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EDITORIAL

Expected clinical applications of circulating tumor cells in breast cancer

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Abstract

Tumor cell invasion and intravascular filtration lead to the presence of circulating tumor cells (CTCs) in peripheral blood. CTCs have, thus, been counted in patients with cancer to analyze metastatic mechanisms or in the hope of developing clinical applications for diagnosis and therapy; various CTC-related studies have been performed. However, the clinical significance of CTCs remains to be established because of the extremely small number of CTCs in peripheral blood as compared with the number of blood cells. Technical problems (e.g. reproducibility and reliability) in the detection of CTCs also remain to be solved. The use of flow cytometric analysis, which can be performed with tumor-cell markers such as anti-epithelial cell adhesion molecule antibodies and anti-cytokeratin antibodies and non-tumor-cell markers such as anti-CD45 antibodies has enhanced specificity for the detection of tumor cells. The CellSearch System® can detect 1 CTC in 7.5 mL of peripheral blood, with high reproducibility. Its detection rate and accuracy for CTCs have been confirmed. In the United States, clinical trials have used this system to detect CTCs in patients with metastatic breast cancer, metastatic colorectal cancer, and metastatic prostate cancer, and CTCs have been confirmed to be a useful prognostic factor. This system was also suggested to be useful for monitoring treatment response in patients with metastatic breast cancer and was approved by the United States Food and Drug Administration in 2004. Measuring CTC counts can facilitate the early prediction of treatment response and thereby avoid unnecessary therapy. CTCs may also be a useful biomarker for molecular targeted agents, enabling the identification of patients most likely to respond to a given treatment and facilitating treatment selection. However, the widespread use of CTC monitoring as a routine examination requires a further improvement in measurement sensitivity, the establishment of criteria for quantitative and qualitative evaluations, and additional clear-cut evidence supporting the clinical significance of CTCs. We expect that CTCs will be established to be a new diagnostic and therapeutic index for breast cancer.

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Key words: Breast cancer; Circulating tumor cells

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INTRODUCTION

Circulating tumor cells (CTCs) have long been known to exist in the peripheral blood of some patients with cancer, particularly those with metastatic disease. The clinical impact of this phenomenon has long been debated. CTCs have been measured in patients with cancer to analyze metastatic mechanisms or in the hope of developing clinical applications for diagnosis and therapy; various CTC-related studies have been performed. We review the historical development of research on CTCs and discuss possibilities for future clinical applications.

METASTATIC MECHANISMS AND CTCS

Intravascular infiltration of tumor cells and the development of distant metastases were previously considered late events in tumor progression. However, recent research has demonstrated that tumor cell infiltration and invasion of the surrounding stroma and blood vessels are early events in tumorigenesis. Subsequently, such metastatic tumor cells remain dormant[1-3]. Tumor cells invade blood vessels *via* fragile tumor vessels. Tumor-cell proliferation reduces the oxygen supply to cells and activates various types of interstitial and inflammatory reactions, such as angiogenesis[4]. Decreased expression of the cell adhesion factor, E-cadherin, reduces adhesive strength between tumor cells, promoting infiltration of surrounding tissue[5].

Infiltrated cells undergo epithelial-mesenchymal transition (EMT) and assume the characteristics of interstitial cells, including migratory ability[6]. Tumor cells that have undergone EMT invade blood vessels and enter the systemic circulation. Some circulating CTCs undergo apoptosis, whereas others continue to exist as dormant tumor cells.

In solitary tumor cells, the cell cycle is thought to be dormant, resulting in the lack of proliferation. However, tumor cells may spread to bone marrow and distant organs and exist as disseminated tumor cells (DTCs). DTCs maintain a state of dormancy and do not proliferate, but can lead to micrometastases or metastatic foci. CTCs may also undergo mesenchymal-epithelial transition, leading to restoration of proliferative ability causing tumor-cell proliferation, angiogenesis, and further metastases in distant organs[6,7] (Figure 1).

Metastatic models have suggested that about 1×10^6 tumor cells per 1 g of tumor enter the bloodstream daily[8]. However, CTCs have very low survival rates in peripheral blood, and 85% of CTCs disappear within 5 min[9,10]. Experiments in animal models have reported that 2.5% of CTCs cause micrometastases, and 0.01% of CTCs proliferate and form macroscopic metastases[11]. Because most CTCs that enter the circulation undergo apoptosis, and angiogenesis is not promoted by micrometastases in distant organs, the proportion of CTCs that cause macroscopic metastases is not necessarily high. In fact, tumor cells may remain dormant for several years because of cessation of the cell cycle in micrometastases[12].

