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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 $\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

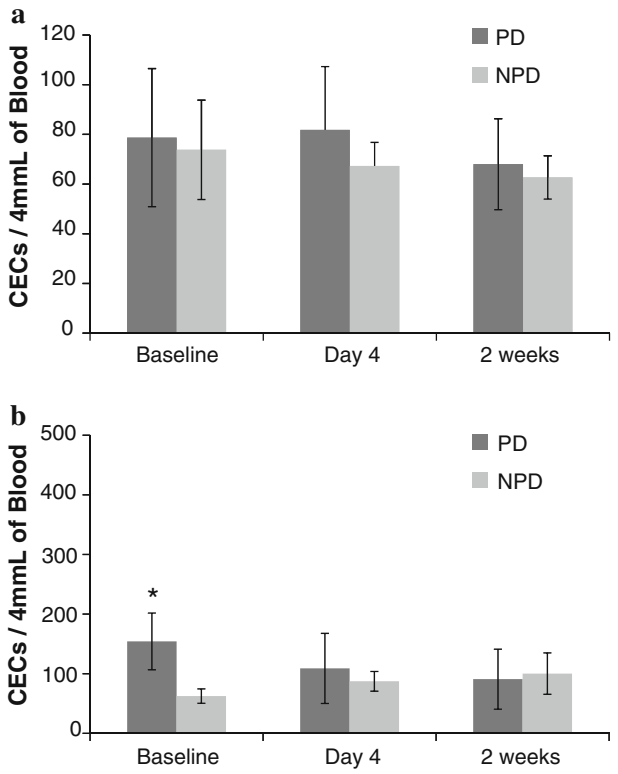

Fig. 1 Relationship between CEC levels of PD cases and CEC levels of NPD cases in FOLFOX4 without bevacizumab (**a**) and FOLFOX4 with bevacizumab (**b**). $*P < 0.05$

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).

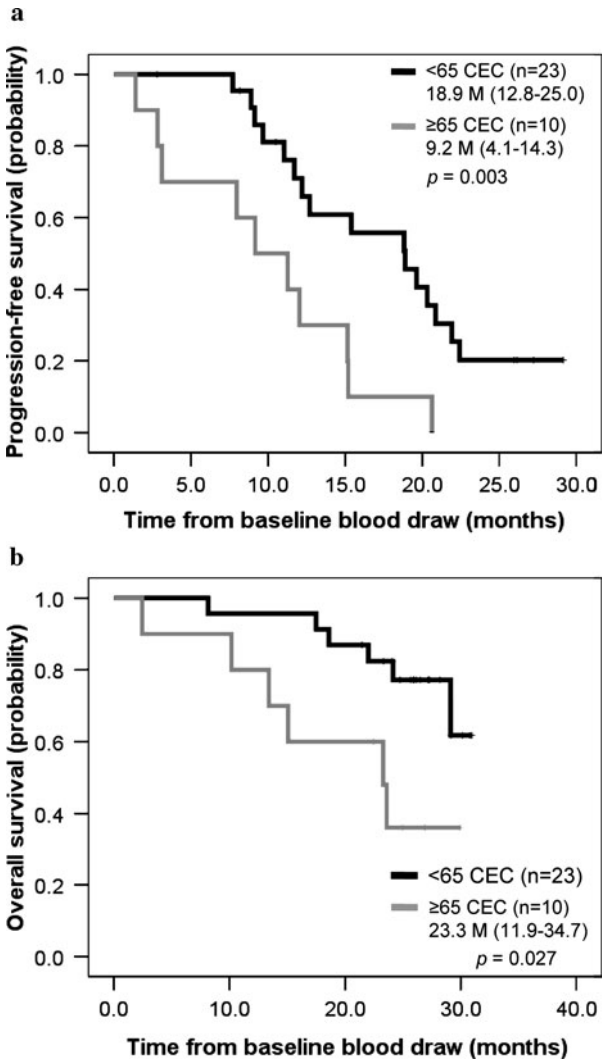

Fig. 2 Kaplan–Meier plots of progression-free survival (PFS) in metastatic colorectal cancer (mCRC) patients with fewer than 65 circulating endothelial cells (CEC, *top line*) or ≥65 CECs (*bottom lines*) at the baseline in FOLFOX4 with bevacizumab (**a**). Kaplan–Meier plots of overall survival (OS)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

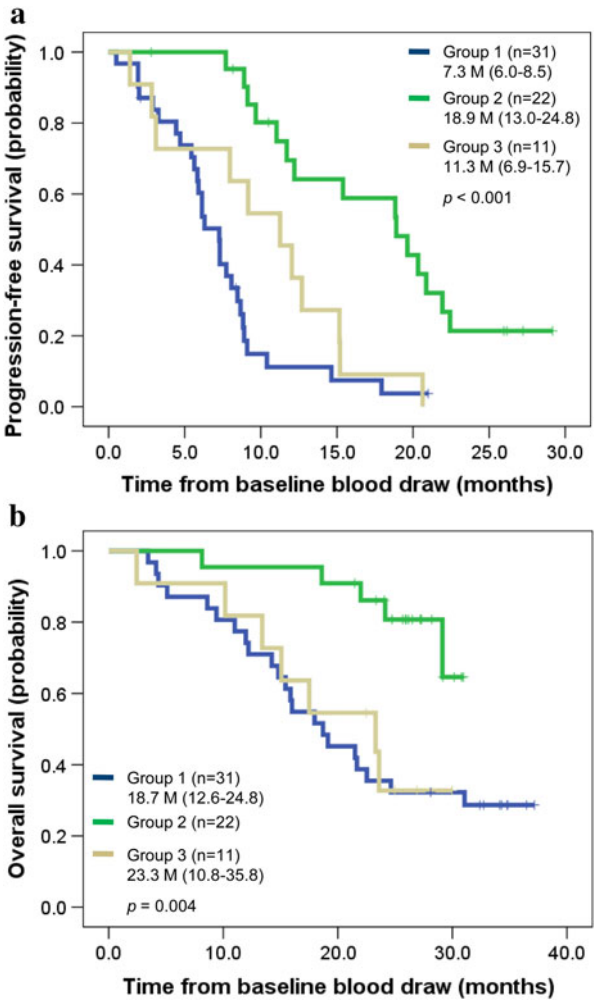

Fig. 3 Patients having CECs at the baseline in the non-combination group (*group* 1), patients having fewer than 65 CECs at the baseline in the bevacizumab-combination group (*group* 2), and patients having 65 or more CECs at the *baseline* in the bevacizumab-combination group (*group* 3). Kaplan–Meier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

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Conflict of interest None.

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