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Original Article

Expression of pattern recognition receptor genes and mortality in patients with colorectal adenocarcinoma

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Abstract: Toll-like receptors (TLRs) and the receptor for advanced glycation end products (AGER) are pattern recognition receptors that regulate intestinal inflammatory homeostasis. However, their relevance in colorectal cancer (CRC) prognosis is unclear. We investigated expression of *TLR*s, *AGER*, and interacting proteins in association with CRC mortality in a retrospective cohort study of 65 males diagnosed with primary resectable CRC between 2002 and 2009. Multiplex quantitative nuclease protection assay was used to quantify the expression of 19 genes in archived tissues of tumor and paired adjacent normal mucosa. We evaluated the association between log2 (tumor/normal) expression ratios for single and combined genes and all-cause mortality using multivariable Cox regression analysis. The false discovery rate adjusted *q*-value less than 0.10 indicated statistical significance for single gene. Five-year survival time was calculated from diagnosis of CRC to death, lost to follow-up, or December 31, 2014. Compared to paired normal mucosa, expression levels of *AGER*, *IL1A*, *MYD88*, and *TLR5* were lower (q = 0.0002); while *CXCL8* and *S100P* were higher (q = 0.0002) in tumor epithelia. Higher tumor expression of *IL1A* (HR*adj* = 0.68, 95% CI: 0.49-0.94), *IL6* (HR*adj* = 0.70, 95% CI: 0.52-0.94), *MyD88* (HR*adj* = 0.53, 95% CI: 0.30-0.93), and *TLR5* (HR*adj* = 0.71, 95% CI: 0.52-0.98) was associated with higher mortality risk. There was a synergistic effect on lower five-year survival in lower co-expressers of *IL-6* and *MyD88* (P < 0.0001). Our findings suggest that a TLRs/MyD88-mediated inflammatory response may play a role in CRC prognosis. The role of pattern recognition receptor-mediated immunity in CRC mortality warrants further research.

Keywords: Toll-like receptors, receptors, pattern recognition, gene expression, colorectal neoplasms, survival, immune, inflammation, tumor microenvironment, receptor for advanced glycation end-products

Introduction

Despite slow decline in mortality during the past two decades, colorectal cancer (CRC) remains the third most commonly diagnosed cancer and cause of cancer death in both men and women in the United States [1]. Chronic inflammation, a characteristic of CRC, acts through a complex tumor-supporting and immunosuppressive microenvironment [2]. Research is needed to explore the complicated relationship between host inflammation, immune function, and CRC outcomes and to identify associated tumor tissue markers for outcome prediction.

Chronic inflammation is believed to result from a sustained response to immune homeostasis disruption. Immune homeostasis in the intestines is maintained through epithelial, innate, and adaptive immune cell defense mechanisms that involve pattern recognition receptors (PRRs) [3]. Toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGE, HUGO nomenclature AGER) are part of host PRRs that recognize endogenous (such as bacterial components) and exogenous (such as dietary nutrients) ligands [4]. The ligation of TLRs and AGER with ligands can trigger NF-kB activation, resulting in inflammatory response [5]. Functional genetic variants of TLR5 have

Table 1. Gene probe IDs for 19 selected and 4 housekeeping genes and assay layout

Gene ID	Accession #	Position	Assay Position	Assay Column	Assay Row
Positive Control			1	1	1
TLR4	NR_024168	95, 254, 386, 2667	2	2	1
IL1B	NM_000576	410, 509, 983, 1340	3	3	1
AGER	NM_001136	246, 421, 589, 828	5	5	1
HMGB1	NM_002128	2209, 2595, 2970, 3265	6	6	1
MYD88	NM_001172567	327, 766, 1345, 1644	8	1	2
NFKB1	NM_003998	1685, 2587, 3272, 3712	10	3	2
NFKB2	NM_001077494	145, 460, 2080	11	4	2
TNFRSF1A	NM 001065	666, 980, 1413, 1851	13	6	2
CD36	NM_001001547	77, 397, 733, 1936	14	7	2
RPL6[a]	NM 000970	1, 200, 593, 837	15	1	3
IL6	NM_000600	101, 282, 732, 1043	16	2	3
MAP3K1	NM_005921	1582, 1681, 3056, 4912	17	3	3
CXCL8	NM_000584	42, 95, 415, 864	18	4	3
IL1A	NM_000575	461, 1090. 1418, 2327	19	5	3
S100A4	NM_002961	138, 218	20	6	3
RPS13[a]	NM 001017	3, 150, 381, 434	21	7	3
TLR2	NM_001318795	259, 870, 1948	22	1	4
TLR3	NM_003265	805, 883, 1531, 2723	24	3	4
TLR5	XM_011509937	627, 2451, 3818	26	5	4
S100P	NM_005980		28	7	4
S100PBP	NM_022753	993, 2322, 3439, 3521	30	2	5
ANT[a]	NM_i 19937	568	42	7	6
ACTA[a]	NM 001101	690, 899, 1177, 1736	43	2	8

