the initial tumor size was also independently associated with the individual size reduction rate ($P < .001$).

COMMENT

It has long been proposed that bulky disease is an adverse prognostic factor during chemotherapy for lymphoma or solid cancer.[8,9] In contrast, the significance of bulky disease when using target therapies has not yet been made clear. For instance, many had assumed that antibody therapy would be ineffective against bulky disease; however, a Phase II study of rituximab (anti-CD20 antibody) for bulky (>10 cm) low-grade lymphoma showed that standard rituximab therapy resulted in a good response rate (43%). In addition, however, they showed that the serum antibody concentration correlated negatively with the baseline tumor bulk.[10] Antiangiogenesis therapy had also been assumed to be ineffective against bulky disease, but this has not been clinically proved. We have demonstrated for the first time that the initial tumor size correlated negatively with the tumor reduction rate in targeted therapy for mRCC.

Up-front cytoreductive nephrectomy, followed by systemic therapy, been established as the standard of care for mRCC in the cytokine era.[1,2] Even for the patients with high-risk and locally advanced RCC, neither preoperative nor postoperative medical treatment has been recommended because of the real lack of effective systemic therapiespreviously available. Therefore, the treatment strategy for RCC must be reconsidered in this targeted therapy era.

According to our results, large tumors will seldom become smaller when TKIs are administered. Therefore, it might be infrequent that an unresectable tumor would become resectable, although no objective criteria exist to define surgical resectability. We believe that the greatest benefit of preoperative approaches in the setting of mRCC is that they can as a litmus test to reserve cytoreductive nephrectomy for only those whowill benefit from the procedure.

Possibly, the resolution of CT scans could affect the tumor size reduction rate. Small lesions might appear to shrink more owing to slice variation and not true size changes. However, we used multidetector CT, which scans every 5 mm. The minimal initial tumor size was 10 mm in the present study, because we excluded tumors that were <10 mm in diameter. Therefore, we believe that the potential issues regarding the resolution of the CT scans did not have a major effect on our conclusions. In addition, when micrometastasis is considered to be of a very small size, adjuvant therapy after radical nephrectomy could meet our expectation of reduced recurrence. Sorafenib and sunitinib have been the focus of adjuvant therapy for patients with resected primary tumors with a high risk of recurrence. Three randomized trials are comparing these agents to placebo in the adjuvant setting: Sunitinib Treatment in Renal Adjuvant Cancer (S-TRAC), Adjuvant Sorafenib or Sunitinib for Unfavorable Renal Carcinoma (ASSURE), and Sorafenib With Placebo in Patients With Resected Primary Renal Cell (SORCE).[11]

Very recently, a study similar to ours was published by Han et al.[12] In their study, the initial tumor enhancement on contrast-enhanced CT could be useful as a clinical predictor during targeted therapy, because it was associated with tumor size reduction of the individual metastases in patients with mRCC who had received targeted therapy.[12] Because of the antiangiogenic therapy, their rationale was quite reasonable. Tumor enhancement was associated not only with tumor size reduction, but also with progression-free survival of the treated patients. However, compared with their study, our study was simpler and could be performed without contrast medium, which can be detrimental to patients with a solitary kidney. In addition, it can be adapted for patients with renal dysfunction, as well as patients who are allergic to contrast medium.

In addition to the treatment paradigm of RCC, our results suggest a weakness in the RECIST, currently the most commonly used system to determine the response in clinical trials and clinical practice. In our study, the longest diameter of large tumor demonstrated a relatively smaller reduction rate than thatof the metastatic small tumor. Therefore, large primary tumors will have an important effect on the overall objective when these are included in the RECIST measurements.Therefore, whether the target lesion includes the large primary and/or metastatic lesions should be considered in calculating the overall response according to the RECIST.

CONCLUSIONS

The initial tumor size was inversely associated with the tumor reduction rate of the individual metastatic sites and primary tumors in patients with mRCC who underwent targeted therapy. Although our small study was preliminary and additional investigations are necessary, we believe that this simple observation might be useful for physicians who treat patients with mRCC, as exemplified by the consideration of the pre- and postoperative approaches. In addition, in assessing clinical trials of targeted agents for metastatic RCC using the RECIST, we might need to consider this association.

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Original Article

Circulating Endothelial Progenitors and CXCR4-Positive Circulating Endothelial Cells Are Predictive Markers for Bevacizumab

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BACKGROUND: Bevacizumab plus chemotherapy is a standard option in the treatment of metastatic colorectal cancer (mCRC). The aim of this study was to investigate the potential of circulating endothelial cell progenitors (CEPs) and phenotypical circulating endothelial cells (CECs) as surrogate markers of clinical outcome in mCRC patients to identify responders to bevacizumab in combination with chemotherapy. **METHODS:** A total of 69 patients with measurable mCRC were enrolled in this prospective study. Whole blood samples were analyzed before initiation of treatment and on days 4 and 14. Phenotypical CECs and CEPs were then isolated and enumerated by using flow cytometry. **RESULTS:** CEP levels of less than 0.04% on day 4 were significantly associated with longer progression-free survival (PFS) and overall survival (OS) ($P < .001$, $P = .002$, respectively) as compared with levels of 0.04% or more. In addition, CXCR4-positive CEC levels of less than 20% at baseline were significantly associated with longer PFS and OS as compared other indicators investigated ($P < .001$, $P = .002$, respectively). **CONCLUSIONS:** Levels of CEPs on day 4 and proportion of CXCR4-positive CECs at baseline were correlated with the prognosis of bevacizumab combination chemotherapy, suggesting that these surrogate markers may play a core role in the selection of candidates for bevacizumab treatment. ***Cancer* 2011;00:000–000.** © *2011 American Cancer Society.*

KEYWORDS: circulating endothelial progenitors, CXCR4-positive circulating endothelial cells, bevacizumab, metastatic colorectal cancer, chemotherapy.

Antiangiogenic agents such as bevacizumab that target the vascular endothelial growth factor (VEGF) pathway have shown promise in the treatment of a variety of malignancies.[1] However, clinical biomarkers are needed for quantitative evaluation of the effect of bevacizumab.

VEGF is known to promote the mobilization of bone-marrow–derived circulating endothelial progenitors (CEPs) and survival by activating antiapoptotic pathways in circulating endothelial cells (CECs),[2–4] which may subsequently differentiate into mature endothelial cells.[5,6] Recently, CEPs were reported to be involved in tumor angiogenesis in tumor implantation models[7–10] and in clinical studies.[11,12] According to several clinical reports, baseline CEC levels in cancer patients have shown higher values compared with those in healthy controls and were correlated with response and outcome.[13–15]

The aim of this study was to investigate the potential of CEPs and phenotypical CECs as surrogate markers of clinical outcome in metastatic colorectal cancer (mCRC) patients to identify responders to chemotherapy with bevacizumab.

MATERIALS AND METHODS

Patients

Principal inclusion criteria were measurable mCRC and commencement of a new systemic therapy. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, adequate organ function, and

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radiographic evidence of disease progression as defined by the Response Evaluation Criteria in Solid Tumors (RECIST). All patients were enrolled on protocols approved by the institutional review board at the Cancer Institute Hospital in The Japanese Foundation for Cancer Research. Written informed consent was obtained from all patients.

Assessment of Biomarkers

Whole blood samples were collected and analyzed at the following times: before initiation of treatment (baseline), immediately after completion of 1 course (day 4), and before commencement of a second cycle (day 14). Blood samples were drawn into 8.5-mL evacuated tubes (BD Biosciences, Franklin Lakes, NJ).

Mononuclear cells isolated by density gradient centrifugation were analyzed using the method established by Duda DG et al.[16] Briefly, Ficoll gradient was used to isolate peripheral blood mononuclear cells (PBMC) and remove red cells and platelets before incubation with antibodies. The following directly conjugated monoclonal antibodies were used for detection of CECs and CEPs by 4-color flow cytometry in peripheral blood: anti-CD31-FITC (BD Pharmingen, San Diego, Calif), anti-CD133-PE (Miltenyi Biotec, Auburn, Calif), anti-CD34-APC (BD Pharmingen), and anti-CD45-PerCP/Cy5.5 (BD Pharmingen). The proportions of CECs (CD31-positive and CD45 negative fractions) and CEPs (CD31-positive, CD34 highly positive, CD133-positive, and CD45 dimly positive fractions) were calculated as percentages of the total number of mononuclear cells after evaluation of at least 50,000 cellular events. Phenotypical CECs expressing VEGFR1, VEGFR2, Tie-2, or CXCR4 were also analyzed. The proportions of these CEC phenotypes were calculated as percentages of the total number of CECs.

Observation of CECs and CEPs

For morphological and immunohistological observation of CECs and CEPs, a small portion of mononuclear cells was fractionated into CXCR4-positive CECs or CEPs by using FACSVantage (Becton Dickinson, Franklin Lakes, NJ). The nuclei of the isolated live CECs and CEPs were stained with DRAQ5 (Alexis, now part of Enzo Life Sciences, Farmingdale, NY) and then observed by confocal laser scanning microscopy (FV1000; Olympus, Center Valley, Penn).

