Expression of cellular retinoic acid binding protein 1 predicts peritoneal recurrence of gastric cancer

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Abstract. To improve the outcome of gastric cancer, novel markers that predict postoperative prognosis are required. For this purpose, the function of cellular retinoic acid binding protein 1 (*CRABP1*) in gastric cancer cells was investigated and it was determined whether it serves as a novel biomarker for gastric cancer. Reverse transcription-quantitative (RT-q) PCR and a PCR-array method were used to determine whether the expression of *CRABP1* mRNA in gastric cancer cell lines correlated with the expression of cancer-related genes. The correlations of *CRABP1* mRNA expression in tissues with clinicopathological factors of 230 patients who underwent radical gastrectomy were further evaluated. *CRABP1* mRNA levels varied among gastric cancer cell lines and showed significant positive correlations with numerous epithelial-mesenchymal transition factors. Additionally, *CRABP1* knockdown significantly suppressed the proliferation, migration and invasion of gastric cancer cell lines. In a mouse xenograft model of peritoneal metastasis of gastric cancer, it was found that the total weight of disseminated nodules was lower in the group, in which *CRABP1* mRNA levels were knocked down compared with those of the untransfected group. Disease-free survival (DFS) was significantly shorter in patients with high expression of *CRABP1*, and multivariate analysis of DFS revealed that high expression of *CRABP1* in the tumor area and lymph node metastasis served as an independent factor associated with poor prognosis. High expression of *CRABP1* in cancer tissues was associated with a greater incidence of peritoneal recurrences after curative gastrectomy. These findings indicated that *CRABP1* contributes to the malignant phenotype of gastric cancer cells and may serve as a biomarker for prognosing recurrence after curative resection, particularly peritoneal dissemination.

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Abbreviations: ATCC, American Type Culture Collection; atRA, all-trans-retinoic acid; CA, carbohydrate antigen; CRABP1, cellular retinoic acid-binding protein 1; CT, computed tomography; DFS, disease-free survival; EMT, epithelial-mesenchymal transition; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; JCRB, Japanese Collection of Research Bioresources Cell Bank; OS, overall survival; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROC, receiver operating characteristic; UICC, Union for International Cancer Control

Key words: gastric cancer, cellular retinoic acid binding protein 1, peritoneal recurrence, biomarker, expression

Introduction

The poor prognosis of gastric cancer contributes to its ignominious standing as the second-leading worldwide cause of cancer-related death with an 8.2% mortality rate in 2018 (1). Gastric cancer, which is clinically and molecularly heterogeneous (2,3), is characterized by the pathways of recurrent metastasis as follows: peritoneal dissemination, hematogenous metastasis and lymph node metastasis. Unfortunately, specific biomarkers for these metastatic pathways are unavailable, hindering the prediction of recurrence when patients undergo standardized adjuvant chemotherapy and postoperative surveillance. Furthermore, the particularly poor prognosis of gastric cancer with peritoneal dissemination may prevent administration of effective treatment.

Efforts to develop effective therapeutic strategies to improve the prognosis of gastric cancer require detailed analyses of the molecular biological mechanisms that determine the malignant phenotypes of gastric cancer cells. In addition, novel markers that predict postoperative prognosis, particularly recurrence, are urgently required. In the present study, genes specifically expressed in association with the metastatic potential of gastric cancer were searched. To this end, comprehensive analyses of genes expressed in tissues of patients with simultaneous distant metastasis were conducted. It was found that cellular retinoic acid-binding protein 1 (*CRABP1*) may serve as a new candidate biomarker. CRABP1, a member of the family of fatty acid-binding proteins, modulates the activity of retinoic acid (4). However, the expression of *CRABP1* in gastric cancer or its involvement in oncogenesis and tumor progression is unknown.

In the present study, the function of *CRABP1* was investigated by regulating its expression in gastric cancer cell lines and by evaluating the correlation of the expression of *CRABP1* in primary gastric cancer tissues with long-term outcomes and the type of recurrence after curative resection.

Materials and methods

Ethics. The present study was approved (approval no. 2014-0043) by the Institutional Review Board of Nagoya University (Nagoya, Japan) and conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki (2013) Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for use of clinical samples and data, as required by the Institutional Review Board, was obtained from all patients.

Transcriptome analysis. Surgically resected gastric tissues from four patients with liver metastasis were subjected to transcriptome analysis. Global expression profiling was conducted using the HiSeq platform (Illumina, Inc.) to compare the expression levels of 57,749 genes in primary gastric cancer tissues with those of the corresponding noncancerous adjacent gastric mucosa as previously described (5).

Sample collection. A total of 14 gastric cancer cell lines (AGS, GCIY, IM95, KATO III, MKN1, MKN7, MKN45, MKN74, NUGC2, NUGC3, NUGC4, N87, OCUM1 and SC-6-JCK) were obtained from the American Type Culture Collection (ATCC) or the Japanese Collection of Research Bioresources Cell Bank. Cells were cultured at 37˚C in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (Corning, Inc.) in an atmosphere containing 5% CO_2. The non-tumorigenic epithelial cell line FHs74 (ATCC) was used as a control. Primary gastric cancer tissues and corresponding normal adjacent tissues were collected from 300 patients who underwent gastric resection for gastric cancer without neoadjuvant therapy at Nagoya University Hospital (Nagoya, Japan) between January 2001 and December 2020. Tissue samples were immediately flash-frozen in liquid nitrogen and stored at -80˚C. Tissue comprising >80% tumor components (H&E staining) without grossly visible necrotic regions (~5 mm^2) was extracted from each tumor sample. Corresponding normal adjacent gastric mucosa samples were obtained from the same patient and were collected >5 cm from the tumor edge.

Specimens were histologically classified according to the guidelines of the Union for International Cancer Control (UICC), 8th edition (6). To determine whether the expression of *CRABP1* differed according to tumor histology, patients were categorized into the histological subtypes of their tumors as follows: differentiated (papillary, well differentiated, and moderately differentiated adenocarcinoma) and undifferentiated (poorly differentiated adenocarcinoma, signet ring cell, and mucinous carcinoma). Since 2006, adjuvant chemotherapy using S-1 (an oral fluorinated pyrimidine) has been administered to all patients with gastric cancer with UICC stages II-III, unless contraindicated by the condition of the patient (7,8).

