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Clinical Significance of Chemokine Receptor CXCR4 and CCR7 mRNA Expression in Patients With Colorectal Cancer

SHINSUKE NAGASAWA[1,2*], KAZUHITO TSUCHIDA[1,2*], MANABU SHIOZAWA[1], YUKIHIKO HIROSHIMA[3], YAYOI KIMURA[4], ITARU HASHIMOTO[1], HAYATO WATANABE[1], KAZUKI KANO[1], MASAKATSU NUMATA[2], TORU AOYAMA[2], SUMITO SATO[2], TAKANOBU YAMADA[1], HIROSHI TAMAGAWA[2], NAOTO YAMAMOTO[1], TAKASHI OGATA[1], SOICHIRO MORINAGA[1], NORIO YUKAWA[2], YASUSHI RINO[2], MUNETAKA MASUDA[2], HIROSHI SAEKI[5], YOHEI MIYAGI[3] and TAKASHI OSHIMA[1]

[1]*Department of Gastrointestinal Surgery, Kanagawa Cancer Center, Yokohama, Japan;*
[2]*Department of Surgery, Yokohama City University, Yokohama, Japan;*
[3]*Kanagawa Cancer Center Research Institute, Yokohama, Japan;*
[4]*Advanced Medical Research Center, Yokohama City University, Yokohama, Japan;*
[5]*Department of Gastrointestinal Surgery, Gunma University, Maebashi, Japan*

Abstract. *Background/Aim: The chemokine receptors C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7) play an important role in the invasion and metastasis of cancer. This study investigated the relationship between relative expression of CXCR4 and CCR7 mRNA, clinicopathological factors, and outcomes in patients with colorectal cancer (CRC). Patients and Methods: We studied 202 patients who underwent surgery for CRC. The expression levels of CXCR4 and CCR7 mRNA in cancerous tissue were measured using quantitative real-time reverse-transcriptase polymerase chain reaction. Results: High CCR7 mRNA expression levels in CRC tissues were positively associated with tumour size and were more frequently associated with cancer of the rectum than of the colon. Moreover, outcomes were significantly poorer in patients with high CCR7 mRNA expression than in those with low expression. On multivariate Cox regression analysis, a higher CCR7 mRNA expression level was a significant independent predictor of poorer overall survival in patients with CRC. Conclusion: Overexpression of CCR7 mRNA may be a useful independent prognostic factor in patients with CRC.*

*These Authors contributed equally to this study.

Correspondence to: Dr. Takashi Oshima, Department of Gastrointestinal Surgery, Kanagawa Cancer Center, 2-3-2 Nakao, Asahi-ku, Yokohama, Kanagawa 241-8515, Japan. Tel: +81 452502222, Fax: +81 452502202, e-mail: oshimat@kcch.jp

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In 2020, colorectal cancer (CRC) was the third most commonly diagnosed cancer worldwide and accounted for 10% of all cancer, with 1,414,259 new cases reported (1). Moreover, in the same year, CRC was the second leading cause of cancer-related mortality, accounting for 9.4% of all cancer-related deaths, with 935,173 deaths recorded (1).

Most cases of CRC are sporadic and not hereditary. In addition, CRC is due to genetic instability and multiple somatic mutations, and about 80% of CRC cases are diagnosed at an early stage and are curable. Surgery is the first choice of treatment for stage I/II CRC. In patients with stage III CRC, the standard treatment is postoperative chemotherapy after curative resection. Multimodality therapy consisting of surgery, chemotherapy and radiotherapy has prolonged survival in patients with resectable stage IV CRC. However, there is still room for improvement in the outcome of treatment, and personalized treatment using biomarkers may improve the outcomes of CRC.

C-X-C chemokine receptor type 4 (CXCR4) and its ligand, C-X-C motif chemokine 12 (CXCL12), are strongly expressed in a variety of tissues, including the lung, liver, and lymph nodes as well as in various types of cancer, where it attracts lymphocytes to these organs (2, 3). On the other hand, interactions between CCR7 and its ligands, C-C motif chemokine ligand 19 (CCL19) and CCL21, play a central role in lymphocyte trafficking and homing to lymph nodes during immune and inflammatory reactions in various organs as well as in various types of cancer (4-6). Furthermore, it has been reported that these chemokine systems play a role in both lymphatic and distant spread of malignant tumours (7-9). There has been a report demonstrating a correlation of CCR7

Table I. *Polymerase chain reaction primers and conditions.*

Gene	Encoded protein	Primer	Annealing temperature (˚C)	Product size (bp)
CXCR4	CXCR4	5'-GCTTTCTTCCACTGTTGTCTG-3' 5'-AATGTCCACCTCGCTTTCC-3'	60	142
CCR7	CCR7	5'-ATGGGAGGAGAGGACAAG-3' 5'-GACAAAGAACAAAGAACAAGC-3'	61	88
ACTB	β-Actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60	171

expression with lymphatic vessel density and lymph node metastasis in an animal model of CRC (9). However, there are few reports on the association between CXCR4 and CCR7 expression levels in CRC tissue and clinicopathological features or on their potential prognostic value in patients with CRC. In this study, we examined the clinical significance of *CXCR4* and *CCR7* mRNA expression in CRC tissues in patients with this disease.

