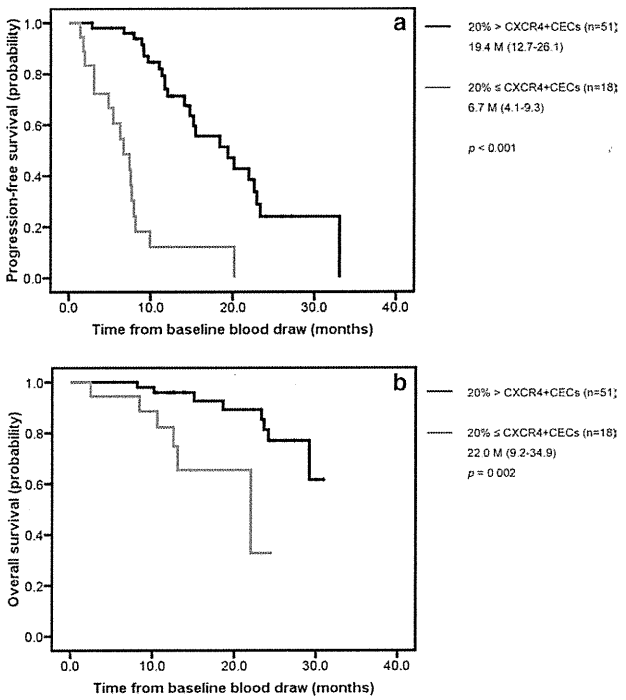

Figure 3. Depicted are Kaplan-Meier plots of (a) progression-free survival (PFS) and (b) overall survival (OS).

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.

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.

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 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

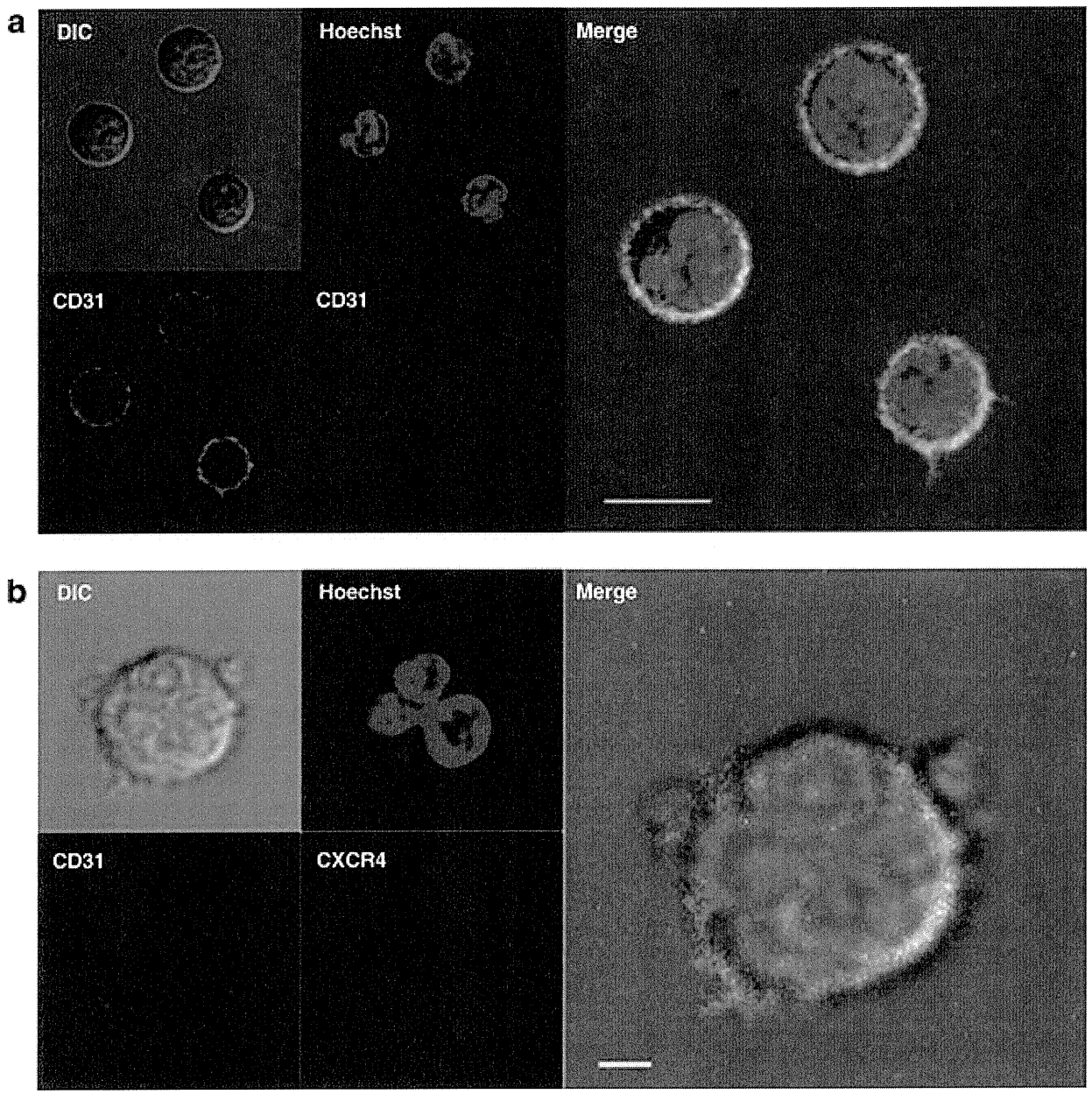

Figure 4. (a) CEPs and (b) CXCR4 + CECs were sorted by flow cytometry as described in the Materials and Methods section and analyzed by confocal microscopy. DIC indicates differential interferences contrast; bar, 5 μm.

