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High Tissue TLR5 Expression Predicts Better Outcomes in Colorectal Cancer Patients

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Running title: High tissue TLR5 predicts a better outcome in colorectal cancer patients

Abbreviations: DAMP, damage-associated molecular pattern; DC, dendritic cell; DSS, disease-specific survival; CI, confidence interval; CME, complete mesocolic excision; CRC, colorectal cancer; CRP, C-reactive protein; HR, hazard ratio; IBD, inflammatory bowel disease; IQR, interquartile range; NPC, nasopharyngeal carcinoma; NSCLC, non-small cell lung cancer; OPSCC, oropharyngeal squamous cell carcinoma; OTSCC, oral tongue squamous cell carcinoma; PAMP, pathogen-associated molecular pattern; PDAC, pancreatic ductal adenocarcinoma; SIR, systemic inflammatory response; SNP, single-nucleotide polymorphism; TLR, toll-like receptor; TMA, tissue microarray; TME, total mesorectal excision

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Abstract

Background: Colorectal cancer (CRC), the third most common cancer globally, caused 881 000 cancer deaths in 2018. Toll-like receptors (TLRs), the primary sensors of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), activate innate and adaptive immune systems and participate in the development of an inflammatory tumor microenvironment. We aimed to explore the prognostic value of TLR3, TLR5, TLR7, and TLR9 tissue expressions in CRC patients.

Methods: Using immunohistochemistry, we analyzed tissue microarray (TMA) samples from 825 CRC patients who underwent surgery between 1982 and 2002 at the Department of Surgery, Helsinki University Hospital, Finland. After analyzing a pilot series of 205 tissue samples, we included only TLR5 and TLR7 in the remainder of the patient series. We evaluated the associations between TLR5 and TLR7 tissue expressions, clinicopathological variables, and survival. Using the Kaplan–Meier method, we generated survival curves, determining significance through the log-rank test. Univariate and multivariate survival analyses relied on the Cox proportional hazards model.

Results: The five-year disease-specific survival (DSS) was 55.9% among TLR5-negative [95% confidence interval (CI) 50.6–61.2%] and 61.9% (95% CI 56.6–67.2%; $p = 0.011$, log-rank test) among TLR5-positive patients. In the Cox multivariate survival analysis adjusted for age, sex, stage, location, and grade, positive TLR5 immunoexpression [hazard ratio (HR) 0.74; 95% CI 0.59–0.92; p

= 0.007) served as an independent positive prognostic factor. TLR7 immunoexpression exhibited no prognostic value in the survival analysis across the entire cohort (HR 0.97; 95% CI 0.78–1.20; p = 0.754) nor in subgroup analyses.

Conclusions: We show for the first time that a high TLR5 tumor tissue expression associates with a better prognosis in CRC patients.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in the world and the second leading cause of cancer mortality, resulting in 881 000 cancer deaths in 2018 (1). The burden of CRC is increasing rapidly and 2.2 million new CRC cases are expected in 2030 (1,2). Although CRC survival has improved in developed countries, approximately 17% of stage II and 36% of stage III patients experience recurrence within 5 years (3).

Inflammation plays a crucial role in gastrointestinal cancers, CRC among them, although the molecular mechanisms behind this relationship are incompletely understood. Numerous biomarkers have been investigated to reveal this connection, among them toll-like receptors (TLRs) (4-6).

TLRs are transmembranous pattern-recognition receptors (PRRs) expressed on the cell surface or intracellularly in the endosomes of innate immune cells such as macrophages, dendritic cells (DCs), neutrophils, mast cells, epithelial cells, and endothelial cells. TLRs can recognize various pathogen-associated molecular patterns (PAMPs) of a bacterial, viral, and fungal origin, and can also be activated by host-derived endogenous damage-associated molecular patterns (DAMPs), ligands released, for example, as stress responses and apoptosis (7-9).

Innate immune responses activated by TLRs are needed to evoke the immune surveillance mechanisms of the adaptive immune system (9,10). In order to grow and progress, tumor cells must overcome these immune surveillance mechanisms (10,11). In the same tumor, both TLR-induced protumorigenic and antitumorigenic processes can be present simultaneously (10,12).

