Functional TLR5 Genetic Variants Affect Human Colorectal Cancer Survival

Sascha N. Klimosch1, Asta Först1,4,9, Jana Eckert4, Jelena Knežević5, Melanie Bevier4, Witigo von Schönfels6, Nils Heitz6, Jessica Walter6, Sebastian Hinz6, Jesús Lascorz7, Jochen Hampe6, Dominik Hartl5, Julia-Stefanie Frick3, Karl Hemminki4,9, Clemens Schafmayer6,8, and Alexander N.R. Weber1,5

Abstract

Toll-like receptors (TLR) are overexpressed on many types of cancer cells, including colorectal cancer cells, but little is known about the functional relevance of these immune regulatory molecules in malignant settings. Here, we report frequent single-nucleotide polymorphisms (SNP) in the flagellin receptor TLR5 and the TLR downstream effector molecules MyD88 and TIRAP that are associated with altered survival in a large cohort of Caucasian patients with colorectal cancer (n = 613). MYD88 rs4988453, a SNP that maps to a promoter region shared with the acetyl coenzyme-A acyl-transferase-1 (ACAA1), was associated with decreased survival of patients with colorectal cancer and altered transcriptional activity of the proximal genes. In the TLR5 gene, rs5744174/F616L was associated with increased survival, whereas rs2072493/N592S was associated with decreased survival. Both rs2072493/N592S and rs5744174/F616L modulated TLR5 signaling in response to flagellin or to different commensal and pathogenic intestinal bacteria. Notably, we observed a reduction in flagellin-induced p38 phosphorylation, CD62L shedding, and elevated expression of interleukin (IL)-6 and IL-1β mRNA in human primary immune cells from TLR5 616LL homozygote carriers, as compared with 616FF carriers. This finding suggested that the well-documented effect of cytokines like IL-6 on colorectal cancer progression might be mediated by TLR5 genotype-dependent flagellin sensing. Our results establish an important link between TLR signaling and human colorectal cancer with relevance for biomarker and therapy development. Cancer Res; 73(24); 7232–42. ©2013 AACR.

Introduction

The innate immune system detects molecular patterns representative for entire classes of microbes by means of germline-encoded receptors of the Toll-like receptor (TLR) family (1). For example, TLR5 senses bacterial flagellin, a component of the bacterial motility apparatus (1). TLRs engage their ligands through leucine-rich repeat (LRR) extracellular domains (ECD; refs. 2, 3). Intracellular signaling is mediated by Toll/interleukin (IL)-1 receptor (TIR) signaling domains, coupling TLR activation to different TLR adaptor molecules, for example, myeloid differentiation 88 (MyD88) and MyD88-adaptor like (Mal; encoded by TIR domain-containing adaptor protein; TIRAP, ref. 1). Subsequent signaling events culminate in the activation of the transcription factor NF-κB and p38 and extracellular signal regulated kinase (ERK) mitogen-activated protein kinases (MAPK), which together regulate a plethora of genes, for example, cytokines, required for subsequent immune responses. Thus, TLRs influence complex inflammatory, autoimmune, and malignant diseases as well as tissue homeostasis, particularly in the gut (1, 4).

The gut represents a unique environment for host–pathogen interactions with a commensal microflora in direct proximity of intestinal epithelial cells, stromal cells, and infiltrating immune cells, all of which express TLRs (5). Although TLR signaling is vital for protection against gut injury and associated mortality (5–7), it is also thought to play a decisive role in the context of a gut-related malignancy, like colorectal cancer, the third most frequent cancer in men and second most frequent in women worldwide (5, 8). However, the exact mechanisms by which TLRs influence colorectal cancer...
progression remain controversial; in some murine models of colorectal cancer, genetic loss of MyD88 (9) or TLR4 (10) reduced the incidence of colorectal cancer suggesting that TLR-dependent signaling promotes colorectal cancer progression (11, 12). Other studies observed the opposite, where the lack of MyD88 or TLR5 enhanced tumor growth (13). Despite this ambiguity, the association of certain sequence variants, for example, single-nucleotide polymorphisms (SNP), of TLRs with different aspects of colorectal cancer, for example, survival or response to therapy, can be highly informative as diagnostic markers to guide decision making on therapeutic approaches in patients with colorectal cancer, which currently relies on clinicopathologic staging in newly diagnosed patients (14). Recently, we reported a TLR3 SNP as a potential prognostic marker for stage II colorectal cancer (15). To explore additional genetic variants in TLR pathway genes as prognostic markers, we analyzed 36 additional SNPs in eight TLR pathway genes in a German colorectal cancer study (n = 613). We identified a total of five SNPs in the MYD88, TIRAP, and TLR5 genes as significantly associated with colorectal cancer survival. MYD88 and TLR5 SNPs were found to have functional effects and thus, apart from serving as biomarkers for colorectal cancer decision making, may directly impact on colorectal cancer survival.

