EUROPEAN JOURNAL OF CANCER 47 (2011) 2265–2272

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Determining circulating endothelial cells using CellSearch system during preoperative systemic chemotherapy in breast cancer patients

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ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 7 June 2011
Accepted 7 June 2011
Available online 5 July 2011

Keywords:
Circulating endothelial cells
CellSearch system
Breast cancer
Chemotherapy
Pathological response

ABSTRACT

Background: Circulating endothelial cells (CECs) have been studied as a biomarker for tumour progression and monitoring therapeutic effects. The CellSearch system is a semi-automated system that allows standardised analysis of CECs. This study assessed the clinical implications of CECs determined by the CellSearch system in breast cancer patients.

Methods: Seventy-six consecutive breast cancer patients (53 operable and 23 metastatic or recurrent) were enrolled for the study. Thirty-five patients with operable breast cancer received preoperative chemotherapy with a regimen based on anthracycline and/or taxane. CECs are defined as $CD146^+CD105^+CD45^-DAPI^+$ cells in the system. CD34 expression was examined using the additional channel in the system.

Results: A majority (4539 of 5183 cells, 88%) of CECs from patients with operable breast cancer were CD34-positive. Triple-negative cancers showed higher baseline CEC and $CD34^+$CEC counts than the other types ($P = 0.0387$ and 0.0377, respectively). Low baseline CEC and $CD34^+$CEC counts, and a low CD34 positive rate were associated with pathological complete response (pCR) of preoperative chemotherapy in patients with primary breast cancer ($P = 0.046$, 0.027 and 0.01, respectively). In multivariate analyses, the CD34 positive rate was significant for pCR ($P = 0.021$). During preoperative chemotherapy, CEC and $CD34^+$CEC counts before each cycle of chemotherapy increased with taxane-based regimens ($P = 0.0018$ and 0.0008, respectively) but not with anthracycline-based regimens.

Conclusions: Baseline CEC, in particular $CD34^+$CEC, counts and the CD34 positive rate might be useful for the prediction of treatment response of preoperative chemotherapy in patients with operable breast cancer.

1. Introduction

Circulating endothelial cells (CECs) and their progenitors, endothelial progenitor cells (EPCs), are being studied with increasing interest in oncology, particularly in relation to tumour angiogenesis. Recent studies have demonstrated elevated CEC count in patients with malignant diseases compared with healthy controls.[1–7] Several pioneering studies

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doi:10.1016/j.ejca.2011.06.015

have demonstrated that CEC elevations are associated with tumour stage, tumour characteristics and prognosis.[4,8–10] It has been experimentally demonstrated that chemotherapy causes a rapid induction of EPCs into the systemic circulation of mice, irrespective of the presence of tumour.[11] EPC mobilisation may support tumour cell survival even during anticancer chemotherapy.

CECs and EPCs are currently determined by several different assay systems including the flow cytometry and immunomagnetic detection system using endothelial cell markers including CD31, CD34, and CD146, and progenitor cell markers including CD133.[12] However, the markers and criteria that are used differ among studies.[13,14] The flow cytometry analysis has some limitations including standardisation between different laboratories and difficulties in fresh blood shipping. Recently, a semi-automated system for the detection of CECs was developed. The CellSearch system (Veridex LLC, Raritan, NJ) is mostly automated but enables researchers to detect endothelial cells visually using the immunofluorescence system. This system allows standardised analyses in different laboratories and shipment of blood samples in special tubes containing preservatives.

In this study, we used the CellSearch system to examine baseline CEC count and CEC alterations during systemic chemotherapy in association with clinicopathological parameters and treatment responses to preoperative chemotherapies.

