Original article: Toll-like receptors 3, 7, 8 and 9 in Gastric Cancer

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Abstract

Eskuri M, Kemi N, Helminen O, Huhta H, Kauppila JH. Toll-like receptors 3, 7, 8 and 9 in Gastric Cancer.

Toll-like receptors (TLRs) have been shown to have anti-tumor, pro-tumor, or even dual effects in cancer, and are thus potential prognostic biomarkers and immunotherapeutic targets. The present study aimed to evaluate associations between endosomal TLRs, namely TLR3, TLR7, TLR8 and TLR9, expression and clinicopathological variables and survival in gastric cancer. A total of 564 gastric adenocarcinoma patients were included in this retrospective cohort study. Samples and clinicopathological data were retrieved and organized into tissue microarray blocks. Protein expressions were detected by immunohistochemical staining. The patients were divided into low expression and high expression groups by median values of expression. Cox regression provided hazard ratios (HR) with 95% confidence intervals (CI), adjusted for confounders. Patients with high nuclear TLR3 expression had significantly poorer 5-year survival compared to the low nuclear TLR3 expression group in the univariable analysis (crude HR 1.31, 95% CI 1.07-1.60). With radically resected patients, poor prognosis was also seen in the multivariable analysis (adjusted HR 1.38, 95% CI 1.08-1.77). Cytoplasmic TLR3, TLR7, TLR8 and TLR9 were not associated with 5-year survival. In conclusion, high nuclear TLR3 expression seems to have prognostic impact in gastric cancer, while TLR7, TLR8 and TLR9 do not.

Key words: Toll-like receptors; TLR3, TLR7; TLR8; TLR9; Gastric cancer

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Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer deaths worldwide (1, 2). In 2020, more than one million new GC were diagnosed (1). GC is usually discovered in advanced stages with an estimated 5-year survival rate of less than 20% (3). In clinical practice, GC prognosis is estimated using the AJCC TNM staging. However, individual estimation of prognosis inside and between certain stages is confounded by several factors, including known and unknown biological properties of cancer such as cellular invasiveness and mutational status. Therefore, new prognostic biomarkers are needed.

Toll-like receptors (TLRs) are pattern recognition receptors of the innate immune system (4). TLRs are activated by several ways, for example, identifying microbial components (pathogen-associated molecular patterns, PAMPs), as well as dead or damaged host cells (damage-associated molecular patterns, DAMPs) (4-5). Therefore, TLRs are involved in the innate immune system identifying foreign material, triggering an immune response (6). GC is a malignancy in which the body's inflammatory response and chronic inflammation are the underlying pathological events (6-8), most notably via *Helicobacter pylori* infection (9). Thus, TLRs could have potential as prognostic biomarkers but may also act as immunotherapeutic targets (4, 10). Various TLR agonists are currently being evaluated in clinical trials as antitumor agents (8, 11-13). Although some studies have shown that TLRs are involved in GC biology, their function still appears to be complex (4). TLR3, TLR7, TLR8 and TLR9 are endosomal RNA/DNA recognition receptors and expression of TLRs has previously been reported in GC with smaller sample sizes, but better powered studies are needed (14-15).

The aim of this study was to evaluate the expression of TLR3, TLR7, TLR8 and TLR9 and their association with clinicopathological parameters and prognostic significance in a large cohort of gastric cancer patients.

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Material and methods

Study design

This study was a retrospective cohort study in a single institution in a tertiary care hospital in Northern Finland. There were 601 consecutive patients who underwent gastrectomy for gastric cancer in Oulu University Hospital between the years 1983 and 2016. Of these, representative samples were available from 564 patients and were included in the cohort.

Data collection

The patients were identified from the archives of the Department of Pathology at the Oulu University Hospital, Finland. Clinical data and pathology reports for each patient were obtained from patient records. The immutable national personal numbers assigned to each resident in the country were used to combine data from the patient records and the 100% complete follow-up data from the Causes of Death Registry at Statistics Finland. Follow-up data were available until the end of 2019.

