Potential Molecular Cross Talk Among CCR5 Pathway Predicts Regorafenib Responsiveness in Metastatic Colorectal Cancer Patients

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Abstract. *Background: Genetic variants in the CCL5/CCR5 pathway have been shown to predict regorafenib efficacy in patients with metastatic colorectal cancer (mCRC). This study investigated the biological role of CCL4 and CCL3 gene polymorphisms in patients with refractory mCRC treated using regorafenib. Patients and Methods: We analyzed the genomic DNA extracted from mCRC patients receiving regorafenib. Serum factor levels at baseline, day 21, and progressive disease (PD) were measured using ELISA. Results: Decreased CCL4 levels at day 21 or increased CCL3 levels at PD were associated with better clinical outcomes. In patients with any CCL5 rs2280789 G allele, CCL3 significantly increased between BL and day 21 compared with the A/A variant (72.7% vs. 23.1%, $p=0.006$), but CCL4 decreased (31.8% vs. 69.2%, $p=0.043$). Conclusion: Increased CCL3 and decreased CCL4 seen in specific genotypes may serve as potential biomarkers of regorafenib in mCRC patients.*

Regorafenib, an oral multi-kinase inhibitor, confers the benefit of longer survival in patients with refractory metastatic colorectal cancer (mCRC) (1, 2). Although in the retrospective exploratory study of the CORRECT trial, circulating DNA was identified as a potential prognostic marker for clinical outcomes (3), there have been few studies to confirm these results. In the previous translational research, baseline serum C–C motif chemokine ligand 5 (CCL5) levels and decreased serum vascular endothelial growth factor-A (VEGF-A) levels after initiation of treatment predicted the efficacy of regorafenib in refractory mCRC (4). CCL5 is characterized by late expression after T cell activation, and it localizes with tumor-infiltrating leukocytes (5, 6); it is also known as RANTES (regulated on activation normal T cell expressed and secreted). The candidate mechanism of action of CCL5/VEGF-A signaling is supported by findings from some preclinical studies (7, 8). CCL5 can promote endothelial progenitor cell (EPC) migration modulated by its receptor C–C motif chemokine receptor 5 (CCR5); CCL5/CCR5 signaling induces VEGF-A production *via* EPC migration (7). An *in vivo* study revealed that only CCL5 induced EPC migration in a dose-dependent manner, in the presence of C–C motif chemokine ligand 3 (CCL3) and 4 (CCL4), CCL5, and C–C motif chemokine receptor 5 (CCR5), whereas CCL3 and CCL4 lacked this activity (8). In another study, mRNA expression of CCL4 and CCL3 were up-regulated in cancer tissues compared to normal tissues, unlike that of CCL5 (9). These findings suggest that CCL5 plays an important role in VEGF-A production among the ligands of CCR5; the potential roles of CCL3 and CCL4 would therefore also be interesting to clarify.

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Table I. Candidate SNPs in *CCL3*, *CCL4*, *CCL5* and *CCR5* genes.

Genes rs number	Allele location	Base exchange	MAF [†] (CEU/JPN)	Function of polymorphism	Forward/Reverse primer (5'-3')
<i>CCL3</i> rs1130371	Synonymous Chromosome 17:36089191	G>A	0.31/0.31	Protein coding, splicing regulation, post translation, conserved	F: GCCTTTCCAGGATAGCCTTC R: CTTTGAGACGAGCAGCCAGT
<i>CCL4</i> rs1634517	Intron Chromosome 17:36105010	C>A	0.33/0.22	Tag SNP	F: CCGATTCTTAAACCGTGCT R: TTCCACCCACTGGATTAGC
<i>CCL5</i> rs2280789	Intron Chromosome 17:35879999	A>G	0.11/0.31	Transcriptional regulation	F: ATCTCCCCAACATGAGTCCA R: CCATATGTCCTGTTGTCCTTGA
<i>CCL5</i> rs3817655	Intron Chromosome 17:35872637	A>T	0.15/0.31	Transcriptional regulation	F: TGATATCGGGGTAGGCATA R: GGCGATTAAAATGCACACAA
<i>CCR5</i> rs1799988	5' prime UTR Chromosome 3:46370768	C>T	0.47/0.48	Transcriptional regulation	F: TGGGATGAGCAGAGAACAAA R: GGCGAAAAGAATCAGAGAACA