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 Japanese patients with metastatic colorectal cancer

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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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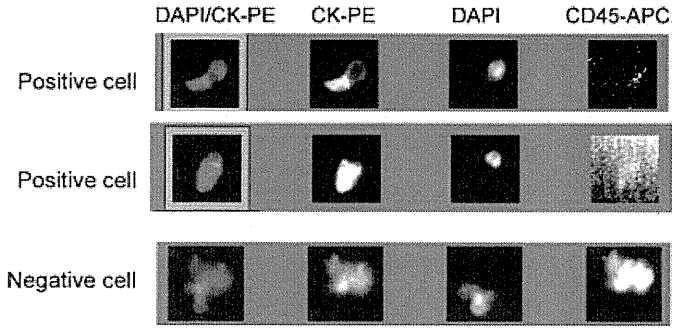

Fig. 1. Image galleries after CellSearch processing. Circulating tumor cells were cytokeratin (CK) and DAPI positive, but CD45 negative.

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).

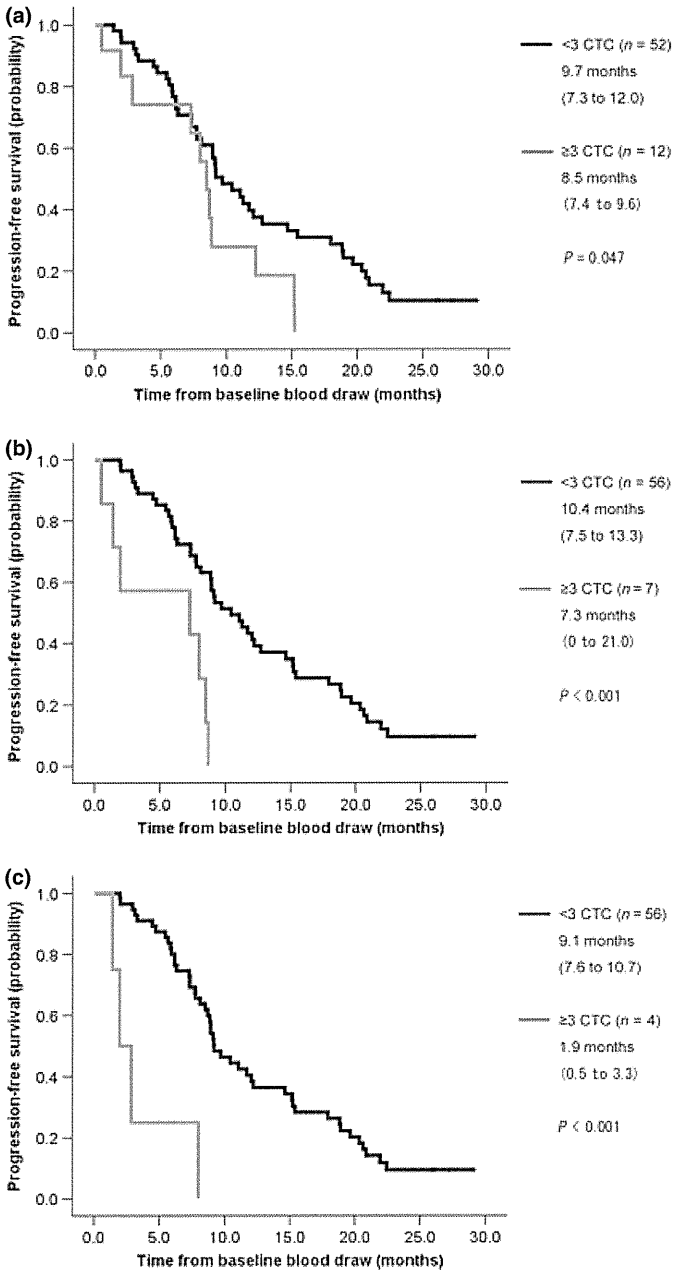

Fig. 2. Kaplan–Meier plots of progression-free survival in metastatic colorectal cancer patients with fewer than three circulating tumor cells (CTC) or ≥3 CTC at baseline (a), 2 weeks (b) and 4 weeks (c).

