The Relationship Between the Tissue Expression of TLR2, TLR4, TLR5, and TLR7 and Systemic Inflammatory Responses in Colorectal Cancer Patients

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Running title: Evaluation of tissue TLRs and plasma CRP in colorectal cancer

Abbreviations: CI, confidence interval; CRC, colorectal cancer; CRP, C-reactive protein; DAMP, damage-associated molecular pattern; DC, dendritic cell; DSS, disease-specific survival; GPS, Glasgow prognostic score; HR, hazard ratio; IQR, interquartile range; LMR, lymphocyte-to-monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PAMP, pathogen-associated molecular pattern; PLR, platelet-to-lymphocyte ratio; SIR, systemic inflammatory response; TIMP-1, tissue inhibitor of metalloproteinase-1; TLR, toll-like receptor; TMA, tissue microarray; TR-IFMA, time-resolved immunofluorometric assay.

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Abstract

Background: Colorectal cancer (CRC) is the third most commonly diagnosed malignancy globally. CRC patients with elevated plasma C-reactive protein (CRP) levels exhibit compromised prognoses. Toll-like receptors (TLRs), activating the innate and adaptive immune system, may contribute to pro- and antitumorigenic inflammatory responses. We aimed to identify a possible link between local and systemic inflammatory responses in CRC patients by investigating the association between tissue TLRs and plasma CRP.

Methods: Tissue expressions of TLR2, TLR4, TLR5, and TLR7 were assessed using immunohistochemistry of tissue microarray (TMA) slides from 549 CRC patients surgically treated between 1998 and 2005. Blood samples were drawn preoperatively, centrifuged, aliquoted, and stored at –80°C until analysis. Plasma CRP was determined through high-sensitivity time-resolved immunofluorometric assay (TR-IFMA). We investigated the association of TLRs to clinicopathological variables, plasma CRP, and survival.

Results: High TLR2 expression [hazard ratio (HR) 0.059; 95% confidence interval (CI) 0.41–0.85; $p = 0.005$], high TLR5 expression (HR 0.60; 95% CI 0.45–0.83; $p = 0.002$), positive TLR7 expression (HR 0.49; 95% CI 0.33– 0.72; $p < 0.001$), and low CRP (HR 1.48; 95% CI 1.08–2.11; $p = 0.017$) associated to a better prognosis. A high TLR2 immunoexpression associated with a better prognosis among low CRP patients (HR 0.53; 95% CI 0.35–0.80; $p = 0.002$), high TLR4expression among high CRP patients (HR 2.04; 95% CI 1.04–4.00; $p = 0.038$), high TLR5 expression among low CRP patients (HR 0.059; 95% CI 0.37–0.92; $p = 0.021$), and positive TLR7 expression among low CRP

patients (HR 0.53; 95% CI 0.28–1.00; p = 0.049). In multivariate analyses, no biomarkers emerged as significant independent variables.

Conclusions: High tissue TLR2, TLR5, and TLR7 levels associate with a better prognosis. Among low CRP patients, those with high TLR2, TLR5, and TLR7 immunoexpressions exhibited a better prognosis. Among high CRP patients, a low TLR4 immunoexpression associated with a better prognosis.

Introduction

Colorectal cancer (CRC) is the second leading malignancy in terms of cancer deaths in the world, and third most common in terms of cancer incidence [1]. Although prognosis among CRC patients has improved, recurrence develops in 17% of stage II and 36% of stage III patients [2]. Reliable prognostic markers are needed to identify individuals at an increased risk who may benefit from tailored adjuvant therapies and intensified surveillance.

Chronic local inflammatory processes represent known risk factors for developing CRC [3]. A higher density of tumor-infiltrating lymphocyte (TIL), however, may promote antitumoral immune mechanisms and, thus, associate with better survival among CRC patients [4].

Transmembranous toll-like receptors (TLRs), expressed by innate immune system cells, B- and T-lymphocytes, epithelial cells of the gastrointestinal tract and the respiratory system, are crucial to activating the host's innate immune responses after recognizing pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) [5–7]. In addition, TLRs form a part of the adaptive immune system by initiating dendritic cell (DC) maturation, migration to lymph nodes, and signaling to naive T-cells [8].

TLRs take part in the pathogenesis of several diseases and contribute to the development of malignancies [5,7]. In carcinogenesis, the role of TLRs vary since the same TLRs may act as tumor-promoting in some and antitumorigenic in other malignancies [6,9,10].

TLRs have not been extensively investigated in CRC, with TLR4 representing the most studied TLR in CRC. In a previous study, we revealed that Dukes B patients with strong TLR4 tissue immunoexpression exhibited a worse prognosis, while a strong TLR2 tissue immunoexpression associated with a favorable prognosis in Dukes C patients [11]. In another study, we demonstrated that a strong TLR5 tissue expression represents an independent positive prognostic factor among CRC patients [12]. To our knowledge, this was the first study to describe the role of TLR5 in CRC. In addition, a low serum TLR2 associated with a high serum C-reactive protein (CRP) in another published study [13].

Several studies have demonstrated that elevated levels of acute-phase protein CRP, a well-known marker for the systemic inflammatory response (SIR), predicts a poorer prognosis in CRC patients [14–17]. A high Glasgow prognostic score (GPS) or modified GPS (mGPS), based on CRP and albumin levels, predict a worse prognosis in CRC patients [18].

The relationship between the local and systemic inflammatory responses in CRC was previously investigated in some studies. A low CD4+ T-lymphocyte infiltration, a predictor of poorer survival, associated with elevated CRP levels in CRC patients [19]. In another study, a significant negative association was detected between a high CRP and intratumoral T-regulatory FOXP3+ cell infiltration [20]. Among stage II and III CRC patients with mild local inflammatory cell infiltration, prognosis appeared better in patients with a low mGPS score [21].

The underlying mechanisms connecting the local and systemic inflammatory responses remain unclear, with further studies needed. Thus, our study aimed to evaluate the possible relationship between local and systemic inflammatory responses in CRC by comparing the tissue immunoexpressions of TLR2, TLR4, TLR5, and TLR7 and plasma CRP values.

