Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

Companion Diagnostics and Cancer Biomarkers

Molecular Cancer Therapeutics

Clinical Significance of *TLR1* I602S Polymorphism for Patients with Metastatic Colorectal Cancer Treated with FOLFIRI plus Bevacizumab

Satoshi Okazaki[1], Fotios Loupakis[2], Sebastian Stintzing[3], Shu Cao[4], Wu Zhang[1], Dongyun Yang[4], Yan Ning[1], Yu Sunakawa[1], Stefan Stremitzer[1], Satoshi Matsusaka[1], Martin D. Berger[1], Anish Parekh[1], Jordan D. West[1], Yuji Miyamoto[1], Mitsukuni Suenaga[1], Marta Schirripa[1], Chiara Cremolini[2], Alfredo Falcone[2], Volker Heinemann[3], R. William DePaolo[5], and Heinz-Josef Lenz[1]

Abstract

The purpose of this study was to evaluate the clinical significance of single-nucleotide polymorphisms in *TLR1*, *TLR2*, *TLR6*, and *TAK1* in patients with metastatic colorectal cancer (mCRC). We genotyped 9 SNPs of *TLR1*, *TLR2*, *TLR6*, and *TAK1* in mCRC patients treated with first-line FOLFIRI (combination therapy of irinotecan, 5-fluorouracil, and folinic acid) plus bevacizumab, using a discovery cohort (TRIBE trial, $n = 228$) and a validation cohort (FIRE-3 trial, $n = 297$), and analyzed for the association with response rate (RR), progression-free survival (PFS), and overall survival (OS). There was a significant association of *TLR1* rs5743618 (T1805G) with the clinical outcome. In the TRIBE cohort, a homozygous wild-type genotype (T/T) associated with a significantly lower RR compared with variant T/G and G/G genotypes (43% vs. 62%, $P = 0.025$), and this observation was validated in the FIRE-3 cohort (46% vs. 65%, $P = 0.021$). In addition, those patients with the T/T genotype had significantly worse PFS (median, 8.2 vs. 10.5 months; HR, 1.57; 95% CI, 1.09–2.28, $P = 0.014$) and OS (median: 19.9 vs. 27.9 months; HR, 1.63; 95% CI, 1.14–2.35, $P = 0.007$), compared with those with other genotypes in the TRIBE cohort. These differences remained statistically significant in multivariate analysis. Our data suggest that *TLR1* rs5743618 could serve as a predictor of clinical response to FOLFIRI plus bevacizumab in patients with mCRC. *Mol Cancer Ther; 15(7); 1740–5. ©2016 AACR.*

Introduction

In recent years, improved anticancer therapies have led to significantly improved clinical outcome of patients with colorectal cancer. However, as not all patients benefit from a chemotherapy regimen, predictive and prognostic biomarkers are critical in identifying patients who most benefit from the chemotherapy. Ras testing is the only validated predictive biomarker of EGFR-inhibitor efficacy (1, 2), and there has been a lot of effort in identifying the biomarker of anti-VEGF therapies (3–5). To date, several studies have suggested that inflammatory and immune responses within the tumor microenvironment may play a role in the efficacy of cytotoxic chemotherapy and targeted antibodies. Most recently, it was shown that colorectal tumors with high microsatellite instability (MSI) likely respond to immune checkpoint inhibitors (6). Key genes involved in immune regulation and inflammatory response may be novel biomarkers predicting clinical responses and tumor prognosis in patients with metastatic colorectal cancer (mCRC) patients.

Toll-like receptors (TLR) play a crucial role in the intestinal mucosal innate and acquired immunity to maintain gut homeostasis (7). TLR, one of the pattern-recognition receptors, recognizes not only exogenous pathogen-associated molecular patterns (PAMP) but also endogenous molecules, termed damage-associated molecular patterns (DAMP), which are released from injured or dying cells (8). Activated TLRs, by PAMPs or DAMPs, initiate signal cascades, such as NFκB, MAPK, and IFN pathways, and promote the secretion of cytokines and chemokines, which regulate immune and inflammatory responses against microbial infection or tissue injury (9). Within the tumor microenvironment, TLRs are expressed not only on immune cells but also on tumor cells and stromal cells (10). Individual TLR signaling in each cell initiates divergent pathways that can influence either tumor promotion (e.g., proinflammation, angiogenesis, and anti-apoptosis) or antitumor immunity (11). These biological responses mediated by TLRs in the tumor microenvironment are thought to influence clinical prognosis, as well as response to anticancer therapy (12).