CRABP1 mRNA levels in primary gastric cancer tissues and corresponding normal adjacent tissues from 300 patients with gastric cancer were evaluated using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Patients included 84 women and 216 men, ranging in age from 26-96 years (mean, 70 years). Patients included those with pathologically diagnosed undifferentiated (n=181) or differentiated gastric cancer (n=119). Patients were diagnosed with stage I (n=50), stage II (n=71), stage III (n=109), or stage IV (n=70) gastric cancer and 230 patients with stages I-III underwent R0 resection. Patients classified with UICC stage IV (n=56 of 70) were assigned this diagnosis due to positive peritoneal lavage cytology, localized peritoneal metastasis, or distant lymph node metastasis. Among patients with stage IV disease, 12 had synchronous liver metastasis and 2 had lung metastasis. These patients underwent gastrectomy to control bleeding or allow ingestion of food.

Expression of CRABP1 mRNA. *CRABP1* mRNA levels in cell lines and clinical samples (n=300) were analyzed using RT-qPCR with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total RNA (10 μg per sample) was purified using RNeasy Plus Mini kit (cat. no. 74136; Qiagen GmbH) according to the manufacturer's protocol. Complementary DNAs were generated using the M-MLV Reverse Transcriptase (cat. no. 28025013; Thermo Fisher Scientific, Inc.), dNTPs Mix (cat. no. U1511; Promega Corporation), the Primer Random pd(N)6 (11034731001, Roche Diagnostics) and RNase inhibitor (cat. no. 3335399001; Roche Diagnostics) according to the manufacturer's protocol, and amplified using primers specific for *CRABP1* (Table I). RT-qPCR was performed using the SYBR-Green PCR Core reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and absolute quantification was performed using the standard curve method. The following thermocycling conditions were used for qPCR: one cycle at 95˚C for 10 min, 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as an internal standard, and the expression level of each sample was determined in triplicate and calculated as the value of *CRABP1* mRNA divided by that of *GAPDH* mRNA (9).

Expression of genes encoding proteins that potentially interact with CRABP1. To identify genes coordinately expressed with *CRABP1* in gastric cancer cell lines, PCR array analysis was performed using the Human Epithelial to Mesenchymal Transition (EMT) RT2 Profiler PCR Array (Qiagen GmbH). This array profiles the expression of 84 key genes including those that encode transcription factors, ECM proteins as well as proteins involved in the EMT, cell differentiation, morphogenesis, growth, proliferation, migration, cytoskeleton and major signaling pathways (10).

siRNA-mediated knockdown of CRABP1 mRNA. A total of two siRNAs specific for *CRABP1* were designed at online sites and were pooled to inhibit *CRABP1* mRNA expression with the aim of obtaining stable knockdown as previously described (Table I) (11,12). si*CRABP1*-1 and si*CRABP1*-2 were designed by siDirect (http://sidirect2.rnai.jp/) and i-Score Designer (https://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html), respectively, and supplied from Hokkaido

Table I. Sequences of primers and siRNAs.

Primer name	Experiment	Primer sequence (5'→3')	Product size (base pairs)	Annealing temperature (˚C)
CRABP1	RT-qPCR	F: CAAAACCTACTGGACCCGTG R: CCGGACATAAATTCTGGTGC	91	60
	siRNA	siCRABP1-1: AGUUUAAUGACUUCGAAACCG siCRABP1-2: UUGAAGUUGAUCUCAGUGGTT		
GAPDH	RT-qPCR	F: GAAGGTGAAGGTCGGAGTC Probe: CAAGCTTCCCGTTCTCAGCC R: GAAGATGGTGATGGGATTTC	221	60

CRABP1, cellular retinoic acid-binding protein 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, quantitative real-time reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; F, forward; R, reverse.

System Science Co., Ltd. MKN1, MKN45 and NUGC4 cells were added to the wells of a 24-well plate ($5x10^4$ cells/ml) and transiently transfected at 37˚C the next day with 30 nM or *CRABP1* siRNA or a control siRNA (siControl with sequence as follows: 5'-GCAAACAUCCCAGAGGUAU-3') combined with LipoTrust EX Oligo (Hokkaido System Science Co., Ltd.); total RNAs were extracted 72 h later. To evaluate the effect of siRNAs on *CRABP1* mRNA expression, RT-qPCR analysis was performed as previously described (11,12). In addition, the knockdown efficacy of si*CRABP1*-1 or si*CRABP1*-2 alone in MKN1, MKN45 and NUGC4 cells was evaluated.

Cell proliferation, invasion, and migration assays. Cell proliferation was evaluated using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) as previously described (11). MKN1, MKN45 and NUGC4 cells (at a density of $1.5x10^3$, $1.5x10^3$ and $5x10^3$ cells per well, respectively) were seeded into 96-well plates in RPMI-1640 medium supplemented with 2% FBS. Cell invasion was determined using BioCoat Matrigel invasion chambers (BD Biosciences,) according to the manufacturer's protocol as previously described (13). MKN1 and MKN45 cells ($2.5x10^4$ cells/well) were suspended in serum-free RPMI-1640 and seeded in the upper chamber. After an appropriate incubation time (24 and 72 h, respectively), cells present on the surface of the membrane were fixed, stained, and counted using a light microscope in eight randomly selected fields as previously described (13). Cell migration was evaluated using wound-healing assays as previously described (14). The width of the wound was measured at 100-μm intervals (20 measurements per well, x400 magnification). The invasion and migration assays were performed in duplicate (n=2; two wells for each assay). For the invasion assay, 8 fields were randomly selected from each well and numbers of invasive cells were counted. Thus, statistical analysis was carried out using 16 values for the untransfected, siControl and si*CRABP1* groups. For the migration assay, the width of the wound was measured at 20 points for each well, indicating that statistical analysis was carried out using 40 values for the untransfected, siControl and si*CRABP1* groups.

Mouse xenograft models of peritoneal metastasis. Animal experiments were performed between October and December 2021 according to the ARRIVE guidelines (15) and were approved (approval no. M210414-001) by the Animal Research Committee of Nagoya University (Nagoya, Japan). A total of 10 six-week-old male NOD/SCID (weight, 24.7 g) and 2 BALBc nu/nu mice (weight, 20.4 g) were obtained from Japan SLC, Inc. and housed at least 1 week before experiments in temperature-controlled rooms at 20-22˚C with free access to food and water supply and a light/dark cycle of 14/10 h. MKN1 and NUGC4 cells transfected with *CRABP1* siRNA or untransfected were implanted into the abdominal cavity of six-week-old male mice (MKN1: n=5 each, NUGC4: n=1 each) to analyze the peritoneal dissemination of the xenografts. MKN1 and NUGC4 cells ($4x10^6$) in 500 μl of phosphate-buffered saline were injected into NOD/SCID and BALBc nu/nu mice, respectively. After 4 weeks of observations, these mice were euthanized after exposure to 100% CO_2 for 5 min and were observed for 20 min after confirmation of respiration cease. The flow rate of CO_2 was 50% of the chamber volume per min. After confirming euthanasia, the formation of peritoneal metastasis was observed under direct viewing.

