in HBsAg negative patients during chemotherapy for breast cancer in spite of avoiding corticosteroids. On the basis of our experience, we have realized the importance of establishing the method of screening before chemotherapy and following data during chemotherapy. Thus, we have set up a guideline to prevent HBV reactivation, referring to a new published guideline [9]. The recommendation is to check not only HBsAg, but also HBsAb and HBcAb before starting chemotherapy. During chemotherapy, we should monitor HBV-DNA in patients with HBsAg positive, either with HBsAg negative and HBsAb and/or HBcAb positive. Monitoring of HBV-DNA is done more effectively with sensitive quantification by real-time PCR. Furthermore, patients with positive HBV-DNA (more than 1.8 log IU/mL) have to commence entecavir 0.5 mg/kg/day (Fig. 2).

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Original Article

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

Satoshi Matsusaka, MD, PhD[1]; Yuji Mishima, PhD[2]; Mitsukuni Suenaga, MD, PhD[1]; Yasuhito Terui, MD, PhD[1]; Ryoko Kuniyoshi, PhD[2]; Nobuyuki Mizunuma, MD, PhD[1]; and Kiyohiko Hatake, MD, PhD[1]

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

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

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

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

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

MATERIALS AND METHODS

Patients

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

Corresponding author: Kiyohiko Hatake, MD, PhD, Department of Medical Oncology, Cancer Institute Hospital of Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan; Fax: (011) 81-3-3570-0343; khatake@jfcr.or.jp

[1]Department of Medical Oncology, Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan; [2]Cancer Chemotherapy Center, Clinical Chemotherapy, Japanese Foundation for Cancer Research, Tokyo, Japan

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

Assessment of Biomarkers

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

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

Observation of CECs and CEPs

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

Table 1. Characteristics of Patients Treated With FOLFOX Plus Bevacizumab

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

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

Statistical Analysis

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

RESULTS

Patient Characteristics

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

Relation Between CEP Levels and Outcome

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

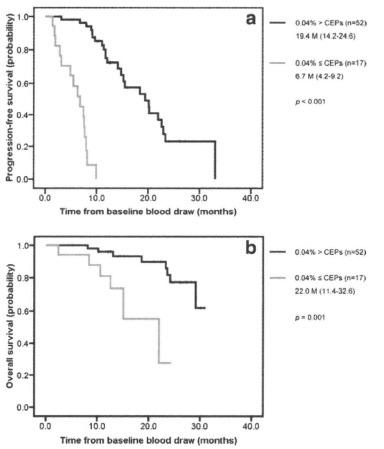

Figure 1. Depicted are (a) Kaplan-Meier plots of progression-free survival (PFS) and (b) Kaplan-Meier plots of overall survival (OS).

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

Relation Between CEC Phenotype and Efficacy

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

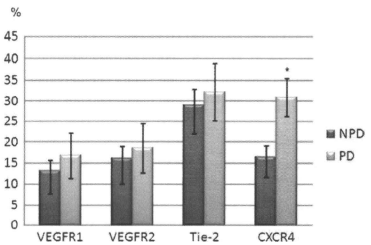

Figure 2. The relation is shown between levels of CEC phenotypes at baseline and bevacizumab efficacy in bevacizumab combination chemotherapy. PD indicates progressive disease; NPD, nonprogression disease. Results are expressed as mean ± standard error of the mean (SE). *$P < .05$

Relation Between CEC Phenotype and Outcome

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

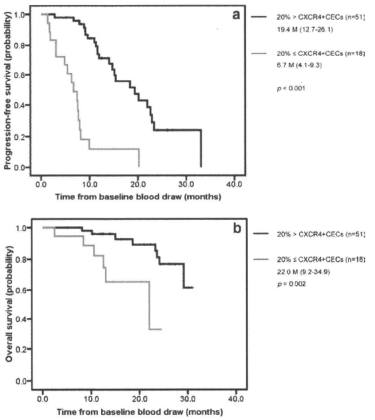

Figure 3. Depicted are Kaplan-Meier plots of (a) progression-free survival (PFS) and (b) overall survival (OS).

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.

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

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

DISCUSSION

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

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

Table 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 level 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

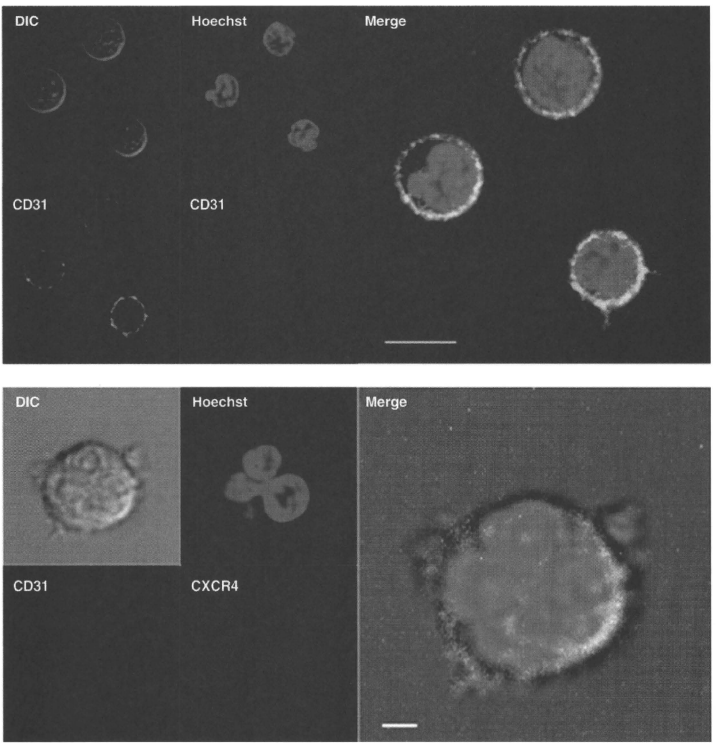

Figure 4. (a) CEPs and (b) CXCR4 + CECs were sorted by flow cytometry as described in the Materials and Methods section and analyzed by confocal microscopy. DIC indicates differential interferences contrast; bar, 5 μm.

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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