Patients and Methods

Patients and samples. The study was approved in advance by the Ethics Committees of Kanagawa Cancer Centre, Yokohama, Japan (approval number: epidemiological study-29) and Yokohama City Medical Centre, Yokohama, Japan (approval number: 18-7A-4). Informed consent was secured from each patient before surgery. A total of 202 patients with CRC and underwent surgery from January 2003 to December 2006 at the Kanagawa Cancer Centre and Yokohama City Medical Centre were selected according to the criteria below. The inclusion criteria were as follows: i) Pathological diagnosis of CRC according to the definitions of the eighth edition of the International Union Against Cancer TNM Classification of Malignant Tumours, (10), and ii) patients undergoing surgery. The exclusion criteria were as follows: i) Death before discharge from hospital, ii) receipt of preoperative treatment, and iii) the presence of multiple cancers within 5 years, iv) refusal to participate in this study. Overall survival (OS) was measured from the day of surgery to death. Each CRC tissue sample was immediately embedded in O.C.T. compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) and stored at −80˚C. All sections sliced from frozen samples were stained with haematoxylin and eosin. Sections that consisted of >80% carcinoma cells were used to extract total RNA.

RNA extraction and complementary DNA (cDNA) synthesis. Total RNA was extracted from CRC tissue samples using TRIzol reagent (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesised using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) from 200 ng of total RNA. The cDNA was diluted 20% with distilled water and stored at –20˚C.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories). PCR was carried out in a total volume of 15 μl consisting of cDNA made from 0.75 mg of RNA, 270 nM of each primer, 7.5 μl of iQ SYBR Green Supermix containing 0.4 mM each of dCTP, dTTP, dATP and dGTP, and 50 U/ml of iTag DNA polymerase. The PCR conditions was performed 3 min at 95˚C, followed by 35 cycles of denaturation of the cDNA for 15 s at 95˚C, annealing for 15 s at an appropriate temperature (Table I), and a primer extension for 30 s at 72˚C, followed by 72˚C for 10 min. The primer sequences of *CXCR4*, *CCR7*, and b-actin, which was used as an internal control, are shown in Table I.

Statistical analysis. The expression levels of *CXCR4* and *CCR7* mRNA were categorised as low (n=101) or high (n=101) according to the median expression of *CXCR4* and *CCR7* mRNA in CRC tissue. The relationship between *CXCR4* and *CCR7* mRNA expression and clinicopathological factors was examined using the chi-squared test. The OS curve was made using the Kaplan-Meier method, and differences in OS were examined using the log-rank test. Univariate and multivariate analyses for predictive factors of OS were carried out using a Cox proportional hazards regression model. All statistical analyses were carried out using IBM SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA). Two-tailed *p*-values were calculated. Differences were considered statistically significant when the *p*-value was less than 0.05.

Results

Relationship between mRNA expression levels of CXCR4 and *CCR7 and clinicopathological factors*. There was no significant association between *CXCR4* mRNA expression and any clinicopathological factor. High *CCR7* mRNA expression levels in CRC tissues were positively associated with tumour size and with cancer of the rectum than of the colon (Table II).

Relationship between CXCR4 and *CCR7 mRNA expression levels in CRC tissues and patient outcomes*. There was no significant difference in OS according to the *CXCR4* expression level in CRC tissue (Figure 1A). In contrast, the 5-year OS rate was significantly poorer in patients with high *CCR7* mRNA expression in CRC tissue than in those with low *CCR7* mRNA expression (p=0.0014, log-rank test; Figure 1B). The median follow-up period was 61.6 months.

