Toll-Like Receptor 9 Is a Novel Biomarker for Esophageal Squamous Cell Dysplasia and Squamous Cell Carcinoma Progression

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Key Words

Epithelium • Pattern recognition receptors • Toll-like receptor 9 • Esophagus • Squamous cell dysplasia • Squamous cell carcinoma

Abstract

Background: Stimulation of Toll-like receptor 9 (TLR9) has been linked to invasion in various cancer cells in vitro. We investigated TLR9 expression in normal, dysplastic and malignant esophageal squamous epithelium. **Methods:** TLR9 expression was analyzed by immunohistochemistry in 46 cases of esophageal squamous cell carcinoma, including 12 cases with adjacent squamous dysplasia and 24 cases with normal esophageal epithelium. TLR9 expression was compared with tumor grade, stage, proliferation, apoptosis and vascular density. **Results:** In normal esophageal squamous epithelium, TLR9 staining intensity decreased linearly from the basal layers to the superficial layers ($p < 0.001$). Strong TLR9 expression was detected across full thickness of high-grade dysplasia, the intensity clearly differing from the normal squamous epithelium and squamous cell carcinoma ($p < 0.001$). All squamous cell carcinomas exhibited TLR9 expression that was positively associated with a high grade

($p < 0.05$), the presence of lymph node metastases ($p < 0.05$) and previously undetected distant metastases ($p < 0.05$). **Conclusions:** Expression of TLR9 in the basal parts of normal esophageal epithelium suggests a role related to cell proliferation and differentiation. TLR9 upregulation detected in dysplastic epithelium and in disseminated carcinomas indicates that this protein may serve as a novel marker for esophageal squamous dysplasia and carcinoma with metastatic potential.

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Introduction

Toll-like receptors (TLR) are transmembrane, pathogen-recognizing receptors that mediate innate immune responses when exposed to pathogen-associated molecular patterns, such as bacterial lipopolysaccharide or microbial RNA or DNA [1]. Thus far, 13 TLRs have been identified in mammals and 10 in humans [2].

H.T. and J.H.K., as well as P.P.L., J.S. and T.J.K. contributed equally to this work.

Members of the TLR9 subfamily (TLR7 to TLR9) and TLR3 are expressed intracellularly, while the other TLRs are expressed on the cell surface. TLR9 is localized to the endoplasmic reticulum, from where it is translocated for ligand recognition to the endosomal/lysosomal compartment. This endosomal localization is essential in avoiding the recognition of 'self'-nucleic acids [3]. Initially, TLR9 was reported to recognize unmethylated CpG sequences within bacterial DNA [4]. Recent findings suggest that in addition to the microbial DNA and synthetic CpG-ODN-containing ligands, also vertebrate DNA or self-DNA can be recognized by TLR9, and this contributes to the pathophysiology of various autoimmune diseases such as psoriasis and systemic lupus erythematosus [5–8]. Currently, very little is known about the regulation of TLR9 expression and its actual role in cancer cells.

High expression of TLR9 has been detected in various cancers, for example lung, breast, gastric and prostate cancers [9–12]. Furthermore, treatment of TLR9-expressing cancer cells with synthetic TLR9 ligands that are reminiscent of bacterial DNA increases the invasion of breast and prostate cancer cells in vitro [10, 11]. Ilvesaro et al. [13] recently demonstrated that this invasive effect is indeed mediated via TLR9. Various TLR subtypes are also widely expressed in the gastrointestinal tract [14–16]. We have previously shown that high TLR9 expression in esophageal adenocarcinoma may indicate metastasis and poor prognosis [17]. In a more complex setting, the CpG nucleotides can be paradoxically used as anticancer reagents [18]. This is due to their capability to activate also the immunological defense mechanisms. Hence the same receptor, if activated in different cell type, can lead to dramatically different consequence.