Materials and Methods

Patients

We retrospectively studied a cohort of 549 consecutive CRC patients surgically treated in the Department of Surgery, Helsinki University Hospital, Finland, between 1998 and 2005 (Table 1). The median age at surgery was 69.2 [interquartile range (IQR) 59.2–77.4], and 260 (47.4%) patients were women. The median follow-up time was 6.44 years (IQR 2.00–14.84). By the end of follow-up, 170 (31.0%) patients were alive and 191 (34.9%) patients had died of CRC. The 5-year disease-specific survival (DSS) for all patients was 69.2% [95% confidence interval (CI) 65.1–63.3]. We used the sixth version of the TNM disease classification [22] for CRC staging, summarized in Table 1. The clinical data were gathered from patient medical records, while survival data and the cause of death of deceased patients were provided by the Population Register Center of Finland (currently, Digital and Population Data Service Agency) and Statistics Finland.

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

Blood samples

Patient blood samples were retrieved before surgery (range 0–30 days), in most cases within 3 days of surgery (92.7%). After centrifuging, serum and plasma components were stored as aliquoted at —80°C until analysis. Plasma CRP was determined through a high-sensitivity method [time-resolved immunofluorometric assay (TR-IFMA) using a monocolonal CRP antibody (anti-hCRP, code 6405, Medix Biochemica, Espoo, Finland)] as described elsewhere [23].

Tissue samples

Formalin-fixed and paraffin-embedded surgical tumor samples were stored in the archives of the Department of Pathology, Helsinki University Hospital. Representative tumor areas were marked on

hematoxylin- and eosin-stained slides by an experienced pathologist (JH). Four 1.0-mm cores were punched from these representative tumor areas and the tissue microarray (TMA) blocks were constructed using a TMA Grand Master 3D instrument (Histech Ltd Budapest, Hungary). For immunohistochemistry, we cut 4-µm sections from the TMA blocks as previously described [24].

Immunohistochemistry

We used the same immunohistochemical staining protocol for each TLR. The 4-µm TMA sections were deparaffinized and rehydrated. For antigen retrieval, the slides were prewarmed in a PreTreatment module (Lab Vision UK Ltd, UK) and treated with a Tris-HCl buffer (pH 9) for 15 min at 98ºC. We used the Autostainer 480 (Lab Vision, Fremont, California, USA), with the REAL EnVision Detection System (peroxidase/DAB+, rabbit/mouse; Dako, Glostrup, Denmark), for staining the TMA slides. The endogenous peroxidases were blocked with a 0.3% Dako REAL Peroxidase-Blocking Solution incubation for 5 min, followed by primary antibody incubation with primary antibodies: 200 µg/ml TLR2 rabbit polyclonal (sc-10739, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted to 1:200; overnight), 200 µg/ml TLR4 mouse monoclonal (sc-293072, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted to 1:2000; 1 hr), 1.0 µg/ml TLR5 mouse monoclonal (NBP2-24787, Novus Biologicals, Centennial, USA; diluted to 1:300; overnight), and 1.0 µg/ml TLR7 rabbit polyclonal (NBP2-24906, Novus Biologicals, Centennial, USA; diluted to 1:300; 1 hr). Finally, we incubated for 30 min with the peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) secondary antibody, and visualized the staining using Dako REAL DAB+ Chromogen for 10 min. Slides were counterstained with Meyer's hematoxylin and mounted in Pertex Mounting (Histolab Products AB, Sweden). In each staining series, we used negative (specimens processed without primary antibody) and positive (tonsillar, skin, and cutaneous squamous cell carcinoma tissue, known to show a high immunoreactivity for the studied antigens) controls.

Scoring of samples

Staining intensities of TLR2, TLR4, TLR5, and TLR7 in CRC TMAs were evaluated by two independent assessors (IB-L and JH), one of whom is an experienced pathologist from the Department of Pathology, Helsinki University Hospital. Both assessors were blinded from the clinical data. The immunoreactivity of the TLRs was scored from 0 to 3 as follows: 0 as negative, 1 as weak positive, 2 as moderate, and 3 as a strong immunoreactivity (Fig. 1). TLR2 and TLR4 immunopositivity appeared as a cytoplasmic brown color. TLR5 immunopositivity was detected on the nuclear membranes and TLR7 as granular cytoplasmic brown immunopositivity (Fig. 1). We scored four cores from each tumor, choosing the core with the highest score for the statistical analysis. The results from both assessors were compared, and any intra-observer disagreements were re-evaluated, with the final result achieved through discussion and consensus.

Statistical analyses

We used the Pearson's chi-square test to evaluate associations and Spearman's correlation test for correlations. Variables were dichotomized for statistical analysis as described below. The cutoff value for CRP was determined by the maximum value for Youden's index. We defined DSS as the time from surgery until death from CRC or until the end of the follow-up period. Survival curves were constructed using the Kaplan–Meier method and compared groups using the log-rank test. The 95% confidence intervals (CIs) were calculated for the survival rates.

We used the Cox proportional hazards model to calculate the hazard ratios (HRs) for the uni- and multivariate survival analyses. For the multivariate analysis, age, gender, tumor stage, and tumor location were used as independent covariates. Tumor stage was processed as a categorical covariate. A two-sided test with $p < 0.05$ was considered significant for all analyses. All statistical analyses

were performed using SPSS version 25.0 (IBM SPSS Statistics, version 25.0 for Mac; SPSS, Inc., Chicago, IL, USA, an IBM Company).

Results

Immunostaining of TLRs

From the 549 TMA samples, TLR2 immunoexpression was scored successfully in 541 (98.5%), TLR4 in 537 (97.8%), TLR5 in 539 (98.2%), and TLR7 in 539 (98.2%) cases. In a few cases, a TLR score was not possible to determine given missing representative cancer tissue or a technical failure. The score distributions for TLRs 2, 4, 5, and 7 are detailed in Supplementary Table 1.

For the statistical analysis, we dichotomized patients as follows: TLR2 low (scores 0–1) and high (scores 2–3; Figs. 1a–d), TLR4 low (scores 0–1) and high (scores 2–3; Figs. 1e–h), TLR5 low (scores 0–2) and high (score 3; Figs. 1i–m), and TLR7 negative (score 0) and positive (scores 1–3; Figs. 1n–r).

