Toll-like receptors TLR-2, TLR-4, TLR-7, and TLR-9 in tumor tissue and serum of the patients with esophageal squamous cell carcinoma and gastro-esophageal junction cancer

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Stimulation of toll-like receptors (TLRs) has been linked to the development of esophageal and gastric cancers.

Objectives. The aim of the study was to evaluate the clinical significance of tissue expression and serum concentration of TLR-2, TLR-4, TLR-7, and TLR-9 in patients with esophageal squamous cell carcinoma and gastro-esophageal junction adenocarcinoma.

Material and methods. The study group consisted of 97 individuals: 32 with esophageal squamous cell carcinoma, 27 with gastro-esophageal junction cancer, and 38 age- and gender-matched controls. The mRNA expression and protein concentration of TLRs in tissues and sera were measured with reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) tests.

Results. In esophageal cancer patients, mRNA expressions of TLR-2, TLR-4 and TLR-7, and protein concentrations of all TLRs were significantly higher in tumor than in control tissue (p < 0.05). In esophageal cancer patients with lymph node metastasis, a tendency toward higher protein concentrations of tumor TLR-4 was observed. In gastro-esophageal junction adenocarcinoma subgroup, only the mRNA expression of TLR-7 and protein concentrations of TLR-4, TLR-7 and TLR-9 were significantly higher in tumors than in normal mucosa (p < 0.05). Protein concentration of TLR-9 was significantly higher in tumors of gastro-esophageal junction cancer with lymph node metastasis and depth of tumor invasion. Diagnostic potential of serum TLR-4 as a marker of gastro-esophageal junction cancer presence was reported.

Conclusions. We demonstrated differences in the expression patterns of TLRs between esophageal squamous cell carcinoma and adenocarcinoma of gastro-esophageal junction, and showed circulating TLR-4 to be a potential marker of gastro-esophageal junction cancer.

Key words: biomarkers, toll-like receptors, gastro-esophageal cancer
Introduction

Esophageal squamous cell carcinoma (ESCC) and gastro-esophageal junction adenocarcinoma (GEJA) are characterized by one of the lowest indicators of 5-year survival rates in Eastern Europe (6%), Western Europe and the USA (14–18%). Poor prognosis of patients with ESCC and GEJA is related to the fact that they are diagnosed in the advanced stage, when the presence of lymph node and/or distant metastases is observed. The pathogenesis of GEJA remains unclear. However, the natural behavior of GEJA and its therapeutic modalities are often similar to those of esophageal adenocarcinoma, as both cancers may develop through metaplasia-dysplasia sequence due to the accumulation of genetic alterations. As such, the decision whether GEJA is classified as esophageal or gastric cancer depends on whether the primary tumor extends to esophagus.

Earlier studies have connected the influence of alcohol, nicotine, acidic gastric contents, and bile from the duodenum with cellular damage of esophageal and gastro-esophageal junction epithelium. The transformation of the epithelial cells results in the loss of epithelial wall integrity and changes in esophageal microbiome. The bacterial products stimulate toll-like receptors (TLRs) in the epithelial and inflammatory cells, inducing persistent innate immune responses, the expression of proinflammatory cytokines and the generation of reactive oxygen species (ROS). Additionally, TLRs may be activated by endogenous ligands from dead or damaged host cells. They contribute to an abnormal increase of inflammatory response, which may lead to the disturbance of cellular homeostasis and the induction of metaplasia-dysplasia sequence. The deregulation of innate and adaptive immune response may play an important role in the development of both ESCC and GEJA.

Toll-like receptors can be either extracellular (such as TLR-1, TLR-2, TLR-4, TLR-5, TLR-6) or intracellular (such as TLR-3, TLR-7, TLR-8, and TLR-9). Extracellular TLRs are located at the plasma membrane, where they recognize macromolecules exposed on the surface of pathogens. Intracellular TLRs are located in endosomes or lysosomes and they detect viral and bacterial nucleic acids, which plays an important role in host immune responses. Recently, the ability of TLRs to heighten the immune response has been exploited for the treatment of cancer disease.