Tumor cell infiltration and entry into blood vessels thus leads to the presence of CTCs in peripheral blood. Measuring CTCs in patients with cancer has thus been expected to contribute to the analysis of metastatic mechanisms and the development of new clinical applications for diagnosis and therapy. Various CTC-related studies have been performed, however, the clinical significance of CTCs remains to be established due to factors such as the extremely small number of CTCs in peripheral blood as compared with the number of blood cells, and technical problems in the detection of CTCs (e.g. reproducibility and reliability).

PRINCIPLES FOR THE IDENTIFICATION OF CTCS

Epithelial-cell adhesion molecule (EpCAM) and cytokeratins (CK) are most commonly used to distinguish between epithelial cells and non-epithelial cells. EpCAM is widely expressed by epithelial cells and cancer cells[13] and is also referred to as Trop1, epithelial surface antigen, or cancer-related antigen. Monoclonal antibodies against EpCAM include HEA125[13], Ber-EP4[14], and KSI/4[15]. Various CK subfractions have been identified, some of which are specifically expressed by certain epithelial cells and epithelial tissue. Studies by Bártek *et al*[16], using various types of anti-CK antibodies, showed that CK subfractions CK4 to CK8, CK10, CK13, and CK18 are expressed in nearly all monolayer cultures of epithelial cells. As for breast cancer, Taylor-Papadimitriou *et al*[17] and Bratthauer *et al*[18] reported that CK7, CK8, CK18, and CK19 are expressed in luminal epithelial cells of terminal duct lobular units, lobular intraepithelial tumors, and ductal intraepithelial tumors.

Braun *et al*[19] used an antibody cocktail of anti-CK8, anti-CK18, and anti-CK20 and the non-cancer-cell marker, anti-CD45 antibody, to identify breast cancer cells. Lymph nodes obtained from patients with breast cancer were stained with these antibodies. They found that only cancer cells entering lymph nodes were positive for CK8, CK18, and CK19 and negative for CD45. Other mesenchymal cells were negative for CK subfractions and positive for CD45.

Combinations of these markers have been used to identify cancer cells entering bone marrow[15,20-24], lymph nodes[19,24-26], or peripheral blood[15]. Although anti-EpCAM antibody and anti-CK antibody target a single antigen molecule, the possibility of false-positive results caused by nonspecific reactions cannot be ruled out. However, the use of flow cytometric analysis, which allows the cancer-cell markers, anti-EpCAM antibody and anti-CK antibody, to be combined with the non-cancer-cell marker, anti-CD45 antibody, has enhanced specificity for cancer-cell detection[27] (Figure 2).