TLR9 and other bacteria-recognizing receptors are of special interest in the normal and especially in the pathological processes in the esophagus, which is exposed to many exogenous molecules and putative pathogens by the oral route or via the gastroesophageal reflux. Esophageal cancer consists of two main histological types, namely squamous cell and adenocarcinoma. They differ from each other both by etiology and histopathological characters. Adenocarcinoma originates from Barrett's esophagus which develops through dysplasia to invasive cancer while squamous cell carcinoma develops directly from epithelial dysplasia to carcinoma. Esophageal squamous carcinoma is associated with smoking and alcohol consumption [19, 20] which both are known to be associated with alterations in oral microbiome [21, 22].

We hypothesized that the TLR9 could be an important link between bacterial exposure and cancer in the esoph-

agus. In order to address this, we studied TLR9 expression in the normal esophageal squamous cell epithelium and during development and progression of esophageal squamous cell cancer.

Materials and Methods

Patients and Samples

The 51 samples of formalin-fixed and paraffin-embedded esophageal squamous cell carcinoma (diagnosed during the years 1984–2001) were collected from the archives of the Department of Pathology, Oulu University Hospital, Oulu, Finland. Of the tumor specimens, 12 contained areas of high-grade dysplasia, and they were all included in the analysis. In 24 cases, normal, non-dysplastic squamous epithelium was also included in the analysis. The histological diagnosis and grading of the tumors were based on the WHO criteria. Forty-six of the patients underwent esophageal resection and 5 of the samples were biopsies. None of the patients received neoadjuvant therapy. Survival and the other relevant clinical data were obtained from the patients' clinical charts. The patient survival data was acquired from Statistics Finland (Helsinki). The clinical data associated with the cases are shown in table 1. The study was approved by the Ethics Committee of Oulu University Hospital.

Immunohistochemistry

Immunohistochemistry was performed as follows. Sections of 4 µm in thickness representing tumor, dysplastic or nonneoplastic areas were immunostained by a commercial mouse monoclonal TLR9 antibody (IMG-305A; Imgenex, San Diego, Calif., USA) with a dilution of 1:200 [23]. CD31 staining to visualize the blood vessels was performed with a monoclonal mouse IgG1 antibody obtained from DakoCytomation (Glostrup, Denmark; M 0823, clone JC70A, using the dilution of 1:300). Monoclonal rabbit IgG antibody, for detection of apoptotic cells, against active caspase-3 (AF835, lot CFZ33) was obtained from R&D Systems (Minneapolis, Minn., USA) and used at 1:1,000 dilution. For assessment of proliferation, we used the Ki67 mouse monoclonal IgG1 antibody (NCL-Ki67-MM1, clone MM1; Novocastra Laboratories, Newcastle Upon Tyne, UK) using a dilution of 1:25. To validate our immunohistochemical stainings, we did two series of negative controls, by omitting the primary antibody and by replacing primary antibody with primary antibody isotype control. Prior to introduction of primary antibody to the specimen, a high-temperature antigen retrieval was done in citrate buffer (caspase-3) or Tris-EDTA buffer (TLR9, CD31, Ki67). Endogenous peroxidase activity was consumed by immersing the sections in 3% hydrogen peroxide in absolute methanol for 15 min, and to prevent non-specific binding 20% fetal calf serum was used. For immunohistochemical detection of the antibody reaction, the Dako Envision kit (Dako, Copenhagen, Denmark) was used. DAB (Zymed Laboratories Inc., San Francisco, Calif., USA) was used as a chromogen. Counterstainings were done with hematoxylin. We had to exclude 5 patients because specimens containing tumor mass from those patients were not available, resulting in a material of 46 patients with 43 resected and 3 biopsy samples.

Table 1. Baseline characteristics of the patients

	Patients	
	n	%
Age at diagnosis		
<60 years	9	17.6
60–65 years	9	17.6
>65 years	33	64.7
Sex		
Male	23	45
Female	28	55
Tumor grade		
1	3	6.3
2	27	56.3
3	18	37.5
Tumor pT stage		
1	4	8.7
2	9	19.6
3	15	32.6
4	18	39.1
Lymph nodes		
Positive	10	21.7
Negative	36	78.3
Distal metastases		
Positive	9	19.6
Negative	37	80.4
Tumor stage		
I	3	6.5
IIA	19	41.3
IIB	1	2.2
III	14	30.4
IVA	4	8.7
IVB	5	10.9