Table 1. Characteristics of Patients Treated With FOLFOX Plus Bevacizumab

Characteristics	Regimen
N=69	FOLFOX+bevacizumab
Median age (range)	61 (27-73)
Sex men/women	38/31
Primary site rectum/colon	24/45
Prior colorectomy +/-	6/63
Metastatic site	
Liver	37
Lung	36
LN	28
Local recurrence	5
Peritoneum	17
Bone	3
Chemotherapy +/-	14/55
5-FU	7
Other	7
CR/PR/SD/PD	2/46/15/6

CR indicates complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Statistical Analysis

Kaplan-Meier survival plots were generated based on CEC levels at each time point of blood sampling, and the curves were compared by using the log-rank test. The Cox proportional hazards regression model was used to determine univariate and multivariate hazard ratios for progression-free survival (PFS) and overall survival (OS).

RESULTS

Patient Characteristics

A total of 69 patients were enrolled. Patient characteristics at baseline are summarized in Table 1. Among 69 patients treated with FOLFOX4 plus bevacizumab assessable for response, we observed complete response in 2 (3%), partial response in 46 (67%), stable disease in 15 (22%), and progressive disease (PD) in 6 (8%) during treatment. Overall response rate was 70%.

Relation Between CEP Levels and Outcome

Univariate Cox regression analysis revealed that CEP levels on day 4 were significantly associated with PFS in 30 of the 69 patients in the training set. To identify the level of CEPs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 0.01%-0.20% of the total number of PBMCs on day 4 were systematically correlated with PFS. Median PFS in patients with levels above or below each threshold differed at 0.04% CEPs of the total number of PBMCs, reaching a plateau at approximately that level. At this level, the Cox

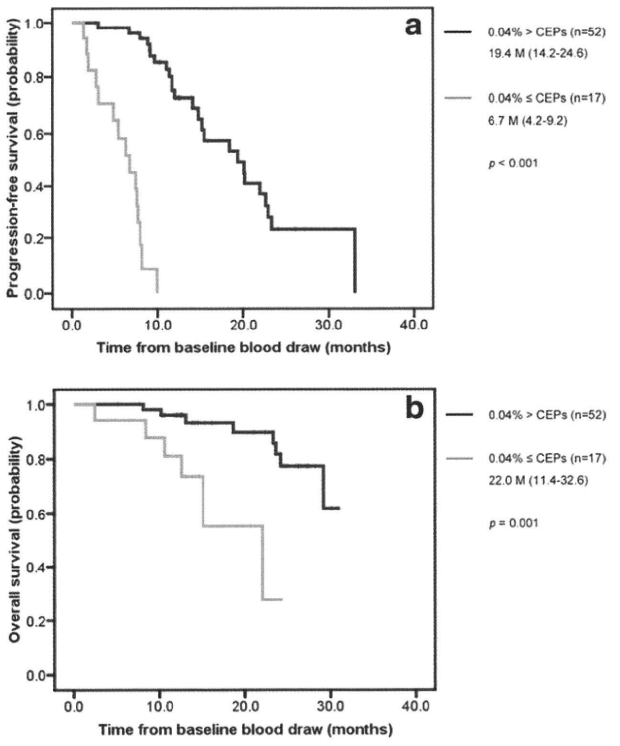

Figure 1. Depicted are (a) Kaplan-Meier plots of progression-free survival (PFS) and (b) Kaplan-Meier plots of overall survival (OS).

proportional-hazard ratio signifying the difference between slow and rapid progression of disease also reached a peak. Therefore, a cutoff of 0.04% CEPs was chosen to distinguish patients. The Kaplan-Meier 0.04% CEP counts were available on day 4 for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 0.04% or more CEPs on day 4 had a shorter median PFS (6.7 months; 95% CI, 4.2-9.2 months) than those with less than 0.04% CEPs on day 4 (19.4 months; 95% CI, 14.2-24.6 months) ($P < .001$) (Fig. 1a). Patients with 0.04% or more CEPs on day 4 had a shorter median OS (22 months; 95% CI, 11.4-32.6 months) than those with less than 0.04% CEPs on day 4 ($P = .001$) (Fig. 1b).

Relation Between CEC Phenotype and Efficacy

Levels of CXCR4 in patients with PD were significantly higher than in those with no PD. Other phenotypes showed no differences between patients with PD and those without (Fig. 2).

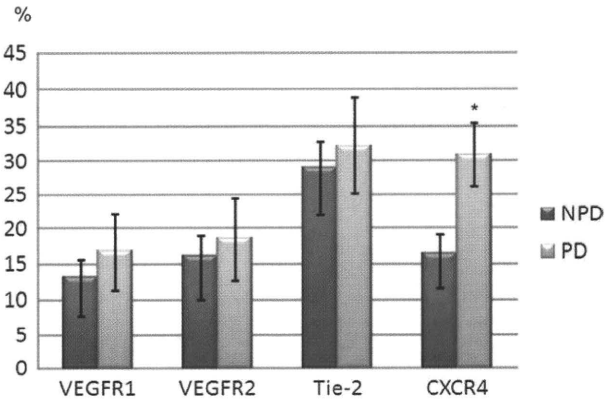

Figure 2. The relation is shown between levels of CEC phenotypes at baseline and bevacizumab efficacy in bevacizumab combination chemotherapy. PD indicates progressive disease; NPD, nonprogression disease. Results are expressed as mean ± standard error of the mean (SE). *$P < .05$

Relation Between CEC Phenotype and Outcome

According to univariate Cox regression analysis, CEC levels at baseline were significantly associated with PFS. To explore the predictive potential of CEC phenotypes at baseline, we analyzed the relation between baseline levels of CEC phenotypes and PFS. Univariate Cox regression analysis revealed that CXCR4-positive CEC levels at baseline were significantly associated with PFS. On the other hand, no correlation was observed between baseline VEGFR1-positive, VEGFR2-positive, or Tie-2–positive CEC levels and PFS. To identify the level of CXCR-positive CECs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 1% to 45% of the total number of CECs at baseline were systematically correlated with PFS. Median PFS in patients with levels of above or below each threshold differed at 20% CXCR4-positive CECs. At this level, the Cox proportional-hazards ratio signifying the difference between slow and rapid progression of disease also reached a plateau. Therefore, a distinguishing cutoff of 20% CXCR4-positive CECs was chosen. The Kaplan-Meier CXCR4-positive CEC count was available at baseline for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. No significant difference was observed in either PFS or OS in either set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 20% or more CXCR4-positive CECs at baseline had a shorter median PFS (6.7 months; 95% CI, 4.1-9.3 months) than those with less than 20% CXCR4-positive

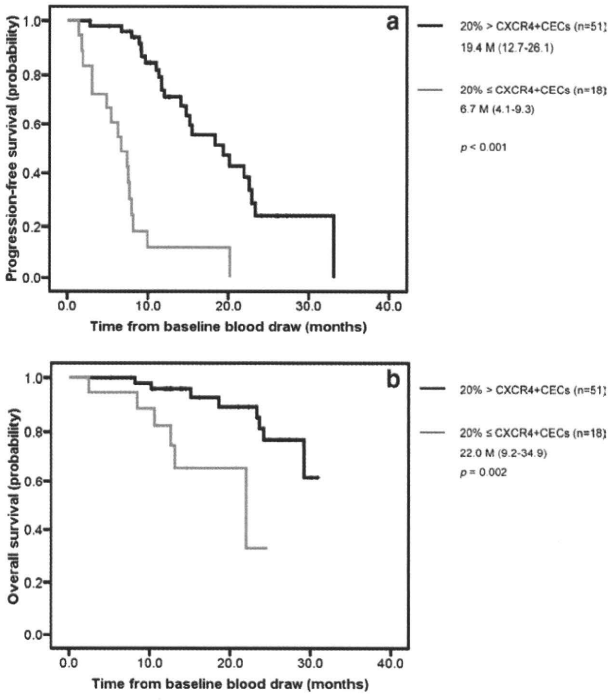

Figure 3. Depicted are Kaplan-Meier plots of (a) progression-free survival (PFS) and (b) overall survival (OS).

CECs at baseline (19.4 months; 95% CI, 12.7-26.1 months) ($P < .001$) (Fig. 3a). Patients with 20% or more CXCR4-positive CECs at baseline had a shorter median OS (22 months; 95% CI, 9.2-34.9 months) than those with less than 20% CXCR4-positive CECs at baseline ($P = .002$) (Fig. 3b).

Univariate and multivariate Cox proportional hazards regression was performed to assess the association between factors of interest and PFS or OS. According to the univariate Cox regression analysis, liver metastasis, lung metastasis, CEP levels on day 4, and CXCR4-positive CEC levels at baseline were associated with PFS; furthermore, peritoneal metastasis, CEP levels on day 4, and CXCR4-positive CEC levels at baseline were associated with OS (Table 2). To evaluate the independent predictive effect of these markers, multivariate Cox regression analysis was carried out (Table 3). Levels of CEP on day 4 and CXCR4-positive CEC levels at baseline were the strongest predictors.

DISCUSSION

Some authors have suggested that CECs are a predictive marker of clinical outcome in cancer patients treated with bevacizumab-based chemotherapy. In breast cancer, most studies[14,17,18] have reported that high CEC levels at baseline indicate a better outcome than low CEC levels. On the other hand, in colorectal cancer, low CEC levels at baseline were reported to indicate a better outcome than high CEC levels.[19,20] These results suggest vascular formation differs according to tumor origin. However, these differences in results between these 2 types of cancer may have resulted from differences in the measurement protocols used. A number of methods and protocols are used to evaluate and count CECs. Two widely used protocols involve the use of flow cytometry. Duda et al[16] reported a cytometry protocol for phenotypic identification and enumeration of CECs and CEPs using 4 surface markers: CD31, CD34, CD133, and CD45. This procedure is believed to allow detection of 0.1% to 6.0% of viable CECs and 0.01% to 0.20% of CEPs from among a blood mononuclear cell population and is mainly used in colorectal cancer. Mancuso et al[21] reported a protocol for the phenotypic identification and enumeration of CECs and CEPs involving 6-color flow cytometry, nuclear staining with Syto16 (Molecular Probes, Eugene, Ore) and 7-AAD (Flow Labs, Irvine, UK) and a panel of monoclonal antibodies, including CD45, CD133, CD31, and CD146. This protocol has been mainly used in breast cancer. In this study, we selected the protocol of Duda et al.