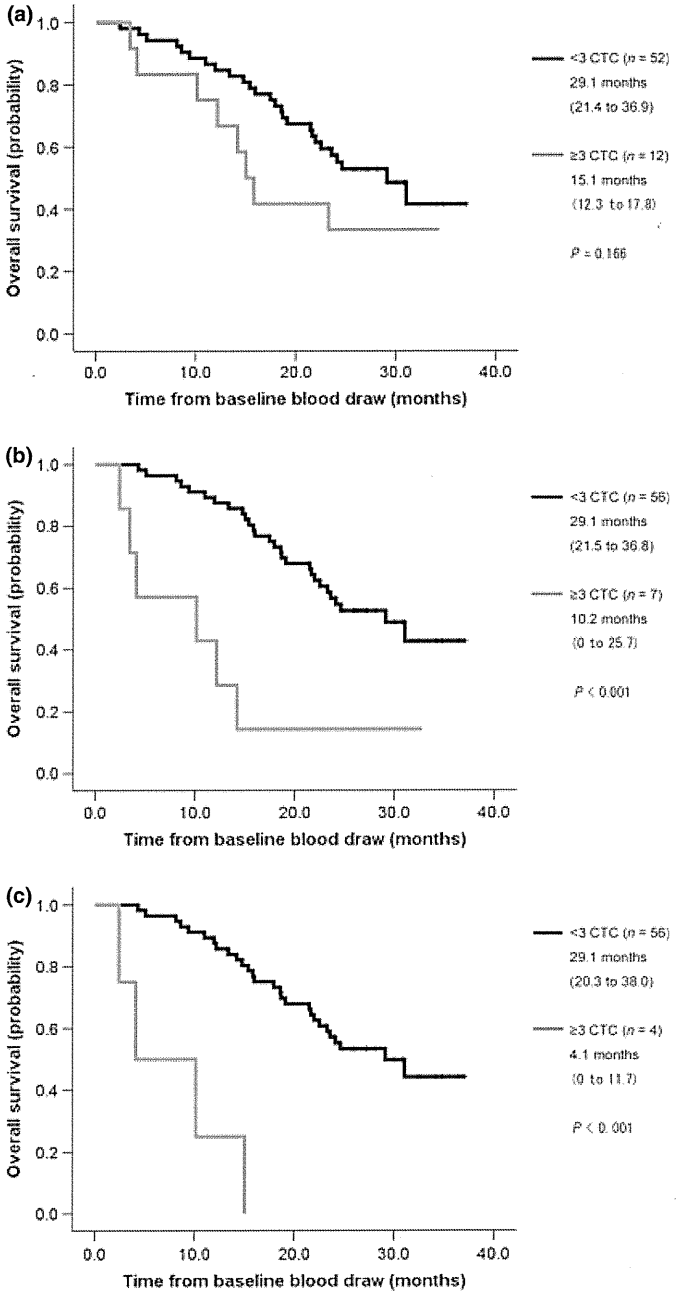

Fig. 3. Kaplan–Meier plots of overall survival in metastatic colorectal cancer patients with fewer than three circulating tumor cells (CTC) or ≥3 CTC at baseline (a), 2 weeks (b) and 4 weeks (c).

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

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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.

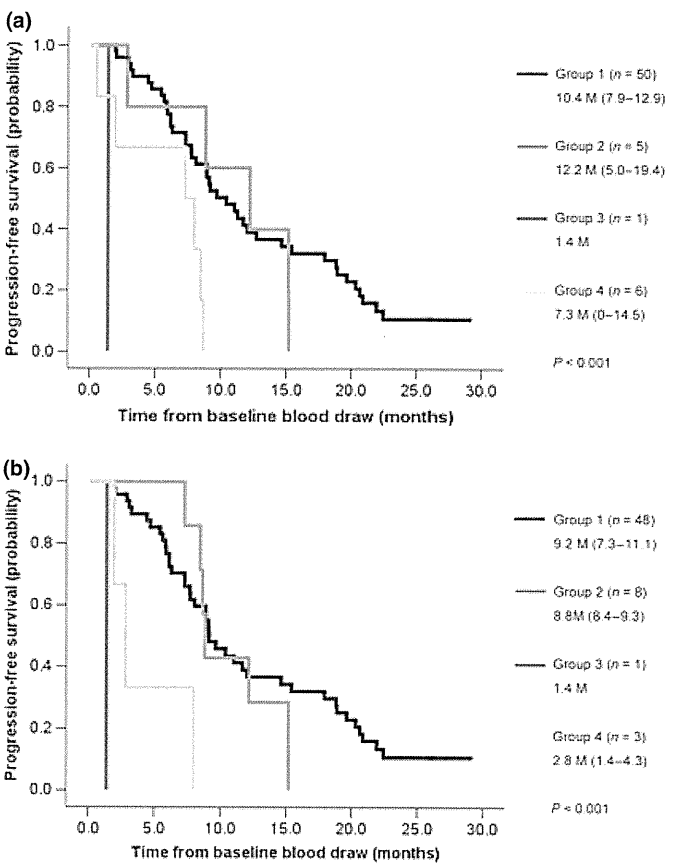

Fig. 4. Kaplan–Meier plots of progression-free survival in metastatic colorectal cancer patients with circulating tumor cell (CTC) change from baseline to 2 weeks (a) and 8–12 weeks (b).

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

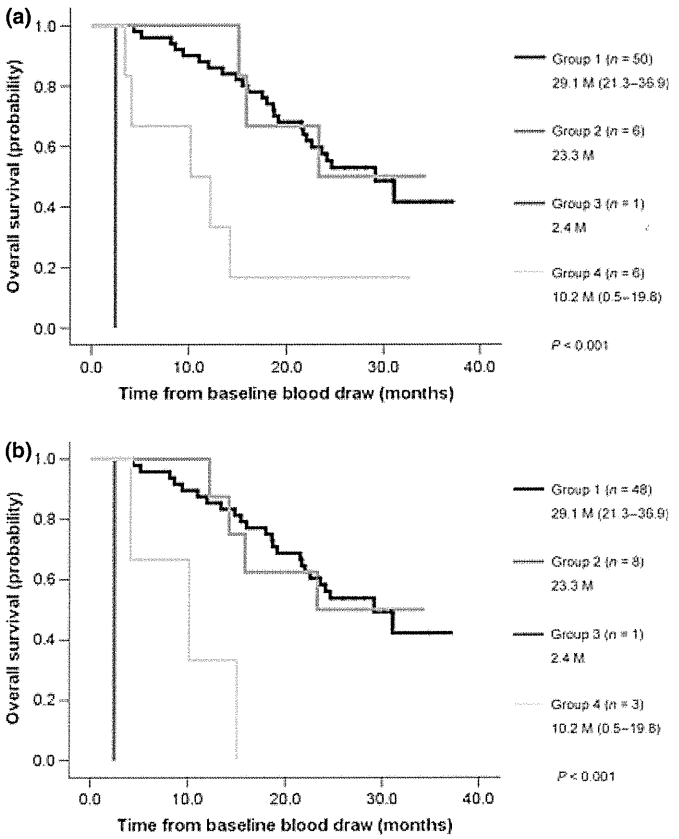

Fig. 5. Kaplan–Meier plots of overall survival in overall survival metastatic colorectal cancer patients with circulating tumor cells (CTC) change from baseline to 2 weeks (a) and 8–12 weeks (b).

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.