Evidence indicating the prognostic value of TLRs in malignancies has proliferated in recent decade. For instance, a recent meta-analysis demonstrated that elevated TLR4 and TLR7 serve as markers of a poorer prognosis in several malignancies (13). In CRC patients, TLRs have not been widely investigated. In our previous study, Dukes B CRC patients with a strong TLR4 tissue expression exhibited a worse prognosis, while Dukes C CRC patients with a strong TLR2 expression experienced a better prognosis (14).

Moreover, to our knowledge, the prognostic role of TLR5 tissue immunohistochemical expression in CRC has not been previously investigated. Among other malignancies, a high TLR5 tissue expression associates with a better prognosis, for example, in non-small cell lung cancer (NSCLC) (15) and gastric cancer (16). By contrast, in HPV-positive oropharyngeal squamous cell carcinoma (OPSCC), a high TLR5 level associates with a poor outcome (17).

In this study, we aimed to study the potential prognostic role of the tissue expression of TLR3, TLR5, TLR7, and TLR9 in CRC patients.

Materials and Methods

Patients

For this study, we selected samples from 825 CRC patients who underwent surgery at the Department of Surgery, Helsinki University Hospital, Finland, between 1982 and 2002. The median age at the time of surgery was 67.5 years [interquartile range (IQR) 57.6–75.3 years] and 457 (55.4%) of all patients were male. Clinical data were collected from patient medical records. The Population Register Center of Finland and Statistics Finland provided follow-up vital statistics data and cause of death information for deceased patients. The median follow-up time was 5.06 years (IQR 1.2–17.1), and 654 (82.9%) patients died by the end of follow-up, 362 (43.9%) from CRC. The 5-year disease-specific survival (DSS) for all patients was 58.8% [95% confidence interval (CI) 55.3–62.35]. The distribution for staging from the modified Dukes classification (18), used for CRC staging in our

clinic during patient recruitment, was as follows: 122 (14.8%) cases were Dukes stage A, 292 (35.4 %) Dukes B, 225 (27.3 %) Dukes C, and 186 (22.5%) Dukes D. Patient's clinicopathological characteristics are shown in Supplementary Table 1.

The Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013) approved the study protocol and the National Supervisory Authority of Health and Welfare granted permission to study the archived tissue samples retrospectively without requiring individual consent (Valvira Dnro 10041/06.01.03.01/2012).

Tissue samples

Formalin-fixed and paraffin-embedded surgical tumor samples originated from the archives of the Department of Pathology, University of Helsinki. An experienced pathologist (JH) marked representative areas on hematoxylin- and eosin-stained slides. From the annotated areas, three 1.0-mm cores were punched using a semiautomatic tissue arrayer (Beecher Instruments Inc., Silver Spring, MD, USA), and embedded in recipient paraffin tissue microarray (TMA) blocks. We cut 4- μ m sections from the TMA blocks for immunohistochemistry as described elsewhere (19).

Immunohistochemistry

The same immunohistochemical staining protocol was applied to each TLR. The 4- μ m TMA sections were deparaffinized in xylene for 15 + 5 min, rehydrated in solutions containing decreasing concentrations of ethanol, beginning with pure alcohol and ending with distilled water, for 5 min in each. For antigen retrieval, the slides were treated in a PreTreatment module (Lab Vision UK Ltd, UK) in a Tris-HCl buffer (pH 8.5) for 20 min at 98°C. We stained the slides in an Autostainer 480 (Lab Vision, Fremont, California, USA) using the REAL EnVision Detection System (peroxidase/DAB+, rabbit/mouse; Dako, Glostrup, Denmark). Endogenous peroxidase blocking was carried out using the 0.3% Dako REAL Peroxidase-Blocking Solution incubated for 5 min. Then, the

primary antibody incubation of TMA slides was carried out using the following primary antibodies: TLR3 rabbit polyclonal (sc-10740, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted to 1:100), TLR5 mouse monoclonal (IMG-664A, Imgenex, San Diego, CA, USA; diluted to 1:200), TLR7 rabbit polyclonal (IMG-581A, Imgenex, San Diego, CA, USA; diluted to 1:300), and TLR9 rabbit polyclonal (sc-25468, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted to 1:100). Finally, the samples were incubated with the peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) secondary antibody for 30 min, visualized by the Dako REAL DAB+ Chromogen for 10 min, and counterstained with Meyer's hematoxylin and mounted in Pertex Mounting (Histolab Products AB, Sweden). In each staining series, a positive control was used from tissue known to exhibit a high immunoreactivity to the studied antigens (tonsillar, skin, and cutaneous squamous cell carcinoma tissue). As a negative control, we used specimens processed without a primary antibody.