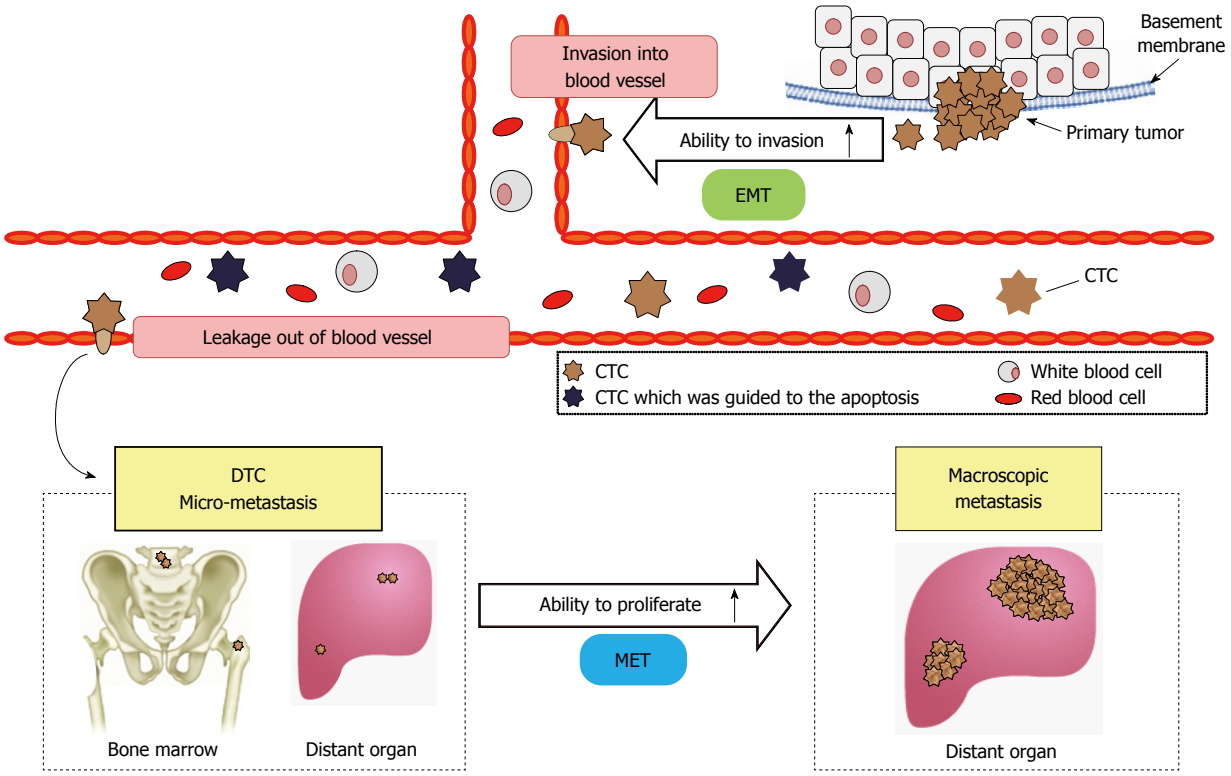
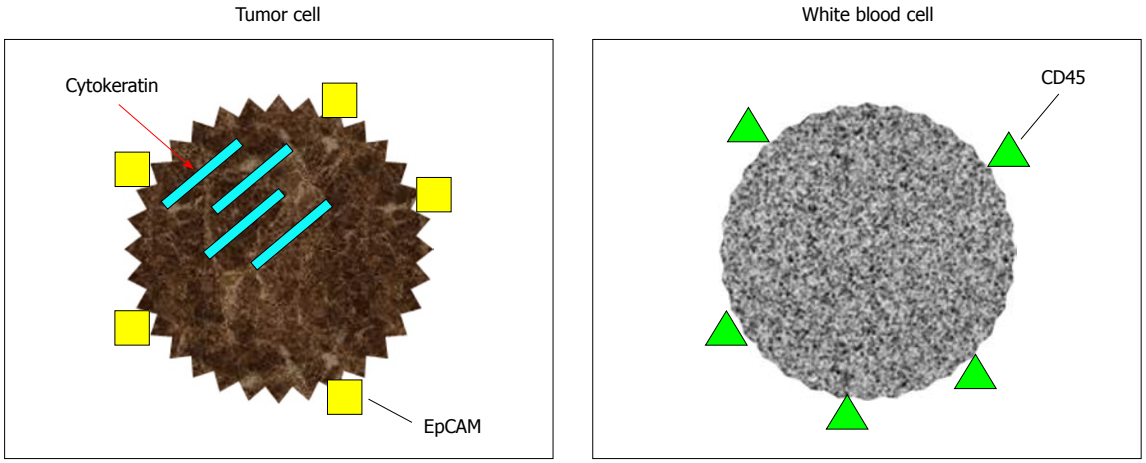


Figure 1 Circulating tumor cell and metastatic process. EMT: Epithelial-mesenchymal transition; CTC: Circulating tumor cell; DTC: Disseminated tumor cell; MET: Mesenchymal-epithelial transition.



	Tumor cell	White blood cell
Anti-EpCAM ferro fluids	+	-
Anti-cytokeratins	+	-
Anti-CD45	-	+
DAPI	+	+

Figure 2 Identification of tumor cells and white blood cells using monoclonal antibodies. EpCAM: Epithelial-cell adhesion molecule; DAPI: 4',6-diamidino-2-phenylindole.

CELLSEARCH SYSTEM®

The CellSearch System® is an automated detection system for CTCs that uses anti-EpCAM antibodies, anti-CK antibodies, and anti-CD45 antibodies. It was developed by Veridex Co., Ltd. (Raritan, NJ, USA). The CellSearch System® is based on a combination of immunomagnetic labeling and automated digital microscopy. The principles of measurement are described below: (1) Cancer cells are labeled with anti-EpCAM antibody magnetic beads that target EpCAM, which is expressed by the cell membrane of tumor cells; (2) Tumor cells are immunomagneti-