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Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL ARTICLE

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

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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 ≥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

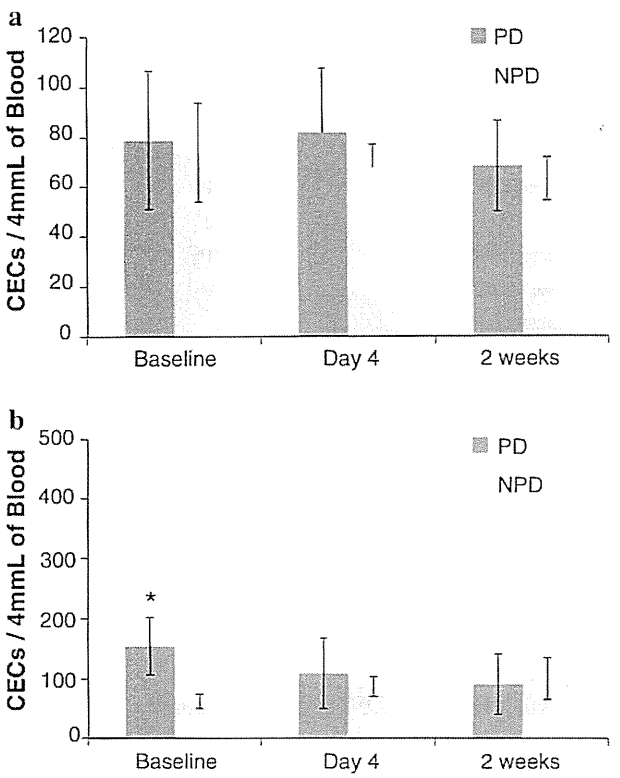

Fig. 1 Relationship between CEC levels of PD cases and CEC levels of NPD cases in FOLFOX4 without bevacizumab (**a**) and FOLFOX4 with bevacizumab (**b**). $^*P < 0.05$

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).

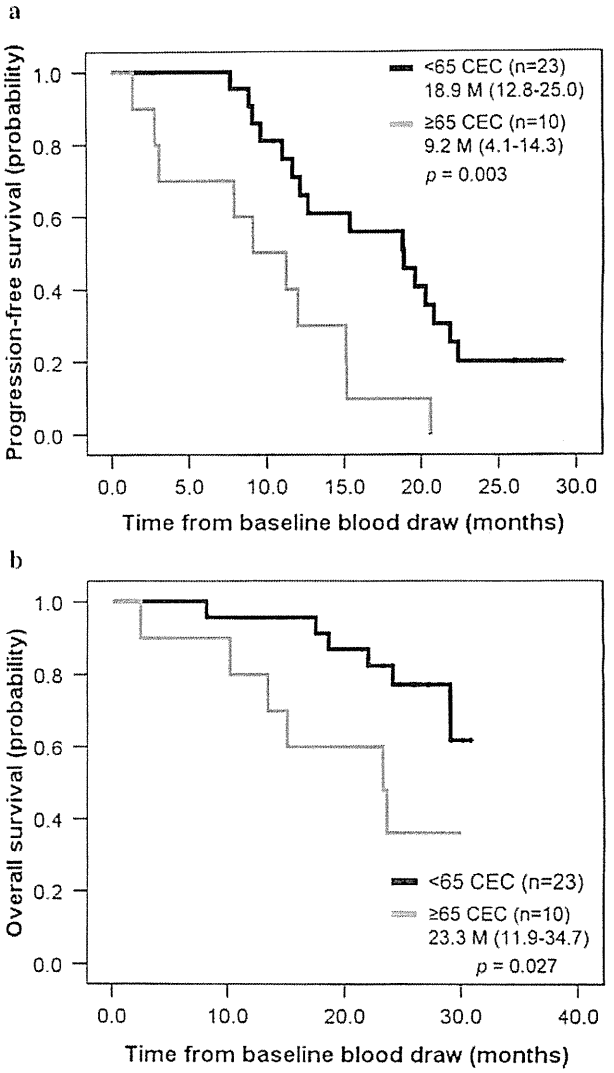

Fig. 2 Kaplan–Meier plots of progression-free survival (PFS) in metastatic colorectal cancer (mCRC) patients with fewer than 65 circulating endothelial cells (CEC, *top line*) or ≥65 CECs (*bottom lines*) at the baseline in FOLFOX4 with bevacizumab (**a**). Kaplan–Meier plots of overall survival (OS)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