2. Patients and methods

2.1. Patients

We enrolled 76 consecutive patients with histologically confirmed breast cancer who were treated at Kyoto University Hospital between 2007 and 2009, comprising 53 patients with operable breast cancer and 23 patients with metastatic or recurrent breast cancer. Other inclusion criteria were age 20–70 years, performance status (ECOG) <3, and estimated survival time >3 months. Blood samples were drawn before the initiation of any treatment in the operable breast cancer group and before the initiation of treatment for the metastatic or recurrent breast cancer in the metastatic or recurrent breast cancer group. Thirty-five patients with operable breast cancer received preoperative chemotherapy with a regimen based on anthracycline, taxane or a combination of both. The anthracycline-based regimen comprises four cycles of FEC (5-FU 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2) tri-weekly or four cycles of EC (epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2) tri-weekly. The taxane-based regimen comprised four cycles of docetaxel alone (75 mg/m^2) tri-weekly or four cycles of TC (docetaxel 75 mg/m^2, cyclophosphamide 600 mg/m^2) tri-weekly. The combination regimen comprises four cycles of an anthracycline-based regimen tri-weekly and four cycles of a taxane-based regimen tri-weekly. Trastuzumab was not administered preoperatively but after surgery to patients with HER2-positive tumours. We analysed alterations in CEC count during treatment in 17 patients who received preoperative chemotherapy by collecting blood samples before each cycle of chemotherapy and 24 h after administration of chemotherapy. For combination regimen, blood samples were drawn during four cycles of the first regimen. Clinical response to chemotherapy was assessed according to the Response Evaluation Criteria in Solid Tumours (RECIST). The study protocol was approved by the Ethics Committee of Kyoto University, and written informed consent was obtained from all the patients.

2.2. Evaluation of CECs by the CellSearch system

Blood samples were drawn into CellSave tubes (Veridex, LLC, NJ) containing a cell preservative. Samples were maintained at room temperature and processed within 24 h of collection. All evaluations were performed without prior knowledge of the clinical status of the patient. The CellSearch system, used for endothelial cell detection, consists of CellSave tubes, CellTracks AutoPrep, a fully automated sample preparation system, the Endothelial Cell Reagent Kit and the CellSpotter Analyzer II, a semi-automated fluorescence microscope.

In brief, 4 ml blood was mixed with 10 ml buffer, centrifuged at 800*g* for 10 min, and placed in the sample preparation system. The instrument aspirated the plasma/buffer layer, and antiCD146 ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and the remaining plasma were aspirated. The enriched cells were fluorescently labelled with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI). Staining reagents (<0.0006% mouse monoclonal antibodies specific to CD105 conjugated to phycoerythrin; <0.0013% mouse antiCD45 monoclonal antibodies conjugated to allophycocyanin in phosphate-buffered saline containing 0.5% BSA and 0.1% sodium azide) together with antiCD34 antibody conjugated to FITC (clone AC136, Miltenyi, Biotech GmbH, Germany) were added in conjunction with a permeabilisation buffer to label the cells fluorescently. After incubation, magnetic separation was repeated to remove the excess staining reagent. After the final processing step, the cells were re-suspended in 300 μL of buffer and transferred to a chamber placed between two magnets that orientate the immunomagnetically labelled cells in a monolayer for analyses. The cells were then examined with a four-colour semi-automated fluorescent microscope, the CellSpotter Analyzer II. A grey-scale charge-coupled device camera was used to scan the entire chamber surface, and each captured frame was then evaluated for potential CEC candidates by image analysis software. CECs were defined as CD146$^+$CD 105$^+$CD45$^-$DAPI$^+$ cells. CECs were stained with an additional antibody against CD34 and its expression was evaluated using an extra channel in the system.

2.3. Pathological analyses

Tumour biopsy specimens before preoperative chemotherapy were examined pathologically for tumour grade according to the Scarff–Bloom–Richardson grading system. Tumour specimens were also examined for oestrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor type 2 (HER2). The antibodies for ER, PgR, and HER2 were ER(SP1), PGR(1E2), and HER2(4B5), respectively (all from Roche Diagnostics, Tokyo, Japan). ER and PgR statuses were defined as positive for tumours having 10% or more positive tumour cells. HER2 positivity was determined by a strong