Materials and Methods

Study populations

Colorectal cancer SNP associations were investigated in a registry- and hospital-based series of 613 patients belonging to the population-based PopGen project in Schleswig-Holstein (Germany, see Supplementary Information, Table S1; ref. 16). Functional analyses were performed with whole blood (see Supplementary Fig. S3) from healthy volunteers from the Department of Immunology, Tübingen, Germany. Written informed consent was obtained from all study participants and all study protocols were approved by the local ethics committees.

Gene and SNP selection

Thirty-six SNPs in candidate TLR pathway genes mapping to 5' and 3'-untranslated regions (UTR) or promoter regions or nonsynonymous coding SNPs with a minor allele frequency more than 0.05 in the HapMap CEPH population or in 96 sequenced samples of the study population, and with a pairwise linkage disequilibrium, r2 < 0.80 (Supplementary Table S1) were selected for genotyping as detailed in Supplementary information.

Genotyping

Genotyping of patients with colorectal cancer was performed using KASPar assays on demand (KBiosciences). Genotyping of the healthy blood donors was conducted using allele-specific TaqMan-based assays (Life technologies) for rs2072493 (N9285), rs5744175 (F616L), rs5744168 (R392X), and rs4988453 (MyD88).

Reagents

All chemicals were from Sigma unless otherwise stated. Cells were stimulated with flagellin from Salmonella typhimurium (S. typhimurium; Imagenex), lipopolysaccharide (LPS), or R848 (InvivoGen) as indicated.

Cell culture

HEK293T Flp-In T-REx (Life Technologies; obtained from A. Pichlmair, CeMM, Vienna; cells were not retested or authenticated) and HCT116 cells (a gift from S. Stevanovic, Department of Immunology, Tübingen, Germany; cells were not retested or authenticated) were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal calf serum, 1-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL; all from Life Technologies) at 37°C and 5% CO2. Flp-In T-REx cells (Life technologies) were cultured with tetracycline-free fetal calf serum (VWR).

Bacterial strains and culture

S. typhimurium [[SALTY LT2; American Type Culture Collection (ATCC) code: 700720] was a gift from S. Wagner (Medical Microbiology, Tübingen, Germany), Escherichia coli Nissle (wt, delta FliC) was obtained from T. Ölschläger (Institute of Molecular Infection Biology, University of Würzburg, Germany), ATCC strains of Alcaligenes faecalis ssp. Faecalis (35655), Enterobacter cloacae (13047), Enterococcus faecalis (19433), Escherichia coli (11229), Exiguobacterium species (Gr. A; 49676), Klebsiella oxytoca (700324), Morganella morganii (25830), Proteus mirabilis (14153), Proteus vulgaris (13315), and Bacillus fragilis (25285) were used. Bacteria were propagated using appropriate conditions, harvested at log phase, and washed in PBS. The cells were heat inactivated and protein levels were measured by bicinchoninic acid.

MYD88/ACAA1 promoter reporter assays

The normalized promoter activities of MYD88 and coenzyme-A acyl-transferase-1 (ACAA1) were studied using custom-made reporter plasmids (GeneCopoeia) containing an inducible Gaussia luciferase (GLuc) MYD88/ACAA1 promoter and a constitutive SEAP reporter. rs4988453 was introduced by Sanger sequencing. HCT116 cells were transiently cotransfected with reporter promoter clones, EGFP plasmids, and transcription factors as indicated using XtremeGene HP (Roche). Following incubation, supernatants were analyzed for secreted GLuc and SEAP.

TLR5 constructs

pcDNA3-TLR5 (NCBI accession no. NM_003268) was from P. Ahmad-Nejad (University Hospital Mannheim, Mannheim, Germany) and pFLAG-CMV-1-hTLR5 from Addgene. Mutations corresponding to nsSNPs were introduced by QuickChange II Site-Directed Mutagenesis (Stratagene) and confirmed by Sanger sequencing. HCT116 cells were transiently cotransfected with promoter reporter clones, EGFP plasmids, and transcription factors as indicated using XtremeGene HP (Roche). Following incubation, supernatants were analyzed for secreted GLuc and SEAP.

Immunoblot analysis

HCT116 cells were transfected with pFLAG-CMV-1 hTLR5 variants and pcEI-EGFP (Clontech). Forty-eight hours later, cells were lysed in radiocommunoprecipitation assay buffer. Lysates were analyzed on 3% to 8% Tris acetate SDS-PAGE (Life Technologies) gels and subsequent anti-FLAG
(Sigma) and anti-GFP (Cell Signaling Technology) immuno-
blot analysis.