MAF, Minor allele frequency; CEU, Caucasian; JPN, Japanese; F, forward primer; R, reverse primer; Tag SNP, tagging SNP. [†]In Caucasians and Japanese from the Ensembl Genome Browser: <http://uswest.ensembl.org/index.html>.

Recently, we reported the results of a unique pharmacogenetic study where the genetic variant in the *CCL5/CCR5* pathway was shown to predict the efficacy of regorafenib in metastatic colorectal cancer patients (mCRC) (10). More specifically, *CCL5* rs2280789 G allele and *CCL5* rs3817655 T allele were associated with longer overall survival (OS) and severe skin toxicity owing to low VEGF-A production *via* endothelial progenitor cells (EPC). *CCL4* rs1634517 C allele and *CCL3* rs1130371 G allele correlated with longer progression-free survival (PFS) and overall survival (OS). However, to deepen the knowledge regarding the network of *CCR5* and the associated ligands in cancer treatment, more comprehensive data of the circulating factors are needed.

To investigate the biological role of *CCL4* and *CCL3* gene polymorphisms, we further checked the circulating protein levels of *CCR5* and its ligands *CCL3*, *CCL4*, and *CCL5*; we analyzed the relationship between SNPs and serial serum factor changes during the treatment and studied the correlation among the tested serum factor levels. To this end, the genetic functionality and biological role of *CCL4* and *CCL3* genotypes were assessed in the *CCR5* network in patients with refractory mCRC treated with regorafenib.

Patients and Methods

Study design and patients. The original pharmacogenetic study investigated genomic DNA extracted from 208 whole blood and 21 tissues of two independent cohorts comprising patients with refractory, histologically-confirmed mCRC; these included an evaluation cohort of 79 patients treated with regorafenib at the Cancer Institute Hospital in Japan and a validation cohort of 150 patients treated with regorafenib at Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy) and Istituto Oncologico Veneto (Padua, Italy) (10). In the current study, we evaluated the clinical impact of serum levels of *CCR5* and its ligands—*CCL3*, *CCL4*, and *CCL5*—in the *CCL5/CCR5* pathway in the evaluation cohort and

investigated the correlation among the SNPs and all serum factor levels. All patients met the eligibility criteria: history of previous standard chemotherapy including 5-fluorouracil, oxaliplatin, irinotecan, bevacizumab, and cetuximab or panitumumab for *KRAS* or *RAS* wild-type; presence of measurable or assessable lesions and signed informed consent. Adverse events were graded according to the Common Terminology Criteria for Adverse Events, version 4.0. Patients received 160 mg of regorafenib (Bayer, Leverkusen, Germany) once daily from day 1 to day 21 every 4 weeks. Doses were adjusted based on adverse events at a physician's discretion, following the manufacturer's recommendations. The study was compliant with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines. The analyses were approved by the Institutional Review Board of each institute and conducted at the University of Southern California/Norris Comprehensive Cancer Center and in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Selection of candidate single-nucleotide polymorphisms (SNPs). The candidate SNPs in *CCL3*, *CCL4*, *CCL5*, and *CCR5* genes were selected depending on the following criteria: i) biological significance of SNPs according to published literature review; ii) tagging SNPs selected using the HapMap genotype data with r^2 threshold=0.8 (<https://snpinfo.niehs.nih.gov/snpinfo/guide.html>) or iii) minor allele frequency with a cut-off of $\geq 10\%$ in Caucasians and East Asians (in the Ensembl Genome Browser: <http://uswest.ensembl.org/index.html>). Significance of functionality of SNPs was predicted based on the functional single-nucleotide polymorphism (F-SNP) database (11) (Table I). The SNP data analyzed in our previous study (10) were used in the analysis of the current study to explore their functionality.

