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

KAZUKI SAKATA, MITSURO KANDA, DAI SHIMIZU, SHUNSUKE NAKAMURA, YOSHIKUNI INOKAWA, NORIFUMI HATTORI, MASAMICHI HAYASHI, CHIE TANAKA, GORO NAKAYAMA and YASUHIRO KODERA

Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8550, Japan

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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.

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.

Key words: gastric cancer, cellular retinoic acid binding protein 1, peritoneal recurrence, biomarker, expression
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, GC1Y, IM95, KATO III, MKN1, MKN7, MKN45, MKN74, NUGC2, NUGC3, NUGC4, N87, OCM1M1 and SC-6-JCK) were obtained from the American Type Culture Collection (ATCC) or the Japanese Collection of Research Bioresources (JCRB) or the Japanese Collection of Research Bioresources 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.

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 oligonucleotide (cat. no. 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 30 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, cytokkeleton 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). siCRABP1-1 and siCRABP1-2 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.

<table>
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<th>Experiment</th>
<th>Primer sequence (5'→3')</th>
<th>Product size (base pairs)</th>
<th>Annealing temperature (°C)</th>
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<td>RT-qPCR</td>
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<td>60</td>
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<tr>
<td>GAPDH</td>
<td>RT-qPCR</td>
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<td>60</td>
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</table>

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.

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 1.5x10^5, 1.5x10^5 and 5x10^5 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 random fields as previously described (13). Cell migration was evaluated using wound-healing assays according to the manufacturer's protocol. 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 siCRABP1 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% CO₂ for 5 min and were observed for 20 min after confirmation of respiration cease. The flow rate of CO₂ 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...
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 $\chi^2$ 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 performed 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, GC1Y, 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.
Effect of CRABP1 knockdown on the biological activities of gastric cancer cells. The efficiency of CRABP1 knockdown by transfection of siCRABP1-1 and siCRABP1-2 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

<table>
<thead>
<tr>
<th>Function</th>
<th>Symbol</th>
<th>Name</th>
<th>GC/Normal</th>
<th>Meta/GC</th>
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<td>Homeobox C10</td>
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</table>

GC, primary gastric cancer tissue; Normal, corresponding adjacent normal gastric tissue; Meta, hepatic metastasis tissue; TNF, Tumor necrosis factor; ETS, erythroblast transformation-specific.
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 siCRABP1 group (Fig. 5). Peritoneal metastasis in the NUGC4-model mice was disseminated to a smaller extent in the siCRABP1 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...
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.
findings suggested that high expression of \( \text{CRABP1} \) is associated with lymph node metastasis and poor differentiation/high grade of pancreatic neuroendocrine tumors (22). Furthermore, a previous study revealed that \( \text{CRABP1} \) 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 \( \text{CRABP1} \) expression and cancer varies among organs, suggesting that \( \text{CRABP1} \) 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 \( \text{CRABP1} \) expression significantly and positively correlated with that of numerous EMT‑promoting factors. Moreover, \( \text{CRABP1} \) expression negatively correlated with the expression of \( \text{TFPI2} \), which is often suppressed during the EMT; and the gene encoding \( \text{TFPI2} \) is frequently methylated in gastric cancers (27,28). These results suggested that \( \text{CRABP1} \) is coordinately expressed with cancer‑related molecules and may promote peritoneal dissemination of gastric cancer through the EMT.

Furthermore, siRNA‑mediated knockdown of \( \text{CRABP1} \) 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 \( \text{CRABP1} \) mRNA levels were knocked down compared with those of the untransfected group. These results suggested that \( \text{CRABP1} \) is involved in the recurrence of peritoneal dissemination of gastric cancer. In the present study, high expression of \( \text{CRABP1} \) in gastric cancer tissues was associated with a higher recurrence
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 summary, it was revealed in the present study that CRABP1 influenced the malignant phenotype of gastric cancer cells, and its high expression in primary tumor tissues may serve as a biomarker for determining the prognosis of recurrence, 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, Y1, 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.

References