HCT116 reporter gene assay
HCT116 cells were transfected with a firefly luciferase reporter construct, a pRL-TK Renilla luciferase reporter plas-
mid for normalization, and pCI-EGFP (Clontech), see Supple-
mentary Information. pcDNA3.1-hTLR5-WT plasmid or indi-
cated mutant plasmids were cotransfected. Twenty-four
hours after transfection cells were stimulated with flagellin
(50 ng/mL) for 18 hours. Luciferase activities were determined
using the Dual Luciferase Assay System (Promega).

Flp-In T-REx TLR5 mutants
To generate HEK293T Flp-In T-REx (Life Technologies) lines
expressing TLR5 or mutants, the respective TLR5 sequences
were cloned into pcDNA™5/FRT/TO (Life Technologies)
and transferred into HEK293T Flp-In T-REX as described in
ref. 17. Stable single-copy integrants were selected with hygro-
mycin and blasticidin. The cells were treated with tetracycline
(1 μg/mL) for 15 hours to induce TLR5 expression.

Quantitative PCR analysis
mRNA was isolated (cell lines: RNeasy Mini Kit; whole
blood: QiAamp RNA Blood Mini Kit; Qiagen) and tran-
scribed to cDNA (High Capacity RNA-to-cDNA Kit; Life
Technologies). The expression of IL-1β, IL-6, IL-8, TLR5, or
TNF mRNA was studied using TaqMan Gene Expression
Assays (see Supplementary Information). Data were nor-
malized to TBP. The samples were analyzed on a real-time
cycler (Applied Biosystems; 7500 fast).

ELISA
The supernatant of Flp-In T-REx TLR5 cells was collected 24
hours poststimulation with flagellin/heat-inactivated bacteria
and tested for the concentration of IL-8 by ELISA (Biosource).

Isolation and analysis of primary blood cells
See Supplementary Fig. S3 and Supplementary Information
for details. In brief, for quantitative PCR (qPCR) analysis
heparinized whole blood was treated as indicated. RNA was
isolated 3 hours poststimulation. For phosflow analysis, whole
blood was treated as indicated for 10 minutes. Red blood cells
were lysed and leukocytes were fixed (Lyse/Fix Buffer, BD
Biosciences). LIVE/DEAD Fixable Aqua was used to stain dead
cells (Life Technologies). Cells were permeabilized with meth-
anol, Fc-receptors were blocked (Flebozima), and cells were
stained with antibodies. For the analysis of CD62L shedding
whole blood was not fixed. Shedding was calculated as follows:

\[
\text{shed CD62L} = 1 - \left(\frac{\text{stimulated}}{\text{unstimulated}}\right)
\]

A standardized protocol and identical flow cytometer settings were used for all donors.

Statistical analysis
SAS software version 9.2 (SAS Institute) was used in the
association study. Unadjusted associations were evaluated by
χ² test. ORs with 95% confidence intervals (CI) were estimated
using logistic regression. The associations were calculated for
codominant and dominant models. Formal adjustment for
multiple comparison was not performed because of possible
biologic effects of the selected polymorphisms in cancer pro-
gression and their probable correlation with each other. Effect
of the different genotypes on colorectal cancer survival were
evaluated using the Kaplan–Meier method and were compared
using log-rank testing. Analysis of different parameters for
prognostic significance was done by univariate and multivar-
tiate Cox proportional hazard models, see Supplementary
Information. Follow-up time was calculated from the date of
colorectal cancer diagnosis to the colorectal cancer-specific
death or death by any cause or to the end of follow-up (date of
last contact with the treating physician). For functional anal-
yses, data were analyzed using JMP (SAS institute) and Graph-
Pad Prism (GraphPad Software, Inc.). For the comparisons
of wild-type (WT) with their respective SNP variants, P values
were determined using an unpaired t test or a Mann–Whitney
test, as indicated. P < 0.05 was generally considered statistically
significant and are given or denoted by * throughout.