DNA extraction and genotyping. In our original study, genomic DNA was extracted from peripheral whole blood or tissue in patients of both cohorts, using the QIAmp Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol (www.qiagen.com). The only exception was formalin-fixed paraffin-embedded tissues of 21 patients from the evaluation cohort. The candidate SNPs were tested with polymerase chain reaction (PCR)-based direct DNA sequence analysis using the ABI 3100A Capillary

Genetic Analyzer and Sequencing Scanner v1.0 (Applied Biosystems, Life Technologies, Grand Island, NY, USA). The forward and reverse primers used for amplification of extracted DNA are listed in Table I. For quality control purposes, a randomly selected 10% of the samples were analyzed by direct DNA sequencing for each SNP, resulting in a genotype concordance rate of 99% or more. The investigators analyzing the SNPs were blinded to the clinical data.

Analysis of serum factor levels. Blood samples were obtained from 58 Japanese patients enrolled in the evaluation cohort at baseline (BL) before the first dose of regorafenib, at day 21 in the first cycle, and at progressive disease (PD). Separated serum was stored at -80°C. The levels of serum factors were measured using Quantikine ELISA kits (R&D Systems). The data regarding VEGF-A and CCL5 were previously reported in the original study (10). In the current study, serum CCL3, CCL4, and CCR5 levels were additionally tested for further analysis of the CCL5/CCR5 signaling network; the relationship between serum factor levels and genotypes was also evaluated. However, the analysis of CCL3, CCL4, and CCR5 was performed in 35 patients, and the other 23 patients were excluded from the analysis owing to the lack of samples.

Statistical analysis. The primary endpoint of the current study was progression-free survival (PFS), and the secondary endpoints were overall survival (OS) and disease control rate (DCR). PFS was defined as the interval between the date of starting treatment and the date of confirmed disease progression or death. The data of patients without disease progression or death were censored at the date of the last follow-up. OS was calculated from the date of starting treatment until the date of death from any cause. In patients who discontinued follow-up, data were censored at the date of the last follow-up. DCR was defined as the proportion of patients who achieved a complete response (CR), partial response (PR), or stable disease (SD) according to the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1. PD was also based on assessment of radiologic images using the RECIST v1.1 and defined as $\geq 20\%$ increase in the sum of diameters of target lesions taking as reference the smallest sum on this study. The tumor shrinkage (TS) was defined as a reduction of the sum of the longest diameters of target by 0% or more when compared with BL. The Chi-square test or Wilcoxon test was used to examine the differences in BL patient characteristics between the two cohorts. The allelic distribution of polymorphisms was tested for deviation from the Hardy–Weinberg equilibrium using Fisher’s exact test, while linkage disequilibrium among SNPs was assessed using D' and r^2 values, and haplotype frequencies of the genes were inferred using HaploView version 4.2 (<http://www.broad.mit.edu/mpg/haploview>). Fisher’s exact test was applied to examine the associations between SNPs and DCR or toxicity. Kaplan–Meier analysis and the log-rank test were performed to evaluate the association between candidate SNPs and PFS or OS. The BL demographic and clinical characteristics that remained statistically significantly associated with PFS and OS in multivariable analyses were included in the final models to reevaluate the independent effect of candidate SNPs. As the true modes for candidate SNPs were not yet established, the analyses used a codominant, dominant, or recessive genetic model as appropriate. Differences in serum factor levels at any point and those between BL and day 21 or PD were analyzed using Student’s unpaired *t*-test for the mean values or the Mann–Whitney *U*-test for

Table II. Baseline patients and tumor characteristics.

