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ORIGINAL ARTICLE

Toll-like receptor 9 expression in the natural history of Barrett mucosa

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Abstract Increased expression of TLR9 in esophageal adenocarcinoma and squamous cell carcinoma correlates with poor prognosis. We have explored the expression and suspected that TLR9 activation might contribute to pathogenesis in esophageal columnar metaplasia-dysplasia-neoplasia sequence, and hence, we have studied the usefulness of TLR9 as a marker for dysplasia. We have determined the expression of TLR9 in specimens with normal esophagus (n=89), gastric (n=71), or intestinal metaplasia (n=56) without dysplasia, and low-grade (n=51) or high-grade dysplasia (n=40), and esophageal adenocarcinoma (n=88). We observed linearly increasing TLR9 expression in specimens to be associated with change from normal epithelium to columnar metaplasia and further to dysplasia. ROC curve analysis showed clinically irrelevant sensitivity of 71 % and specificity of 67 % for TLR9 intensity in detection of low-grade dysplasia. Membrane-associated TLR9 expression detected by immunohistochemistry and immunofluorescence was predominantly associated with foveolar-type dysplasia as detected by HE staining (p=0.015). TLR9 is expressed in Barrett's esophagus, and dissolution of TLR9 staining increases from nondysplastic epithelium to dysplastic. TLR9 may serve as a new way of recognizing the histopathological origin of dysplasia (adenomatous vs foveolar) with observed subcellular pattern of TLR9.

Keywords Toll-like receptor 9 · Barrett's esophagus · Esophageal columnar dysplasia · Columnar metaplasia · Gastric pathway · Intestinal pathway · Immunohistochemistry · Immunofluorescence

Introduction

Incidence of esophageal adenocarcinoma in western world is rising rapidly with low survival rates even after initially curative surgery [1]. Most important risk factor for adenocarcinoma is Barrett's esophagus defined as replacement of normal squamous epithelium with columnar metaplastic cells [2, 3]. The estimated risk for Barrett's esophagus progression is less than 1 % per year [4, 5]. However, when low-grade dysplasia is detected in biopsy samples, progression rate to high-grade dysplasia or adenocarcinoma rises up to 13 % per year [6, 7]. Diagnosis of low-grade dysplasia leads to endoscopic surveillance with an interval rate of 6–12 months [8]. For more efficient identification of low-grade dysplasia, new biomarkers are needed.

Toll-like receptors (TLR) are pathogen-associated molecular pattern (PAMP)-recognizing receptors. The innate immune system recognizes and senses invasion of microorganism via TLRs [9, 10]. The expression of TLRs has also been reported in various cancers, such as prostate, colon, esophageal, and tongue cancer [11–15]. TLR9 is localized in endoplasmic reticulum, where translocation for ligand recognition to the endosomal-lysosomal compartment occurs [10]. TLR9 recognizes CpG sequences within bacterial DNA [10,

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Table 1 Baseline characteristics of patients with esophageal adenocarcinoma (EAC), high-grade dysplasia (HGD), and low-grade dysplasia (LGD)

Patientclinical data	EAC *n*=88	%	HGD *n*=11	%	LGD *n*=21	%	
Age at diagnosis							
<60 years	27	31	5	46	5	24	
60–65 years	21	24	2	18	4	19	
>65 years	40	46	4	36	12	57	
Sex							
Male	72	82	9	82	13	62	
Female	16	18	2	18	8	38	
Lesiontype							Total
NE	68		8		13		89
GM	49		8		14		71
IM	39		5		12		56
LGD	24		6		21		51
HGD	29		11				40
EAC	88						88

Other evaluated lesions from the patients were normal epithelium (NE), gastric metaplasia (GM), and intestinal metaplasia (IM)

A total of 88 patients with EAC, 11 patients with HGD and 21 with LGD are pictured in the table. Several lesions were analyzed from single patient but no more than one of each type

16] and also endogenous ligands [14]. TLR9 expression is associated with poor prognosis in patients with esophageal adenocarcinoma and squamous carcinoma [13, 17]. We [18] and others [17] have shown that TLR9 ligands induce esophageal cancer cell invasion in vitro. The mechanisms by which TLR9 acts in carcinogenesis remain unclear. The increased expression of TLR9 in various neoplasia leads to suggestion that TLR9 may also overexpressed in dysplastic lesions and it may play a role in carcinogenesis.

Increased expression of TLR9 has been reported in oral epithelial dysplasia [19], in esophageal squamous cell dysplasia [20], and recently in Barrett's metaplasia dysplasia adenocarcinoma sequence [21]. The aim of the study was to determine TLR9 expression in normal esophagus, esophageal columnar metaplasia, dysplasia, and esophageal adenocarcinoma. We also wanted to evaluate if TLR9 could be used as a marker for esophageal columnar low-grade dysplasia and in determination of cell lineage of dysplastic columnar epithelium.