Scoring of samples

Immunostainings of TLR3, TLR5, TLR7, and TLR9 were interpreted by two independent researchers, including an experienced pathologist from the Department of Pathology, University of Helsinki (IB-L and JH), both blinded to the clinical data. A brown cytoplasmic color in the tumor cells reflected the immunopositivity of TLR3, TLR7, and TLR9. TLR5 immunopositivity was indicated as brown staining of the nuclear membranes of the tumor cells. The immunoreactivity was scored from 0 to 3: negative staining was scored as 0, weak positive immunoactivity as 1, moderate as 2, and strong as 3 (Figure 1). If the scores for a patient differed, the highest score was chosen for the final calculations. After comparing the scores from both researchers, all spots with different scoring results were re-evaluated and the final score was reached through discussion and consensus.

Statistical analysis

For the statistical analyses, the immunoexpression for each TLR in the pilot series was dichotomized into mild (0–1) and high (2–3) expression levels. In the larger cohort, TLR5 immunoexpression was dichotomized into negative (0) and positive (1–3) expression levels and TLR7 immunoexpression was dichotomized into mild (0–2) and strong (3) expression levels, based on the analysis of DSS curves of all scores (Supplementary Figure 1). The associations between the TLR5 and TLR7 immunoexpressions and clinicopathological variables were evaluated using the chi-square test. DSS was calculated from surgery to death from CRC or until the end of follow-up. We used the Kaplan–Meier method to generate survival curves and the log-rank test to compare different groups. The 95% confidence intervals (CI) were calculated for survival rates.

In addition, we used the Cox proportional hazards model for the univariate and multivariate survival analyses. For the multivariate analysis, we entered the following covariates: age, gender, Dukes stage, WHO grade, tumor location, and TLR5 immunoexpression. The Dukes stage was processed as a categorical covariate. A time-dependent variable was included for Dukes stage D, tumor grade, and tumor location to fulfill the Cox’s assumption. Interaction terms were considered, for which we found no significant interactions following a Bonferroni correction for multiple comparisons. All tests were two-tailed, and we considered $p < 0.05$ as statistically significant. All statistical analyses were performed using SPSS version 24.0 (IBM’s SPSS Statistics, version 24.0 for Mac; SPSS, Inc., Chicago, IL, USA) and SPSS version 25.0 (IBM’s SPSS Statistics, version 25.0 for Mac; SPSS, Inc., Chicago, IL, USA).

Results

Pilot staining

The immunostainings for TLR3, TLR5, TLR7, and TLR9 were initially completed on a smaller cohort of 205 CRC patients. Among that cohort, we successfully scored the TLR3 immunostaining for 198 (96.6%) samples, TLR5 for 166 (81%), TLR7 for 199 (97.1 %), and TLR9 for 196 (95.6%)

samples. Some samples were lost due to technical reasons, such as an inadequate position of the spot or the lack of a tumor in the TMA spot. Representative images for the different TLR staining patterns appear in Figure 1.

For statistical analysis from the pilot series, the immunoexpression of each TLR was grouped into mild (scores 0–1) and strong (scores 2–3) expression levels. In the Kaplan–Meier analysis of the pilot cohort, we found no differences in prognosis between the different TLR3 (HR 0.91; 95% CI 0.55–1.51; $p = 0.722$), TLR5 (HR 0.66; 95% CI 0.93–1.12; $p = 0.126$), TLR7 (HR 0.76; 95% CI 0.48–1.21; $p = 0.245$), and TLR9 (HR 1.38; 95% CI 0.432–4.37; $p = 0.589$) immunoexpression groups (Figure 2). We then continued examining TLR5 and TLR7 expressions in a larger study population, setting aside TLR3 and TLR9 from further analysis, as from pilot cohort DSS results we concluded that TLR5 and TLR7 were promising markers in CRC but TLR3 and TLR9 did not provide any prognostic information.