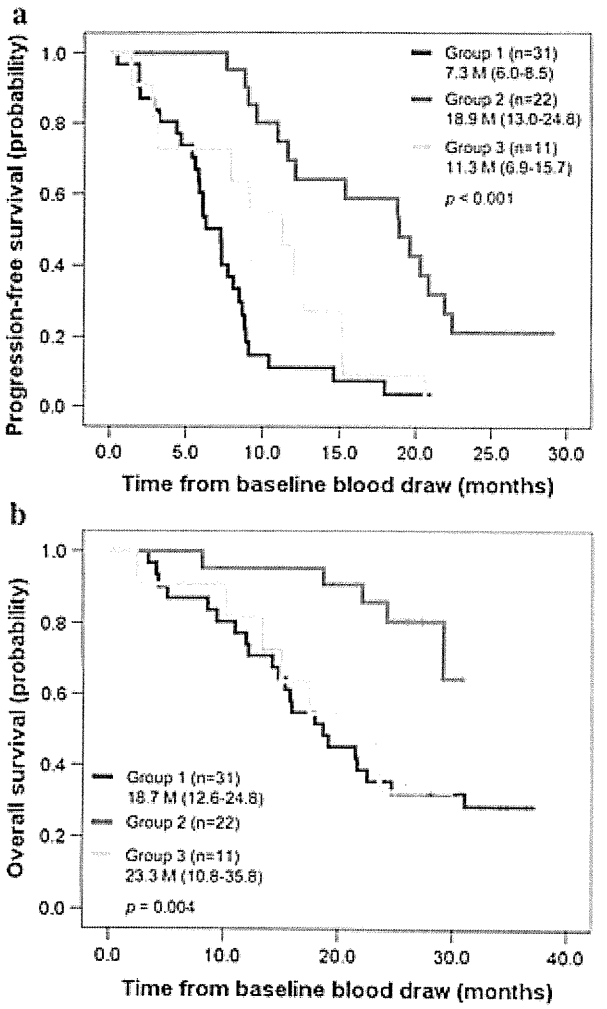

Fig. 3 Patients having CECs at the baseline in the non-combination group (*group* 1), patients having fewer than 65 CECs at the baseline in the bevacizumab-combination group (*group* 2), and patients having 65 or more CECs at the *baseline* in the bevacizumab-combination group (*group* 3). Kaplan–Meier 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.

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Conflict of interest None.

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RESEARCH PAPER

Treatment outcome and prognostic factors in renal cell cancer patients with bone metastasis

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Abstract We retrospectively analyzed treatment outcomes and factors for poor prognosis for patients with renal cell cancer (RCC) bone metastases. Patients with bone metastases at initial diagnosis of metastasis secondary from RCC, treated at our hospital between 1984 and 2009, were retrospectively reviewed and statistically analyzed. Among 214 RCC patients with metastasis, 71 patients (33%) were found to have bone metastases at initial diagnosis of metastasis. The median follow-up was 21.1 months (intra-quartile range: IQR, 9.1–47.4 months). The estimated median overall survival time from the diagnosis of bone metastasis was 27.7 months. The probability of patients surviving at 1, 2, and 5 years was 63.7, 52.2, and 19.3%, respectively. When they were stratified by MSKCC scores, the probability of the median overall survival of the populations classified as favorable, intermediate, and poor was not reached, 32.9, and 10.5 months, respectively ($P = 0.002$). In addition, poor performance status (PS) (hazard ratio [HR]: 1.938, $P = 0.035$) and no prior nephrectomy (HR: 3.008, $P = 0.004$) were extracted as independent poor prognostic factors by multivariate analysis. All treatment modalities—including radical en bloc surgery, radiation therapy, cytokine therapy, molecular targeted therapy, and administration of zoledronic acid—seemed to contribute to favorable survival. More than half of the patients with bone metastases secondary from RCC were predicted to survive more than 24 months. In this population, MSKCC scores were valid predictors of survival. With increased treatment options, RCC patients with bone metastasis may benefit further from subsequent modalities and/or agents.

Keywords Renal cell cancer · Sunitinib · Outcome · Bone metastasis · Prognostic factor

Abbreviations

RCC	Renal cell cancer
PS	Performance status
Ca	Calcium
Lower Hb	Hemoglobin
LDH	Lactate dehydrogenase
MSKCC	Memorial Sloan Kettering Cancer Center
CT	Computed tomography
MRI	Magnetic resonance imaging
ECOG	Eastern Cooperative Oncology Group
HRs	Hazard ratios
CIs	Confidence intervals

Introduction

In patients with metastatic renal cell cancer (RCC), bone is a common metastatic site, second only to the lung, with estimates of frequency ranging from 24 to 51% [1–3]. Bone metastases create serious problems for these patients as they often bring poor performance status (PS) due to pathologic fractures, spinal cord compression, and intractable pain [4]. Although bone metastasis secondary from RCC would appear to predispose toward poor prognosis, until now it has been controversial [4–6]. Neither of two large retrospective studies has identified bone metastasis as an independent

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poor prognostic factor [3, 7]. Instead, the time from the RCC diagnosis to the initial systemic treatment being less than one year, poor PS, elevated serum adjusted calcium (Ca), lower hemoglobin (Hb), and elevated serum lactate dehydrogenase (LDH) are well-known independent prognostic factors in the cytokine era, as measured by MSKCC scores (named for Memorial Sloan Kettering Cancer Center investigators). These factors are often used as stratification factors in various clinical trials for patients with metastatic RCC [7].

Because RCC is commonly sensitive neither to chemotherapy nor radiation therapy, treatment options for these RCC patients with bone metastasis have long been limited. Recently, several new agents have been introduced in the clinical treatment of metastatic RCC. The third-generation bisphosphonate, zoledronic acid, demonstrated significant reduction in skeletal-related complications in these patients [8]. In addition, various molecularly targeted agents for metastatic RCC may possibly result in improved survival [9, 10] although their efficacies remain to be established. In this study, we retrospectively analyzed treatment outcomes and the factors for poor prognosis in patients with RCC bone metastases.

Materials and methods

Patients and treatment

The medical records of patients with bone metastases secondary from RCC, who were treated in our hospital between 1980 and 2009, were retrospectively reviewed. In all patients, bone metastasis was confirmed by bone scans and computed tomography (CT) and/or magnetic resonance imaging (MRI). We considered clinical and geometric factors including age, gender, Eastern Cooperative Oncology Group (ECOG) PS, presence or absence of extra-osseous metastases, solitary or multiple bone metastases, pain due to osseous metastasis, the interval from the diagnosis of RCC to the initial systemic therapy, surgical treatment, and radiation therapy of bone metastasis, as well as systemic medical treatment including cytokine therapy, zoledronic acid, and targeted agents (sorafenib and sunitinib). In addition, common laboratory blood and serum data, including Hb, LDH, and adjusted Ca levels, were determined by using a multichannel autoanalyzer (LX-20, Beckman-Coulter, Los Angeles, CA). The corrected serum Ca level was calculated using Payne's formula [11]. Fundamentally, administered radiation dose was 30 Gy given over two weeks with single fractions of 3 Gy. Regarding the medical therapy, three million international unit (IU) of interferon-alpha (Sumiferon, Dainippon Sumitomo Pharma, Osaka, Japan) were administered subcutaneously three times a week to the most patients who were treated by cytokine therapy. Sunitinib (Sutent; Pfizer Inc, New York, NY) was administered orally at a dose of 50 mg daily, consisting of four weeks of treatment followed by a two-week rest period. Sorafenib (Nexaval, Bayer Pharmaceuticals Corporation, West Haven, CT) was administered orally at a dose of 800 mg daily, continuously. In addition, 4 mg of zoledronic acid (Zometa, Novartis Pharma AG, Basel, Switzerland) was administered once a 3/4 weeks. Dose reduction of each agent was performed depending on the type and severity of adverse events.