Assessment of TLR9 Expression

The immunostaining results were evaluated by two investigators (H.T. and J.H.K.). In case of a discrepancy in the assessment, a consensus was reached with a participation of a third investigator (T.J.K.). We assessed the intensity of immunostaining using a four-point scale (0 = absent, 1 = weak, 2 = moderate and 3 = strong) and evaluated the percentage of positive cells in the tumor specimen at 10% accuracy. We also calculated the staining intensity score (histoscore) by multiplying the percentage of TLR9-expressing cells (0–100) with the staining intensity (0–3). In the analysis of normal epithelium, the staining intensity was assessed by using a four-point scale, as described above, and the estimate was based on impression of the whole area of normal squamous epithelium. In addition to the epithelial cells, the presence or absence of TLR9 expression in endothelial cells within the tumor and adjacent to normal epithelium was assessed.

Computerized Analysis of TLR9 in Epithelium

We used the MCID-Image analysis (InterFocus Imaging Ltd., Cambridge, UK) to objectively analyze the staining in normal and dysplastic esophageal squamous epithelium. A threshold level for

the staining intensity and color were first adjusted subjectively to match the staining pattern of DAB. The epithelium was then divided into the basal, middle and luminal layers, each one of them consisting of one third of the thickness of the epithelium. These layers were then analyzed and the percentage of pixels exceeding the threshold for intensity counted resulting in a staining intensity value between 0.000 and 1.000.

Assessment of Cell Proliferation

The determination of cell proliferation activity was based on the assessment of Ki-67/MIB-1 immunoreactive cells. The percentage of positively stained cells within the tumor tissue was evaluated in 10 high-power fields (HPF) for each tumor. Based on the median value of all cases, the proliferation rates were dichotomized to low or high levels of proliferation.

Assessment of Apoptosis

To identify apoptotic cells, 3'-end labeling of DNA (Apop-Tag™ In situ apoptosis detection kit; Oncor, Gaithersburg, Md., USA) was used according to the manufacturer's instructions. Briefly, the sections were dewaxed and dehydrated, after which they were incubated with 20 mg/ml proteinase K (Sigma Chemical Co., St. Louis, Mo., USA) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in PBS, pH 7.2. The slides were then treated with terminal transferase enzyme and digoxigenin-labeled nucleotides, after which the anti-digoxigenin peroxidase solution was applied onto the slides. The color was developed with diaminobenzidine, after which the slides were counterstained with hematoxylin. As a positive control, a hyperplastic lymph node with germinal centers was used. The percentage of apoptotic cells was assessed in 10 HPF in hot spot areas of each tumor. To estimate the percentage of apoptotic cells, the number of tumor cells in an HPF was calculated and the number of apoptotic events in the same HPF was then divided by the number of all tumor cells. Caspase-3 staining was assessed similarly to confirm the result given by 3'-end labeling of DNA, by counting the percentage of apoptotic cells in 10 HPF. For each case, the mean apoptosis rate was determined and the rates were also dichotomized based on the median value of all cases.

Assessment of Vascular Density

The vascular density was estimated as the number of positively stained (CD31) blood vessels in 1 HPF. Any endothelial cell cluster consisting of two or more cells was considered a single, countable microvessel. In each tumor section a minimum of 10 HPF were analyzed.

Statistical Analysis

SPSS for Windows (SPSS Inc., Chicago, Ill., USA) and Prism 4 for Macintosh (Graphpad, La Jolla, Calif., USA) were used for statistical analysis. The significance of associations was determined using Fisher's exact probability test, Pearson χ^2 test, Mann-Whitney test, two-tailed t test and paired-samples t test. Correlations were analyzed by using the Spearman rank correlation test. Survival was analyzed with the Kaplan-Meier curve, and significance of associations with the log-rank test. Probability values of <0.05 were considered significant.