a: Housekeeping genes. Abbreviation: Toll-like receptor 4, TLR4; Interleukin 1, beta, IL1B; Advanced glycation end product-specific receptor, AGER; High motility group box 1, HMGB1; Myeloid differentiation primary response 88, MYD88; Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, NFKB1; Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, NFKB2; Tumor necrosis factor receptor superfamily, member 1A, TNFRSF1A; CD36 molecule [thrombospondin receptor], CD36; ribosomal protein L6, RPL6; Interleukin 6, IL6; Mitogen-activated protein kinase kinase1, E3 ubiquitin protein ligase, MAP3K1; chemokine (C-X-C motif) ligand 8, CXCL8; Interleukin 1, alpha, IL1A; S100A4; ribosomal protein S13, RPS13; Toll-like receptor 2, TLR2; Toll-like receptor 3, TLR3; Toll-like receptor 5, TLR5; S100 calcium binding protein P, S100P; S100 binding protein, S100PBP; S100 calcium binding protein A4; ANT; actin beta, ACTB.

been shown to significantly affect survival in CRC patients [6].

Despite these findings, the potential relevance of PRRs as tumor markers for CRC prognosis is largely unknown. We, therefore, investigated the relationship between the expression of TLRs and AGER, their ligands and interacting proteins, and their inflammatory effectors and all-cause mortality in primary CRC. We hypothesized that differential gene expression between tumor and normal tissue would be associated with differences in mortality risk. We used a quantitative nuclease protection assay (qNPA) to measure the expression of 19 *a priori* selected genes simultaneously using archived sections of CRC.

Materials and methods

Study subjects and tissue samples

We conducted a retrospective cohort study using an established registry of surgical patients seen between 2002 and 2009 at the Department of Surgery at Michael E. DeBakey VA Medical Center (MEDVAMC) in Houston, TX. We randomly selected 65 male patients who had undergone tumor resection for primary colorectal adenocarcinoma (Stage 0-IV; ICD-9 codes 153.1, 153.6, 153.9, and 154.1). Formalin-fixed, paraffin-embedded (FFPE) tissue blocks for both colorectal adenocarcinoma and paired adjacent normal tissue were retrieved from all 65 patients. All FFPE blocks

were fixed in 10% neutral-buffered formalin and stored at room temperature according to standard procedures. Upon participant selection and block retrieval, multiple 5-μm tissue slides were reviewed and cut to select sections with tumor volumes > 75%. The adjacent normal areas were identified from a separate slide from the same patient. Colorectal adenocarcinoma diagnosis and the area of macrodissection were confirmed by two independent pathologists at Baylor College of Medicine and HTG Molecular Diagnostics (Tucson, AZ). The Institutional Review Board of both Baylor College of Medicine and the MEDVAMC approved the research protocol.

Gene expression detection

We used the customized multiplex quantitative nuclease protection assay (qNPA) to examine the expression of TLRs (*TLR2-TLR5*), *AGER*, their ligands or interacting proteins (*HMGB1*, *MAP3K1*, *MYD88*, *NFKB1-2*, *S100P*, *S100PBP*, and *S100A4)*, and inflammatory effectors (*CD36*, *CXCL8*, *IL1A*, *IL1B*, *IL6*, and *TNFRSF1A*), comprising 19 genes in total [7]. We selected the qNPA for gene expression analysis [8] (HTG Molecular Diagnostics, Tucson, AZ) because it can simultaneously measure very small amounts of RNA from multiple genes without RNA extraction from FFPE samples [7]. The complete qNPA methodology has been documented [7].

Tumor and normal slides were processed on the same day but separately to prevent contamination. Selected tissue area was scraped from each slide and placed in a well containing HTG lysis buffer to permeabilized cells and denature macromolecules. A 50-mer nuclease protection probe specific for each of 19 genes (**Table 1**) were hybridized with soluble sample RNA. These probe-mRNA duplexes were formed from in-situ hybridization on the ArrayPlate™. S1 nuclease digestion destroyed all non-hybridized probes; and alkaline hydrolysis separated mRNA-probe duplexes, which were then transferred to the customized ArrayPlate™ for probe capture, detection, and quantification. The resulting chemiluminescent signals were quantified with a CCD-based Omix Imager and Vuescript software (HTG molecular diagnosis). Expression levels were calculated as average pixel intensity for the spot image on the array plate. Each sample was run in triplicate and assayed with input tumor area 0.5 cm^2/well. Tumor volumes from 2-3 slides were sufficient for the gene-expression assay.