Results

Five TLR pathway SNPs are associated with colorectal
cancer survival
To gain an insight into the influence of TLR pathway SNPs on
human colorectal cancer survival, 36 SNPs (see Supplementary
Table S1) in TLR receptors (TLR1, 2, 4, 5, 6, and 9) and adaptors
(MyD88, TIRAP) were genotyped in a German colorectal cancer
cohort (Supplementary Table S2; ref. 16). As shown in Table 1
and Supplementary Fig. S3, five variants were associated with
colorectal cancer survival in a dominant model: the nonsynon-
ymous SNP rs2072493 (coding for N592S) in TLR5 was associ-
ated both with colorectal cancer-specific survival (HR = 1.89;
95% CI, 1.27–2.80) and with overall survival (HR = 1.57; 95% CI,
1.08–2.27), whereas another nonsynonymous SNP in TLR5,
rs5744174 (coding for F616L), was associated only with col-
orectal cancer-specific survival (HR = 0.66; 95% CI, 0.45–0.98).
Both rs2072493 and rs5744174 were associated exclusively in the
group of patients with cancer located in the colon (rs2072493
HR = 3.12; 95% CI, 1.84–5.28 vs. rs5744174 HR = 0.47; 95% CI,
0.28–0.80). In TIRAP, two 3’UTR SNPs, rs619153 and rs625413
(pairwise linkage disequilibrium, r² = 0.95), were associated
with colorectal cancer-specific survival in a dominant model
(HR = 1.46; 95% CI, 1.01–2.13; HR = 1.55; 95% CI, 1.06–2.27,
respectively). In MyD88, the promoter SNP rs4988453 was
associated both with colorectal cancer-specific and overall
survival in a dominant model (HR = 1.66; 95% CI, 0.99–2.78;
HR = 1.67; 95% CI, 1.05–2.66, respectively). The significant effect
of these five variants on colorectal cancer patient survival was
also evidenced using Kaplan–Meier survival analysis (Fig. 1).

TLR5 and TIRAP SNPs are associated with colorectal
cancer clinical characteristics
Some survival-associated SNPs were also associated with
different International Union against Cancer (UICC) criteria “T”
(size of primary tumor), “N” (lymph node involvement), or “M”
distant metastases) (Supplementary Table S4) at the time of
diagnosis. In TLR5, rs2072493 (N592S) was associated with
distant metastasis (OR = 1.81; 95% CI, 1.13–2.90) and with a
higher tumor stage in a dominant model (OR = 1.52; 95% CI,
1.06–2.20, not shown). In contrast, homozygous carriers of the

\[
1.06
\]

\[
1.20
\]
Table 1. Association of selected polymorphisms with colorectal cancer survival

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th># Died (%)</th>
<th>HR (95% CI)</th>
<th>P</th>
<th># Died (%)</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>rs2072493 TLR5 (N592S)</td>
<td>AA</td>
<td>468</td>
<td>16 (16.45)</td>
<td>1.00</td>
<td>148</td>
<td>31 (31.62)</td>
<td>1.00</td>
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<td></td>
<td>AG</td>
<td>135</td>
<td>36 (26.67)</td>
<td>1.92</td>
<td>(1.29–2.87)</td>
<td>0.001</td>
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<tr>
<td></td>
<td>GG</td>
<td>5</td>
<td>1 (20.00)</td>
<td>1.13</td>
<td>(0.16–8.14)</td>
<td>0.90</td>
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<tr>
<td></td>
<td>AG+GG</td>
<td>140</td>
<td>37 (26.43)</td>
<td>1.89</td>
<td>(1.27–2.80)</td>
<td>0.002</td>
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<tr>
<td>rs5744174 TLR5 (F616L)</td>
<td>TT</td>
<td>191</td>
<td>42 (21.99)</td>
<td>1.00</td>
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<td></td>
<td>TC</td>
<td>278</td>
<td>50 (17.99)</td>
<td>0.73</td>
<td>(0.48–1.10)</td>
<td>0.13</td>
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<td></td>
<td>CC</td>
<td>128</td>
<td>17 (13.28)</td>
<td>0.51</td>
<td>(0.29–0.93)</td>
<td>0.03</td>
<td></td>
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<tr>
<td></td>
<td>TC+CC</td>
<td>406</td>
<td>67 (16.50)</td>
<td>0.66</td>
<td>(0.45–0.98)</td>
<td>0.04</td>
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<tr>
<td>rs611953 TIRAP*</td>
<td>GG</td>
<td>364</td>
<td>58 (15.93)</td>
<td>1.00</td>
<td></td>
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<tr>
<td></td>
<td>GA</td>
<td>211</td>
<td>45 (21.33)</td>
<td>1.39</td>
<td>(0.93–2.06)</td>
<td>0.11</td>
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<tr>
<td></td>
<td>AA</td>
<td>35</td>
<td>10 (28.57)</td>
<td>1.93</td>
<td>(0.98–3.78)</td>
<td>0.06</td>
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<tr>
<td></td>
<td>GA+AA</td>
<td>246</td>
<td>55 (22.36)</td>
<td>1.46</td>
<td>(1.01–2.13)</td>
<td>0.05</td>
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<tr>
<td>rs625413 TIRAP*</td>
<td>CC</td>
<td>354</td>
<td>55 (15.54)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>CT</td>
<td>204</td>
<td>47 (23.04)</td>
<td>1.54</td>
<td>(1.03–2.29)</td>
<td>0.04</td>
<td></td>
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<td>TT</td>
<td>32</td>
<td>8 (25.00)</td>
<td>1.66</td>
<td>(0.79–3.49)</td>
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<tr>
<td></td>
<td>CT+TT</td>
<td>236</td>
<td>55 (23.31)</td>
<td>1.55</td>
<td>(1.06–2.27)</td>
<td>0.02</td>
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<tr>
<td>rs4988453 MYD88</td>
<td>CC</td>
<td>538</td>
<td>96 (17.84)</td>
<td>1.00</td>
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<tr>
<td></td>
<td>CA</td>
<td>59</td>
<td>15 (25.42)</td>
<td>1.53</td>
<td>(0.89–2.64)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>3</td>
<td>2 (66.67)</td>
<td>4.54</td>
<td>(1.12–18.49)</td>
<td>0.03</td>
<td></td>
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<tr>
<td></td>
<td>CA+AA</td>
<td>62</td>
<td>17 (27.42)</td>
<td>1.66</td>
<td>(0.99–2.78)</td>
<td>0.06</td>
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</tbody>
</table>