[1]Department of Medical Oncology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California. [2]U.O. Oncologia Medica 2, Azienda Ospedaliero-Universitaria Pisana, Istituto Toscano Tumori, Pisa, Italy. [3]Department of Medical Oncology and Comprehensive Cancer Center, University of Munich, Munich, Germany. [4]Department of Preventive Medicine, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California. [5]Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Heinz-Josef Lenz, Division of Medical Oncology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, 1441 Eastlake Avenue, Suite 3456, Los Angeles, CA 90033. Phone: 323-865-3967; Fax: 323-865-0061; E-mail: lenz@usc.edu

doi: 10.1158/1535-7163.MCT-15-0931

Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

In recent studies, TLR1 and TLR6, each of which forms a heterodimer with TLR2, were suggested to be essential in regulating mucosal immune response within the gut (13–16). We herein focused on TLR1, TLR2, TLR6, and their common downstream signal molecule TAK1 (Supplementary Fig. S1), and hypothesized that genetic variations in these genes may cause inter-individual differences of clinical outcome in mCRC patients treated with chemotherapy. Single-nucleotide polymorphisms (SNP) are substantial germline genetic variations, some of which may alter the gene function and/or activity. In this study, we tested whether SNPs in these genes are associated with clinical outcome in mCRC patients treated with FOLFIRI (combination therapy of irinotecan, 5-fluorouracil, and folinic acid) plus bevacizumab across independently different cohorts.

Materials and Methods

Patients and samples

This study enrolled two independent cohorts with mCRC patients who were enrolled in a prospective randomized phase III clinical trial, TRIBE (17) or FIRE-3 (18), and underwent FOLFIRI plus bevacizumab as the first-line chemotherapy. The TRIBE study consisted a total of 508 patients with untreated mCRC, from 34 Italian centers, were enrolled and randomly assigned to receive either FOLFIRI plus bevacizumab (arm A, $n = 256$) or FOLFOXIRI plus bevacizumab (arm B, $n = 252$). In the FIRE-3 study, 752 patients with mCRC, from centers in Germany and Austria, were randomized to receive FOLFIRI plus cetuximab ($n = 380$) or FOLFIRI plus bevacizumab ($n = 372$) as the first-line chemotherapy. Although patients were initially enrolled without regard to KRAS status, the enrollment was restricted to only patients with KRAS exon 2 wild-type tumors due to the emerging evidence on the negative predictive value of KRAS exon 2 mutations for cetuximab. Eligibility criteria of our study included patients with histologically confirmed colorectal adenocarcinoma, measurable metastatic disease according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1, and no previous exposure to systemic chemotherapy except for adjuvant chemotherapy, and all patients received irinotecan-based regimen (FOLFIRI). In this study, 228 patients with sufficient samples from arm A of TRIBE (89% of 256 enrolled patients) and 297 patients with sufficient samples from the bevacizumab arm of FIRE-3 (80% of 372 enrolled patients) were enrolled as discovery and validation cohorts, respectively.

All patients signed an informed consent before entering the randomized trials that included information regarding the use of their blood or tumor tissue to explore relevant molecular parameters. This study was conducted adhering to the REporting recommendations for tumor MARKer prognostic studies (REMARK). The tissue analysis was approved by the University of Southern California (USC) Institutional Review Board of Medical Sciences and conducted at the USC/Norris Comprehensive Cancer Center in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Selected polymorphisms and genotyping

Candidate SNPs in *TLR1*, *TLR2*, *TLR6*, and *TAK1* were selected for analyses when having a minor allele frequency of $\geq$10% in Europeans according to the Ensembl database (19). Among the candidate SNPs, we focused on 9 SNPs that had a biological significance reported in literature reviews or were considered potentially functional according to the F-SNP database (20). The characteristics of the selected polymorphisms are shown in Table 1.

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) specimens in patients enrolled in FIRE-3, and from blood in patients enrolled in TRIBE using the QIAamp DNAeasy Kit (Qiagen) according to the manufacturer's instructions. The primers used for polymerase chain reaction analyses are listed in Table 1. DNA sequences were analyzed using the ABI Sequencing Scanner version 1.0 (Applied Biosystems). Investigators involved in SNP analyses were blinded to patients' clinical data.