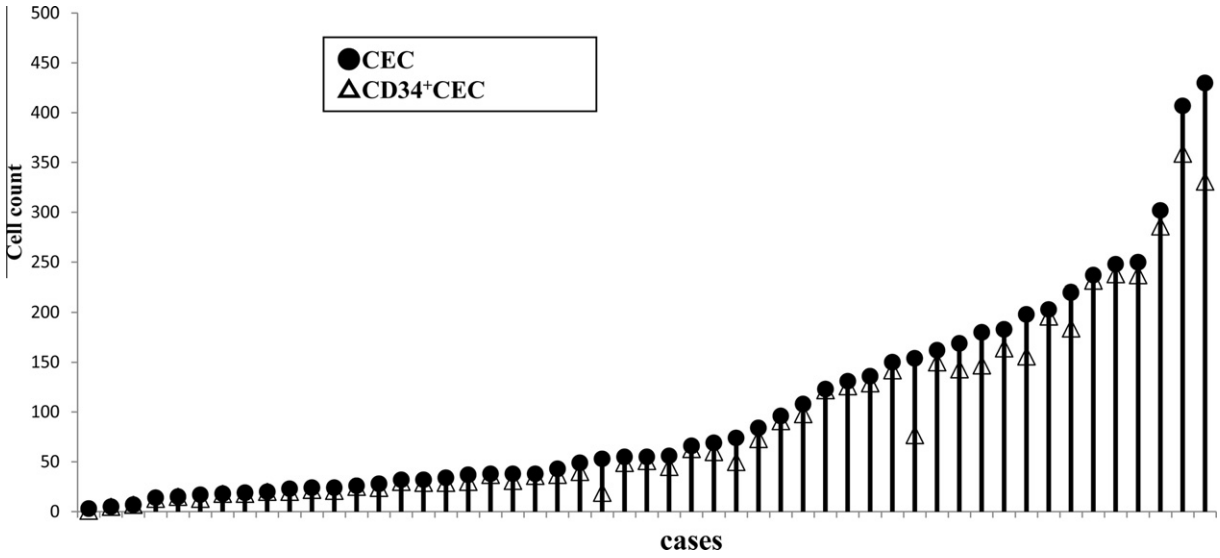

Fig. 1 – Distribution of CECs and CD34⁺ CECs in individual patients with operable breast cancer. CEC and CD34⁺ CEC counts in individual patients with operable breast cancer are shown. Eighty-eight percent (4539 of 5183 cells) of CECs detected by the CellSearch system are CD34 positive CECs.

expression (3+) of HER2 by the HercepTest or by an HER2:CEP17 ratio >2.2 by fluorescence in situ hybridisation (FISH). Triple negative was defined as ER negative, PgR negative, and HER2 negative tumours.

The pathological response was assessed after surgery following preoperative chemotherapy. A pathological complete response (pCR) was defined as no residual invasive tumour cells in mammary glands and lymph nodes.

The MIB1/Ki67 labelling index was calculated by counting positively stained tumour cells per 1000 tumour cells in the hot spots. Tumours having an MIB1/Ki67 index $\geqslant$20% were categorised as rapidly proliferative (positive), and those having an index <20% were defined as slowly proliferative (negative).

2.4. *Statistical analyses*

Correlation analyses were performed to assess the associations between baseline CEC counts and tumour size, nodal status, grade, stage, ER, PgR and HER2 statuses, tumour phenotype, and tumour response. Correlation analysis was performed using the Mann–Whitney test for two independent samples and the Kruskal–Wallis test for more than two independent samples. Logistic regression analysis was used to identify parameters associated with pathological response. Changes in CEC and CEP numbers were analysed using repeated measures ANOVA. Statistical analyses were performed using JMP (ver. 8.0.1; SAS Institute Japan, Tokyo, Japan). *P* values of <0.05 were considered statistically significant.

3. Results

3.1. *Characteristics of CECs detected by the CellSearch system*

The expression of CD34, which is a commonly used marker for endothelial cells, was examined in CECs detected by the CellSearch system. As shown in Fig. 1, 88% (4539 of 5183 cells) of CECs from patients with operable breast cancer before treatment were CD34 positive.

3.2. *Patient characteristics and correlations with clinicopathological parameters*

Table 1 shows the characteristics of the patients and their baseline CEC and CD34⁺CEC counts in relation to clinicopathological parameters. CD34 expression was not measured in two patients with operable breast cancer. CEC count was higher in metastatic or recurrent breast cancer patients than in patients with operable breast cancer ($P = 0.0275$). Among patients with operable breast cancer, those with triple-negative cancers had significantly higher CEC and CD34⁺CEC counts than those with other types of cancer ($P = 0.0387$ and 0.0377, respectively). Similarly, patients with PR-negative cancers showed higher CEC and CD34⁺CEC counts than those with PR-positive cancers ($P = 0.0413$ and 0.0437, respectively). In patients with metastatic or recurrent breast cancer, patients with lung, liver or bone metastasis showed higher CEC counts than those with lymph node or skin metastasis ($P = 0.037$).