Clinical significance of CRABP1 expression. The optimal cut-off value (0.0000325) of *CRABP1* mRNA levels in primary gastric cancer tissues was determined using receiver operating characteristic curve analysis for evaluating the significance of the association of their levels with metastasis or recurrence. Patients were stratified according to the cut-off value of *CRABP1* mRNA levels in gastric cancer tissues as follows: high *CRABP1* expression (>cut-off value) and low *CRABP1* expression (≤cut-off value). Correlations between the patterns of *CRABP1* mRNA expression and clinicopathological parameters were evaluated. Correlation analysis of *CRABP1* mRNA expression and recurrence patterns after curative surgery was applied to 230 patients who underwent curative surgery (i.e., stages I-III). Thus, the analysis of recurrence pattern specifically focused on initial recurrence after curative surgery. Outcome analyses of the overall survival and disease-free survival (DFS) rates and multivariate analysis were applied to 230 patients who underwent curative surgery. To validate the present data, an integrated microarray dataset comprising tissues of 1065 patients [Berlin, Bethesda, and Melbourne

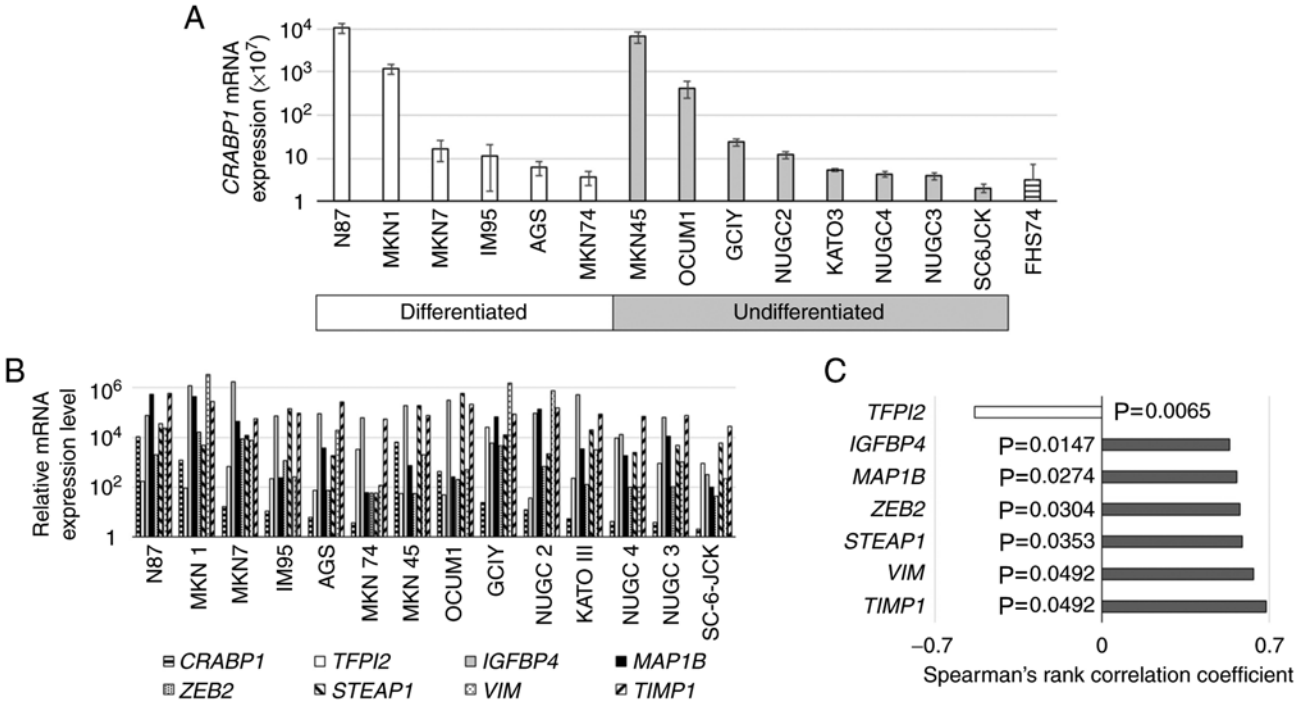

Figure 1. Expression analysis of *CRABP1* mRNA in cell lines. (A) *CRABP1* mRNA expression in 14 gastric cancer cell lines and the nontumorigenic intestinal cell line FHs74. Error bars indicate standard deviation. (B) The relative levels of *CRABP1* mRNA and those of mRNAs encoding potential CRABP1-interacting proteins in gastric cancer cell lines. (C) Cancer-related genes expressed in concert with *CRABP1* expression were identified by PCR array analysis. CRABP1, cellular retinoic acid binding protein 1.

datasets (http://kmplot.com/analysis/)] was analyzed as previously described (16).

Statistical analysis. The significance of differences of the relative mRNA levels (*CRABP1*/*GAPDH*) between the two groups were analyzed using the Mann-Whitney test. The significance of a correlation between two variables was assessed using the Spearman's rank correlation coefficient. The χ^2 test was used to analyze the associations between the expression levels of *CRABP1* and clinicopathological parameters. DFS rates were calculated using the Kaplan-Meier method, and the differences in the slopes of the survival curves were analyzed using the log-rank test. Multivariable regression analysis was preformed to identify prognostic factors using the Cox proportional hazards model, and variables with $P<0.05$ were entered into the final model. All statistical analyses were performed using JMP 15 software (SAS Institute, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of CRABP1 as a candidate gastric cancer-related gene. Transcriptome analysis of gastric tissues compared with corresponding noncancerous adjacent gastric mucosa from four patients with metastatic gastric cancer was first performed. Transcriptome analysis identified 26 candidate genes that were: i) Overexpressed in gastric cancer compared with the corresponding normal tissues and ii) Expressed at comparable expression levels in primary gastric cancer and metastatic tissues (Table II). A literature review of the functions of the identified genes was conducted and *CRABP1* was selected for subsequent analyses for the following reasons: i) Insufficient evidence was available on the oncological roles of *CRABP1*; ii) CRABP1 mediates the activity of retinoid, which is involved in cancer progression; and iii) nucleotide sequence of *CRABP1* is available from the United States National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Expression of CRABP1 and genes encoding potential CRABP1-interacting proteins by gastric cancer cell lines. The relative levels of *CRABP1* mRNA and those of mRNAs encoding potential CRABP1-interacting proteins in gastric cancer cell lines are presented in Fig. 1B. There were large differences in the levels of *CRABP1* mRNA and those of other genes among gastric cancer cell lines. *CRABP1* mRNA levels positively correlated with those encoding IGFBP4, MAP1B, ZEB2, STEAP1, VIM and TIMP1 and negatively with TFPI2 (Fig. 1C).