RPL6 and *RPS13* were selected from four potential housekeeping genes (**Table 1**) because their expression signals were relatively stable across the tested slides, regardless of tissue type (normal versus tumor) or sample age. For quality-control purposes, we calculated coefficient of variation (CV) for each gene. We excluded data points with CV > 20% after triplicate measurement for each gene. We also assessed interplate variability via normalized CV [(CV = (SD/√n)/AVG)], where n is the number of wells per test compound, and no significant differences were found ($P > 0.05$). Samples were measured on three plates. The average CVs for each plate were 8%, 11%, and 16% respectively. Tumor and adjacent normal tissues were assayed side by side on the same plate.

Data collection and outcomes ascertainment

Using the electronic medical record at the MEDVAMC, we used a structured form to abstract data on patients' characteristics (age, race/ethnicity, body mass index (BMI, kg/m^2)), clinical characteristics (history of type 2 diabetes, CRC diagnosis date, and date of death or last clinical visit through December 31, 2014), and tumor characteristics at time of surgery (American Joint Committee on Cancer Tumor Nodes Metastasis (TNM) stage [9], size, location, degree of differentiation [10], liver metastasis, and neoadjuvant therapy).

Statistical analysis

Survival time was calculated from the date of CRC diagnosis to death, lost to follow-up, or December 31, 2014. Five-year all-cause survival time was the major outcome variable. We compared the tumor characteristics according to five-year survival status using log-rank tests for categorical variables and Cox proportional hazard ratios for continuous variables. The following variables were assessed as potential confounders or effect modifiers: age of diagnosis, TNM stage (0 vs 1, 2, 3, and 4), tumor differentiation degree (poor with < 50% gland formation vs. moderate 50-95% gland formation to well- differentiated > 95% (gland formation), tumor location (proximal vs. distal colon), tumor size (mm), liver metastasis at time of diagnosis (no vs. yes), tumor neoadjuvant therapy (no vs.

yes), and BMI category (BMI < 18.5 kg/m^2, BMI 18.5-24.9, BMI 25.0-29.9, and BMI ≥ 30 kg/m^2).

Data imputation

Normalized gene expression data that did not meet HTG Molecular Diagnostics manufacturer quality standards were excluded from the analysis. To improve data quality, we used the *K* nearest neighbor (KNN) imputation module (Impute MissingValues. KNN, Version 13) to impute the approximately 10% of missing array data. KNN is one of the best known and most frequently used imputation algorithms, and the missing value is imputed based on pairwise information between the target gene with missing values and the K nearest reference genes [11]. This approach was selected because KNN performs well when strong local correlation exists between genes in the data, such as our customized assay [11]. The KNN module was applied separately to tumor and adjacent normal tissue in samples using Gene Pattern (www.broadinstitute.org) and was calculated using 10 neighbors for the imputed values [12]. The average number of imputed genes was higher for normal than for tumor samples, with an overall average of 3.12 genes for all samples.

Single gene survival analysis

Normalized gene expression ratios [log2 (tumor)/(normal)], equivalent to gene expression differences, [12] were calculated for each of the 19 genes and used as a continuous predictor in Cox proportional hazard regression models to estimate hazard ratios (HR) and 95% confidence intervals (CI) for risk of five-year all-cause mortality. Patient and tumor characteristics with $P < 0.20$ were assessed in multivariable Cox Proportional Hazards analysis and were retained if their individual inclusion in the model changed the multivariable HR estimates by > 10%. Genes with false discovery rate FDR adjusted q-values < 0.10 in the univariate analysis were entered into multivariable analysis. Final multivariable models included gene expression values adjusted for TNM tumor stage (0 (referent) vs. 1, 2, 3, or 4) and neoadjuvant therapy prior to surgery (no vs. yes). We assessed the interaction effect between gene expression and BMI, TNM stage on CRC mortality risk.

Multigene survival analysis

Best subset analysis: In an exploratory analysis of combined gene multivariable models, we used best subset selection, which determines the "best" model based on the highest global chi-square statistic for a given number of covariates. To assess genes that might be significant in cooperative pathways rather than individually, we selected candidate genes that achieved nominal significance ($q < 0.05$) in multivariable Cox regression analysis and dichotomized each gene into high- or low-expression categories based on the median. We selected the top combined gene models, based on the smallest combined set of genes with the highest global chi-square value. For presentation purposes, we listed the overall best identified model.

TLR gene set analysis

Candidates for the TLR gene set analysis were identified from multigene best subset selection analysis with the largest global chi-square increases over single-gene models. Patients were assigned into high or low gene expression categories based on median cut-points. Each gene was assessed separately first and then reexamined in combination. Patients were grouped by their total number of gene-set candidates (0, 1, 2 or more) and overall survival was re-examined using the log-rank test. A test for trend was performed to assess whether the presence of additional genes, compared with 1 or no genes, etc, affected survival. We used a web-based enrichment analysis from WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) to explore the gene set analysis and KEGG pathway for the direct and indirect interactions of the genes comprising this set [13].