Univariate and multivariate analyses for OS of clinicopathological factors and CCR7 mRNA expression

Table II. *Relationship between C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7) mRNA expression levels and clinicopathological features.*

Variable		*CXCR4* expression, n (%)			*CCR7* expression, n (%)		
	Category	Low (n=101)	High (n=101)	*p*-Value	Low (n=101)	High (n=101)	*p*-Value
Age, years		66.1±10.8	65.3±10.9	0.590	67.1±10.1	64.3±11.3	0.067
Gender	Male	54 (53.5)	56 (55.4)	0.778	56 (55.4)	54 (53.5)	0.778
	Female	47 (46.5)	45 (44.6)		45 (44.6)	47 (46.5)	
Size	<5 cm	60 (59.4)	52 (51.5)	0.257	67 (66.3)	30 (29.7)	**0.002**
	≥5 cm	41 (40.6)	49 (48.5)		34 (33.7)	71 (70.3)	
Histological type	Wel	31 (30.7)	28 (27.7)	0.821	35 (34.7)	24 (23.8)	0.222
	Mod	57 (56.4)	58 (57.5)		52 (51.4)	63 (62.4)	
	Poor	5 (5.0)	8 (7.9)		5 (5.0)	8 (7.9)	
	Muc	8 (7.9)	7 (6.9)		9 (8.9)	6 (5.9)	
Depth of invasion	T1	11 (10.9)	6 (5.9)	0.464	9 (8.9)	8 (7.9)	0.147
	T2	16 (15.8)	17 (16.8)		21 (20.8)	12 (11.9)	
	T3	35 (34.7)	43 (42.6)		41 (40.6)	37 (36.6)	
	T4	39 (38.6)	35 (34.7)		30 (29.7)	44 (43.6)	
Tumor location	Colon	60 (59.4)	50 (49.5)	0.158	62 (61.4)	48 (47.5)	**0.048**
	Rectum	41 (40.6)	51 (50.5)		39 (38.6)	53 (52.5)	
LN metastasis	Absent	54 (53.5)	49 (48.5)	0.482	55 (54.5)	48 (47.5)	0.325
	Present	47 (46.5)	52 (51.5)		46 (45.5)	53 (52.5)	
Lymphatic invasion	Absent	67 (66.3)	65 (64.4)	0.767	68 (67.3)	64 (63.4)	0.554
	Present	34 (33.7)	36 (35.6)		33 (32.7)	37 (36.6)	
Venous invasion	Absent	38 (37.6)	37 (36.6)	0.884	44 (43.6)	31 (30.7)	0.058
	Present	63 (62.4)	64 (63.4)		57 (56.4)	70 (69.3)	
Distant metastasis	Absent	74 (73.3)	79 (78.2)	0.412	82 (81.2)	71 (70.3)	0.071
	Present	27 (26.7)	22 (21.8)		19 (18.8)	30 (29.7)	

LN: Lymph node; Mod: moderately differentiated adenocarcinoma; Muc: mucinous carcinoma; Poor: poorly differentiated adenocarcinoma; Well: well-differentiated adenocarcinoma. Significant *p*-Values are shown in bold.

levels. On univariate Cox regression analysis, tumour diameter, histological type, depth of invasion, lymph node metastasis, lymphatic invasion, distant metastasis, pStage, and high expression level of *CCR7* mRNA were selected as significant predictors of OS (Table III). On multivariate Cox regression analysis, high expression of *CCR7* mRNA was identified as a significant independent predictor of poorer OS (p=0.037; Table III).

Discussion

In this study, we examined whether *CXCR4 and CCR7* mRNA expression in CRC tissue was associated with clinicopathological factors and outcomes in patients with CRC. High *CCR7* mRNA expression levels in CRC tissues were positively associated with tumour size and with rectal rather than colonic cancer. In addition, the 5-year OS rate was significantly poorer in patients with high *CCR7* mRNA expression than in those with low *CCR7* mRNA expression. Furthermore, multivariate analysis demonstrated that overexpression of *CCR7* mRNA was an independent predictive factor of outcomes in patients with CRC.

Several chemokines and their receptors have been found to play important roles in tumour progression (7). Furthermore, interactions between CXCR4 and its ligand CXCL12 may direct several key processes in the directional regulation of haematopoiesis and migration of metastatic tumour cells (11). CCR7, stimulated by its ligand CCL21, may induce invasion and migration of overexpression of CCR7 cancer cells into lymph nodes in a manner similar to that found for lymphocyte homing (4-6). Therefore, the chemokine receptors CXCR4 and CCR7 are suggested to play an important role in lymphatic invasion and tumour progression in cancer. We examined whether *CXCR4* and *CCR7* mRNA expression levels were associated with clinicopathological features and outcomes in patients with CRC.