Analyses of CRABP1 mRNA levels in gastric cancer cell lines. To characterize *CRABP1* in gastric cancer, the levels of *CRABP1* mRNA in 12 gastric cancer cell lines were next compared with those of a nontumorigenic epithelial cell line. *CRABP1* mRNA levels were >2-fold higher in MKN1, MKN7, N87, IM95, GCIY, MKN45, NUGC2 and OCUM1 cells compared with FHs74 cells (Fig. 1A). *CRABP1* mRNA levels did not significantly differ according to the extent of differentiation of the gastric cancer cells. MKN1, MKN45 and NUGC4 cells were selected for subsequent analyses, since MKN1 and MKN45 cells expressed relatively high levels of *CRABP1* mRNA, and these three cell lines were easy to use in functional analyses.

Table II. Genes overexpressed in primary cancerous tissues from patients with metastatic gastric cancer.

			GC/Normal		Meta/GC	
Function	Symbol	Name	Log_2	P-value	Log_2	P-value
Regulator of cell cycle	*CRABP1*	Cellular retinoicacid-binding protein 1	3.66	0.0048	0.81	0.3022
	CCNE1	Cyclin E1	3.41	<0.0001	-1.06	0.0709
	CDC25B	Cell division cycle 25B	3.17	0.0006	-0.66	0.3947
Cell membrane receptor	*GRB7*	Growth factor receptor bound protein 7	3.98	<0.0001	-0.03	0.9716
	UTS2R	Urotensin 2 receptor	4.50	<0.0001	0.50	0.5675
	TNFRSF11B	TNF receptor superfamily member 11b	4.57	<0.0001	0.53	0.4265
Cell-surface glycoprotein	*MELTF*	Melanotransferrin	3.27	<0.0001	-0.19	0.7380
Cellular adhesin	*CLDN1*	Claudin 1	3.27	<0.0001	0.71	0.1568
	COMP	Cartilage oligomeric matrix protein	3.15	0.0003	0.91	0.1072
	THBS2	Thrombospondin 2	3.76	<0.0001	0.20	0.7759
	THBS4	Thrombospondin 4	4.01	<0.0001	0.95	0.2787
Growth factor	*INHBA*	Inhibin beta A subunit	3.76	<0.0001	-0.37	0.5028
Mediator of neural transmission	*CPLX2*	Complexin 2	4.36	0.0007	1.88	0.2436
	NPY	Neuropeptide Y	4.86	<0.0001	0.09	0.9008
	VSNL1	Visinin like 1	4.04	<0.0001	1.09	0.1528
Metabolic enzyme	*AKR1C4*	Aldo-keto reductase family 1-member C4	3.28	0.0009	0.59	0.4064
	KLK10	Kallikrein related peptidase 10	3.26	0.0003	-0.76	0.2984
	PADI2	Peptidyl arginine deiminase 2	3.01	<0.0001	-1.29	0.0758
	PLA2G2A	Phospholipase A2 group IIA	3.70	<0.0001	-0.43	0.4529
Trafficking protein	*DNAJC12*	DnaJ heat shock protein family member C12	4.15	<0.0001	-1.16	0.1038
	RBP4	Retinol binding protein 4	4.25	<0.0001	1.51	0.0515
	SYT7	Synaptotagmin 7	4.29	<0.0001	0.30	0.6281
Transcription factor	*ELF5*	E74 like ETS transcription factor 5	5.00	0.0001	-0.85	0.3319
	FNDC1	Fibronectin type III domain containing 1	4.50	<0.0001	-0.89	0.1592
	GNG4	G protein subunit gamma 4	4.84	<0.0001	0.29	0.7296
	HOXC10	Homeobox C10	6.49	0.0001	1.68	0.0752

GC, primary gastric cancer tissue; Normal, corresponding adjacent normal gastric tissue; Meta, hepatic metastasis tissue; TNF, Tumor necrosis factor; ETS, erythroblast transformation-specific.

Effect of CRABP1 knockdown on the biological activities of gastric cancer cells. The efficiency of *CRABP1* knockdown by transfection of siCRABP1-1 and si*CRABP1*-2 alone was evaluated in MKN1, NUGC4 and MKN45 cells (Fig. S1). These two siRNAs were pooled to constitute a *CRABP1*-specific siRNA. To evaluate the function of *CRABP1* in gastric cancer cells, MKN1 and NUGC4 cells were transfected with a *CRABP1*-specific siRNA. It was first determined that the knockdown efficacy of the *CRABP1* siRNA in MKN1, MKN45 and NUGC4 cells was sufficient for analysis (Figs. 2A and S2). The proliferation of siRNA-transfected MKN1, MKN45 and NUGC4 cells as well as the invasiveness and migration of