Cohort	Evaluation cohort (N=79)	
	N	%
Gender		
Male	37	46.8
Female	42	53.2
Age (year)		
Median (range)	62 (34-83)	
≤ 65	48	60.8
> 65	31	39.2
ECOG Performance status		
ECOG 0	44	55.7
ECOG 1-2	35	44.3
Primary tumor site		
Right	23	29.1
Left	56	70.9
Liver metastasis		
Yes	53	67.1
No	26	32.9
Lung metastasis		
Yes	46	58.2
No	33	41.8
Lymph node metastasis		
Yes	40	50.6
No	39	49.4
Peritoneal metastasis		
Yes	20	25.3
No	59	74.7
Number of metastases		
< 2	24	30.4
≥ 2	55	69.6
Primary tumor resected		
Yes	69	87.3
No	10	12.7
Adjuvant history		
Yes	26	32.9
No	53	67.1

the median values. Spearman’s rank correlation coefficient was calculated for correlations among serum factors and Pearson’s correlation coefficient was used for data with normal distribution. All analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC, USA). All tests were two-sided at a significance level of 0.05.

Results

Patient and tumor BL characteristics. The median follow-up time was 15.3 months and median PFS and OS were 2.0 and 8.7 months, respectively. The BL characteristics are summarized in Table II. Median age was 62 years and 35 out of 79 patients (44.3%) had PS 1 or 2. All candidate SNPs were within the Hardy–Weinberg equilibrium.

Association between serum factor and clinical outcomes. Association between serum factors and clinical outcomes are

shown in Table III and Table IV. The median serum CCL3 change between BL and day 21 was higher in patients with TS compared to those without TS (13.0 vs. -4.1 pg/ml, $p=0.045$), and the pattern of increased CCL3 levels at PD was associated with longer OS than that of decreased CCL3 levels (12.6 vs. 4.8 months, $p=0.003$). In contrast, patients with TS tended to have lower changes in serum CCL4 levels from BL to day 21 (-13.2 vs. 7.6 pg/ml, $p=0.054$) and PD (-15.4 vs. -2.5 pg/ml, $p=0.064$) than those without TS. In addition, patients with the pattern of decreased CCL4 levels at day 21 had a trend toward longer PFS compared to those with increased CCL4 levels (2.7 vs. 2.0 months, $p=0.09$). Concerning CCR5, patients with TS had lower mean changes in serum CCR5 levels between BL and day 21 compared with those without TS (-0.1 vs. 0.2 pg/ml, $p=0.041$) (Table III). There were no significant differences in DC and TS in BL serum factor levels, whereas serum CCL3 and CCR5 levels at day 21 were higher in patients with TS compared to those without TS (Table IV).

Correlations among serum factors. Spearman's rank correlation coefficient between different serum factors during treatment are shown in Table V. Moderate correlations were observed in some pairs at day 21 including CCL3-CCL5 and CCL4-CCL5 and at PD including CCL4-CCR5, CCL4-VEGF, and CCL5-VEGF. A moderate correlation was only found in a pair of CCL3-CCL5 for changes between BL and day 21. However, there were no significant pairs at BL.

Associations between SNPs and serum factor levels. In our previous study, we demonstrated that the *CCL3* rs1130371 G/G variant and *CCL4* rs1634517 C/C variant are associated with better clinical outcome and we analyzed the correlation between SNPs and CCL5 or VEGF (10). Briefly, patients with *CCL3* rs1130371 G/G variant and *CCL4* rs1634517 C/C variant tended to have increased CCL5 levels at day 21, whereas those with the *CCR5* rs1799988 variant mainly had decreased CCL5 levels. In this study, the serum factors CCL3, CCL4 and CCR5 were analyzed for association with SNPs, and the results are shown in Table VI. Patients with the G/G variant in *CCL3* rs1130371 had increased CCL3 levels (median, 9.5 vs. -4.6 pg/ml, $p=0.041$) at day 21 compared to those with any A allele. In contrast, the *CCL4* rs1634517 C/C variant was associated with higher CCR5 changes between BL and day 21 compared to any A allele in *CCL4* rs1634517 (median, 0.17 vs. -0.035, $p=0.042$) but did not correlate with CCL4 levels. In patients with any *CCL5* rs2280789 G allele, CCL3 tended to increase between BL and day 21 (72.7%, $p=0.006$), but CCL4 conversely decreased (31.8%, $p=0.043$). Similar results were obtained in *CCL5* rs3817655. In partial correlation analysis with CCL5 as controlled variable, negative correlation between CCL3 and CCL4 for change between BL and day 21 were observed in the *CCL3* rs1130371 G/G variant ($r=-0.542$, $p=0.030$)

Table III. Serum factor levels and clinical outcomes.