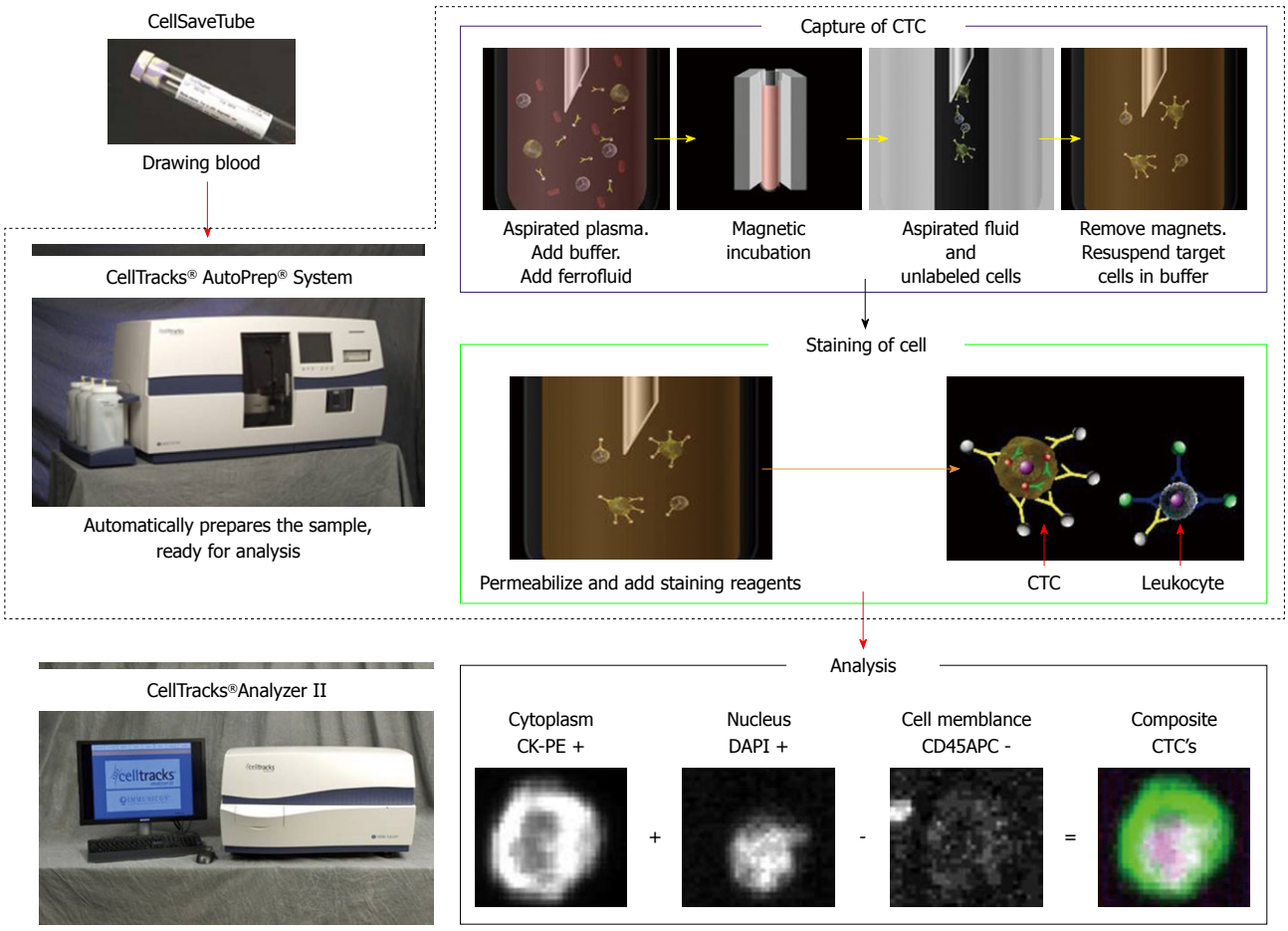


Figure 3 Measurement of circulating tumor cells by the CellSearch® System. CTC: Circulating tumor cell; DAPI: 4',6-diamidino-2-phenylindole; CK: Cytokeratins; PE: Phycoerythrin.

cally captured and concentrated; and (3) Tumor cells are stained with phycoerythrin (PE)-labeled anti-CK antibodies (CK-PE). Cell nuclei are then immunofluorescently stained with 4',6-diamino-2-phenylindole. Leukocytes are stained with allophycocyanin-labeled anti-CD45 antibodies (CD45-APC), and cellular fixation is performed; and (4) Concentrated, stained tumor cells are examined by fluorescence microscopy to assess labeling by PE, 4',6-diamidino-2-phenylindole (DAPI), and allophycocyanin (APC) and thereby distinguish tumor cells from leukocytes (Figure 3).

