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Serum CCL7 Is a Novel Prognostic Biomarker of Metastatic Colorectal Cancer

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Abstract. *Background/Aim: Colorectal cancer is the third most common cancer globally, and the poor prognosis of patients with metastatic colorectal cancer (mCRC) warrants urgent attention. We previously obtained 10 candidate serum biomarkers for mCRC. Our aim with this study was to determine the prognostic performance of the pre-treatment serum C-C motif chemokine ligand 7 (CCL7) concentration in patients with mCRC. Patients and Methods: Protein concentrations of CCL7 were examined using ELISA and immunohistochemistry for serum (n=110) and surgical specimens (n=85), respectively, of patients with mCRC. The relationship between protein concentration and prognosis was examined using Cox regression analysis, receiver operator characteristic curve analysis and the Kaplan-Meier method. Results: The overall survival (OS) of patients with high concentrations of serum CCL7 was significantly poorer than that of patients with low concentrations. Patients with a high CCL7 concentration in the stroma had significantly poorer outcomes than those with a low concentration. The concentrations of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 were significantly higher in the high-CCL7 group, compared to those in the low-CCL7 group. Univariate and multivariate analysis revealed that serum CCL7 concentration was a significant prognostic factor for mCRC. The combination of serum CCL and CEA concentrations was also useful in this regard (area under the curve=0.71). Conclusion: The combined pre-treatment serum levels of CCL7 and CEA are useful prognostic biomarkers for mCRC.*

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally (1). In patients with CRC, accurate staging is important to make treatment choices (2, 3). Metastatic CRC (mCRC) is defined as CRC with distant metastasis, and the rate of mCRC at diagnosis is about 20% according to the US Surveillance, Epidemiology, and End Results Program (4). In patients with stage I-III CRC, post-relapse treatment is important (5), but similarly, improving the prognosis of mCRC is an urgent issue. Over the last decade, doublet treatments, such as those comprising fluorinated pyrimidine with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), with or without monoclonal antibodies such as anti-vascular endothelial growth factor antibodies or anti-epidermal growth factor receptor antibodies, have markedly improved the prognosis of patients with mCRC (6-10). Furthermore, triplet fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) plus bevacizumab has been associated with improved outcomes for patients with mCRC relative to doublet chemotherapy (11). In addition, immune checkpoint inhibitors have yielded breakthrough clinical results in cancer treatment (12). Nevertheless, responses to

chemotherapy and immunotherapy vary from person to person, and the prognosis of patients with mCRC remains extremely poor (13).

In a study of FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab (the TRIBE study), progression-free survival, the response rate, overall survival, and the resection rate of metastases were significantly better with FOLFOXIRI plus bevacizumab compared to FOLFIRI plus bevacizumab; however, the rates of grade 3 or 4 neutropenia, diarrhea, stomatitis and neurotoxicity (peripheral neuropathy) were significantly higher in FOLFOXIRI plus bevacizumab (14). Therefore, prognostic biomarkers for patients with mCRC would be useful. Moreover, non-invasive sampling, such as obtaining a blood or stool sample, is preferable to invasive sampling, such as a tissue biopsy. Biomarkers identified via the analysis of cell-free DNA (15), circulating tumor DNA (15, 16), microRNAs (17), and inflammatory molecules (18, 19), in the peripheral blood (*i.e.*, a liquid biopsy) may be useful for prognostication in patients with mCRC (20).

Previously, we obtained 10 candidate biomarkers for prognostication as a result of a comprehensive analysis, using the SOMAscan assay, of 1,129 protein concentrations in the sera of 24 patients with stage IV mCRC (21). Of the 10 candidates, myeloperoxidase (22), interleukin (IL)-8 (23), heterogeneous nuclear ribonucleoproteins (24), and vascular endothelial growth factor (VEGF)121 (25) are known prognostic markers in patients with CRC. Moreover, we revealed serum lectinlike oxidized low-density lipoprotein receptor-1 (LOX-1) as a novel prognostic biomarker of stage III/IV CRC (21). Among the remaining candidates, we focused on serum C-C motif chemokine ligand 7 [CCL7; also known as monocyte chemotactic protein 3 (MCP-3)] in this study. CCL7 is highly expressed in liver metastases of CRC (26, 27). CCL7 is reportedly related to cancer progression (26, 28, 29); however, we are aware of no reports on the prognostic performance of serum or tissue CCL7 concentrations in patients with CRC. Therefore, this study aimed to examine the prognostic performance of the serum CCL7 concentration in patients with stage IV mCRC.