Fig. 1 Microphotographs showing expression of TLR9 in different esophageal lesions. **a** Gastric metaplasia with a strong reaction polarized to the basal cytoplasm and membrane staining in majority of epithelial cells. **b** Intestinal metaplasia with less evidence of staining polarization and absence of membrane staining in most cells. **c** High magnification detail of polarized TLR9 staining in intestinal (*left*) and gastric (*right*) metaplasia. **d** Gastric metaplasia and low-grade dysplasia with retained membrane staining and of some loss of polarization of TLR9 expression as shown by extension of TLR9 expression even to apical parts of the cells. **e** Intestinal metaplasia and low-grade dysplasia showing intensive and diffuse TLR9 staining in the lower part of the figure. **f** Gastric metaplasia and high-grade dysplasia showing presence of membrane expression in majority of cells and diffuse staining extending to apical parts of the cells. **g** Intestinal metaplasia and high grade dysplasia. TLR9 staining is mainly cytoplasmic and extending to apical parts of the cells. **h** High magnification detail of lost polarization in dysplasia. **i** Adenocarcinoma with a weak TLR9 expression in the tumor epithelium. **j** Adenocarcinoma with strong TLR9 expression. **k** Normal ventricle epithelium with moderate TLR9 expression. **l** Normal duodenum with strong TLR9 expression

Table 2 Association of strong and weak membrane expression of TLR9 in esophageal columnar dysplasia with expression of gastric (MUC5AC) and intestinal (CDX2) specific markers and intestinal (adenomatous) or gastric (foveolar) histological morphology

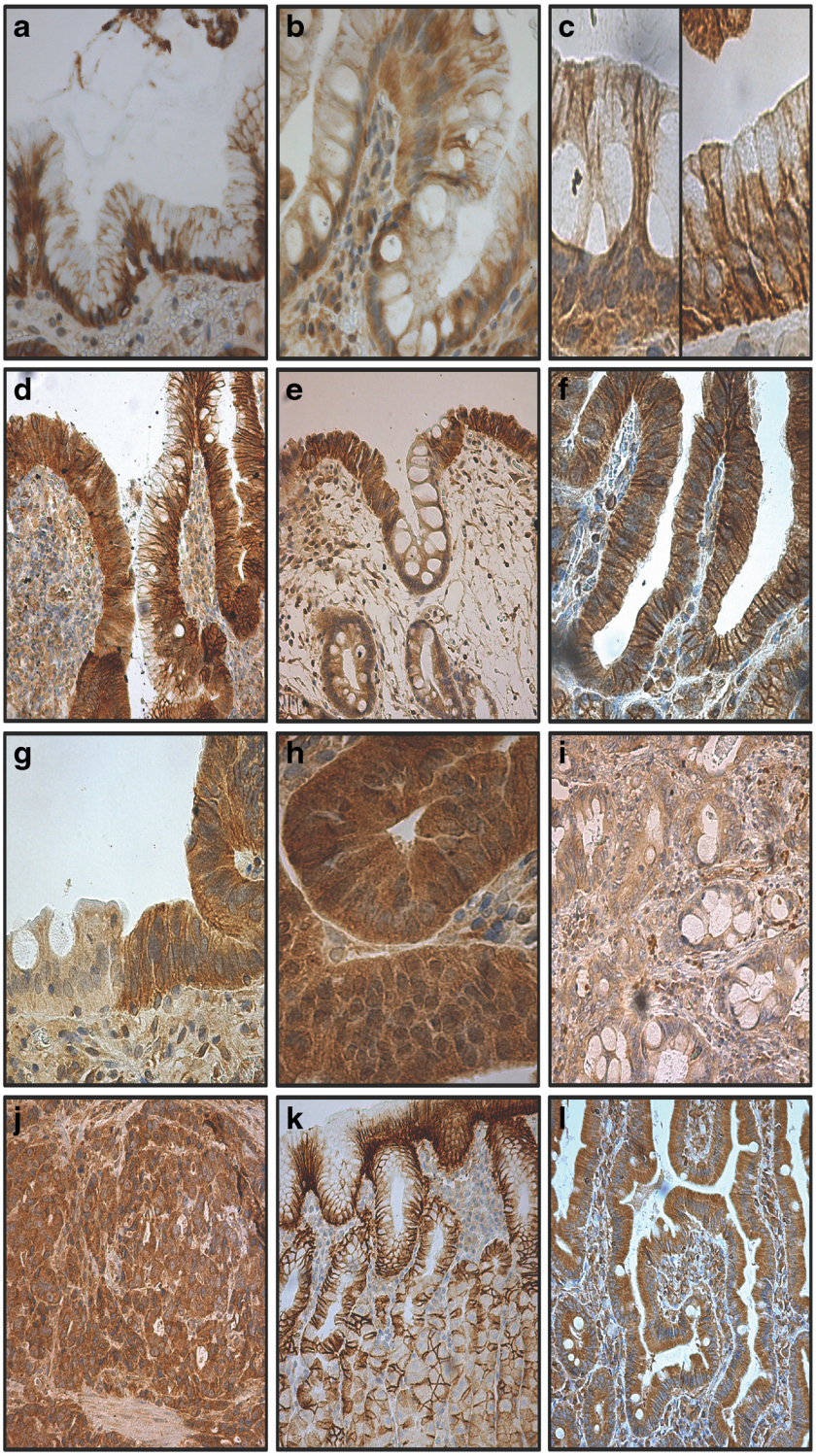
Dysplasia[a]	Membrane expression[b]		
MUC5AC	Strong TLR9 *N*=8	Weak TLR9 *N*=9	*p* value[c]
intensity	2.0 (SD 1.0)	1.3 (SD 0.6)	0.186
percentage	83 (SD 41)	47 (SD 35)	0.385
CDX2			
intensity	2.0 (SD 1.0)	2.7 (SD 0.4)	0.282
percentage	82 (SD 34)	93 (SD 10)	0.053
HISTOLOGY	*N*=9	*N*=10	
Adenomatous	4	9	
Foveolar	4	0	0.015