An overexpression of TLR-3, TLR-4, TLR-5, TLR-7, and TLR-9 in ESCC tumors has been demonstrated both on mRNA and, qualitatively, on protein level. The potential usefulness of their evaluation as prognostic markers for ESCC has been suggested. However, the quantitative analyses of TLRs concentrations have not been previously performed. Also, there is no data concerning TLR-2 mRNA expression in ESCC. With regard to adenocarcinomas, the expression of TLRs has been evaluated in adenocarcinomas of esophagus and stomach, but not in the area of gastro-esophageal junction. A potential correlation between tissue expression and circulating levels of TLRs has not been determined, either.

Therefore, the aim of the present study was to evaluate mRNA copy numbers for TLR-2, TLR-4, TLR-7, and TLR-9, and their protein concentrations in ESCC and GEJA tumors as compared to patient-matched normal tissues with reference to the disease advancement and circulating levels.

Material and methods

Patients’ characteristics

The study population consisted of 97 individuals: 59 cancer patients and 38 healthy individuals. Cancer patients (45 male, 14 female, median of age: 62 years, 95% confidence interval (CI) = 58–67 years) were admitted to the Department of Gastrointestinal and General Surgery of Wroclaw Medical University (Poland) from 2010 to 2015 for curative resection of upper gastrointestinal tract tumors. Patients with any systemic illness, prior radio- or chemotherapy and stage IV cancer (distant metastases) were excluded from the study. Preoperative evaluation was conducted by physical examination and imaging techniques, such as ultrasonography, computed tomography and magnetic resonance. There were 32 patients with histologically confirmed ESCC and 27 patients with histologically confirmed GEJA. In the present study, all adenocarcinomas of gastro-esophageal junction extended into the esophagus and, in line with the 7th edition of the Union for International Cancer Control (UICC) TNM classification, were classified as esophageal cancers. Resected tumors were staged pathologically using the UICC TNM classification. There were 8 patients with stage I cancer (3 with ESCC and 5 with GEJA), 14 patients with stage II (8 with ESCC and 6 with GEJA), and 37 with stage III (21 with ESCC and 16 with GEJA).

Sera of 38 apparently healthy blood donors (27 male, 11 female, median age 59 years, 95% CI = 55–63 years) from the Lower Silesian Center of Blood Donation and Therapeutics, Wroclaw, Poland, were used as a reference in the analysis of circulating TLRs. The control group was age- and gender-matched to the study group (p = 0.129 and p = 0.890, respectively).

The study was planned according to the ethical standards detailed in the Declaration of Helsinki, as revised in 1983. The study protocol was approved by the Medical Ethics Committee, Wroclaw Medical University, Poland (signature numbers: KB 28/2011 and 203/2014). Informed consent was obtained from all participants.

Collection and preparation of samples

Tissue samples

Fresh samples of tumor and normal mucosa, taken approx. 10 cm from the tumor, were collected after the...
resection and divided into 2 groups of samples: the first one, subsequently used for transcriptional analysis, was soaked in RNAlater (Ambion, Inc., Austin, USA) and stored at −80°C; the second one, subsequently used for protein analysis, was rinsed with 0.9% NaCl and stored at −45°C.

To determine TLRs protein concentration, tissue samples were homogenized in 10 mM Tris-HCl with 150 mM KCl and 1 mM EDTA, pH 7.4 buffer (proportion 1:2 w/v), using Fastprep-24 homogenizer (2 min, 4.0 m/s). The homogenates were centrifuged at 14,500 × g, 10 min, 6°C, and the supernatants were collected and stored at −45°C.