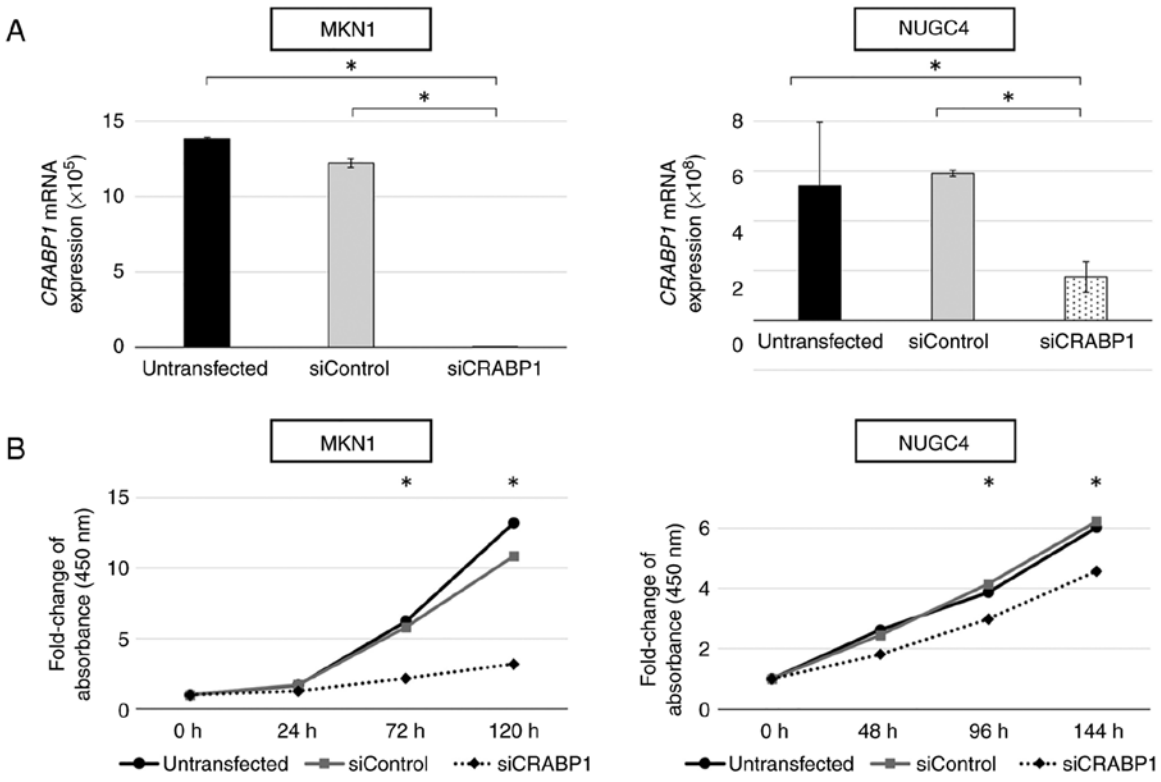

Figure 2. *CRABP1* knockdown and proliferation of gastric cancer cells. (A) Knockdown efficacy of the *CRABP1* siRNA in MKN1 and NUGC4 cells. (B) Proliferation of MKN1 and NUGC4 cells subjected to siRNA-mediated knockdown of *CRABP1*. *P<0.05. Error bars indicate standard deviation. si-, small interfering; CRABP1, cellular retinoic acid binding protein 1.

MKN1 and MKN45 cells were then evaluated. The proliferation of MKN1, MKN45 and NUGC4 cells was decreased as a result of CRABP1 knockdown starting from 72 h after transfection compared with the siControl-transfected cells (Figs. 2B and S2). Furthermore, the invasiveness of MKN1 and MKN45 cells was reduced by inhibiting *CRABP1* expression (Fig. 3). The migration of MKN1 and MKN45 cells was reduced by inhibiting *CRABP1* expression (Fig. 4).

Effect of CRABP1 knockdown on peritoneal metastasis in mouse xenograft models of gastric cancer. MKN1 and NUGC4 cells transfected with *CRABP1* siRNA or untransfected were injected into mice to identify the function of *CRABP1* in recurrence and metastasis of gastric cancer. Observations in the abdominal cavity of the mice were performed after euthanasia. In the MKN1 xenograft model, peritoneal dissemination was not observed in the si*CRABP1* group (Fig. 5). Peritoneal metastasis in the NUGC4-model mice was disseminated to a smaller extent in the si*CRABP1* group compared with the untransfected group (Fig. S3).

Prognostic impact of CRABP1 expression. The DFS rate of the *CRABP1*-high group was significantly lower compared with that of the *CRABP1*-low group (5-year DFS rates; 59.6% and 77.8%, respectively; P=0.012) (Fig. 6A) and were consistent with those of the extra-validation cohort (Fig. 6B).

Next, gastric cancer recurrence patterns were analyzed according to *CRABP1* mRNA levels of 230 patients who underwent R0 resection (stages I-III). Among them, 57 (24.7%) experienced postoperative recurrence at 65 initial recurrence sites. Analysis of recurrence patterns revealed that high expression of *CRABP1* mRNA was significantly associated with peritoneal recurrence (P=0.016) (Fig. 6C), but not with the other two recurrence patterns.

The correlations between *CRABP1* expression and clinicopathological characteristics of patients were next examined (Table III). High *CRABP1* expression was significantly associated with lymph node metastasis. Univariate analysis of DFS demonstrated that carbohydrate antigen 19-9 (37 IU/ml), tumor size ≥50 mm, macroscopic type (Borrmann type 4/5), pT4, lymphatic involvement, vascular invasion, invasive growth, lymph node metastasis and high *CRABP1* mRNA expression in gastric cancer tissues were significant prognostic factors for adverse outcomes (Table IV). Multivariable analysis identified high *CRABP1* mRNA expression as an independent prognostic factor of poor outcome (hazard ratio 1.89; 95% confidence interval, 1.15-3.09; P=0.012).

Discussion

In the present study, biomarkers of the malignant phenotype of gastric cancer that predict postoperative recurrence were searched. As a result, it was identified that the expression levels of *CRABP1* mRNA correlated with those of genes encoding EMT-related molecules. Furthermore, knockdown of *CRABP1* influenced the proliferation, invasiveness, and migration of gastric cancer cell lines. The results of these *in vitro* analyses are consistent with the demonstration that *CRABP1* expression in primary tumor tissues of gastric cancer was an independent predictor for

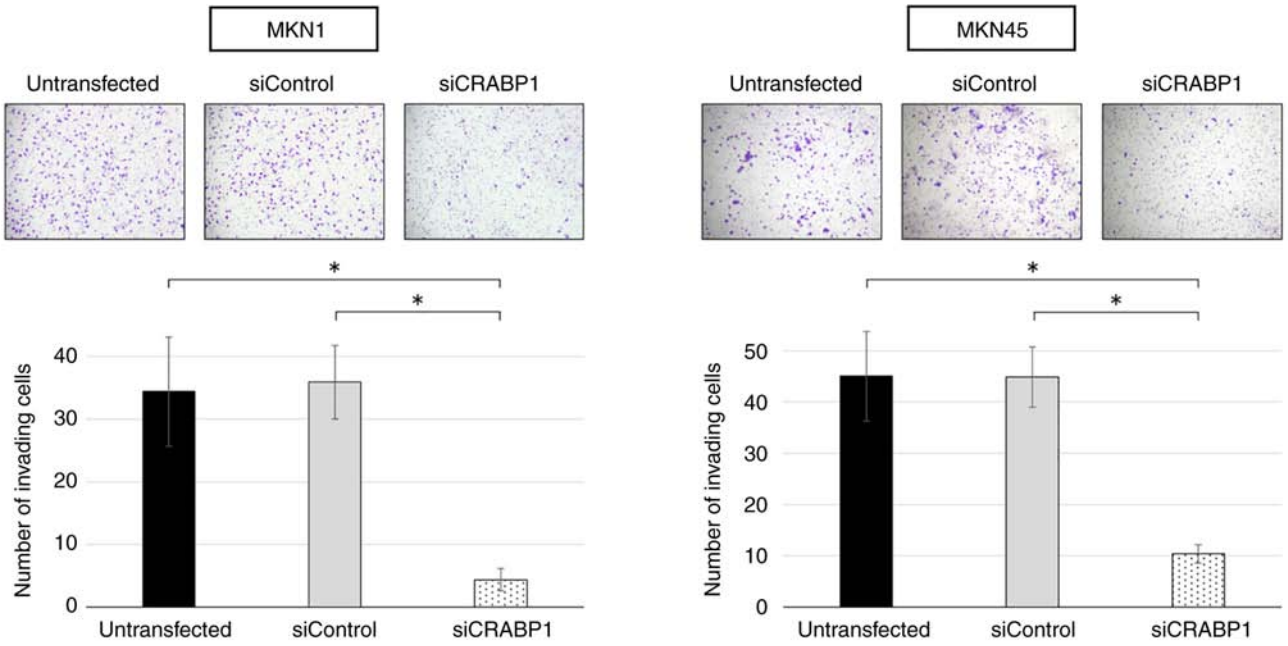

Figure 3. Effect of knockdown of *CRABP1*: Invasion assay of MKN1 and MKN45 cells. Top panels show representative images of stained cancer cells (magnification, x200), and the bottom graph shows the mean numbers of invading cells in eight randomly selected fields. *P<0.05. Error bars indicate standard deviation. si-, small interfering; CRABP1, cellular retinoic acid binding protein 1.