Statistical analysis

The endpoints of the current study were objective response rate (RR), progression-free survival (PFS), and overall survival (OS). Tumor responses based on RECIST were grouped into responders, including complete or partial response, and nonresponders,

Table 1. SNPs and Primers

	SNP	Location	MAF[a]	Base change (amino acid change)	Primer sequence	
TLR 1	rs5743618	Exon 4	0.25	T > G (missense)	F	5′-TGGCACACCATCCTGAGATAC-3′
					R	5′-ACCCGGAAAGTTATAGAGGAACC-3′
	rs5743565	5′-UTR	0.19	T > C	F	5′-TGGCCTGAGAAACAGAAGGAC-3′
					R	5′-CCCGCCATTTGTATTCTCTTC-3′
TLR 2	rs3804099	Exon 3	0.44	C > T (synonymous)	F	5′-CCTTGAGGAACTTGAGATTGATG-3′
					R	5′-CCAAACATTCCACGGAACTTG-3′
	rs4696480	Promoter	0.48	T > A	F	5′-ATGGTTCTGGAGTCTGGGAAG-3′
					R	5′-CCAAGGGAGCAGTTTATTGTGAG-3′
TLR 6	rs3821985	Exon 1	0.32	G > C (synonymous)	F	5′-CCTTCGTCATGAGACCTACTTTG-3′
					R	5′-CTCATGCACCAAGCACATTC-3′
	rs5743818	Exon 1	0.27	A > C (synonymous)	F	5′-CCCAGGCAGAATCATGTTCAC-3′
					R	5′-TTGGATCTGCCCTGGTATCTC-3′
TAK1	rs1145727	Intron	0.30	A > G	F	5′-GCTAAGATGAGAGTCAAGACAGAGAC-3′
					R	5′-GCTGAGTTAATTCTGACAAAAGGAC-3′
	rs157688	5′-UTR	0.31	T > C	F	5′-TCCTCAAATTAGACAAGGAACAGAG-3′
					R	5′-AGAAGCCTAGGCCTTAAAGGTG-3′
	rs157432	Intron	0.30	T > G	F	5′-AAGAATGGACCCCTGCCTTC-3′
					R	5′-GCCTTCATCATTAGCCCTTACC-3′

Abbreviation: 5′-UTR, 5′-untranslated region.
[a]Minor allele frequency (MAF), according to the Ensembl database (phase I of the 1000 Genomes Project) for Europeans.

Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

including stable or progressive disease. PFS was defined as the period from the first day of starting first-line chemotherapy to the first day of documented disease progression or death. If progression or death was not observed, PFS was censored on the day of the last CT scan. OS was defined as the period from starting therapy to the date of death or censored on the date of last contact if alive. The differences in baseline patient characteristics between the two cohorts were examined using a χ^2 test or the Wilcoxon rank-sum test when appropriate. Allelic distribution of polymorphisms by ethnicity was tested for deviation from the Hardy–Weinberg equilibrium (HWE) using the exact test. Linkage disequilibrium among selected SNPs was assessed using D′ and r^2 values, and the haplotype frequencies were inferred using Haploview version 4.2 (www.broad.mit.edu/mpg/haploview). The power to detect an association between a SNP and PFS would be 80% when the minimum hazard ratio (HR) varied from 1.53 to 2.05 using a two-sided log-rank test at the 0.05 significance level in the training cohort ($n = 228$, 174 PFS events). We assumed that the minor allele frequency ranged from 0.05 to 0.4, and the dominant model was considered. The power would be from 88% using the same test to detect the same ranged HRs with the same allele frequencies under the dominant model in the validation cohort ($n = 297$, 252 PFS events). The associations between polymorphisms and PFS, OS, and RR were investigated using Kaplan–Meier curves, log-rank test, and Fisher exact test. A Cox proportional hazards regression model with stratification factors was fitted to reevaluate the association between SNPs and PFS and OS considering imbalances in the distributions of baseline characteristics among cohorts. The baseline demographic and clinical characteristics that remained significantly associated with endpoints in the multivariable analysis ($P < 0.1$) were included in the final model. All analyses were performed with two-sided tests at a significance level of 0.05 by using the SAS 9.4 (SAS Institute).

Results

The baseline characteristics of the two cohorts included in this study were summarized in Table 2. Compared with the FIRE-3 cohort, the TRIBE cohort comprised younger patients and better performance status, fewer patients received primary tumor resection, more patients with synchronous metastasis, and more patients with KRAS mutant. The median PFS, OS, and follow-up period were 9.7, 26.1, and 49.3 months in the TRIBE cohort; 10.1, 23.8, and 40.8 months in the FIRE-3 cohort, respectively. Genotyping was successful in at least 90% of cases in each polymorphism analyzed. In failed cases, genotyping was not successful because of limited quantity and/or quality of extracted genomic DNA. The allelic frequencies for all SNPs were within the