NOTE: Statistically significant (P < 0.05) values shown in bold.
*High observed linkage disequilibrium between the SNPs with r² = 0.95.

TLR5 alleles modulate flagellin responsiveness in vitro

The TLR5 SNPs N592S and F616L map to the C-terminal part of the TLR5 ECD (Fig. 3A), and represent substitutions unique for humans (Fig. 3B). On the basis of structural evidence (3) and modeling studies (18, 19), both positions are not in direct contact with the flagellin D1 regions, which were proposed to induce receptor activation, and do not lead to structural perturbations (Supplementary Fig. S2A and S2B) but might rather affect receptor dimerization or interaction with the flexible flagellin D3 domain (Fig. 3C). Because of the reported

The MYD88/ACAA1 SNP rs4988453 decreases promoter activity

rs4988453 maps to the promoter region shared by MYD88 and ACAA1 (Fig. 2A). To test whether rs4988453 affected MYD88 and/or ACAA1 transcription, MYD88 or ACAA1 promoter reporter constructs containing the rs4988453 Caucasian major (WT) or minor allele (VAR; Fig. 2) were tested for transcriptional activity in colorectal cancer HCT116 cells. There was no significant difference in the basal transcription level (control; Fig. 2B and C). In cotransfections with different transcription factors, MYD88 and ACAA1 promoter activities were upregulated by C/EBP-α, NF-kB p65, IRF3 and IRF7 (Fig. 2B and C), and MYD88 only by IRF1. The MYD88 reporter showed SNP-dependent significant differences when C/EBP-α and NF-kB p65 were cotransfected (Fig. 2B), with the minor allele reporter showing reduced activity. For ACAA1, a difference was observed for IRF3-CA expression (Fig. 2C) but not starvation or hypolipidic substances (Supplementary Fig. S1A and S1B). Activation of C/EBP-α, IRF3-CA, and NF-kB p65 might thus differentially regulate ACAA1 and/or MYD88 in rs4988453 major versus minor allele carriers.

Minor C allele of TLR5 rs5744174 (F616L) were less likely to have lymph node metastasis (OR 0.59; 95% CI 0.36–0.95) and more likely to have tumors with lower stage (OR 0.72; 95% CI 0.52–1.00; TC+CC vs. TT, not shown) than homozygotes for the major allele. Carriers of the minor allele of the TIRAP SNPs rs611953 and rs625413 were more likely to have distant metastases than homozygous carriers of the major allele (OR = 1.92; 95% CI, 1.26–2.95 and OR = 1.98; 95% CI, 1.29–3.04, respectively). Subsequently, we tested the value of the TLR5, TIRAP, and MYD88 polymorphisms as independent prognostic factors in a multivariate analysis. The HRs of carriers of the minor allele of TLR5 rs2072493 remained significantly increased after adjustment for age at diagnosis, T and N: HR = 1.82; 95% CI, 1.21–2.71 for all patients with colorectal cancer, see Table 2, and HR = 2.40; 95% CI, 1.41–4.08 for patients with colon cancer, not shown. Also for the TIRAP SNPs, the HRs remained similar after adjustment for age, T and N (rs611953 HR = 1.44; 95% CI, 0.99–2.11; rs625413 HR = 1.49; 95% CI, 1.02–2.19; Supplementary Table S5). Collectively, our data show a genetic association of TLR5, TIRAP, and MYD88 SNPs and colorectal cancer survival. In the following, we determined whether the associations of MYD88 rs4988453 TLR5 rs2072493 (N592S) and rs5744174 (F616L) might be due to a functional phenotype.