RNA extraction, quantitation and quality assessment

Tissue samples (30–40 mg) were homogenized in TRIzol Reagent (Invitrogen Life Technologies, Waltham, USA), using Fastprep 24 Homogenizer (MP Biomedical, Solon, USA), and total RNA was extracted using the phenol-chloroform method. Isolated RNA was purified using RNaseasy Mini Kit (Qiagen, Valencia, USA) with DNase treatment, in accordance with the manufacturer’s instructions. Purified RNA was quantified using NanoDrop 2000 (ThermoScientific, Rockford, USA). Its purity was assessed by calculating 260:280 and 260:230 ratios. The integrity of RNA was evaluated using the Experion platform incorporating LabChip microfluidic technology and Experion RNA StdSens analysis kits (BioRad, Hercules, USA).

Blood samples

Samples of peripheral blood were collected into sterile vacuum tubes from healthy controls and cancer patients (prior to surgery) after overnight fasting. Blood was clotted (30 min, room temperature) and centrifuged (1,500 × g, 10 min, room temperature). Obtained sera were stored at −45°C.

Analytical methods

Determination of protein concentrations

Concentrations of TLRs in tissue homogenates and sera were determined using enzyme-linked immunosorbent assay (ELISA) tests (Cloud-Clone Corp., Houston, USA). Detection ranges and minimum detectable doses were respectively: 0.312–20 ng/mL and 0.117 ng/mL for TLR-2; 0.312–20 ng/mL and 0.133 ng/mL for TLR-4; 0.780–50 ng/mL and 0.290 ng/mL for TLR-7; 0.625–10 ng/mL and 0.236 ng/mL for TLR-9. In the case of tissue samples, TLRs concentrations were adjusted to total protein level, measured using the Bradford method and BioRad Protein Assay (BioRad) with bovine serum albumin as a standard. Concentrations of TLRs were expressed as µg per g of total protein content.

cDNA synthesis

According to the manufacturer’s instruction, 0.5 µg of RNA was transcribed by Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Negative transcription controls were performed and tested for all samples. All incubation steps were carried out in C1000 thermocycler (BioRad).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) mixture consisted of: 2 µL of cDNA (diluted 1:5), 10 µL of SsoFast EvaGreen Supermix (BioRad), 1 µL of each 10 nM forward and reverse target-specific primer, and 6 µL of water. The list of primers (GeneSys Sp. z o.o., Wrocław, Poland) used in this study is shown in Table 1. Reactions were conducted in triplicates, using CFX96 RT-PCR system (BioRad) with the following cycling conditions: 95°C for 30 s, 95°C for 5 s, 61°C for 5 s, 40 cycles. Specificity of the product was confirmed by melting curve analysis (60–90°C with fluorescent reading every 0.5°C) and by electrophoresis in a high-resolution agarose (SeaKem LE agarose; Lonza, Basel, Switzerland) in TBE with SYBR Green (Lonza) detection. For each sample, the expression of TLRs was examined and normalized to the GAPDH reference gene, and relative induction was calculated using the 2^(-ΔΔCt) method.

Statistical analysis

Data distribution was analyzed using the Shapiro-Wilk normality test. Descriptive data was presented as medians (Me) and minimum–maximum (min–max) values. Independent samples were analyzed using the Mann-Whitney U test, the Kruskal-Wallis H test with post-hoc Dunn’s test, and paired samples were tested by the Wilcoxon test. Chi-squared test was conducted in order to analyze the compliancy of the control group with the study group (variable “gender”). Receiver operating characteristic (ROC) analysis was used to test the diagnostic utility of serum TLR-4. All values of p < 0.05 were considered as statistically significant. The statistical analyses were performed using STATISTICA v. 12.0 software (StatSoft, Tulsa, USA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-2</td>
<td>forward CTCGAGGAGCAACAGCAAGCA, reverse ACACCAGTGCTTGTGTGGACAG</td>
</tr>
<tr>
<td>TLR-4</td>
<td>forward CCGTGAGGACTTGGAGCCAGCT, reverse AGTGTAGAGCTGCTTGGAGCTT</td>
</tr>
<tr>
<td>TLR-7</td>
<td>forward CTGAGCTCAGCGCCACAAC, reverse CGCAACGTGAAAGCAGCACATG</td>
</tr>
<tr>
<td>TLR-9</td>
<td>forward TGAACGAACTGACGATCGTGG, reverse CAGTGCGTGGTACTCGTCAGT</td>
</tr>
</tbody>
</table>

qPCR – quantitative polymerase chain reaction; TLR – toll-like receptor.
Results

Pairwise comparison of toll-like receptors protein levels between tumor and adjacent normal tissue