3.3. *CEC and CD34⁺ CEC counts and responses to chemotherapy*

In 35 patients with operable breast cancer, CEC and CD34⁺CEC counts were examined according to pathological and clinical responses to preoperative chemotherapy. The pCR group showed lower numbers of baseline CD34⁺CEC counts than the non-pCR group ($P = 0.0416$) (Fig. 2). In addition, the pCR group showed a lower CD34-positive rate (CD34⁺CEC count/total CEC count) than the non-pCR group ($P = 0.0356$) (Fig. 2). In the logistic regression analysis, CEC, CD34⁺CEC, and CD34-positive rates were significantly associated with pCR in univariate analyses ($P = 0.046$, 0.027, and 0.01, respectively) (Table 2). In

Table 1 – Clinicopathological characteristics and baseline CEC and CD34$^+$CEC counts.

Variables	CEC		P value	CD34$^+$CEC		P value
	n	Median		*n*	Median	
Cancer status						
Operable breast cancer	53	55	0.0275	51	49	0.072
Recurrent or metastatic breast cancer	23	122		23	96	
Operable breast cancer						
Menopausal status						
Premenopausal	23	55	NS	23	49	NS
Postmenopausal	30	52		28	50	
Tumour size (UICC)						
T1	13	56	NS	12	54	NS
T2	29	49		29	37	
T3	10	82		9	98	
T4	1	55		1	51	
Clinical nodal status						
Negative	22	46	NS	21	37	NS
Positive	28	90		27	77	
Histological grade						
1	6	26	NS	6	22	NS
2	19	56		19	45	
3	28	55		26	55	
Oestrogen receptor (ER)						
Negative	26	62	0.1445	24	66	0.0715
Positive	27	43		27	36	
Progesterone receptor (PgR)						
Negative	34	62	0.0413	32	66	0.0437
Positive	19	28		19	24	
HER2 status						
Negative	41	56	NS	41	49	NS
Positive	11	38		9	37	
Tumour phenotype[a]						
Triple negative	17	96	0.0387	17	91	0.0377
Non-triple negative	36	40		34	36	
Ki-67 index						
Negative	7	38	NS	7	36	NS
Positive	28	55		28	49	
Metastatic or recurrent breast cancer						
Major metastatic site						
Lymph node	5	26	0.037	5	25	0.102
Lung	6	227		6	148	
Liver	7	156		4	102	
Bone	4	163		3	146	
Skin	1	40		1	40	
Oestrogen receptor						
Negative	12	90	0.065	12	71	0.124
Positive	11	172		11	152	
Progesterone receptor						
Negative	13	104	0.217	13	70	0.285
Positive	9	156		9	140	
Unknown	1	271		1	163	
HER2 status						
Negative	17	122	0.834	17	102	0.972
Positive	6	135		6	81	

[a] Triple negative: ER, PgR, and HER2 negative.

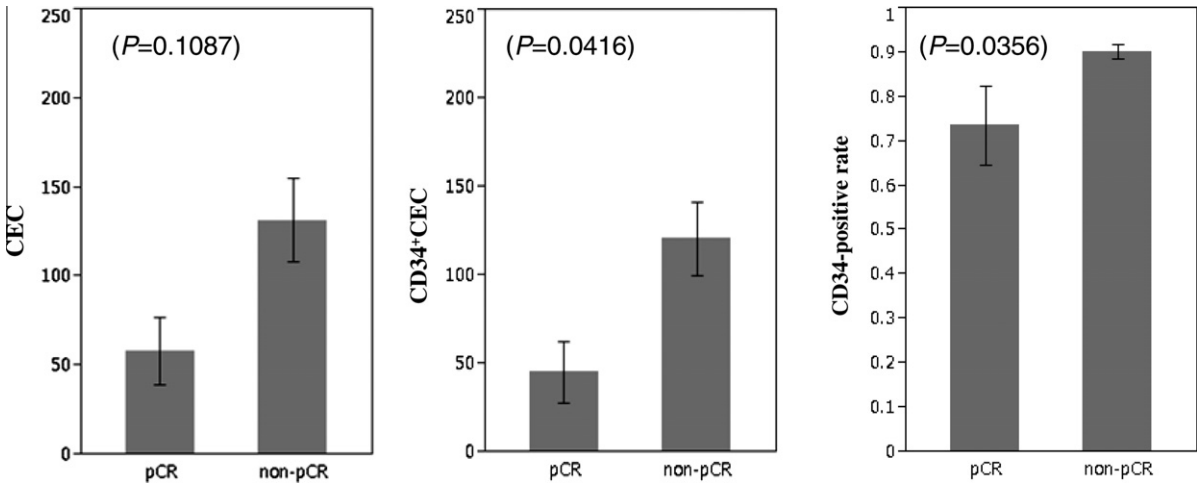

Fig. 2 – CEC and CD34$^+$ CEC counts and pathological responses. The pCR group had lower baseline counts of CD34$^+$ CECs than the non-pCR group ($P = 0.0416$). CEC count showed a similar trend ($P = 0.1087$). The pCR group showed a lower CD34 positive rate than the non-pCR group ($P = 0.0356$).