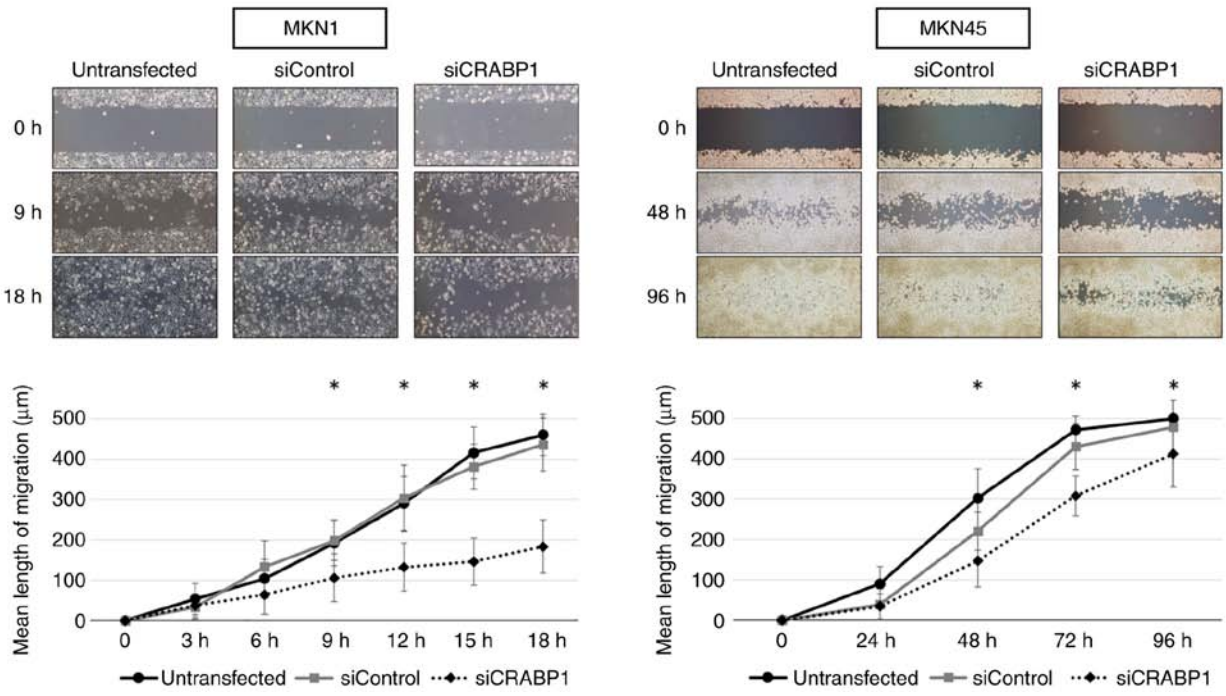

Figure 4. Effect of siRNA-mediated knockdown of *CRABP1* expression: Wound-healing assays of MKN1 and MKN45 cells. Top panels show representative images from assays at the indicated times, and the bottom graph shows the mean length of migration at the indicated times. *P<0.05. Error bars indicate standard deviation. si-, small interfering; CRABP1, cellular retinoic acid binding protein 1.

worse postoperative recurrence-free survival, which significantly correlated with an increased rate of peritoneal recurrence.

CRABP1 specifically binds retinoic acid, an activator of ERK1/2, which in turn, activates protein phosphatase 2A through binding to *CRABP1* to lengthen the cell cycle (17). This effect sensitizes cancer cells to apoptosis by triggering the homeostatic action of retinoic acid on the genome via the retinoic acid receptor (18). Thus, *CRABP1* may encode a tumor suppressor, as indicated by findings that *CRABP1* inhibits the growth of cancers such as those of the esophagus and thyroid (19-21). Conversely, evidence has indicated that the tumor suppressive effect of *CRABP1* is independent of its retinoic acid-binding activity and may contribute to the malignant transformation of mesenchymal tumors (22). Moreover, these