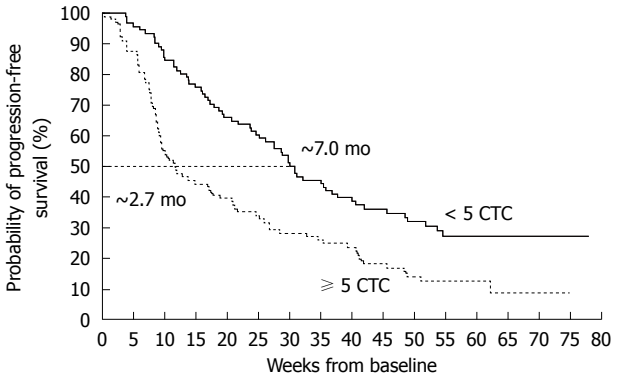
The CellSearch System® is a semiautomated system for the detection and enumeration of CTCs. The main advantages of this system are its ease of use and high reproducibility. A validation study conducted by Riethdorf *et al*[28] showed that CTC counts remained stable for 72 h after blood sample collection, even at room temperature. CTC counts did not differ among hospitals, and reproducibility was high.

The CellSearch System® can detect 1 CTC per 7.5 mL of peripheral blood, with high reproducibility. This system has been confirmed to accurately identify and enumerate CTCs. In the United States, clinical trials of the CellSearch System® have been performed in patients with metastatic breast cancer, metastatic colorectal cancer, and metastatic prostate cancer, confirming that CTC counts are a useful prognostic factor. In metastatic breast cancer, the CellSearch System® has been suggested to be useful for monitoring treatment response. This system was approved by the United States Food and Drug Administration in 2004.

STUDIES OF CTCS AS A PROGNOSTIC FACTOR IN BREAST CANCER

A prospective, multicenter clinical trial performed by Cristofanilli *et al*[29] counted the number of CTCs in 7.5-mL peripheral blood samples obtained from 145 healthy subjects, 200 patients with benign breast tumor, and 177 patients with metastatic breast cancer. The mean CTC count in the patients with metastatic breast cancer was 54 ± 138. When outcomes were assessed according to the CTC counts before the start of treatment, median progression-free survival was found to be 7.0 mo in patients with less than 5 CTCs per 7.5 mL of blood, as compared with only 2.7 mo in those with 5 or more CTCs ($P < 0.001$) (Figure 4). Moreover, median overall survival was significantly longer in patients with less than 5 CTCs (> 18 mo) than in those with 5 or more CTCs (10.1 mo, $P < 0.001$) (Figure 5)[29].

In Japan, a multicenter trial has been conducted in 38

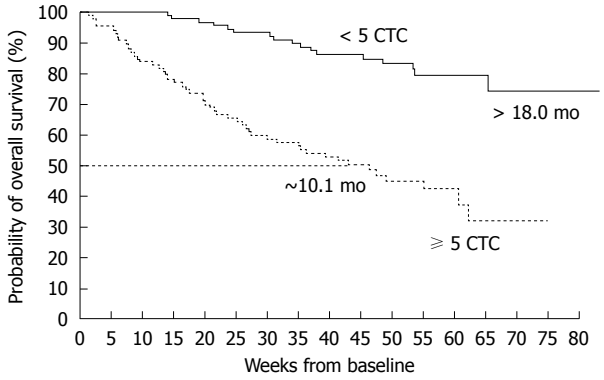


No. at risk

< 5 CTC	90	87	77	69	59	52	44	39	33	26	22	16	12	5	4	2	0
⩾ 5 CTC	87	76	48	38	34	29	24	22	17	12	9	8	4	1	1	1	0

Log-lank test: $P < 0.001$

Figure 4 Kaplan-Meier estimates of probabilities of progression-free survival in patients with metastatic breast cancer who had less than 5 circulating tumor cells and those who had 5 or more circulating tumor cells (before initiation of a new line of therapy)[29]. CTC: Circulating tumor cell.



