressive degrees of dysplasia [26, 45]. Although we were unable to

reproduce the differences between LGD and HGD, we did find a significant decrease in EAC relative to HGD. E-cadherin is more likely related to the development of invasive activity and, as such, a marker of EAC. A role in the discrimination between BE and LGD on one hand and HGD on the other is unlikely.

Nuclear β -catenin is an indicator of canonical Wnt activation. When accumulating in the nucleus, β -catenin forms a complex with lymphoid enhancer factor (LEF1), T-cell factor (TCF4), and other transcription factors [13]. Unlike in many other carcinomas, mutations in the β -catenin gene do not play a role in EAC [46], the mechanisms involved in Wnt activation having been incompletely resolved to date. The key regulators of the Wnt pathway in EAC are probably different from those in other carcinomas as is stated by Clement et al. [12]. We found no statistically significant differences in β -catenin expression between BE and LGD or HGD. The Wnt pathway is likely to be involved in the development of EAC [12, 13] but in EAC invasion rather than in the progression from BE to dysplasia.

In conclusion, our data indicate that loss of CD44 and gain of DKK1 expression characterizes the transition from LGD to HGD, which might go along with an increased risk for developing an EAC. This observation warrants their inclusion into a follow-up study. The first goal of this study would be to confirm our findings in a larger series of cases. Ultimately, when these findings are confirmed, these markers should be included in a prospective clinicopathological study with long-term patient follow-up to confirm their significance as indicators for an increased risk for the development of EAC from BE.

Conflict of interest We declare that we have no conflict of interest.

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