Immunostaining for TLRs in a larger population

Among 825 CRC tumor samples represented in the TMA, TLR5 immunoexpression on nuclear membranes was successfully interpreted in 734 (89%) cases, while TLR7 cytoplasmic immunostaining was successful in 769 (93.2%) samples. The detailed distribution of immunostainings appears in Table 1. The evaluation failed in 11% of TLR5 and 6.8 % of TLR7 samples due to technical staining problems such as the folding of tissue or overlapping spots. For the statistical analyses, patients were divided into two groups consistent with the pilot series.

Association with clinicopathological parameters

An association emerged between mild TLR5 and mild TLR7 immunoexpressions and mucinous histological type ($p < 0.001$ and $p = 0.22$, respectively; chi-square test, Table 2). Mild TLR7 immunoexpression associated with a higher tumor grade ($p < 0.001$; chi-square test, Table 2) and

Dukes stage A ($p = 0.048$; chi-square test, Table 2). No association emerged between TLR5 and TLR7 immunoexpressions with age, gender, side, or location of the primary tumor. TLR5 immunoexpression did not associate with the tumor stage.

Survival analysis

Patients with a strong TLR5 immunoexpression (HR 0.76, 95% CI 0.61–0.93; $p = 0.011$; Figure 3a and Suppl. Figure 1) exhibited a better prognosis. The 5-year DSS was 55.9% among mild TLR5 patients (95% CI 50.6–61.2%) and 61.9% (95% CI 56.6–67.2%; $p = 0.011$, log-rank test; Figure 3a) among strong TLR5 expression patients. TLR7 immunoexpression provided no prognostic value in the survival analysis (HR 1.04, 95% CI 0.83–1.29; $p = 0.754$; Figure 3b and Suppl. Figure 1).

In the subgroup analysis, patients with a left-sided tumor and a strong TLR5 immunoexpression exhibited a better prognosis (HR 0.76, 95% CI 0.59–0.98; $p = 0.035$; Figure 3d and Table 4). Also among adenocarcinoma subgroup, patients with a strong TLR5 immunoexpression showed a better survival (HR 0.73, 95% CI 0.58–0.93; $p = 0.010$; Table 4). This same trend emerged in female patients (HR 0.71, 95% CI 0.51–0.99; $p = 0.042$; Table 4) and among older patients (HR 0.67, 95% CI 0.50–0.90; $p = 0.007$; Table 4). In the subgroup analysis for the Dukes stage with borderline significance, Dukes B patients with a strong TLR5 immunoexpression exhibited a better prognosis (HR 0.61, 95% CI 0.37–1.00; $p = 0.050$; Figure 3c and Table 4). TLR7 provided no prognostic value in the subgroup analysis (Table 4).

In the Cox multivariate survival analysis, in addition to age, sex, Dukes stage, and WHO grade, a strong TLR5 immunoreactivity (HR 0.074, 95% CI 0.59–0.92; $p = 0.007$) emerged as an independent positive prognostic factor (Table 3).

Discussion

Our findings demonstrate that a high TLR5 tissue expression may identify CRC patients with more favorable prognoses. That is, a strong TLR5 tissue expression level emerged as an independent prognostic factor. To our knowledge, this is the first study to demonstrate the prognostic role of TLR5 among CRC patients. TLR3, TLR7, and TLR9 showed no prognostic significance here.

The prognostic value of various TLRs has been studied in several malignancies. However, according to a recent meta-analysis, not very many studies had been published in this field by 2017, with TLR4 emerging as the most widely studied TLR (13). This meta-analysis concluded that the elevated expressions of TLR4 and TLR7 predicted worse outcomes in several malignancies.