Presented values are medians and standard deviations (SD)

[a] Dysplasia included 9 low-grade and 10 high-grade dysplasia. There were no statistical differences between dysplasia grades, and therefore, these were combined. Paraffin blocks were available for MUC5AC and CDX2 staining only from 17 patients

[b] Weak membrane expression was defined as percentage of ≤10 % of cell with membrane expression, and strong expression as ≥57 % with membrane expression

[c] Significance of membrane expression and MUC5AC or CDX2 were tested with Mann-Whitney *U* and in histological morphology Pearson's χ^2 was used



Materials and methods

Patients The use of the samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee and by the National Authority for Medicolegal Affairs (VALVIRA). Paraffin-embedded archival specimens of esophageal adenocarcinoma or dysplasia of esophageal columnar metaplasia were collected from the Department of Pathology, Oulu University Hospital, between the years 1987–2009. The esophageal adenocarcinoma series has been previously described [13, 22]. The final series consisted of 88 patients with esophageal adenocarcinoma, 11 with high-grade

dysplasia, and 21 with low-grade dysplasia as the most advanced lesion. All other evaluated lesions were obtained from these same patients (Table 1). The median age of the patients was 65 years (range 38–93). For comparison of TLR9 expression in esophageal intestinal and gastric metaplasia and that in normal duodenal and gastric mucosa, we also stained additional specimens from selected cases. Relationship of TLR9 and survival of the cancer patients has been described previously [13] and therefore has been left out from this report.

Assessment of dysplasia High- and low-grade dysplasia of columnar epithelium were distinguished by the presence of less severe cytological abnormalities, and no or very mild architectural abnormalities in the latter [23, 24]. Diagnosis of dysplasia was confirmed by expert gastrointestinal pathologists re-evaluating the type of the lesions, which had been originally analyzed according to the routine diagnostic protocol by two pathologists working in the University Hospital of Oulu. Twenty cases of dysplasia were also classified from hematoxylin-eosin stained samples to foveolar (gastric) or adenomatous (intestinal) type dysplasia by morphological features previously documented [25–27].

Immunohistochemistry Formalin-fixed and paraffin-embedded sections were pretreated by heating with microwaves in Tris-EDTA (pH 9) 15 min (TLR9, CDX2) or sodium citrate (pH 6) for 10 min (MUC5AC) for antigen retrieval. A representative tissue block was selected for immunostaining on the basis of hematoxylin-eosin stained section. The immunohistochemistry was performed with mouse monoclonal antibodies against TLR9 (1:150, IMG-305A, Imgenex, San Diego, CA, USA), CDX2 (1:200, ab76541, Abcam, UK), and MUC5AC (1:200, NCL-MUC-5 AC, Novocastra, Leica BiosystemsNewcasIteHd). Incubation time for primary antibodies was 60 min for TLR9 and 30 min for CDX2 and MUC5AC. For immunohistochemical detection of the antibody reaction, Dako Envision kit (Dako, Copenhagen, Denmark) was used. Diaminiobenzidine (Dako basic DAB-kit) was used as a chromogen. All stainings were done with Dako Autostainer (Dako, Copenhagen, Denmark). Validation of our immunohistochemical analysis was performed through two series of negative controls by omitting the primary antibody and by replacing primary antibody with mouse primary antibody isotype control.

Immunofluorescence Formalin-fixed and paraffin-embedded esophageal sections were deparaffinized followed by treatment with 1 % Triton X-100 in PBS for 5 min. Non-specific staining was blocked by treatment with 1 % bovine serum albumin for 20 min. Incubation with primary antibodies for 30 min at 37 °C or 120 min at room temperature was then performed. The primary antibody used was mouse monoclonal antibody against TLR9 (1:150 dilution) (IMG-305A, Imgenex, San Diego, CA, USA). After several washes, Alexa Fluor 488 or Alexa Fluor 568 conjugated to goat anti anti-mouse IgG (Life Technologies) was applied at appropriate dilutions and incubated for 60 min at 37 °C. Samples were mounted with Immumount (Thermo Scientific) and examined by using a Zeiss LSM510 confocal microscope. Images were analyzed with LSM510 Pascal software (Carl Zeiss, Jena, Germany).