Patients and Methods

Comprehensive analysis of serum proteins. A comprehensive analysis of serum protein concentrations was performed using the SOMAscan platform (SomaLogic, Boulder, CO, USA), which was used to measure 1,129 proteins related to mCRC, as previously described (21). Briefly, serum samples were obtained on the day of admission, immediately before chemotherapy, from 24 consecutive patients with mCRC treated with FOLFOX as first-line chemotherapy at our hospital from February 2009 to November 2012. Excluding 4 patients who survived for 2-3 years, we analyzed serum samples from 20 patients and divided them into two groups: the good prognosis group, surviving for more than 3 years (n=9), and the poor prognosis group, surviving for less than 2 years (n=11).

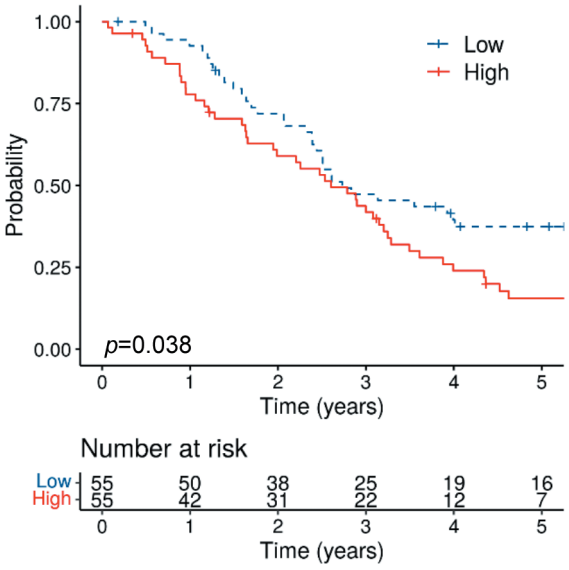

Figure 1. *Kaplan–Meier curves for OS of 110 patients with mCRC (stage IV) according to serum CCL7 concentrations. The cut-off serum CCL7 concentration (44.25 pg/ml) was determined using the time dependent ROC curve analysis. OS, overall survival; mCRC, metastatic colorectal cancer; CCL7, C-C motif chemokine ligand 7; ROC, receiver operator characteristic.*

Sample collection. From February 2007 to December 2017, serum samples were obtained from 110 consecutive patients with mCRC (stage IV) in our hospital, excluding those from 24 patients used for the SOMAscan analysis, and stored at –80˚C until further use. These were obtained at the time of admission or initial medical examination, shortly before surgery or chemotherapy. For immunohistochemical staining (IHC), 85 tissue samples were collected and evaluated; these were from patients with mCRC who underwent surgery, including two with multiple cancers. This study was approved by the Ethics Committee of Yamaguchi University Hospital (approval numbers: H17-83 and H23-135). All samples were obtained with the patients' written informed consent.

Measurement of serum CCL7 concentrations. Serum CCL7 concentrations were determined using the Human CCL7 ELISA Kit (ab 193769; Abcam, Cambridge, UK). The samples were collected by centrifugation, aliquoted, and preserved at –80˚C until analysis. They were assayed according to the manufacturer's instructions. The absorbances at 450 nm were immediately measured using EnVision Multilabel Plate Readers (PerkinElmer, Waltham, MA, USA). A standard curve was constructed for the stock standard. The sensitivity of the assay was 10.3 pg/ml.

IHC staining for CCL7. Of the 110 cases for which the serum CCL7 concentrations were measured, IHC was performed for the 85 cases in which primary lesion resection was performed. IHC was performed on 4-μm-thick formalin-fixed paraffin-embedded sections. Antigen retrieval was performed in 10 mM Tris-EDTA buffer (pH=9.0) by incubating at 95˚C in a microwave for 40 min. After blocking endogenous peroxidase activity with 3% H_2O_2 methanol for 10 min, the sections were incubated with serum-free protein block (Agilent Technologies, Santa Clara, CA,