Factor/ change	DC	N	Mean±SD (pg/ml)	p-Value*	Median (pg/ml)	p-Value*	TS	N	Mean±SD (pg/ml)	p-Value*	Median (pg/ml)	p-Value*	Change pattern**	N	PFS (months)	p-Value	OS (months)	p-Value
CCL3 BL-day 21	N	17	-4.6	18.7	0.92	0.0	0.32	N	24	-6.1	19.7	0.96	DEC	17	2.3	0.27	6.7	0.62
	Y	18	-6.0	54.4		3.2		Y	10	-5.4	71.9		INC	20	3.7		12.0	
CCL3 BL-PD	N	17	4.7	35.7	0.57	2.4	0.74	N	24	4.1	31.4	0.43	DEC	14	2.3	0.20	4.8	0.003
	Y	18	-3.2	45.4		5.6		Y	10	-8.5	59.8		INC	23	3.2		12.6	
CCL4 BL-day 21	N	17	-8.9	40.9	0.55	6.4	0.44	N	24	-4.9	48.2	0.07	DEC	20	2.7	0.09	7.8	0.78
	Y	18	-19.2	58.1		-8.8		Y	10	-39.3	50.1		INC	17	2.0		10.3	
CCL4 BL-PD	N	17	10.8	50.5	0.076	-6.4	0.35	N	24	8.3	50.5	0.02	DEC	23	2.7	0.52	10.8	0.58
	Y	18	-23.5	59.6		-13.0		Y	10	-41.4	62.3		INC	14	2.5		5.9	
CCR5 BL-day 21	N	17	0.1	0.3	0.43	0.12	0.70	N	24	0.2	0.3	0.041	DEC	15	2.7	0.84	10.3	0.73
	Y	18	0.1	0.3		0.03		Y	10	-0.1	0.4		INC	22	2.3		6.3	
CCR5 BL-PD	N	17	0.1	0.4	0.33	0.07	0.80	N	24	0.1	0.4	0.53	DEC	16	2.5	0.72	11.8	0.82
	Y	18	0.02	0.3		0.11		Y	10	0.01	0.4		INC	21	2.7		6.7	

BL, Baseline; PD, progressive disease; DC, disease control; TS, tumor shrinkage; DEC, decreased; INC, increased; SD, standard deviation; N, no; Y, yes. *Differences in the mean and median cytokine levels were tested using Student's unpaired *t*-test and Mann-Whitney *U*-test, respectively. **Pattern of changes in serum factor levels between BL and day 21 or PD. *p*-Values <0.05 are shown in bold.

Table IV. Serum factor levels and clinical outcomes.