The role of TLRs in cancer development is diverse, since they can carry both tumor-inducing and antitumorigenic effects (12). *In vitro* studies of different carcinomas confirmed that TLR5 plays a different role depending upon the malignancy. For example, TLR5 activation enhances the proliferation of gastric cancer cells (20). In contrast, knocking down TLR5- or MyD88-induced responses in mouse xenograph models of human colon adenocarcinoma resulted in tumor growth, while in flagellin-treated tumors a TLR5-induced antitumor activity was observed leading to tumor necrosis and a decrease in the tumor size (21). This finding agrees with our results, demonstrating that TLR5 might carry an antitumorigenic effect in CRC. However, previous studies relied on *in vitro* studies and we conducted an *in vivo* study, rendering these results not directly comparable.

Moreover, in a study among 70 patients with colorectal polyps, no TLR2, TLR3, TLR4, and TLR5 expression was observed in the polyps (22). Yet, TLR7 and TLR9 expressions were higher in the polyps compared to the normal colon mucosa in controls, especially in hyperplastic and adenomatous polyps, the more benign polyp types. Furthermore, within this cohort, colon cancer was diagnosed in 38 patients either before, during, or after a polypectomy. These CRC patients exhibited lower TLR7 and TLR9 expressions in their polyps, indicating an antitumorigenic role for TLR7 and TLR9 as well. Moreover, these CRC patients exhibited no TLR5 expression in the

polyps, possibly reflecting that TLR5 may become more important following malignant development and, thus, not as relevant during the premalignant phase. However, TLR7 may play an important antitumorigenic role during the premalignant phase, possibly explaining why we found no relevant role of TLR7 in our CRC patient cohort.

In addition, the expression of genes related to TLRs were investigated using multiplex quantitative nuclease protection assay (qNPA) in 63 male CRC patients, during which the downregulation of TLR5, MyD88, IL1A, and IL6 in the tumor appeared to worsen overall survival (23). That study supports our findings of a strong TLR5 tissue immunoexpression as a positive prognostic marker. Unfortunately, that cohort was small, including only male patients without access to cause of death data for all patients, making it impossible to determine or analyze DSS.

Although the prognostic role of tumoral TLR5 has not been studied in CRC patients previously, studies on other malignancies have yielded promising results. In 313 gastric cancer patients, Kasurinen et al. examined TLR1, TLR2, TLR5, TLR7, and TLR9 tissue immunoexpressions (16). They found that patients with a high TLR5 tissue expression exhibited a better prognosis, in agreement with our findings among CRC patients. Stage III gastric cancer patients with a high TLR7 tissue expression exhibited a better DSS than patients with a low TLR7 tissue expression. Thus, Kasurinen et al. observed both nuclear and cytoplasmic expressions for all TLRs, while we observed TLR5 nuclear membrane expression and TLR7 cytoplasmic granulosomatous expression, confirming that in various cancers TLRs can play different roles and exhibit different expression patterns. The antibodies used may also influence different expressions patterns. For instance, we used mouse monoclonal TLR5 antibodies and TLR7 rabbit polyclonal antibodies in our study like Kasurinen et al., although the manufacturers of the antibodies differed.

In contrast to our study, among 154 pancreatic ductal adenocarcinoma (PDAC) patients, TLR5 tissue immunoexpression provided no prognostic value (24), possibly explaining the versatile

role of TLRs in different cancers. Mouse monoclonal TLR5 and rabbit polyclonal TLR7 were used, provided by different manufacturers compared to the antibodies we used.

In oral tongue squamous cell carcinoma (OTSCC) patients, moderate and strong TLR5 tissue expressions exhibited better outcomes, although TLR5 did not serve as an independent prognostic factor (25). Interestingly, using the same antibody that we used, Mäkinen et al. observed cytoplasmic TLR5 positivity, while in our CRC patients the TLR5 staining appeared in the nuclear membrane. By contrast, our TLR7 positivity was cytoplasmic, while in the OTSCC patients, TLR7 expression appeared in the nuclear membrane. In oropharyngeal squamous cell carcinoma (OPSCC), a high TLR5 immunoexpression associated with a worse prognosis among HPV-positive tumors (17). Similar results emerged for nasopharyngeal tumors (NPC) (26), where a high TLR7 tissue expression predicted a better prognosis and patients with a strong TLR5 expression experienced a worse prognosis.