Table I. *Relation between CCL7 level and clinicopathological characteristics.*

	Serum CCL7			Stromal CCL7		
	Low (n=55)	High (n=55)	*p*-Value	Low (n=26)	High (n=59)	*p*-Value
Age, years						
Median (range)	66 (37-83)	68 (25-87)	0.365	67 (38-84)	68 (25-87)	0.345
Sex						
Male	28	30	0.849	13	31	1.000
Female	27	25		13	28	
CEA (ng/ml)						
Median (IQR)	15.5 (7-91)	48.1 (13-348)	0.028	19.6 (12-258)	39.5 (12-150)	0.801
CA19-9 (U/ml)						
Median (IQR)	26.6 (6-84)	84.4 (18-1,938)	0.010	64.4 (16-1,788)	45.5 (10-248)	0.296
Tumor location						
Right colon	23	26	0.239	10	30	0.559
Left colon	22	14		10	17	
Rectum	10	15		6	12	
Surgery						
Performed	42	43	1.000	26	59	-
Unresectable	13	12		-	-	
Depth						
pT1-3	33	24	0.127	16	27	0.240
pT4a, 4b	22	31		10	32	
Lymph nude						
pN0, 1	34	29	0.441	16	31	0.486
pN2, 3	21	26		10	28	
Distant metastasis						
pM1a	36	31	0.283	20	28	0.012
pM1b	16	15		2	19	
pM1c	3	9		1	8	
Serum CCL7						
Low	-	-	-	15	27	0.353
High	-	-		11	32	

p-Values were determined using the Mann–Whitney *U*-test or Fisher's exact test. For unresectable cases, the clinicopathological characteristics were assessed *via* clinical imaging. CCL7, C-C motif chemokine ligand 7; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; IQR, IQR interquartile range.

USA) for 10 min and an anti-CCL7 antibodies (AV07048; Merck KGaA, Darmstadt, Germany; dilution, 1:1,500) at 4˚C overnight. The reactions were visualized with 3,3'-diaminobenzidine chromogen (DAB; Agilent Technologies) and counterstained with Mayer's hematoxylin solution. Photographs were obtained using the all-in-one fluorescence microscope BZ-X710 (KEYENCE, Osaka, Japan). An experienced and independent pathologist, blinded to patient status, assessed the slides to estimate the CCL7 level in the tumor cytoplasm and stromal cells. Staining intensity in the tumor cytoplasm was divided in turn into strong, moderate, and weak, with at least 10% of stained tumor cells at that intensity; high-CCL7 was defined as the staining intensities of strong and moderate, low-CCL7 was defined as weak and negative staining. In the stroma, we counted the number of CCL7-positive cells in the five most abundant fields under a magnification of 400×.

Statistical analysis. The cut-off values for biomarkers were determined by using the time-dependent receiver operating characteristic (ROC) curve and the closest-to-the-top-left index. Differences between two groups were estimated using Welch's *t*-test or the Mann–Whitney *U*-test. Categorical variables were compared using the Fisher's exact test. Survival curves for overall survival (OS) were calculated using the Kaplan–Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were performed using the Cox regression model. Statistical analyses were performed using R software (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria). $p<0.05$ was considered to indicate statistical significance.

Results

Serum CCL7 concentration, prognosis and clinicopathological characteristics of patients with mCRC. Using the cut-off serum CCL7 concentration of 44.25 pg/ml based on time-dependent ROC curve analysis (n=110), patients with mCRC who had high concentrations of CCL7 had significantly poorer outcomes in terms of OS than those with low CCL7 concentrations ($p=0.038$, Figure 1). The concentrations of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) were significantly higher in the high-CCL7 group than in the low-CCL7 group, although there were no