The MYD88 and/or ACAA1 transcription, MYD88 or ACAA1 promoter reporter constructs containing the rs4988453 Caucasian major (WT) or minor allele (VAR; Fig. 2) were tested for transcriptional activity in colorectal cancer HCT116 cells. There was no significant difference in the basal transcription level (control; Fig. 2B and C). In cotransfections with different transcription factors, MYD88 and ACAA1 promoter activities were upregulated by C/EBP-α, NF-kB p65, IRF3 and IRF7 (Fig. 2B and C), and MYD88 only by IRF1. The MYD88 reporter showed SNP-dependent significant differences when C/EBP-α and NF-kB p65 were cotransfected (Fig. 2B), with the minor allele reporter showing reduced activity. For ACAA1, a difference was observed for IRF3-CA expression (Fig. 2C) but not starvation or hypolipidic substances (Supplementary Fig. S1A and S1B). Activation of C/EBP-α, IRF3-CA, and NF-kB p65 might thus differentially regulate ACAA1 and/or MYD88 in rs4988453 major versus minor allele carriers.

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role of TLR5 in the gut and the observed association with colorectal cancer, it seemed plausible that N592S and F616L might affect TLR5 signaling. Using Flp-In T-REx 293T cells, which endogenously express very low TLR5 (as verified by qPCR) and allow the stable integration of a single copy of TLR5 WT or variants, we observed that TNF mRNA induction (Fig. 3D) and IL-8 secretion (Supplementary Fig. S2C) were slightly but significantly increased for TLR5 N592S but drastically reduced for F616L compared with WT, respectively, upon stimulation with purified *S. typhimurium* flagellin. TLR5 rs5744168 R392X, a known hyporesponsive TLR5 variant (20), served as negative control. Although *S. typhimurium* flagellin is commonly used in studies addressing TLR5 function, many other intestinal bacterial species (see Materials and Methods) express flagellin genes and in this system elicited entirely TLR5- and flagellin-dependent responses when heat killed (Supplementary Fig. S2D). Interestingly, responses for the gut-resident *E. cloacae, A. faecalis*, and *P. mirabilis* were similarly strong for TLR5 WT versus N592S (Fig. 3E), whereas for *E. coli* and *P. vulgaris*, N592S showed significantly lower responsiveness compared with WT TLR5. F616L showed poor responsiveness to all tested bacteria. This also pertained to a probiotic bacterial strain used for therapy of gut disorders, *E. coli* strain Nissle 1917 (21), which interestingly, showed a 4-fold higher stimulatory capacity than normal *E. coli*. Collectively, TLR5 F616L thus represents a strongly attenuated TLR5, whereas N592S shows differential functional modulation dependent on the nature of flagellin preparation.

**TLR5 F616L affects TLR5 function in primary immune cells**

We next sought to investigate whether the functionally attenuated phenotype of N592S and F616L might pertain to colon epithelial cells or possibly infiltrating immune cells. We therefore tested the functional activity of TLR5 variants in colorectal cancer cell lines and in primary immune cells from healthy volunteers. Initially, NF-κB activation in response to flagellin was analyzed in HCT116 and DLD1 colorectal cancer cells. In transient transfections with Flag-TLR5 WT or variants, expression levels (Fig. 4A) and flagellin-induced NF-κB reporter activation (Fig. 4B) were similar, suggesting that in colorectal cancer epithelial cells, the TLR5 SNPs do not lead to attenuated signaling. Similar results were obtained in DLD-1 colorectal cancer cells (not shown). On the other hand, a functional impact of TLR5 alleles on colorectal cancer progression may also be exerted by immune cells in the gut setting (22). We therefore investigated TLR5 function in whole blood drawn from healthy homozygous 616FF major allele and homozygous TLR5 616LL minor (rs5744174) allele carriers and stimulated with flagellin, LPS (TLR4), or R848 (TLR7/8) as controls (see Supplementary Fig. S3).

![Survival distribution function](image)

**Figure 1.** TLR SNP carriage affects colorectal cancer survival. Kaplan–Meier estimates of colorectal cancer-specific survival according to genotypes. A, TLR5 rs2072493, all patients (n = 608); B, TLR5 rs2072493, colon cancer patients (n = 287); C, TLR5 rs5744174, all patients (n = 597); D, TIRAP rs625413, all patients (n = 590); and E, MYD88 rs4988453, all patients (n = 600).
and rs5744168 (R392X) genotypes were identical in all tested individuals. To exclude or minimize secondary effects and cross-talk, stimulation times were kept to 10 minutes for phospho-flow cytometry of phospho-p38 and CD62 ligand (CD62L) gated on monocytes (Fig. 4C and D); and 3 hours for qPCR of IL-6 and IL-1β mRNA (Fig. 4E and F). Compared with TLR5 616FF gated monocytes, 616LL monocytes showed reduced phospho-p38 and CD62L shedding upon flagellin but not R848 stimulation (Fig. 4C and D). Furthermore, R848 (Fig. 4E and F, right) and LPS (Supplementary Fig. S4A) initiated comparable IL-6 and IL-1β mRNA in 616FF versus 616LL whole blood, whereas for flagellin stimulation (Fig. 4E and F, left) homozygous 616LL minor allele carriers showed significantly reduced mRNA levels for both cytokines. Thus, homozygous carriage of rs5744174 is characterized by a selective hyporesponsiveness to the TLR5 ligand flagellin in blood immune cells. Unfortunately, due to its lower frequency, rs2072493 (N592S) could not be analyzed functionally in this way. Nevertheless, our data indicate that the epidemiologic association of TLR5 and MYD88 SNPs with colorectal cancer survival may be due to a functional effect of some of the associated SNPs on signal transduction or gene expression levels, respectively.