Willet et al[19] reported that CEP levels decreased on day 3 after initiation of bevacizumab with chemoradiation in rectal cancer patients. On the basis of this earlier report, we decided, in this study, to collect samples at 3 days (day 4) after initiation of chemotherapy with bevacizumab. We

Table 2. Independent Predictive Factors by Univariate Cox Regression Analysis for Progression-Free Survival and Overall Survival

Parameter	No. of Patients	HR	95% CI	*P*	X^2
PFS					
CEP	69	7.01	3.5-14.05	<.001	<.001
CXCR4+CEC	69	22.96	8.52-61.87	<.001	<.001
Liver metastasis	69	2.71	1.36-5.38	.004	.003
Lung metastasis	69	2.44	1.22-4.90	.012	.009
OS					
CEP	69	5.45	1.71-17.4	.004	.002
CXCR4+CEC	69	5.26	1.64-16.9	.005	.002
Peritoneal metastasis	69	3.46	1.16-10.33	.026	.018

HR indicates hazard ratio; CI, confidence interval; PFS, progression-free survival; CEP, circulating endothelial progenitor; CEC, circulation endothelial cell; OS, overall survival.

Table 3. Independent Predictive Factors by Multivariate Cox Regression Analysis for Progression-Free Survival and Overall Survival

		HR	95% CI	*P*	Model χ^2
PFS					
No. of patients	69				<.001
CEP		27.71	9.51-80.72	<.001	
Liver metastasis		2.95	1.46-5.95	.002	
No. of Patients	69				<.001
CXCR4+CEC		15.71	6.31-39.13	<.001	
Liver metastasis		2.71	1.33-5.55	.006	
Bone metastasis		0.09	0.02-0.48	.005	
OS					
No. of patients	69				<.001
CEP		8.90	2.48-31.93	.001	
Peritoneal metastasis		5.49	1.71-17.66	.004	
No. of Patients	69				<.001
CXCR4+CEC		6.14	1.85-20.41	.003	
Peritoneal metastasis		9.85	2.59-37.43	.001	

HR indicates hazard ratio; CI, confidence interval; PFS, progression-free survival; CEP, circulating endothelial progenitor; CEC, circulation endothelial cell; OS, overall survival.

found that bevacizumab combination therapy resulted in a marked and significant decrease in CEP levels on day 4 in comparison with those at the other time points selected. Levels of CEP on day 4 were the strongest predictor of PFS and OS. These results suggest that bevacizumab inhibits bone marrow-dependent tumor vasculogenesis by reducing endothelial progenitor cells mobilizing from bone marrow into the peripheral blood and reducing the proliferation of CEPs. Based on these results, we believe that if CEP levels do not decrease immediately after initiation of bevacizumab, then the patient must be considered unresponsive, and that it would not be beneficial to continue.

These results support the view of Ronzoni et al[20] that low CECs at baseline are indicative of longer PFS. Ronzoni reported that low levels of total CECs at baseline were correlated with improved PFS, but not significantly so. However, analysis of resting CEC levels at baseline revealed a significant correlation with improved PFS, indicating the potential of phenotypical subgroups of CECs as biological markers. Torrisi et al[18] reported that VEGFR-1–positive CEC levels showed a significant increase with bevacizumab-combination treatment. To explore the predictive potential of CEC phenotypes that express markers such as VEGFR1, VEGFR2, Tie-2, and CXCR4 at baseline, we analyzed the relation between baseline levels of CEC phenotypes and bevacizumab efficacy. We found that a lower ratio of CXCR4-positive CECs at baseline may indicate a beneficial effect for bevacizumab treatment. Xu et al[22] reported that bevacizumab upregulated stromal cell-derived factor 1alpha (SDF-1alpha) and its receptor, CXCR4, and that higher SDF-1alpha plasma levels during bevacizumab treatment were significantly associated with distant metastasis at 3 years. Siegel et al[23] reported that SDF-1 levels decreased from baseline in all patients after 8 weeks of bevacizumab, with an increase noted at time of progression. Their results suggest that SDF-1 is a resistance factor for bevacizumab, with SDF-1 inducing CXCR4-positive CECs in peripheral blood. Several studies[24,25] reported that the SDF-1/CXCR4 axis may contribute to functional vascular establishment and that the antiangiogenic effects of the blockade of CXCR4 are related to a reduction in the establishment of tumor endothelium independent of VEGF inhibition. Therefore, we confirmed differentiation by pathology between CEPs and CXCR4-positive CECs. Live CEPs sorted by flow cytometry were observed by using confocal microscopy, and cell surface expression of CD31 and CD34 was confirmed (Fig. 4a). Similarly, live CXCR4-positive CECs were also observed. The nuclear/cytoplasm ratio of CEPs was higher than that of CXCR4-positive CECs (Fig. 4b). The cell nuclei of the CEPs were mononuclear, but those of CXCR4-positive CECs were lobulated. These results indicate that the CEPs and CXCR4-positive CECs were different populations and that the CEPs were more immature than the CXCR4-postive CECs. Our findings suggest that activation of CXCR4-positive CECs may be responsible for

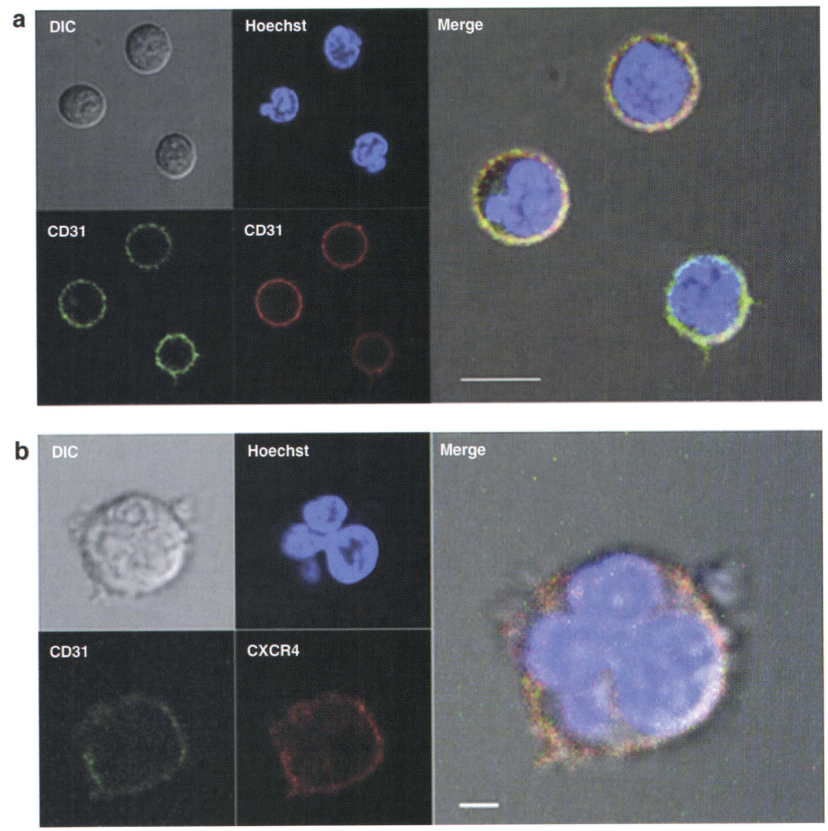

Figure 4. (a) CEPs and (b) CXCR4 + CECs were sorted by flow cytometry as described in the Materials and Methods section and analyzed by confocal microscopy. DIC indicates differential interferences contrast; bar, 5 μm.

angiogenesis occurring in cases where the VEGF antibody, bevacizumab, has proved ineffective. However, this also suggests that resistance to the antiangiogenic effects of bevacizumab may be neutralized by administration of SDF-1/CXCR4.

In conclusion, CEP levels on day 4 and proportions of CXCR4-positive CECs at baseline showed a correlation with prognosis in bevacizumab combination chemotherapy. This indicates the potential of these surrogate markers in the selection of candidates for bevacizumab treatment. Further research in the form of large-scale clinical trials is needed, however, to confirm these results.

