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Predictive value of *TLR7* polymorphism for cetuximab-based chemotherapy in patients with metastatic colorectal cancer

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The TLR7 and TLR9 signalings are implicated in the regulation of the immune system through type-I interferon induction. Preclinical studies have demonstrated the immunomodulatory and antitumor effects of TLR7 and TLR9 agonists in combination with cetuximab. We tested the hypothesis that genetic variations in TLR7 and TLR9 and their downstream molecules IRF5 and IRF7 were associated with outcomes in metastatic colorectal cancer (mCRC) patients receiving cetuximab-based chemotherapy. Six single nucleotide polymorphisms (SNPs) in *TLR7, TLR9, IRF5* and *IRF7* were tested for the association with RR, PFS, and OS in *KRAS*-wild type mCRC patients. Patients treated with FOLFIRI + cetuximab or FOLFIRI + bevacizumab in the FIRE-3 trial served as a discovery set (*FIRE3-Cet*, $n = 244$) or a control set (*FIRE3-Bev*, $n = 246$), respectively. Patients treated with FOLFOX or SOX + cetuximab in the JACCRO-CC05/06 trial served as a validation set (*JACCRO*, $n = 76$). Genomic DNA isolated from tumor tissue samples was analyzed by PCR-based direct sequencing. In the discovery cohort, patients with the *TLR7* rs3853839 G/G variant showed a trend toward longer PFS than those with any C variants (median 10.0 *vs*. 11.8 months, HR 1.39, $p = 0.092$). This preliminary association was confirmed in the validation cohort, and those with the G/G genotype showed a PFS benefit compared with others (univariate: 9.1 *vs*. 11.6 months, HR 2.04, $p = 0.005$, multivariate: HR 2.02, 95% CI: 1.14–3.55, $p = 0.015$). This association was not observed in the control cohort. Our findings suggest that *TLR7* rs3853839 predicts the outcome of cetuximab-based chemotherapy in mCRC patients.

Tumor Markers and Signatures

Key words: cetuximab, metastatic colorectal cancer, polymorphism, predictive marker, TLR7

Abbreviations: TLR: toll like receptor; IRF: interferon regulatory factor; mCRC: metastatic colorectal cancer; SNP: single nucleotide polymorphism; RR: response rate; PFS: progression-free survival; OS: overall survival; FOLFIRI: combination therapy of irinotecan, 5-fluorouracil and folinic acid; FOLFOX: combination therapy of oxaliplatin, 5-fluorouracil and folinic acid; SOX: combination therapy of oxaliplatin and S-1; PCR: polymerase chain reaction; HR: hazard ratio; CI: confidence interval; IFN: interferon; MyD88: myeloid differentiation factor 88; IRAK: IL-1R associated kinase; EGFR: epidermal growth factor receptor; IMO: immunomodulatory oligonucleotide; ADCC: antibody-dependent cellular cytotoxicity; RQ: relative quantification; RECIST: Response Evaluation Criteria in Solid Tumors; HWE: Hardy–Weinberg equilibrium; SLE: systemic lupus erythematosus

Additional Supporting Information may be found in the online version of this article.

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What's new?
For patients with colorectal cancer, a particular genetic variant of *TLR7* could mean the difference between life and death. Preclinical data have shown that *TLR7* enhances the tumor-killing ability of cetuximab; these authors tested six genetic variants of *TLR7* and downstream molecules to find which ones affected survival of metastatic colorectal cancer. They found that patients who carried a G/G variant at one locus survived longer when treated with cetuximab than patients with any C variant. This marker could be useful in predicting which patients would benefit most from cetuximab.

Introduction

Immune responses and inflammatory changes within the tumor microenvironment are widely known to play critical roles in carcinogenesis and cancer progression.[1,2] Recent studies have also shown that these biological responses influence the efficacies of anticancer therapies.[3–5]

Toll like receptors (TLRs) are primary innate immune sensors that recognize conserved microbial structures and host alarmins. Among the TLR family, TLR7 and TLR9 are endosomally localized and limitedly expressed on certain types of immune cells.[6] It is thought that the major functional role of TLR7 and TLR9 signals is to induce type-I interferon (IFN) secretion, including IFN-α and IFN-β, by activating the downstream MyD88/IRAK/IRF pathway (Supporting Information Fig. S1).[7] Therefore, activation of TLR7 and TLR9 can lead to stronger immune responses through the production of type-I IFN and can exert not only antiviral effects but also antitumor activities. Indeed, several studies have shown promising results that TLR7 and TLR9 agonists enhance the anti-tumor activity in combination with cytotoxic agents and target antibodies, including cetuximab, an anti-EGFR monoclonal antibody.