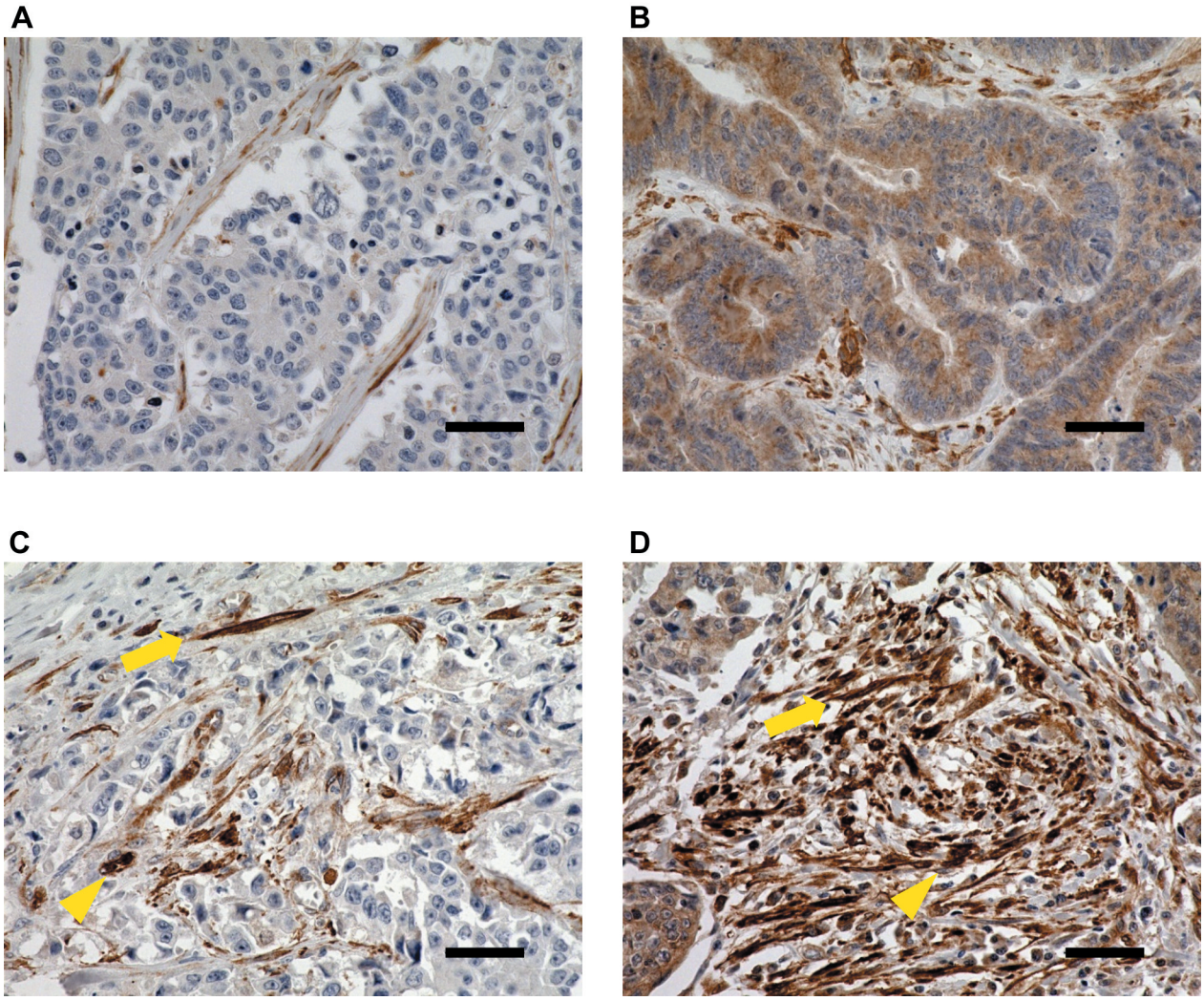

Figure 2. *Immunohistochemistry of CCL7 in colorectal cancer tissues. (A, B) Representative images of low and high CCL7 expression in the tumor, respectively. (C, D) Representative images of a few and many CCL7-positive stromal cells around the tumor, respectively. Arrows and arrowheads indicate fibroblasts and macrophages, respectively. The scale bars represent 50 μm.*

significant differences in clinicopathological characteristics (age, sex, tumor location, lymph node metastasis, distant metastasis) between these groups (Table I).

Expression of CCL7 in tissues and prognosis of mCRC. We also evaluated the CCL7 protein level in surgically obtained specimens (n=85). IHC analysis revealed that CCL7 was expressed in the cytoplasm of the tumor cells and the stromal cells surrounding the tumor cells (Figure 2). There was no significant difference in the OS between the low- and high-CCL7 groups (p=0.41, Figure 3A). Regarding stroma cells positive for CCL7, the cut-off value was set at 40.8 cells/field based on the ROC curve analysis. Interestingly, the group with a higher CCL7 level in the stroma had a significantly poorer prognosis than the group with a lower level (p=0.043, Figure 3B). Moreover, the CCL7 level in the stroma was significantly correlated with the number of distant metastatic organs (p=0.012, Table I). However, no statistically significant correlation was observed between the CCL7 level in the serum and that in the stroma.