Factor	DC	N	Mean±SD (pg/ml)		<i>p</i> -Value*	Median <i>p</i> -Value* (pg/ml)		TS	N	Mean±SD (pg/ml)		<i>p</i> -Value*	Median (pg/ml)	<i>p</i> -Value*
CCL3 BL	N	17	28.6	22.2	0.61	22.9	0.82	N	24	29.3	21.9	0.40	22.9	0.99
	Y	18	35.8	53.2		21.8		Y	10	42.7	69.3		21.8	
CCL3 day 21	N	17	24.0	11.4	0.24	20.8	0.24	N	24	23.2	11.6	0.007	20.8	0.009
	Y	18	29.8	16.5		28.4		Y	10	37.3	17.2		34.8	
CCL3 PD	N	17	33.2	37.4	0.95	19.0	0.25	N	24	33.4	32.7	0.94	26.4	0.35
	Y	18	32.6	17.7		33.4		Y	10	34.2	16.6		28.6	
CCL4 BL	N	17	49.7	29.2	0.30	39.4	0.96	N	24	50.5	35.2	0.27	39.4	0.58
	Y	18	68.2	68.0		41.6		Y	10	81.7	81.4		43.8	
CCL4 day 21	N	17	40.8	30.6	0.59	29.3	0.95	N	24	45.6	44.6	0.85	32.2	0.75
	Y	18	49.0	53.8		32.9		Y	10	42.4	45.4		29.9	
CCL4 PD	N	17	60.6	52.6	0.40	54.8	0.08	N	24	58.8	50.8	0.39	47.0	0.092
	Y	18	44.8	57.6		14.7		Y	10	40.4	66.7		13.1	
CCR5 BL	N	17	0.59	0.50	0.21	0.45	0.18	N	24	0.54	0.43	0.55	0.46	0.24
	Y	18	0.42	0.21		0.41		Y	10	0.45	0.26		0.39	
CCR5 day 21	N	17	0.73	0.62	0.14	0.58	0.16	N	24	0.71	0.53	0.066	0.55	0.008
	Y	18	0.48	0.23		0.45		Y	10	0.38	0.13		0.37	
CCR5 PD	N	17	0.74	0.82	0.18	0.68	0.58	N	24	0.65	0.72	0.45	0.49	0.68
	Y	18	0.45	0.26		0.44		Y	10	0.47	0.28		0.40	

BL, Baseline; PD, progressive disease; DC, disease control; TS, tumor shrinkage; SD, standard deviation; N, no; Y, yes. *Differences in the mean and median serum factor levels were tested using Student's unpaired *t*-test and Mann-Whitney *U*-test, respectively. *p*-Values <0.05 are shown in bold.

Table V. Correlations among serum factors.

Pairs of factors	CCL3–CCL4	CCL3–CCL5	CCL3–CCR5	CCL4–CCL5	CCL4–CCR5	CCL5–CCR5	CCL3–VEGF	CCL4–VEGF	CCL5–VEGF	CCR5–VEGF
Points										
BL	$\rho=-0.242$	$\rho=-0.069$	$\rho=0.084$	$\rho=0.133$	$\rho=-0.135$	$\rho=0.275$	$\rho=-0.048$	$\rho=-0.133$	$\rho=0.033$	$\rho=-0.118$
Day 21	$\rho=-0.124$	$\rho=-0.337^*$	$\rho=0.168$	$\rho=0.351^*$	$\rho=0.107$	$\rho=0.191$	$\rho=-0.088$	$\rho=0.241$	$\rho=0.238$	$\rho=-0.035$
PD	$\rho=0.117$	$\rho=0.058$	$\rho=-0.025$	$\rho=0.352$	$\rho=0.411^*$	$\rho=-0.131$	$\rho=0.146$	$\rho=0.381^*$	$\rho=0.373^*$	$\rho=0.077$
Changes										
BL–day21	$\rho=-0.260$	$\rho=0.029$	$\rho=0.220$	$\rho=-0.205$	$r=-0.171$	$\rho=0.065$	$\rho=0.010$	$\rho=-0.017$	$\rho=0.163$	$\rho=0.262$
BL–PD	$\rho=0.153$	$\rho=0.377^*$	$\rho=0.105$	$\rho=0.039$	$\rho=0.011$	$\rho=-0.068$	$\rho=-0.167$	$\rho=-0.013$	$\rho=0.105$	$\rho=0.111$

BL, Baseline; PD, progressive disease. Correlations were estimated using Spearman's rank correlation coefficient (ρ) or Pearson's correlation coefficient (r) according to data distribution. **p*-Values are <0.05.

and *CCL4* rs1634517 C/C variant ($r=-0.475$, $p=0.034$), whereas no correlation was observed in any A allele in either *CCL3* or *CCL4* SNPs ($\rho=-0.173$, $p=0.492$; $\rho=-0.099$, $p=0.736$). In addition, *CCL5* SNPs showed no significant correlation between *CCL3* and *CCL4*.