For example, the TLR9 agonist IMO (immunomodulatory oligonucleotide) has been demonstrated to increase the antitumor effects in combination with cetuximab compared with IMO or cetuximab monotherapies in a colon cancer xenograft model.[8] TLR7 agonist R-848 has also been shown to enhance the antitumor effects via antibody-dependent cellular cytotoxicity (ADCC) in cetuximab-coated colorectal cancer (CRC) cell lines.[9] These preclinical data suggest that the TLR7 and TLR9 signals contribute to the cetuximab efficacy.

Single nucleotide polymorphisms (SNPs) are germline genetic variations, some of which may alter the gene function of both immune and cancer cells. We herein tested the hypothesis that SNPs of TLR7 and TLR9 and those of their downstream molecules IRF5 and IRF7 will be associated with the clinical efficacy of cetuximab-based chemotherapy in metastatic CRC (mCRC) patients.

Methods

Patients and samples

Tissue samples examined in this study were obtained from mCRC patients who were enrolled in a prospective randomized phase III clinical trial [FIRE-3[10]] and phase II clinical trials [JACCRO-CC05[11] and JACCRO-CC06[12]]. The FIRE-3 trial comprised a total of 592 patients with *KRAS* exon 2 wild-type tumors, from centers in Germany and Austria, who were randomly assigned to receive either FOLFIRI + cetuximab ($n = 297$) or FOLFIRI + bevacizumab ($n = 295$) as first-line chemotherapy. In the JACCRO-CC05 and JACCRO-CC06 trials, a total of 76 patients with *KRAS* exon 2 wild-type mCRC were enrolled at centers in Japan to receive first-line FOLFOX or SOX plus cetuximab. To evaluate the association of target SNPs with clinical outcome for cetuximab, we used samples from the cetuximab arm of FIRE-3 as a discovery set (*FIRE3-Cet*, $n = 244$), those from the bevacizumab arm of FIRE-3 as a control set (*FIRE3-Bev*, $n = 246$), and those from the JACCRO-CC05 and JACCRO-CC06 trials as a validation set (*JACCRO* cohort, $n = 76$). Patients with *KRAS*-mutant type tumors or those without available samples were excluded from the study population. All patients signed an informed consent form before participating in the randomized trials, which included information regarding the use of their tumor tissue to explore relevant molecular parameters.

Selected polymorphisms and genotyping

Candidate SNPs in *TLR7, TLR9, IRF5* and *IRF7* with a minor allele frequency of $\geq$5% in both European and Japanese populations according to the Ensembl database[13] were selected for analyses. Among the candidate SNPs, we focused on six SNPs (Table 1) that previously had their biological significance reported in literature reviews or were considered potentially functional according to the F-SNP database.[14] Genomic DNA was extracted from formalin-fixed paraffin-embedded specimens using the QIAamp DNAeasy Kit (Qiagen, Valencia, CA). The primers used for polymerase chain reaction (PCR) analyses are listed in Supporting Information Table S1. 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.

mRNA expression assay

RNA was isolated from both normal epithelium and tumor tissue of patients enrolled in the JACCRO-CC05 and JACCRO-CC06 trials using histologic macrodissection techniques. cDNAs were synthesized using the miScript II RT Kit (Qiagen) and amplified by PCR using a fluorescence-based real-time detection method with the ABI Prism 7500 Real-

Table 1. SNPs genotyped in the present study

Gene	Gene location	SNP	SNP location	Base change	Allele frequency[1]	
					European	Japanese
TLR7	Xp22.3	rs3853839	3′-UTR	C/G	17/83 (%)	70/30 (%)
		rs179010	intron	T/C	32/68 (%)	33/67 (%)
TLR9	3p21.3	rs187084	promoter	G/A	43/57 (%)	51/49 (%)
		rs5743836	promoter	G/A	13/87 (%)	5/95 (%)
IRF5	7q32	rs2280714	3'-UTR	C/T	31/69 (%)	50/50 (%)
IRF7	11p15.5	rs12272434	intron	T/A	27/73 (%)	5/95 (%)

[1]Allele frequency, According to the Phase 1 of the 1000 Genomes Project for Europeans and Japanese, 3′-UTR: 3′-untranslated region.