Assessment of TLR9 immunostaining Immunoreaction was analyzed by three independent researchers (H.H., O.H., and T.J.K.) blinded for the clinical data. The overall staining intensity in epithelial cells was assessed on a four point scale (0=negative; 1=weak; 2=moderate; 3=strong). In addition, the proportion of positive cells and membrane staining were assessed. This evaluation was performed separately in all types of lesions visible in each specimen described in Table 1. All available lesions were analyzed from all patients, but no more than one of each type from a single patient [13, 22].

Assessment of MUC5AC and CDX2 immunostaining To get insight whether the characteristic expression differences of TLR9 seen between gastric and intestinal metaplasia would show similar discriminating features between foveolar and adenomatous dysplasia, a subset of dysplasia samples with high and low membrane expression were re-evaluated by an expert gastrointestinal pathologist (T.J.K.). Samples were divided into groups of adenomatous and foveolar dysplasia according to histological morphology. Evaluator was blinded from TLR9 membrane expression data. Group selection was based on strong (percentage of membrane positive cells ≥57 %) or weak (≤10 %) membrane expression of TLR9. To further characterize cell lineage of dysplastic epithelium with low or high membrane staining, we stained these samples representing dysplasia for gastric (MUC5AC) and intestine-specific (CDX2) markers and evaluated intensity and percentage of cells (Table 2). This evaluation was performed blinded from TLR9 data.

Statistical analysis For statistical analyses, we used IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. To compare TLR9 expression between different lesions, we used Kruskall-Wallis due to skewed distributions. To define the sensitivity and specificity of TLR9 expression in differentiating between metaplasia and dysplasia, we used ROC-curve analysis. Mann-Whitney *U* was used in evaluating the association of TLR9 membrane expression to gastric and intestinal specific markers. To evaluate correlation between TLR9 membrane expression and dysplasia histological type (adenomatous/foveolar), Pearson's $\chi 2$ test was used. To test differences of intensity between HGD and T1a-bN0M0 adenocarcinoma, Mann-Whitney *U* was used.

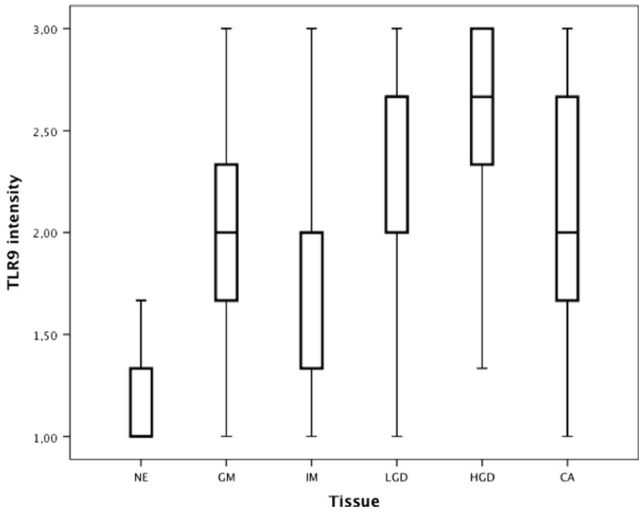


Fig. 2 Distribution of TLR9 expression intensity in normal esophageal squamous epithelium and in different esophageal columnar lesions. *The bottom and top of the box* are the first and third quartiles. *The band inside the box* is median and whiskers show the min and max of the data

Results

Characteristics of TLR9 expression in normal esophageal squamous epithelium and in nondysplastic columnar lesions In normal esophageal squamous epithelium, TLR9 expression was predominantly cytoplasmic and diffuse and at least weak expression in some squamous epithelial cells was present in all cases. Expression was most often present in the basal third of the epithelium. Intensity of cytoplasmic staining was weaker compared to columnar nondysplastic epithelial cells (Figs. 1 and 2; Table 3).

In esophageal intestinal metaplasia and gastric metaplasia, cytoplasmic expression was usually strongest in the basal third of the cells and expression turned gradually weaker to the apical part of the cell showing weak cytoplasmic expression (Fig. 1). As compared with intestinal metaplasia, intensity of TLR9 expression was significantly higher in gastric type metaplasia as was the extent of membrane expression ($p<0.001$; $p<0.001$; Fig. 2; Table 3). In the control samples from the normal duodenal and gastric mucosa, the expression was higher in duodenum than in the gastric epithelium (intensity score 3 vs 2) and in intestinal smaples extended to apical cytoplasm of the enterocytes (Fig. 1). Normal duodenal mucosa showed higher expression than Barrett's intestinal metaplasia, whereas similar stainings were observed in normal gastric mucosa and esophageal gastric type metaplasia.

