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

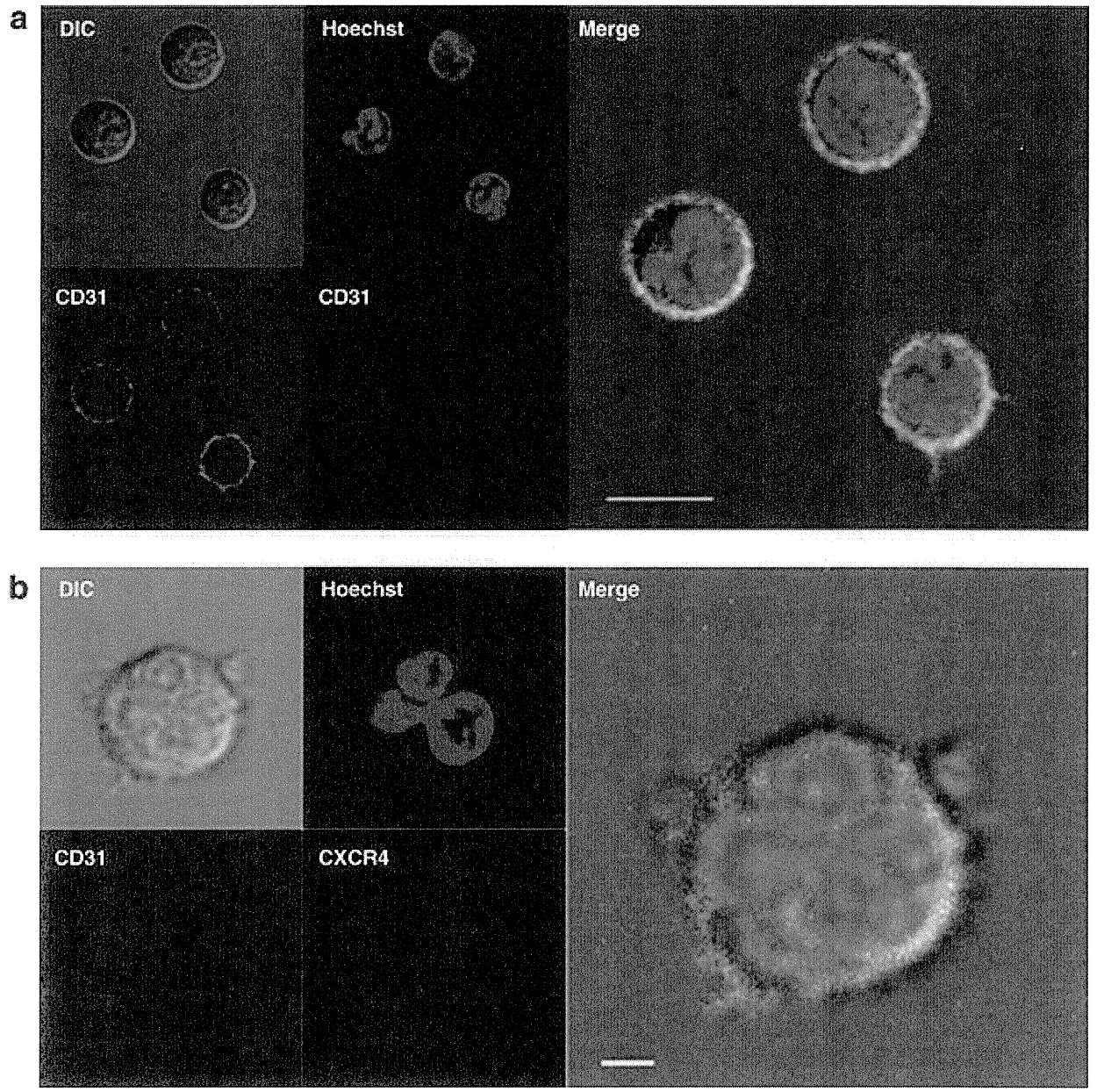

Figure 4. (a) CEPs and (b) CXCR4 + CECs were sorted by flow cytometry as described in the Materials and Methods section and analyzed by confocal microscopy. DIC indicates differential interferences contrast; bar, 5 μm.

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

This work was supported by an AstraZeneca Research Grant 2007, the Kobayashi Institute for Innovative Cancer Chemotherapy, and a Grant-in-Aid for Scientific Research (Japan Society for the Promotion of Science) (grant numbers 19790963, 21591741, 17016077).

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Cancer Science
The official journal of the Japanese Cancer Association JCA

Circulating tumor cells as a surrogate marker for determining response to chemotherapy in Japanese patients with metastatic colorectal cancer

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(Received November 10, 2010/Revised February 17, 2011/Accepted February 28, 2011/Accepted manuscript online March 14, 2011/Article first published online April 11, 2011)

The purpose of this study was to investigate the potential of circulating tumor cells (CTC) as a surrogate marker of the clinical outcome in metastatic colorectal cancer (mCRC) patients in order to identify Japanese patients responsive to oxaliplatin-based chemotherapy. Between January 2007 and April 2008, 64 patients with mCRC were enrolled in this prospective study. The treatment regimen was oxaliplatin-based chemotherapy. Collection of CTC from whole blood was performed at baseline and at 2 and 8–12 weeks after initiation of chemotherapy. Isolation and enumeration of CTC was performed using immunomagnetics. Patients with ≥3 CTC at baseline and at 2 and 8–12 weeks had a shorter median progression-free survival (8.5, 7.3 and 1.9 months, respectively) than those with <3 CTC (9.7, 10.4 and 9.1 months, respectively) (log-rank test: $P = 0.047$, $P < 0.001$ and $P < 0.001$, respectively). Patients with ≥3 CTC at 2 and 8–12 weeks had a shorter median overall survival (10.2 and 4.1 months, respectively) than those with <3 CTC (29.1 and 29.1 months, respectively) ($P < 0.001$ and $P = 0.001$, respectively). A spurious early rise in carcinoembryonic antigen level was observed in 11 patients showing a partial response. In contrast, no rise in early CTC level was observed among responders. Our data support the clinical utility of CTC enumeration in improving our ability to accurately assess treatment benefit and in expediting the identification of effective treatment regimens for individual Japanese patients. (*Cancer Sci* 2011; 102: 1188–1192)

Circulating tumor cells (CTC) have been documented in the peripheral blood from patients with various cancers[1–3]. Attempts to isolate CTC have led to the development of two leading procedures: density–gradient centrifugation[4–6] and flow cytometry[7]. The number of CTC, as quantified by the CellSearch (Veridex LLC, Raritan, NJ, USA) methodology, has been shown to have prognostic significance in patients with breast cancer, prostate cancer and colorectal cancer, so recent efforts have concentrated on detecting CTC in the peripheral blood of cancer patients.

Cohen *et al.*[8] reported that the number of CTC before and during treatment was an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with metastatic colorectal cancer (mCRC). Detection of three or more CTC versus fewer than three CTC before and after initiation of a new systemic treatment regimen was associated with shorter median PFS and OS. These observations led us to conduct a validation study with the hypothesis that the number of CTC in Japanese patients relative to a threshold of three would correlate strongly with disease progression, allowing decisions on treatment efficacy to be made earlier than would normally be possible with imaging alone.

The American Society for Clinical Oncology recommends carcinoembryonic antigen (CEA) as the marker of choice for monitoring the response of metastatic disease to systemic therapy. However, Sorbye and Dahl[9] reported a transient increase in CEA level despite an objective response among patients receiving oxaliplatin-based chemotherapy for colorectal cancer.

In the present study, using the CellSearch system, we investigated the potential of CTC level in comparison with CEA level as a surrogate marker of clinical outcome in order to identify Japanese patients responsive to chemotherapy.

Materials and Methods

Patients. All patients were enrolled using institutional review board-approved protocols at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. Informed consent was obtained from all patients. The study population consisted of patients aged 18 years or older with histologically proven mCRC. Other inclusion criteria were an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1 and adequate organ function. The chemotherapy regimen was FOLFOX4 with or without bevacizumab.

Sample preparation for isolation of CTC from blood. For isolation of CTC from mCRC patients, 10-mL samples of blood were drawn into a Cell Save Preservative Tube (Veridex LLC). Blood was drawn before initiation of treatment (baseline) and at 2 and 8–12 weeks after administration of FOLFOX4 with or without bevacizumab. The CellSearch system (Veridex LLC) consists of the CellPrep system, the CellSearch Epithelial Cell kit (for 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 performed as described by Allan *et al.*[10] Briefly, 7.5 mL blood was mixed with 6 mL buffer, centrifuged at 800 *g* for 10 min and then placed on the CellPrep system. After aspiration of the plasma and buffer layer, 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, magnetic separation was repeated and

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doi: 10.1111/j.1349-7006.2011.01926.x

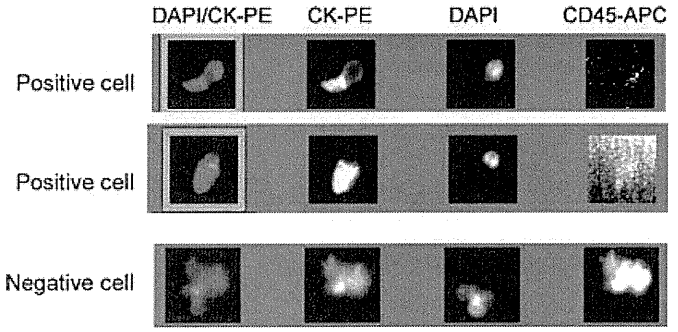

Fig. 1. Image galleries after CellSearch processing. Circulating tumor cells were cytokeratin (CK) and DAPI positive, but CD45 negative.

excess staining reagents aspirated. As the final step in the procedure, the cells were resuspended in the MagNest Cell Presentation Device (Veridex LLC). 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 is placed on the CellSpotter Analyzer, a four-color, semi-automated fluorescence microscope. Image frames covering the entire surface of the cartridge are captured. Captured images containing objects that meet predetermined criteria are automatically presented in a web-enabled browser; an operator makes the final selection of cells. 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 blood (Fig. 1).

Statistical analysis. Progression-free survival was defined as the time elapsed from blood collection to progression. Each time blood was collected, Kaplan–Meier survival plots were generated based on CTC levels and curves were compared using log-rank testing. A P-value of <0.05 was considered significant. The Cox proportional-hazards regression model 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 their clinical response were compared using the Fisher exact test.

Results

Patient characteristics. A total of 64 patients were enrolled. Patient characteristics at baseline, which are summarized in Table 1, were as follows: median age, 59 years (range, 18–72 years); PS 0/1, 61/3; primary site rectum/colon, 36/28; and bevacizumab +/−, 31/33. Among the 64 patients, the objective response rate was 56%.