Tissue microarray

Representative tumor areas with the deepest tumor invasion were defined on diagnostic hematoxylin-eosin slides. The slides were scanned using Aperio AT2 (Leica Biosystems, Wetzlar, Germany), and the representative areas of tumor were selected, two cores from the tumor front and two cores from the tumor center. To avoid loss of participants during the experiments and to achieve representative samplings from different parts of the tumor, a total of four cores were taken from each patient tissue block, using the chosen scanned slides as a guideline. The cores were punched from paraffin-embedded tissue blocks and transferred to a receiver block, which were used for further staining analysis and construct tissue microarray (TMA). Computer-driven TMA-device Galileo TMA CK4500 (Integrated Systems Engineering, Milan, Italy) was used for construction.

Immunohistochemistry

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TLR protein expression were detected by immunohistochemical (IHC) staining. TMAs were cut in 4 µm slices, placed on glass slides, deparaffinized in xylene and rehydrated through graded alcohols. Rehydrated samples were submitted into a microwave oven for antigen retrieval with tris-EDTA-buffer (pH 9) 800 W for 2 min and 150 W for 15 min and then cooled to room temperature for 20 min. Samples were rinsed in distilled water and phosphate-buffered saline with Tween (PBS-T) and endogenous peroxidase was then neutralized in peroxidase blocking solution (Dako S2023) for 5 min. After a wash in PBS-T, sections were incubated with antibodies (Dako S2022); TLR3 (diluted 1:30, NOVUS NBP-2-24875), TLR7 (diluted 1:500, NOVUS NB 100-56682), TLR8 (diluted 1:850, NBP-2-24917), and TLR9 (1:300, NOVUS NBP-2-24729). After another wash in PBS-T, samples were incubated with En-vision polymer (Dako K5007) for 3-5 min. After the final wash in PBS-T, Diaminobenzidine (Dako basic DAB-kit) was used as a chromogen. Lastly, the samples were counterstained in hematoxylin for 1 min. All staining was done with Dako Autostainer (Dako, Copenhagen, Denmark). Cancer tissues with high expression of TLR3, TLR7, TLR8 and TLR9 were used as an external positive control. For negative control, the primary antibody was omitted.

Assessment of immunostaining

Sections were scanned and digitized using Aperio AT2 (Leica Biosystems, Wetzlar, Germany). Cores were analyzed from scanned slides using QuPath (16). Immunohistochemical staining was evaluated by two independent researchers (M.E. and N.K.) who were blinded to the clinical and outcome data. It was decided a priori that the cores for each staining would be analyzed by one researcher (M.E.) if good interobserver agreement could be achieved. This was indicated by a kappa value of at least 0.7 in assessment of a sample of 100 cores from randomly selected cases. As this was not achieved, both M.E. and N.K. analyzed all stainings.

Patients were included in this study as they had at least one assessable core of each staining available. The core was excluded from the analysis if it was incomplete or clearly defectively stained. All available cores were evaluated individually, up to four cores from each patient. Cytoplasmic and

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nuclear staining was evaluated independently. We assessed the intensity of staining from 0 (absent) to 3 (strong intensity) and the percentage of positive tumor cells (0-100%) for each core. The mean intensity and mean percentage of assessable cores for each patient cores were used to obtain a histoscore for staining intensity, which was calculated by multiplying the mean intensity and the mean percentage of the cancer cells (values 0-300). The mean value of all evaluated cores was calculated, and finally, the mean value from two investigators was obtained. For statistical evaluation, each staining was dichotomized into two equal-sized groups (low and high expression) based on median values. In nuclear TLR8 staining, the median value was 0, and thus it was divided into positive and negative expression groups.

Statistical analysis

The primary outcome of this study is 5-year survival, defined as death for any cause during 5 years after surgery. All statistical analyses were performed using the IBM SPSS Statistics 26.0. (IBM Corp., Armonk, NY). Cohen's kappa was calculated to analyze interobserver agreement. The Chi-squared test was used to compare categorical variables. The T-test was used to compare continuous variables. The Kaplan-Meier method was used to obtain Kaplan-Meier curves. A Cox regression model was used to perform univariate and multivariable analysis, providing hazard ratios (HR) with 95% confidence intervals (CI). Cox regression was adjusted for potential confounding variables: (1) year of surgery (<2000 or ≥ 2000), (2) age at diagnosis (continuous variable), (3) sex (male or female), (4) administration of preoperative chemotherapy (yes or no), (5) tumor stage (stage I+II or stage III+IV), (6) Lauren classification (intestinal, diffuse, or mixed), and (7) radical resection (R_0 or $R_{1/2}$). Subgroup analyses were conducted in intestinal and diffuse histological types. Also, a subgroup analysis was conducted including only R_0 resected patients to isolate only curatively operated patients. Ro resection was defined as no cancer cells seen microscopically at the tumor border. $R_{1/2}$ resection was defined as tumor growth on the border of the resected specimen, or macroscopic residual disease. p values less than 0.05 were accepted as statistically significant.