Table 2. Baseline clinical characteristics of the TRIBE and FIRE-3 cohorts

	TRIBE cohort ($N = 228$) *n* (%)	FIRE-3 cohort ($N = 297$) *n* (%)	*P*[a]
Gender			
Male	138 (61)	195 (66)	
Female	90 (39)	102 (34)	0.23
Age			
Median (range)	60 (29–75)	65 (31–76)	<0.001
<65	163 (71)	156 (53)	
≥65	65 (29)	141 (47)	<0.001
Performance status			
ECOG 0	188 (83)	163 (55)	
ECOG 1–2	39 (17)	134 (45)	<0.001
Unknown[b]	1 (0.4)		
Primary tumor site			
Right side	57 (25)	63 (21)	
Left side	156 (68)	180 (61)	0.84
Unknown[b]	15 (7)	54 (18)	
Liver limited disease			
Yes	72 (32)	96 (32)	
No	156 (68)	201 (68)	0.86
Number of metastatic sites			
<2	99 (43)	107 (36)	
≥2	129 (57)	142 (48)	0.92
Unknown[b]		48 (16)	
Time to metastasis			
Synchronous	188 (82)	185 (62)	
Metachronous	40 (18)	63 (21)	0.038
Unknown[b]		48 (16)	
Primary tumor resection			
Yes	144 (63)	257 (87)	
No	84 (37)	40 (13)	<0.001
Adjuvant chemotherapy			
Yes	28 (12)	54 (18)	
No	200 (88)	243 (82)	0.065
KRAS status			
Wild-type	96 (42)	249 (84)	
Mutant	93 (41)	48 (16)	<0.001
Unknown[b]	39 (17)		

[a]Based on the χ^2 test or the Wilcoxon rank-sum test whenever appropriate.
[b]Not included in the test.

Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

Table 3. Association of *TLR1* rs5743618 with clinical outcome in the TRIBE and FIRE-3 cohorts

		RR		PFS				OS					
				Univariate[a]		Multivariate[b]		Univariate[a]			Multivariate[b]		
Genotype	*n*	*n* (%)	*P*	Median month (95% CI)	HR (95% CI)	*P*	HR (95% CI)	*P*	Median month (95% CI)	HR (95% CI)	*P*	HR (95% CI)	*P*
TRIBE													
G/G	50	29 (60%)		10.8 (8.8–12.6)	1 (Reference)		1 (Reference)		23.2 (16.4–37.6)	1 (Reference)		1 (Reference)	
G/T	132	81 (63%)	0.077	10.5 (9.4–11.3)	0.92 (0.63–1.34)	**0.045**	0.89 (0.60–1.34)	0.120	28.6 (25.6–33.5)	0.95 (0.65–1.39)	**0.024**	0.94 (0.63–1.40)	0.077
T/T	44	18 (43%)		8.2 (7.5–9.7)	1.48 (0.93–2.35)		1.38 (0.85–2.25)		19.9 (15.1–24.0)	1.57 (0.99–2.49)		1.46 (0.91–2.36)	
Any G	182	110 (62%)	**0.025**	10.5 (9.5–11.2)	1 (Reference)	**0.014**	1 (Reference)	**0.046**	27.9 (25.0–33.0)	1 (Reference)	**0.007**	1 (Reference)	**0.025**
T/T	44	18 (43%)		8.2 (7.5–9.7)	1.57 (1.09–2.28)		1.50 (1.01–2.22)		19.9 (15.1–24.0)	1.63 (1.14–2.35)		1.53 (1.06–2.23)	
FIRE-3													
G/G	147	95 (68%)		10.7 (9.1–12.3)	1 (Reference)		1 (Reference)		24.2 (19.4–27.4)	1 (Reference)		1 (Reference)	
G/T	87	45 (58%)	**0.020**	10.1 (9.0–13.2)	0.94 (0.70–1.26)	0.466	0.98 (0.73–1.31)	0.559	26.9 (21.3–31.0)	0.88 (0.63–1.22)	0.335	0.84 (0.61–1.18)	0.332
T/T	52	22 (46%)		10.1 (8.5–11.3)	1.18 (0.84–1.67)		1.19 (0.84–1.67)		23.1 (15.1–28.0)	1.19 (0.83–1.72)		1.15 (0.79–1.67)	
Any G	234	140 (65%)	**0.021**	10.4 (9.3–11.9)	1 (Reference)	0.244	1 (Reference)	0.286	24.8 (21.5–27.6)	1 (Reference)	0.205	1 (Reference)	0.265
T/T	52	22 (46%)		10.1 (8.5–11.3)	1.21 (0.87–1.68)		1.20 (0.86–1.66)		23.1 (15.1–28.0)	1.25 (0.88–1.77)		1.22 (0.86–1.74)	

NOTE: Correlations with $P < 0.05$ are marked with bold text.