Discussion

In this study, we used an epidemiologic approach and identified five SNPs that were associated with colorectal cancer survival. According to databases of genetic disease associations, our report is the first to describe an association of MYD88, TIRAP, or TLR5 with any aspects of human colorectal cancer, thus finally providing strong evidence for a link between TLR pathways and colorectal cancer gleaned from mouse models in humans. Some of the associations were based on heterozygous minor allele carriers and our data thus warrant the verification in additional/larger cohorts to comprehensively review effects in homozygotes. We did not correct for multiple comparisons, however, the associations between the SNPs and colorectal cancer survival, clinical tumor characteristics, and multivariate analyses were consistent and supported each other. The TLR5 and TIRAP SNPs were associated with classical prognostic markers and supported the associations with survival. Except for TLR5 rs5744174/F616L, all SNPs were linked with a more severe disease. The strongest association was observed for TLR5 rs2072493/N592S, with the minor allele carriers having an 89% increased risk of dying compared with the homozygote major allele carriers, strengthened by a multivariate analysis. As currently, 27% of newly diagnosed stage III patients suffer from later recurrence (23, 24) and predictive and prognostic markers in colorectal cancer are limited (25), rs2072493 may serve as a valuable new biomarker in decision making in colorectal cancer therapy. An "immune score" encompassing tumor immune cell infiltration, their activation status, and cytokine milieu has been proposed as a staging instrument in colorectal cancer superior to classical UICC criteria (22). On the basis of our study, it would be of special interest to compare immune function-related SNPs, for example, in TLR5, TIRAP, and MYD88, with patients’ immune scores in future clinical studies.

Table 2. Multivariate analysis of TLR5 rs2072493 and colorectal cancer survival

<table>
<thead>
<tr>
<th>Parameter</th>
<th># At risk</th>
<th># Died (%)</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tr>
<td>Unadjusted model</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AA</td>
<td>468</td>
<td>77 (16.45)</td>
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<td>AG+GG</td>
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<td>1.89 (1.27–2.80)</td>
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<tr>
<td>AA</td>
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<td>77 (16.45)</td>
<td>1.00</td>
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<tr>
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<td>Pathologic tumor stage (T)</td>
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<tr>
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<td>75 (18.29)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
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<td>37 (31.36)</td>
<td>1.81 (1.21–2.69)</td>
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<td>Pathologic lymph nodes (N)</td>
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<tr>
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<tr>
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<td>37 (31.36)</td>
<td>1.82 (1.21–2.71)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

NOTE: Statistically significant (P < 0.05) values shown in bold.
Figure 2. SNP rs4988453 affects the promoter activity of MyD88 and ACAA1. A, rs4988453 maps to the dual promoter region shared by MYD88 and ACAA1. B and C, Gaussia luciferase promoter reporter assay of MyD88 (B) or ACAA1 (C) with the major allele sequence (WT) or rs4988453 (VAR). HCT116 cells were transiently cotransfected with promoter reporter clones and the indicated transcription factors or an empty control. Gaussia luciferase activity was normalized to secreted alkaline phosphatase. Data from three independent experiments with three biologic replicates each, relative to the corresponding major allele control, and shown as box- and whiskers-plots (minimum to maximum). *, P < 0.05 (unpaired t test).
To investigate whether the observed association could be direct, we functionally characterized the SNPs in MYD88/ACAA1 and TLR5. Because the population frequency for rs4988453 in MYD88/ACAA1 is very low (AA = 0.3%, AC = 9.2%, CC = 90.5%), we used a promoter reporter assay to investigate its influence on the promoter activity. Differences between WT and SNP promoter were observed in the presence of C/EBP-α, IRF-3CA, or NF-κB p65 (cf. Fig. 2B and C), for example, transcription factors activated upon immune activation. Given that high MYD88 levels correlate with poor colorectal cancer prognosis (26), future work outside the scope of this present study may be able to address whether rs4988453 modulates MyD88 or ACAA1 levels in primary cells and/or colorectal cancer tissue arrays.