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Results

Patients

There were 564 patients included, with median age 69 years (range 27-90) and 61.0% being male. Of these 564 patients, 420 (74.5%) underwent microscopically confirmed R_0 resection, and 144 (25.5%) had $R_{1/2}$ resection, including patients with palliative intent, as well as 33 (5.9%) patients that had distant metastases discovered at the time of surgery. Only 22 (3.9%) of patients underwent perioperative chemotherapy (Table 1).

TLRs immunohistochemistry

TLR3, TLR7, TLR8, and TLR9 were all expressed in GC. Staining was cytoplasmic in all TLRs. The staining was nuclear in TLR3 and TLR8, and nuclear staining was assessed separately. In addition, TLR9 staining was found occasionally on the membranes. Representative images of immunostainings are shown in Figure 1 and Supplementary Figure 1. There was no association between the intensity of immunostainings and sample age.

Toll-like receptor 3

Cytoplasmic TLR3

A total of 554 patients were included in TLR3 staining analysis. For cytoplasmic TLR3, the kappa value was 0.64, with median of 28.8. Those in high cytoplasmic TLR3 expression group were more often operated after year 2000 and had more often perioperative chemotherapy compared to low expression group (Supplementary table 1). Cytoplasmic TLR3 expression was not associated with 5-year survival (Table 2, Supplementary Figure 2). In the R0 resected patients, the results were similar to the main analysis.

Nuclear TLR3

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For nuclear TLR3, the kappa value was 0.65, with a median of 75.0. High nuclear TLR3 expression was associated with operation after 2000, perioperative chemotherapy, higher T-class, and positive lymph node status, compared to low expression group (Supplementary Table 1).

The primary outcome occurred in 389 (70.2%) of the 554 patients. The 5-year survival was significantly worse in high nuclear TLR3 expression group (24.6%) compared to low nuclear TLR3 expression group (35.2%) in univariable analysis (HR 1.31, 95% CI 1.07-1.60), but not in the multivariable analysis (Figure 2, Table 2). In the subgroup analysis of intestinal type of histology, the 5-year survival was worse in high nuclear TLR3 expression group (24.3%) compared to low nuclear TLR3 expression group (35.3%) in univariable analysis (HR 1.36, 95% CI 1.03-1.80), but not in the multivariable analysis (Figure 2, Table 2). In the diffuse type of histology subgroup, no significant differences between the groups were present (Table 2).

In subgroup analysis of R0 resected patients, high nuclear TLR3 expression group was associated with significantly worse 5-year survival compared to low nuclear TLR3 expression group in both univariable and multivariable (adjusted HR 1.38, 95% CI 1.08-1.77) analysis (Table 2). Similarly, high nuclear TLR3 expression group was independently associated with worse survival in intestinal type histology subgroup (adjusted HR 1.40, 95% CI 1.02-1.94, Table 2), but not in diffuse type subgroup (Table 2).

Toll-like receptor 7

A total of 559 patients were included in TLR7 staining analysis, with kappa value of 0.66 and median value of 137.5. High cytoplasmic TLR7 expression was associated with surgery after 2000, perioperative chemotherapy and poorly differentiated histology (Supplementary Table 2). Cytoplasmic TLR7 expression was not associated with 5-year survival (Table 2, Supplementary Figure 2). In the subgroup analysis of R0 resected patients, the results were similar to the main analysis.

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Toll-like receptor 8

Cytoplasmic TLR8

A total of 554 were included in TLR8 staining analysis, with kappa value of 0.59 and median value of 175.0. High cytoplasmic TLR8 expression was associated with surgery before year 2000, lower T-class, negative lymph node status and older age (Supplementary Table 3). Cytoplasmic TLR8 expression was not associated with 5-year survival (Table 2, Supplementary Figure 2). In the subgroup analysis of R0 resected patients, the results were similar.