Discussion

In this study, we aimed to evaluate the *CCL3*, *CCL4*, *CCL5*, and *CCR5* network more comprehensively by further analyzing serum *CCL3*, *CCL4*, and *CCR5* levels in addition to *CCL5* and VEGF-A. We demonstrate the candidate

biological role of *CCL3* and *CCL4* SNPs, based on the analysis of circulating protein levels in mCRC patients receiving regorafenib.

The *CCL5/CCR5* axis is involved in the immune microenvironment and promotes tumor progression (12, 13). *CCL3* and *CCL4* are produced particularly by macrophages, dendritic cells, and lymphocytes that activate *CCR5* downstream (14, 15). In a gene expression analysis study for colorectal cancer, *CCL4* was the most strongly expressed in cancer tissues compared to those expressed in non-neoplastic mucosal tissues. *CCL3* was also highly expressed in cancer tissues. In contrast, *CCL5* was widely expressed not only in

Table VI. Associations between SNPs and serum factor levels.

SNP	Variant	Median CCL3 levels (pg/ml)				Median CCL4 levels (pg/ml)				Median CCR5 levels (pg/ml)			
		BL	Day 21	BL-day 21	INC (%)*	BL	Day 21	BL-day 21	INC (%)*	BL	Day 21	BL-day 21	INC (%)*
<i>CCL3</i> rs1130371	G/G	16.7	30.1	9.5	70.6	67.7	48.4	-17.5	41.2	0.40	0.49	0.17	64.7
	Any A	22.9	20.8	-4.6	38.9	35.3	26.7	2.2	50.0	0.47	0.40	0.03	55.6
	<i>p</i> -Value	0.27	0.49	0.041	0.092	0.077	0.060	0.43	0.74	0.41	0.14	0.083	0.73
<i>CCL4</i> rs1634517	C/C	20.8	30.1	5.6	57.1	39.4	35.5	-8.8	42.9	0.48	0.49	0.17	71.4
	Any A	19.2	20.7	-0.12	50.0	44.1	26.9	1.0	50.0	0.40	0.40	-0.035	42.9
	<i>p</i> -Value	0.74	0.27	0.14	0.74	0.61	0.45	0.53	0.74	0.35	0.12	0.042	0.16
<i>CCL5</i> rs2280789	A/A	22.9	20.8	-7.1	23.1	34.2	43.8	12.9	69.2	0.45	0.45	0.12	69.2
	Any G	16.4	31.1	7.81	72.7	59.1	27.8	-19.7	31.8	0.42	0.45	0.03	54.5
	<i>p</i> -Value	0.097	0.35	0.034	0.006	0.15	0.53	0.017	0.043	0.99	0.67	0.57	0.49
<i>CCL5</i> rs3817655	A/A	22.88	20.83	-7.11	23.1	34.24	43.82	12.88	69.2	0.45	0.45	0.12	69.2
	Any T	16.4	31.1	7.81	72.7	59.1	27.8	-19.7	31.8	0.42	0.45	0.025	54.5
	<i>p</i> -Value	0.097	0.35	0.034	0.006	0.15	0.53	0.017	0.043	0.99	0.67	0.57	0.49
<i>CCR5</i> rs1799988	Any C	20.8	20.8	1.62	52.0	44.1	35.1	-4.4	48.0	0.43	0.45	0.03	60.0
	T/T	22.7	26.4	7.5	60.0	37.47	17.5	-4.4	40.0	0.44	0.59	0.085	60.0
	<i>p</i> -Value	0.83	0.61	0.47	0.72	0.69	0.19	0.93	0.72	0.84	0.16	0.58	1.0

BL, Baseline; PD, progressive disease; INC, increased. *Increased at day 21 from BL. *p*-Values <0.05 are shown in bold. Differences in the median serum factor levels were tested using Mann-Whitney *U*-test.

cancer tissue but also in non-neoplastic mucosal tissues (9) and was reported to play an important role in activating CCL5 signaling via CCR5 (7, 16, 17).