Associations between TLRs, CRP, and clinicopathological parameters

A low TLR5 associated with a higher tumor stage ($p < 0.001$; Table 2), higher pT classification ($p < 0.001$; Table 2), and lymph node positivity ($p = 0.004$; Table 2). Positive TLR7 associated with left-sided disease ($p = 0.003$; Table 2), a lower tumor stage ($p < 0.001$; Table 2), lower pT classification ($p = 0.043$; Table 2), lower WHO grade ($p = 0.035$; Table 2), and with pN0 disease ($p < 0.001$; Table 2). TLR2 and TLR4 did not associate with any clinicopathological parameters (Table 1). CRP was lower in stage I–III patients ($p < 0.001$, Supplementary Table 2) and patients with pT1–2 disease ($p < 0.001$, Supplementary Table 2). We observed no association between TLRs, age, gender, and CRP (Tables 1 and 2).

A high TLR2 immunoexpression associated with a high expression of all other TLRs ($p < 0.001$ for all; data not shown) and comparisons also revealed weak or moderate positive correlations

($p < 0.001$ for all; Table 3). A high TLR4 expression associated with a high TLR5 expression ($p = 0.004$; data not shown) and a positive TLR7 expression ($p < 0.001$; data not shown), also here weak positive correlations revealed ($p < 0.001$ for both, Table 3). TLR5 did not associate with TLR7 ($p = 0.266$; data not shown), although we noted a weak positive correlation ($p = 0.006$, Table 3). No correlations emerged between TLRs and CRP (Table 3).

Survival analysis

The 5-year DSS reached 55.9% among patients with a low TLR2 expression (95% CI 44.9–68.9), compared to 71.2% (95% CI 66.9–75.5; $p = 0.005$; Fig. 2a) among those with a high TLR2 expression. TLR4 immunoexpression exhibited no prognostic value (HR 0.92; 95% CI 0.67–1.22; $p = 0.504$; Fig 2b). Among patients with a low TLR5 expression, 5-year DSS reached 63.5% (95% CI 57.8–69.2) and 77.5% (95% CI 71.6–83.4; $p = 0.002$, Fig. 2c) among patients with a high TLR5 expression. Among negative TLR7 immunoexpression patients, 5-year DSS fell to 51.1% (95% CI 38.0–64.2), compared to 71.1% (95% CI 67.4–76.0; $p < 0.001$; Fig. 2d) among those with a positive TLR7 expression. Among patients with a low CRP, 5-year DSS was 74.1% (95% CI 68.8–79.4) compared to 64.3% among high CRP patients (95% CI 54.6–72.2; $p = 0.017$; Fig. 2e).

In the subgroup analysis, among patients with stage III disease (HR 0.40; 95% CI 0.24–0.65; $p < 0.001$; Supplementary Table 3), a lower WHO grade (HR 0.73; 95% CI 0.35–0.80; $p = 0.003$; Supplementary Table 3), a younger age (HR 0.47; 95% CI 0.26–0.83; $p = 0.009$; Supplementary Table 3), female gender (HR 0.40; 95% CI 0.25–0.67; $p < 0.001$; Supplementary Table 3), a higher pT stage (HR 0.61; 95% CI 0.40–0.94; $p = 0.024$; Supplementary Table 3), and a low CRP (HR 0.53; 95% CI 0.35–0.80; $p = 0.002$; Fig. 3a and Supplementary Table 3), a high TLR2 immunoexpression served as positive prognostic factors.

Among patients with a high CRP (HR 2.04; 95% CI 1.04–4.00; $p = 0.038$; Fig. 3d and Supplementary Table 3), those with a high TLR4 immuoexpression exhibited a worse prognosis.

Among patients with a lower WHO grade (HR 0.59; 95% CI 0.41–0.83; p = 0.003; Supplementary Table 4), female gender (HR 0.52; 95% CI 0.34–0.86; p = 0.009; Supplementary Table 4), a higher pT stage (HR 0.67; 95% CI 0.48–0.94; p = 0.020; Supplementary Table 4), location in the rectum (HR 0.54; 95% CI 0.34–0.86; p = 0.009; Supplementary Table 4), and a low CRP (HR 0.059; 95% CI 0.37–0.92; p = 0.021; Fig. 3e and Supplementary Table 4), those with a high TLR5 immunoexpression experienced a better prognosis.

Patients with a positive TLR7 immunoexpression exhibited a better prognosis among subgroups of older patients (HR 0.47; 95% CI 0.29–0.75; p = 0.002; Supplementary Table 4), female gender (HR 0.38; 95% CI 0.22–0.64; p < 0.001; Supplementary Table 4), a lower WHO grade (HR 0.53; 95% CI 0.33–0.86; p = 0.010; Supplementary Table 4), a higher pT stage (HR 0.59; 95% CI 0.39–0.89; p = 0.012; Supplementary Table 4), location in the right colon (HR 0.32; 95% CI 0.17–0.60; p < 0.001; Supplementary Table 4), and among patients with a low CRP (HR 0.53; 95% CI 0.28–1.00; p = 0.049; Fig. 3g and Supplementary Table 3).

In the multivariate Cox regression analysis including age, gender, tumor location, and tumor stage as covariates, none of the biomarkers under investigation served as significant independent factors of DSS, while age and tumor stage significantly associated with survival (data not shown).

Discussion

The prognostic role and possible relationship between the tissue immunoexpression of TLRs (local inflammatory response) and plasma CRP (SIR) among CRC patients have not previously been studied. Here, we observed a favorable prognosis among patients with a high TLR2 expression, a high TLR5 expression, a positive TLR7 expression, and a low CRP. We did not detect any association or correlation between the different TLRs and CRP. However, among patients with a low CRP, those with a high TLR2, TLR5, and TLR7 tumor expression exhibited a better prognosis and among high CRP patients, those with a low TLR4 immunoexpression exhibited a better prognosis. Unfortunately,

none of the biomarkers examined remained independent prognostic factors in the multivariate analysis.

The biological mechanisms behind and interaction between systemic and local inflammation and carcinogenesis are complex and remain poorly understood. In epidemiological studies, an elevated level of circulating CRP serves as a marker of cancer as well as indicating a predisposition to the development of future malignancies [25]. Yet, some genetic epidemiological studies do not agree with this theory [25]. CRC patients with an increased CRP (14,15,26), high mGPS [18,26], an elevated neutrophil-to-lymphocyte ratio (NLR) [27], elevated lymphocyte-to-monocyte ratio (LMR) [28], elevated platelet-to-lymphocyte ratio (PLR) [29], high IL-6 [30], and elevated tissue inhibitor of metalloproteinase-1 (TIMP-1) [31], all markers of SIR, associate with a worse prognosis.