In the total group of patients, relative protein concentrations of TLR-2, TLR-4, TLR-7, and TLR-9 were significantly higher in tumor tissue than in the corresponding control tissue (p < 0.05 for all) (Fig. 1).

When analyzed separately, protein content of all TLRs was significantly higher only in the tumor tissue of ESCC. In GEJA, tumor concentrations of TLR-4, TLR-7 and TLR-9 were significantly higher, but there was no difference in the concentration of TLR-2 protein between tumor and normal tissue (Table 2).

Tumor concentrations of TLRs in ESCC and GEJA were comparable, except for TLR-4, whose concentration was significantly higher in ESCC than in GEJA patients (p = 0.00002) (Table 2).

Table 2. Pairwise comparison of protein concentrations of TLRs between tumor and control tissue in ESCC and GEJA subgroups of patients. Data in each subgroup was analyzed with the Wilcoxon test for paired samples.

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>ESCC (n = 32)</th>
<th>GEJA (n = 27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-2 [µg/g protein]</td>
<td>tumor tissue – Me (min–max)</td>
<td>control tissue – Me (min–max)</td>
<td>p-value</td>
</tr>
<tr>
<td></td>
<td>10.09 (6.76–13.64)</td>
<td>8.83 (4.95–16.50)</td>
<td>0.014*</td>
</tr>
<tr>
<td>TLR-4 [µg/g protein]</td>
<td>34.36 (10.57–97.57)</td>
<td>9.86 (4.49–69.98)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>TLR-7 [µg/g protein]</td>
<td>6.10 (0.82–8.70)</td>
<td>3.98 (0.51–9.26)</td>
<td>0.001*</td>
</tr>
<tr>
<td>TLR-9 [µg/g protein]</td>
<td>0.83 (0.45–1.73)</td>
<td>0.71 (0.40–1.25)</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

ESCC – esophageal squamous cell carcinoma; GEJA – gastro-esophageal junction adenocarcinoma; Me – median; * statistically significant; protein concentration of TLR-4 in ESCC tumor vs protein concentration of TLR-4 in GEJA tumor, p = 0.00002 (the Mann-Whitney test).
Relations of tumor toll-like receptors concentrations to clinical and histopathological parameters

Tumor concentrations of TLRs were evaluated in relation to age, gender, stage of cancer, primary tumor progression, and lymph node invasion. In ESCC, a tendency toward higher protein concentrations of tumor TLR-4 in patients with lymph node metastasis was observed. No similar association for other TLRs was detected. Also, there was no relationship between TLRs and other clinic-pathological parameters (Table 3).

In GEJA patients, tumor protein concentration of TLR-9 was significantly higher in more advanced cancers (stage III, pT3/4 and pN1). No such association was observed for other TLRs. Protein content of TLR was not affected by patients’ age or gender (Table 4).