Nuclear TLR8

The kappa value was 0.80 for nuclear TLR8, with median value 0. For this reason, nuclear TLR8 was dichotomized as positive (316, 57.0%) and negative (238, 42.0%) expression. Positive nuclear TLR8 expression associated with younger age and diffuse-type histology. (Supplementary Table 3). Nuclear TLR8 expression was not independently associated with 5-year survival (Table 2, Supplementary Figure 2). No statistically significant results were obtained in the subgroup analysis of R0 resected patients.

Toll-like receptor 9

A total of 559 patients were included in TLR9 staining analysis, with kappa value of 0.59 and median of 150.0. High cytoplasmic TLR9 expression associated with older age, male sex, positive organ metastases, poorly differentiated histology, and intestinal-type histology (Supplementary Table 4).

Cytoplasmic TLR9 expression was not associated with 5-year survival (Table 2, Supplementary Figure 2) in the main analysis. In the subgroup analysis of R0 resected patients, the results were similar.

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Discussion

In this study, TLR3, TLR7, TLR8 and TLR9 were abundantly expressed in gastric adenocarcinoma. While only nuclear TLR3 had independent prognostic impact in radically resected GC, also TLR8 and TLR9 were associated with known prognostic factors in GC. Therefore, this study cannot exclude that TLR8 and TLR9 could have prognostic relevance.

There are some strengths and limitations that should be considered before interpreting the results. The strengths of the study include the large size of the study and the lack of selection bias. The retrospective single-institution design might limit its applicability for larger populations. Nevertheless, this study is larger than any of the previous studies on the topic. Patients with unradical resections were also included to minimize selection bias and maximize the power this study. On the other hand, subgroup analyses excluding palliative and non-radically resected patients showed largely similar results to the main analysis. With many statistical tests, chance findings are always possible. However, the results of the pre-specified analyses were reasonably consistent among the different TLRs analyzed. Although our study was larger than other studies, the statistical power was somewhat limited, and even larger studies are needed to confirm the findings. Long inclusion period can cause some confounding due to varying staging methods, operative techniques, and surveillance strategies. However, the year of surgery was taken into account in adjusted analyses. The assessment of staining was at times challenging to replicate, as seen in the kappa-value 0.59 for cytoplasmic TLR8 and TLR9 indicating moderate agreement, 0.64 in cytoplasmic TLR3, 0.65 in nuclear TLR3 and 0.66 in cytoplasmic TLR7 indicating substantial agreement, potentially limiting their applicability in clinical practice. The use of only immunohistochemistry for defining could be considered a possible weakness, but it is the main method in clinical assessment of protein expression. IHC consistency and quality was ensured by performing antigen retrieval before immunohistochemical staining. Unfortunately, *H. pylori* infection status was not taken into account as a confounding variable in this study. Atrophy reduces the prevalence of *H. pylori*, causing severe difficulties in determining *H.*

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pylori status reliably from the resected specimen. For the majority of the patients, there were no information available on *H. pylori* infection.

Two previous studies with smaller sample sizes have described TLR3, TLR7 and TLR9 in GC, both of them using immunohistochemistry and tissue microarrays. These studies did not evaluate the nuclei separately, so they are not entirely comparable with the present study. In a Spanish study (N=106), TLR3 and TLR9 were not independent prognostic factors of GC, but high TLR3 expression was significantly associated with a poor overall survival in patients with resectable tumors (N=63) (15). In a Finnish study (N=313), TLRs 7 and 9 were associated with intestinal histology, high TLR7 expression being associated with better prognosis in stage III disease, and high TLR9 expression with better prognosis in stage II disease, but no stage-independent prognostic value was confirmed for either TLR7 or TLR9 (14). These studies are mostly consistent with our results. While none of the markers were independently associated with prognosis in analyses including all patients, TLR3, TLR8 and TLR9 were associated with poor prognostic factors, suggesting that they might have biological relevance in GC, requiring further assessment. However, in patients with R0 resection, nuclear TLR3 was independently associated with 5-year survival.

TLRs induced inflammatory cascades may disrupt homeostasis and compromise tissue integrity (5, 17), which may promote tumor invasion, (neo-)vascularization, cell survival, chemoresistance, tumor progression and metastasis in cancer (6, 18). On the other hand, TLRs also have antitumor capability via activation of a tumor-specific immune response, including stimulation of NK-, T-helper-, and cytotoxic T-cell migration and transition of tumor-stimulating macrophages to tumor-suppressing macrophages (8). TLRs are increasingly being used as potential immunotherapeutic targets for cancer treatment (13). Since TLRs have been shown to have both pro- and anti-cancer functions, and GC is a heterogenous disease (4), their specific functions in GC need to be clarified. TLR3 appears to be one of the most promising immunotherapeutic targets in various cancers (10), and based on our study, it could be relevant in GC too.