Univariate and multivariate analyses for OS of patients with mCRC. Univariate analysis (Table II) with factors obtained before treatment revealed that the serum CCL7 concentration was significantly associated with the OS of patients with mCRC [hazard ratio (HR)=1.61, p=0.04]. Regarding factors obtained after surgery, lymph nodes, distant metastasis, and stromal CCL7 level were significantly associated with OS (p<0.001, p<0.001, and p=0.047, respectively).

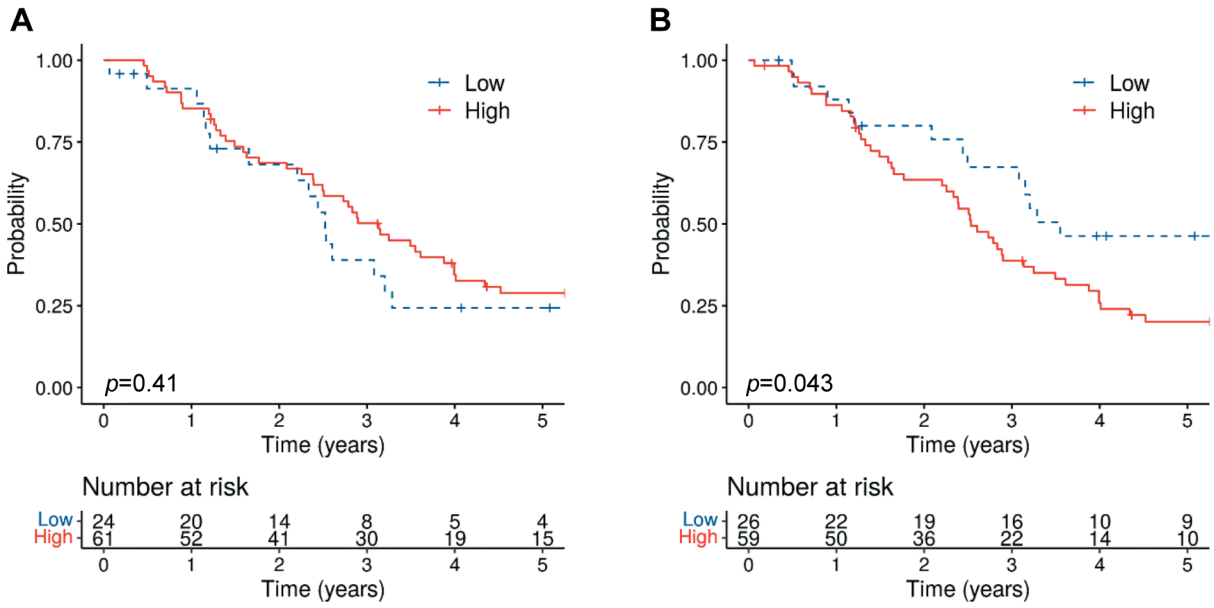

Figure 3. *Kaplan–Meier curves for OS of patients according to CCL7 levels in colorectal cancer tissues. (A, B) Patients with metastatic colorectal cancer were divided into two groups, low and high, based on the CCL7 levels in tumor (left) and stromal (right) cells. The cut-off stromal CCL7 expression, 40.8 cells/field, was determined using the time-dependent ROC curve analysis. OS, overall survival.*

Table II. *Univariate analysis using Cox regression models.*

Factor (test *vs*. control, or numerical)	HR	95% CI	*p*-Value
Before treatment			
Age (years)	1.012	0.992-1.032	0.239
Sex (Male *vs*. Female)	0.849	0.543-1.329	0.474
Serum CCL7 (High *vs*. Low)	1.607	1.022-2.527	0.040
CEA (ng/ml, logarithmic)	1.261	0.996-1.598	0.055
CA19-9 (U/ml, logarithmic)	1.120	0.915-1.370	0.274
Surgery (Performed *vs*. Unresectable)	0.870	0.518-1.461	0.598
After surgery			
Depth (pT1-4)	1.213	0.819-1.797	0.335
Lymph node (pN0-3)	1.481	1.172-1.871	<0.001
Distant metastasis (pM1a-1c)	1.721	1.254-2.362	<0.001
Tumor CCL7 (score 2-3 *vs*. 0-1)	0.692	0.396-1.209	0.196
Stromal CCL7 (High *vs*. Low)	1.874	1.009-3.481	0.047

HR, Hazard ratio; CI, confidence interval; CCL7, C-C motif chemokine ligand 7; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

Table III. *Multivariate analysis using Cox regression models.*

Factor (test *vs*. control, or numerical)	HR	95% CI	*p*-Value
Age (years)			
Sex (Male *vs*. Female)			
Serum CCL7 (High *vs*. Low)	1.611	1.022-2.540	0.040
CEA (ng/ml, logarithmic)	1.236	0.970-1.576	0.088

Stepwise multivariate Cox regression analysis was performed based on the Akaike information criterion. HR, Hazard ratio; CI, confidence interval; CCL7, C-C motif chemokine ligand 7; CEA, carcinoembryonic antigen.