Sensitivity analysis

We performed two separate sensitivity analyses by excluding participants who received neo-adjuvant therapy (n = 19) because gene expression levels could potentially be affected by treatment. We also repeated univariate and multivariable gene expression analysis using non-imputed data.

All analysis was performed in SAS 9.4 (SAS Institute Inc., Cary, NC). All tests were two sided and *P* value < 0.05 indicates statistical significance. We used the FDR to account for multiple testing by calculating the *q*-value. Q-value <

Table 2. Characteristics of 65 patients with colorectal adenocarcinoma according to five year mortality

	Vital status 5 years post-CRC diagnosis		
	Alive (n = 39)	Deceased (n = 26)	*P* value
Patient Characteristics			
N or mean (SD)			
Age at diagnosis (mean, SD)	63.2 (6.5)	61.6 (5.5)	0.9445
Days from diagnosis to surgery (mean, SD)	55.8 (68.9)	49.4 (53.9)	0.6333
Race			0.2068
White	24	20	
Black	15	6	
Diabetes			0.3858
No	25	14	
Yes	14	12	
Body mass index (BMI)[a]			< 0.0001[b]
< 18.5, Underweight	0	3	
18.5-24.9, Normal	5	13	
25.0-29.9, Overweight	20	5	
> 30.0, Obese	12	4	
Tumor Characteristics			
Neoadjuvant therapy[a]			0.9581
No	26	17	
Yes	12	7	
Tumor metastasis node (TMN) stage			< 0.0001[b]
0	2	0	
1	6	2	
2	14	10	
3	16	6	
4	1	8	
Differentiation[a]			0.7151
Poor (< 50%)	23	17	
Moderate to well (≥ 50)	15	9	
Size in mm (mean (SD))[a]	42.5 (21.4)	52.5 (24.1)	0.0578[b]
Location in colon[a]			0.1080[b]
Proximal	14	16	
Distal	24	10	
Liver metastasis			0.7416
No	34	22	
Yes	5	4	

[a]The numbers don't add up due to missing values. [b]Variables with *P*-value < 0.20 were assessed in multivariable Cox Proportional Hazards analysis.

0.05 indicated statistical significance, unless otherwise indicated. The *q*-value provides the expected proportion of false positives among the results [14].

Results

Study characteristics

Mean survival time was 46.2 months (Standard deviation (SD)D = 19.9), and 26 (40%) participants died during the five year follow-up period. Among those that died, almost 60% (n = 15) died within 36 months after CRC diagnosis. BMI category ($P < 0.0001$) and TNM Stage ($P < 0.0001$) were significantly associated with mortality in univariate analysis (**Table 2**). Age, race, diabetes status, and other tumor characteristics were not significantly different between deceased and non-deceased patients ($P > 0.05$).

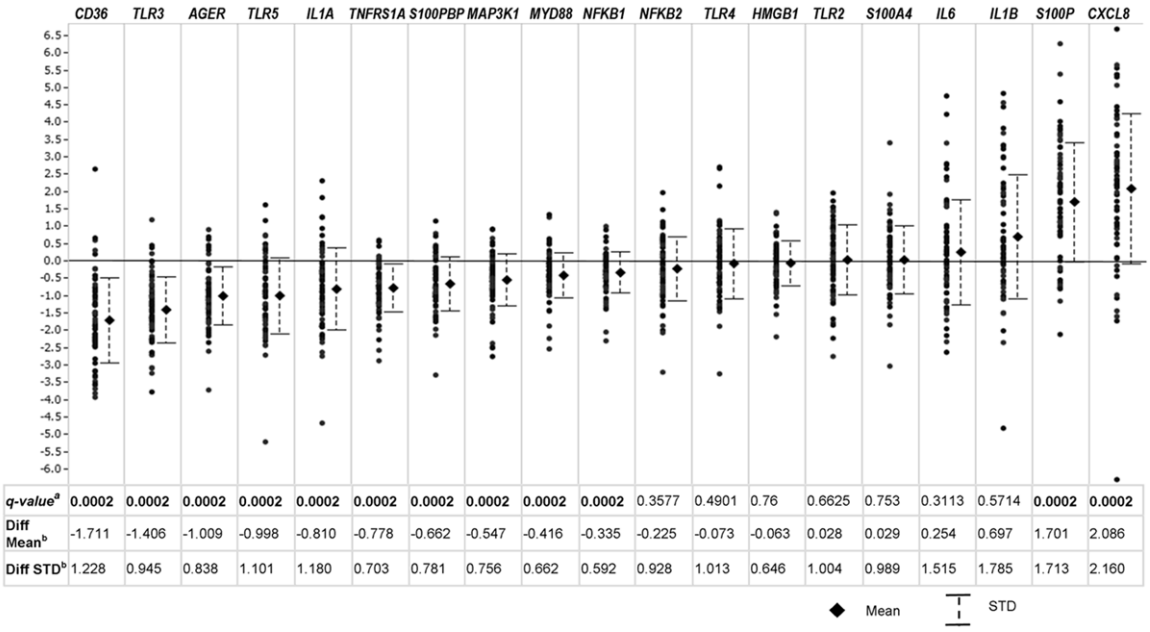

Figure 1. The difference between log2 gene expression (tumor-normal) in 65 paired colorectal tumor and adjacent normal tissue for 19 genes; [a]Paired T-Test; [b]Mean difference or relative expression is [log2 (Diff[i])=log2 (Tumor[i])-log2 (Adjacent Normal[i])] Standard Deviation.