Expression of TLR9 evaluated with immunofluorescence To confirm the subcellular localization of TLR9 expression observed by immunohistochemistry, we used an immunofluorescence marker with confocal microscopy and studied gastric type metaplasia samples with high membrane expression, samples of intestinal metaplasia and carcinoma without membrane staining according to immunohistochemical stainings. Gastric type metaplasia showed clear expression of TLR9 on the cell membranes (Figs. 1a and c, 3c and 4), whereas intestinal metaplasia and adenocarcinoma were negative for membrane staining. In contrast, nuclear staining of TLR9 as occasionally seen in immunohistochemistry was constantly absent in all lesions according to IF analysis.

Characteristics of TLR9 expression in dysplastic lesion Barrett's epithelium Expression was significantly more extensive and intensive in dysplastic columnar epithelium as compared with columnar metaplasia without dyspalsia. Nearly all dysplastic cells expressed TLR9, and moderate to strong expression was most prevalent (Figs. 1 and 2; Table 3). In dysplastic cells, expression was more often diffuse extending homogenously throughout the cell cytoplasm with no apparent basal polarization as seen in benign columnar metaplastic

Table 3 Baseline characteristics of TLR9 expression in normal esophageal squamous epithelium and in different esophageal lesions

	Intensity	25th–75th	Percentage	25th–75th	Membrane	25th–75th
Normal epithelium	1.0□○◊	1.0 – 1.3	100□◊	93 – 100	3.3□○	0 – 20
Gastric metaplasia	2.0□○	1.7 – 2.3	97□○◊	97 – 100	87□○◊	77 – 90
Intestinal metaplasia	2.0□○♦	1.3 – 2.0	100■●♦	100 – 100	27■○◊	17 – 50
Low-grade dysplasia	2.7♦	2.0 – 2.7	100	100 – 100	17●◊	10 – 33
High-grade dysplasia	2.7◊	2.3 – 3.0	100♦	100 – 100	7	0 – 28

Intensity was assessed with a four point scale from negative (0) to weak (1), moderate (2), and strong intensity (3). The extent of the staining was expressed as percentage of positive cells and positive cell membranes (0–100 %). Values are presented as median and25th–75th percentiles

■Compared to low-grade dysplasia, $p<0.05$

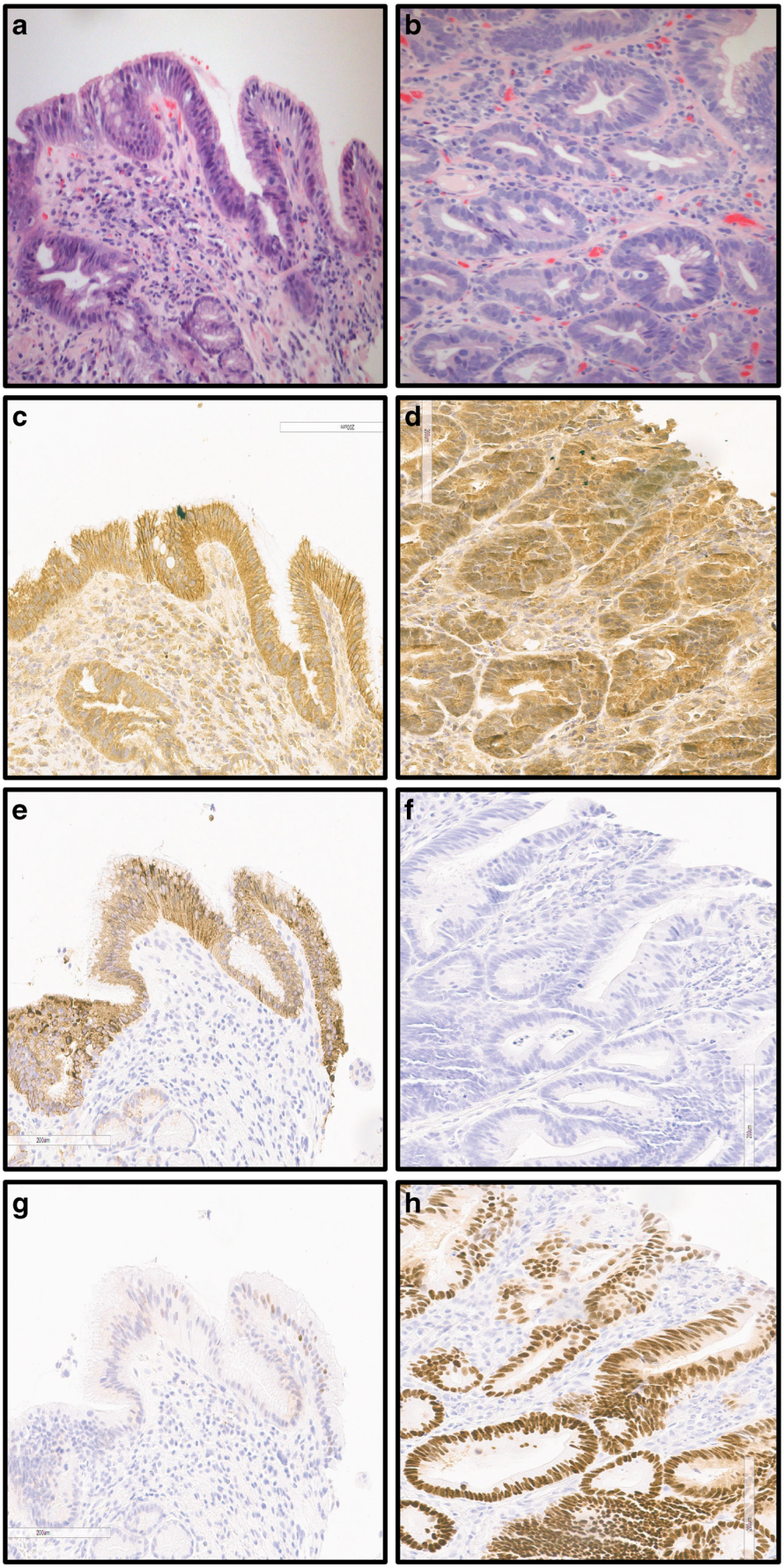
●Compared to high-grade dysplasia, $p<0.05$