Multivariate analysis revealed that the pre-treatment serum CCL7 concentration (HR=1.61, p=0.040) was an independent risk factor for mCRC prognosis obtained before treatment (Table III).

Predictive performance of CCL7 and CEA concentrations for OS in patients with mCRC. In the time-dependent ROC analysis, the combination of serum CCL7 and CEA concentrations yielded the highest areas under the ROC curves (AUCs) for all except for the third year after the cancer diagnosis (Figure 4A). The combination of serum CCL7 and CEA concentrations (both low, either high, both high) yielded an AUC equal to 0.71, for OS of patients with mCRC 5 years after the cancer diagnosis (Figure 4B). Survival analysis revealed significant differences in OS between the combined CCL7 and CEA concentrations (p=0.006, Figure 4C). The group of patients with high concentrations of both CCL7 and CEA had a significantly poorer OS than those with low concentrations of both (p=0.001, Figure 4C).

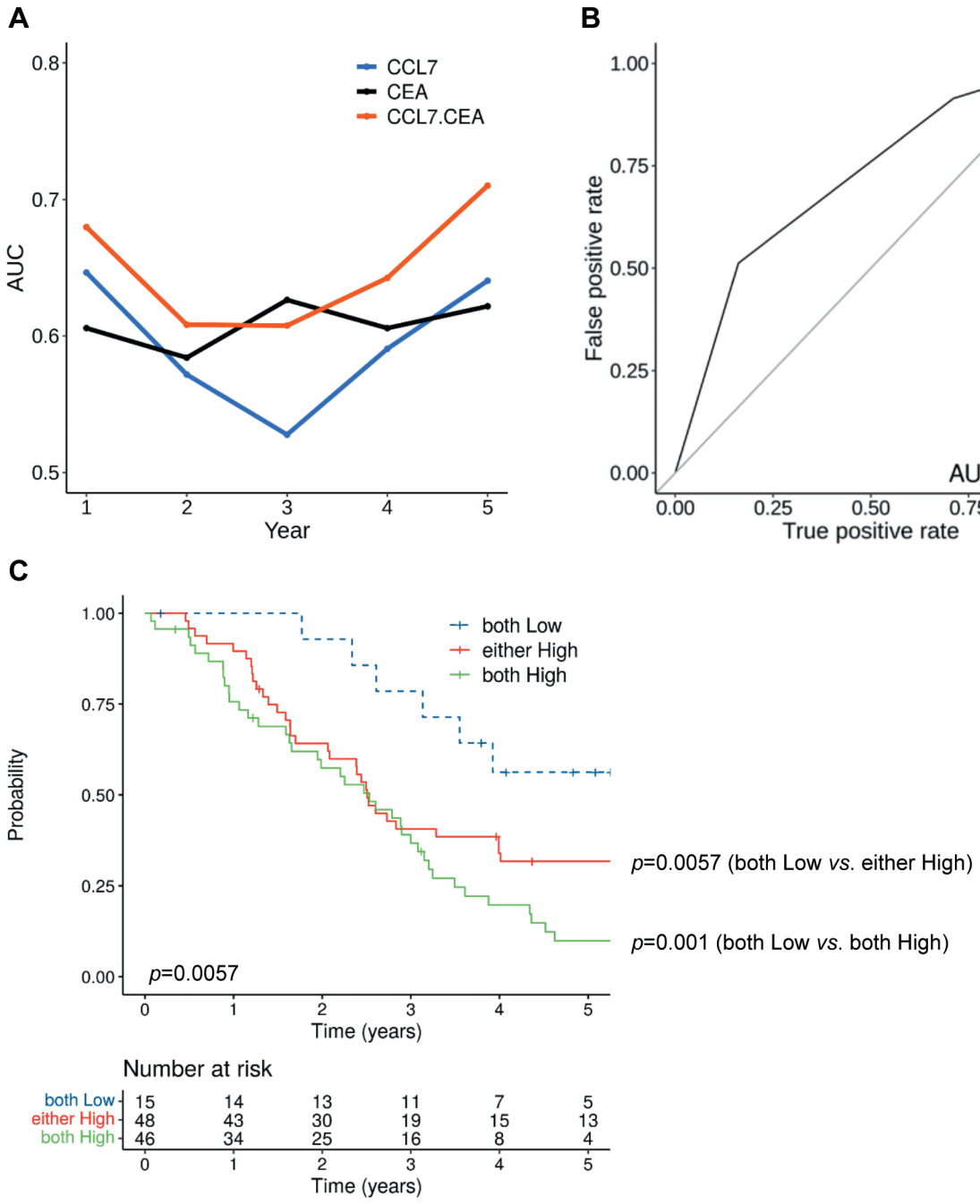

Figure 4. *OS times of patients according to combined concentrations of serum CCL7 and CEA. (A) A plot of AUCs of time-dependent ROC curves for OS time of patients with mCRC (stage IV). (B) A ROC curve for combined values of serum CCL7 and CEA concentrations 5 years after the cancer diagnosis. (C) Kaplan–Meier curves for OS of patients according to combined serum CCL7 and CEA concentrations. The cut-off values of 44.25 pg/ml and 8.32 ng/ml for CCL7 and CEA, respectively, were determined using the time-dependent ROC curve analysis. CEA, carcinoembryonic antigen; AUC, area under the curve; OS, overall survival.*

Discussion

The aim of this study was to determine the prognostic performance of a candidate biomarker for patients with mCRC. This is, to our knowledge, the first report of the clinical significance of the serum CCL7 concentration in patients with mCRC. Patients with a high concentration of serum CCL7 had a significantly poorer prognosis than those

Table IV. *Associations between CCL7 mRNA expressions and OS.*