CTC level and imaging to assess response to therapy. Fifty-six of 64 patients were classified as having no progressive disease (PD) (non-PD, including stable disease, partial or complete response), with 47 of these patients having <3 CTC and nine patients having ≥3 CTC before initiation of therapy. Eight patients were classified as having PD, with five of these having <3 CTC and three having ≥3 CTC before initiation of therapy. The difference between the clinical response and CTC level was not significant. In contrast, 55 of 63 patients were classified as having non-PD, with 51 of these patients having <3 CTC and four patients having ≥3 CTC at 2 weeks. Eight of 63 patients were classified as having PD, with five of these having <3 CTC and three having ≥3 CTC at 2 weeks. The difference between the clinical response and CTC level was highly significant (P = 0.038, Fisher's exact test). Fifty-three of 60 patients were classified as having non-PD, with 52 of these patients having <3 CTC and one patient having ≥3 CTC at 8–12 weeks. Seven of 60 patients were classified as having PD, with four of these having <3 CTC and three having ≥3 CTC at 8–12 weeks. The difference between best overall response and CTC level was highly significant (P = 0.004, Fisher's exact test) (Table 2).

Table 1. Patient characteristics

	Oxaliplatin-based regimen
Median age (range) (years)	59 (18–72)
Sex (male/female)	31/33
PS: 0/1	61/3
Primary site: rectum/colon	36/28
No. lines: 1st/2nd	49/15
Bevacizumab: +/−	33/31
Site of metastasis	
Liver	34
Lung	32
Bone	4
Lymph node	25
Local	9
Peritoneum	20
Metastases to more than two organs	46
Best objective response (CR/PR/SD/PD)	2/34/20/8

CR, complete response; PD, progressive disease; PR, partial response; PS, performance status; SD, stable disease.

Table 2. CTC and correlation with response assessment by imaging

	Non-PD			PD			Fisher's exact P
	No. patients	CTC <3	CTC ≥3	No. patients	CTC <3	CTC ≥3	
Baseline	56	47	9	8	5	3	0.164
2 weeks	55	51	4	8	5	3	0.038
8–12 weeks	53	52	1	7	4	3	0.004

CTC, circulating tumor cells; PD, progressive disease.

Table 3. Spurious early rise in CEA level or CTC level

	No. patients with a transient rise	
	CEA level	CTC level
CR	0	0
PR	11	0

CEA, carcinoembryonic antigen; CR, complete response; CTC, circulating tumor cells; PR, partial response.

Spurious early rise in CEA and CTC levels. A spurious early rise in CEA level was observed in 11 patients showing a partial response. In contrast, no rise in CTC levels at 2 weeks was observed in any patient showing either a partial or complete response (Table 3).

Analysis of PFS according to CTC level. Figure 2 shows the Kaplan–Meier plots for prediction of PFS using the CTC counts at baseline (Fig. 2a) and at 2 weeks (Fig. 2b) and 8–12 weeks (Fig. 2c). Patients with ≥3 CTC at baseline had a shorter median PFS (8.5 months; 95% CI, 7.4–9.6 months) than those with <3 CTC at baseline (9.7 months; 95% CI, 7.3–12.0 months) (P = 0.047) (Fig. 2a). Patients with ≥3 CTC at 2 weeks had a shorter median PFS (7.3 months; 95% CI, 0–21.0 months) than those with <3 CTC at 2 weeks (10.4 months; 95% CI, 7.5–13.3 months) (P < 0.001) (Fig. 2b). Patients with ≥3 CTC at 8–12 weeks had a shorter median PFS (1.9 months; 95% CI, 0.5–3.3 months) than those with <3 CTC at 8–12 weeks (9.1 months; 95% CI, 7.6–10.7 months) (P < 0.001) (Fig. 2c).

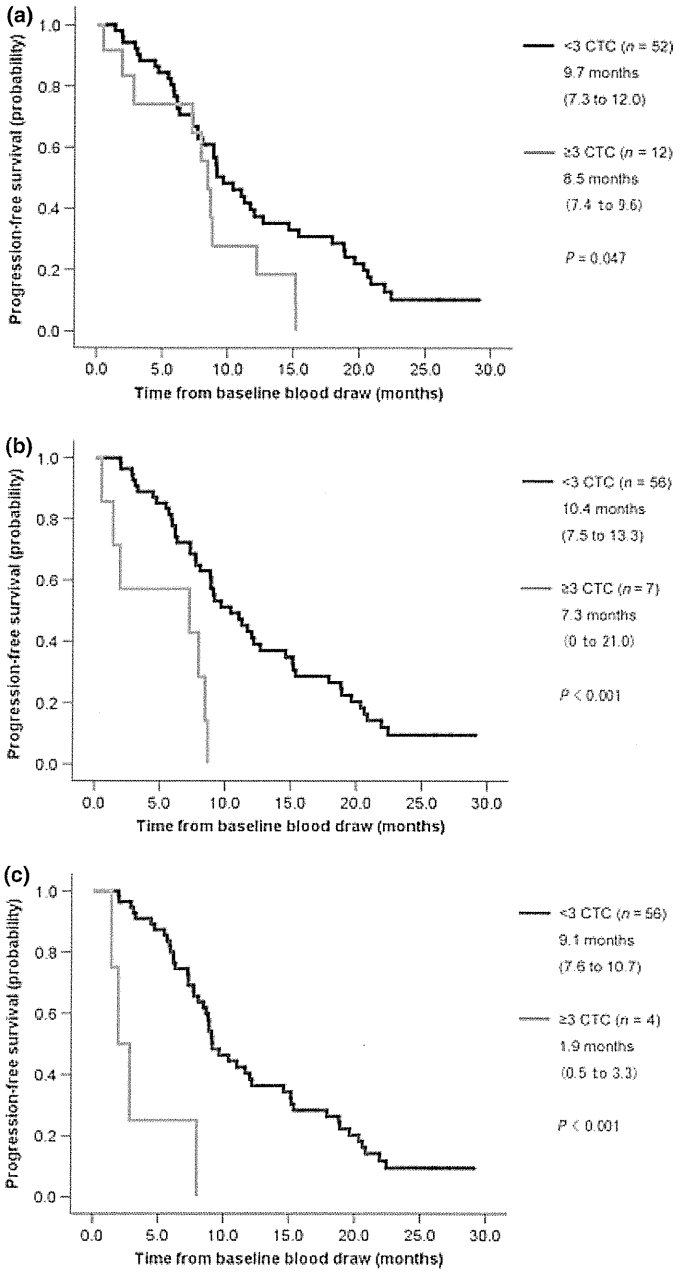

Fig. 2. Kaplan–Meier plots of progression-free survival in metastatic colorectal cancer patients with fewer than three circulating tumor cells (CTC) or ≥3 CTC at baseline (a), 2 weeks (b) and 4 weeks (c).

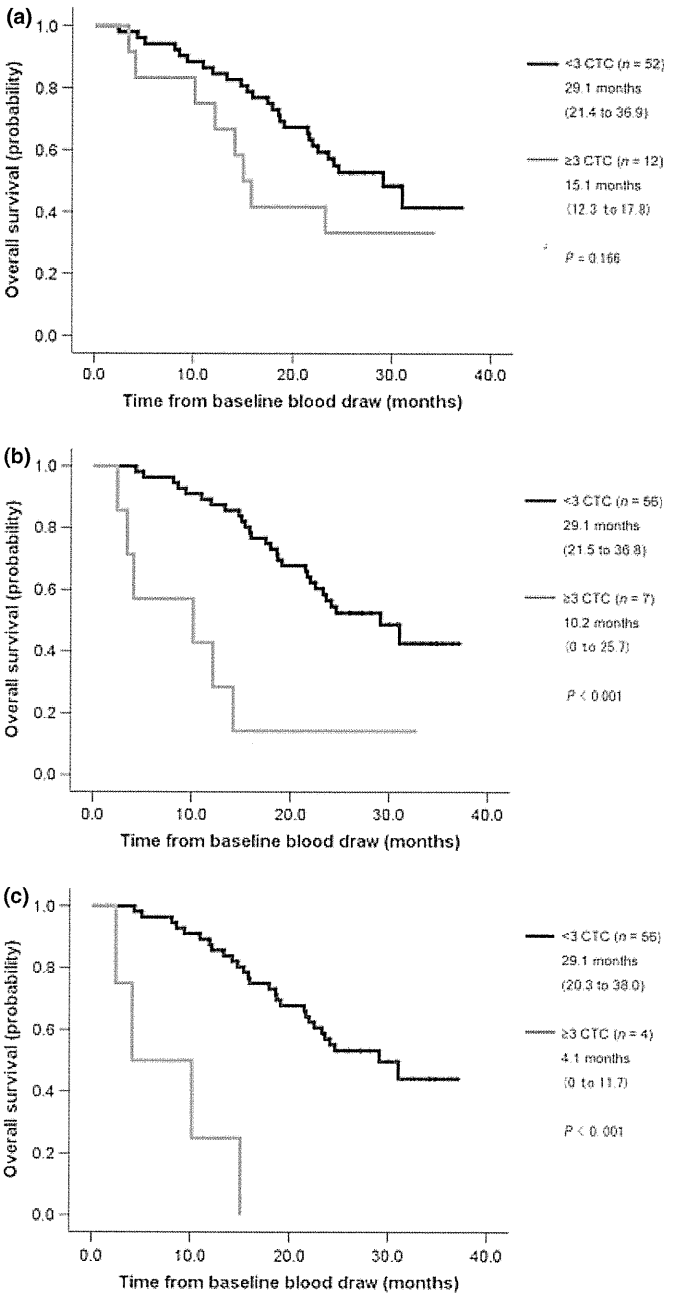

Fig. 3. Kaplan–Meier plots of overall survival in metastatic colorectal cancer patients with fewer than three circulating tumor cells (CTC) or ≥3 CTC at baseline (a), 2 weeks (b) and 4 weeks (c).

Analysis of OS according to CTC level. Figure 3 shows the Kaplan–Meier plots for prediction of OS using baseline CTC counts (Fig. 3a) at 2 weeks (Fig. 3b) and at 8–12 weeks (Fig. 3c). A shorter median OS was observed in patients who had ≥3 CTC at all time points. Patients with ≥3 CTC at 2 weeks had a significantly shorter median OS (10.2 months; 95% CI, 0–25.7 months) than those with <3 CTC at 2 weeks (29.1 months; 95% CI, 21.5–36.8 months) ($P < 0.001$) (Fig. 3b). Patients with ≥3 CTC at 8–12 weeks had a significantly shorter median OS (4.1 months; 95% CI, 0–11.7 months) than those with <3 CTC at 8–12 weeks (29.1 months; 95% CI, 20.3–38.0 months) ($P = 0.001$) (Fig. 3c).