♦Compared to adenocarcinoma, $p<0.05$

□Compared to low-grade dysplasia, $p<0.001$

○Compared to high-grade dysplasia, $p<0.001$

◊Compared to adenocarcinoma, $p<0.001$


a
b
c
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e
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g
h

◀ **Fig. 3** TLR9 expression patterns and histopathological origin of esophageal columnar dysplasia. **a**, **c**, **e**, **g** From the same representing a foveolar (gastric) type dysplasia; **b**, **d**, **f**, **h** from the same sample representing a adenomatous (intestinal) type dysplasia. **a** Hematoxylin-eosin staining showing features of foveolar type of dysplasia. **b** Hematoxylin-eosin staining showing features of adenomatous type dysplasia. **c** TLR9 staining in foveolar dysplasia shows strong and extensive TLR9 membrane expression with no apparent basal polarization. **d** TLR9 staining in adenomatous dysplasia shows lack of TLR9 membrane expression and diffuse cytoplasmic staining. **e** MUC5AC staining shows strong positive expression on dysplastic cells consistent with foveolar differentiation. **f** Absence of expression of MUC5AC in dysplastic cells consistent with intestinal differentiation. **g** Absence of CDX2 staining in foveolar dysplasia. **h** Strong positive CDX2 staining in adenomatous type dysplasia

epithelial cells (Fig. 1; Table 3). Median percentage of membrane positive cells in dysplastic lesions (n=91) was 13 % with a high standard deviation of 24 and range from 0 to 87 %, indicating that the membrane expression varied extensively between different cases of dysplasia.

TLR9 expression in differentiation of nondysplastic and dysplastic columnar epithelium We used a receiver operating characteristic curve (ROC curve) analysis to determine the optimal cutoff values and to test the sensitivity and specify of TLR9 staining in distinguishing Barrett's dysplasia from metaplasia. When we assessed the diagnostic value of intensity of expression, ROC curve analysis showed an optimal TLR9 intensity of 2.2 to differentiate between subjects with low-grade dysplasia and columnar metaplasia; 69 of the 91 dysplastic lesions and 42 of the 127 columnar metaplasias had TLR9 expression higher than this cutoff value. Accordingly, sensitivity of TLR9 staining in distinguishing low-grade dysplasia from metaplasia was 71 % and the specificity 67 %.

We also assessed whether the adjacent adenocarcinoma affected the staining of nondysplastic and dysplastic lesions found in these patients. We found no statistical differences in staining intensity, percentage of positive cells, or membrane positivity when compared to patients without adenocarcinoma (data not shown).

TLR9 expression in esophageal adenocarcinoma All esophageal adenocarcinomas expressed TLR9. Staining intensity was significantly weaker in adenocarcinoma than in dysplasia. Staining was dominantly cytoplasmic and diffuse with no visible polarization (Fig. 1; Table 3). Association of TLR9 with clinicopathological variables has been previously reported [13] and indicates that advanced tumor stage was associated with high TLR9 expression. In addition to previous work [13], we compared TLR9 expression in HGD with that in T1N0M0 adenocarcinoma. Median TLR9 intensity in patients with HGD (n=11) was higher (2.7; 25th–75th percentiles 1.8–2.9) than in patients with T1N0M0 adenocarcinoma (1.7; 25th–75th percentiles 1.0–2.2; p=0.04). Furthermore, we divided T1 adenocarcinoma to T1a (N=4) and T1b (N=5). A similar trend for higher expression in HGD as compared to T1a adenocarcinomas was seen (p=0.294), and significant difference in comparison of HGD and T1b (p=0.038). T1a and T1b carcinomas showed nearly similar intensities of TLR9 expression (median 1.7 and 1.7; p=0.800).