[a]*P* value was based on Fisher exact test for tumor response, log-rank test for PFS and OS in the univariable analysis.

[b]*P* value was based on Wald test in the multivariable Cox proportional hazards regression model adjusting age, ECOG performance status, primary tumor site, number of metastatic sites, resection of the primary tumors, RAS mutation status, adjuvant chemotherapy in TRIBE cohort; adjusting for sex, ECOG performance status, liver limited metastasis, primary tumor resection, and KRAS mutation status in FIRE3 cohort.

probability limits of HWE ($P > 0.001$), with exception of *TLR6* rs3821985 in the FIRE-3 cohort. High linkage disequilibrium was found between *TLR6* rs5743818 and *TLR6* rs3821985 ($D' = 0.95$, $r^2 = 0.53$) in the TRIBE cohort.

Association of SNPs with clinical outcome

We first evaluated the relation of each SNP to RR, PFS, and OS, using the TRIBE cohort as a discovery study. When there were statistically significant associations between SNP and clinical outcome, a validation study was subsequently performed using the FIRE-3 cohort.

TLR1. Table 3 shows the association between *TLR1* rs5743618 and clinical outcome. In TRIBE, the homozygous wild-type T/T genotype was associated with a significantly lower RR compared with other variant T/G and G/G genotypes (43% vs. 62%, $P = 0.025$). In addition, those patients with the T/T genotype showed significantly worse PFS and OS as compared with those with the T/G or G/G genotypes (Fig. 1). The median PFS was 8.2 months for patients with the T/T genotype compared with 10.5 months for patients with other genotypes (HR, 1.57; 95% CI, 1.09–2.28, $P = 0.014$), and the median OS were 19.9 and 27.9 months, respectively (T/T vs. any G, HR, 1.63; 95% CI, 1.14–2.35, $P = 0.007$). These differences remained statistically significant in multivariate analyses (PFS; HR, 1.50; 95% CI, 1.01–2.22, $P = 0.046$, and OS; HR, 1.53; 95% CI, 1.06–2.23, $P = 0.025$). In FIRE-3, the same association was observed in RR (T/T: 46% vs. any G: 65%, $P = 0.021$). However, significant differences between genotypes were not observed in PFS and OS. For *TLR1* rs5743565, there was no association with clinical outcome (Supplementary Table S1).

TLR2. For *TLR2* rs3804099 and rs4696480, both polymorphisms significantly associated with PFS in univariate analyses, but these significances were lost when a multivariable testing was applied (Supplementary Table S1).

TLR6. In TRIBE, the *TLR6* rs5743818 A/A genotype was associated with a significantly lower RR compared with the A/C and C/C genotypes. Univariate analysis showed that patients with the A/A genotype had significantly shorter PFS compared with those with A/C or C/C genotype. However, this significance did not retain statistical significance in multivariate analysis. The association between this SNP and RR was not validated in FIRE-3. For rs3821985, there was no association with clinical outcome (Supplementary Table S1).

TAK1. In TRIBE, patients homozygous (A/A) for rs1145727 showed significantly shorter OS compared with those with A/G or G/G genotypes, which retained statistical significance in multivariate analysis. However, these results were not validated in FIRE-3. For rs157688, although the C/C genotype was associated with a significantly longer PFS and OS than other genotypes in univariate analyses, these differences did not remain significant in multivariate analyses. For rs157432, no association with clinical outcome was observed (Supplementary Table S1).

Discussion

Our data showed for the first time that the SNP in *TLR1* was associated with clinical outcome in patients with mCRC. *TLR1* rs5743618 was significantly associated with clinical response to chemotherapy FOLFIRI plus bevacizumab, which was validated in an independent cohort. This polymorphism also significantly correlated with PFS and OS in the TRIBE cohort in both univariate and multivariate analyses. These findings indicate that a cellular

Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

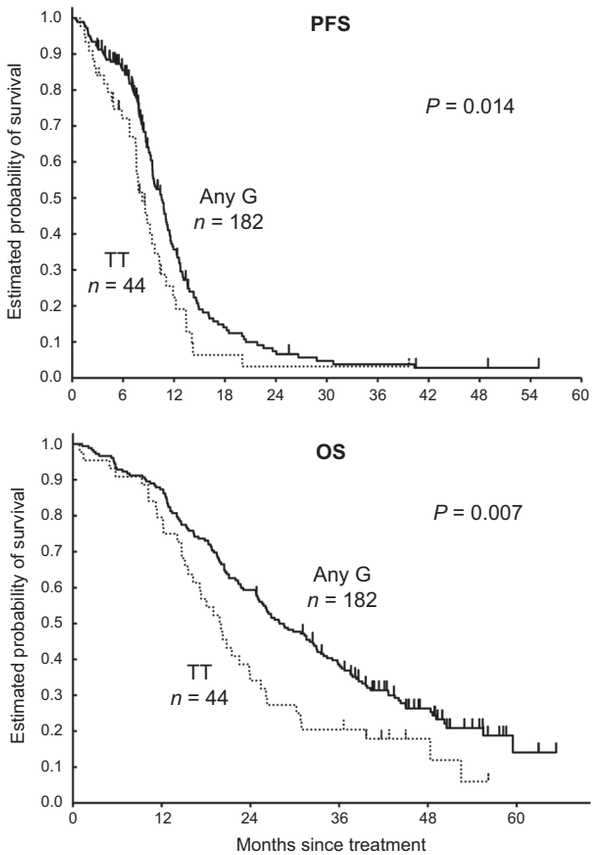

Figure 1.
Prognostic significance of *TLR1* rs5743618 in the TRIBE cohort.

TLR1 signaling plays a critical role in the efficacy of FOLFIRI plus bevacizumab and may be a novel target for drug development.

TLR1 rs5743618 (base pair change: T1805G, amino acid change: I602S) is a common nonsynonymous SNP lying just at the junction of the transmembrane and cytoplasmic domain of TLR1. Although the mechanism by which I602S affects the function of TLR1 remains unclear, it has been suggested that a structural change induced by the substitution of a serine (S) for an isoleucine (I) within the transmembrane domain affects the extracellular ligand-binding domain or the intracellular domain that binds to adaptor proteins (21). Indeed, several studies have shown that I602S is associated with decreased cytokine responses. Hawn and colleagues (21) demonstrated that the individuals with a variant genotype (602S) showed a significantly decreased IL6 level compared with those with a wild-type genotype (602I) in a ligand-stimulated whole-blood cytokine assay. In another study, homozygous for the 602S allele exhibited significantly lower levels of TNFα release in response to the ligand (22). These pieces of evidence indicate that the variant 602S genotype relates to impaired TLR1 signal and subsequent decreased cytokine production.

However, the functional role of the TLR1 signal in the tumor microenvironment is not fully understood. Specific TLRs are known to recognize DAMPs released from stressed or dying tumor cells upon use of chemotherapy agents. The TLR activated by DAMP generates various biological responses, including inflammation, immune response, angiogenesis, and antiapoptosis in the tumor microenvironment, which contribute to create an ideal condition for cancer cell survival and result in chemoresistance (23, 24). Recent studies have also described the biological functions of TLR1, which may lead to cancer promotion and survival.

One possible role is to promote angiogenesis. TLR1/2 heterodimers on endothelial cells recognize a molecular pattern of a lipid oxidation product, ω-(2-carboxyethyl1)pyrrole (CEP), which is generated as a consequence of oxidative stress (25). TLR1/2 signaling triggered by CEP activates the downstream NFκB pathway, and eventually promotes angiogenesis. Notably, the CEP-induced TLR1/2 signal was demonstrated to be independent of the VEGF pathway and have a proangiogenic effect comparable with VEGF in an *in vitro* study (26). This evidence implies that the TLR1/2 signal may act as an important alternative proangiogenic pathway independent of VEGF. Therefore, oxidative stress induced by chemotherapy may activate the TLR1/2 signal and promote angiogenesis, which results in the resistance to bevacizumab.

Another possible role of TLR1/2 is regulating T-helper 17 (Th17) polarization in the gut. Th17 is known to have a protective immune response against bacteria in the gut, and the TLR1/2 signal in dendritic cells contributes to the Th17 polarization at mucosal surface by inducing IL6 and IL23 (15). However, there is still controversy over whether Th17 has a tumor-promoting function or has a tumor-suppressing function in the tumor microenvironment. Recent studies have shown that Th17 infiltrate in the tumor microenvironment negatively influenced the prognosis of colorectal cancer patients (27, 28). The mechanism underlying this association is considered to be that Th17-related cytokines, such as IL17, IL21, and IL22, stimulate the STAT3 and NFκB pathways in cancer cells along with IL6 and TNFα, which leads to CRC cell growth and survival (29). Therefore, the TLR1/2 signal may also play a key role in inducing Th17 polarization in the tumor microenvironment.