Among many malignancies including CRC, local and systemic inflammation have been studied separately, with very few studies assessing the association between local inflammation and SIR. Paarnio et al. studied both the serum and tumoral expression of TLR2 and TLR4 finding no association with each other, although patients with detectable serum TLR2 levels exhibited higher TLR2 immunoexpression in the normal colorectal mucosa [13]. They found that patients with undetectable TLR2 serum levels had higher CRP values, indicative of some connection between local and systemic inflammation [13]. Neither serum nor tissue TLR2 nor TLR4 expressions carried a prognostic value in their survival analysis [13,32]. However, they did not investigate the association between the tissue expression of TLRs and SIR as we did here. In our study, among the high CRP subgroup, patients with a low tumor TLR4 immunoexpression exhibited a better prognosis. In addition, a high TLR2, a high TLR5 and a positive TLR7 expression linked with a better prognosis among patients with low CRP, further suggesting a connection between local and systemic inflammation.

TLRs play an important role in carcinogenesis, although that role varies since the same TLR can act differently in differing malignancies [10]. Very few studies exist describing the role of TLRs

in CRC. We previously demonstrated that patients with lymph node–positive disease with a strong tissue expression of TLR2 exhibited a better DSS [11]. The current study supports this finding, since CRC cases with a high TLR2 expression exhibited a better prognosis across the entire cohort. As mentioned above, Paarnio et al. observed no significant association between TLR2 and TLR4 tissue expressions and prognosis [32]. In our previous work, a strong TLR4 expression indicated a worse prognosis among Dukes B CRC patients [11], although such patients constituted a quite small proportion of cases in the present study, possibly explaining why TLR4 expression did not associate with prognosis. This finding agrees with previous work by Paarnio et al. [32].

In addition, we have demonstrated that a high TLR5 expression serves as a positive prognostic factor in a cohort of 825 CRC patients [12], with the current study supporting this finding, since patients with a high TLR5 expression exhibited a better prognosis, also in the low CRP subgroup as well. Previously, we found no association between TLR7 immunoexpression and survival [12], despite patients with a positive TLR7 immunoexpression in the current study experiencing a better prognosis. Perhaps this discrepancy results from a difference in the study population, since in current study we included only patients for whom CRP information was available. In addition, the antibodies we used for immunostaining differed between the previous and the current study. A similar finding for the TLR7 association with a better prognosis was noted among stage III gastric cancer patients [33] and in patients with nasopharyngeal [34] and oropharyngeal tumors [35].

Guidelines for CRC treatment rely on TNM (tumor, node, and metastasis) staging, which takes into account only the tumor-associated factors and does not include the host's immune responses [36,37]. Patients with the same TNM stage may have different prognoses. For instance, in some patients, we see a relapse soon after curative intent treatment, while others may have stable disease for years or even show regression of their lymph node–positive or metastasized disease without additional treatment [38,39]. The diagnosis and treatment of stage II and III tumors remains under debate, since 17% of stage II and 36% of stage III patients relapse within 5 years [2,38]. This

variation in outcomes among patients with the same stage disease shows that malignancies are complex and depend not only on tumor-related factors, but also on the tumor's interaction with its microenvironment and the host's immune response to the tumor. When the host's immunosurveillance mechanisms over-compete, the tumor begins to progress and metastasize [39]. TLRs as the first-line immune activators certainly play a role in immunosurveillance. In our present study, TLRs and CRP associate with prognosis, TLR2 specifically associating with prognosis among patients with stage III disease. Further studies are necessary in order to clarify the exact role of and interaction between local TLR immunoexpressions and SIR manifested as an elevated CRP in CRC, especially among patients whose disease recurs.

This large and well-characterized patient cohort with a long follow-up time represents a strength to our study. Missing reliable data about neoadjuvant or adjuvant therapy, however, can be seen as a limitation. Using TMA for immunohistochemistry is sometimes criticized, although this technique also carries several advantages, enabling the study of large numbers of immunostained samples. Punching tumor cores from several locations within the tumor sample overcomes the risk of misinterpretation because of the focal expression of antigens.

Conclusions

To our knowledge, this is the first study to evaluate the association between tissue TLR expression and CRP. A high tissue TLR2, high TLR5, positive TLR7, and low plasma CRP levels all associated with a better prognosis. However, no association or correlation was observed between different TLRs and CRP. Further research is still needed to understand the role of TLRs in CRC and the interaction between systemic inflammation and the local activation of TLRs to better understand tumor development.

Notes

Statement of Ethics

The Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013) approved the study protocol. The National Supervisory Authority of Health and Welfare (Valvira Dnro 10041/06.01.03.01/2012) permitted the use of archival tissue and blood samples without requiring individual consent. The study complied with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

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Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

Author Contributions

IB-L, CB, CH, JH, UG, RP, and KS participated in conceptualizing and designing the study. CH collected the blood and tissue samples. IB-L and TK collected and processed the data. JH contributed to the specimen preparation. IB-L and JH scored the samples. Serum samples were analyzed by US. IB-L analyzed the results, drafted the manuscript, and prepared the original figures and tables. All authors agreed to the content of the manuscript, and provided their final approval of the current version of the submitted manuscript. CB and CH equally participated in supervision.

Figure legends

Fig. 1. Images of the TLR2, TLR4, TLR5, and TLR7 immunohistochemistry stainings representing colorectal cancer tumors with negative (**a, e, i, m**), mild (**b, f, k, o**), moderate (**c, g, l, p**), and strong (**d, h, m, r**) staining. Original magnification: 20x.

Fig. 2. Colorectal cancer patients' disease-specific survival analysis for different TLRs and CRP using the Kaplan–Meier method; (**a**) TLR2, (**b**) TLR4, (**c**) TLR5 and TLR7 (**d**), and CRP (**e**). *p* value for the log-rank test.

Fig. 3. Colorectal cancer patients' disease-specific survival analysis by subgroup using the Kaplan–Meier method. A high versus low TLR2 among patients with (**a**) a low CRP and (**b**) a high CRP. A high versus low TLR4 among patients with (**c**) a low CRP and (**d**) a high CRP. A high versus low TLR5 among patients with (**e**) a low CRP and (**f**) a high CRP. A positive versus negative TLR7 among patients with (**g**) a low CRP and (**h**) a high CRP. *p* value for the log-rank test.