Time PCR System. TaqMan Gene Expression Assays-on-demand (Assay ID Hs01933259_s1, Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) were used for PCR amplification. The mRNA expression level in each sample was normalized to the beta-actin content in each sample as an internal standard. Relative quantification (RQ) of mRNA expression was calculated by the ddCt method by SDSv1.2 with RQv1.0 software (Applied Biosystems). Each experiment was performed in duplicate.

Statistical analysis

To explore the predictive value of SNPs for cetuximab-based chemotherapy, the primary outcome measure was defined as progression-free survival (PFS), which was calculated from the date of randomization until the first observation of disease progression or death. If progression or death was not observed, PFS was assessed on the day of the last computed tomography scan. We also evaluated the association with response rate (RR) and overall survival (OS), which are defined as the percentage of patients experiencing complete responses or partial responses according to the response evaluation criteria in solid tumors (RECIST) and the period from randomization to the date of death or censored on the date of last contact if alive, respectively. The differences in baseline patient characteristics between the three cohorts were examined using χ^2 test or the Kruskal–Wallis 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. The power to detect an association between a SNP and PFS would be 80% when the minimum hazard ratio (HR) varied from 1.48 to 1.66 using a two-sided log-rank test at 0.05 significance level in the training cohort ($n = 244$, 206 PFS events). We assumed that the minor allele frequency ranged from 0.1 to 0.4, and the dominant model was considered. The HRs would be from 2.13 to 2.62 using the same test with 80% power with the same allele frequencies under the dominant model in the validation cohort ($n = 76$, 59 PFS events). The associations between polymorphisms and PFS and OS were investigated using Kaplan–Meier curves and log-rank test. A Cox proportional hazards regression model adjusted for baseline patient and disease characteristics and previous treatment fitted to evaluate the independent effects of SNPs on PFS and OS. The baseline demographic and clinical characteristics that remained significantly associated with endpoints in the multivariate analysis ($p < 0.1$) were included in the final model. For mRNA expression, RQ values were compared between groups using nonparametric tests. All analyses were performed with two-sided tests at a significance level of 0.05 by using the SAS 9.4 (SAS Institute, Cary, NC).

Results

The baseline characteristics of the three cohorts included in this study are given in Table 2. Compared with the *FIRE3-Cet* discovery cohort, the *JACCRO* validation cohort comprised better performance status, more patients with liver metastases, and fewer patients receiving adjuvant chemotherapy. There were no differences in characteristics between the discovery and control cohorts. The median PFS, OS, and follow-up periods were 9.9, 30.0 and 34.1 months, respectively in the *FIRE3-Cet*; 10.3, 24.7 and 39.9 months in the *FIRE3-Bev*; and 10.0, 33.9 and 24.7 months in the *JACCRO* cohort. Genotyping was successful in at least 90% of cases in each polymorphism analyzed. The allelic frequencies for all SNPs were within the probability limits of HWE ($p > 0.05$). High linkage disequilibrium was not found.

Association of SNPs with clinical outcome

Table 3 and Figure 1 show the association between *TLR7* rs3853839 and clinical outcome. In the *FIRE3-Cet* discovery cohort, patients with the *TLR7* rs3853839 G/G variant showed a borderline significant trend toward longer PFS [10.0 *vs.* 11.8 months, HR: 1.38, 95% confidence interval (CI): 0.94–2.04, $p = 0.092$] and OS (27.6 *vs.* 36.4 months, HR: 1.05, 95% CI: 0.70–1.58, $p = 0.095$) than those with any C variants. This preliminary association with PFS was confirmed in the *JACCRO* validation cohort, and patients with the G/G genotype showed a PFS benefit compared with those carrying any C variants, both in univariate and multivariate analyses (Univariate model, median 9.1 *vs.* 11.6 months, HR: 2.04, 95% CI: 1.18–3.55, $p = 0.006$, multivariate model, HR: 2.02, 95% CI: 1.14–3.55, $p = 0.015$). In the *FIRE3-Bev* control