Taken together, our findings suggest that the protumorigenic effects mediated by TLR1/2 were impaired in the individuals with variant T/G or G/G genotypes, which resulted in better responses against FOLFIRI plus bevacizumab. However, this study is hypothesis generating. As the biological role of TLR1 in the tumor microenvironment remains unclear, further functional studies are warranted to fully elucidate the underlying biological mechanisms of TLR1. This study has limitations such as sample size and the retrospective design of this study. Larger prospective studies are critical to validate our findings.

In conclusion, this is the first study to show the association of genetic variations in *TLR1*, *TLR2*, *TLR6*, and *TAK1* with clinical outcome of mCRC patients treated with chemotherapy. Our findings suggest that *TLR1* rs5743618 could serve as a predictive biomarker of clinical response to FOLFIRI plus bevacizumab. As the long-term goal of pharmacogenetic studies is to use genotype data to predict the efficacy of drugs and to individualize the treatment of patients, if our findings are validated prospectively, this SNP could be helpful in the selection of appropriate patients who would benefit from FOLFIRI plus bevacizumab in mCRC patients.

Disclosure of Potential Conflicts of Interest

S. Stintzing has received speakers bureau honoraria from Amgen, Merck KgaA, Roche, Bayer, and Sanofi and is a consultant/advisory board member for Amgen, Bayer, Merck KgaA, Sanofi, and Roche. V. Heinemann is a consultant/advisory board member for Merck, Amgen, SIRTEX, Sanofi, and Baxalta. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Okazaki, W. Zhang, A. Falcone, R.W. DePaolo, H.-J. Lenz

Development of methodology: S. Okazaki, W. Zhang, Y. Ning, H.-J. Lenz

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Okazaki, F. Loupakis, S. Stintzing, Y. Ning, Y. Sunakawa, S. Stremitzer, J.D. West, M. Schirripa, C. Cremolini, A. Falcone, V. Heinemann, H.-J. Lenz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Cao, W. Zhang, D. Yang, S. Stremitzer, V. Heinemann, H.-J. Lenz

Writing, review, and/or revision of the manuscript: S. Okazaki, S. Stintzing, S. Cao, D. Yang, S. Stremitzer, S. Matsusaka, M.D. Berger, A. Parekh, Y. Miyamoto, M. Suenaga, Marta Schirripa, A. Falcone, R.W DePaolo, H.-J. Lenz

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Sunakawa, S. Stremitzer, V. Heinemann, H.-J. Lenz

Other (provision of data from the FIRE-3 study): V. Heinemann

Grant Support

This work was supported by the NIH (P30CA014089-27S1 to H.-J. Lenz), the Gloria Borges Wunderglo Project, the Dhont Family Foundation (H.-J. Lenz), the Dave Butler Research Fund (H.-J. Lenz), and the Call to Cure Research Fund (H.-J. Lenz). S. Stintzing was supported by a postdoctoral fellowship from German Cancer Aid (the Mildred-Scheel Foundation).S. Stremitzer is the recipient of an Erwin Schrödinger fellowship from the Austrian Science Fund. M.D. Berger received a grant from the Swiss Cancer League (BIL KLS-3334-02-2014). Y. Miyamoto received a grant from Japan Society for the Promotion of Science (S2606).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 7, 2015; revised April 1, 2016; accepted April 22, 2016; published OnlineFirst May 11, 2016.