Fig. 1. Computerized quantitative analysis of TLR9 expression intensity in normal and dysplastic esophageal squamous epithelium. TLR9 expression in normal esophageal epithelium (n = 24) is linearly decreased from the basal layer towards the apical layer ($p < 0.001$, paired samples t test). In squamous high-grade dysplasia (n = 12), TLR9 expression was strong in full thickness of the epithelium. HGD = High-grade dysplasia. *** $p < 0.001$.

Results

TLR9 Expression in Normal Esophageal Squamous Epithelium

In normal squamous epithelium, intense cytoplasmic TLR9 immunoreaction was always present in the basal layer. Image analysis confirmed that the intensity of the immunoreaction decreased constantly linearly from the basal layers towards the superficial layers ($p < 0.001$), and the latter were negative in the majority of cases suggesting that in normal esophageal epithelium these cells lose TLR9 expression as they mature (fig. 1, 3a–d).

TLR9 Expression in Squamous Dysplasia of Esophagus

Squamous dysplasia was high grade in all 12 cases. In all of these cases, cytoplasmic TLR9 expression was present throughout the full thickness of the dysplastic epithelium, with moderate to strong intensity. Image analysis confirmed that there was no decreasing basal-luminal gradient as seen in the normal squamous epithelium (fig. 1, 3c–e). Such difference in the staining pattern and intensity allowed us to easily pinpoint the margin between dysplastic and normal squamous epithelium (fig. 3e). The overall expression of TLR9 was more intensive in dysplasia than in normal epithelium ($p < 0.001$) and also higher than in the corresponding carcinomas ($p < 0.001$; fig. 2).

Fig. 2. Estimated TLR9 expression intensity in normal esophageal squamous epithelium and in neoplastic squamous cell lesions. TLR9 staining intensity in dysplasia (n = 12) is significantly stronger than in normal esophageal epithelium (n = 24) or esophageal squamous cell carcinoma (n = 46). Mann-Whitney test. HGD = High-grade dysplasia. *** $p < 0.001$.

TLR9 Expression in Squamous Cell Carcinoma of Esophagus

TLR9 expression was detected in all carcinomas, with weak immunoreactivity in 11/46 (23.9%), moderate in 33/46 (71.7%) and strong in 2/46 (4.3%) cases (fig. 3h, f, j). The reactivity for TLR9 was cytoplasmic, but also some nuclear positivity was observed in 7/46 (15.6%) cases (fig. 3f). TLR9 expression was present in the majority of cancer cells (mean 89.7% of tumor cells).

High-grade (grades II and III) carcinomas exhibited higher TLR9 histoscore than low-grade (grade I) tumors ($p < 0.05$; table 2). Among the patients, the depth of invasion was not associated with TLR9 staining intensity or TLR9 histoscore. Moderate and strong TLR9 intensity were associated with the presence of nodal ($p < 0.05$) or distant metastases ($p < 0.05$; table 2). However, the TLR9 staining intensity was not associated with stage of esophageal squamous cell carcinoma (0–IIa/IIb–IVb; table 2) or with 5-year survival (data not shown).

TLR9 Expression and Tumor Angiogenesis, Proliferation and Apoptosis

There was a negative correlation between TLR9 expression in tumor epithelium as measured by either histoscore or intensity, and the mean vessel density (CD31 staining; $\rho = -0.33$; $p < 0.05$). The mean vessel density in the tumor area was not related with tumor grade, or with

Fig. 3. Hematoxylin-eosin staining and TLR9 immunohistochemical stainings of normal esophageal squamous epithelium (**a, b**; 40 $\times$) demonstrating an intensive expression in the basal third of the epithelium and a gradual decrease in TLR9 expression intensity towards esophageal lumen. In esophageal squamous epithelium-dysplasia transition zone (**c, d**, 40 $\times$; **e**, 100 $\times$; transition marked with arrows, normal esophagus with an asterisk and dysplasia with a plus), an abrupt increase in TLR9 intensity is seen in the dysplasia (**d, e**). Examples of esophageal squamous cell carcinomas with different TLR9 expression patterns (**f-j**). Moderate TLR9 expression and nuclear positivity (**f**; 100 $\times$), weak TLR9 expression (**g, h**; 100 $\times$) and strong TLR9 expression (**i, j**; 100 $\times$) are shown.