	Poorer OS	HR	95% CI	*p*-Value
Cervical squamous cell carcinoma (n=304)	High-CCL7	1.87	(1.15-3.02)	0.010
Kidney renal clear cell carcinoma (n=530)	High-CCL7	2.28	(1.68-3.10)	<0.001
Liver hepatocellular carcinoma (n=370)	Low-CCL7	0.44	(0.31-0.63)	<0.001
Lung adenocarcinoma (n=504)	High-CCL7	1.55	(1.10-2.20)	0.012
Ovarian cancer (n=373)	Low-CCL7	0.71	(0.52-0.98)	0.034
Pancreatic ductal adenocarcinoma (n=177)	High-CCL7	1.87	(1.15-3.05)	0.010

The association between CCL7 mRNA expressions from RNA-sequencing data and OS of 21 tumor types were examined using the Kaplan Meier plotter, a web-based survival analysis tool. Of the 21 tumor types, the significant associations by best cut-off were listed. *p*-Values were determined using the log-rank test. HR, Hazard ratio; CI, confidence interval; CCL7, C-C motif chemokine ligand 7; OS, overall survival.

with a low concentration (Figure 1). The serum CCL7 concentration was correlated with CEA and CA19-9 concentrations (Table I). Moreover, predictive performance for the prognosis of mCRC was highest for the combination of CCL7 and CEA concentrations (Figure 4). Patients with a poor prognosis predicted by using novel biomarkers may be assigned triplet chemotherapy, such as FOLFOXIRI, for maximum treatment efficacy, while treatments with fewer side effects, such as capecitabine plus bevacizumab, may be used for long-term control of the tumors in patients with a favorable prognosis (30).

CCL7 is a pro-inflammatory cytokine and a powerful chemotactic protein that elicits infiltration of monocytes, eosinophils, and basophils (31). The systemic inflammatory response may suppress systemic tumor immunity (32), increase resistance to chemotherapy, and lead to a poor prognosis (33). Pro-inflammatory cytokines, such as IL-6 and IL-8 are reportedly associated with a poor prognosis in various cancers, including colon cancer (19, 34). Similarly, we demonstrated that CCL7 is a potential marker of poor prognosis in patients with mCRC.