Table 2 – Univariate and multivariate analysis for pCR (logistic regression analysis) ($n = 35$).

Parameters	P value
Univariate analysis	
Age	0.144
Tumour size (T3–T4 versus T1–T2)	0.303
N (positive versus negative)	0.350
ER (positive versus negative)	0.207
PgR (positive versus negative)	0.625
HER2 (positive versus negative)	0.385
Grade (grade3 versus grade 1–2)	0.633
CEC	0.046
CD34$^+$CEC	0.027
CD34-positive rate	0.01
Multivariate analysis	
ER	0.14
HER2	0.459
CD34$^+$CEC	0.066
CD34-positive rate	0.021

multivariate analyses, the CD34-positive rate remained significant for pCR ($P = 0.021$) (Table 2). CEC counts, CD34$^+$CEC counts, and CD34-positive rate did not show any association with clinical responses (data not shown).

3.4. *Changes in CEC and CD34$^+$CEC counts during systemic chemotherapy*

Alterations in CEC and CD34$^+$CEC counts during the first four cycles of chemotherapy were analysed in 17 patients with operable breast cancer who received preoperative chemotherapy as either a taxane-based or an anthracycline-based regimen. Patients who received taxane-based regimens showed increasing numbers of pretreatment CECs and CD34$^+$CECs during the treatment cycles ($P = 0.0018$ and 0.0008, respectively) (Fig. 3a) whereas those who received anthracycline-based regimens did not show such increases ($P = 0.97$ and 0.77, respectively) (Fig. 3b). This indicates that changes in CEC and CD34$^+$CEC counts depend on the type of chemotherapy. CEC and CD34$^+$CEC counts showed a rapid increase 24 h after each cycle of chemotherapy. Unlike anthracycline-based regimens (Fig. 3d), taxane-based regimens showed an incremental pattern in CEC count after repeated cycles of chemotherapy (Fig. 3c).

4. Discussion

At present, no standardised method is available to determine CEC and EPC counts, which makes reported data on CEC variable. The CellSearch system is a commercially available semi-automated system that enables standardised determination of CECs. A recent study reported that increases in CECs detected by the CellSearch system during antiangiogenic treatment were associated with improved outcome in metastatic breast cancer patients treated with bevacizumab and standard chemotherapy.[15] However, CEC count by the CellSearch system is yet to be examined in patients with operable breast cancer. Thus, we examined clinical utility of CEC count by this system in patients with operable breast cancer, in particular during preoperative systemic chemotherapy.

Our results showed that patients with triple-negative tumours had higher CEC and CD34$^+$CEC counts compared with those who had other types of breast cancer. Intratumoural expression levels of vascular endothelial growth factor (VEGF)-A, stromal-derived growth factor (SDF)-1α and granulocyte colony-stimulating factor (G-CSF), all of which are known to mobilise EPCs,[16,17] are reported to be higher in basal-like tumours, which are a major subtype of triple-negative breast cancers.[18] A cDNA microarray study with a series of 138 tumours (80 luminal A, which is an ER-positive subtype, and 58 basal-like) showed that basal-like tumours overexpressed genes associated with angiogenesis, such as *VEGF* genes compared with luminal-type tumours. In contrast, genes associated with antiangiogenesis, such as thrombospondin, type I, domain containing 1 (*THSD1*) and *THSD4*, were underexpressed in basal-like tumours.[19] Patients with ER-positive tumours have been noted to have higher serum