Table 2. Relationship between TLR9 immunopositivity and tumor characteristics

Variable	Pa- tients	TLR9 immunopositivity			p
		weak	moderate	strong	
pT					
T _{1, 2}	12	2 (4.7)	10 (23.3)	0	NS
T _{3, 4}	31	9 (20.9)	20 (46.5)	2 (4.7)	
N					
Negative	34	10 (23.3)	24 (55.8)	0	<0.05
Positive	9	1 (2.3)	6 (14)	2 (4.7)	
M					
Negative	35	10 (23.3)	25 (58.1)	0	<0.05
Positive	8	1 (2.3)	5 (11.6)	2 (4.7)	
Stage					
I	3	0	3 (7.0)	0	NS
IIA or IIB	19	5 (11.6)	14 (32.6)	0	
III	13	5 (11.6)	8 (18.6)	0	
IVA or IVB	8	1 (2.3)	5 (11.6)	2 (4.7)	
Caspase 3					
Low	32	9 (22.0)	22 (53.7)	1 (2.4)	NS
High	9	1 (2.4)	8 (19.5)	0	
Ki67					
Low	17	3 (7.3)	14 (34.1)	0	NS
High	24	8 (19.5)	15 (36.6)	1 (2.4)	
Grade	TLR9 histoscore				p
I	150.7				<0.05 ¹
II, III	176.7				

Figures in parentheses are percentages. NS = Not significant. Fisher's exact test was used for p values except where indicated otherwise.

¹ Mann-Whitney test.

presence of lymph node or distant metastases. Endothelial cells within the tumor appeared to be TLR9 positive in 43% (15/35) of cases, while in the area of normal esophagus they were positive in 90% (20/22) of cases ($p < 0.001$).

There was no correlation between TLR9 and proliferation, the percentage of apoptotic cells or caspase-3-positive cells. A positive correlation was found between the percentage of apoptotic cells and caspase-3-positive cells ($\rho = 0.57$, $p < 0.001$), as expected.

Discussion

TLR9-activating CpG nucleotides are being evaluated as immunological anticancer agents [24], but they have also been shown to promote in vitro invasion of various

cancers [11–13, 25]. The aim of our study was to evaluate the expression of TLR9 in normal, dysplastic and neoplastic esophageal squamous epithelium. In the gastrointestinal tract, TLR9 has been linked to inflammation-associated gastric cancer, but the expression and role of TLR9 in esophageal squamous cell dysplasia and cancer have not been established [9]. We detected a constant staining pattern of strong TLR9 expression basally in the normal esophageal squamous cells. Furthermore, we detected that in squamous cell dysplasia this staining pattern changes to an intensive TLR9 expression that traverses the epithelium in full thickness. In addition, we show here that TLR9 expression is constantly present in esophageal squamous cell carcinoma, where strong TLR9 staining also associates with factors related to carcinoma progression.

The biological significance of the TLR9 abundance at the basal layer could be related to the epithelial proliferation and maturation status. We have earlier shown that TLR9 is expressed in mesenchymal stem cells [26]. There are no previous studies on squamous epithelial progenitor cells, but it is obvious that similar mechanisms could exist also in these cells. The fact that the most intensive TLR9 expression was seen in the proliferating basal cell layer might be linked to self-organization of these cells to produce the mature squamous cell structure. During the epithelial maturation and transit of cells toward the esophageal lumen, the expression of TLR9 appeared to decrease and eventually to disappear in the uppermost layers. There are no previous studies on TLR9 expression in esophageal squamous epithelium either. In normal cervical squamous epithelium TLR9 expression has been reported to be present in about 44% of cases, but no information staining pattern was provided [27]. In human skin, predominant TLR9 expression in the upper layers of normal epidermal keratinocytes has been reported [28]. In addition to methodological issues including different monoclonal antibody used, differences might be related to functional differences between keratinizing and nonkeratinizing squamous epithelia. The full biological significance of this decrement in the staining must be clarified in vitro. It is, however, evident that the loss of TLR9 at the apical surface leads to decreased TLR9 signaling and hence to tolerance towards bacterial DNA at the upper cell layer. On the other hand, if bacterial DNA manages to penetrate deeper layers of epithelium, this might inflict cell division and production of new epithelium in response to tissue wear. Interestingly, a similar expression pattern related to differentiation as in esophageal squamous epithelium has been recently reported in

mesenchymal cells. Nurmenniemi et al. [26] discovered that undifferentiated human mesenchymal stem cells express TLR9 and that during differentiation into osteoblasts this expression decreases.