Table 3. Hazard ratio (HR) and 95% confidence interval (CI) for overall mortality risk according to [Log2(tumor/normal)] gene expression values among 65 patients with colorectal adenocarcinoma

	Univariate				Multivariable[a]			
Gene	HR	95% CI		*q*-value[b]	HR	95% CI		*q*-value[b]
AGER	0.73	0.48	1.11	0.145				
CD36	0.79	0.57	1.11	0.176				
CXCL8	0.98	0.83	1.16	0.815				
HMGB1	0.86	0.44	1.66	0.645				
IL1A	**0.72**	**0.54**	**0.96**	**0.042**	**0.68**	**0.49**	**0.94**	**0.0238**
IL1B	0.94	0.76	1.17	0.592				
IL6	**0.77**	**0.58**	**1.02**	**0.070**	**0.70**	**0.52**	**0.94**	**0.0177**
MAP3K1	0.72	0.45	1.13	0.153				
MYD88	**0.51**	**0.30**	**0.89**	**0.042**	**0.53**	**0.30**	**0.93**	**0.0278**
NFKB1	0.62	0.34	1.13	0.119				
NFKB2	**0.70**	**0.49**	**1.02**	**0.060**	0.70	0.46	1.07	0.1018
S100A4	1.04	0.67	1.61	0.873				
S100P	1.17	0.92	1.48	0.198				
S100PBP	**0.68**	**0.44**	**1.06**	**0.086**	0.77	0.47	1.28	0.314
TLR2	0.88	0.62	1.25	0.465				
TLR3	0.82	0.54	1.23	0.331				
TLR4	**0.73**	**0.51**	**1.05**	**0.088**	0.70	0.48	1.02	0.060
TLR5	**0.73**	**0.55**	**0.96**	**0.042**	**0.71**	**0.52**	**0.98**	**0.036**
TNFRS1A	0.68	0.40	1.15	0.151				

[a]Adjusted for TNM stage (0-4, with 0 as ref), neo-adjuvant therapy (Yes vs. No).
[b]Genes in-bold with q < 0.05 were included in best subset Cox Proportional Hazards analysis.

Gene expression in paired CRC versus normal paired samples

Log2 gene expression differences (**Figure 1**) and ratios were calculated. Overall, gene downregulation in colorectal tumor epithelia was present in 10 of the 19 genes tested, with paired mean expression differences significantly higher (q < 0.0002) in adjacent normal tissue than in tumor tissue (*AGER*, *CD36*, *IL1A*, *MAP3K1*, *MYD88*, *NFKB1*, *TLR3*, *TLR5*, *TNFRS1A*, and *S100PBP*) (**Figure 1**). Only *CXCL8* and *S100P* were significantly overexpressed in tumor versus paired adjacent normal tissue (q < 0.0002).

Single and multigene cox regression analysis

In univariate analysis, paired gene expression ratios for 7 of 19 genes were associated with mortality in CRC (q < 0.10) and assessed in multivariable models (**Table 3**). Four of seven genes were significant after ad-