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TLR3 is an endosomal receptor recognizing viral double-stranded RNA and nucleotides from necrotic tissues (19). Gastric tissue is often exposed to exogenous signals, which is why TLR3 might be constantly activated in the gastric environment, leading to increased inflammatory activity, and towards a tumor supporting microenvironment. In contrast to other TLRs, TLR3 signals via a myeloid differentiation factor 88 (MyD88)-independent pathway, and through the TIR-domain-containing adapter-inducing interferon-B (TRIF) protein (15). This leads to the activation of transcription factors, for example, NF-κB activation, eventually leading to the production of inflammatory cytokines and chemokines (20). NF-kB activation is known to participate in carcinogenic promotion and progression by different mechanisms, for example, cell proliferation, anti-apoptosis, angiogenesis, invasion, and metastasis (15, 20), explaining the mechanism why a high TLR3 expression was associated with a worse prognosis in our study as well. In addition, TLR3 was predictive in early-stage disease, which is most likely explained by increased inflammatory activity in premalignant stages, and thus increased expression of TLR3. On the other hand, it might be reflecting a resistance to TLR3-mediated apoptosis acquired by tumor cells during tumor progression, also speculated in lung cancer (21).

Nuclear localization and the possibility of non-specific IHC staining have been discussed previously (20). Nuclear expression of TLR3 and intracellular trafficking in GC is not entirely clear. However, nuclear location has been reported in several previous studies (19-22), and in addition, we have previously described and characterized nuclear localization of cell membrane and endosomal localized TLRs (19). NucPred score for TLR3 was 0.55, suggesting that is somewhat likely that TLR3 translocate to the nucleus (23).

Conclusion

TLRs 3, 7, 8 and 9 are expressed in GC and all but TLR7 associate with aggressive tumor biology. High nuclear TLR3 expression seems to associate with poor prognosis in GC, but is limited to

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radically resected patients. Further studies on the function of TLRs in GC, as well as confirmation of TLR3 as a marker of poor prognosis are needed.

Ethics declarations

Ethics approval

The use of patient samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee (15.2.2016 §51). The need to obtain a written or oral consent from the patients was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA). This study was performed in accordance with the Declaration of Helsinki.

Competing interests

The authors have no potential conflicts of interest to declare.

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analysis, or interpretation of the results, writing of the manuscript, or the decision to submit the manuscript for publication.

Authors' contributions

M.E., N.K. and J.H.K. conceived and designed the study. N.K., O.H., H.H. and J.H.K. acquired the data. M.E. and N.K. performed the experiments. M.E. and J.H.K. analyzed the data. M.E. drafted the manuscript. All authors critically reviewed, edited, and approved the manuscript. J.H.K provided funding, supervised the study and is the guarantor of the study.

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References

(1) Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71(3):209-249.

(2) Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol 2019; 5(12):1749-1768

(3) Asplund J, Kauppila JH, Mattsson F, Lagergren J. Survival Trends in Gastric Adenocarcinoma: A Population-Based Study in Sweden. Ann Surg Oncol 2018; 25(9):2693-2702

(4) Cui L, Wang X, Zhang D. TLRs as a Promise Target Along With Immune Checkpoint Against Gastric Cancer. Frontiers in cell and developmental biology. Front Cell Dev Biol 2021; 8:611444

(5) Diakowska D, Nienartowicz M, Grabowski K, Rosińczuk J, Krzystek-Korpacka M. 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. Adv Clin Exp Med 2019; 28(4):515-522

(6) Braunstein MJ, Kucharczyk J, Adams S. Targeting Toll-Like Receptors for Cancer Therapy. Target Oncol 2018; 13(5):583-598

(7) El-Omar EM, Ng MT, Hold GL. Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 2008; 27(2):244-52

(8) Shcheblyakov DV, Logunov DY, Tukhvatulin AI, Shmarov MM, Naroditsky BS, Gintsburg AL. Toll-like receptors (TLRs): The role in tumor progression. Actanaturae 2010; 2(3):21-29