TLR9 expression was clearly more intensive in high-grade squamous dysplasia than in the normal squamous epithelium. In addition, expression was evenly present throughout the full thickness of the epithelium. Quite a similar change in the staining pattern of TLR9 in accordance with the histopathological grade of cervical intraepithelial neoplasia has been reported [27]. In contrast, in the study of Schmausser et al. [29], TLR9 was detected in normal gastric epithelium, but expression was lost in premalignant lesions including intestinal metaplasia and gastric dysplasia. These findings suggest that the role of TLR9 is different in squamous and columnar dysplasia. In squamous dysplasia, overexpression may be related to either lack of differentiation, increased proliferation or both [27]. In our material, the intensive TLR9 expression clearly helped to locate the dysplastic lesions. Accordingly, the intensive TLR9 immunoreaction could be used as a marker of dysplastic esophageal squamocellular lesions.

In our study, all esophageal squamous cell carcinomas exhibited a widespread expression of TLR9. Moderate to strong TLR9 expression was associated with poor differentiation and presence of lymph node metastases or distant metastases. Similarly, extensive TLR9 expression and association with poor differentiation has also been noticed in breast and prostate cancers [23, 30]. In esophageal squamocellular carcinoma, TLR9 expression could be used as a marker of aggressive biological behavior. Inverse association was seen with the vascular density suggesting that possible tumor-promoting properties of TLR9 expression are not linked to induction of angiogenesis. Activation of tumor cell TLRs has been reported to promote tumor cell proliferation and resistance to apoptosis [12, 31]. In this study, the TLR9 expression showed no association with tumor cell proliferation or apoptosis. No conclusions can be drawn from this finding because the natural TLR9 ligand in cancer remains unknown. However, there are other potential mechanisms linking TLR9 expression and prognosis of carcinoma, including modification of tumor cell invasion and metastasis by regulation of metalloproteinases [26].

The role and possible ligand of the originally microbial DNA recognizing TLR9 in the pathogenesis of esophageal squamous cell dysplasia and cancer remain unknown. Because of the short transit time of esophagus, its epithelial cells are normally exposed to oral microbes only for short periods of time. This suggests that there

could also be a microbe-independent but TLR9-related pathway contributing to esophageal squamous cell carcinoma and disease progression. Whether the TLR9 senses DNA of dying cells from injured esophageal tissue or cancer tissue, or some other currently unknown ligand contributing to cancer development, is unknown, and should be evaluated in future studies. Endothelial TLR9 expression within esophageal carcinomas was downregulated compared with vessels outside the cancer. Previously, TLR9 has been detected in both animal and human endothelial cells, but characteristics of endothelial TLR9 expression in malignant tissue has remained unknown [32, 33]. During inflammation, microbial recognition by endothelial TLRs contributes to structural and functional changes in endothelial cells including cytokine production and increased vascular permeability [34].

In conclusion, we show that in the normal esophageal squamous epithelium TLR9 is expressed in the basal parts of the epithelium. TLR9 is upregulated in high-grade dysplasias, which suggests that TLR9 might serve as a marker of esophageal squamous cell intraepithelial neoplasia. In esophageal squamous cell carcinomas, extensive TLR9 expression associated with high tumor grade and presence of lymph node and distant metastases. This finding should be validated in independent patient material using the same criteria for TLR9 analysis. Mechanisms of the upregulation of TLR9 in esophageal squamous malignancy remain speculative. However, our findings suggest that TLR9 might contribute to esophageal squamous cell carcinogenesis.

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