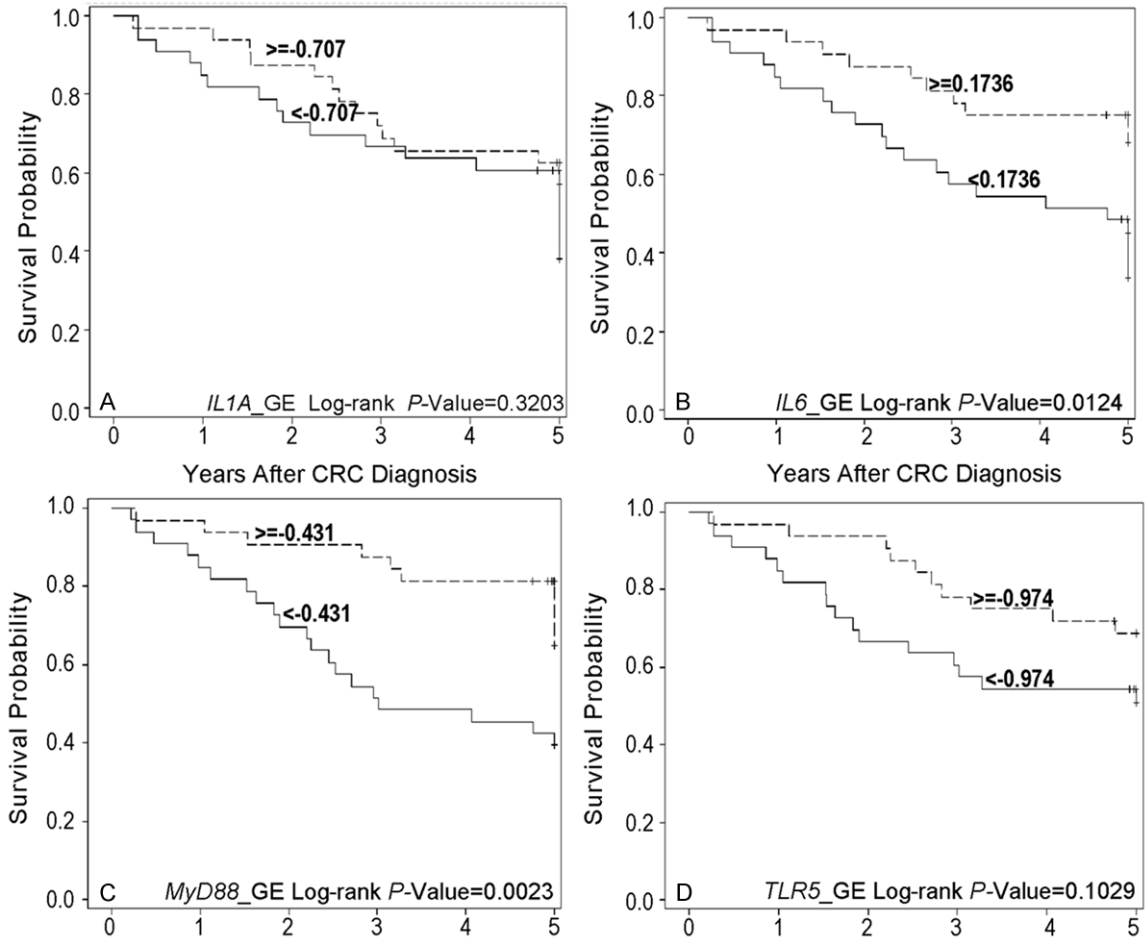

Figure 2. Kaplan Meier survival curves based on dichotomized median expression (above and below) for Log2 gene expression ratios (tumor/normal) of *IL1A* (A), *IL6* (B), *MyD88* (C), and *TLR*5 (D).

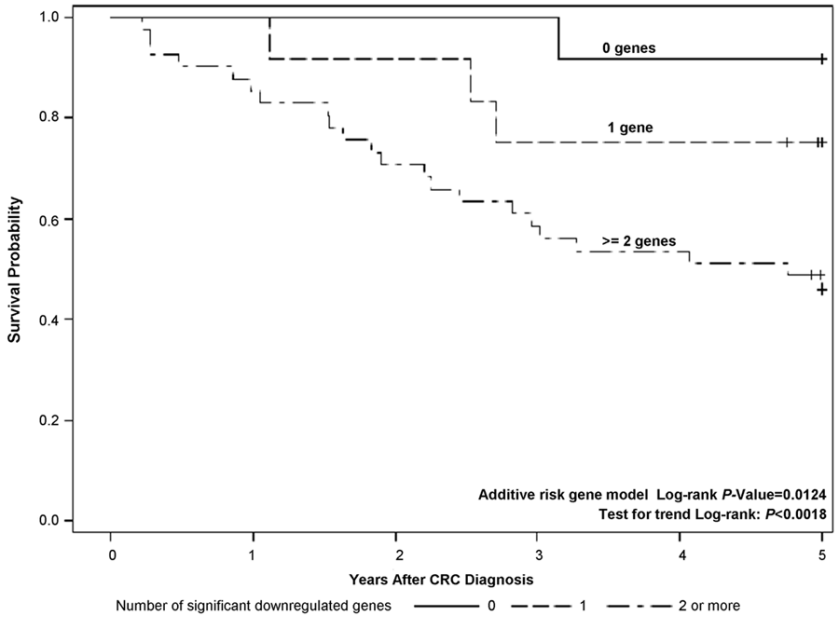

Figure 3. Kaplan Meier curves for combined *TLR* gene set analysis. Gene expression based on combined median log2 gene expression ratios for *IL1A, and IL6, MyD88,* and *TLR5.*

justment for TNM stage and neo-adjuvant therapy, including *IL1A* (HR*adj* = 0.68, 95% CI: 0.49-0.94), *IL6* (HR*adj* = 0.70, 95% CI: 0.52-0.94), *MyD88* (HR*adj* = 0.53, 95% CI: 0.30-0.93), and *TLR5* (HR*adj* = 0.71, 95% CI: 0.52-0.98). The associations