CONFLICT OF INTEREST DISCLOSURES

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Circulating tumor cells as a surrogate marker for determining response to chemotherapy in patients with advanced gastric cancer

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The purpose of this study was to quantify circulating tumor cells (CTCs) in advanced gastric cancer (AGC) patients, and to demonstrate the role of CTCs in cancer therapy. This study investigates the hypothesis that CTCs can predict clinical outcomes in patients with AGC. From November 2007 to June 2009, 52 patients with AGC were enrolled into a prospective study. The chemotherapy regimen was an S-1-based regimen (S-1 with or without cisplatin) or paclitaxel. CTCs of whole blood at baseline, 2 weeks, and 4 weeks after initiation of chemotherapy, were isolated and enumerated using immunomagnetics. Patients with ≥4 CTCs at 2-week points and 4-week points had a shorter median progression-free survival (PFS) (1.4, 1.4 months, respectively) than those with the median PFS of <4 CTCs (4.9, 5.0 months, respectively) (log-rank test; $P < 0.001$, $P < 0.001$, respectively). Patients with ≥4 CTCs at 2-week points and 4-week points had shorter median overall survival (OS) (3.5, 4.0 months, respectively) than those with the median PFS of <4 CTCs (11.7, 11.4 months, respectively) (log-rank test; $P < 0.001$, $P = 0.001$, respectively). In conclusion, this study demonstrates that CTC measurement may be useful as a surrogate marker for determining response to S-1-based or paclitaxel regimens in AGC. (*Cancer Sci* 2010; 101: 1067–1071)

Gastric cancer is more prevalent in Asia, Eastern Europe, and Central and South America than in other areas. In Japan, this cancer is one of the most common causes of cancer-related mortality, despite dramatic advances in diagnosis and treatment. Outcomes are extremely poor in patients with unresectable gastric cancer, with the median survival ranging from 3 to 5 months with the best supportive care.[1–3] The ability to identify patients with the worst prognoses or those destined to progress quickly could have broad clinical applications.

Circulating tumor cells (CTCs) or disseminated tumor cells (DTCs) in bone marrow and peripheral blood from patients with cancers have been documented.[4–6] Braun *et al.*[7,8] reported that ~30% of women with primary breast cancer have DTCs in bone marrow, and a 10-year follow-up of these patients revealed a significantly decreased disease-free survival and overall survival (OS) when compared with patients without DTCs. However, aspiration of bone marrow is time consuming and, in many cases, uncomfortable for the patients precluding multiple samplings for therapy monitoring studies. Therefore, recent efforts have concentrated on the detection of CTCs in the peripheral blood of cancer patients. Cristofanilli *et al.*[9,10] showed in a prospective study that CTC detection provided significant prognostic information for patients with metastatic breast cancer. Cohen *et al.*[11] showed that the number of CTCs before and during treatment was an independent predictor of PFS and OS in patients with metastatic colorectal cancer. It is not clear whether CTC detection using this system provides prognostic information for patients with advanced gastric cancer. We initiated this study to evaluate whether CTCs could serve as a prognostic and/or predictive marker in patients with AGC.

Materials and Methods

Patients. All patients were enrolled using institutional review board-approved protocols at the Cancer Institute Hospital at the Japanese Foundation for Cancer Research and provided informed consent. The study population consisted of patients aged 18 years or older with histologically proven AGC. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 2; adequate organ function; and S-1-based (S-1 with or without cisplatin) or paclitaxel chemotherapy regimen. The subjects were five patients treated with S-1 (40 mg/m^2, twice daily, days 1–28, repeated every 6 weeks), 26 patients treated with S-1 plus CDDP (S-1 40 mg/m^2, twice daily, days 1–21, CDDP 60 mg/m^2, day 8, repeated every 5 weeks), and 21 patients treated with paclitaxel (80 mg/m^2, weekly).

Sample preparation for isolation of CTCs from blood. Blood was drawn from advanced gastric cancer patients into 10 mL of evacuated blood for CTC in a Cell Save Preservative Tube (Veridex, Raritan, NJ, USA). Blood was always drawn from cancer patients before treatment initiation (baseline), 2 weeks, and 4 weeks after the administration of an S-1-based or paclitaxel regimen. The CellSearch system (Veridex) consists of the CellPrep system, the CellSearch Epithelial Cell Kit (for the measurement of CTC), and the CellSpotter Analyzer. The CellPrep system is a semi-automated sample preparation system, and the CellSearch Epithelial Cell Kit consists of ferrofluids coated with epithelial cell-specific EpCAM antibodies to immunomagnetically enrich epithelial cells; a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratin 8, 18, and 19; an antibody to CD45 conjugated to allophycocyanin; nuclear dye 4′,6- diamidino-2-phenylindole (DAPI) to fluorescently label the cell; and buffers to wash, permeabilize, and resuspend the cells. Sample processing and evaluation were done as described by Allard *et al.* Briefly, 7.5 mL of blood for CTCs were mixed with 6 mL of buffer, centrifuged at 800*g* for 10 min, and then placed on the CellPrep system. After aspiration of the plasma and buffer layer by instrument, ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were then added in conjunction with a permeabilization buffer to fluorescently label the immunomagnetically labeled cells. After incubation in the system, the magnetic separation was repeated, and

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excess staining reagents were aspirated. In the final processing step, the cells were resuspended in the MagNest Cell Presentation Device (Veridex). This device consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis using the CellSpotter Analyzer.

Sample analysis. The MagNest was placed on the CellSpotter Analyzer, a four-color semi-automated fluorescence microscope. Image frames covering the entire surface of the cartridge for each of the four fluorescent filter cubes were captured. The captured images containing objects that met predetermined criteria were automatically presented in a web-enabled browser from which final selection of cells was made by the operator. The criteria for an object to be defined as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratin, and negative staining for CD45. Results of cell enumeration are always expressed as the number of cells per 7.5 mL of blood for CTCs.

Statistical analysis. Progression-free survival (PFS) was defined as the elapsed time from blood collection to progression. Kaplan–Meier survival plots were generated based on CTC levels each time blood was collected, and the curves were compared using a log-rank testing. A P-value <0.05 was considered significant. Cox proportional hazards regression was used to determine univariate and multivariate hazard ratios for selected potential predictors of PFS and OS. The distribution of patients above and below the CTC threshold and clinical response was compared using Fisher's exact test.

Results

Patient characteristics. A total of 52 patients were enrolled. Patients' characteristics at baseline are summarized in Table 1. Patients' characteristics were as follows: median age, 62 years (range, 24–78 years); PS 0/1/2, 39/12/1; primary tumor +/−, 33/19; and regimen S-1/S-1 with cisplatin/paclitaxel, 5/26/21. Thirty-five patients had diffuse-type histology (67.3%). Seventeen patients (32.7%) had intestinal type. Among 52 patients, the best response rates were 28.8% (complete response [CR]/partial response [PR]/stable disease [SD]/progressive disease [PD]: 0/15/19/18). Of 31 patients treated with the S-1-based regimen (S-1 alone or S-1/cisplatin [CDDP]) assessable for response, we observed 14 PR (45.2%), 11 patients (35.5%) with SD, and six patients (19.4%) with PD during treatment. The overall response rate was 45.2%. On the other hand, of 21 patients treated with the weekly paclitaxel regimen assessable for response, we observed one PR (4.8%), eight patients (38.1%) with SD, and 12 patients (57.1%) with progression of disease during treatment, for an overall response rate (RR) of 4.8% (Table 2).

Table 1. Patient demographics

Demographic	Number or median (range)
Median age (range)	62 (24–78)
Male/female	44/8
PS: 0/1/2	39/12/1
S1-based/PAC regimen	31/21
Line: 1st/2nd	34/18
Histopathology: diffuse/intestinal type	35/17
Primary tumor: +/−	33/19
Sites of metastasis: +/−	
Liver	24/28
Lung	3/49
Bone	1/51
Peritoneum	22/30
Lymph node	37/15

Table 2. Objective response

	S1-based regimen (31) S1 alone (5), S1/CDDP (26) 1st line (31)	PAC (21) Weekly PAC (21) 1st line (3), 2nd line (18)
CR	0	0
PR	14	1
SD	11	8
PD	6	12

CDDP, cisplatin; CR, complete response; PAC, paclitaxel; PD, progressive disease; PR, partial response; SD, stable disease.

Stratification according to CTC levels. To select a level of circulating tumor cells that most clearly distinguished patients with a response of chemotherapy, thresholds of 1 to 88 cells for 2-week point were systematically correlated with PFS for 26 of the 30 patients in the training set. The median PFS among patients with levels above or below each threshold differed at the level of one circulating tumor cell per 7.5 mL of blood, and reached a plateau at approximately four cells per 7.5 mL of blood. At the latter level, the Cox proportional hazards ratio signifying the difference between slow and rapid progression of disease also reached a plateau. Thus, a cut-off of four circulating tumor cells per 7.5 mL of blood was chosen to distinguish patients.[12] The Kaplan–Meier circulating tumor-cell counts were available at a 2-week point for 26 of the thirty patients in the training set and for 21 of the 22 patients in the validation set. Neither PFS nor OS was significantly different in the two sets (data not shown). Because the two sets of data were nearly identical, they were combined for the estimation of PFS and OS for the entire population.

CTCs and imaging to assess response to therapy. Thirty-four (65.4%) of 52 patients were classified as having non-progressive disease (non-PD), with 24 of these patients (46.2%) having <4 CTCs and 10 patients (19.2%) having ≥4 CTCs before the initiation of therapy. Ten (19.2%) of 52 patients were classified as having PD, with 11 of these patients (21.2%) having <4 CTCs and seven patients (13.4%) having ≥4 CTCs before the initiation of therapy. The difference between the clinical responses and CTC levels were not significant. In contrast, 33 (64.7%) of 51 patients were classified as having non-PD, with 33 of these patients (64.7%) having <4 CTCs and no patients (0%) having ≥4 CTCs at 2 weeks. Eighteen (35.3%) of 51 patients were classified as having PD, with 11 of these patients (21.6%) having <4 CTCs and seven patients (13.7%) having ≥4 CTCs at 2 weeks. The difference between the clinical responses and CTC levels was highly significant. (P = 0.001, Fisher's exact test). Thirty-two (64%) of 48 patients were classified as having non-PD, with 31 of these patients (64.6%) having <4 CTCs and one patient (2.0%) having ≥4 CTCs at 4 weeks. Sixteen (33.3%) of 48 patients were classified as having PD, with eight of these patients (16.7%) having <4 CTCs and eight patients (16.7%) having ≥4 CTCs at 4 weeks. The difference between the clinical responses and CTC levels were highly significant (P < 0.001, Fisher's exact test) (Table 3).