(9) Moss SF, Malfertheiner P. Helicobacter and Gastric Malignancies. Helicobacter 2007; 12 Suppl 1:23-30

(10) Cuzzubbo S, Mangsbo S, Nagarajan D, Habra K, Pockley AG, Mcardle SEB. Cancer Vaccines: Adjuvant Potency, Importance of Age, Lifestyle, and Treatments. Front Immunol 2021; 11:615240

(11) Schmoll H, Wittig B, Arnold D, Riera-Knorrenschild J, Nitsche D, Kroening H, et al. Maintenance treatment with the immunomodulator MGN1703, a Toll-like receptor 9 (TLR9) agonist, in patients with metastatic colorectal carcinoma and disease control after chemotherapy: a randomised, double-blind, placebo-controlled trial. J Cancer Res Clin Oncol 2014; 140(9):1615-1624

(12) Ishihara M, Tono Y, Miyahara Y, Muraoka D, Harada N, Kageyama S, et al. First-in-human phase I clinical trial of the NY-ESO-1 protein cancer vaccine with NOD2 and TLR9 stimulants in patients with NY-ESO-1-expressing refractory solid tumors. Cancer Immunol Immunother 2020; 69(4):663-675

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(13) Murphy A, Kelly RJ. Immunotherapy in Upper GI Malignancies. Curr Treat Options in Oncol 2015; 16(5):20

(14) Kasurinen A, Hagström J, Laitinen A, Kokkola A, Böckelman C, Haglund C. Evaluation of toll-like receptors as prognostic biomarkers in gastric cancer: high tissue TLR5 predicts a better outcome. Sci Rep 2019; 9(1):12553

(15) Fernandez-Garcia B, Eiró N, González-Reyes S, González L, Aguirre A, González LO, et al. Clinical Significance of Toll-like Receptor 3, 4, and 9 in Gastric Cancer. J Immunother 2014; 37(2):77-83

(16) Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open-source software for digital pathology image analysis. Sci Rep 2017; 7(1):16878

(17) Castano-Rodríguez N, Kaakoush NO, Mitchell HM. Pattern-recognition receptors and gastric cancer. Front Immunol 2014; 5:336

(18) Huhta H, Helminen O, Lehenkari PP, Saarnio J, Karttunen TJ, Kauppila JH. Toll-like receptors 1, 2, 4 and 6 in esophageal epithelium, Barrett's esophagus, dysplasia and adenocarcinoma. Oncotarget 2016; 7(17):23658-67

(19) Helminen O, Huhta H, Lehenkari PP, Saarnio J, Karttunen TJ, Kauppila JH. Nucleic acid-sensing toll-like receptors 3, 7 and 8 in esophageal epithelium, barrett's esophagus, dysplasia and adenocarcinoma. OncoImmunology 2016; 01-25;5(5)

(20) Muresan XM, Culig Z, Soucek K, Bouchal J. Toll-Like Receptor 3 in Solid Cancer and Therapy Resistance. Cancers 2020; 12(11):3227

(21) Bianchi F, Milione M, Casalini P, Centonze G, Le Noci VM, Storti C. Toll-like receptor 3 as a new marker to detect high risk early stage Non-Small-Cell Lung Cancer patients. Sci Rep 2019; 9(1):14288

(22) Helminen O, Huhta H, Leppänen J, Kauppila JH, Takala H, Lehenkari PP, et al. Nuclear localization of Toll-like receptor 5 in Barrett's esophagus and esophageal adenocarcinoma is associated with metastatic behavior. Virchows Arch 2016; 469(4):465-470

(23) Huhta H, Helminen O, Kauppila JH, Salo T, Porvari K, Saarnio J, et al. The Expression of Toll-like Receptors in Normal Human and Murine Gastrointestinal Organs and the Effect of Microbiome and Cancer. J Histochem Cytochem 2016; 64(8):470-82

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Tables

Table 1. Patient characteristics

Characteristics		No.
Age		
	< 69	285 (50.5%)
	≥69	279 (49.5%)
Sex		
	Male	344 (61.0%)
	Female	220 (39.0%)
Radicality of resection		
	R0	420 (74.5%)
	R1 or R2	144 (25.5%)
Perioperative chemotherapy		
	Yes	22 (3.9%)
	No	542 (96.1%)
Metastases		
	Yes	33 (5.9%)
	No	531 (94.1%)
Year of surgery		
	≥2000	248 (44.0%)
	<2000	316 (56.0%)
Lauren		
	Intestinal	286 (50.7%)
	Diffuse	259 (45.9%)
	Other*	19 (3.4%)
Stage		
	I+II	345 (61.2%)
	III+IV	219 (38.8%)

Other*: mixed, no classified

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Table 2. Univariable and multivariable analysis of TLRs expressions and 5-year survival in 564 patients with gastric adenocarcinoma.