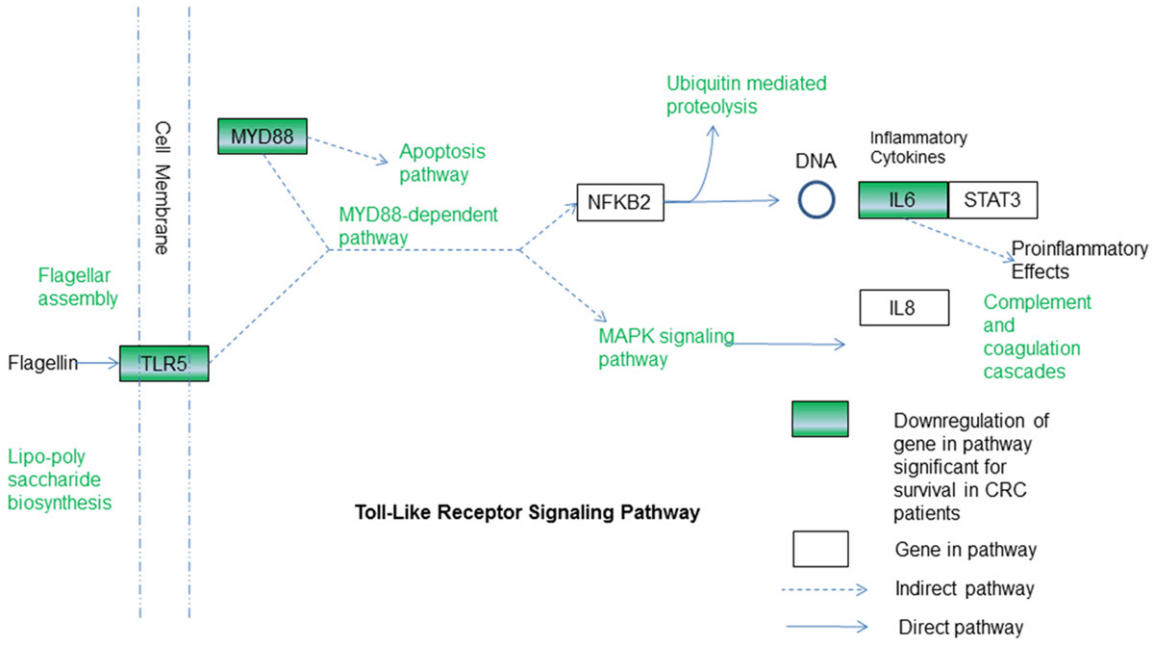

Figure 4. A pathway-based analysis of resulting gene set using KEGG Toll-Like Receptor Pathway information.

did not differ significantly by TNM stage and BMI category when interaction was assessed (interaction $P > 0.05$; data not shown).

In an exploratory multi-gene analysis, best-subset Cox regression was done for mortality and *IL1A, IL6, NFKB2, MYD88, TLR4,* and *TLR5* genes because they met nominal significance after multivariable adjustment ($q < 0.01$, **Table 3**). Compared with a single-gene model, the 2 variable model containing *IL6* and *MyD88* had the largest increase in the global Chi-Square per variable among six genes that were assessed (21.9%, Chi-square = 17.1), and the 2 variable model containing *IL1A* and *TLR5* (Chi-square = 14.3 and 13.5) had the second largest global Chi-square increases. Therefore, *IL6*, *MyD88, IL1A,* and *TLR5* were dichotomized and used for the TLR gene set analysis.

Log-rank survival differences for dichotomized single genes in the *TLR* gene set analysis were only significant for two of four genes (*MyD88*, $P = 0.0023$ and *IL6*, $P = 0.0124$) (**Figure 2B** and **2C**). Participants with gene expression values less than the median were used to build and assess TLR gene set survival models for *IL1A, IL6, MyD88,* and *TLR5*, with the reference category greater than or equal to the median for each gene. Survival was lowest for those with downregulation in at least 2 of 4 genes ($P < 0.0124$) compared with those that were low expressers for only one of the genes or none (five-year survival, 44% vs 57% and 88%, respectively). This comparison was also significant for trend ($P < 0.0001$) (**Figure 3**). After adjustment for TNM Stage and neoadjuvant therapy, risk of death for patients with low expression for *MyD88* or *IL6* was eight times higher (HR*adj*: 8.27; 95% CL 1.40-48.88) than those without low expression, and even higher in those with low expression with both genes (HR*adj*: 12.96 95% CL 2.20-76.52). After multivariable adjustment, the addition of either *TLR5* or *IL1A* did not significantly change estimates compared with assessment of *IL6* and *MyD88* alone. The KEGG pathway analysis showed direct and indirect interactions of the genes comprising this set that may affect CRC survival (**Figure 4**).

In sensitivity analysis after removal of 19 patients who received neoadjuvant therapy prior to surgery, only *TLR5* was significantly associated with overall mortality ($q < 0.01$). There were no changes in the direction of estimates for overall mortality risk compared with imputed data (data not shown).