The abovementioned studies demonstrate that the TLR5 staining pattern differs and TLR5 associates with prognosis in a varying manner, depending upon the cancer type and likely on the antibody as well. The precise biology behind this remains unknown, although it is obvious that TLRs play diverging roles in different cancers (12,27,28). Single-nucleotide polymorphism (SNP) within the TLR genes could partly explain these divergent roles among TLRs (29).

A TLR-targeted therapy has been studied in several malignancies including CRC and several clinical phase I and II trials are ongoing (30,31). To date, only the TLR7 agonist imiquimod has received approval for clinical use in basal cell carcinoma, used topically as a monotherapy or combined with other agents (32). Obviously, cancer immunotherapy represents a promising weapon to combat malignancies in the future. We need to identify solid biomarkers to distinguish tumors with different clinical behaviors and assist in developing targeted therapy for CRC patients. Furthermore, suitable biomarkers could help identify stage II CRC patients at a higher risk of

recurrence and simultaneously identify stage III patients at a lower risk and, thus, spared from treatment. TLR5 tissue expression could represent a promising biomarker in the future.

The biology behind TLR activation and following cascades is very complex and still unknown. TLRs may participate in activation of metalloproteinases and cytokines, and other intracellular cascades. The TLR activation may be different depending on activation by DAMP or PAMP signals. The biological explanation for our results dealing with TLR5 expression having effect on prognosis is still unrevealed. We can only speculate that possibly, if TLR5 is not present and active the cellular cascades will stay inactivated, and this phenomenon might lead to worse prognosis. Our large and well-characterized patient material dates back several years, representing one of the strengths of our study, allowing us a long follow-up period and providing reliable subgroup analyses. Adjuvant therapy was not that common at that time, giving us a valuable opportunity to investigate the more natural course of CRC. In addition, the tumor tissue microenvironment could be remarkably affected by neoadjuvant treatment causing a bias. Using TMA slides for the analysis provided us with several advantages, by saving the primary tissue material and allowing us the opportunity to study large patient cohorts at less expense. Furthermore, the TMA technique requires an experienced specialist to minimize folding or missing spots. Thus, debate continues regarding whether TMA is sufficiently representative to rely on small 1.0-mm tumor cores, although previous studies demonstrated that even a few cores, or even just one, could be representative of the tumor (33,34). Using archival TMA blocks could be seen as disadvantage as older tissue blocks might show different staining pattern compared to younger tissue blocks. We have investigated TLR5 and TLR7 stainings in newer tissue samples, which will be published separately with other projects. We did not see impressive differences in staining patterns between older and newer tissue samples (data not shown). Also missing of validation series in this study can be criticized, but as mentioned above, we have investigated TLR5 and TLR7 stainings in newer cohort and the results are in line with the results of current study (unpublished data).

Surgical techniques have changed in recent decades. The gold standard for colorectal surgical technique remains a total mesorectal excision (TME) for rectal tumors and complete mesocolic excision (CME) for colon tumors, both following the principles of operating along embryological planes (35,36). Using these techniques, the amount of retrieved lymph nodes has increased, thereby improving the prognostic value for the outcome of CRC patients (37). Thus, the pathology reports of resected tumors have significantly improved over the years. In our cohort, unfortunately, less detailed reports were available for most patients than in the structured synoptic report that currently represents the gold standard (38), leading to a lack of histopathological information, such as perineural, vascular, and lymphatic invasion. In addition, the amount of harvested lymph nodes from a specimen impacts the bias and harvesting fewer lymph nodes can cause mistakes in the tumor staging in an older cohort (39).

Conclusions

In conclusion, we showed for the first time that a strong TLR5 tumor tissue expression could be useful in identifying CRC patients likely to enjoy a better prognosis. Specifically, a strong TLR5 tissue expression served as an independent prognostic factor. Thus, TLR5 represents a promising biomarker, and further multicenter studies are needed to validate our results.

Notes

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Statement of Ethics

The study protocol was approved by The Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013). The National Supervisory Authority of Health and Welfare granted permission to use tissue archive samples retrospectively without requiring individual informed consent (Valvira Dnro 10041/06.01.03.01/2012).

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Conflict of Interest Statement

The authors have no conflict of interest to declare.