Statistical analysis

Survival time was defined as the time from the diagnosis of bone metastasis to death or the last follow-up date. The duration of follow-up was calculated from the date at the initial diagnosis of bone metastasis to death or the last follow-up. In this study, we consider the initial cytokine or molecular targeted therapy as the start of the systemic treatments. Overall survival was estimated by using the Kaplan–Meier method. The relationship between survival and each of the variables was analyzed by using the log-rank test for categorical variables. The associations of the pre-treatment and treatment features with death from RCC were assessed by using the Cox proportional hazards regression model and summarized with hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical analyses were performed using the Statistical Package for Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, Ill). Two-tailed $P < 0.05$ was considered significant.

Results

Characteristics of the patients and their bone metastases

Among 214 RCC patients with metastasis, 71 (33%) patients were diagnosed with bone metastasis. The median follow-up period was 21.1 months (intra-quartile range: IQR, 9.0–47.3 months). Patient characteristics and demographic data are shown in Table 1. The estimated median overall survival time from the diagnosis of bone metastasis was 27.7 months. The probability of patients surviving at 1, 2, and 5 years was 63.7, 52.2, and 19.3%, respectively (Fig. 1). While bone metastases were diagnosed at the same time as the initial diagnosis of RCC in 53 patients, metastatic bone lesions were detected after nephrectomy in the remaining 18 patients, 13 of whom were found to have bone metastasis 12 months or more postoperatively. Among these patients, 41 (58%) of the 71 patients had extra-osseous metastasis at the time of diagnosis of bone metastasis. These extra-osseous metastatic sites included 35 lung (85%), 15 lymph nodes (37%), 5 liver (12%), 4 adrenal gland (10%), and 2 brain (5%) metastases.

Table 1 Patient characteristics

Gender	
Male *n* (%)	55 (77)
Female *n* (%)	16 (23)
Age at Dx of bone metastasis	
Median (IQR)	62 (55.8–69.9)
Interval from initial Dx of RCC to bone metastasis	
Bone metastasis at initial Dx n (%)	53 (75)
<12 month *n* (%)	3 (4)
≥12 month *n* (%)	15 (21)
ECOG PS	
PS0 *n* (%)	22 (31)
PS1 *n* (%)	28 (39)
PS ≥ 2 *n* (%)	21 (30)
Hb at Dx of bone metastasis	
Male Median (IQR) g/dl	13.4 (12.5–14.4)
Female Median (IQR) g/dl	11.5 (10.7–13.6)
Serum LDH at Dx of bone metastasis	
Median (IQR) IU/l	222 (167–315)
Serum Ca at Dx of bone metastasis	
Median (IQR) g/dl	9.5 (9–9.8)
Follow-up	
Median (IQR) month	20.4 (8.7–44.5)

Dx diagnosis, *IQR* interquartile range, *ECOG* Eastern Cooperative Oncology Group, *PS* performance status

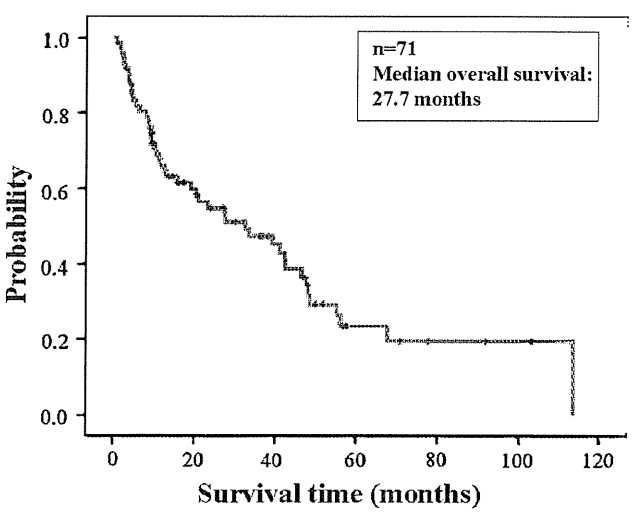

Fig. 1 Kaplan–Meier estimates of overall survival of 71 patients with bone metastasis secondary from renal cell cancer

Meanwhile, the primary bone metastatic sites were 34 vertebra (48%) including 16 lumbar (23%), 14 thoracic (20%), and 3 lumbar bones (4%), 26 lower extremities (37%), 24 thoracic cage (34%), 19 pelvis (27%), 13 upper extremities (18%), and 3 skull bone (4%). There was only a single metastasis in 39 patients (55%), whereas the remaining 32 patients (45%) had multiple bone metastases. Surgical management for bone metastasis was performed in 33 patients, including 13 (18%) and 20 (28%) for radical en bloc resection and palliative curettage procedure, respectively. In addition, radiation therapy was performed in 24 patients (34%). As systemic therapy, 48 patients (68%) and 18 (25%) patients received immune and molecular targeted therapies for their metastatic disease, respectively. Among these patients, 13 patients (18%) received both immune and molecular targeted therapies. Regarding the molecular targeted therapies, sorafenib and sunitinib, sorafenib alone, and sunitinib alone were administered to 3, 7, and 8 patients, respectively. In addition, 29 patients (41%) received zoledronic acid for their bone metastases.