Analysis of PFS according to CTC level. Figure 1 shows the Kaplan–Meier plots for prediction of PFS using the baseline CTC counts (Fig. 1a), at 2 weeks (Fig. 1b), and at 4 weeks (Fig. 1c). Seventeen of the patients (32.7%) had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with that of patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.4 months; 95% confidence interval [CI], 1.2–1.6) than the median PFS of <4 CTCs at 2 weeks (4.9 months; 95% CI, 4.0–5.8) (P < 0.001) (Fig. 1b). Patients with ≥4 CTCs at the 4-week point had a shorter median

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Table 3. CTCs and correlation with response assessment by imaging

	Non-PD			PD			Fisher's exact *P*-values
	No. of patients	CTCs <4 (%)	CTCs ≥4 (%)	No. of patients	CTCs <4 (%)	CTCs ≥4 (%)	
Baseline	34	24 (46.2)	10 (19.2)	18	11 (21.2)	7 (13.4)	0.544
2 week	33	33 (64.7)	0 (0)	18	11 (21.6)	7 (13.7)	0.001
4 week	32	31 (64.6)	1 (2.0)	16	8 (16.7)	8 (16.7)	<0.001

CTCs, circulating tumor cells; PD, progressive disease.

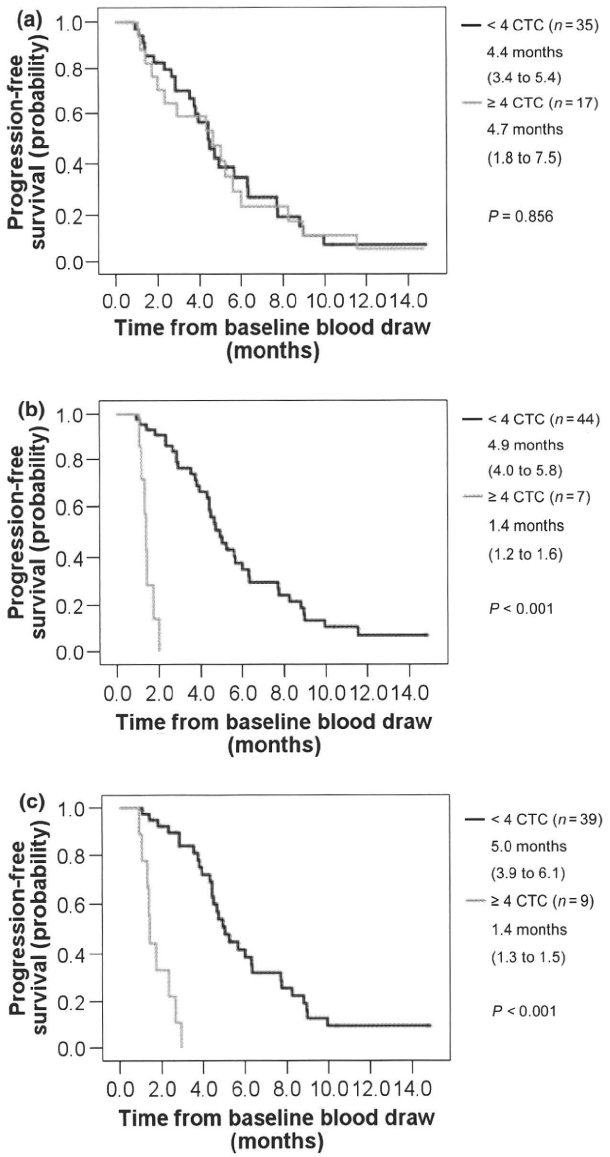

Fig. 1. Kaplan–Meier plots of progression-free survival (PFS) in advanced gastric cancer patients with less than four circulating tumor cells (CTCs) or ≥4 CTCs at baseline (a), 2 weeks (b), and 4 weeks (c).

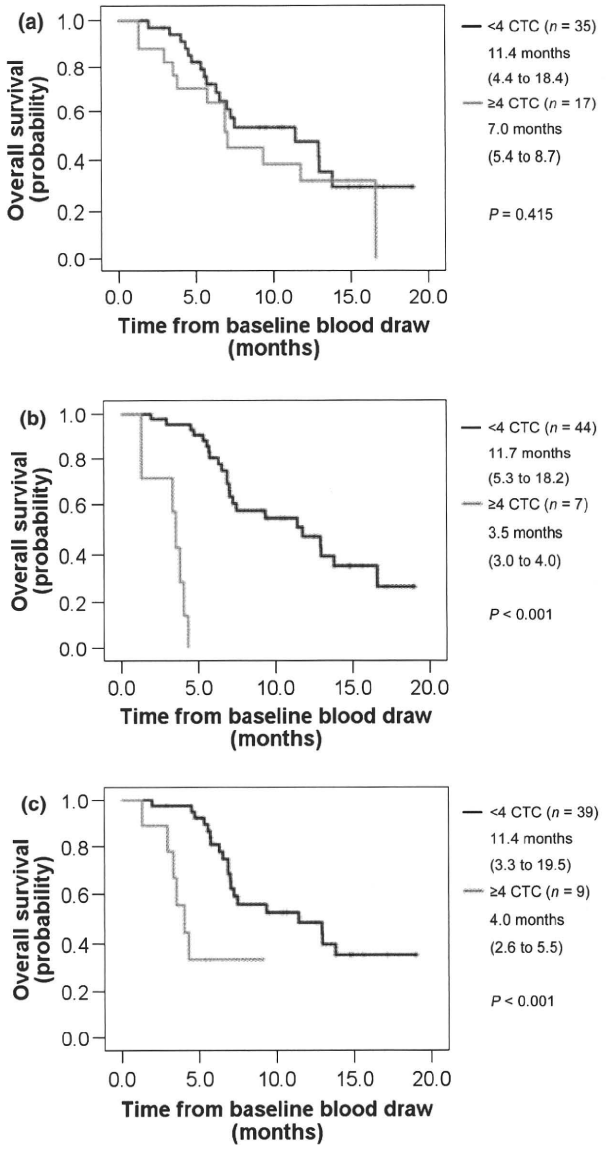

Fig. 2. Kaplan–Meier plots of overall survival (OS) in advanced gastric cancer patients with less than four circulating tumor cells (CTCs) or ≥4 CTCs at baseline (a), 2 weeks (b), and 4 weeks (c).

PFS (1.4 months; 95% CI, 1.3–1.5) than the median PFS of <4 CTCs at 4 weeks (5.0 months; 95% CI, 3.9–6.1) ($P < 0.001$) (Fig. 1c). With the S-1-based regimen, 10 patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with 21 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.2 months) than the median PFS of <4 CTCs at 2 weeks (6.0 months; 95% CI, 4.3–7.7) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median PFS (2.3 months; 95% CI, 0.7–3.9) than the median PFS of <4 CTCs at 4 weeks (6.3 months; 95% CI, 3.0–9.7) ($P < 0.001$). With the paclitaxel regimen, seven patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significantly different PFS compared with 14

patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median PFS (1.4 months; 95% CI, 1.4–1.5) than the median PFS of <4 CTCs at 2 weeks (4.3 months; 95% CI, 3.5–5.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median PFS (1.4 months; 95% CI, 1.0–1.8) than the median PFS of <4 CTCs at 4 weeks (4.4 months; 95% CI, 3.6–5.3) ($P < 0.001$).

Analysis of OS according to CTC level. Figure 2 shows the Kaplan–Meier plots for prediction of OS using baseline CTC counts (Fig. 2a), at 2 weeks (Fig. 2b), and at 4 weeks (Fig. 2c). Seventeen of the patients (32.7%) with ≥4 CTCs per 7.5 mL of blood at baseline had no significant different OS compared with patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (3.5 months; 95% CI, 3.0–4.0) than the median OS of <4 CTCs at 2 weeks (11.7 months; 95% CI, 5.3–18.2) ($P < 0.001$) (Fig. 2b). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (4.0 months; 95% CI, 2.6–5.5) than the median OS of <4 CTCs at 4 weeks (11.4 months; 95% CI, 3.3–19.5) ($P = 0.001$) (Fig. 2c). With the S-1 based regimen, 10 patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significant different OS compared with 21 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (1.3 months) than the median OS of <4 CTCs at 2 weeks (13.8 months; 95% CI, 9.4–18.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (4.0 months; 95% CI, 2.3–5.7) than the median OS of <4 CTCs at 4 weeks (>11.7 months) ($P = 0.031$). With the paclitaxel regimen, seven patients had ≥4 CTCs per 7.5 mL of blood at baseline. These patients had no significant different OS compared with 14 patients with <4 CTCs per 7.5 mL of blood at baseline. Patients with ≥4 CTCs at the 2-week point had a shorter median OS (3.5 months; 95% CI, 3.1–4.0) than the median OS of <4 CTCs at 2 weeks (6.5 months; 95% CI, 5.9–7.2) ($P < 0.001$). Patients with ≥4 CTCs at the 4-week point had a shorter median OS (3.5 months; 95% CI, 2.3–4.7) than the median OS of <4 CTCs at 4 weeks (6.5 months; 95% CI, 5.5–7.5) ($P = 0.013$).