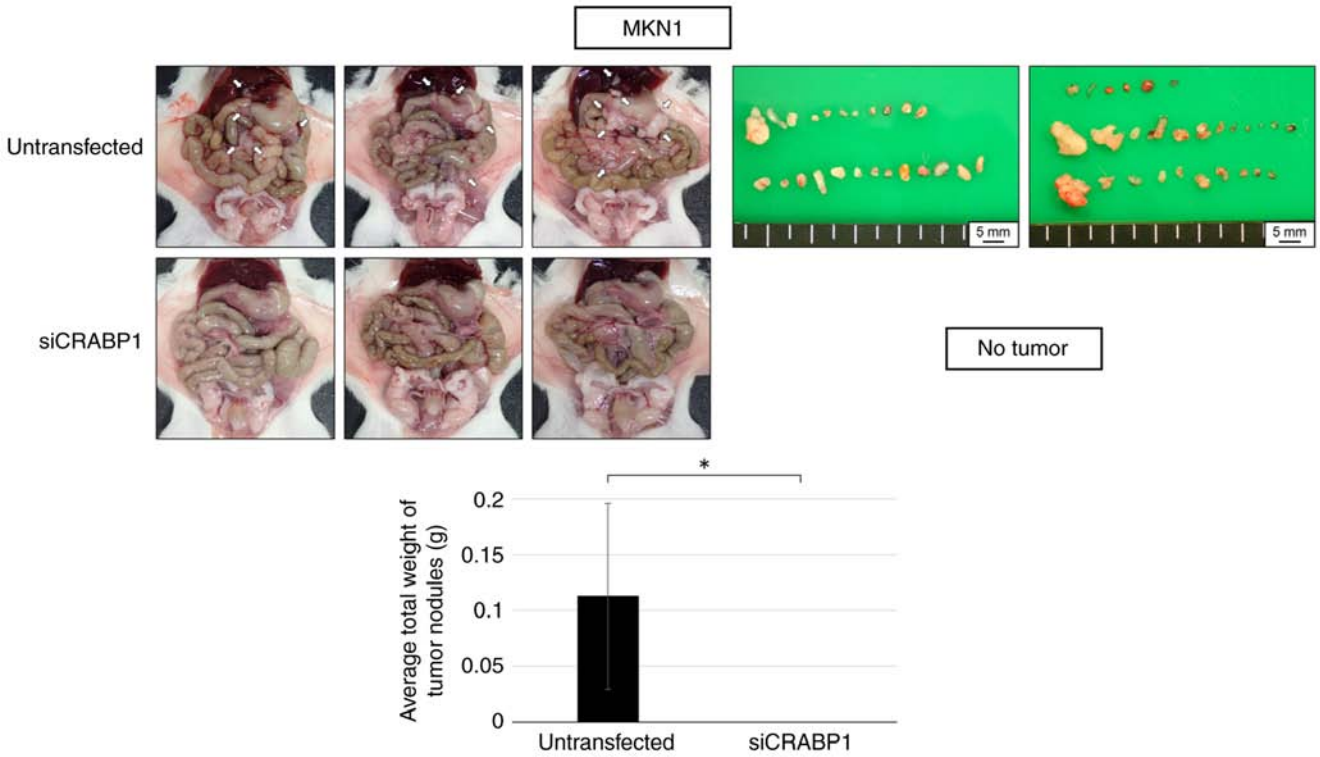

Figure 5. Effect of *CRABP1* knockdown on peritoneal metastasis formation in mouse xenograft models of MKN1 cells. Left images show dissemination of representative tumors in the peritoneal cavities of mice. Right panels present all tumor nodules and the bottom graph shows the average total weight of tumor nodules. *P<0.05. Error bars indicate standard deviation. si-, small interfering; CRABP1, cellular retinoic acid binding protein 1.

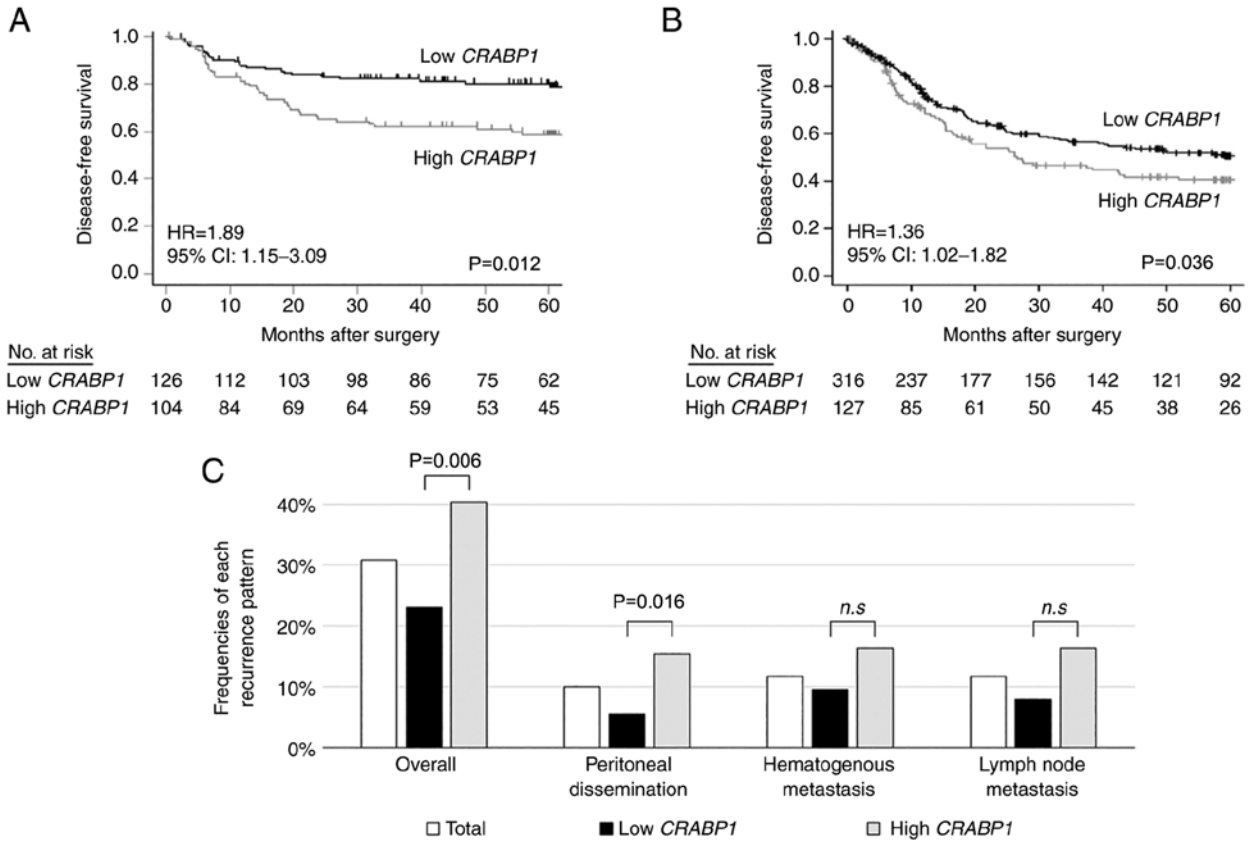

Figure 6. Prognostic implications of *CRABP1* mRNA expression in patients with gastric cancer. (A) Kaplan-Meier analysis of disease-free survival in the institutional cohort. The present dataset consisted of 230 clinical samples who underwent surgical resection for stages I-III gastric cancer. (B) Kaplan-Meier analysis of disease-free survival in the external validation cohort from the integrated Kaplan-Meier plotter dataset (http://kmplot.com/analysis/). (C) Frequencies of the sites of initial recurrence after curative gastrectomy according to *CRABP1* expression. CRABP1, cellular retinoic acid binding protein 1; CI, confidence interval; HR, hazard ratio; n.s, not significant.

Table III. CRABP1 expression and the clinical characteristics of patients with gastric cancer.