Table 3. Relationships between clinical and histopathological parameters and protein concentrations of TLRs in tumor tissue of ESCC patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLR-2 [µg/g protein]</th>
<th>TLR-4 [µg/g protein]</th>
<th>TLR-7 [µg/g protein]</th>
<th>TLR-9 [µg/g protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Me (min–max)</td>
<td>p-value</td>
<td>Me (min–max)</td>
<td>p-value</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male (n = 23)</td>
<td>10.1 (6.9–13.3)</td>
<td>0.933</td>
<td>35.3 (12.0–86.6)</td>
<td>0.923</td>
</tr>
<tr>
<td>female (n = 9)</td>
<td>9.7 (6.2–13.6)</td>
<td></td>
<td>30.9 (10.6–97.6)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 (n = 16)</td>
<td>9.2 (6.2–13.6)</td>
<td>0.122</td>
<td>32.9 (12.0–86.3)</td>
<td>0.624</td>
</tr>
<tr>
<td>≥60 (n = 16)</td>
<td>10.7 (6.8–13.3)</td>
<td></td>
<td>35.8 (10.6–97.6)</td>
<td></td>
</tr>
<tr>
<td>Stage (TNM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II (n = 11)</td>
<td>9.4 (6.8–13.6)</td>
<td>0.936</td>
<td>24.3 (10.6–54.9)</td>
<td>0.451</td>
</tr>
<tr>
<td>III (n = 21)</td>
<td>10.1 (6.2–13.3)</td>
<td></td>
<td>38.0 (10.6–97.6)</td>
<td></td>
</tr>
<tr>
<td>Depth of tumor invasion (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+2 (n = 9)</td>
<td>9.4 (6.2–13.6)</td>
<td>0.899</td>
<td>33.4 (13.2–54.9)</td>
<td>0.644</td>
</tr>
<tr>
<td>3+4 (n = 23)</td>
<td>10.1 (6.9–13.3)</td>
<td></td>
<td>38.0 (10.6–97.6)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis (N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (n = 13)</td>
<td>9.4 (6.8–13.6)</td>
<td>0.489</td>
<td>23.3 (10.6–54.9)</td>
<td>0.071</td>
</tr>
<tr>
<td>1 (n = 19)</td>
<td>10.1 (7.0–13.3)</td>
<td></td>
<td>38.3 (15.6–97.6)</td>
<td></td>
</tr>
</tbody>
</table>

TLR – toll-like receptor; ESCC – esophageal squamous cell carcinoma; Me – median.

Pairwise comparison of toll-like receptors mRNA expressions between tumor and adjacent normal tissue of cancer patients

The analysis of TLR expression on the mRNA level showed the up-regulated levels of all receptors in tumor tissues as compared to normal ones: by 3.9-fold for TLR-2, by 2.6-fold for TLR-4, by 3.6-fold for TLR-7, and by 2-fold for TLR-9 (Table 5). When analyzed separately, the expressions of TLR-2, TLR-4, and TLR-7, but not that of TLR-9, were significantly elevated in ESCC tumors. In GEJA, only the expression of TLR-7 was significantly higher in tumor tissue as compared to the normal one (Table 5).

Table 4. Relationships between clinical and histopathological parameters and protein concentrations of TLRs in tumor tissue of GEJA patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLR-2 [µg/g protein]</th>
<th>TLR-4 [µg/g protein]</th>
<th>TLR-7 [µg/g protein]</th>
<th>TLR-9 [µg/g protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Me (min–max)</td>
<td>p-value</td>
<td>Me (min–max)</td>
<td>p-value</td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>male (n = 22)</td>
<td>8.6 (5.2–12.4)</td>
<td>0.230</td>
<td>18.0 (6.2–30.3)</td>
<td>0.925</td>
</tr>
<tr>
<td>female (n = 5)</td>
<td>9.7 (8.0–12.8)</td>
<td></td>
<td>17.6 (6.4–23.2)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt;60 (n = 9)</td>
<td>10.1 (6.6–12.4)</td>
<td>0.571</td>
<td>17.6 (8.9–26.6)</td>
<td>0.662</td>
</tr>
<tr>
<td>≥60 (n = 18)</td>
<td>8.7 (5.2–12.8)</td>
<td></td>
<td>17.0 (6.2–30.3)</td>
<td></td>
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<tr>
<td>Stage (TNM)</td>
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<td></td>
</tr>
<tr>
<td>I+II (n = 11)</td>
<td>10.0 (5.2–12.6)</td>
<td>0.786</td>
<td>15.5 (8.9–25.4)</td>
<td>0.902</td>
</tr>
<tr>
<td>III (n = 16)</td>
<td>8.6 (5.7–12.8)</td>
<td></td>
<td>18.1 (6.2–30.3)</td>
<td></td>
</tr>
<tr>
<td>Depth of tumor invasion (T)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1+2 (n = 8)</td>
<td>9.9 (6.6–12.6)</td>
<td>0.599</td>
<td>17.6 (8.9–25.4)</td>
<td>0.811</td>
</tr>
<tr>
<td>3+4 (n = 19)</td>
<td>8.7 (5.2–12.8)</td>
<td></td>
<td>18.3 (6.2–30.3)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis (N)</td>
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<tr>
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<td>8.6 (5.7–12.8)</td>
<td></td>
<td>18.1 (6.2–30.3)</td>
<td></td>
</tr>
</tbody>
</table>