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 the univariate analyses, PS, lung metastasis, bevacizumab and CTC levels at baseline and at 2 and 8–12 weeks predicted PFS, and PS, bevacizumab and CTC levels at 2 and 4 weeks predicted OS (Table 4). In order to evaluate the independent predictive effect of chemotherapy, multivariate Cox regression analysis was carried out (Table 5). Levels of CTC at 2 and 4 weeks were the strongest predictors.

Discussion

To our knowledge, this is the first study to validate the clinical use of CTC for monitoring the response of mCRC to systemic therapy in Japanese patients. A cut-off of three CTC was chosen based on the results of an earlier study by Cohen *et al.*[8] We determined the relationship among patients with no CTC, those

doi: 10.1111/j.1349-7006.2011.01926.x

Table 4. Independent predictive factors by univariate Cox regression analysis for PFS and OS

Parameter	No. patients	HR	95% CI	*P*-value	χ^2
PFS					
PS	64	4.418	1.338–14.589	0.015	0.008
Bevacizumab: +/−	64	0.276	0.155–0.493	<0.001	<0.001
Lung metastasis	64	1.988	1.138–3.473	0.016	0.016
CTC at baseline	64	1.085	1.026–1.147	0.004	0.003
CTC at 2 weeks	63	1.179	1.089–1.276	<0.001	<0.001
CTC at 8–12 weeks	61	1.211	1.099–1.334	<0.001	<0.001
OS					
PS	64	20.416	5.172–80.592	<0.001	<0.001
Bevacizumab: +/−	64	0.449	0.222–0.905	0.025	0.022
CTC at 2 weeks	63	1.192	1.090–1.303	<0.001	<0.001
CTC at 8–12 weeks	61	1.339	1.119–1.601	<0.001	<0.001

CI, confidence interval; CTC, circulating tumor cells; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; PS, performance status.

Table 5. Independent predictive factors by multivariate Cox regression analysis for PFS and OS

Parameter	No. patients	HR	95% CI	*P*-value	Model χ^2
PFS					
PS	64	74.42	10.063–550.35	<0.001	<0.001
Liver metastasis		1.897	1.057–3.406	0.032	
Bone metastasis		0.136	0.024–0.759	0.023	
Bevacizumab: +/−		0.169	0.088–0.324	<0.001	
CTC at baseline		1.058	0.977–1.145	0.164	
PS	63	0.08	0.014–0.462	0.005	<0.001
LN metastasis		0.542	0.297–0.986		
Bevacizumab: +/−		0.162	0.081–0.322	<0.001	
CTC at 2 weeks		1.144	1.047–1.251	0.003	
Lung metastasis	61	1.836	1.013–3.329	0.045	<0.001
Bevacizumab: +/−		0.269	0.147–0.492	<0.001	
CTC at 8–12 weeks		1.211	1.092–1.344	<0.001	
OS					
PS	63	46.194	9.401–226.971	<0.001	<0.001
Peritoneum		2.787	1.331–5.839	0.007	
Bevacizumab: +/−		0.468	0.221–0.990	0.047	
CTC at 2 weeks		1.236	1.100–1.387	<0.001	
PS	61	22.142	2.415–203.035	0.006	<0.001
Bevacizumab: +/−		0.346	0.154–0.777	0.010	
CTC at 8–12 weeks		1.441	1.143–1.817	0.002	

CI, confidence interval; CTC, circulating tumor cells; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; PS, performance status.

Table 6. Relationship between CTC levels and outcome

	0 CTC	1 or 2 CTC	≥3 CTC	*P*-value
	Median PFS (95% CI)			
Baseline	11.7 (9.5–13.9)	7.3 (5.4–9.2)	8.5 (7.4–9.6)	0.002
2 weeks	11.3 (7.9–14.2)	9.7 (2.2–17.1)	7.3 (0–21.0)	<0.001
8–12 weeks	9.7 (6.3–13.0)	8.7 (2.6–14.7)	1.9 (0.5–3.3)	<0.001
	Median OS (95% CI)			
Baseline	31.1 (27.2–34.9)	18.7 (5.8–31.6)	15.1 (12.3–17.8)	0.058
2 weeks	29.1 (22.1–36.2)	22 (12.9–31.1)	10.2 (0–25.7)	0.001
8–12 weeks	31.1 (20.2–41.9)	23.3 (20.4–26.2)	4.1 (0–11.7)	<0.001

CI, confidence interval; CTC, circulating tumor cells; OS, overall survival; PFS, progression-free survival.

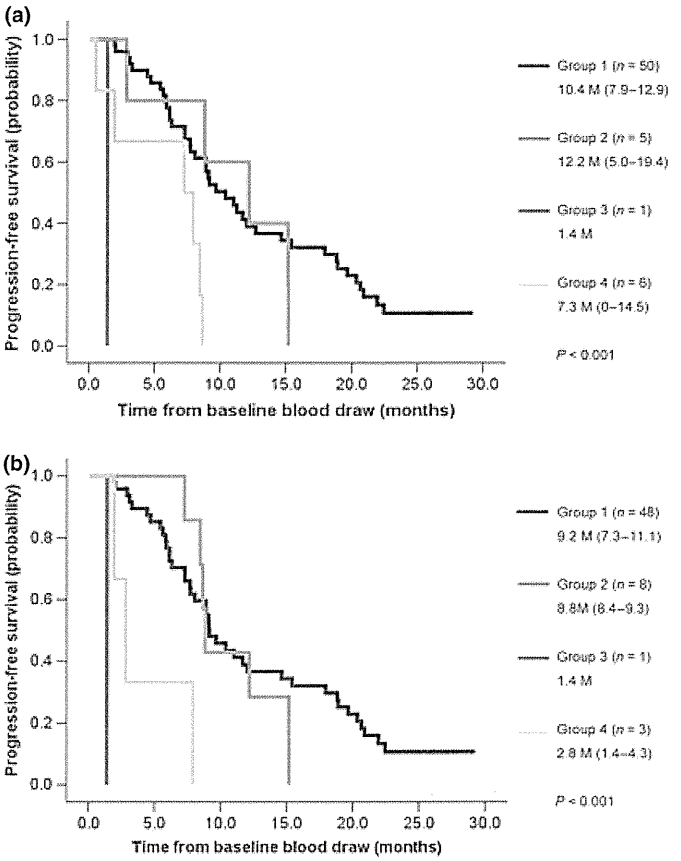

Fig. 4. Kaplan–Meier plots of progression-free survival in metastatic colorectal cancer patients with circulating tumor cell (CTC) change from baseline to 2 weeks (a) and 8–12 weeks (b).

with one or two CTC and those with three or more CTC on the clinical outcome. Patients with one or two CTC had a similar PFS and OS to those with more than three CTC at baseline. In contrast, patients with one or two CTC at 2 and 8–12 weeks had a similar PFS and OS to those with no CTC (Table 6). A cut-off of three CTC not before but during treatment was an independent predictor of PFS and OS in patients with mCRC in the present study. Therefore, we analyzed the relationship between the change in CTC levels from baseline to 2 or 8–12 weeks and the clinical outcome in oxaliplatin-based chemotherapy. Kaplan–Meier plots were generated for patients with <3 CTC at both time points (group 1), patients with three or more CTC at baseline and fewer than three CTC at 2 or 8–12 weeks (group 2), patients with fewer than three CTC at baseline and three or more CTC at 2 or 8–12 weeks (group 3), and patients with three or more CTC at both time points (group 4). Median PFS in group 2 was not significantly different from that in group 1. However, the median PFS in group 2 was significantly longer than that in groups 3 or 4 (Fig. 4). Median OS in group 2 was not significantly different from that in group 1. However, the median OS in group 2 was significantly longer than that in groups 3 or 4 (Fig. 5). The results of the present study clearly show that persistent achievement of fewer than three CTC at 2 weeks after initiating chemotherapy is a strong indicator that the current therapy is effective, whereas three or more CTC is a strong

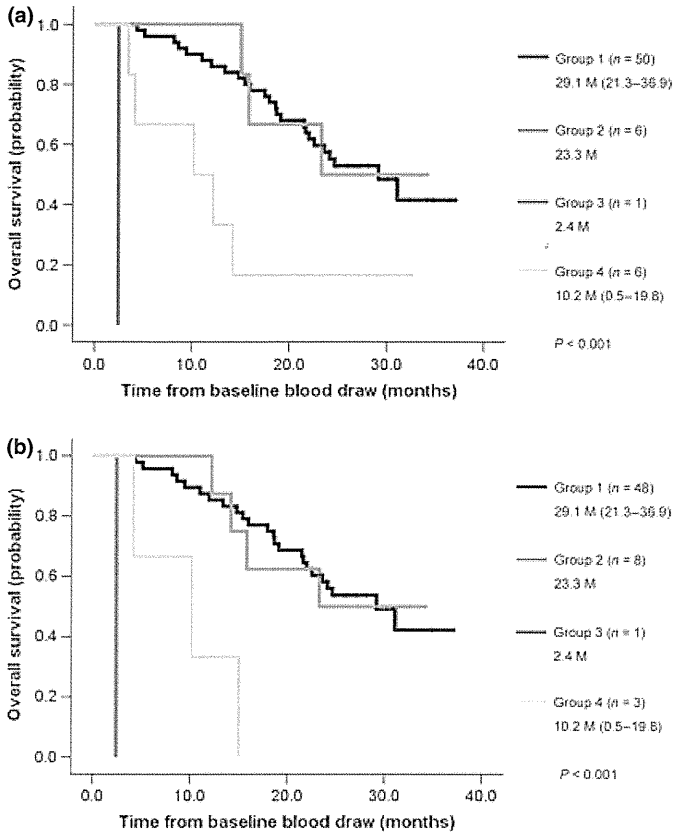

Fig. 5. Kaplan–Meier plots of overall survival in overall survival metastatic colorectal cancer patients with circulating tumor cells (CTC) change from baseline to 2 weeks (a) and 8–12 weeks (b).

indicator that any benefits are likely to be short-term only. These data suggest that CTC counts are valuable in the identification of chemotherapy-resistant patients, irrespective of ethnicity, who could thus benefit from early treatment change and/or different investigational approaches.

The 2006 update of ASCO recommended CEA as the marker of choice for monitoring the response of metastatic disease to systemic therapy. However, caution should be exercised in interpreting a rise in CEA level during the first 4–6 weeks of a new therapy, as a spurious rise might occur early on in treatment, especially with oxaliplatin[11]. We observed a transient increase in CEA level in 11 patients, despite an objective response among those receiving oxaliplatin-based chemotherapy. The observation here of a transient increase in CEA level, even among patients responsive to oxaliplatin-based chemotherapy, agrees with the results of Locker *et al.*[11] In contrast, to our knowledge, no other studies to date have reported such a surge phenomenon in the CTC levels in patients receiving oxaliplatin-based chemotherapy for mCRC. These results suggest that CTC are a more effective marker than CEA for monitoring the response of metastatic disease to systemic therapy.