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Figure 1

Figure 2

a

Cumulative survival vs Time (in years)

TLR2 high
TLR2 low

$p = 0.005$
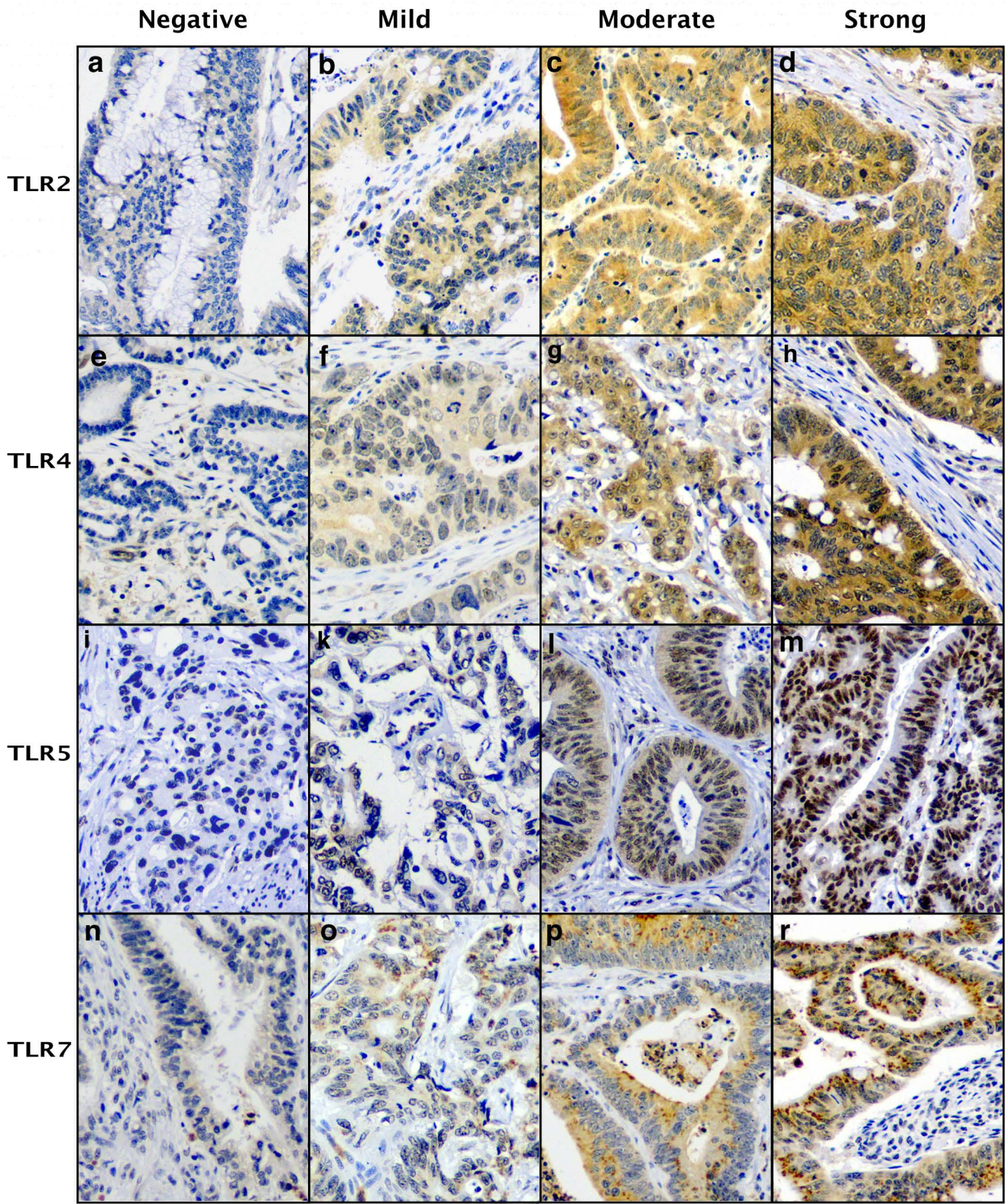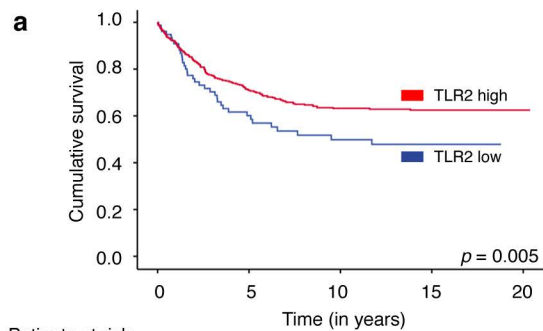
Patients at risk

TLR2 low:	71	35	23	14	0
TLR2 high:	468	271	197	112	3

b

Cumulative survival vs Time (in years)

TLR4 high
TLR4 low

$p = 0.504$
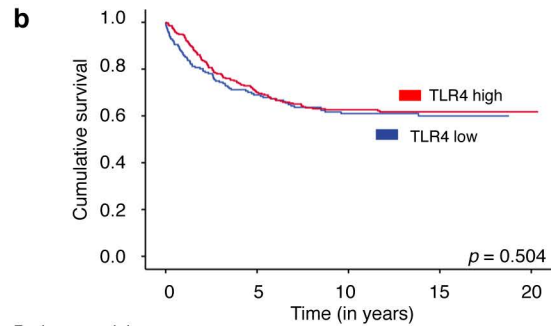
Patients at risk

TLR4 low:	212	123	86	36	0
TLR4 high:	347	195	142	94	3

c

Cumulative survival vs Time (in years)

TLR5 high
TLR5 low

$p = 0.002$
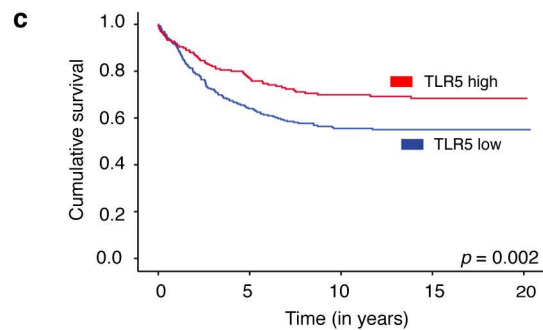
Patients at risk

TLR5 low:	314	161	116	66	2
TLR5 high:	223	144	103	59	1

d

Cumulative survival vs Time (in years)