	Cytoplasmic TLR3		Nuclear TLR3		Cytoplasmic TLR7		Cytoplasmic TLR8		Nuclear TL8		Cytoplasmic TLR9	
	No. patients	High TLR3 HR (95% CI)	No. patients	High TLR3 HR (95% CI)	No. patients	High TLR7 HR (95% CI)	No. patients	High TLR8 HR (95% CI)	No. patients	positive TLR8 HR (95% CI)	No. patients	High TLR9 HR (95% CI)
5-year survival												
All patients (Crude)	554	0.96 (0.78-1.17)	554	**1.31 (1.07-1.60)**	559	0.89 (0.73-1.09)	554	0.87 (0.71-1.06)	554	0.99 (0.81-1.21)	559	1.13 (0.92-1.38)
All patients (Adjusted)[a]	554	0.99 (0.81-1.22)	554	1.21 (0.99-1.49)	559	0.89 (0.73-1.10)	554	0.87 (0.71-1.07)	554	0.96 (0.78-1.19)	559	1.16 (0.94-1.44)
R0 resected patients (Crude)	412	0.98 (0.77-1.25)	412	**1.41 (1.11-1.80)**	417	1.00 (0.78-1.27)	410	0.88 (0.69-1.13)	410	0.96 (0.75-1.23)	416	1.17 (0.91-1.49)
R0 resected patients (Adjusted)[a*]	412	0.98 (0.76-1.25)	412	**1.38 (1.08-1.77)**	417	0.97 (0.75-1.25)	410	0.89 (0.69-1.14)	410	0.98 (0.76-1.28)	416	1.15 (0.89-1.49)
Subgroup analysis												
Intestinal type (Crude)	284	0.97 (0.74-1.28)	284	**1.36 (1.03-1.80)**	284	0.85 (0.64-1.12)	285	0.85 (0.65-1.13)	285	1.27 (0.95-1.71)	285	1.22 (0.90-1.66)
Intestinal type (Adjusted)[b]	284	1.02 (0.77-1.36)	284	1.31 (0.98-1.74)	284	0.82 (0.61-1.10)	285	0.80 (0.60-1.07)	285	1.16 (0.86-1.56)	285	1.26 (0.93-1.72)
R0 resected patients Intestinal type (Crude)	231	0.98 (0.71-1.34)	231	1.37 (1.00-1.88)	231	0.89 (0.65-1.22)	232	0.94 (0.69-1.30)	232	1.31 (0.94-1.83)	232	1.11 (0.78-1.56)
R0 resected patients Intestinal type (Adjusted)[b*]	231	0.95 (0.69-1.32)	231	**1.40 (1.02-1.94)**	231	0.79 (0.57-1.10)	232	0.91 (0.65-1.28)	232	1.26 (0.90-1.77)	232	1.20 (0.84-1.69)
Diffuse type (Crude)	251	0.91 (0.67-1.22)	251	1.31 (0.98-1.76)	256	0.91 (0.68-1.22)	250	0.91 (0.68-1.23)	250	0.77 (0.58-1.04)	255	1.09 (0.81-1.46)
Diffuse type (Adjusted)[c]	251	0.91 (0.67-1.24)	251	1.17 (0.86-1.59)	256	0.90 (0.66-1.23)	250	0.98 (0.72-1.32)	250	0.86 (0.63-1.16)	255	1.07 (0.79-1.44)
R0 resected patients Diffuse type (Crude)	167	0.91 (0.61-1.34)	167	**1.53 (1.03-2.27)**	172	1.09 (0.74-1.60)	164	0.82 (0.56-1.22)	164	0.70 (0.47-1.04)	170	1.17 (0.79-1.72)
R0 resected patients Diffuse type (Adjusted)[c*]	167	0.92 (0.61-1.37)	167	1.29 (0.85-1.95)	172	1.19 (0.78-1.80)	164	0.84 (0.56-1.27)	164	0.70 (0.47-1.05)	170	0.99 (0.65-1.49)