Risk factors for poor outcome in univariate analysis

Univariate analysis of several clinical features associated with poor prognosis is summarized in Table 2. Among the factors in MSKCC scores, the time between RCC diagnosis and the initial systemic therapy of less than 1 year, poor PS (PS ≥ 2), elevated serum adjusted Ca were associated with short survival period, whereas lower Hb and elevated serum LDH were not statistically significant (Table 2). When we applied the MSKCC model, which stratifies patients into three risk groups: favorable: 0 risk factors ($n = 8$); intermediate: 1 or 2 risk factors ($n = 44$); and poor: 3, 4, or 5 risk factors ($n = 19$), the estimated median overall survival time from the diagnosis of bone metastasis of the populations classified as favorable, intermediate, and poor was not reached, 32.9, and 10.5 months, respectively, and resulted in distinctly separate overall survival curves ($P = 0.002$) (Fig. 2a).

In addition, elevated serum level of CRP and prior nephrectomy were associated with short survival period, whereas multiple bone metastasis, extra-osseous metastasis, and osseous pain due to metastatic lesion seemed not to be significant poor prognostic factors.

Regarding the treatment factors, all treatment modalities—including radiation therapy, cytokine therapy, molecular targeted therapy, and administration of zoledronic acid—seemed to contribute to longer survival (Table 2). As to surgical treatment, radical and palliative surgery were performed in 13 (18%) and 20 (28%) patients, respectively, which also seemed to contribute to longer survival ($P = 0.05$) (Table 2).

Multivariate analysis for predictors of poor prognosis

Among the pre-treatment factors, the time between RCC diagnosis and the initial systemic therapy of less than one year, poor PS, elevated serum adjusted Ca and CRP level,

Table 2 Univariate analysis of various prognostic factors

Factors	Yes			No			
	n	mOS	95%CI	*n*	mOS	95%CI	*P*
Gender	16	42.5	4.2–80.8	55	27.7	9.0–46.4	0.322
Age (> 64)	36	42.4	15.4–69.4	35	27.6	9.6–45.7	0.341
From Dx of RCC to initial Tx < 1 year or no treatment	60	20.4	3.9–37.0	11	NR		0.014
PS > 1	50	11.2	5.5–16.8	21	42.5	33.6–51.4	<0.001
Anemia	22	12.6	0.0–25.7	49	41.2	28.9–53.5	0.088
Serum LDH > 1.5xULN	32	20.4	0.0–42.1	36	42.4	26.7–58.1	0.091
Serum adjusted Ca > ULN	13	4.5	0.5–8.6	56	39.3	23.1–55.5	0.004
Serum CRP > ULN	33	10.0	6.3–13.8	33	44.2	33.9–54.5	0.016
Multiple bone metastases	32	27.6	4.9–50.4	39	42.4	16.1–68.7	0.164
Extra-osseus metastasis	41	11.2	0.0–26.6	30	42.5	20.7–64.3	0.087
Pain	48	32	10.0–55.8	23	13.7	3.1–24.2	0.415
Prior nephrectomy	45	48.3	44.9–51.7	26	6.4	1.4–11.5	<0.001
Radiation therapy	24	64	44.5–83.6	47	20.4	2.6–38.3	0.006
Cytokine therapy	48	41.2	30.0–52.3	23	11.2	8.1–14.2	0.018
Molecular targeted therapy	13	81.3	55.8–106.7	58	20.4	7.2–33.7	0.002
Zoledronic acid	29	77.9	56.9–99.0	42	11.7	7.2–16.2	<0.001
MSKCC score							
Good	8	NR					0.002
Intermediate	44	32.9	10.6–55.1				
Poor	19	10.7	6.6–14.9				
Surgical treatment							
Radical en bloc resection	13	48.7	21.4–75.9				0.05
Palliative curettage procedure	20	32.6	6.8–58.5				
No surgery	38	12.6	4.8–20.5				

mOS median overall survival, 95%CI: 95% confidence interval, *Dx* diagnosis, *Tx* systemic treatment, *NR* not reached, *ULN* upper limit of normal range, *MSKCC* Memorial Sloan Kettering Cancer Center

and prior nephrectomy were associated with short survival period by univariate analysis. Then a multivariate analysis was performed using Cox proportional hazard model and it showed that poor PS (HR: 1.938, 95% CI: 1.048–3.584, $P = 0.035$) and no prior nephrectomy (HR: 3.008, 95%CI: 1.416–6.390, $P = 0.004$) were extracted as independent poor prognostic factors. When we applied the model using these two risk factors, which stratifies patients into three risk groups: favorable: 0 risk factors ($n = 21$); intermediate: 1 risk factor ($n = 32$); and poor: 2 risk factors ($n = 18$), the estimated median overall survival time from the diagnosis of bone metastasis of the populations classified as favorable, intermediate, and poor was not reached, 21.1 and 10.1 months, respectively, and resulted in distinctly separate overall survival curves ($P = 0.001$) (Fig. 2b).

Discussion

Motzer et al. and recently Naito et al. [3, 7] analyzed the prognosis of 463 Western and 1463 Japanese metastatic RCC patients in the cytokine era. These large, multicenter, retrospective studies demonstrated similar poor prognostic factors, using MSKCC scores [3, 7]. In the Japanese study, the median overall survival times from diagnosis and the favorable, intermediate, and poor prognoses as classified by the MSKCC criteria were 21.5 months and 55.3, 29.6, and 9.8 months, respectively [3]. Interestingly, these median overall survival times are quite similar to those in our study (Figs. 1, 2a), suggesting that bone metastasis might not be a poor prognostic factor among Japanese RCC patients with metastasis.