The strongest data have been provided by analyses from several prospective studies[12–14] that used the US Food and Drug Administration-approved CellSearch system. A previous study showed that CTC detection also provided significant prognostic information for patients with advanced gastric cancer.[15] However, the CellSearch system is yet to be approved for use in Japan. We anticipate that CTC counts for monitoring patients with colorectal, gastric, breast and prostate cancer will eventually be approved in Japan.

In conclusion, our data support the clinical utility of CTC enumeration in improving our ability to accurately assess the treatment benefit and in expediting the identification of effective treatment regimens for individual Japanese patients. In further studies, patients should be randomly assigned to continue current therapy or start a new treatment regimen if they have three or more CTC at 2 weeks before typical imaging intervals.

Acknowledgments

This work was supported by AstraZeneca Research Grant 2007, the Kobayashi Institute for Innovative Cancer Chemotherapy and a Grant-in-Aid for Scientific Research (Japan Society for the Promotion of Science) (grant numbers 19790963, 21591741 and 17016077). The excellent technical assistance of Sayuri Minowa, Harumi Shibata and Mariko Kimura (Cancer Chemotherapy Center, Clinical Chemotherapy, Japanese Foundation for Cancer Research, Tokyo, Japan) is greatly appreciated.

Disclosure Statement

The authors have no conflict of interest.

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doi: 10.1111/j.1349-7006.2011.01926.x

Cancer Chemother Pharmacol (2011) 68:763–768
DOI 10.1007/s00280-010-1543-2

ORIGINAL ARTICLE

Circulating endothelial cells predict for response to bevacizumab-based chemotherapy in metastatic colorectal cancer

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Received: 8 September 2010 / Accepted: 26 November 2010 / Published online: 18 December 2010

Abstract
Purpose Standardized enumeration of CEC counts is required to minimize variability and allow cross-studies comparisons. The purpose of this paper is to identify CEC threshold proposal, by CellSearch system, for determining response to bevacizumab-based chemotherapy in metastatic colorectal cancer.
Methods From July 2007 to June 2008, 33 patients treated with FOLFOX4 plus bevacizumab were enrolled in a prospective study. From January 2007 to June 2007, before bevacizumab was approved by the government in Japan, 31 patients treated with FOLFOX4 as a control were enrolled. CECs of whole blood at the baseline, day 4, 2 weeks after initiation of chemotherapy were isolated and counted using CellSearch system.
Results There was no correlation between CEC levels and the outcome in the FOLFOX4. In the bevacizumab-based chemotherapy, CEC levels at the baseline were significantly associated with the outcome. Patients with 65 or more CECs at the baseline had a shorter median PFS and OS, than the median PFS and OS of less than 65 CECs at the baseline in the bevacizumab-based chemotherapy ($P = 0.003$, $P = 0.027$, respectively). By univariate and multivariate Cox proportional-hazards regression, CEC levels (cut-off; 65) at the baseline indicated the strongest predictor for the outcome to bevacizumab-based chemotherapy.
Conclusion A threshold of lower than 65 CECs, by the CellSearch System, at the baseline was a significant predictor of the outcome for colorectal cancer patients treated with bevacizumab-based chemotherapy.

Keywords Circulating endothelial cell · Metastatic colorectal cancer · Bevacizumab · FOLFOX4

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Introduction

Bevacizumab, a humanized monoclonal antibody against VEGF, has been proven as an effective antiangiogenic agent in cancer [1, 2]. Giantonio et al. [3] have reported the ameliorative effects of FOLFOX4 [L-OHP/5-FU/LV] with bevacizumab therapy in a phase III clinical trial that compared FOLFOX4 alone. In a large observational bevacizumab treatment study (the BRiTE study) in patients who had metastatic colorectal cancer (mCRC), the use of bevacizumab beyond the first progression (BBP) was strongly and independently associated with improved survival compared with post-progression disease treatment without bevacizumab (no BBP) [4]. At present, clinical biomarkers are needed to establish for quantitatively evaluating bevacizumab effects.

Circulating endothelial cell (CEC), derived from endothelial cells that have separated from local vessel walls, is a term that collectively refers to endothelial cells that circulate in the peripheral blood. CEC levels are increased in the peripheral blood of some cancer patients at diagnosis, and these cells return to normal values in patients achieving a complete remission [5–8]. Some authors [9–11] suggest

CECs are a predictive marker of the clinical outcome for cancer patients treated with bevacizumab-based chemotherapy. However, multiple methods and protocols were used to evaluate and count CECs by different laboratories [12, 13]. Standardized enumeration of CEC counts is required to minimize variability and allow cross-studies comparisons. The introduction of monoclonal antibodies with specificity for endothelial cells has led to the development of two main procedures: immunomagnetic bead selection and flow cytometry. These approaches are to first exclude haematopoietic cells by using the pan-haematopoietic marker CD45, and then to confirm the endothelial nature of the remaining CD45-negative cells by using some endothelial markers, such as CD146, CD31 or VEGFR2.

The CellSearch System was developed to accurately and reliably enumerate CECs. CEC sorting is based on a $CD146^{+}CD105^{+}DAPI^{+}CD45^{-}$ phenotypes of CECs. It had been thought for some years that CD146 was an endothelial-specific marker. But there is now evidence that CD146 is also expressed on activated lymphocytes, which are frequently increased in cancer patients. Therefore, the endothelial nature of cells counted using the CellSearch system should be confirmed by the lack of haematopoietic antigen expression. CD45 expression can be used to exclude haematopoietic cells from the analysis. The use of a nuclear-staining molecule can be useful to exclude aggregated platelets and/or endothelial microparticles. CD105 expression, which is expressed in activated endothelial cells, can be used to confirm the endothelial nature of the remaining CD45-negative cells in the CellSearch system. It has been reported that CECs, by the CellSearch system, were elevated in metastatic carcinomas compared with health subjects [14]. As established by the seminal study of Bidard FC et al. [15], CEC count, by the CellSearch system, could be a significant early surrogate marker of time to progression for breast cancer patients treated with bevacizumab combined with standard chemotherapy. Further validation studies are needed to investigate the different CEC threshold proposals in different cancers.

The present investigation was conducted to identify CEC threshold proposal, by CellSearch system, for prediction of the outcome for colorectal cancer patients treated with bevacizumab-based chemotherapy.

Materials and methods

Patients

Principal inclusion criteria were measurable mCRC, and commencement of a new systemic therapy. All patients were enrolled using institutional review board-approved protocols at the Cancer Institute Hospital in the Japanese Foundation for Cancer Research and provided informed consent. From July 2007 to June 2008, 33 patients treated with FOLFOX4 plus bevacizumab were enrolled in a prospective study. From January 2007 to June 2007, before bevacizumab was approved by the government in Japan, 31 patients treated with FOLFOX4 as a control were enrolled. The study population consisted of patients aged 18 years or older with histologically proven mCRC. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, adequate organ function, and radiographic evidence of disease progression as defined by Response Evaluation Criteria in Solid Tumors (RECIST).

Sample preparation for isolation of CECs from blood

Blood (10 ml) was drawn from metastatic colorectal cancer patients into evacuated Cell-Save Preservative Tubes (Veridex LLC, Raritan, NJ). Blood was always drawn from cancer patients before treatment initiation (baseline) and immediately after one course had been completed (day 4), before starting the second cycle (2 weeks) after the administration of chemotherapy.

Sample preparation for isolation of CECs from blood

The CellSearch system (Veridex LLC) consists of the CellPrep system, the CellSearch Endothelial Cell Kit, and the CellSpotter Analyzer. CellPrep is a semi-automated sample preparation system, and the CellSearch Endothelial Cell Kit consists of ferrofluids coated with CD146 antibody and phycoerythrin-conjugated antibodies that bind to CD105 antibody; 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. The 4 ml of blood for CECs was mixed with 6 ml of buffer, centrifuged at $800\times g$ for 10 min, and then placed on the CellPrep system. After aspiration of the plasma and buffer layer by the instrument, ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining re-agents were then added in conjunction with a permeabilization buffer to fluorescently label the immunomagnetically labeled cells. After incubation on the system, the magnetic separation was repeated, and excess staining re-agents were aspirated. In the final processing step, the cells were resuspended in the MagNest Cell Presentation Device (Veridex LLC). This consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis using the CellSpotter Analyzer.

Sample analysis

The MagNest is placed on the CellSpotter Analyzer, a four-color semiautomated fluorescence microscope. Image frames covering the entire surface of the cartridge for each of the four fluorescent filter cubes are captured. The captured images containing objects that meet predetermined criteria are automatically presented in a web-enabled browser from which final selection of cells is made by the operator. The criteria for an object to be defined as a CEC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for CD105, and negative staining for CD45. Results of cell enumeration are always expressed as the number of cells per 4 ml of blood for CECs.

Statistical analysis

Kaplan–Meier survival plots were generated based on CEC levels; at each time, blood was collected, and the curves were compared using log-rank testing. $P < 0.05$ was considered significant. Cox proportional-hazards regression was used to determine univariate and multivariate hazard ratios for selected potential predictors of progression-free survival (PFS) and overall survival (OS).

Results

Patient characteristics

The characteristics of 64 patients diagnosed with mCRC are listed in Table 1. Of 33 patients treated with FOLFOX4 plus bevacizumab assessable for response, we observed two complete responses (CR; 6%), 21 partial responses (PR; 64%), seven patients (21%) with stable disease (SD), and three patients (9%) with progressive disease (PD) during treatment. The overall response rate was 70%. On the other hand, of 31 patients treated with FOLFOX4 assessable for response, we observed 13 PRs (42%), 13 patients (42%) with SD, and five patients (16%) with progression of disease during treatment, for an overall RR of 42%.

Relationship between CEC and optimal therapeutic effects

There was no significant difference in CEC levels for each point between PD cases and non-PD cases in FOLFOX4 without bevacizumab (Fig. 1a). On the other hand, CEC levels of PD cases at the baseline in FOLFOX4 with bevacizumab were significantly higher compared to those in non-PD (Fig. 1b).