No. at risk

< 5 CTC	90	90	90	87	85	80	80	77	67	59	50	39	28	15	10	4	2
⩾ 5 CTC	87	83	73	68	62	57	52	49	40	33	24	18	9	2	2	1	0

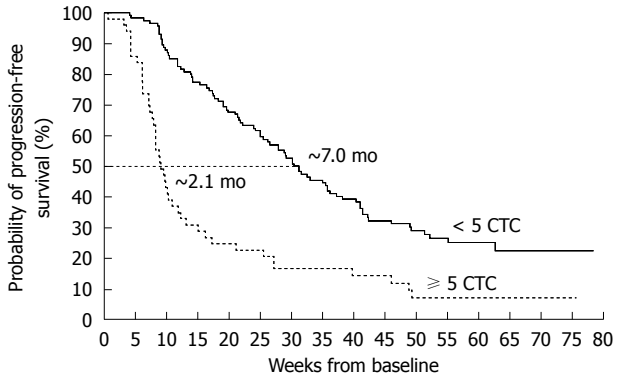
Log-lank test: $P < 0.001$

Figure 5 Kaplan-Meier estimates of probabilities of overall survival in patients with metastatic breast cancer who had less than 5 circulating tumor cells and those who had 5 or more circulating tumor cells (before initiation of a new line of therapy)[29]. CTC: Circulating tumor cell.

patients with metastatic breast cancer. Overall survival ($P = 0.04$) and progression-free survival ($P = 0.04$) were both significantly shorter in the 11 patients who had 5 or more CTCs than in those who had less than 5 CTCs. These results were consistent with those obtained in the United States[30].

ROLE OF CTC COUNTS AS A PROGNOSTIC FACTOR FOR BREAST CANCER

Cristofanilli *et al*[29] counted CTCs at baseline and at the first follow-up visit (mean ± SD, 4.5 ± 2.4 wk) after the

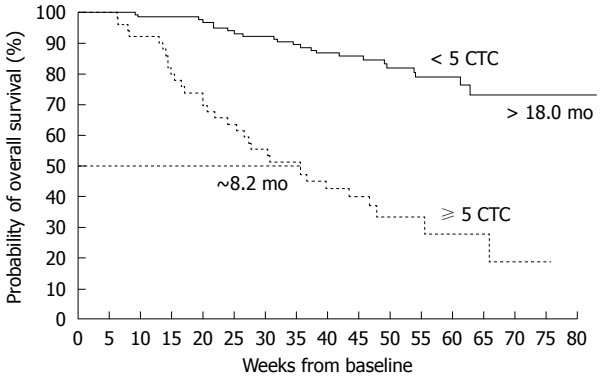


No. at risk

< 5 CTC	114	112	99	88	77	67	57	50	41	29	25	19	13	4	4	2	0
⩾ 5 CTC	49	42	20	14	12	11	8	8	6	6	3	3	1	1	1	1	0

Log-lank test: $P < 0.001$

Figure 6 Kaplan-Meier estimates of probabilities of progression-free survival in patients with metastatic breast cancer who had less than 5 circulating tumor cells and those who had 5 or more circulating tumor cells (at the first follow-up visit after initiation of a new line of therapy)[29]. CTC: Circulating tumor cell.



No. at risk

< 5 CTC	114	114	112	111	108	103	102	99	86	75	62	48	32	13	10	4	2
⩾ 5 CTC	49	49	45	39	35	31	27	24	18	14	9	6	3	3	2	1	0

Log-lank test: $P < 0.001$

Figure 7 Kaplan-Meier estimates of probabilities of overall survival in patients with metastatic breast cancer who had less than 5 circulating tumor cells and those who had 5 or more circulating tumor cells (at the first follow-up visit after initiation of a new line of therapy)[29]. CTC: Circulating tumor cell.

initiation of therapy in 163 patients. Patients who had 5 or more CTCs per 7.5 mL blood at the first follow-up visit, as compared with those who had less than 5 CTCs per 7.5 mL blood, had a shorter median progression-free survival (2.1 mo *vs* 7.0 mo, $P < 0.001$, Figure 6) and shorter median overall survival (8.2 mo *vs* > 18 mo, $P < 0.001$, Figure 7). A similar difference was seen between the groups on the basis of the baseline values.

The median progression-free survival and median overall survival in the 33 patients with 5 or more CTCs at baseline, but less than 5 CTCs at follow-up differed significantly from the respective values in the 25 patients who