Univariate and multivariate analysis of predictors of PFS and OS. Univariate and multivariate Cox proportional hazards regression was performed to assess the association between factors of interest and PFS or OS. In univariate analysis, PS, treatment regimen, line of chemotherapy, and CTC levels (cut-off, 4) at 2 and 4 weeks predicted PFS and OS (Table 4). In order to evaluate the independent predictive effect of chemotherapy, multivariate Cox regression analysis was carried out (Table 5). CTC levels at 2 and 4 weeks were the strongest predictors.

Discussion

The CellSearch system is designed to enrich and enumerate CTCs from peripheral blood. Furthermore, it is the first system to validate the clinical use of CTCs in patients with advanced gastric cancer. Our results show that the system is a suitable tool for assessment of CTCs in these patients, enabling reliable detection of CTCs in whole blood.

Approaches for isolation of CTCs in a research setting range from enrichment of tumor cells using density-gradient centrifugation[13–15] and flow cytometry.[16,17] CTC number as quantified by the CellSearch methodology[18–21] has been shown to have prognostic significance, and post-therapy decreases and increases in CTC number are associated with a superior and inferior survival, respectively, in patients with breast cancer, prostate cancer, and colorectal cancer. In this study, a finding of <4 CTCs in 7.5 mL of peripheral blood at 2 and 4 weeks after initiation of chemotherapy was associated with significantly longer PFS and OS as compared with these patients with ≥4 CTCs in 7.5 mL of peripheral blood. The results of this analysis demonstrated that the presence of four or more CTCs in 7.5 mL of blood before initiation of chemotherapy is not associated with PFS and OS. But the levels of CTCs at 2 and 4 weeks after initiation of chemotherapy are predictive of treatment efficacy, PFS,

Table 4. Univariate Cox regression analysis of independent parameters for prediction of PFS and OS

Parameter	No. of patients	PFS				OS			
		HR	95% CI	*P*-values	χ^2	HR	95% CI	*P*-values	χ^2
ECOG, 2 *vs* 1 *vs* 0	52	1.817	1.010–3.268	0.046	0.042	2.795	1.416–5.516	0.003	0.002
Treatment regimen	52	0.422	0.225–0.792	0.007	0.006	0.239	0.106–0.538	0.001	<0.001
Line of therapy	52	3.155	1.577–6.311	0.001	0.001	4.527	2.031–10.088	<0.001	<0.001
CTCs at the 2nd week	51	22.633	6.214–82.429	<0.001	<0.001	42.796	8.382–218.515	<0.001	<0.001
CTCs at the 4th week	48	15.947	5.380–47.271	<0.001	<0.001	4.699	1.751–12.609	0.002	0.001

CI, confidence interval; CTCs, circulating tumor cells; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

Table 5. Multivariate Cox regression analysis for prediction of PFS and OS

Parameter	PFS				OS			
	No. of patients	HR	95% CI	*P*-values	No. of patients	HR	95% CI	*P*-values
No. of patients	51				51			
Line of therapy, 1st *vs* 2nd		0.463	0.219–0.977	0.043		0.307	0.129–0.731	0.008
Lymph node metastasis		0.458	0.214–0.980	0.044				
CTCs at the 2nd week		0.049	0.012–0.199	<0.001		0.037	0.007–0.191	<0.001
Model χ^2			<0.001				<0.001	
No. of patients	48				48			
Line of therapy, 1st *vs* 2nd		0.412	0.192–0.880	0.022		0.217	0.089–0.504	<0.001
CTCs at the 4th week		0.082	0.027–0.224	<0.001		0.216	0.077–0.607	0.004
Model χ^2			<0.001				<0.001	

CI, confidence interval; CTCs, circulating tumor cells; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

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and OS. The presence of at least four CTCs at 2 and 4 weeks is a strong independent prognostic factor for inferior PFS and OS. These data demonstrate that CTC measurement may be a useful biomarker for monitoring response to therapy in AGC.

Outcomes are extremely poor in patients with ≥4 CTCs at 2 and 4 weeks, with the median OS ranging from 2 to 5 months. These data suggest the value of this technology in the identification of chemotherapy-resistant patients who could benefit from early treatment change and/or more investigational. Further study should prospectively address whether a change of treatment based on ≥4 CTCs at 2 or 4 weeks after initiation of chemotherapy early in the course of treatment will result in improvement in OS. CTC levels drawn at 2 and 4 weeks, before typical imaging intervals, may have the potential to suggest treatment choices and spare unnecessary toxicity by suggesting that an early change in therapy is warranted. Because the CellSearch system has not been approved in Japan, the price of one sample costs about ¥80 000 as in the case of the extra laboratory in the clinical trial. Several prospective trials led to the FDA approval of CTC counts for monitoring of patients with breast, colorectal, and prostate cancer. We expect CTC counts for monitoring of patients with gastric, breast, colorectal, and prostate cancer to be approved in Japan.

In conclusion, this study demonstrates the independent predictive value of CTCs for patients initiating chemotherapy for AGC. The data obtained in this clinical trial of the CellSearch system were for enumeration of CTCs in AGC. Our study was not designed to assess whether a change in therapy based on ≥4 CTCs is beneficial. However, clinical trials to explore this hypothesis are warranted.

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Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL PAPER

Management of venous thromboembolism in colorectal cancer patients treated with bevacizumab

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Abstract Venous thromboembolism associated with use of a central venous access system is an urgent problem in patients treated with bevacizumab (bev). We investigated the effectiveness of Doppler ultrasound imaging (DUS) in the early detection of catheter-related thrombosis for avoidance of severe venous thromboembolism. Patients with metastatic colorectal cancer received either FOLFOX-4 + bev or FOLFIRI + bev. DUS was performed on the deep venous system for detection of thrombus formation during the initial cycle of treatment, followed by re-evaluation after the third cycle in patients with asymptomatic thrombus formation. All patients were followed up until treatment was interrupted. Median duration of follow-up was 484 days (range 72–574). Among 41 enrolled patients, curable symptomatic thrombosis occurred in one, and asymptomatic thrombosis in 21 (51.2%). Of 21 patients undergoing re-evaluation, thrombi remained without progression in 17 patients, and enlargement in 4 patients. In two of the patients in whom there was progression, pulmonary embolism occurred after the sixth cycle. In the asymptomatic group, no thrombi developed as far as the superior vena cava in any patient. In the cases of progression, thrombotic enlargement was observed in all the 4 patients, with decreased vascular flow in 2. Using DUS, we were able to detect asymptomatic thrombosis in the early cycles of treatment, indicating its potential in the monitoring of venous thrombi. In the event of an enlarging asymptomatic thrombosis developing into the superior vena cava along with decreased vascular flow, careful follow-up and appropriate anticoagulant therapy may be recommended without increased risk of bleeding.

Keywords Venous thromboembolism · Bevacizumab · Colorectal cancer

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Introduction

Bevacizumab (bev) is a recombinant, humanized monoclonal antibody against vascular endothelial growth factor (VEGF). The combination of bev and chemotherapy for first- and second-line treatment of metastatic colorectal cancer has been shown to improve survival [1–4]. Furthermore, a large observational study indicated that use of bev beyond first progression correlated with improved survival [5]. However, use of bev in conjunction with chemotherapy is associated with an increased risk of arterial thromboembolism, and there is also some controversy as to whether bev contributes to the development of venous thromboembolism (VTE) [6]. Pulmonary embolism (PE) occurs in 2–5% of cases where bev and chemotherapy are

used together [1, 3]. A recent meta-analysis of 15 randomized controlled trials [7] found that bev significantly increased risk of VTE in cancer patients and anticoagulant therapy is indicated in the event of VTE.

An implantable central venous access system (CVAS) is a risk factor for VTE [8]. Many VTEs, although asymptomatic, can be as serious as PEs in terms of morbidity [9, 10]. Based on the results of studies on the prevention of catheter-related thrombosis, anticoagulant prophylaxis is not generally recommended with a CVAS [11–13].

In our hospital, symptomatic venous thrombosis associated with a CVAS occurred in patients treated with bev plus chemotherapy during the initial cycle. Appropriate screening and management of patients after detection of either symptomatic or asymptomatic VTE remain to be clarified.

We evaluated the effectiveness of Doppler ultrasound imaging (DUS) in the early detection of CVAS-associated venous thrombosis to determine its potential in the prevention of further development into severe VTE.

Patients and methods

Study design

This was a prospective cohort study conducted at a single institute. Patients were enrolled from July 2007 onward after approval of bev in June 2007 in Japan. The study protocol, including the use of DUS, was approved by the institutional review board of our institute. All the patients provided written informed consent before treatment.

The study design is shown in Fig. 1. DUS was performed on the deep venous system in the upper extremities where catheterization had been carried out to detect thrombus formation during the early cycles of chemotherapy. The first DUS was performed after the initial cycle of bev. Only patients with asymptomatic thrombus formation underwent follow-up evaluation by DUS after the third cycle of bev. During DUS, location and dimension of thrombus, vascular flow, and collateral vascular flow were evaluated and diagnosed by a radiologist at our institute. In addition, dimension of thrombus, location, whether it extended as far as the junction of the external jugular vein (EJV), suprascapular vein (SSV) or subclavian vein (SCV), collateral vascular flow, and retention of peripheral vascular flow were evaluated as important factors directly affecting vascular flow.