TLR – toll-like receptor; GEJA – gastro-esophageal junction adenocarcinoma; Me – median; * statistically significant.
Circulating levels of TLR-2, TLR-4, TLR-7, and TLR-9

Only the concentrations of circulating TLR-4 were higher, both in cancer patients and healthy controls, than the limit of detection of the assay. In the case of TLR-2, 82% of control samples and 25% of cancers yielded results that were below the detection limit. Similarly, 87% of cancers and all of controls in TLR-7 assay, and 75% of cancers and all of controls in TLR-9 assay were below the limit. Therefore, further analysis was limited to TLR-4.

We demonstrated significantly higher concentrations of serum TLR-4 in cancer patients in general than in healthy controls. Patients with GEJA had significantly higher circulating TLR-4 than these with ESCC (Table 6).

Circulating TLR-4 was significantly elevated in more advanced tumors of ESCC, but not in GEJA patients (stage III, pT3/4). A similar tendency was observed for lymph node metastasis (Table 7).

Diagnostic potential of circulating TLR-4 level in ESCC and GCC

To assess the strength of association and potential diagnostic utility of circulating TLR-4 as a marker of ESCC and GEJA, ROC analysis was performed. Circulating TLR-4 was significantly associated exclusively with GEJA presence with overall 79% accuracy, defined as area under ROC curve (AUC). At a cut-off of 1,338 pg/mL, TLR-4 as GEJA marker was characterized by 70% sensitivity and 78% specificity (Fig. 2).

Discussion

To our knowledge, this is the first study addressing the issue of TLR-2, TLR-4, TLR-7, and TLR-9 expression, either on mRNA and protein level, as well as their circulating levels in adenocarcinoma of gastro-esophageal junction. We showed that, similarly to ESCC, the protein expression...
of TLR-4, TLR-7 and TLR-9 was up-regulated in GEJA tumors as compared to normal tissue. However, on the mRNA level, only the expression of TLR-7 was significantly higher. The observation of increased protein content of TLR-4 and TLR-9 in tumor tissue is not accompanied by equally pronounced up-regulation of their mRNA. This may be explained by enhanced accumulation of TLR proteins due to their increased stability.

Corroborating our findings on TLR-4 in GEJA, Huhta et al. showed increased TLR-4 protein expression in Barrett’s esophagus. TLR-4 immunoreactivity, both in the cytoplasm and nucleus, was correlated with the degree of dysplasia, its progression to esophageal adenocarcinoma, as well as with poor prognosis. Up-regulation of the receptor has also been reported due to *Helicobacter pylori* (HP), gastritis and subsequent metaplasia, dysplasia and gastric adenocarcinoma. TLR-4 might contribute to neoplastic transformation by activating nuclear factor-kappaB (NF-κB) pathway and the production of proinflammatory cytokines and cyclooxygenase-2 in the epithelial and immune cells, as well as by mitochondrial ROS production. Contrary to GEJA, TLR-4 in ESCC tumors was overexpressed both at protein and mRNA levels. Neither in ESCC nor in GEJA, protein expression of TLR-4 was correlated with TNM stage, except for a tendency toward higher protein content in ESCC with lymph node involvement. Also, Sheyhidin et al. reported an association of high mRNA and protein expression of tumor TLR-4 with the lymph node metastasis of ESCC. They found that high TLR-4 expression in stromal mononuclear inflammatory cells was significantly associated with a risk of lymph node metastasis and poor prognosis of ESCC patients. Correspondingly, circulating levels of TLR4 in our ESCC patients were positively correlated with the disease advancement. The current study is the first one to assess the diagnostic usefulness of serum TLR-4. Its elevated levels were associated with GEJA presence with good accuracy, warranting its further evaluation as a potential GEJA marker.