All TLR expressions: Low expression HR (95% CI) 1.00 (Reference)
a Adjusted for year of diagnosis, age, sex, tumor stage, Lauren classification, perioperative chemotherapy and radical resection
b Adjusted for year of diagnosis, age, sex, tumor stage, tumor grade, perioperative chemotherapy, and radical resection
c Adjusted for year of diagnosis, age, sex, tumor stage, perioperative chemotherapy, and radical resection
* Not adjusted for radical resection

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Figure legends

Figure 1. Representative images of nuclear TLR3 expression immunostaining in gastric adenocarcinoma. Low nuclear TLR3 expression (A), high nuclear TLR3 expression (B).

Figure 2. The Kaplan-Meier figures presenting 5-year survival stratified by nuclear TLR3 expression in gastric adenocarcinoma (A), 5-year survival stratified by nuclear TLR3 expression in the intestinal-type gastric adenocarcinoma (B), 5-year survival stratified by nuclear TLR3 expression in the diffuse type gastric adenocarcinoma (C).

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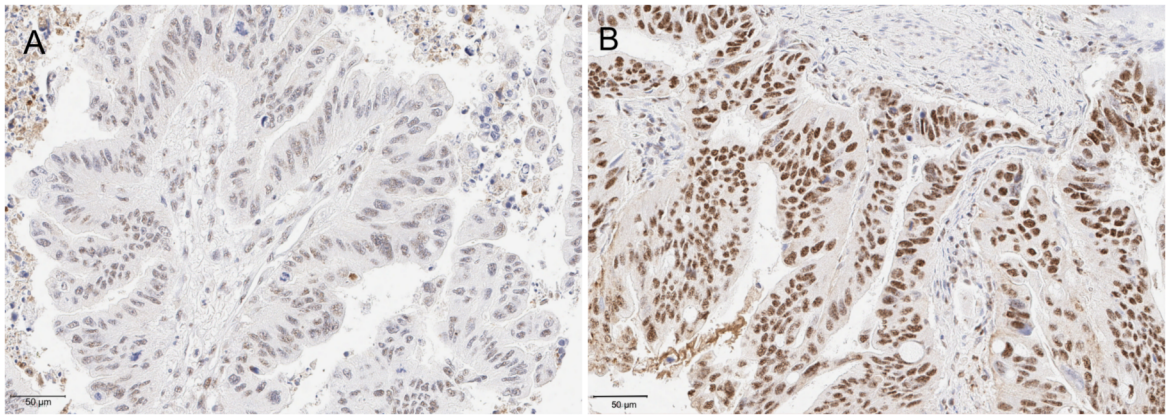

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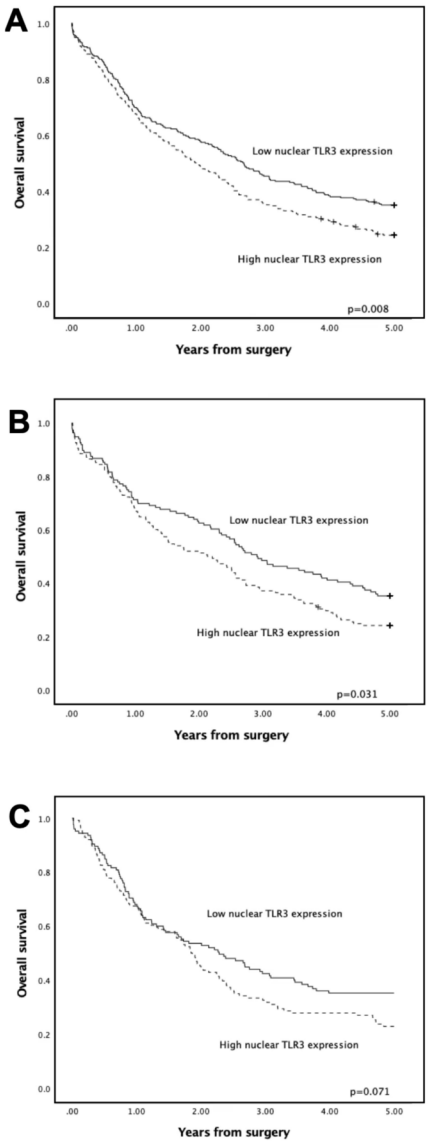

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