Table 2. Baseline clinical characteristics of *FIRE3-Cet*, *FIRE3-Bev* and *JACCRO* cohorts

	FIRE3-Cet ($n = 244$) [*n* (%)]	*FIRE3-Bev* ($n = 246$) [*n* (%)]	*JACCRO* ($n = 76$) [*n* (%)]	*p* value[1]
Gender				
Male	170 (69.7)	161 (65.4)	44 (57.9)	0.16
Female	74 (30.3)	85 (34.6)	32 (42.1)	
Age				
Median (range)	64 (38–79)	65 (31–76)	63 (39–79)	0.39
≤65	129 (52.9)	129 (52.4)	44 (57.9)	0.69
>65	115 (47.1)	117 (47.6)	32 (42.1)	
Performance status				
ECOG 0	124 (50.8)	131 (53.3)	68 (89.5)	< 0.001
ECOG 1	120 (49.2)	115 (46.7)	8 (10.5)	
Primary tumor site				
Right	45 (18.4)	63 (25.6)	11 (14.5)	0.046
Left	194 (79.5)	177 (72.0)	63 (82.9)	
Unknown[2]	5 (2.0)	6 (2.4)	2 (2.6)	
Synchronous tumor				
Yes	182 (74.6)	185 (75.2)	58 (76.3)	0.99
No	59 (24.2)	60 (24.4)	18 (23.7)	
Unknown[2]	3 (1.2)	1 (0.4)		
Liver metastasis				
Yes	83 (34.0)	81 (32.9)	47 (61.8)	<0.001
No	161 (66.0)	165 (67.1)	29 (38.2)	
Number of metastatic sites				
<2	104 (42.6)	107 (43.5)	33 (43.4)	0.98
≥2	140 (57.4)	139 (56.5)	43 (56.6)	
Adjuvant chemotherapy				
Yes	50 (20.5)	45 (18.3)	6 (7.9)	0.040
No	192 (78.7)	201 (81.7)	70 (92.1)	
Unknown[2]	2 (0.8)			

[1]Based on χ^2 test, or the Kruskal–Wallis test whenever appropriate.
[2]Not included in the test.

cohort, this correlation of *TLR7* rs3853839 with PFS was not observed.

TLR9 rs187084 showed a significant association with PFS (13.3 *vs.* 9.6 month, $p = 0.021$) in univariate analysis, which remained marginally significant ($p = 0.093$) in multivariate analysis. However, these observations were not confirmed in the validation cohort (Supporting Information Table S2). For other SNPs, no associations with outcomes were observed (Supporting Information Table S3).

Subgroup analysis for TLR7 rs3853839 by primary tumor location

Because distinct molecular profiles based on the primary tumor location have been reported,[15] subgroup analysis based on the tumor location was performed. Primary tumor locations were grouped into right (cecum and ascending and transverse colon) and left (descending and sigmoid colon and rectum) sides. In the *FIRE3-Cet* cohort, *TLR7* rs3853839 showed an association with PFS also in patients with left-sided tumors, whereas no association between the two was observed in patients with right-sided tumors. In patients with left-sided tumors in the *JACCRO* cohort, this association was validated (Table 4).

Subgroup analysis for TLR7 polymorphisms by gender

Because the *TLR7* gene is encoded on the X chromosome (Xp22.3) and several studies reported gender-specific associations between *TLR7* polymorphisms and disease susceptibility,[16–18] we conducted a subgroup analysis by gender. There was no evidence for an association between *TLR7* polymorphisms and outcome in either male or female subgroups in the discovery cohort (Supporting Information Table S4).