Author contributions

IBL, CB, CH, and JH conceived and designed the study. IBL, CB, HM, and SK collected and processed the data. JH contributed to specimen preparation. IBL and JH scored the histological samples. IBL and HM analyzed the data and interpreted the results. IBL drafted the manuscript and prepared the original figures and tables. All authors revised the manuscript and provided their final approval to the current version of the submitted manuscript.

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Figure legends

Figure 1 Images of TLR3, TLR5, TLR7, and TLR9 immunohistochemistry stainings representing colorectal cancer tumors with mild (**a, c, e, g**) and strong (**b, d, f, h**) staining. Images a–b stained with a TLR3 polyclonal antibody, images c–d stained with a TLR5 monoclonal antibody, images e–f stained with a TLR7 polyclonal antibody, and images g–h stained with a TLR9 polyclonal antibody. Original magnification: 20x.

Figure 2 Disease-specific survival analysis of different TLRs in colorectal cancer patients using the Kaplan–Meier method. (**a**) TLR3, (**b**) TLR5, (**c**) TLR7, and (**d**) TLR9. The log-rank test was used.

Figure 3 Disease-specific survival analysis of colorectal cancer patients using the Kaplan–Meier method. A mild versus strong (**a**) TLR5 expression among all patients, (**b**) TLR7 expression among all patients, (**c**) TLR5 expression among Dukes B patients, and (**d**) TLR5 expression among left-sided tumor patients. The log-rank test was used.

Supplementary Figure 1 Disease-specific survival analysis of colorectal cancer patients using the Kaplan–Meier method. (**a**) TLR5 expression among all patients, (**b**) TLR7 expression among all patients. The log-rank test was used.

Figure 1

Figure 2

Figure 3

Supplementary Figure 1

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Supplementary Table 1. Clinicopathological characteristics of 825 colorectal cancer patients

Clinicopathological variable	n (%)
Age	
<65	352 (42.5)
≥65	474 (57.5)
Gender	
Male	457 (55.4)
Female	368 (44.6)
Dukes stage	
A	122 (14.8)
B	292 (35.4)
C	225 (27.3)
D	186 (22.5)
Grade (WHO)	
1	26 (3.2)
2	563 (68.4)
3	201 (24.4)
4	33 (4.0)
Location	
Colon	424 (51.4)
Rectum	401 (48.6)
Side	
Right	225 (27.3)
Left	600 (72.7)
Histological type	
Adenocarcinoma	734 (89.1)
Mucinous	90 (10.9)

Table 1. Distribution of immunoactivity of TLRs among colorectal cancer patients

	Negative (%)	Low (%)	Moderate (%)	Strong (%)
Pilot series (n = 205)				
TLR3	1 (0.5)	54 (27.3)	125 (63.1)	19 (9.1)
TLR5	9 (5.4)	41 (24.7)	58 (34.9)	58 (34.9)
TLR7	18 (9.0)	84 (42.2)	75 (37.7)	22 (11.1)
TLR9	0 (0.0)	72 (36.7)	118 (60.2)	6 (3.1)
Whole cohort (n = 825)				
TLR5	154 (21.0)	209 (28.5)	216 (29.4)	155 (21.1)
TLR7	148 (19.2)	318 (41.4)	243 (31.6)	60 (7.8)

Abbreviations: TLR, toll like receptor

Table 2. Associations between TLR5 and TLR7 immunointensity and clinicopathological variables in 825 colorectal cancer patients