Table 1 Patient characteristics

	FOLFOX4 ($n = 31$)	FOLFOX4 + bevacizumab ($n = 33$)
Median age (range)	62 (35–72)	58 (18–71)
Gender (male/female)	16/15	15/18
PS: 0/1	29/2	32/1
Primary site: rectum/colon	15/16	21/12
No. of line: 1st/2nd	22/9	25/8
Site of metastasis		
Liver	17	17
Lung	17	15
Bone	3	1
Lymph node	14	11
Local	6	3
Peritoneum	8	12
Metastases to more than two organs	24	22
Best objective response (CR/PR/SD/PD)	0/13/13/5	2/21/7/3

Relationship between CEC and the outcome

By univariate Cox regression analyses, CEC levels for each point were not significantly associated with PFS in the FOLFOX regimen; however, in the FOLFOX with bevacizumab regimen, CEC levels at the baseline were significantly associated with PFS. At the baseline, CEC prognostic value was assessed using different thresholds to define CEC positivity. To select a level of CECs that most clearly distinguished patients with response to FOLFOX with bevacizumab regimen, thresholds of 1–200 cells for the baseline point were systematically correlated with PFS. The median PFS among patients with levels above or below each threshold differed at the level of 65 CECs of blood and reached a plateau at approximately 65 cells 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 65 CECs was chosen to distinguish patients. A threshold of $\geq$65 CECs was a significant predictor of PFS. Patients with 65 or more CECs at the baseline had shorter median PFS (9.2 months; 95% CI, 4.1–14.3), than the median PFS of fewer than 65 CECs at the baseline (18.9 months; 95% CI, 12.8–25.0) in the bevacizumab-based chemotherapy ($P = 0.003$) (Fig. 2a). Patients with 65 or more CECs at the baseline had shorter median OS (23.3 months; 95% CI, 11.9–34.7), than the median OS of fewer than 65 CECs at the baseline in the bevacizumab-based chemotherapy ($P = 0.027$) (Fig. 2b). Therefore, we analyzed the relationship between CECs at the baseline and clinical outcome in the bevacizumab-based chemotherapy and non-combination chemotherapy. Kaplan–Meier plots were generated for those patients treated in

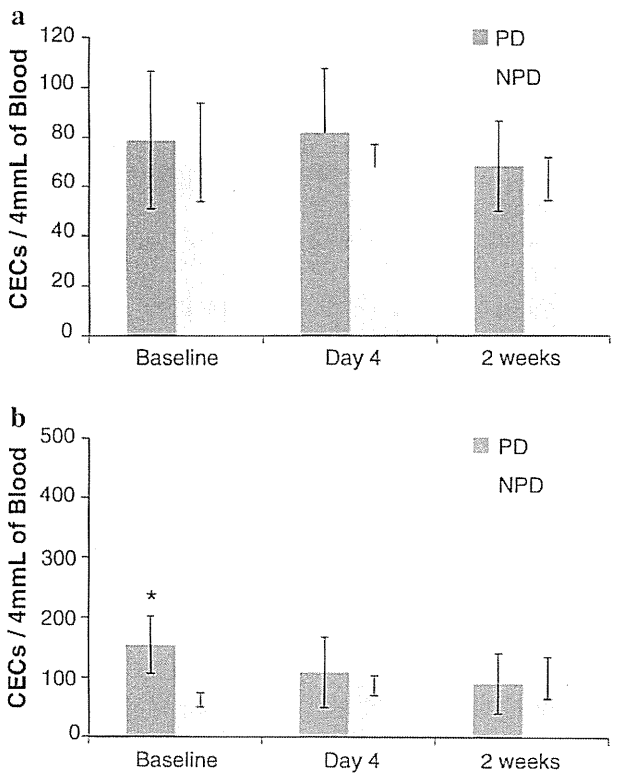

Fig. 1 Relationship between CEC levels of PD cases and CEC levels of NPD cases in FOLFOX4 without bevacizumab (**a**) and FOLFOX4 with bevacizumab (**b**). $*P < 0.05$

the non-combination chemotherapy (group 1), those who had fewer than 65 CECs at the baseline in the bevacizumab-based chemotherapy (group 2), and those who had 65 or more CECs at the baseline in the bevacizumab-based chemotherapy (group 3). Patients in group 2 had the longest median PFS or OS among the three groups ($P < 0.001$, $P = 0.004$, respectively) (Fig. 3a, b). PFS and OS show no difference between group 1 and group 3 ($P = 0.145$, $P = 0.776$, respectively) (Fig. 3).

Univariate and multivariate analysis of predictors of the outcome

Univariate and multivariate Cox proportional-hazards regression was performed to assess the association between factors of interest and PFS or OS in the bevacizumab combination chemotherapy. In the univariate analysis, lung metastasis, lymph node metastasis, and CEC levels (cut-off; 65) at the baseline predicted PFS (Table 2). In the univariate Cox regression analyses, peritoneal metastasis and CEC levels (cut-off; 65) at the baseline were associated with OS (Table 3). In order to evaluate the independent predictive effect of chemotherapy, multivariate Cox regression analysis was carried out. CEC levels at the baseline were the strongest predictor (Tables 2, 3).

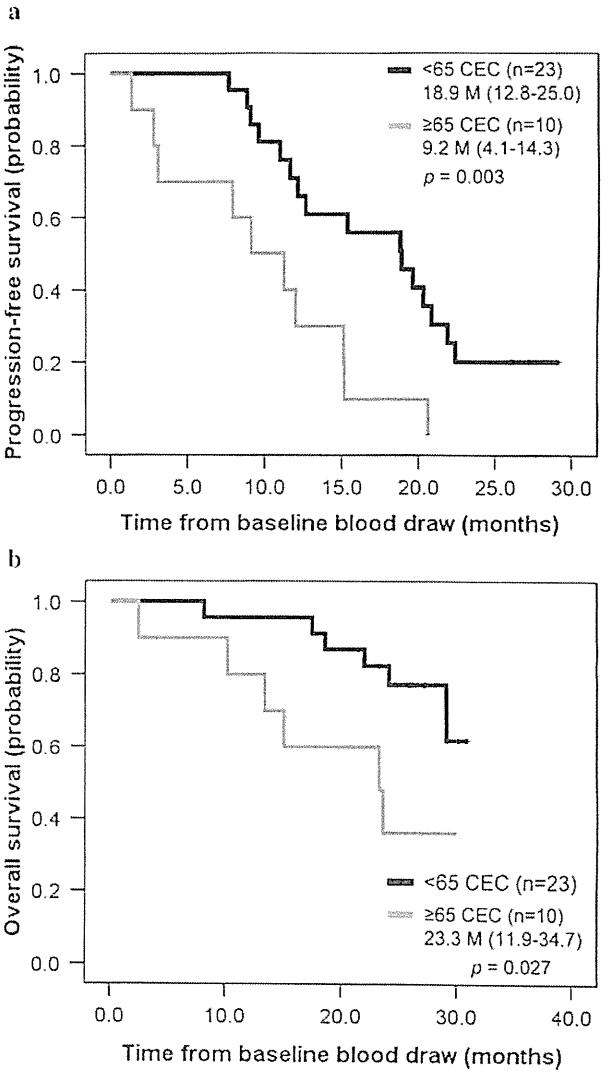

Fig. 2 Kaplan–Meier plots of progression-free survival (PFS) in metastatic colorectal cancer (mCRC) patients with fewer than 65 circulating endothelial cells (CEC, *top line*) or ≥65 CECs (*bottom lines*) at the baseline in FOLFOX4 with bevacizumab (**a**). Kaplan–Meier plots of overall survival (OS) in mCRC patients with fewer than 65 circulating endothelial cells (CEC, *top line*) or ≥65 CECs (*bottom lines*) at the *baseline* in FOLFOX4 with bevacizumab (**b**)

Discussion

By univariate and multivariate Cox proportional-hazards regression, CEC levels (cut-off; 65) at the baseline indicated the strongest predictor for the outcome to bevacizumab-based chemotherapy. Further, patients with fewer than 65 CECs threshold at the baseline in the bevacizumab-based chemotherapy had the longest median PFS or OS. The outcomes between those who had 65 or more CECs threshold at the baseline in the bevacizumab-based chemotherapy and those who treated non-combination chemotherapy showed no difference. These results suggest

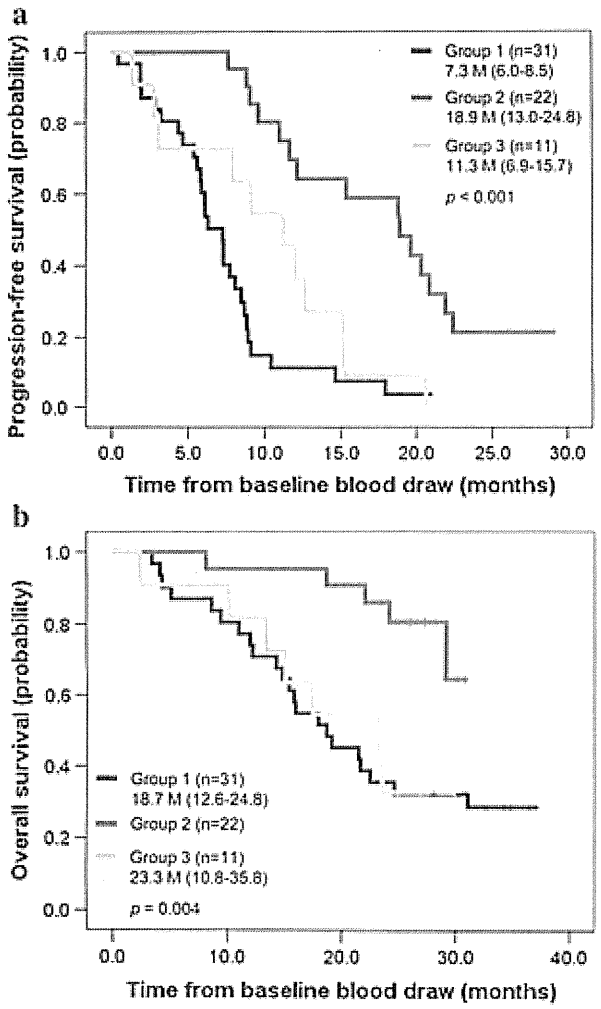

Fig. 3 Patients having CECs at the baseline in the non-combination group (*group* 1), patients having fewer than 65 CECs at the baseline in the bevacizumab-combination group (*group* 2), and patients having 65 or more CECs at the *baseline* in the bevacizumab-combination group (*group* 3). Kaplan–Meier plots of progression-free survival (PFS) (a). Kaplan–Meier plots of overall survival (OS) (b)

Table 2 Cox regression analysis for prediction for PFS

Parameter	No. of pts	HR	95% CI	*P* value	χ^2
Univariate Cox regression analyses					
CEC (cut-off; 65)	33	3.32	1.42–7.74	0.006	0.003
Lung metastasis	33	2.79	1.23–6.32	0.014	0.011
LN metastasis	33	2.44	0.11–0.84	0.022	0.016
Multivariate Cox regression analyses					
No. of patients	33				<0.001
CEC (cut-off; 65)		0.24	0.09–0.69	0.007	
LN metastasis		4.37	1.76–10.84	0.001	

Table 3 Cox regression analysis for prediction for OS

Parameter	No. of pts	HR	95% CI	*P* value	χ^2
Univariate Cox regression analyses					
CEC (cut-off; 65)	33	3.36	1.07–10.84	0.038	0.027
Peritoneal metastasis	33	4.41	1.28–15.18	0.019	0.010
Multivariate Cox regression analyses					
No. of patients	33				0.003
CEC (cut-off; 65)		3.77	1.18–12.03	0.025	
Peritoneal metastasis		4.88	1.40–17.1	0.013	

that patients with 65 or more CECs threshold at the baseline are not beneficial to administer bevacizumab.