Table 3. Association of *TLR7* rs3853839 with clinical outcome in three cohorts

		RR		PFS					OS				
				Univariate[1]			Multivariate[2]		Univariate[1]			Multivariate[2]	
Genotype	*n*	(%)	*p* value	Median month (95% CI)	HR (95% CI)	*p* value		*p* value	Median month (95% CI)	HR (95% CI)	*p* value		*p* value
Discovery cohort													
G/G	37	81%	0.16	11.8 (8.8, 15.0)	1 (Reference)	**0.073**	1 (Reference)	0.37	36.4 (29.8, 70.8)	1 (Reference)	**0.042**	1 (Reference)	0.19
G/C	20	56%		6.3 (3.9, 12.2)	1.90 (1.08, 3.35)		1.33 (0.71, 2.50)		16.0 (9.5, 45.0)	2.43 (1.20–4.92)		1.96 (0.90, 4.26)	
C/C	172	72%		10.3 (9.0, 12.2)	1.33 (0.90, 1.97)		1.32 (0.89, 1.96)		28.7 (23.8, 37.5)	1.47 (0.87, 2.50)		1.52 (0.89, 2.61)	
G/G	37	81%	0.30	11.8 (8.8, 15.0)	1 (Reference)	**0.092**	1 (Reference)	0.16	36.4 (29.8, 70.8)	1 (Reference)	**0.095**	1 (Reference)	0.10
Any C	192	70%		10.0 (8.7, 11.1)	1.38 (0.94, 2.04)		1.32 (0.90, 1.96)		27.6 (22.6, 37.1)	1.05 (0.70, 1.58)		1.56 (0.92, 2.66)	
Validation cohort													
G/G	39	81%	0.39	11.6 (9.4, 16.3)	1 (Reference)	**0.006**	1 (Reference)	**0.015**	36.2 (26.5, 42.8)	1 (Reference)	0.53	1 (Reference)	0.52
Any C	31	70%		9.1 (5.6, 13.8)	2.04 (1.18, 3.55)		2.02 (1.14, 3.55)		30.5 (19.0, 38.3)	1.25 (0.60, 2.60)		1.27 (0.61, 2.68)	
Control cohort													
G/G	30	67%	0.83	11.1 (8.8, 13.5)	1 (Reference)	0.69	1 (Reference)	0.53	30.3 (26.1, 40.0)	1 (Reference)	0.15	1 (Reference)	0.24
Any C	200	62%		10.2 (9.2, 11.7)	0.92 (0.61, 1.39)		0.88 (0.58, 1.33)		23.6 (20.6, 25.9)	1.45 (0.87, 2.38)		1.35 (0.81, 2.27)	

p values was based on Fisher's exact test for tumor response, log-rank test for PFS and OS in the univariate analysis (a) and Wald test for PFS and OS in the multivariate Cox regression model adjusted for sex, ECOG performance status, liver limited metastasis, primary tumor resection in the training and control cohort; adjusted for tumor site, number of metastases, lymph nodes involvement in the validation cohort (b). Correlations with $p < 0.1$ are marked with bold text style.