TLR9 membrane staining in detection of the histopathological origin of dysplasia Considering the abundant TLR9 membrane expression constantly present in nondysplastic gastric metaplasia (Fig. 1; Table 2) and the variation in the extent of membrane expression dysplastic epithelium, we hypothesized that TLR9 membrane expression could be used to distinguish the histopathological origin of dysplasia, i.e., adenomatous or foveolar [26]. We divided dysplasia cases in two categories according to membrane expression. Weak TLR9 membrane expression was observed in 38 dysplasia and strong in 12 dysplasia samples. In total, 19 randomly selected cases representing the groups with different pattern of membrane expression were taken in to analysis of cell lineage (Table 2).

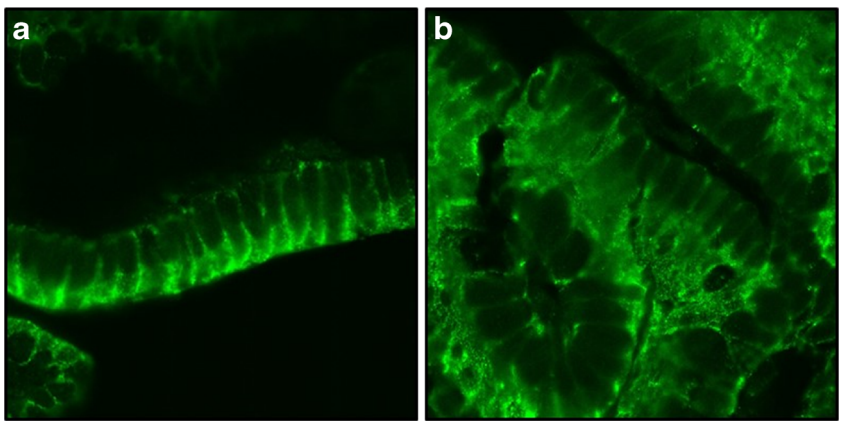

Fig. 4 Immunofluorescence staining of gastric metaplasia with clear membrane staining of TLR9. Similar membrane pattern was observed also with immuhistochemistry

Abundant TLR9 membrane expression associated with foveolar type dysplasia as detected by HE staining (p=0.015; Fig. 3, Table 2). There was a nonsignificant trend for association between gastric marker (MUC5AC) intensity and strong TLR9 membrane expression (p=0.186) and, respectively, between intensity and percentage of positive cells of intestinal marker (CDX2) and weak TLR9 membrane expression (p=0.282; p=0.053; Table 2).

Discussion

In this study, we report for the first time the immunohistochemical expression of TLR9 in intestinal and gastric metaplasia and in dysplastic lesions of Barrett's esophagus. We show that TLR9 immunoreaction linearly increases from normal squamous epithelium to columnar metaplasia and further to dysplasia. In addition, we show that gastric and intestinal metaplasia present with a characteristic subcellular localization of TLR9 expression, and present preliminary evidence that this lineage specific difference may be retained even in dysplasia in Barrett's esophagus.

TLR9 expression was constantly present in esophageal gastric and intestinal metaplasia. Expression in metaplastic gastric epithelium in the esophagus was similar to that in normal gastric mucosa both showing high expression on cell membranes with predominant expression in the basal parts of the cells and differing significantly from the expression in normal duodenal epithelium. Duodenal sample showed highest expression of all analyzed samples differing from less intensive expression Barrett's intestinal metaplasia. More studies are needed to evaluate the variation of TLR9 expression in the duodenal mucosa and to assess whether such difference between duodenal enterocytes and esophageal intestinal metaplasia is related with differences in the regulation of expression or different exposure to ligands. In nondysplastic esophageal gastric metaplasia, TLR9 expression was usually present in the lateral cell membranes of the surface and foveolar epithelium. Similar pattern has been documented in normal gastric mucosa [28, 29]. In addition to expression in lateral cell membranes, the expression was accentuated in basal parts of epithelial cells, and there was clear polarization in staining. In contrast, in esophageal intestinal metaplasia, the subcellular localization of TLR9 was predominantly cytoplasmic while expression in the lateral cell membranes was only rarely present.

Previous studies about TLR9 expression in intestinal epithelium are not conclusive. In intestinal metaplasia of the gastric mucosa, TLR9 expression was not detected [28], but normal human colon epithelium showed cytoplasmic as well as cell membrane expression [30] and normal murine duodenal epithelium showed expression in the apical cell membrane [31]. Although it is possible that esophageal intestinal metaplasia is biologically different in terms of TLR9 expression, these discrepancies may also be related with methodological differences including those in primary antibody and the detection methods.