A few studies, by flow cytometry, suggested CEC predictive value are different in each kind of cancer. In breast cancer, most studies [7, 9, 16] have reported that high CEC levels at baseline indicated 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 [10, 17]. These results suggest a vascular turnover differs according to tumor origin. However, these differences in the results between these two 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. [13] reported a cytometry protocol for phenotypic identification and enumeration of CECs and CEPs using four surface markers: CD31, CD34, CD133, and CD45. This protocol has been mainly used in colorectal cancer. Mancuso et al. [12] reported a protocol for the phenotypic identification and enumeration of CECs and CEPs involving six-color flow cytometry and nuclear staining with Syto16 and 7-AAD and a panel of monoclonal antibodies, including CD45, CD133, CD31, and CD146. This protocol has been mainly used in breast cancer. In breast cancer experiences, the results, by CellSeach system, reported by Bidard [15] are in line with those, by flow cytometry, reported by Calleri [16]. In colorectal cancer experiences, our results support the view, reported by Ronzoni [17], that patients with lower baseline CEC values have better PFS. These results evaluated by the CellSearch system in each kind of cancer are compatible with those results evaluated by flow cytometry, respectively. The possible reasons might be related to a different vascular turnover in breast vs colorectal cancer.

We conclude that a threshold of fewer than 65 CECs, by CellSearch system, is a significant predictor of the outcome for colorectal cancer patients treated with bevacizumab-based chemotherapy. Further studies are needed to be

validated in large-scale, prospective, biomarker-embedded clinical trials.

Acknowledgments This work was supported by AstraZeneca Research Grant 2007, the Kobayashi Institute for Innovative Cancer Chemotherapy, and a Grant-in-Aid for Scientific Research (Japan Society for the Promotion of Science) (grant number 19790963, 21591741, 17016077). The excellent technical assistances of Sayuri Minowa, Harumi Shibata, and Mariko Kimura are greatly appreciated.

Conflict of interest None.

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International Journal of Cancer

Antitumor effect of sunitinib against skeletal metastatic renal cell carcinoma through inhibition of osteoclast function

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We investigated the inhibitory effect of sunitinib, a newly approved multitargeted tyrosine kinase inhibitor, against the progression of renal cell cancer (RCC) bone metastases *in vivo*. *In vitro* cell proliferation was determined using the MTS assay. To investigate the inhibitory effects of sunitinib *in vivo*, we established luciferase-labeled ACHN^{Luc} cells derived from papillary RCC. Mice in which ACHN^{Luc} cells had been transplanted into the left ventricle to establish bone metastases were treated orally with 40 mg/kg/day sunitinib or vehicle control for 3 weeks. Growth of the cancer cells was monitored using an *in vivo* imaging system. In addition, 16 patients with metastatic RCC were treated with sunitinib, and serum and urine levels of amino-terminal telopeptide (NTx) were measured as markers of bone resorption. Sunitinib did not inhibit the growth of RCC cells *in vitro* at clinically or experimentally achievable serum levels (100 nM–1 μM). To investigate the inhibitory effect of sunitinib *in vivo*, we established luciferase-labeled human RCC cells (ACHN^{Luc}). Sunitinib prevented the growth of ACHN^{Luc} RCC cells in the bone metastatic mouse model. The number of osteoclasts in sunitinib-treated mice was significantly less than that in control mice. Serum and urine levels of NTx in patients with metastatic RCC declined significantly during the first 4 weeks of sunitinib treatment ($p = 0.027$). Sunitinib is a potent anticancer agent for RCC bone metastases, at least for papillary RCC.

Bone is a common site of metastasis, with the frequency of solitary or multiple metastases to bone ranging from 24 to 51% in patients with metastatic renal cell cancer (RCC).[1–3] Although bone metastasis is not an independent prognostic factor associated with poor survival, the prognosis of patients with bone metastasis is not favorable when they are treated with cytokines, with an average life expectancy of 8–16 months.[2–4] Moreover, bone metastases are associated with poor performance status due to intractable pain and pathological fractures.[5] Because treatment options for RCC patients with bone metastasis are limited, appropriate treatment strategies are desired.

Sunitinib is a newly approved, multitarget, small-molecule tyrosine kinase inhibitor for the treatment of metastatic RCC. It inhibits various receptor tyrosine kinases, including vascular endothelial growth factor (VEGF) receptors 1, 2 and 3; stem cell factor receptor (KIT) and PDGF receptors α and β.[6–8] Moreover, sunitinib has been known to inhibit the phosphorylation of colony-stimulating factor (CSF)-1R, resulting in the prevention of osteoclast function and CSF-1R-dependent osteolysis in an experimental breast cancer bone metastasis model.[9,10] These findings led us to propose the hypothesis that sunitinib may inhibit tumor growth and osteolysis in bone metastatic lesions in RCC patients.

Although establishing a treatment strategy for bone metastases from RCC is important for urologists, the assessment of inhibitory effects on the growth of bone metastases is often difficult in clinical practice. In this study, we show that sunitinib has anticancer as well as inhibitory activities against osteolysis in an experimental mouse model of bone metastasis of RCC cells.

Key words: renal cell carcinoma, bone metastases, sunitinib, *in vivo* imaging system
Grant sponsors: Takeda Science Foundation, The Kobayashi Institute for Innovative Cancer Chemotherapy, The Shimadzu Science Foundation, The Sagawa Foundation for Promotion of Cancer Research, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and the GCOE program of the Ministry of Education, Culture, Sports, Science and Technology, Japan
DOI: 10.1002/ijc.26034
History: Received 15 Jun 2010; Revised 4 Jan 2011; Accepted 26 Jan 2011; Online 8 Mar 2011
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Material and Methods

Animals, cell lines and reagents

Approval for these studies was obtained from the institutional review board at Akita University School of Medicine. Specific pathogen-free BALB/c *nu/nu* mice (CLEA, Kyoto, Japan) aged 7 weeks were used. The human RCC lines ACHN, CCFRC-1, CCFRC-2 and NC65 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and sunitinib was obtained from Pfizer (New York, NY).

Patients

A total of 16 native Japanese patients with metastatic RCC, who were treated at the Department of Urology at Akita University School of Medicine between 2008 and 2009, were enrolled, and the serum and urine levels of amino-terminal telopeptide (Serum NTx, normal range: 9.5–17.7 nmol/l) were measured as markers of bone resorption. The patients' characteristics are shown in Table 1. The median dose was 37.5 (25–50) mg/day and the median number of treatment cycles was 4.6 (1–21). Written informed consent was provided according to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Akita University Graduate School of Medicine. The response was assessed by computed tomography (CT) after at least every two cycles of treatment, according to the Response Evaluation Criteria in Solid Tumors (RECIST ver. 1.0).[11]

Growth inhibitory effects of sunitinib in vitro

Cell proliferation was determined by the MTS assay using CellTiter96 (Promega Corporation, Madison) as described previously.[12]

Generation of a stable luciferase-expressing cancer cell line

Among the RCC cell lines we tested (ACHN, CCFRC-1, CCFRC-2 and NC65), ACHN was the only line that was transplanted into the left ventricle and formed bone metastases successfully. Therefore, we used ACHN^{Luc} in the *in vivo* experiment. ACHN cells were stably transfected with the pGL3 control vector (Promega Corporation, Madison) and with pSV2Neo (ATCC), as described previously.[12] In brief, the cells were treated with 10 μg pGL3 control vector and 1 μg pSV2Neo vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen) and selected using geneticin (400 μg/ml). Stable clones expressing luciferase were isolated and the clone with the highest level of luciferase expression (as determined by bioluminescence) was selected using luciferin (Xenogen, Alameda, CA) and an *in vivo* imaging system (IVIS; Xenogen).

In vivo effects of sunitinib

To produce bone metastasis models, RCC cell suspensions (3×10^6/100 μl phosphate-buffered saline) were injected into the left ventricle of mice under inhalation anesthesia with isoflurane (Abbott Japan, Tokyo, Japan). From 21 days after implantation, 14 mice with bone metastases were selected and divided into two matched groups on the basis of bioluminescence quantified by IVIS. On the same day, we started daily oral administration of 40 mg/kg (body weight) sunitinib or the solution used to dissolve sunitinib as vehicle control. According to the human 4 weeks on/2 weeks off schedule, mice were treated with sunitinib for 4 weeks before being sacrificed. Mice were observed by IVIS once per week.

Table 1. Patients characteristics

Factors			
Age (years old)	Median	60.5	Range: 37–80
Sex (*n*)	Male	13	(81%)
	Female	3	(19%)
Metastatic sites	Lung	12	(75%)
	Liver	4	(25%)
	Bone	5	(31%)
	Brain	3	(19%)
	Lymph node	3	(19%)
Follow-up period (month)	Median	4.5	Range: 1–37
Performance status	0 or 1	15	(94%)
	>1	1	(6%)
Diagnosis to initial treatment	>1 year	5	(31%)
	<1 year	11	(69%)
Blood hemoglobin	Normal range	7	(44%)
	ULN>	9	(56%)
Serum calcium	<10 mg/dl	15	(94%)
	≥10 mg/dl	1	(6%)
Serum LDH	<1.5 × ULN	16	(100%)
	≥1.5 × ULN	0	(0%)
MSKCC risk classification	Favorable	6	(38%)
	Intermediate	9	(56%)
	Poor	1	(6%)

ULN: upper limit of normal range.

Measurement of bone metastatic lesions by in vivo imaging

An aqueous solution of luciferin (150 mg/kg) was injected intraperitoneally 10 min before imaging. The animals were anesthetized with isoflurane and placed in the light-tight chamber of a CCD camera system (Xenogen) and photons emitted from the luciferase-expressing cells within the animal were quantified for 5 min using the software program Living Image (Xenogen) as an overlay on Igor (Wavemetrics, Seattle, WA). Using this *in vivo* imaging system, we evaluated the efficacy of sunitinib by measuring the photon counts of the metastatic lesions in the mandible and both hip joints in a blinded manner as described previously.[13]

Measurement of serum VEGF and M-CSF in the mouse bone metastasis model in vivo

The serum concentrations of VEGF and M-CSF in mice were determined using Quantikine ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. To investigate the serum concentrations of VEGF and M-CSF, sera from each of seven treated and seven untreated mice were collected and analyzed 4 weeks after $ACHN^{Luc}$ inoculation.