At our institute, time to treatment from implantation of a CVAS is usually just 2 days. This made it difficult to perform DUS prior to initiation of treatment and we could not delay treatment for that purpose in the patients enrolled in this study. Therefore, as a subordinate examination, we performed additional pre-treatment DUS between implantation of the CVAS and induction of bev in those patients who consented to the procedure.

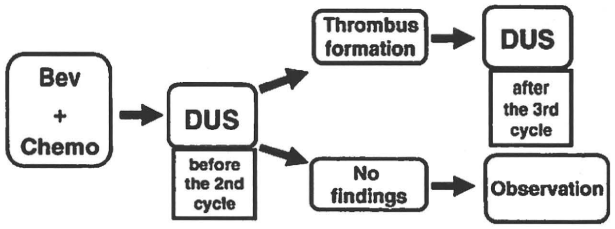

Fig. 1 Timing of DUS: study schema

Patients

All the patients conformed to the following criteria: histologically confirmed colorectal cancer; advanced metastatic colorectal cancer; age ≤ 70 years; Eastern Cooperative Oncology Group performance status of 0, 1, or 2; no history of thromboembolic events; no prior use of bev; no increased risk factors for bleeding; hypertension, if present, controlled with a single agent.

All the patients received the initial cycle of treatment when they were in the hospital, and additional treatment cycles at an ambulatory center. Complete blood count, international normalized ratio (INR), and D-dimer were measured biweekly or before treatment in all the patients. Deficiencies of protein C, protein S, and antithrombin III as congenital risk factors for thrombosis were examined, as well as the presence of acquired risk factors before initial bev administration.

Chemotherapy treatment

Treatment regimens were as follows: FOLFOX-4 + bev (biweekly cycles of 85-mg/m^2 intravenous oxaliplatin for 2 h on day 1 plus 100-mg/m^2 L-leucovorin (L-LV) for 2 h and 400-mg/m^2 bolus 5-FU, followed by a 22-h infusion of 600 mg/m^2 5-FU on days 1 and 2, plus 5–10-mg/kg bev on day 1 every 2 weeks); or 5-mg/kg FOLFIRI + bev (biweekly cycles of 150-mg/m^2 intravenous irinotecan for 1.5 h on day 1 plus 200-mg/m^2 L-LV for 2 h and 400-mg/m^2 bolus 5-FU, followed by a 46-h infusion of 1200 mg/m^2 5-FU on days 1 and 2 plus 5-mg/kg bev on day 1 every 2 weeks). Treatment continued until progression, unmanageable toxic effects, or patient refusal. Antiemetic agents were provided at the discretion of the treating physician. No prophylactic use of colony-stimulating factor was permitted. No anticoagulant therapy before initial evaluation by DUS was permitted. If an asymptomatic thrombus that could potentially cause a PE was identified on DUS, anticoagulant therapy was permitted. The anticoagulant treatment regimen was at the discretion of the physician.

Evaluation of toxicity and efficacy

Patient data were recorded and reviewed on electronic clinical records. Adverse effects were graded in all the patients biweekly or before treatment using the National Cancer Institute Common Toxicity Criteria, version 3.0. Cancer response was assessed every 12 weeks using computed tomography according to the response evaluation criteria for solid tumors. Data on toxicity and tumor evaluation were analyzed using electronic medical records and by examination of films of each patient. A radiologist examined the films and made an assessment of status, and the evaluations were recorded in electronic medical records.

Differences in baseline characteristics and clinical features between patients with and without catheter-related thrombosis were analyzed. We used the Chi-square test (without the Yates correction) and Fisher's exact probability test for categorical comparisons of data. Differences in the means of continuous measurements were tested by the Student's t test and checked by Mann–Whitney U test. Quantitative variables such as time between two points were summarized using medians. A two-way repeated-measures analysis of variance was used to evaluate differences between sequential continuous variables. A P value of <0.05 was considered significant.

Results

Characteristics of patients

Forty-one patients were enrolled in the study. The baseline characteristics of the patients are shown in Table 1. Median follow-up time from the date of initial bev administration was 484 days (range 72–574 days). Eight patients (19.5%) received an antihypertensive agent at baseline. In addition, no congenital factors for thrombosis were seen, but anticardiolipin antibody IgG and lupus anticoagulant were observed in 5 (12.2%) and 1 (2.4%) patients, respectively.

Effectiveness of DUS

The results of DUS are shown in Table 2. Catheter-related thrombosis was observed on initial DUS in 22 patients (53.7%), including both asymptomatic ($n = 21$ [51.2%]) and symptomatic ($n = 1$ [2.4%]) thrombi. No thrombus formation was detected in 19 patients (46.3%). Twenty-two patients received a follow-up DUS, one of whom received anticoagulant therapy after initial DUS. Thrombi disappeared completely in 3 (13.6%) of these 22 patients without anticoagulant therapy.

Comparisons of initial and follow-up DUS findings in asymptomatic thrombi are shown in Table 3. In 21 patients with asymptomatic thrombi, none of the thrombi extended to the superior vena cava, and complete disappearance was seen in 3. Thrombus size was <20 mm in 16 patients (76.2%) on initial DUS, compared to in 16 patients (76.2%) on follow-up DUS. Decreased vascular flow was observed

Table 1 Baseline characteristics of patients ($N = 41$)

Characteristics	N (%)
Sex: male/female	17/24
Mean age (range), years	58.4 (16–69)
Chemotherapy regimen	
FOLFOX4 + bev 5 mg/kg	28 (68.3)
FOLFOX4 + bev 10 mg/kg	1 (2.4)
FOLFIRI + bev 5 mg/kg	12 (29.3)
ECOG performance status	
0	39 (95.1)
1	2 (4.9)
Treatment set as systemic chemotherapy for metastatic disease	
First-line	28 (68.3)
Second-line	13 (31.7)
Prior adjuvant fluorouracil	5 (12.2)
No. of involved organs	
1	16 (39)
2	19 (46.3)
3	5 (12.2)
4	1 (2.4)
Sites of metastases	
Liver	19 (46.3)
Lung	20 (48.8)
Peritoneum	8 (19.5)
Nodes	17 (41.5)
Local recurrence	7 (17.1)
Bone	1 (2.4)
Previous history/complication	
Thromboembolic events	0
Hypertension	8 (19.5)
Diabetes	2 (4.9)
Hyperlipidemia	1 (2.4)
Hyperuricemia	1 (2.4)
Liver function failure	1 (2.4)
Congenital risk factors	
Protein C deficiency	0
Protein S deficiency	0
Antithrombin III deficiency	0
Acquired risk factors	
Anticardiolipin antibody IgG	5 (12.2)
Anticardiolipin antibody β2-glycoprotein 1	0
Lupus anticoagulants	1 (2.4)

Bev bevacizumab, *ECOG* Eastern Cooperative Oncology Group, *INR* international normalized ratio, *CRP* C-reactive protein, *IgG* immunoglobulin G

Table 2 Results of DUS ($N = 41$)

Median length, days (range)	
IP-CVAS—induction of bev	7 (2–695)
IP-CVAS—initial DUS	18 (7–700)
Induction of bev—initial DUS	7 (4–14)
Induction of bev—follow-up DUS	35 (14–49)
Results of initial DUS, *n* (%)	
Thrombus formation	22 (53.7)
Symptomatic thrombosis	1 (2.4)
Asymptomatic thrombosis	21 (51.2)
No thrombus formation	19 (46.3)
Results of follow-up DUS ($n = 22$), *n* (%)	
Thrombus formation	19 (86.4)
No thrombus formation (disappeared)	3 (13.6)

IP-CVAS implantation of central venous access system, *bev* bevacizumab

in 3 patients (14.3%) on initial DUS that showed no progression on follow-up DUS; however, another 2 patients in whom adequate vascular flow was detected on initial DUS showed decreased vascular flow on follow-up DUS. We defined overall improvement as at least one improved finding without progression in location, maximum size, or (collateral) vascular flow and progression as at least one progressed finding; those fitting neither category were defined as the remainder. Overall, thrombi improved or remained stable in 17 patients (81%), and progressed without symptoms in 4 patients (19%). Correlations between vascular flow and other findings are shown in Table 4. Incidence of thrombi extending into the junction of the SCV, ECV, and SSV was significantly higher in patients with inadequate vascular flow than in patients with adequate vascular flow on initial DUS (66.7 vs. 5.6%, respectively; $P = 0.0414$) and on follow-up DUS (80 vs. 0%, respectively; $P = 0.0016$).

Symptomatic thrombosis was revealed on DUS in 1 patient (a DUS image with a diagram is shown in Fig. 2). This thrombus extended into the superior vena cava, was >40 mm in diameter, and resulted in decreased vascular flow. This patient received anticoagulant therapy after initial DUS and re-started bev after a follow-up DUS revealed that the thrombus had not progressed.

Characteristics of patients with and without thrombi are shown in Table 5. Median follow-up time from date of initial bev administration was 518 days (range 232–574 days) and 487 days (72–559), respectively, in these patients. No significant difference was observed in performance status or age. Presence of acquired risk factors showed no effect on thrombus formation or outcome in patients with asymptomatic thrombus. Incidence of thrombus formation was significantly higher in the FOLFOX + bev treatment group than in the FOLFIRI + bev group ($P = 0.0047$). Median length of time between implantation of CVAS and induction of bev was significantly shorter in patients with thrombus formation than in patients without thrombus formation (5 vs. 107.5 days, respectively; $P = 0.0048$). Similarly, median length of time between implantation of CVAS and initial DUS was significantly shorter in patients with thrombus formation than in patients without thrombus formation (13.5 vs. 116 days, respectively; $P = 0.0059$). In further follow-up after completion of the protocol, 1 patient in whom no thrombus was detected in the planned DUS experienced asymptomatic thrombosis of the inferior vena cava during the 12th cycle. However, we were able to continue FOLFOX in this patient without bev using warfarin.