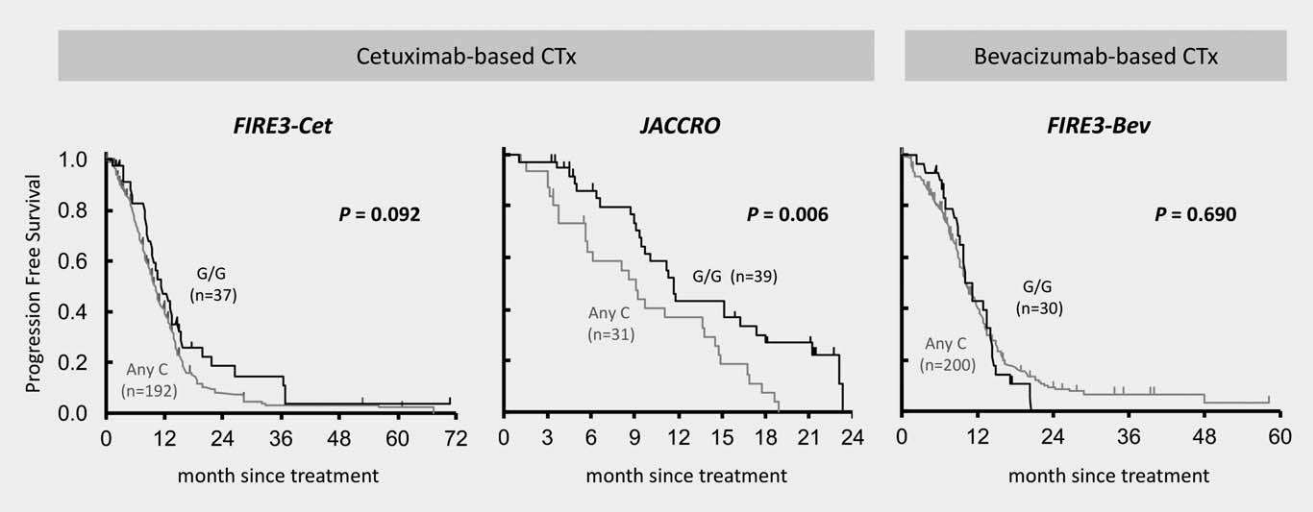

Figure 1. Progression free survival in each cohort. Kaplan–Meier estimates of progression free survival in patients with G/G and those with G/C or C/C genotypes for *TLR7* rs3853839.

TLR7 mRNA expression in cancer cells

To assess whether *TLR7* rs3853839 genotypes correlate with gene expression in cancer cells, we compared TLR7 expression levels between genotypes, as well as between normal epithelium and tumor tissue. Quantitative data on TLR7 expression were available from tumor tissues and adjacent normal epithelia of 55 patients from the *JACCRO* cohort. The TLR7 mRNA expression was not relatively increased in tumor tissues (median RQ = 0.99) compared with that in adjacent normal epithelia (median RQ = 1, Wilcoxon's signed rank test, $p = 0.13$, Fig. 2*a*). In addition, no association was observed between TLR7 mRNA levels and rs3853839 genotypes (Mann–Whitney *U* test, $p = 0.92$, Fig. 2*b*).

Discussion

In this study, we found an association between *TLR7* rs3853839 and PFS in patients who received cetuximab-based chemotherapy in two independent clinical trials, suggesting that this polymorphism predicts the efficacy of cetuximab.

TLR7 rs3853839 is located on the 3′-untranslated region of the *TLR7* gene, and the function is thought to possibly downregulate mRNA expression through affected miRNA binding activity.[19] Indeed, several studies demonstrated distinct mRNA expression levels and cytokine profiles between rs3853839 genotypes in patients with autoimmune diseases.[19–21] In an *in vivo* study using peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE), those carrying wild-type homozygous G/G genotype had a significantly higher TLR7 mRNA expression level in monocytes as well as increased IFNα secretion than those with other G/C and C/C genotypes.[21] In our study, patients with a G/G genotype showed a favorable PFS for cetuximab-based chemotherapy compared with other patients, indicating that TLR7 signals might contribute to enhancing the efficacy of cetuximab.

However, the exact role of TLR7 in the tumor microenvironment has not yet been defined. Because the major antitumor effect of cetuximab is via the blockade of the EGFR downstream pathway, one possible mechanism by which TLR7 enhances the cetuximab efficacy may be the direct effect on the EGFR pathway. Cross-talks between the EGFR and TLR signaling pathways have also been suggested,[22–24] and TLR9, for example, is demonstrated to directly impair EGFR and its downstream proteins.[8] Regarding TLR7, several kinds of cancer cells, including those causing pancreatic, prostate, breast, and lung cancer, highly express TLR7,[25] which affects signal pathways within a tumor cell and leads to tumor progression. In a pancreatic cancer model, elevated TLR7 expression levels in tumor cells have shown to lead to STAT3 activation and contribute to a loss of PTEN, p16, and cyclin D1, resulting in tumor growth and anti-apoptosis.[26] The mediation of anti-apoptotic effects of TLR7 by the induction of Bcl-2 in lung cancer cells have also been proposed.[27] However, to the best of our knowledge, the functional expression of TLR7 in CRC cells has not been fully investigated. In our study, we demonstrated that TLR7 was not highly expressed in CRC cells compared with normal colorectal epithelia. Additionally, no association was observed between TLR7 mRNA levels in tumor cells and SNP genotypes. These results imply that the direct impact of TLR7 signals in CRC cells may not be as important, however, they do not eliminate the possibility that this receptor is altered in a selected population of TLR7-expressing cells. Further studies at a subcellular level such as the analysis of mRNA expression using *in situ* hybridization are warranted.