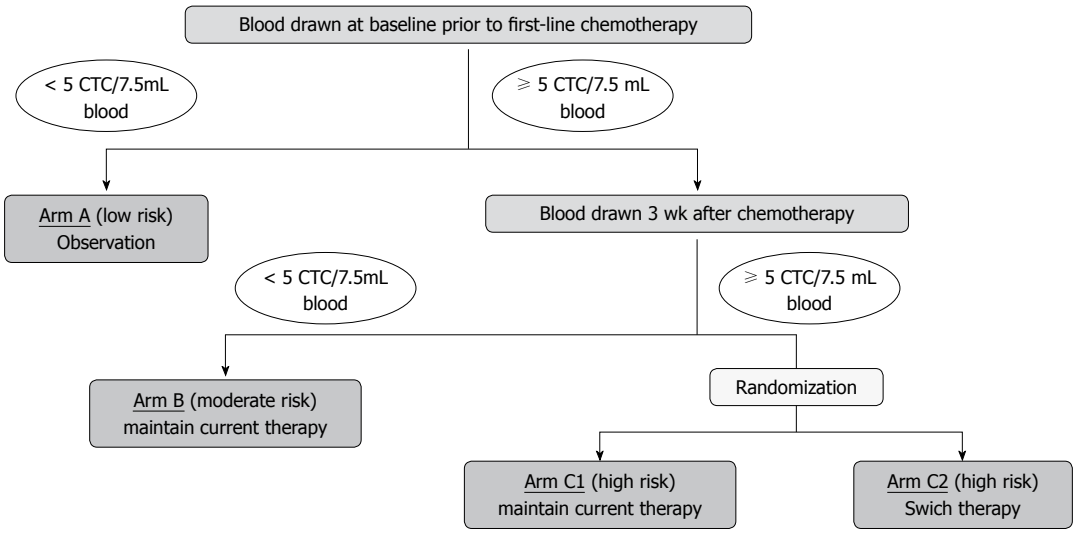


Figure 8 Study design of the Southwest Oncology Group Trial S0500. CTC: Circulating tumor cell.

had a decrease in CTCs from baseline, but had 5 or more CTCs at follow-up (progression-free survival, 7.6 mo *vs* 2.1 mo, $P = 0.002$; overall survival, 14.6 mo *vs* 9.2 mo, $P = 0.006$)[29].

Hayes *et al*[31] studied the prognostic implications of CTC counts before treatment and after each treatment cycle. Even if the CTC count was 5 or more before treatment, patients who had CTC counts of less than 5 at any follow-up time point during treatment had good outcomes. In contrast, patients with CTC counts of 5 or higher at any time point during treatment had poor outcomes. These studies showed that blood CTC counts at initial follow-up after chemotherapy can be used to evaluate the response to treatment and suggest that the results can contribute to reducing stress on patients caused by long-term treatment with ineffective anticancer drugs.

In the United States, a phase Ⅲ clinical trial (SWOG S0500) is ongoing to determine whether CTC counts can be used to facilitate decisions concerning whether to change or maintain therapy. The feasibility or using CTC counts to compare outcomes is also being assessed. In that study, the number of CTCs is counted before the first course of chemotherapy, and patients with 5 or more CTCs are given a specified regimen of chemotherapy. If the CTC count is less than 5 after the first course of chemotherapy (after 3 wk), current therapy is maintained. If the CTC count is 5 or more at this time, patients are randomly assigned to maintain current therapy or to switch to a new regimen of chemotherapy. This study is designed to determine whether CTC monitoring can be used to predict the response to treatment at an early time, and thereby promptly discontinue ineffective anticancer drugs and switch to another therapy if a response is unlikely. Another important objective is to determine whether outcomes can be improved by adhering to this procedure (Figure 8).

Epithelial growth factor receptor (EGFR) is overexpressed in many cancers. EGFR includes 4 families: EGFR (HER1), HER2, HER3, and HER4. Molecular targeted agents that target these receptors have been developed. *HER2* gene amplification or protein overexpression is found in about 20% to 30% of patients with breast cancer. Treatment with trastuzumab, a humanized monoclonal antibody against *HER2* receptor, has been shown to improve outcomes in such patients[32].

Meng *et al*[33] studied 24 patients with HER2-negative primary breast cancer who had recurrence. *HER2* gene amplification on CTCs during tumor progression was confirmed in 9 (37.5%) of 24 patients. Trastuzumab was given to 4 of the 9 patients. One had a complete response, 2 had a partial response, and 1 had progressive disease. In these 4 patients, the primary tumor was HER2 negative, but *HER2* gene amplification was confirmed on CTCs.

Trastuzumab may be effective in patients in whom resected or biopsy specimens are not available and those with *HER2*-negative primary breast cancer in whom *HER2* on CTCs is positive. We believe that molecular expression analysis of CTCs combined with increased sensitivity for the detection and enumeration of CTCs will provide important biomarkers for molecular targeted agents.