A comparison of patients with improved thrombus findings on follow-up DUS ($n = 5$) with those showing thrombus progression ($n = 4$) revealed that median follow-up time from date of initial bev administration was 505 days (range 446–563 days) and 395.5 days (range 256–484 days), respectively. No significant differences were observed in findings on initial DUS, median time to induction of bev from implantation of CVAS (5 vs. 6 days; $P = 0.9004$), or laboratory data between the two groups. The results of a two-way repeated-measures analysis of variance to evaluate differences between sequential continuous variables such as platelet count, INR, and D-dimer showed no significant differences. Changes in thrombus size, as well as decreased vascular flow, were mainly related to outcomes of thrombus on initial DUS. However, two patients showing thrombus progression developed pulmonary embolism requiring urgent treatment with a thrombolytic agent followed by warfarin, after which, they were able to continue with FOLFOX without bev until progression of disease. None of the patients experienced sequelae, including post-thrombotic syndrome, and there were no deaths related to thromboembolic events or anticoagulant therapy.

Discussion

In this study, we assessed the effectiveness of DUS in the early identification of catheter-related thrombosis and variations in asymptomatic venous thrombosis under use of bev.

According to the American Society of Clinical Oncology [14], the presence of a central venous catheter is a risk factor for VTE in cancer patients. Active chemotherapy [15, 16] and antiangiogenic therapy [2, 3] also carry risk of VTE. With the newer antiangiogenic agents, the use of a prophylaxis for VTE is controversial [2, 3, 17, 18].

Although a CVAS makes it easier to administer chemotherapy in ambulatory patients, its use is associated with

Table 3 Findings associated with asymptomatic thrombosis ($n = 21$)

Findings	Initial DUS	Follow-up DUS
Location, *n* (%)		
Distal (not extended to SVC)	21 (100)	18 (85.7)
Central (extended to SVC)	0	0
Comparison	Improved (disappeared) in 3 patients (14.3)	
Maximum size, *n* (%)		
0–<10 mm	14 (66.7)	12 (57.1)
10–<20 mm	2 (9.5)	4 (19)
20–<30 mm	3 (14.3)	3 (14.3)
>30 mm	2 (9.5)	2 (9.5)
Comparison	Improved in 5 patients (23.8) (disappeared in 3 and reduced in 2)	
	Progressed in 4 patients (19)	
Vascular flow, *n* (%)		
Adequate	18 (85.7)	13 (61.9)
Inadequate	3 (14.3)	5 (23.8)
Comparison	Improved in 3 patients (14.3)	
	Progressed in 2 patients (9.5)	
Collateral vascular flow, n (%)		
Adequately increased	2 (9.5)	2 (9.5)
Inadequately increased	1 (4.8)	3 (14.3)
Comparison	Progressed in 2 patients (9.5)	
Overall evaluation[a]	Improved in 5 patients (23.8)	
	Stable in 12 patients (57.1)	
	Progressed in 4 patients (19)	

[a] Overall improvement was defined as at least one improved finding without progression in location, maximum size, or (collateral) vascular flow and progression as at least one progressed finding; those fitting neither category were defined as the remainder. One patient receiving anticoagulant therapy after initial DUS showed a thrombus 45 mm in diameter that developed into the brachiocephalic vein; no progression was noted. One patient with thrombus progression experienced a symptomatic pulmonary embolism after the sixth cycle, and one progressed patient experienced an asymptomatic pulmonary embolism after the sixth cycle

SVC superior vena cava

Table 4 Correlation between vascular flow and other findings of asymptomatic thrombosis ($n = 21$)

Findings on DUS	Initial DUS ($n = 21$)		Follow-up DUS ($n = 18$)	
	Adequate ($n = 18$)	Inadequate ($n = 3$)	Adequate ($n = 13$)	Inadequate ($n = 5$)
Location, *n* (%)				
SCV–ECV–SSV junction[a]	1 (5.6)	2 (66.7)	0	4 (80)
P value	0.0414		0.0016	
Maximum size, *n* (%)				
<30 mm	18(100)	1 (33.3)	13 (100)	3 (60)
≥30 mm	0	2 (66.7)	0	2 (40)
P value	0.0143		0.0654	

[a] Thrombi extended into junction of SCV, ECV, and SSV in two inadequate patients at initial DUS; both thrombi sizes were ≥30 mm

DUS Doppler ultrasound imaging, *SCV* subclavian vein, *ECV* external jugular vein, *SSV* suprascapular vein, *N.A* not applicable

an increased risk for VTE and PE [8–10]. According to a review by Vescia et al. [19], the incidence of catheter-related thrombosis varied from 12 to 64% in four retrospective studies [20–24]. In a recent prospective trial using phlebography in patients with a CVAS, Verso et al. [25] found that the incidence of thrombosis in two groups receiving low molecular weight heparin (LMWH) or placebo for 6 weeks was 14.1 and 18%, respectively (95% CI 0.47–1.31; $P = 0.35$), with symptomatic upper limb thrombosis seen only in 1.0% of the LMWH group and 3.1% of the placebo group (hazard ratio 0.32; 95% CI 0.07–1.66). In another trial by Couban et al. [12], the rate of symptomatic thrombosis in a group received 1-mg warfarin for 9 weeks was 4.6% when compared with 4.0% in the placebo group (hazard ratio 1.20; 95% CI 0.37–3.94).

We summarized thromboembolic events reported in nine pivotal studies of bev plus chemotherapy [1–4, 17, 18, 26–28]. According to the results, the incidence of thromboembolism ranged from 3 to 26% in these studies, and PE was reported in 1–4% of cases. Prophylactic anticoagulant treatment was not permitted in any study, except for maintenance of CVAS in four studies [1, 2, 4, 18].

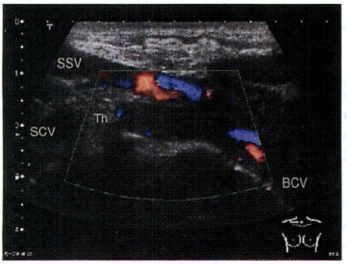

Fig. 2 Findings of DUS image and illustration in symptomatic case. This thrombus (Th) extended into superior vena cava (SVC) through brachiocephalic vein (BCV), was >40 mm in diameter, and resulted in clearly decreased vascular flow

Table 5 Comparison between patients with and without thrombus formation

	With thrombus (n = 22)	Without thrombus (n = 19)	P value
Sex: male/female	9/13	8/11	>0.9999
Mean age (range), years	62 (16–69)	60.1 (47–69)	0.9896
ECOG performance status, n (%)			
0	22 (100)	17 (89.5)	0.2308
1	0	2 (10.5)	
Chemotherapy regimen, n (%)			
FOLFOX4 + bev	20 (90.9)	9 (47.4)	0.0047
FOLFIRI + bev	2 (9.1)	10 (52.6)	
Prior treatment, n (%)			
FOLFOX	2 (9.1)	10 (52.6)	0.0047
Hepatic arterial infusion	3 (13.6)	4 (21.1)	0.6847
Radiation	3 (13.6)	0	0.2354
No. of involved organs, n (%)			
1/2/3/4	10/10/2/0	6/9/3/1	0.3899
≥3	2 (9.1)	4 (21.1)	
Baseline laboratory data, mean ± SD			
Platelets ($\times 10^4$ μl)	22.6 ± 5.68	18.89 ± 6.84	0.0652
INR	1.05 ± 0.49	1.05 ± 0.12	0.9811
D-dimer	1.15 ± 1.52	1.21 ± 0.83	–
Acquired risk factors, n (%)			
Anticardiolipin antibody IgG	3 (13.6)	2 (10.5)	>0.9999
Lupus anticoagulants	1 (4.5)	0	>0.9999
Median length (range), n (%)			
IP-CVAS—induction of bev	5 days (2–252)	107.5 days (2–695)	0.0048
IP-CVAS—initial DUS	13.5 days (7–259)	116 days (7–700)	0.0059

ECOG Eastern Cooperative Oncology Group, *bev* bevacizumab, *INR* international normalized ratio, *IP-CVAS* implantation of central venous access system, *DUS* Doppler ultrasound imaging, *SD* standard deviation

According to these studies, the incidence of thromboembolic events was not high and routine prophylactic anticoagulant treatment for thromboembolism did not appear necessary.

Patient characteristics in our study were similar to those in previous reports, and no specific characteristics related to thrombus formation were seen. However, we observed a higher rate of thrombi than expected using DUS and almost all of them were asymptomatic. Indeed, this study was designed to detect diagnostic findings, not clinical findings.

This study had a number of limitations. First, there was no control population (with no administration of bev). In other words, thromboembolic events may have been due to prior chemotherapy rather than bev, as only small doses of bev had been given at first screening. Second, the study protocol did not provide true baseline DUS at pre-treatment, as the time to treatment from implantation of the CVAS was usually just 2 days or more. Therefore, it was difficult to establish whether there was a correlation between the treatment drugs and catheter-related thrombosis, or when