Firstly, we examined the relationship between *CXCR4* and *CCR7* mRNA expression levels in CRC tissues and clinicopathological features. The effects of CXCR4 and its ligands on lymphatic spread and of CCR7 on distant metastasis have been demonstrated in various types of cancer (9, 12-23). As for associations between their expression levels, it was reported that CXCR4 expression levels were positively correlated with TNM stage, lymph node

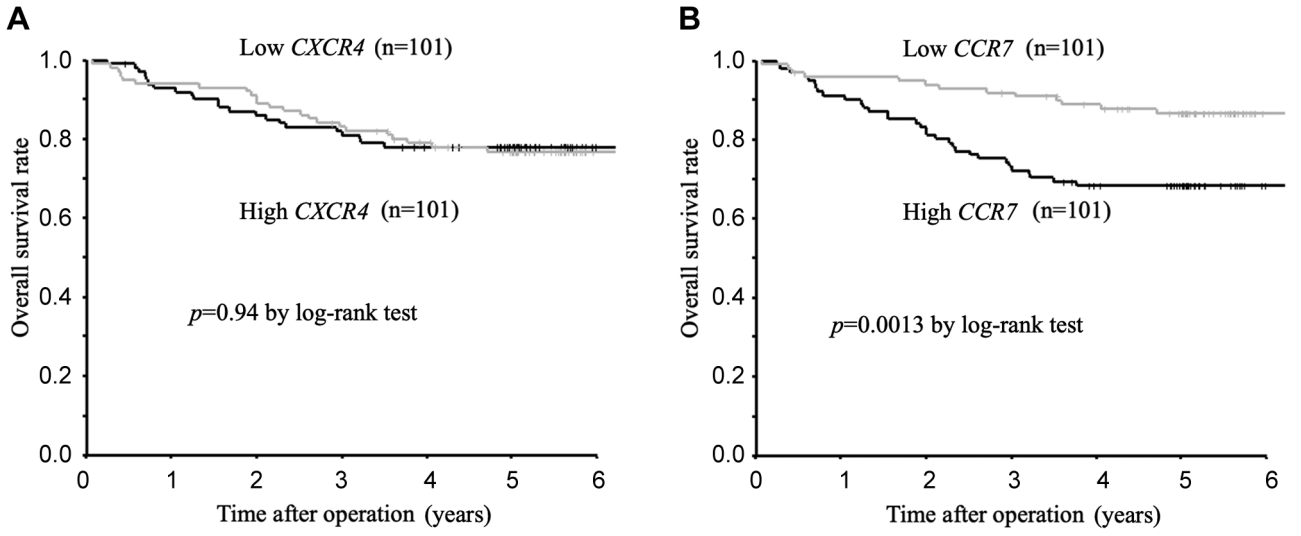

Figure 1. *Comparison of overall survival after surgery according to C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7) mRNA expression levels in patients with colorectal cancer. A: Overall survival did not differ significantly according to the CXCR4 expression level. B: The overall survival rate was significantly lower in patients with high CCR7 gene expression than in those with low CCR7 gene expression (p=0.0013, log-rank test).*

metastasis, and distant metastasis in 68 patients with CRC (24). A further study demonstrated that strong expression of CXCR4 was significantly associated with advanced tumour stage (Union for International Cancer Control), lymph node metastasis, and distant metastasis in 92 patients with CRC (24). It was reported that high expression of the chemokine receptor CCR7 at the tumour invasive front was an independent and powerful predictor of regional lymph node metastases in 94 patients with CRC without distant metastasis. In addition, overexpression of CCR7 correlated positively only with location of the primary tumour in the rectum and not with any other clinicopathological features (22). In our present study, there was no relationship between *CXCR4* gene expression levels and clinicopathological features. In contrast, a high *CCR7* mRNA expression level was related to greater tumour size and location in the rectum.

We then examined the relationship between *CXCR4* and *CCR7* mRNA expression levels in CRC tissue and outcomes in patients with CRC. High expression of both *CXCR4* and *CCR7* correlated with poor outcomes in various types of cancer (11-14, 22-25). However, a clear association between *CXCR4* or *CCR7* mRNA expression in CRC tissue and outcomes has yet to be established in patients with CRC. It was reported that high *CXCR4* mRNA expression in primary CRC was significantly associated with recurrence, poorer survival, and liver metastasis (30, 31). Another study demonstrated thatin immunohistochemical experiments, CCR7 expression at the tumour invasion front was a significant prognostic factor, whereas CXCR4 expression was not observed (32). Furthermore, another study found no significant relationship between *CXCR4* and *CCR7* expression levels and patient survival (25). In our study, *CXCR4* mRNA expression was unrelated to survival in patients with CRC; however, high *CCR7* mRNA expression in cancerous tissue was significantly associated with poorer OS. Moreover, on multivariate analyses, high expression of *CCR7* mRNA in CRC tissue was a significant independent predictor of poorer OS in patients with CRC.