Histological analysis

After imaging studies, the femora of the mice were removed, frozen immediately and stored at −80°C. To detect osteoclasts, 4-μm-thick sections were stained with tartrate-resistant acid phosphatase (TRAP) using the TRAP and ALP double-stain kit (Takara Bio, Otsu, Japan), as described previously.[14] Three sections were examined in each femur. The number of TRAP-positive osteoclasts was counted per ten high-power microscope fields by two blinded examiners, as described previously.[14]

Statistical analysis

The influence of sunitinib on the growth of bone metastases was analyzed by Student's *t* test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 13.0; SPSS, Chicago, IL), and two-sided *p* values <0.05 were considered statistically significant.

Results

Effect of sunitinib on RCC growth in a mouse bone metastasis model in vivo

Injection of cancer cells via the left ventricle is an established method of inducing bone metastases, as reported previously.[12,13] In the present study, all mice that were successfully implanted with $ACHN^{Luc}$ cancer cells developed bone metastases 3 weeks after injection. Of these mice, we excluded those that showed brilliant bioluminescence in the lungs. The remaining 14 mice were then divided into two matched groups according to bioluminescence quantified by IVIS, and we administered either sunitinib or vehicle control for 4 weeks and monitored the growth of bone metastases in the lesions in the maxilla and bilateral hip joints, as described previously[12,13] (Fig. 1*b*). Metastatic bone lesions in the control group progressed during the 3 weeks. On the other hand, photon emission was significantly suppressed in the sunitinib treatment group ($p < 0.001$) (Figs. 1*a* and 1*c*). The mean body weights of the mice did not differ significantly between the two groups.

Serum VEGF and M-CSF in a mouse bone metastasis model in vivo

To examine the indirect antitumor effect of sunitinib, we measured the concentrations of VEGF and M-CSF. However, no significant difference was present in the serum concentrations of these growth factors between the two groups (Fig. 1*d*).

Effect of sunitinib on osteoclasts in a mouse bone metastasis model

Next, we investigated the efficacy of sunitinib against osteoclasts in the tumor-bearing mice. Femoral bone sections were stained with TRAP to enable counting of the number of osteoclasts, as described previously.[13] The mean number of TRAP-positive osteoclasts in mice treated with sunitinib was significantly lower than that in mice treated with vehicle control (23.1 ± 4.7 *vs.* 33.2 ± 7.9 osteoclasts/100 high-power fields, respectively; $p = 0.013$).

Sunitinib did not inhibit cell proliferation in vitro at a clinically achievable serum concentration

To assess the direct antitumor effect of sunitinib, four RCC cell lines (ACHN, CCFRC-1, CCFRC-2 and NC65) were cultured in the presence of various concentrations of sunitinib (0.1 nM–10 μM). Sunitinib inhibited the proliferation of these cell lines in a concentration-dependent manner (Fig. 2). However, sunitinib was not effective *in vitro* at the clinically achievable serum concentration (~80 nM), as demonstrated previously.[8] On the other hand, the serum concentration of sunitinib was reported to be ~100 nM on administration to mice at 40 mg/kg/day.[14] The IC50s of sunitinib for these cell lines were estimated to be >1 μM. These results suggest the involvement of an indirect growth inhibitory mechanism of sunitinib, at least partially, for bone metastatic lesions in mice.

Effect of sunitinib on serum and urine levels of NTx in patients with metastatic RCC

The characteristics and demographic data of the patients are shown in Table 1. As shown in Figure 3, both serum and urine levels of NTx significantly declined during the first 4 weeks of treatment with sunitinib ($p = 0.027$). During the holiday period when the administration was discontinued following 4 weeks of administration of sunitinib, the serum and urine levels of NTx showed gradual recovery (Fig. 3). Of these 16 patients, five had bone metastatic lesions, but we could not evaluate the efficacy of sunitinib quantitatively. Regarding the extraosseous sites, nine of 14 patients demonstrated a partial response (PR) or stable disease (SD) whereas the remaining five demonstrated progressive disease (PD). The reduction rate of the serum NTx level from the baseline in patients with favorable efficacy (PR/SD; 30.8%) was higher than that in patients with poor efficacy (PD; 22%), although the difference was not significant ($p = 0.6404$).

Discussion

In patients with metastatic RCC, bone is the major metastatic organ, second only to the lung.[1–3] Bone metastases were shown to be associated with severe bone pain, pathological fractures, spinal cord compression and a short survival

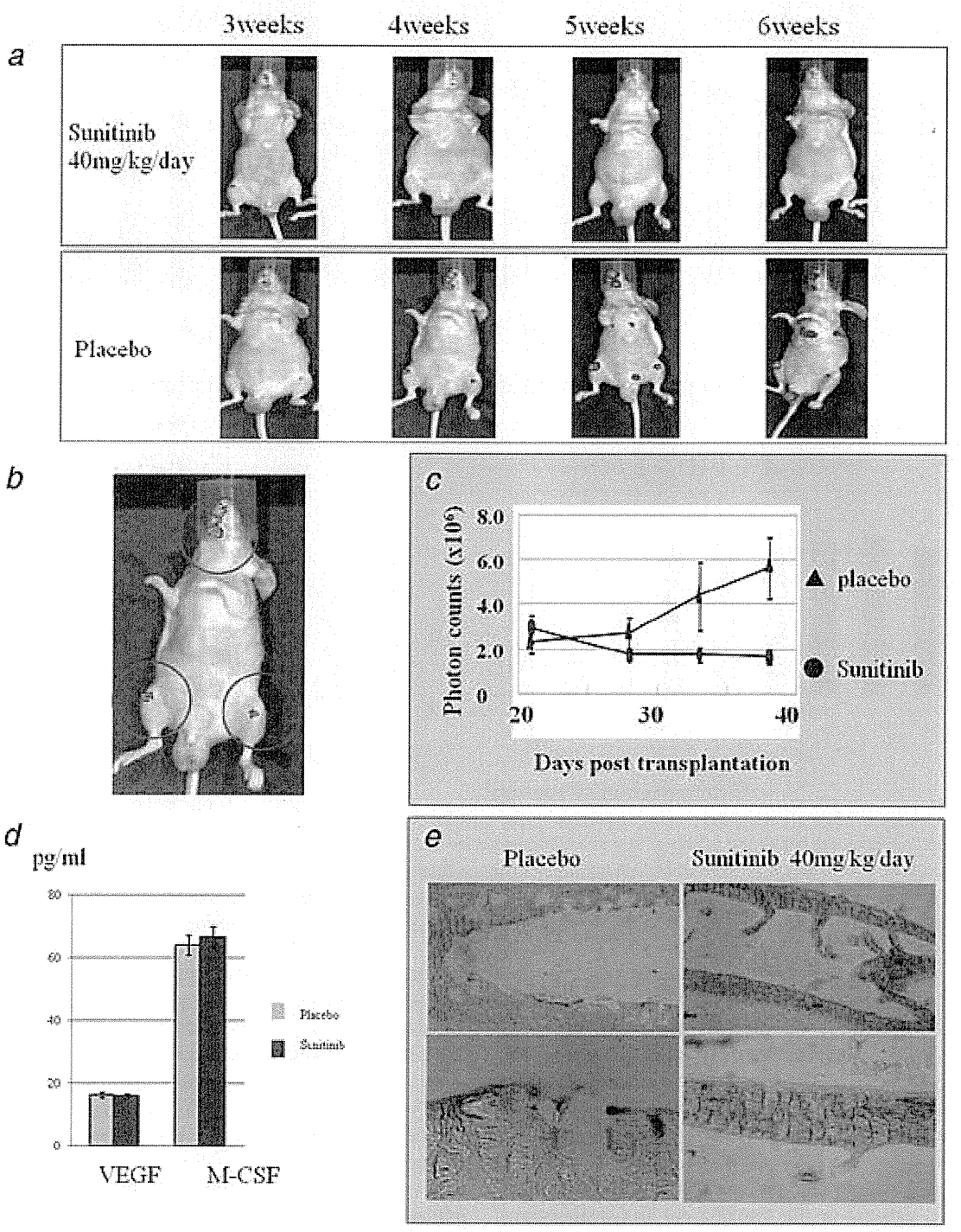

Figure 1. Growth inhibitory effect of orally administered sunitinib in an RCC bone metastatic mouse model. We established an RCC bone metastatic mouse model using the cell line ACHN^{Luc}. Images were obtained using an *in vivo* imaging system 3–6 weeks after cell transplantation by intracardiac injection (*a*). To evaluate the growth inhibitory effect of orally administered sunitinib, we selected metastatic lesions from the maxilla and bilateral hip joints as examples of bone metastasis (*b*). Average real-time growth curves of ACHN^{Luc} cells of bone metastatic lesions in sunitinib- and control vehicle-treated groups demonstrated that sunitinib significantly prevented the growth of metastatic bone lesions ($p < 0.001$; *c*). Serum levels of VEGF and M-CSF did not differ significantly between sunitinib-treated and control mice (*d*). The mean number of TRAP-positive osteoclasts in mice treated with sunitinib was significantly lower than that in mice treated with vehicle control ($p = 0.013$; *e*).