In the analysis of serum factor levels in our study, we could confirm the relationship between different CCL3 and CCL4 changes and efficacy after the administration of regorafenib. Furthermore, we examined the changes in serum factors for each SNP variant, and the *CCL3* rs1130371 G/G and *CCL4* rs1634517 C/C variants were associated with longer PFS and OS. CCL3 and CCL4 are known to be specifically expressed in cancer tissue, but lack the ability to induce EPC migration. In contrast, CCL5 expression is not limited to cancer tissue and induces EPC migration where CCR5 and the ligands are expressed (8, 9). In this study, serum CCL3 levels increased in the G/G variant of *CCL3* rs1130371, which suggests that the G/G variant serves as a prognostic factor. For CCL4, there was no significant correlation between serum levels and the SNP. The most interesting finding was the additional analysis of *CCL5* SNPs, which has been previously reported to be associated with serum CCL5 and VEGF-A levels (10). Namely, an increase in serum CCL3 and a decrease in CCL4 after regorafenib administration were observed only in the *CCL5* rs2280789 G/G variant and *CCL5* rs3817655 T/T variant, suggesting that these genotypes may be prognostic factors.

Concerning drug-related toxicities, we had previously revealed that *CCL5* SNPs act as prognostic markers of severe hand-foot skin reaction (HFSR), based on the capacity of VEGF-A production, which is an important factor in wound

healing (18, 19). In this study, there was no significant correlation between CCL3, CCL4, CCR5 and HFSR. Therefore, CCL5-CCR5-VEGF-A signaling seems to play an important role in the development of severe HFSR in the CCR5 network. Thus, given that most CCL5 is derived from the host (20, 21), CCL5 genotyping remains a predictor of severe HFSR due to regorafenib.

Our findings can be summarized in three key points: (i) Both increased CCL3 and decreased CCL4 appear to be involved in antitumor effects; (ii) The prognostic value of *CCL3* and *CCL4* SNPs shown in our previous study (10) depends on the variation in CCL3 and CCL4 responses to regorafenib; that is, negative correlations between CCL3 and CCL4 were observed in both the *CCL3* rs1130371 G/G variant and *CCL4* rs1634517 C/C variant; and (iii) *CCL5* SNPs may impact the opposing reactions of CCL3 and CCL4. However, our study has some limitations: the retrospective study design, lack of preclinical data regarding the function of SNPs and limited number of serum factors testing and samples. In contrast, we demonstrated the relationship between serum factor levels and SNPs in CCL5/CCR5 network more systematically in patients receiving regorafenib, and the mechanism should be clarified through further research.

In conclusion, both increased CCL3 and decreased CCL4 are considered to be one of the mechanisms of action of regorafenib efficacy, and differences between CCL3 and CCL4 changes were found in a specific genotype of *CCL5* SNPs. This suggests an alternative mechanism of action in

the CCR5 network besides CCL5-VEGF-A signaling *via* EPC in mCRC patients receiving regorafenib. Furthermore, *CCL3* and *CCL4* SNPs remain prognostic markers for the efficacy of regorafenib. Development of new therapeutic targeting molecules in this network is expected in the future.

Conflicts of Interest

The Authors have no conflicts of interest to declare in this work.

Authors' Contributions

Conceptualization: MS (Mitsukuni Suenaga), WZ, TM, HL. Data curation: MS (Mitsukuni Suenaga), TM. Formal analysis: SC. Funding acquisition: MS (Mitsukuni Suenaga), HL. Investigation: MS (Mitsukuni Suenaga), WZ. Methodology: MS (Mitsukuni Suenaga), WZ, TM. Project administration: MS (Marta Schirripa), SO, MB, YM. Resources: MS (Mitsukuni Suenaga), WZ, TY. Software: SC, AB. Supervision: HL. Writing—original draft: MS (Mitsukuni Suenaga). Writing—review and editing: All Authors.

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