We demonstrated overexpression of mRNA and an increase of protein concentration of TLR-7 in tumor of GEJA patients. However, no association between tumor TLR-7 concentration and clinic-pathological parameters was observed. Although a possible association might be obscured by the limited number of observations in subgroup analysis, analogous results were presented by Helminen et al., who evaluated TLR-7 expression in esophageal adenocarcinoma, using immunohistochemistry. Much research points at a dual or controversial role of TLR-7 in cancer development. Whereas Lin et al. showed down-regulation of TLR-7 gene expression in hepatocellular adenocarcinoma and hepatitis, Vaz and Andersson demonstrated its overexpression in pancreatic ductal adenocarcinoma.

In summary, the role of TLR-7 expression and activation in adenocarcinomas remains unclear and requires further studies. In ESCC, TLR-7 was overexpressed both at mRNA and protein levels. Similarly, Sheyhidin et al. demonstrated a positive association between TLR-7 immunoreactivity and tumor grade. Distinctive patterns of TLR-7 expression were observed in tumor cells and fibroblast-like cells in oral squamous cell carcinoma. High expression of tumor TLR-7 associated with its low expression in stromal fibroblast-like cells has been reported to predict worse clinical outcome. It has been speculated that expression of TLR-7 in tumor cells may stimulate cancer development, whereas the expression of TLR-7 in fibroblast-like cells may play a protective role in oral squamous cell carcinoma. We also demonstrated significantly higher protein concentration of tumor TLR-9 in GEJA, which corresponded with disease progression, depth of tumor invasion, and lymph node metastasis. Correspondingly, Kauppila et al. showed that an increase in tumor TLR-9 expression may contribute to the growth, metastasis and poor prognosis of patients with esophageal adenocarcinoma. It has been suggested that changes in bacterial flora and apoptotic reactions in esophagus and gastro-esophageal junction can induce TLR-9 production, which stimulates the early steps of the cancer development. However, the possible role of endogenous ligands for TLR-9 in the pathogenesis of esophageal adenocarcinoma requires further study. Herein, high protein concentration of TLR-9 was observed in ESCC tumors, whereas its gene expression did not differ significantly between tumor and normal mucosa. Previous qualitative studies demonstrated the predictive role of TLR-9 in esophageal squamous cell dysplasia and carcinoma, oral tongue squamous cell carcinoma, and cervical neoplasms. Additionally, Mäkinen et al. reported...
that high expression of TLR-9 was associated with oral tongue squamous cell carcinoma recurrence. Our results are the first to demonstrate the high gene expression and protein concentration of TLR-2 in ESCC patients. Previous reports have described TLR-2 expression in cervical neoplasia and squamous cell carcinoma of cervix. The predictive role of TLR-2 as a marker of invasive tumor growth has been shown in the early stage of oral tongue squamous cell carcinoma. In the advanced stage of this cancer, nuclear TLR-2 expression level may be a marker of neck metastasis and tumor recurrence. TLR-2 is considered as a promising marker in squamous cell carcinomas.

In conclusion, our results expand the current knowledge on TLRs in cancers of the upper digestive tract, adding information about cancers of gastro-esophageal junction. We demonstrated differences in the expression patterns of TLRs between ESCC and GEJA, and presented circulating TLR-4 as a potential marker of GEJA.

References