TLR7 positive
TLR7 negative

$p < 0.001$
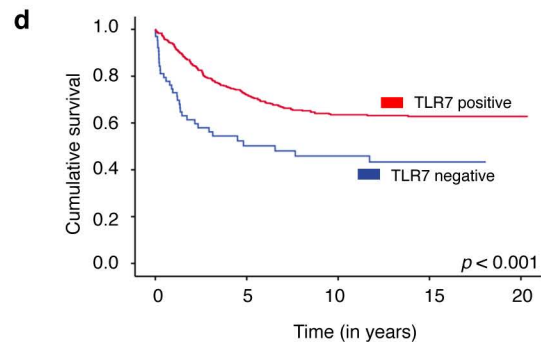
Patients at risk

TLR7 negative:	64	23	18	8	0
TLR7 positive:	473	282	202	119	3

e

Cumulative survival vs Time (in years)

CRP low
CRP high

$p = 0.017$
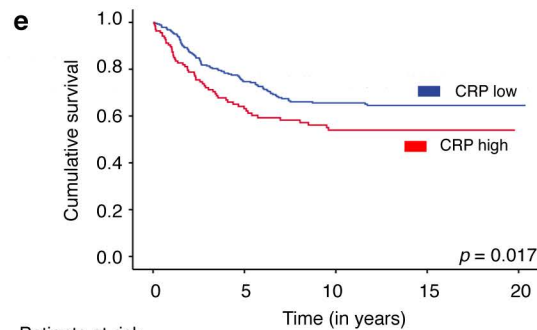
Patients at risk

CRP low:	286	180	129	70	3
CRP high:	139	64	48	35	0

Figure 3

Table 1. Association between TLR2 and TLR4 immunointensity and clinicopathological variables among 549 colorectal cancer patients

Clincopathological variable	n (%)	TLR2			TLR4		
		Low (%)	High (%)	*p* value[1]	Low (%)	High (%)	*p* value[1]
Age							
<65	220 (40.1)	28 (13.0)	188 (87.9)	0.847	84 (39.4)	129 (60.6)	0.412
≥65	329 (59.9)	44 (13.5)	281 (86.5)		116 (35.8)	208 (64.2)	
Gender							
Male	289 (52.6)	38 (13.4)	245 (86.6)	0.932	98 (34.8)	184 (65.2)	0.212
Female	260 (47.4)	34 (13.2)	224 (86.8)		102 (40.0)	153 (60.0)	
Location							
Right colon	155 (28.2)	14 (9.3)	137 (90.7)	0.073	53 (35.1)	98 (64.9)	0.783
Left colon	126 (23.0)	14 (11.1)	112 (88.9)		46 (37.1)	78 (62.9)	
Rectum	268 (48.8)	44 (16.7)	220 (83.3)		101 (38.5)	161 (61.5)	
Tumor stage							
I	108 (19.7)	14 (13.2)	92 (86.8)	0.481	37 (34.6)	70 (65.4)	0.840
II	153 (27.9)	15 (9.9)	137 (90.1)		58 (38.4)	93 (61.6)	
III	201 (36.7)	28 (14.1)	170 (85.9)		75 (38.7)	119 (61.3)	
IV	86 (15.7)	14 (16.7)	70 (83.3)		29 (34.5)	55 (65.5)	
Tumor classification (pT)							
pT1–pT2	134 (24.8)	16 (12.1)	116 (7.9)	0.768	49 (36.8)	84 (63.2)	0.520
pT3–pT4	407 (75.2)	54 (13.5)	347 (86.5)		147 (37.1)	249 (62.9)	
Lymph node metastasis (pN)							
pN0	276 (51.2)	32 (11.7)	241 (88.3)	0.369	97 (35.5)	176 (64.5)	0.369
pN1–2	263 (48.8)	38 (14.7)	220 (85.3)		100 (39.4)	154 (60.6)	
Tumor grade (WHO)							
1	43 (8.7)	3 (7.1)	39 (92.9)	0.522	14 (32.6)	29 (67.4)	0.515
2	389 (78.9)	52 (13.4)	335 (86.6)		145 (38.1)	236 (61.9)	
3	29 (5.9)	4 (13.8)	25 (86.29		9 (31.0)	20 (69.0)	
4	32 (6.)	4 (13.3)	26 (86.7)		8 (26.7)	22 (73.3)	
Systemic inflammatory response (CRP)							
≤8.7	287 (67.4)	41 (14.5)	242 (85.5)	0.453	97 (34.3)	186 (65.7)	0.739
>8.7	139 (32.6)	16 (11.7)	121 (88.3)		43 (32.3)	90 (67.7)	