Another possible mechanism arises via the contribution of TLR7 signals in immune cells to enhancing cetuximab-mediated ADCC. Antitumor effects by cetuximab are thought to not only block EGFR downstream pathways but also induce ADCC.[28] ADCC is a lytic reaction characterized by cetuximab coating EGFR on the surface of tumor cells and binding to the Fc-gamma receptor (FcγR) expressed on immune effector cells such as NK cells. In addition, ADCC

Table 4. Association of *TLR7* rs3853839 with clinical outcome in patients with left-sided CRC in three cohorts

		RR		PFS					OS				
				Univariate[1]			Multivariate[2]		Univariate[1]			Multivariate[2]	
Genotype		(%)	*p* value	Median month (95% CI)	HR (95% CI)	*p* value	HR (95% CI)	*p* value	Median month (95% CI)	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Discovery cohort													
G/G	31	80%	0.63	12.6 (9.4, 15.7)	1 (Reference)	0.10	1 (Reference)	0.38	40.0 (30.6, 70.8)	1 (Reference)	0.10	1 (Reference)	0.20
G/C	16	67%		7.0 (5.7, 12.2)	1.95 (1.03, 3.66)		1.42 (0.70, 2.91)		23.7 (6.4, 49.8)	2.39 (1.05, 5.44)		2.21 (0.88, 5.59)	
C/C	136	75%		10.6 (9.6, 12.9)	1.38 (0.89, 2.14)		1.36 (0.87, 2.11)		36.6 (23.9, 42.8)	1.57 (0.85, 2.91)		1.62 (0.87, 3.04)	
G/G	31	80%	0.64	12.6 (9.4, 15.7)	1 (Reference)	**0.094**	1 (Reference)	0.17	40.0 (30.6, 70.8)	1 (Reference)	0.10	1 (Reference)	0.10
Any C	152	74%		10.4 (9.5 (12.2)	1.43 (0.93, 2.21)		1.36 (0.88, 2.11)		36.6 (23.8, 42.8)	1.65 (0.90, 3.04)		1.67 (0.90, 3.11)	
Validation cohort													
G/G	32	79%	1.00	11.8 (10.0, 18.0)	1 (Reference)	**0.010**	1 (Reference)	**0.027**	42.8 (26.5, 42.8[1])	1 (Reference)	0.53	1 (Reference)	0.49
Any C	25	76%		9.7 (6.1, 14.5)	2.09 (1.12, 3.90)		2.03 (1.09, 3.79)		30.5 (20.0, 38.3[1])	1.29 (0.56, 2.98)		1.34 (0.58, 3.09)	
Control cohort													
G/G	23	67%	0.81	11.3 (8.8, 14.2)	1 (Reference)	0.74	1 (Reference)	0.64	35.0 (26.1, 42.9)	1 (Reference)	0.23	1 (Reference)	0.37
Any C	142	63%		10.4 (9.6, 12.0)	0.93 (0.58, 1.47)		0.90 (0.56, 1.43)		25.4 (21.5, 28.8)	1.44 (0.78, 2.63)		1.32 (0.72, 2.44)	

p values was based on Fisher's exact test for tumor response, log-rank test for PFS and OS in the univariate analysis (a) and Wald test for PFS and OS in the multivariate Cox regression model adjusted for sex, ECOG performance status, liver limited metastasis, primary tumor resection in the training and control cohort; adjusted for tumor site, number of metastases, lymph nodes involvement in the validation cohort (b). Correlations with $p < 0.1$ are marked with bold text style.

[1]Estimates were not reached yet.