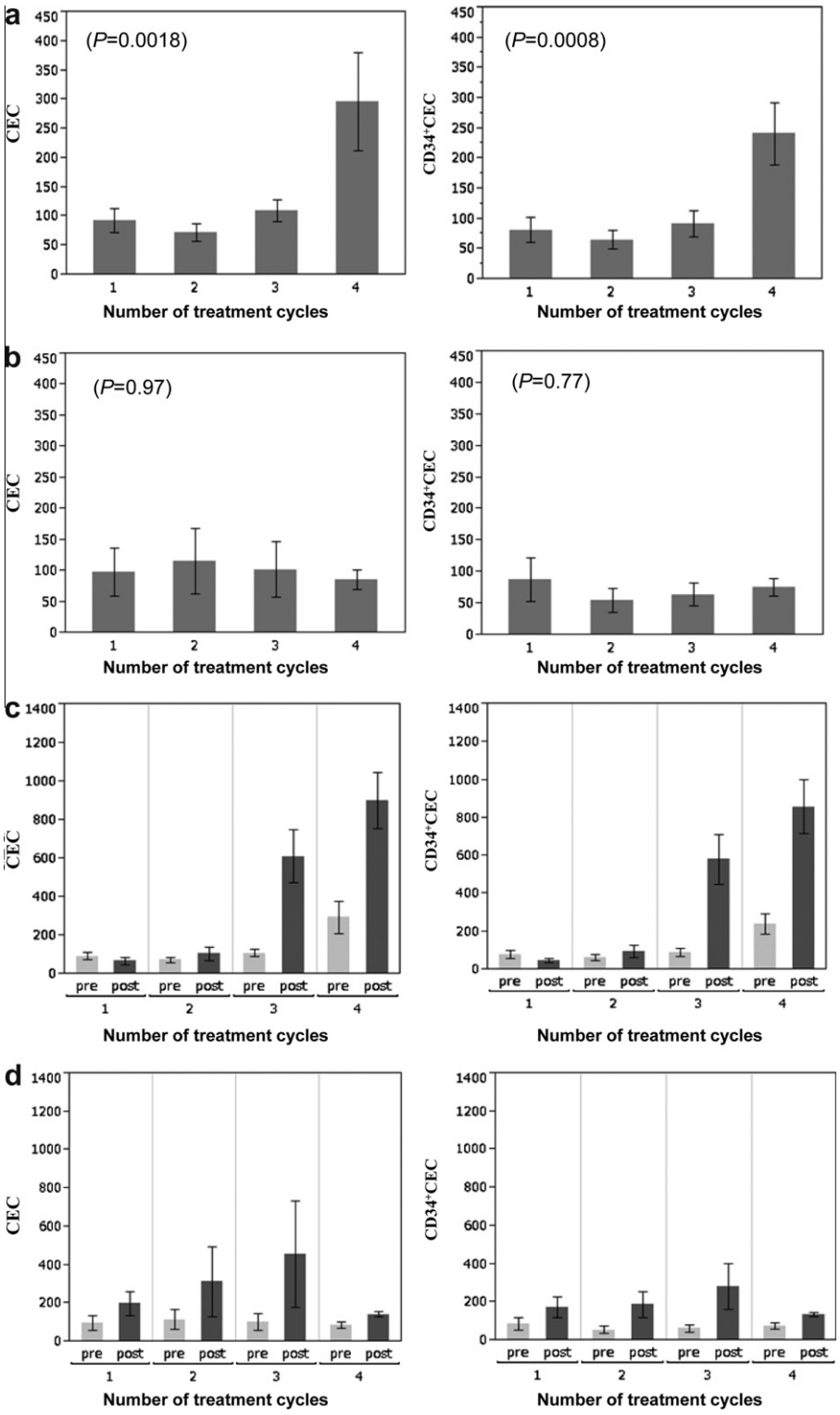

Fig. 3 – Changes in CEC and CD34$^+$ CEC counts during preoperative chemotherapy. CEC and CD34$^+$ CEC counts before each cycle of chemotherapy were measured during preoperative chemotherapy. (a) Patients receiving a taxane-based regimen showed increasing numbers of CEC and CD34$^+$ CEC during chemotherapy cycles (P = 0.0018 and 0.0008, respectively). (b) Patients receiving an anthracycline-based regimen did not show increases in CEC and CD34$^+$ CEC counts during preoperative chemotherapy (P = 0.97 and 0.77, respectively). CEC and CD34$^+$ CEC counts were repeatedly measured before and 24 h after each cycle of chemotherapy in 17 patients. (c) Patients receiving taxane-based chemotherapy showed an incremental pattern of CEC and CD34$^+$ CEC counts during chemotherapy. (d) Patients receiving anthracycline-based chemotherapy did not show an incremental pattern of CEC and CD34$^+$ CEC elevation after chemotherapy.

levels of endostatin, an intrinsic negative regulator of angiogenesis, compared to those with ER-negative tumours.[20] Although the origin of CECs determined by the CellSearch system is unclear, our results are in agreement with these reports and suggest that triple-negative breast cancers have more angiogenic properties than other types of breast cancer.