The pro-tumorigenic properties of CCL7 have been previously confirmed in CRC cells (28, 35). CRC cell proliferation, migration, and invasion are increased by the overexpression of CCL7 *in vitro* (36) and *in vivo* (22). CRC cells stably overexpressing CCL7 exhibited enhanced expression of C-C motif chemokine receptor 3 (35). We discovered that the patients with a high frequency of CCL7 in the stroma, in which fibroblasts and macrophages were abundant, had a significantly poorer prognosis than those with a low frequency (Figure 3B), although the tumorous CCL7 frequency was not significantly related to prognosis (Figure 3A). Interestingly, it has been reported that intracellular survival of cancer-associated *Fusobacterium nucleatum* in macrophages was facilitated by upregulation of CCL7 expression (37). Stromal CCL7 frequency was significantly correlated with the number of distant metastatic organs and lymph nodes (Table I), which may indicate that the high expression of CCL7 in stromal cells may promote distant metastasis. In addition to CRC (36), CCL7 is also reportedly associated with renal cell carcinoma (38) and oral squamous cell carcinoma (39). In human oral squamous cell carcinoma, IL-1α, released by cancer cells, stimulates cancer-associated fibroblast proliferation, and increases the CCL7 expression (39). In mice, CT26 colorectal cancer cells co-cultured with mesenchymal stem cells exhibited increased expression of CCL7 compared to CT26 monocultures. CCL7 has immunoglobulin- and B cell-dependent metastasis-promoting effects, and cells overexpressing CCL7 had a higher metastasis rate than control mice; it accelerated the initial growth of tumors and caused a higher rate of lung metastasis compared to control mice in one experiment study (36). In a recent study, co-culture with macrophage cells in the presence of CCL7 reportedly promoted migration of CT26 cells via epithelial mesenchymal transition (37). Thus, CCL7 may promote the local migration and invasion of cancer cells as an initial step of metastasis (40). Ren *et al*. reported that CCL7 secreted by monocytic myeloid-derived suppressor cells (MDSCs) plays an important role in initiating the outgrowth of metastatic latent CRC cells (41), and that a high serum CCL7 concentration was significantly correlated to metastasis and short-term recurrence in patients with CRC (41). CCL7 secreted by monocytic MDSCs binds to CCR2 of tumor cells and stimulates the Janus kinase (JNK)/signal transducer and activator of transcription 3 pathway to promote the proliferation of dormant CRC cells (41). It has also been reported that CCL7 downstream of the JNK pathway is an important regulator for early osteoclast precursors during bone metastasis of CRC (42). CCL7-expressing stromal cells may similarly affect the behavior of CRC cells and influence the prognosis of patients with mCRC. Interestingly, the Kaplan Meier plotter (43), a web-based survival analysis tool, showed significant association of CCL7 mRNA expressions and OS of several tumor types (Table IV). As for gastrointestinal cancers, the CCL7-high group had a significant poorer OS in stroma-rich pancreatic ductal adenocarcinoma than the CCL7-low group, and conversely, the CCL7-high group had a significant better prognosis in stroma-poor hepatocellular

carcinoma. Furthermore, preventive administration of CCL7 inhibitor may significantly inhibit CRC cell proliferation and metastasis and reduce tumor recurrence (41). Taken together, the inhibition of CCL7 may be a potential therapeutic target to prevent disease progression in patients with a high concentration of this protein.

However, there was no correlation between the expression of CCL7 in the tumor and that in the serum. Therefore, the expression of interstitial CCL7 is not directly reflected by the concentration of serum CCL7. This may be because CCL7 is an inflammatory cytokine secreted by various cells (35), and the serum CCL7 concentration may include its secretion owing to chronic inflammation caused by cancer. This study was limited by the small sample, because of restriction to patients with stage IV mCRC, as well as by its retrospective and single-center nature. Furthermore, detailed molecular mechanisms were not clarified; hence, the mechanism by which CCL7 reflects the prognosis of mCRC needs to be further investigated.

Conclusion

In conclusion, the combined serum CCL7 and CEA concentrations are a useful prognostic biomarker for patients with mCRC. They can be obtained prior to treatment and may be useful in planning with respect to the aggressiveness or lightness of treatment for mCRC at the time of diagnosis. The role of CCL7 in cancer tissue may be more important in stromal than in tumor cells.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

HC, SH, and HN designed the study. HC, RT, MX, MN, CNN, STo, SY, NS, YW, HM, YS, YT, MI, STa, TI, TU, TT, and YH contributed to patient recruitment and collection, analysis, and interpretation of data. RT and YN performed the statistical analysis. HC, RT, and HN wrote the manuscript. All Authors read and approved the final manuscript.

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