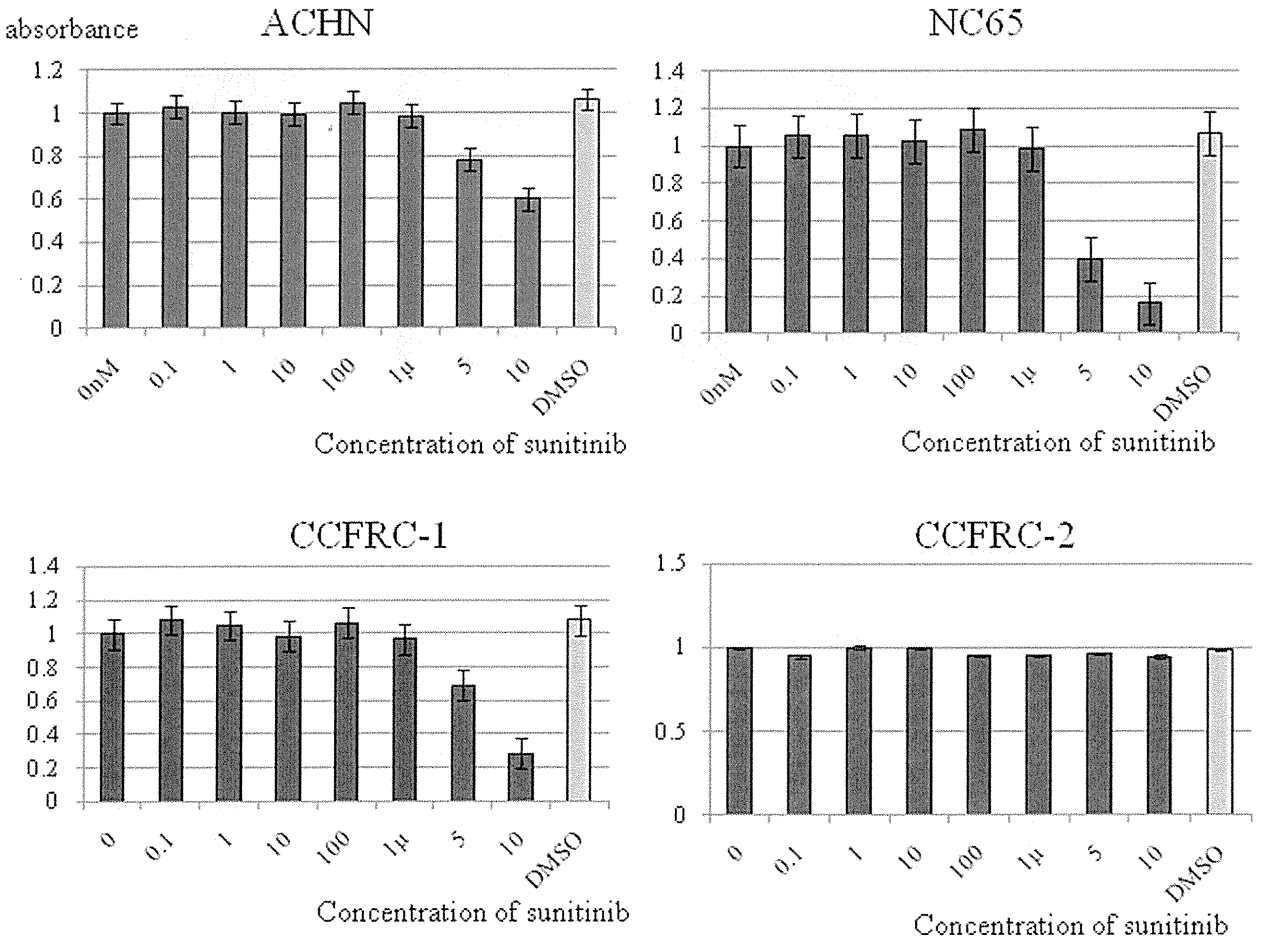

Figure 2. Sunitinib does not inhibit the growth of RCC at a clinically achievable concentration *in vitro*. Cells of the RCC lines ACHN, NC65, CCFRC-1 and CCFRC-2 were plated at 3,000 cells/well in 96-well plates, incubated for 24 hr, and then treated with various concentrations (0–100 mM) of sunitinib. After 72 hr of incubation, relative cell growth was measured in an MTS assay. Data are Mean ± SD. Sunitinib did not inhibit the growth of any of the four RCC cell lines at the clinically achievable concentration (~80 nM) *in vitro*.

period.[4,11] Several studies have demonstrated that bone metastasis is one of the risk factors for poor prognosis in the cytokine era, although it was not identified as an independent prognostic factor.[1–4] Négrier *et al.* investigated the prognostic factors of 782 metastatic RCC patients treated with cytokines and found that 32% (248/776) had bone metastases, and that these patients had a significantly worse prognosis than those without bone metastases ($p = 0.008$).[2] Recently, Naito *et al.* retrospectively analyzed the prognosis of 1,463 Japanese metastatic RCC patients in the cytokine era and demonstrated that 24.6% (320/1,302) had bone metastases, and that these patients also had a significantly worse prognosis than those without bone metastases ($p = 0.003$).[3] Accumulated evidence suggests that systemic immunotherapy is not effective in the management of bone metastasis of RCC.

The efficacy of sunitinib against RCC bone metastasis, however, remains to be established and is difficult to evaluate in clinical practice. Thus, we sought to investigate the efficacy of sunitinib against bone metastatic RCC in the preclinical setting. The dose of sunitinib used in this study (40 mg/kg/day) was intended to provide a serum level of sunitinib similar to that attained in the clinical setting.[8,15] Pharmacokinetic and pharmacodynamic analyses showed that the clinical dose of 50 mg/day led to plasma concentrations ranging from 50 to 100 ng/ml in humans.[8] This dose is equivalent to the plasma concentration in mice administered sunitinib at 40 mg/kg/day.[15] Data from VEGF-induced vascular permeability assays also support 50–100 ng/ml as the range, including the minimum plasma concentrations required to inhibit VEGFR and PDGFR *in vivo*.[8] Therefore, our results obtained in the RCC bone metastatic model used in this study might be reflective of those obtained in the clinical setting.

Similar to several other *in vitro* analyses, our results showed that sunitinib at concentrations of 50–100 ng/ml did not inhibit the proliferation of RCC cells *in vitro*.[10,16] Therefore, we sought an indirect mechanism for this *in vivo* growth inhibition of RCC bone metastases. Bone is an abundant repository for immobilized growth factors, including

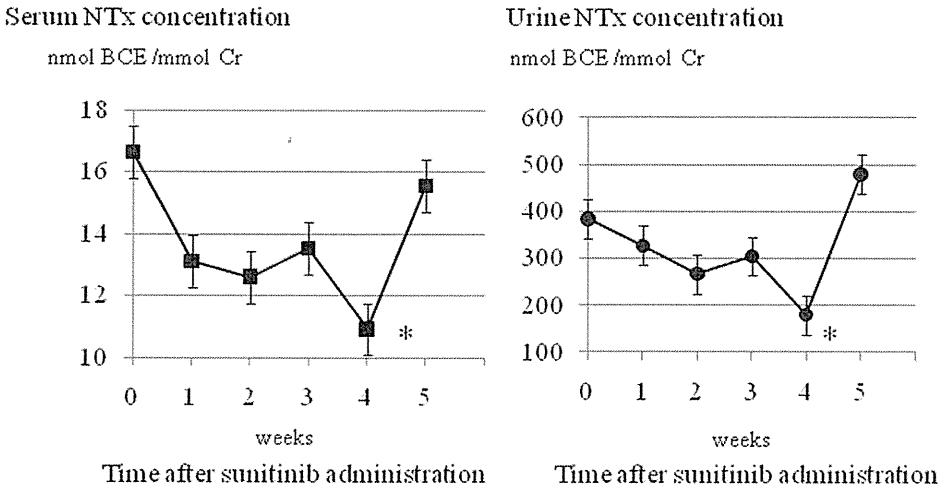

Figure 3. Alteration of bone resorption markers in sunitinib-treated patients with metastatic RCC. Serum and urine levels of NTx 28 days after oral administration of sunitinib were significantly lower than initial levels ($*p < 0.01$). Characteristics of the 16 sunitinib-treated patients are shown in Table 1.

transforming growth factor beta, fibroblast growth factor, insulin-like growth factors I and II, PDGF and bone morphogenetic proteins.[17] When osteoclasts absorb bone by secreting protons and proteases, these growth factors are released and they provide fertile ground for the growth of cancer cells. Therefore, osteoclasts are a suitable therapeutic target in the treatment of bone metastases. In this study, there were significantly fewer TRAP-positive osteoclasts in the mice treated with sunitinib than in those treated with vehicle control (Fig. 1*e*). This observation is consistent with previous reports.[10,18] Zwolak *et al.* reported that treatment with sunitinib decreased the percentage of active osteoclasts to 45.6% ± 5.8% compared with the percentage in untreated tumor-bearing mice (79.4% ± 8.6%), suggesting that sunitinib treatment (40 mg/kg/day) may inhibit osteoclast maturation.[18] Murray *et al.* reported that sunitinib inhibited osteoclast development and function mediated by M-CSF, which is one of the differentiating factors for osteoclasts and is a target tyrosine kinase of sunitinib, both *in vitro* and *in vivo*.[10] Our clinical observation of decreases in serum and urine NTx is also in line with these reports (Fig. 3).

NTx is a degradation product of Type I collagen and is often used as a marker of bone resorption both in serum and urine. Some clinical studies have suggested that levels of NTx correlate with the presence and extent of bone metastases, prognosis and response to treatment.[19,20] Although our data did not show an association between the reduction rate of NTx and the efficacy of sunitinib, further investigation is necessary to clarify this association, especially in bone metastatic lesions.

During the completion of this manuscript, we found that ACHN originated from papillary renal cancer in a 22-year-old patient (Reference [21] and by personal communication from Dr. Ernest Borden). Recent studies have suggested the possible clinical efficacy of sunitinib for patients with clear and non-clear cell cancer.[22,23] However, there are no prospective Phase 2 or Phase 3 studies clarifying this question. We therefore have to wait for the results of a large prospective study on the use of sunitinib for non-clear cell cancer. Since bone is the second most common site of metastases for RCC, we reported an indirect mechanism that may partly help to elucidate the reasons for the clinical efficacy of sunitinib.

Mesenchymal-epithelial transition factor (MET) and fumarate hydratase (FH) are considered to be the genes responsible for Type 1 and Type 2 papillary RCC, respectively.[24,25] MET, which is a proto-oncogene, encodes a tyrosine kinase membrane receptor, and activation of MET can indirectly promote angiogenesis and tumor growth through overexpression of VEGF.[26,27] FH is an enzyme in the mitochondrial tricarboxylic acid (TCA) cycle. Loss of FH leads to a state of pseudohypoxia through overexpression of hypoxia-inducible factor (HIF), resulting in an increase in downstream targets, including VEGF.[26,28] Therefore, activation of MET and loss of FH, which are considered to be responsible for Type 1 and Type 2 papillary RCC, lead to angiogenesis. Clinically, Ljungberg *et al.* demonstrated that the mRNA levels of VEGF, VEGF-receptor Type 1 and VEGF-receptor Type 2 above the median were related to adverse survival in papillary RCC.[29] Therefore, it is relevant to measure VEGF in a clear or non-clear cell RCC model.

To elucidate whether sunitinib has any other indirect effects, we measured the concentrations of VEGF and M-CSF. However, we found no significant difference between the two groups in the serum concentrations of these growth factors. This observation is consistent with previous findings. Ebos *et al.* reported a significant increase in the serum VEGF level on administration of 60–120 mg/kg sunitinib.[30] While it has been shown that sunitinib is a multikinase inhibitor that