CTCS AND IMAGING STUDIES IN BREAST CANCER

De Giorgi *et al*[34] retrospectively studied the relation between CTC counts and therapeutic response by performing[18] fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) in patients with metastatic breast cancer. In 115 patients with metastatic breast cancer who newly started therapy, CTCs in blood were counted and FDG-PET/CT was performed at the time of starting treatment and 9 to 12 wk after treatment had begun. The mean survival time in 102 patients with assessable lesions was 14 mo. In 68 patients

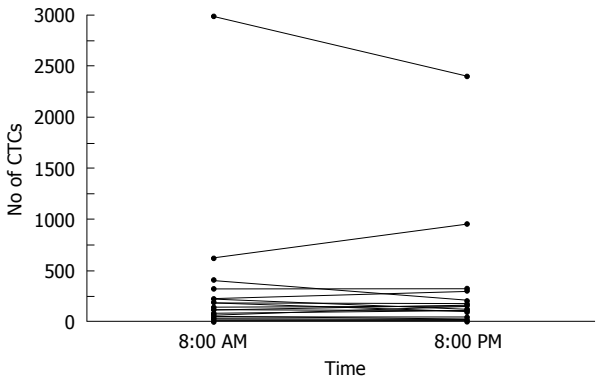


Figure 9 Circulating tumor cell values at 8:00 AM and at 8:00 PM in the 51 assessable patients[36]. CTC: Circulating tumor cell.

(67%), CTC counts during treatment correlated with the tumor response on FDG-PET/CT. On univariate analysis, better overall survival was significantly associated with less than 5 CTCs ($P < 0.001$) and a treatment response as evaluated by FDG-PET/CT ($P = 0.001$). Multivariate analysis showed that the CTC count was an important factor for overall survival ($P = 0.004$). They concluded that prospective studies are needed to confirm the results and assess cost-effectiveness.

Liu *et al*[35] conducted a prospective study to clarify the relation between CTC counts and radiographic disease progression. They serially measured CTC levels in patients starting a new treatment regimen for radiographically measurable metastatic breast cancer. Blood samples were collected before starting treatment and at 3- to 4-wk intervals after treatment began. The presence of 5 or more CTCs per 7.5 mL blood was defined as the cut-off value. Clinical outcomes were based on imaging studies performed at 9- to 12-wk intervals. Correlations between CTC counts and treatment response on imaging studies were assessable in 68 patients. Progression-free survival was assessable in 74 patients. Mean follow-up was 13.3 mo. In these patients, CTC counts were associated with radiographic disease progression. As compared with patients who had less than 5 CTCs, odds ratios for radiographic disease progression in patients who had 5 or more CTCs were 4.9 when CTCs were enumerated 7 to 9 wk before imaging, 3.1 when CTCs were enumerated 3 to 5 wk before imaging, and 6.3 when CTCs were enumerated at the time of imaging. As compared with patients who had less than 5 CTCs at each time point, progression-free survival was slightly, but not significantly shorter in patients who had 5 or more CTCs 3 to 5 wk after the start of treatment (5.1 mo *vs* 3.1 mo, $P = 0.07$) and was significantly shorter in patients who had 5 or more CTCs 7 to 9 wk after the start of treatment (6.7 mo *vs* 2.5 mo, $P < 0.001$). The study concluded that CTC monitoring combined with imaging studies provided a more accurate assessment of disease progression than imaging studies alone.

CTC counts combined with imaging studies can decrease the risk of radiation exposure, as well as facilitate the early assessment of treatment response. One study reported no significant circadian variations in CTC counts[36] (Figure 9). CTC counts are expected to be useful as a stable biomarker of disease progression and treatment response.

CONCLUSION

Breast cancer consists of diverse subtypes with different biologic characteristics. Individualized therapy matched to these characteristics is being developed. Appropriate treatment for patients requires a more accurate diagnosis. The enumeration of CTCs can help to predict response treatment at an early period and thereby avoid unnecessary therapy. When used as a biomarker for molecular targeted agents, CTC counts can facilitate the identification of patients most likely to respond to a given treatment and thereby provide the best-suited therapy to patients with breast cancer. Although CTC counts are associated with an extremely low false-positive rate in patients with metastatic breast cancer, the sensitivity has been reported to be only 26% to 49%[28,20,31,37-39]. The routine use of CTC monitoring as a diagnostic test would require the establishment of measurement techniques with improved sensitivity by, for example, the discovery of new detection targets, the establishment of quantitative and qualitative evaluation criteria to determine appropriate cut-off values, and further evidence clearly supporting the clinical significance of CTC counts. We expect that CTC counts will be established to be a new diagnostic and therapeutic index for patients with breast cancer.

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