The expression of TLR9 was strongest in Barrett's associated dysplasia. Elliott and colleaques describe increased TLR9 expression in Barrett's esophagus and adenocarcinoma compared to normal epithelium. High TLR9 expression was also associated with survival in metastatic disease, although specific information was not included in their congress abstract [21]. Similar increase of TLR9 expression has been reported in esophageal squamous cell dysplasia [20] but not in low-grade dysplasia of the gastric mucosa [28]. In addition, in dysplastic lesions, subcellular distribution of TLR9 expression changed to diffuse, and basal polarization seen nondysplastic columnar epithelium was lost. We have recently shown that similar alteration of subcellular expression pattern for TLR5 accompanies development of dysplasia [22]. Accordingly, increase of TLR expression levels may be a common reaction pattern in epithelial premalignant lesions of the GI tract and may support the role of luminal agents in their pathogenesis.

ROC curve analysis showed that moderate to high intensity of TLR9 indicates low-grade dysplasia with 71 % sensitivity and 67 % specificity. However, the optimal discriminating expression intensity score of 2.2 may not be practical in clinical work since evaluation was performed with integers from 0 to 3. Also, median intensity of nondysplastic intestinal metaplasia (2.0) was close to suggested optimal cutoff value. Regardless of these caveats, intensive TLR9 expression highlights dysplastic regions (Fig. 1) and may be of help to spot foci for a closer inspection. Currently, predictive markers for neoplastic progression in Barrett's esophagus are needed [32]. Aberrant or overexpression of p53 and overexpression of Ki67 appears to be more powerful predictors for neoplastic progression than histological diagnosis of low-grade dysplasia [33–35]. Although TLR9 seems to play a role in neoplastic progression of Barrett's esophagus, additional studies including follow-up studies are needed to confirm the role of TLR9 in progression of dysplasia.

As the subcellular TLR9 expression patterns differed between nondysplastic gastric and intestinal metaplasia, we assessed the potential of subcellular TLR9 staining patterns in the differentiation gastric or intestinal origin the dysplastic lesions. Our analysis showed that extensive TLR9 membrane expression seen in nondysplastic gastric metaplasia was associated with the gastric type dysplasia, while weak or absent membrane staining and extensive cytoplasmic staining typical for intestinal metaplasia was characteristic for intestinal type dysplasia, when classification of cell lineage of dysplasia was recognized by conventional criteria based on H&E staining [26]. However, when we used immunohistochemical markers for gastric (MUC5AC) and intestinal differentiation (CDX2)

of dysplasia, we detected only a nonsignificant trend for association of the two staining patterns of TLR9 with the cell lineage of dysplastic epithelium (Table 2). In addition to low number of cases included in this analysis, heterogenous and commonly mixed expression of gastric and intestinal lineage markers reported in Barrett's dysplasia [26, 33] might explain the lack of significant association. However, our observations suggest that TLR9 has a potential for a marker for recognition of cell lineage of dysplastic columnar epithelium.

Association of high TLR9 expression and adverse prognosis or high tumor stage has been reported in several cancer types [13, 14, 17, 19, 28, 29, 36, 37]. However, the role of TLR9 expression in the development and progression of premalignant lesions has not been studied, and role of TLR9 in carcinogenesis in Barrett's esophagus remains speculative. According to our data, the significant decline in expression occurs between HGD and $T_1N_0M_0$ adenocarcinoma, thereafter starting to rise again toward carcinoma with adverse prognosis [13].

Transformation of the microbiome in Barrett's metaplasia from gram-positive to mostly gram-negative bacteria has been described [38, 39], but there are no studies of microbiome in Barrett's dysplasia. We have previously shown that bacterial DNA induces invasion of cancer cell lines, and that interaction of bacterial DNA with TLR9 is an important mechanism of this induction [18]. Interestingly, for some cell lines, the level of invasive response was dependent on the bacterial species suggesting that microbiome shift might modify TLR9 response [18]. Additionally, there might be indirect links between the microbiome shift and TLR9 based on amplifying cross talk between TLR4 and TLR9 [40]. Accordingly, we speculate that shift to dominance of gram-negative bacteria would be accompanied by LPS-induced upregulation of TLR4 and secondary upregulation of TLR9. Also, the changes in the subcellular localization of TLR9 observed in the present study might promote the progression of the carcinogenic cascade, since the direction of access of TLR ligands may affect the reaction type. For example, apical access of TLR9 ligands promotes suppression of inflammatory response [41] and regulatory Th1 effector immune response [42]. More studies are needed to assess the significance of microbiome changes and increased expression and alteration of subcellular distribution of TLR9 in the progression of Barrett's esophagus, and even explain different progression tendencies of gastric and intestinal metaplasia.

In conclusion, we have showed that expression of TLR9 linearly increases in columnar esophageal dysplasia declining again in $T_1N_0M_0$ adenocarcinoma. Our assessment of subcellular localization of TLR9 expression indicates that presence of expression in the lateral cell membranes tends to mark gastric and the lack of this pattern intestinal cell lineage of the dysplastic epithelium. However, further studies are needed to confirm these findings and to estimate the value of assessment of TLR9 expression in prospective follow-up of patients with Barrett's esophagus or in an independent patient material. Finally, our findings favor the idea that interaction of TLR9 and the luminal ligands of TLR9 may have a pathophysiological role in the progression of Barrett's lesions.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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