References

1. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:1626–34.
2. Van Cutsem E, Lenz HJ, Köhne CH, Heinemann V, Tejpar S, Melezínek I, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. J Clin Oncol 2015;33:692–700.
3. Ince WL, Jubb AM, Holden SN, Holmgren EB, Tobin P, Sridhar M, et al. Association of k-ras, b-raf, and p53 status with the treatment effect of bevacizumab. J Natl Cancer Inst 2005;97:981–9.
4. Kopetz S, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY, et al. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. J Clin Oncol 2010;28:453–9.
5. Ronzoni M, Manzoni M, Mariucci S, Loupakis F, Brugnatelli S, Bencardino K, et al. Circulating endothelial cells and endothelial progenitors as predictive markers of clinical response to bevacizumab-based first-line treatment in advanced colorectal cancer patients. Ann Oncol 2010;21:2382–9.
6. Xiao Y, Freeman GJ. The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy. Cancer Discov 2015;5:16–8.
7. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol 2010;10:131–44.
8. Sato Y, Goto Y, Narita N, Hoon DS. Cancer cells expressing toll-like receptors and the tumor microenvironment. Cancer Microenviron 2009;2Suppl 1:205–14.
9. Li TT, Ogino S, Qian ZR. Toll-like receptor signaling in colorectal cancer: carcinogenesis to cancer therapy. World J Gastroenterol 2014;20:17699–708.
10. Ridnour LA, Cheng RY, Switzer CH, Heinecke JL, Ambs S, Glynn S, et al. Molecular pathways: toll-like receptors in the tumor microenvironment–poor prognosis or new therapeutic opportunity. Clin Cancer Res 2013;19:1340–6.
11. Pradere JP, Dapito DH, Schwabe RF. The Yin and Yang of Toll-like receptors in cancer. Oncogene 2014;33:3485–95.
12. Jinushi M, Yagita H, Yoshiyama H, Tahara H. Putting the brakes on anticancer therapies: suppression of innate immune pathways by tumor-associated myeloid cells. Trends Mol Med 2013;19:536–45.
13. Kamdar K, Nguyen V, DePaolo RW. Toll-like receptor signaling and regulation of intestinal immunity. Virulence 2013;4:207–12.
14. DePaolo RW, Kamdar K, Khakpour S, Sugiura Y, Wang W, Jabri B. A specific role for TLR1 in protective T(H)17 immunity during mucosal infection. J Exp Med 2012;209:1437–44.
15. Sugiura Y, Kamdar K, Khakpour S, Young G, Karpus WJ, DePaolo RW. TLR1-induced chemokine production is critical for mucosal immunity against Yersinia enterocolitica. Mucosal Immunol 2013;6:1101–9.
16. Morgan ME, Koelink PJ, Zheng B, den Brok MH, van de Kant HJ, Verspaget HW, et al. Toll-like receptor 6 stimulation promotes T-helper 1 and 17 responses in gastrointestinal-associated lymphoid tissue and modulates murine experimental colitis. Mucosal Immunol 2014;7:1266–77.
17. Loupakis F, Cremolini C, Masi G, Lonardi S, Zagonel V, Salvatore L, et al. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. N Engl J Med 2014;371:1609–18.
18. Heinemann V, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. Lancet Oncol 2014;15:1065–75.
19. Ensemble [Internet]. Release 84. UK: EMBL-EBI and the Wellcome Trust Sanger Institute. c2000- [updated 2016 Mar] Available from: http://www.ensembl.org/index.html
20. Lee PH, Shatkay H. F-SNP: computationally predicted functional SNPs for disease association studies. Nucleic Acids Res 2008;36:D820–4.
21. Hawn TR, Misch EA, Dunstan SJ, Thwaites GE, Lan NT, Quy HT, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. Eur J Immunol 2007;37:2280–9.
22. Johnson CM, Lyle EA, Omueti KO, Stepensky VA, Yegin O, Alpsoy E, et al. Cutting edge: a common polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy. J Immunol 2007;178:7520–4.
23. Mittal D, Saccheri F, Vénéreau E, Pusterla T, Bianchi ME, Rescigno M. TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. EMBO J 2010;29:2242–52.
24. Cherfils-Vicini J, Platonova S, Gillard M, Laurans L, Validire P, Caliandro R, et al. Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance. J Clin Invest 2010;120:1285–97.
25. Saeed AM, Duffort S, Ivanov D, Wang H, Laird JM, Salomon RG, et al. The oxidative stress product carboxyethylpyrrole potentiates TLR2/TLR1 inflammatory signaling in macrophages. PLoS One 2014;9:e106421.
26. West XZ, Malinin NL, Merkulova AA, Tischenko M, Kerr BA, Borden EC, et al. Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. Nature 2010;467:972–6.
27. Fridman WH, Galon J, Pagès F, Tartour E, Sautès-Fridman C, Kroemer G. Prognostic and predictive impact of intra- and peritumoral immune infiltrates. Cancer Res 2011;71:5601–5.
28. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. Cancer Res 2011;71:1263–71.
29. De Simone V, Franzè E, Ronchetti G, Colantoni A, Fantini MC, Di Fusco D, et al. Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. Oncogene 2015;34:3493–503.

Published OnlineFirst May 11, 2016; DOI: 10.1158/1535-7163.MCT-15-0931

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Clinical Significance of *TLR1* I602S Polymorphism for Patients with Metastatic Colorectal Cancer Treated with FOLFIRI plus Bevacizumab

Satoshi Okazaki, Fotios Loupakis, Sebastian Stintzing, et al.

Mol Cancer Ther 2016;15:1740-1745. Published OnlineFirst May 11, 2016.