Clinicopathological variable	TLR5			TLR7		
	Mild	Strong	<i>p</i> value ¹	Mild	Strong	<i>p</i> value ¹
Age						
<65	155 (48.9)	162 (51.1)	0.823	193 (59.2)	133 (40.8)	0.503
≥65	208 (49.9)	209 (50.1)		273 (61.6)	170 (38.4)	
Gender						
Female	161 (49.7)	163 (50.3)	0.941	271 (62.4)	163 (37.6)	0.235
Male	202 (49.3)	208 (50.7)		195 (58.2)	140 (41.8)	
Side						
Right	111 (54.1)	94 (45.9)	0.119	141 (66.2)	72 (33.8)	0.058
Left	252 (47.6)	277 (52.4)		325 (58.5)	231 (41.5)	
Location						
Colon	205 (52.6)	185 (47.4)	0.076	242 (60.7)	157 (39.3)	0.975
Rectum	158 (45.9)	186 (54.1)		224 (60.5)	146 (39.5)	
Histological type						
Adenocarcinoma	299 (45.8)	354 (54.2)	<0.001	406 (59.1)	281 (40.9)	0.022
Mucinous	63 (78.8)	17 (21.3)		59 (72.8)	22 (27.2)	
Dukes stage						
A	46 (43.0)	61 (57.0)	0.388	77 (70.6)	32 (29.4)	0.048
B	130 (48.3)	139 (51.7)		153 (55.4)	123 (44.6)	
C	103 (52.3)	94 (47.7)		128 (60.7)	83 (39.3)	
D	84 (52.2)	77 (47.8)		108 (62.4)	65 (37.6)	
Tumor grade (WHO)						
1–2	251 (47.6)	276 (52.4)	0.118	297 (54.1)	252 (45.9)	<0.001
2–3	112 (54.4)	94 (45.6)		167 (76.6)	51 (23.4)	

Abbreviations: TLR, toll-like receptor

¹Chi-square test

Table 3. Univariate and multivariate Cox regression analyses for disease-specific survival among colorectal cancer patients

	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
Age						
<65	1.00			1.00		
≥65	1.02	1.01–1.02	0.004	1.03	1.02–1.05	<0.001
Gender						
Female	1.00			1.00		
Male	0.96	0.78–1.18	0.706	1.05	0.84–1.31	0.680
Dukes stage						
A	1.00			1.00		
B	2.18	1.29–3.69	0.004	2.15	1.22–3.78	0.008
C	5.29	3.17–8.82	<0.001	6.07	3.50–10.5	<0.001
D	38.3	21.4–68.5	<0.001	46.2	24.7–86.6	<0.001
Tumor grade (WHO)						
1–2	1.00			1.00		
3–4	2.48	1.80–3.42	<0.001	2.38	1.69–3.35	0.001
Location						
Colon	1.00			1.00		
Rectum	0.78	0.59–1.04	0.094	1.15	0.72–1.34	0.906
TLR5						
Mild	1.00			1.00		
Strong	0.75	0.60–0.94	0.011	0.74	0.59–0.92	0.007
TLR7						
Mild	1.00					
Strong	0.97	0.78–1.20	0.754			

Multivariate analysis included adjustments for Dukes stage, tumor grade, and tumor location.
Abbreviations: TLR, toll-like receptor, CI, confidence interval; HR, hazard ratio

Table 4. Survival analysis by subgroups, strong TLR5 and TLR7 expression compared strong to mild, in 825 colorectal cancer patients

	Strong TLR5			Strong TLR7		
	HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
Age						
<65	0.88	0.63–1.24	0.462	1.10	0.75–1.48	0.768
≥65	0.67	0.50–0.90	0.007	0.92	0.69–1.23	0.557
Gender						
Female	0.71	0.51–0.99	0.042	1.19	0.86–1.64	0.1301
Male	0.79	0.59–1.10	0.119	0.80	0.59–1.09	0.161
Dukes stage						
A	0.55	0.20–1.55	0.257	0.18	0.02–1.36	0.096
B	0.61	0.37–1.00	0.050	0.96	0.59–1.54	0.856
C	0.96	0.64–1.42	0.821	1.11	0.75–1.64	0.604
D	0.76	0.54–1.06	0.104	0.89	0.64–1.24	0.500
Tumor grade (WHO)						
1–2	0.76	0.59–1.01	0.062	1.01	0.78–1.31	0.934
3–4	0.76	0.52–1.12	0.167	1.08	0.70–1.68	0.720
Location						
Colon	0.82	0.60–1.21	0.213	1.06	0.78–1.45	0.708
Rectum	0.67	0.49–0.92	0.012	0.87	0.64–1.20	0.396
Histological type						
Adenocarcinoma	0.73	0.58–0.93	0.010	0.91	0.72–1.15	0.435
Mucinous	1.25	0.61–2.55	0.544	1.88	0.98–3.60	0.058

Abbreviations: TLR, toll-like receptor, CI, confidence interval, HR, hazard ratio