Several recent studies have reported that elevated CEC count in cancer patients returns to normal levels in response to systemic treatment.[6,7,20–23] In the present study, the pCR group showed lower $CD34^+$CEC count and a lower CD34-positive rate at baseline compared to the non-pCR group. In the logistic regression analysis, $CD34^+$CEC count and the CD34-positive rate showed higher predictive power for pCR compared to CEC count. Furthermore, the CD34-positive rate remained significant for pCR in the multivariate analyses, suggesting that detection of CD34-positive population in CECs determined by the CellSearch system would increase their clinical utility. Further investigations are required to validate the clinical significance of CEC count, particularly by using larger prospective clinical studies that validate these findings in CD34-positive populations using the CellSearch system.

In this study, as opposed to anthracycline-based regimens, taxane-based regimens caused increasing numbers of pretreatment CEC and $CD34^+$CECcounts during chemotherapy. Although the origin of CECs is not completely understood, evidence suggests that CECs determined by the CellSearch system originated from damaged vasculature since CEC count increased after venesection and cannulation.[24] Thus, our results suggest that different chemotherapeutic agents may cause vascular or tumour damage in different ways. Various chemotherapeutic agents have been suggested to induce different ways of mobilising endothelial progenitor cells from bone marrow.[11] Chemotherapeutic agents such as paclitaxel are suggested to upregulate angiogenic cytokines and chemokines such as CXCL8 (IL8), probably through NF-κB activation.[25–27] These cytokines and chemokines would also affect CEC count after chemotherapy. We also showed a rapid increase of CEC and $CD34^+$CEC count 24 h after chemotherapy, which may be due to acute damage of tumour or normal vasculature by chemotherapy. It was demonstrated that a rapid elevation of EPCs after chemotherapy resulted in the colonisation of tumours by the bone marrow-derived cells and the promotion of tumour angiogenesis, which would result in tumour recovery.[11] Even in the absence of tumours, chemotherapy alone was shown to induce EPC mobilisation, although induced levels might differ depending on the type of chemotherapy. As the origin of CECs by the CellSearch system is not fully understood, further investigations are warranted to elucidate the mechanisms of chemotherapy-induced increases in CECs. Since the sample size is small and this is not a randomised trial, conducting a larger prospective randomised study is necessary to validate these results.

In conclusion, we studied the clinical significance of CECs determined by the CellSearch system in patients with operable breast cancer during preoperative systemic chemotherapy. CEC count, $CD34^+$CEC count and CD34-positive rates at baseline were significantly associated with pCR and the CD34-positive rate remained significant in multivariate analyses, suggesting that the CD34-positive rate may predict therapeutic responses to preoperative chemotherapy. Our results indicate that alterations in CEC and $CD34^+$CEC counts during systemic chemotherapy show different patterns depending on the type of chemotherapy. Because angiogenesis may possibly play an important role in cancer progression and therapeutic responses, conducting further studies is essential to clarify the origin of CECs determined by different assays and how angiogenic reactions are involved in therapeutic responses to anticancer treatment. The results of such studies will improve the understanding of how antiangiogenic treatment should be combined with conventional chemotherapies for improved treatment efficacy and ultimately lead to the achievement of personalised treatment.

Sources of support (Grants)

Japan's Ministry of Health, Labor, and Welfare for a study on construction of an algorithm for multimodality therapy with biomarkers for primary breast cancer during the formulation of the decision-making process, led by Masakazu Toi (H18-3JIGAN-IPPAN-007, H19-3JIGAN-IPPAN-007).The Innovative Techno-Hub for Integrated Medical Bio-imaging Project of the Special Coordination Fund for Promoting Science and Technology, from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflict of interest statement

None declared.

Acknowledgements

This study was funded by a research grant from Japan's Ministry of Health, Labor, and Welfare for a study on constructing an algorithm for multimodality therapy with biomarkers for primary breast cancer during the formulation of the decision-making process, led by Masakazu Toi (H18-3JIGAN-IPPAN-007, H19-3JIGAN-IPPAN-007).

This work was supported in part by the Innovative Techno-Hub for Integrated Medical Bio-imaging Project of the Special Coordination Fund for Promoting Science and Technology, from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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