Clinical characteristics	Expression level of CRABP1 Low (n=126)	High (n=104)	P-value
Age, years			0.687
<70	74	64	
≥70	52	40	
Sex			0.769
Male	89	76	
Female	37	28	
CEA (ng/ml)			0.850
≤5	107	90	
>5	19	14	
CA19-9 (IU/ml)			0.382
≤37	102	89	
>37	24	15	
Tumor location			0.992
Entire	4	4	
Upper third	34	27	
Middle third	43	37	
Lower third	45	36	
Tumor size (mm)			0.562
<50	68	56	
≥50	58	48	
Macroscopic type			0.376
Borrmann type 4/5	10	12	
Others	116	92	
Multifocal lesions			0.823
Absent	115	94	
Present	11	10	
Tumor depth (UICC)			0.581
pT1-3	83	64	
pT4	43	40	
Differentiation			1.000
Differentiated	54	45	
Undifferentiated	72	59	
Lymphatic involvement			0.288
Absent	24	14	
Present	102	90	
Vascular invasion			0.077
Absent	55	33	
Present	71	71	
Infiltrative growth			0.886
Absent	58	29	
Present	68	75	
Lymph node metastasis			0.006
Absent	58	29	
Present	68	75	
UICC stage			0.056
I	34	16	
II	40	31	
III	52	57	

CRABP1, cellular retinoic acid-binding protein 1; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control.

findings suggested that high expression of *CRABP1* is associated with lymph node metastasis and poor differentiation/high grade of pancreatic neuroendocrine tumors (22). Furthermore, a previous study revealed that *CRABP1* expression is associated with poor prognosis of patients with breast cancer, which reflects high Ki67 immunoreactivity and a high pathological grade (23). Thus, the relationships between *CRABP1* expression and cancer varies among organs, suggesting that *CRABP1* may possess unidentified functions.

Metastasis that leads to cancer recurrence involves factors such as adhesion, infiltration, and angiogenesis, as the EMT contributes to cancer progression and metastasis (24-26). For example, the present PCR array results showed that *CRABP1* expression significantly and positively correlated with that of numerous EMT-promoting factors. Moreover, *CRABP1* expression negatively correlated with the expression of *TFPI2*, which is often suppressed during the EMT; and the gene encoding TFPI2 is frequently methylated in gastric cancers (27,28). These results suggested that *CRABP1* is coordinately expressed with cancer-related molecules and may promote peritoneal dissemination of gastric cancer through the EMT.

Furthermore, siRNA-mediated knockdown of *CRABP1* expression reduced the proliferative, invasive and migratory capacities of gastric cancer cells. Proliferation and invasion of gastric cancer cells are required for their migration from the primary tumor site, passage through endothelial cells, and invasion of lymphatic and blood vessels, which culminates in the colonization of lymph nodes and target organs, as well as the proliferation of cancer cells in the parenchyma (29).

In a mouse xenograft model of peritoneal metastasis of gastric cancer, it was found that the total weight of disseminated nodules was lower in the group, in which *CRABP1* mRNA levels were knocked down compared with those of the untransfected group. These results suggested that *CRABP1* is involved in the recurrence of peritoneal dissemination of gastric cancer. In the present study, high expression of *CRABP1* in gastric cancer tissues was associated with a higher recurrence

Table IV. Prognostic factors for disease-free survival of patients with gastric cancer.

	Univariate			Multivariable		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥70 years)	0.82	0.50-1.34	0.420			
Sex (female)	1.06	0.63-1.76	0.834			
CEA (>5 ng/ml)	1.39	0.74-2.58	0.304			
CA 19-9 (>37 IU/ml)	2.35	1.37-4.03	0.002	1.82	1.03-3.22	0.040
Tumor location (lower third)	0.76	0.46-1.27	0.297			
Tumor size (≥50 mm)	1.95	1.21-3.15	0.006	1.44	0.88-2.35	0.145
Macroscopic type (Borrmann type 4/5)	2.32	1.27-4.24	0.007	1.26	0.65-2.45	0.487
Multifocal lesions	0.91	0.39-2.09	0.816			
Tumor depth (pT4, UICC)	2.55	1.59-4.08	<0.001	1.63	0.96-2.78	0.073
Tumor differentiation (undifferentiated)	1.59	0.97-2.60	0.068			
Lymphatic involvement	4.12	1.50-11.30	0.006	0.93	0.29-3.04	0.932
Vascular invasion	2.66	1.52-4.65	<0.001	1.34	0.72-2.48	0.359
Invasive growth	1.66	1.03-2.69	0.038	1.12	0.64-1.97	0.687
Lymph node metastasis	7.97	3.63-17.49	<0.001	4.94	2.03-12.03	<0.001
High *CRABP1* expression	2.07	1.28-3.35	0.003	1.89	1.15-3.09	0.012

CRABP1, cellular retinoic acid-binding protein 1; CI, confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control.

rate, shorter DFS and significantly more frequent peritoneal dissemination, leading to recurrence. These results indicated that preoperative and intraoperative analysis of *CRABP1* expression may predict the risk of peritoneal dissemination recurrence after curative resection.

Thus, evaluating the expression of *CRABP1* as a biomarker of patients at high risk of peritoneal dissemination may inform decisions on implementing a surveillance plan that considers the course of peritoneal dissemination after surgery. Specifically, closely spaced abdominal echocardiography and computed tomography of the pelvis can be used to detect small amounts of ascites and small peritoneal nodules. Furthermore, the present data have important clinical implications for administering adjuvant chemotherapy to patients with high tissue levels of *CRABP1* mRNA after resection of gastric cancer to reduce their risk of recurrence.

There are several limitations to the present study. First, the clinical impact of *CRABP1* expression was retrospectively evaluated. Second, the clinical samples of the present study were insufficient to evaluate *CRABP1* as a biomarker to detect disseminated metastasis. A prospective observational study of clinical samples, including disseminated metastasis, is therefore required to evaluate the prognostic ability of *CRABP1* expression levels. Third, the detailed molecular mechanisms underlying the correlation between high *CRABP1* expression and postoperative prognosis, including disseminated recurrence, must be determined. Identification of the relevant signal transduction pathways is required to fully understand the role of *CRABP1* in tumor progression. In breast cancer cells, *CRABP1* sequesters all-trans-retinoic acid (atRA) in the cytosol, inhibiting its nuclear action (23). Evaluating the expression levels of *CRABP1* in gastric cancer cells and the effects of atRA on the tumor may further illuminate their mechanism of action related to malignancy.

In summary, it was revealed in the present study that *CRABP1* influenced the malignant phenotype of gastric cancer cells and that its high expression in primary tumor tissues may serve as a biomarker for determining the prognosis of recurrence after curative resection, particularly that of patients with peritoneal dissemination.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

KS, MK, and SN performed the experiments and data analysis. KS, MK, DS, SN, YI, NH, MH, CT, GN, and YK collected

cases and clinical data. KS and MK confirm the authenticity of all the raw data. KS and MK conceived and designed the study and prepared the initial draft of the manuscript. YK supervised the project. All authors contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (2013). The present study was approved (approval no. 2014-0043) by the Institutional Review Board of Nagoya University (Nagoya, Japan). Written informed consent was obtained from all patients. Animal experiments were approved (approval no. M210414-001) by the Animal Research Committee of Nagoya University (Nagoya, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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