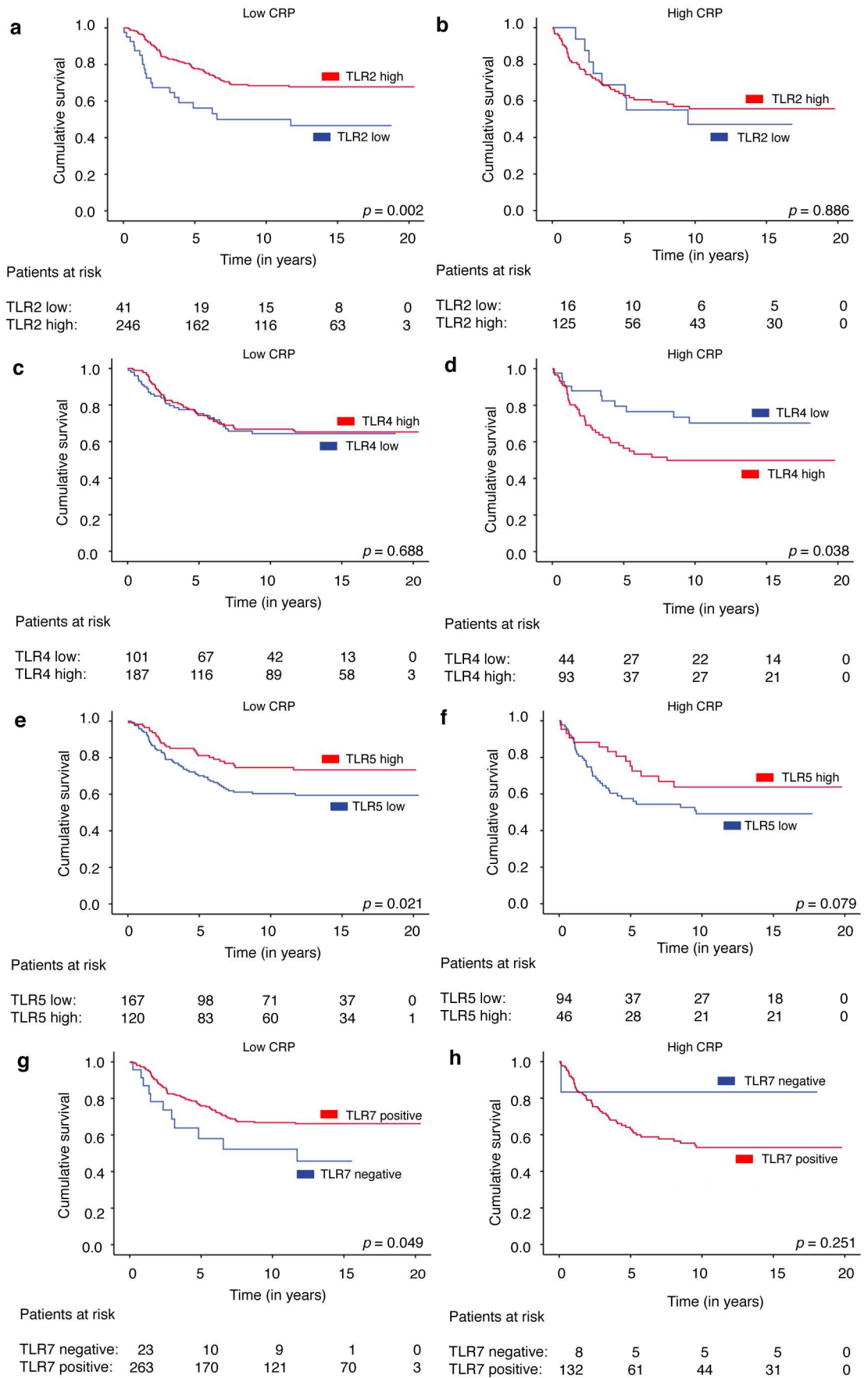Abbreviations: CRP, C-reactive protein; TLR, toll-like receptor

Table 2. Association between TLR5 and TLR7 immunointensity and clinicopathological variables among 549 colorectal cancer patients

Clincopathological variable	TLR5			TLR7		
	Low (%)	High (%)	p value[1]	Negative (%)	Positive (%)	p value[1]
Age						
<65	118 (54.9)	97 (45.1)	0.154	23 (10.6)	193 (89.4)	0.500
≥65	198 (61.6)	126 (38.9)		41 (12.79	282 (87.3)	
Gender						
Male	164 (58.4)	117 (41.6)	0.930	34 (12.1)	248 (87.9)	0.499
Female	152 (58.9)	106 (41.1)		30 (11.7)	227 (88.3)	
Location						
Right colon	87 (57.2)	65 (42.8)	0.836	29 (19.3)	121 (80.7)	**0.003**
Left colon	72 (57.6)	53 (42.4)		13 (10.3)	113 (89.7)	
Rectum	157 (59.9)	105 (40.1)		22 (8.4)	241 (91.6)	
Tumor stage						
I	44 (41.1)	63 (58.9)	**<0.001**	8 (7.5)	99 (92.5)	**<0.001**
II	89 (58.9)	62 (41.1)		6 (4.0)	145 (96.0)	
III	123 (62.8)	73 (37.2)		33 (16.8)	163 (83.2)	
IV	59 (70.2)	25 (29.8)		16 (19.0)	68 (81.0)	
Tumor classification (pT)						
pT1–pT2	54 (40.6)	79 (59.4)	**<0.001**	9 (6.8)	124 (93.2)	**0.043**
pT3–pT4	260 (65.3)	138 (34.7)		53 (13.3)	345 (86.7)	
Lymph node metastasis (pN)						
pN0	146 (53.5)	127 (46.5)	**0.004**	14 (5.1)	259 (94.9)	**<0.001**
pN1–2	168 (65.6)	88 (34.4)		47 (18.4)	209 (81.6)	
Tumor grade (WHO)						
1	24 (55.8)	19 (44.2)	0.966	4 (9.3)	39 (90.7)	**0.035**
2	224 (58.2)	161 (41.8)		37 (9.6)	348 (90.4)	
3	18 862.1)	11 (37.9)		6 (21.4)	22 (78.6)	
4	17 (58.6)	12 (41.4)		7 (23.3)	23 (76.7)	
Systemic inflammatory response (CRP)						
≤8.7	165 (58.3)	118 (41.7)	0.135	23 (8.2)	258 (91.8)	0.435
>8.7	90 (66.2)	46 (33.8)		8 (5.9)	128 (94.1)	

Abbreviations: CRP, C-reactive protein; TLR, toll-like receptor

[1]Chi-square test

Table 3. Correlations for TLR2, TLR4, TLR5, and TLR7 among each other and with CRP in 549 colorectal cancer patients

	TLR2		TLR4		TLR5		TLR7	
	r_s	*p* value	r_s	*p* value	r_s	*p* value	r_s	*p* value
TLR4	0.219	**<0.001**						
TLR5	0.157	**<0.001**	0.317	**<0.001**				
TLR7	0.171	**<0.001**	0.248	**<0.001**	0.109	**0.006**		
CRP	0.069	0.161	0.033	0.505	-0.039	0.421	-0.012	0.813

Abbreviations: CRP, C-reactive protein; TLR, toll-like receptor

r_s = Spearmans's correlation coefficent

Supplementary Table 1. Expression distribution for TLR2, TLR4, TLR5, and TLR7 among 549 colorectal cancer patients