The mechanism of the association between *CCR7* mRNA overexpression in CRC tissue and poor patient survival remains unclear. Several previous studies reported that CCR7 has important roles in tumor formation, invasion, migration, and metastasis (33-36). In addition to chemotaxis by chemokines, mechanisms for the enhancement of tumorigenesis and cell survival by chemokines have been described. One study demonstrated that activation of CCR7 through ligands present in the tumour microenvironment contributed directly to tumour formation *via* immunologically mediated mechanisms (33). Furthermore, CCR7 was shown to activate phosphoinositide-3 kinase to promote tumour invasion (34). Another report suggested that CCR7 promotes expression of c2H2-zinc finger type transcription factor SNAI1 and induces epithelial–mesenchymal transition with, as a result, cell cycle progression, proliferation, invasion, and metastasis of gastric cancer (35). CCR7 was reported to increase invasion and migration of cancer cells, and lymph node metastasis by facilitating expression of vascular endothelial growth factors, thereby facilitating the Wnt and phosphorylated extracellular signal-regulated kinase pathways (36). While only a hypothesis, it is possible that the above mechanism of tumour formation,

Table III. *Univariate and multivariate analyses to identify clinicopathological factors affecting overall survival.*

			Univariate analysis			Multivariate analysis		
Variable	Category	n	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age, years	<65 Years	84	1					
	≥65 Years	118	1.132	0.623-2.055	0.685			
Gender	Gender							
	Male	110	1					
	Female	92	0.854	0.472-1.543	0.600			
Tumour diameter	<5 cm	112	1					
	≥5 cm	90	3.626	1.902-6.913	**<0.001**	2.034	1.056-3.917	**0.034**
Histological type	Well, Mod	174	1					
	Poor, Muc	28	2.753	1.284-5.010	**0.007**	1.574	0.777-3.188	0.208
Depth of invasion	T1, T2	50	1					
	T3, T4	152	16.981	2.339-123.276	**0.005**	4.881	0.634-37.568	0.128
Location	Colon	110	1					
	Rectum	92	1.549	0.860-2.789	0.145			
LN metastasis	Absent	103	1					
	Present	99	5.868	2.731-12.610	**<0.001**	2.308	0.951-5.598	0.064
Lymphatic invasion	Absent	132	1					
	Present	70	3.47	1.910-6.305	**<0.001**	1.882	0.912-3.887	0.087
Venous invasion	Absent	75	1					
	Present	127	1.796	0.928-3.478	0.082	1.322	0.628-2.782	0.462
Distant metastasis	Absent	153	1					
	Present	49	7.536	4.109-13.823	**<0.001**	4.35	2.290-8.262	**<0.001**
CXCR4	Low	101	1					
	High	101	0.976	0.544-1.751	0.935			
CCR7	Low	101	1					
	High	101	2.761	1.449-5.264	**0.002**	2.064	1.045-4.077	**0.037**

CI: Confidence interval; CCR7: C-C chemokine receptor type 7; CXCR4: C-X-C chemokine receptor type 4; HR: hazard ratio; Mod: moderately differentiated adenocarcinoma; Muc: mucinous carcinoma; Poor: poorly differentiated adenocarcinoma; Well: well-differentiated adenocarcinoma. Significant *p*-Values are shown in bold.

invasion, migration, and lymph node metastasis involving CCR7 is associated with poor survival after surgery in patients with CRC and overexpression of *CCR7* mRNA.

This study has several limitations. Firstly, we only examined the expression of *CXCR4* and *CCR7* mRNA in CRC tissues of patients with CRC. We believe that the association between CCR7 and CXCR4 protein expression and *CXCR4* and *CCR7* mRNA expression should be analysed by immunohistochemical studies using the same specimens. Secondly, heterogeneity in the CRC specimens was an important problem. The specimens used for mRNA extraction were about 5-mm square CRC tissue specimens; although these specimens were collected from the central part of the entire tumour, they did not faithfully represent the entire tumour.

In conclusion, overexpression of *CCR7* mRNA in cancerous tissue may be a useful independent prognostic marker in patients with CRC.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

KT, SN, MS and TO designed the study. Data collection and literature searches were performed by HW, KK, MN, SS, TY HT, NY, NY, TO, SM, and RY. Data analysis was performed by KT, SN, IH, TA, YH, YK, MM, HS, YM, and TO. Data interpretation was performed by all investigators. The article and figures were drafted by KT, SN, and TO. Finally, the article was revised and approved by all investigators. Thus, all of the Authors actively participated in this study.

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