In this study, although MSKCC scores had been previously identified as prognostic in patients treated with cytokines, these factors were significantly or marginally associated with poor OS (Table 2). This is similar to recent reports that have identified patients who are more likely to benefit from the tyrosine kinase inhibitors [12–14]. Their results suggest that MSKCC scores are associated with the behavior of the disease rather than with a specific form of therapy. Our study suggests that MSKCC prognostic factors are still valid to predict survival in patients with bone

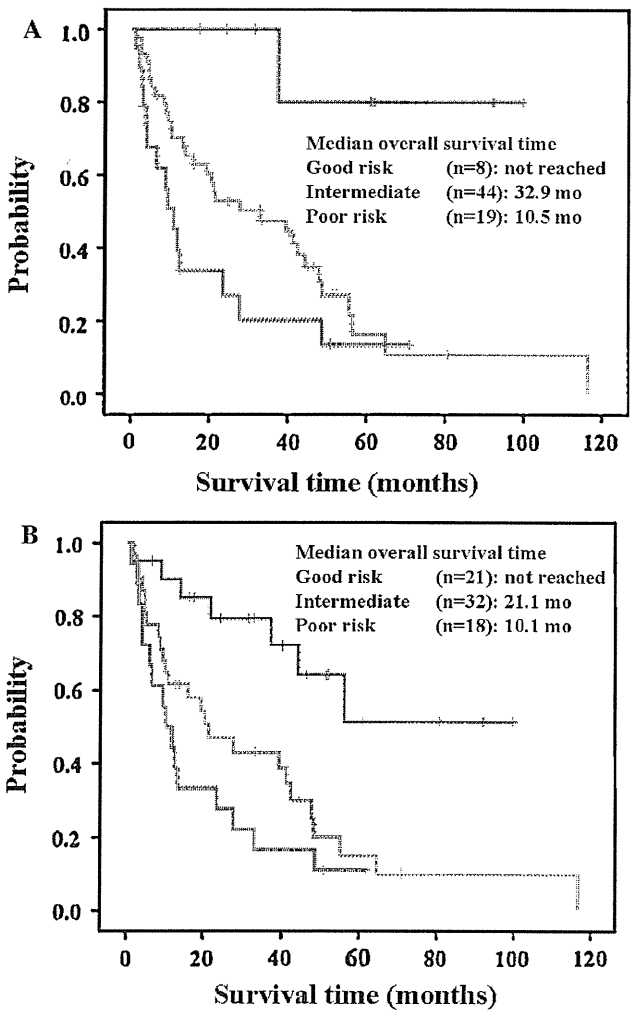

Fig. 2 A Kaplan–Meier estimates of overall survival of patients stratified by the risk factors into favorable, intermediate, and poor risk categories. Kaplan–Meier survival curves stratified by MSKCC score (**a**) and stratified by two risk factors, the performance status and prior nephrectomy, which were associated the poor prognosis in this study (**b**)

metastasis secondary from RCC (Fig. 2a). However, the distribution of patients according to the MSKCC model is uneven: in our series, 15, 58, and 27% patients belonged to favorable, intermediate, and poor risk groups, respectively. The disproportionately large number of patients in the intermediate group suggests that it may be somewhat heterogeneous and could be divided into subclasses. On the basis of our results, we classified the risk by combining the two prognostic factors, poor PS and no prior nephrectomy. In the present study, 21 (30%), 32 (45%), and 18 (25%) of 71 patients had a good, intermediate, and poor prognosis, respectively. This classification distinctly separated overall survival curves and seemed to classify proportionately ($P = 0.001$, Fig. 2b).

It is remarkable that all of the treatment modalities—including radical en bloc surgery, radiation therapy, prior nephrectomy, cytokine therapy, molecular targeted therapy, and administration of zoledronic acid—were identified as factors contributing to longer survival (Table 2) although this is quite a matter of course as various types of treatment tend to be administrated for the patients who survive longer. In other words, we can say that patients who underwent several types of treatment seemed to survive longer. Regarding surgical treatment, when radical resection surgery was performed, excellent local tumor control was reported [15, 16]. Lin et al. [15] reported that the local relapse-free survival rates at one and five years were 94 and 91% for 117 patients with en bloc resections. Jung et al. [16] also reported the superiority of surgical treatment on the basis of records from 99 patients. In this study, the eight patients who had radical en bloc surgical resection for a solitary metastasis in combination with a nephrectomy had a cancer-specific survival rate of 100% (mean follow-up, 69 months; range, 24–76 months). These studies, along with ours, suggest that when patients have good PS and are good candidates for surgical treatment with curative intent, we may need to consider aggressive surgical treatment including nephrectomy in order to achieve complete remission.

In prospective studies for patients with metastatic RCC, palliative radiotherapy can result in significant relief of local symptoms and improved quality of life [17, 18]. Therefore, external beam radiation therapy has been standard palliative treatment for symptomatic bone metastases. In addition, our recent retrospective study demonstrated that combining radiation therapy with administration of zoledronic acid achieved a higher objective response rate and prolonged skeletal related event-free survival than radiation therapy alone in patients with bone metastases from RCC [19]. Although further prospective investigation is necessary, a combination of radiation therapy and zoledronic acid, with or without targeted therapy, may prove effective.

Recently available agents, such as zoledronic acid and molecular targeted agents, might possibly be administered to long-time survivors. With increased treatment options, these RCC patients may benefit further from subsequent modality and/or agents. Zoledronic acid has been demonstrated to exhibit anti-tumor effects, not only inhibiting proliferation but also inducing apoptosis of various cancer cells in vitro and in vivo, and demonstrating survival benefits in recent clinical trials [20–22]. Regarding RCC, zoledronic acid was shown to significantly reduce the risk of skeletal complications in a large randomized, double-blind, placebo-controlled Phase III trial [8]. In this trial, zoledronic acid was also demonstrated to delay time to