Discussion

We performed a novel investigation of the association between expression of genes related to *TLRs* and *AGER* pathways and all-cause mortal-

ity in male patients following CRC surgical resection. Our study suggested increased mortality risk for patients with decreased tumor expression of *TLR5*, *MyD88*, *IL1A, and IL6* independent of TNM stage and neoadjuvant therapy. CRC is immunogenic and provokes the host's immune system. An insufficient response due to concerted downregulation in key immune system genes may predict poorer outcomes in those patients with resectable tumor [15].

Immune evasion is an emerging hallmark in cancer research. Understanding the interaction between cancer and the immune system can potentially help predict CRC outcomes [16]. A recent meta-data analysis identified six subtypes of CRC. Two of these identified subtypes were composed of deregulated immune signaling pathways, including KEGG TLR [17]. The dynamic interaction among the tumor's immunogenicity, the host's immune response, and the cancer cells' proficiency in evading is an ongoing area of research [15]. We found that CRC patients with lower tumor expression of *TLR5*, *MyD88*, *IL1A*, and *IL-6* genes had higher risk of mortality, which may indicate an insufficient immune response.

MyD88 acts downstream of *TLRs* or *IL1R* signaling in transformed, stromal, or inflammatory cells in the tumor microenvironment and it contributes to both tissue repair and regeneration to maintain homeostasis. *MyD88*-coupled TLRs induce the synthesis of key mediators of the inflammatory cytokines, such as *TNF-α*, *IL-6* and *IL-1* [18, 19]. *MyD88* can either promote or inhibit carcinogenesis. In CRC tumors, *MyD88* is often found to be downregulated compared to nearby normal tissue expression, as was also shown in our study [20, 21]. One study found altered or delayed colon tissue repair [22] and liver regeneration [23] in *MyD88*$^{-/-}$ mice. This could result from an insufficient signaling of *MyD88* repair mechanisms in cells that express TLRs, as well as an inability to respond to the IL-1 cytokine family.

We found that mortality was higher in patients who were low expressers for both *MyD88* and *IL-6*, suggesting a synergistic effect that may result from multiple pathway deregulations. The cooperative role of *MyD88* and *IL-6* may not be surprising because of the pleotropic role of *IL-6* in CRC progression and outcomes as a major contributor to proinflammatory effects [24]. We also found that downregulation in both *TLR5* and *MyD88* resulted in higher mortality, although their additive joint effect was not significant. Our findings are in contrast to several previous studies that have found tumor upregulation in *TLRs* and *IL-6* to be markers for CRC progression [25-27]. The precise mechanisms that dictate whether signaling of *TLR/MyD88/STATA3/IL6* in tumor will promote or suppress cancer may differ by tumor stage, although our observed associations did not differ by TNM stage.

Our results did not support previous studies that suggest an important role of *TLR4* and downstream genes in CRC [6]. Reasons for this discrepancy could include sample size and study population differences, as well as differences in methodology. We also did not find a significant association between *AGER* expression and CRC mortality risk, although previous studies have implicated the *AGER* (RAGE) pathway in colon carcinogenesis and CRC survival [28, 29]. Additional research will be needed to clarify their potential roles in CRC progression.

Our study has several strengths. We performed a novel evaluation of PPRs and their interacting proteins in association with CRC mortality. An important practical aspect of our study is that we used a quantitative methodology that requires only a very small amount of tissue and thus is applicable to clinical studies for assessing gene expression patterns in single biopsies. A further advantage of the HTG qNPA assay is that it allows assessment of gene expression of multiple genes simultaneously in the same tissue processed for routine histology. We accounted for multiple comparisons via calculation of the FDR-adjusted *q* value, which precluded the false positive findings.

This study has several limitations. Our study was restricted to men. Most study participants were White/Caucasian or African American/Black. Therefore, the generalizability of our findings to men of other races/ethnicities and women is unknown. Our analysis was limited to five-year all-cause mortality as opposed to CRC-specific mortality because we did not have access to the data on cause of death for all study participants. We were unable to assess some factors that may have modulated mortality outcomes in our cohort, including physical activity, history of alcohol consumption and cigarette smoking. Finally, our sample size was

limited for detection of multi-gene interactions.

In summary, our findings suggest that tumor downregulation in *TLR5, MyD88*, *IL1A, and IL6* are associated with increased risk of mortality in men with primary CRC after tumor resection. Further understanding of the mechanistic actions of *TLR5* and other PRRs in modulating immunity, chronic inflammation, and bacterial populations in the tumor microenvironment to affect CRC outcomes is warranted. Replication of our findings in a large prospective clinical study could support creation of predictive immunologic signature biomarkers, particularly in patients who will most likely benefit from current immunotherapies.

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Disclosure of conflict of interest

None.

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