	Negative (%)	Low (%)	Moderate (%)	Strong (%)
TLR2	10 (1.8)	62 (11.5)	252 (46.6)	217 (40.1)
TLR4	48 (8.9)	152 (28.3)	246 (45.8)	91 (16.9)
TLR5	60 (11.1)	58 (10.8)	198 (36.7)	223 (41.4)
TLR7	64 (11.9)	175 (32.5)	248 (46.0)	52 (9.6)

Abbreviations: TLR, toll-like receptor

Supplementary Table 2. Association between CRP and clinicopathological parameters among 549 colorectal cancer patients

Clincopathological variable	CRP		
	≤8.7	>8.7	p value[1]
Age			
<65	119 (64.7	65 (35.3)	0.348
≥65	168 (69.4)	74 (30.6)	
Gender			
Male	140 (69.3)	62 (30.7)	0.469
Female	147 (65.6	77 (34.4)	
Location			
Right colon	63 (61.8)	39 (38.2)	0.150
Left colon	51 (63.0)	30 (37.0)	
Rectum	173 (71.2)	70 (28.8)	
Tumor stage			
I	68 (81.9)	15 (18.1)	**<0.001**
II	81 (67.5)	39 (32.5)	
III	107 (68.6)	49 (31.4)	
IV	31 (46.3)	36 (53.7)	
Tumor classification (pT)			
pT1–pT2	86 (81.9)	19 (18.1)	**<0.001**
pT3–pT4	196 (62.0)	120 (38.0)	
Lymph node metastasis (pN)			
pN0	155 (71.1)	63 (28.9)	0.062
pN1–2	126 (62.4)	76 (37.6)	
Tumor grade (WHO)			
1	24 (70.6)	10 (29.4)	0.316
2	207 (68.1)	97 (31.9)	
3	19 (79.2)	5 (20.8)	
4	13 (54.2)	11 (45.8)	

Abbreviations: CRP, C-reactive protein

[1]Chi-square test

Supplementary Table 3. Survival analysis by subgroups, high TLR2 and TLR4 tissue expressions compared to low in 549 colorectal cancer patients

	High TLR2			High TLR4		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age						
<65	0.47	0.26–0.83	**0.009**	0.74	0.46–1.19	0.209
≥65	0.69	0.43–1.11	0.128	1.01	0.69–1.48	0.949
Gender						
Male	0.85	0.50–1.45	0.541	1.22	0.80–1.87	0.357
Female	0.40	0.25–0.67	**<0.001**	0.66	0.43–1.00	0.050
Location						
Right colon	0.55	0.25–1.24	0.149	0.56	0.31–1.02	0.058
Left colon	0.51	0.25–1.01	0.069	0.74	0.42–1.28	0.277
Rectum	0.63	0.38–1.04	0.070	1.35	0.87–2.10	0.185
Tumor stage						
I	0.54	0.11–2.59	0.438	0.42	0.11–1.56	0.194
II	0.91	0.28–3.02	0.878	0.63	0.30–1.33	0.225
III	0.40	0.24–0.65	**<0.001**	1.04	0.66–1.63	0.862
IV	1.64	0.81–3.31	0.172	0.86	0.57–1.761	0.859
Tumor classification (pT)						
pT1–pT2	1.16	0.27–5.03	0.846	0.56	0.22–1.41	0.218
pT3–pT4	0.61	0.40–0.94	**0.024**	1.03	0.75–1.42	0.862
Lymph node metastasis (pN)						
pN0	0.73	0.33–1.62	0.436	0.77	0.43–1.40	0.377
pN1–2	0.86	0.47–1.60	0.640	1.04	0.73–1.45	0.814
Tumor grade (WHO)						
1–2	0.73	0.35–0.80	**0.003**	0.93	0.6–1.30	0.665
3–4	0.89	0.30–2.61	0.829	0.91	0.37–2.22	0.837
Systemic inflammatory response (CRP)						
≤8.7	0.53	0.35–0.80	**0.002**	0.92	0.59–1.41	0.688
>8.7	0.95	0.45–2.01	0.886	2.04	1.04–4.00	**0.038**

Abbreviations: CI, confidence interval; CRP, C-reactive protein; HR, hazard ratio; TLR, toll-like receptor

Supplementary Table 4. Survival analysis by subgroups, high TLR5 expression compared to low and positive TLR7 expression compared to negative in 549 colorectal cancer patients

	High TLR5			Positive TLR7		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age						
<65	0.61	0.38–0.99	**0.044**	0.55	0.28–1.07	0.078
≥65	0.63	0.42–0.94	**0.025**	0.47	0.29–0.75	**0.002**
Gender						
Male	0.67	0.45–1.02	0.063	0.63	0.36–1.11	0.111
Female	0.54	0.34–0.86	**0.009**	0.38	0.22–0.64	**<0.001**
Location						
Right colon	0.66	0.36–1.22	0.187	0.32	0.17–0.60	**<0.001**
Left colon	0.67	0.38–1.17	0.160	0.55	0.25–1.23	0.146
Rectum	0.54	0.34–0.86	**0.009**	0.63	0.32–1.26	0.195
Tumor stage						
I	0.89	0.24–3.31	0.860	0.55	0.07–1.43	0.576
II	0.64	0.29–1.42	0.272	0.52	0.12–2.21	0.379
III	0.78	0.49–1.22	0.268	0.68	0.40–1.18	0.168
IV	1.02	0.59–1.76	0.956	0.70	0.37–1.35	0.291
Tumor classification (pT)						
pT1–pT2	1.16	0.45–3.0	0.757	0.45	0.10–1.95	0.284
pT3–pT4	0.67	0.48–0.94	**0.020**	0.59	0.39–0.89	**0.012**
Lymph node metastasis (pN)						
pN0	0.55	0.30–1.01	0.054	1.24	0.30–5.11	0.768
pN1–2	0.72	0.50–1.04	0.083	0.68	0.44–1.04	0.075
Tumor grade (WHO)						
1–2	0.59	0.41–0.83	**0.003**	0.53	0.33–0.86	**0.010**
3–4	0.94	0.40–2.21	0.881	0.55	0.21–1.39	0.205
Systemic inflammatory response (CRP)						
≤8.7	0.59	0.37–0.92	**0.021**	0.53	0.28–1.00	**0.049**
>8.7	0.58	0.31–1.07	0.079	3.19	0.44–23.1	0.251

Abbreviations: CI, confidence interval; CRP, C-reactive protein; HR, hazard ratio; TLR, toll-like receptor