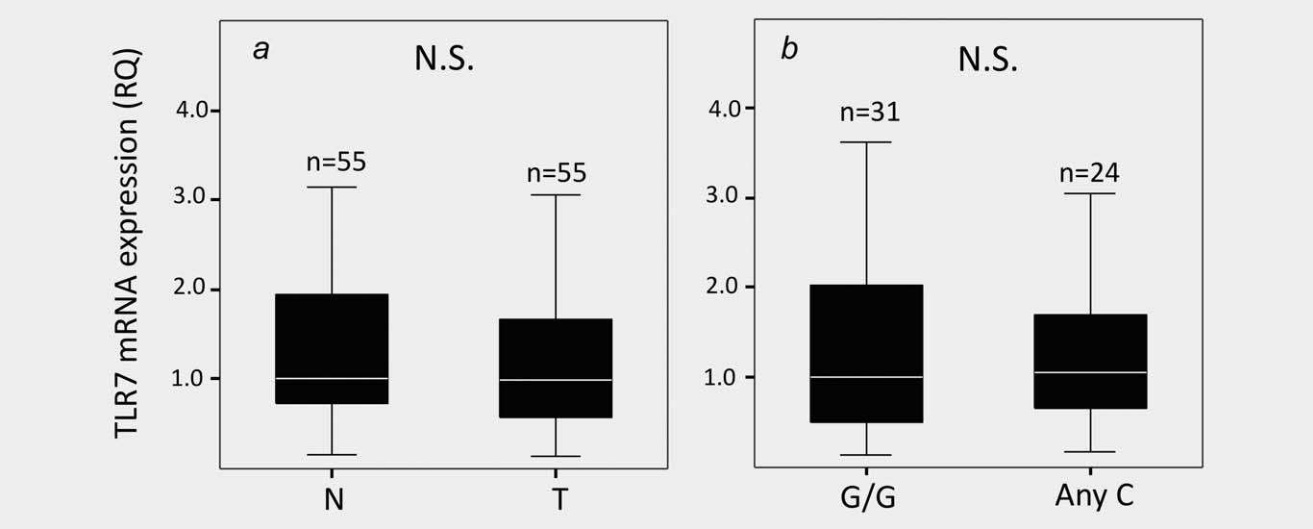

Figure 2. Comparison of TLR7 mRNA expression between *(a)* tumor tissue (T) and adjacent normal epithelium (N), and (*b*) between G/G genotype (G/G) and G/C + C/C (Any C) genotype. The white bar represents the median value.

reactions are known to be facilitated by several kinds of cytokines (*i.e.*, IFN-α, IFN-β, IL-2, IL-12 and IL-21) through activating immune effector cells.[29,30] Interestingly, unlike other TLRs, TLR7 has an expression pattern restricted to defined subtypes of immune cells and is mostly expressed on plasmocytoid dendritic cells (pDCs) and B cells.[6] Upon engagement of TLR7, pDC secretes large amounts of type I IFNs (IFN-α and IFN-β), which are crucial stimulators for NK cells.[31] Therefore, TLR7 signals in pDC could play a critical role in enhancing cetuximab-mediated ADCC. In a recent *in vivo* study,[9] cetuximab-mediated ADCC was shown to be enhanced when a monocyte was stimulated by the TLR7 agonist. The authors also demonstrated the high expression of FcγR in monocytes and suggested that cytokines secreted by monocytes upregulated the FcγR expression levels and led to more potent ADCC. Taken together, *TLR7* rs3853839 might influence the cytokine production from pDC, which resulted in the enhancement of cetuximab-mediated ADCC.

However, this hypothesis-generating study has some limitations that need to be considered, particularly the difference between discovery and validation cohorts. Patients in the *FIRE3-Cet* cohort were treated with a FOLFIRI backbone, whereas those in the *JACCRO* cohort received a FOLFOX or SOX backbone. In the most recent study by Gonzalez-Nicolini, cetuximab-mediated ADCC has shown to be reduced in the presence of irinotecan as opposed to no reduction in the combination with platinum drugs such as cisplatin and carboplatin.[32] This difference in chemotherapy partners for cetuximab could be responsible for the observation that the difference in PFS between genotypes by *TLR7* rs3853839 did not reach a statistical significance in the *FIRE3-Cet* cohort. The difference in allele frequencies of *TLR7* rs3853839 among mCRC patients of different ethnicities also impacts the outcomes of these cohorts. In addition, we could not correlate *TLR7* polymorphisms and serum cytokine levels that may affect the ADCC activity. Further functional studies are warranted to fully elucidate the underlying biological mechanisms of TLR7.

In conclusion, this is the first study to show the association of genetic variations in *TLR7, TLR9, IRF5 and IRF7* with clinical outcomes in mCRC patients treated with chemotherapy. Our findings suggest that *TLR7* rs3853839 could serve as a predictive marker for cetuximab-based chemotherapy in mCRC patients. Furthermore, our findings would provide an insight for the ongoing clinical studies testing the immunomodulatory and antitumor effects of TLR agonists in combination with conventional chemotherapeutic agents.

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