TLR5 variants have not been linked to human colorectal cancer so far and for rs2072493/N592S disease associations have not been reported. rs5744174/F616L has been associated with increased risk for gastric cancer and higher IFN levels upon vaccination and in viral disease (see Supplementary Table S6) and, of note, represents one of the most promising candidates of positive selection in the human genome in recent human history (27) so that the insights gained here may have importance beyond colorectal cancer. The two TLR5 SNPs disparately affected colorectal cancer risk with rs2072493 being associated

Figure 3. Coding TLR5 SNPs modulate responsiveness to flagellin. A, schematic model of TLR5, which is composed of a LRR ectodomain (EC), a transmembrane domain (TM), and an intracellular (IC) part with a TIR. Positions of the rs5744168 (R392X), rs2072493 (N592S), and rs5744174 (F616L) SNPs are indicated. B, multiple sequence alignment of amino acids of TLR5 at position 592 or 616 (green), see Materials and Methods for details. C, 3D modeling of TLR5 (gray) and flagellin (blue). D, Flp-In T-REx 293T cells expressing TLR5 variants were stimulated with flagellin. The activation of TLR5 was analyzed on the level of TNF mRNA by qPCR. One representative experiment out of three (triplicate mean ± SD). E, TNF mRNA in TLR5 cell lines upon stimulation with heat-killed bacterial preparations. One representative experiment out of two (triplicate mean ± SD). *P < 0.05 (unpaired t test).
Figure 4. TLR5 616L reduces TLR5 function in primary immune cells but not in a colorectal cancer cell line. A, expression levels of Flag-TLR5 WT and variants is comparable in HCT116 cells transiently cotransfected with EGFP, pcDNA, or the indicated TLR5 plasmids as assessed by immunoblot analysis and quantification (bracketed areas). One representative experiment of three. B, activation of NF-κB by TLR5 gene variants. HCT116 cells were cotransfected with indicated TLR5 WT or mutant plasmids for 24 hours and stimulated with flagellin (50 ng/mL) overnight. NF-κB activity was analyzed by a dual luciferase assay and normalized to pcDNA. Combined data from three independent experiments, measured in biologic triplicates, and shown as box- and whiskers-plots (minimum to maximum). C, phospho-flow analysis of p38 phosphorylation in gated monocytes in whole blood treated with flagellin (100 ng/mL; left), or R848 (5 μg/mL; right) for 10 minutes. Mean fluorescence intensity (MFI) ratios of stimulated cells versus unstimulated cells. Each dot represents one individual donor. D, blood samples were treated similar to C and monocytes were analyzed for CD62L shedding by flow cytometry. E and F, whole blood from healthy donors was treated with the indicated concentrations of flagellin (left) or R848 (right) for 3 hours. mRNA levels of IL-1β (E) and IL-6 (F) were analyzed by qPCR, relative to TBP (2^−ΔΔCt method). Each dot indicates the mean of technical triplicates of an individual donor. In C–F, dashes indicate the median of each group. *, donors with TLR5 616 FF (major allele); ○, donors with TLR5 616 LL (minor allele). *, P < 0.05 (Mann–Whitney test).
with worse, rs5744174 with better survival. This clear difference was not fully reflected at the functional level, where both SNPs showed dependence on the cellular context. F616L caused significant hypersensitivity in stable FlpIn cells but not transiently transfected HCT116 (this study), CHO, and HEK293 cells (20) and Jurkat T cells (27). N592S significantly deviated from WT stimulus dependently in stable FlpIn cells but not transient HCT116 (this study), HEK293, or CHO (20). These discrepancies could reflect differences in the experimental setup (stable vs. transient) and a sensitivity of TLR5 to cellular context in these cellular model systems. Therefore the first analysis of rs5744174/F616L in human primary cells presented here is of particular value and unequivocally showed reduced TLR5 responsiveness in 616L carriers at the level of monocyte p38 phosphorylation, CD62L shedding, and proinflammatory cytokine induction (e.g., IL-6, IL-1β). Although we here analyzed whole blood, effects in epithelial cells from colon or colon/ colorectal cancer-infiltrating immune cells may be comparable as both express TLR5 and respond to flagellin (1, 5). This will require verification in future studies outside the current scope of this study. Because high IL-6 levels in serum and tumor tissue link with an unfavorable colorectal cancer prognosis due to a tumor-promoting role of IL-6 via STAT3 (28), it is tempting to speculate that sensing of intestinal microbiota via TLR5 might be a contributing factor for colorectal cancer (31). We speculate that sensing of intestinal microbiota via TLR5 might be a contributing factor for colorectal cancer (31). We

References

Functional TLR5 Genetic Variants Affect Human Colorectal Cancer Survival

Sascha N. Klimosch, Asta Försti, Jana Eckert, et al.


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