

Circulating Tumor Cells in Metastatic Breast Cancer: Timing of Blood Extraction for Analysis

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Abstract. Purpose: Circulating tumor cells (CTCs) can be detected in the peripheral blood of around 50% of patients with metastatic breast cancer. Their numbers are an independent predictor of the patient's progression-free survival (PFS) and of overall survival (OS). However, to date, none of the studies carried out with the most commonly used system of CTC determination (the CellSearch System, approved by the US Food and Drug Administration) has examined the intra-patient variation in CTC numbers, a variation that could impact on prognosis assessment. Experimental design: To evaluate possible circadian variations in the number of CTCs in patients with breast cancer a pilot study was conducted in which these cells were quantified 12 h apart (at 8:00 a.m. and 8:00 p.m. of the same day) in a cohort of hospitalized patients with metastatic breast cancer. Results: Out of the 58 patients included in the study, 51 were evaluable. No statistically significant differences between day-time and night-time CTC numbers were observed ($p=0.8427$, Wilcoxon matched pair test). Only two of the patients were classified in different prognostic categories in the morning and night determinations (5 or more CTCs=poor prognosis group; <5 CTCs=good prognosis group). The prognostic classification of the remaining 49 patients was the same at 8:00 a.m. and 8:00 p.m. Conclusion: The number of peripheral blood CTCs in metastatic breast cancer patients is not significantly different at 8:00 a.m. from that at 8:00 p.m. and, as such, indicates a lack of circadian rhythm with respect to CTC numbers in these patients.

The detection and quantification of circulating tumor cells (CTCs) in patients with metastatic breast cancer is, currently, undergoing intense investigation. CTCs can be detected in the peripheral blood of 10-15% of patients with early breast cancer and in 40-50% of patients with metastatic disease (1, 2). In patients with metastatic breast cancer, the baseline number of CTCs is an independent predictor of progression-free survival (PFS) and overall survival (OS) (2-4). The US Food and Drug Administration has approved a semi-automatic immuno-magnetic method, the CellSearch System (from Veridex, LLC, Warren, NJ, USA), specifically for this purpose.

Despite the increasing investigational and clinical use of CTC measurement in breast cancer, little attention has been paid to a particular aspect of the method *i.e.* the time of extraction of the blood sample used for the measurements. Indeed, the majority of studies published to date make no mention of the time of blood sampling for the CTC measurement (1-4), the assumption being that the timing of blood extraction has no impact on CTC number *i.e.* that the CTC number remains constant throughout the day. This is despite the observation of several circadian variations in tumor cell biology (5). Therefore, a circadian variation in CTC numbers in patients with metastatic breast cancer cannot be ruled out, *a priori*.

To acquire some insight into the possible variation in CTC numbers in patients with breast cancer, a pilot study was conducted in which these cells were quantified 12 h apart (at 8:00 a.m. and at 8:00 p.m. of the same day) in a cohort of hospitalized patients with metastatic breast cancer. The preliminary results of this study have been published (6) and the present report represents the definitive findings of the pilot study.

Patients and Methods

Consecutive breast cancer patients with advanced metastatic disease who were hospitalized in our Medical Oncology Service for treatment of disease complications (including pain, pleural effusion, medullary cord compression, bone fractures) were included in the trial. The study was approved by the institutional Ethical Committee. All the patients provided written informed consent and

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all the study procedures conformed to Good Clinical Practice.

Method for the isolation of circulating tumor cells. Two samples (7.5 ml each) of peripheral venous blood were collected from the patients. The first sample was collected at 8:00 a.m. and the second was collected at 8:00 p.m. Also, an equivalent blood sample at 8:00 a.m. was collected from healthy age-matched control individuals and processed in parallel with the patients' samples. The blood was drawn into a 10 ml test tube with EDTA and cellular preservatives (Cellsav tubes; Veridex™ LLC) and processed according to the manufacturer's instructions at room temperature within 72 h. Briefly, the blood was mixed with a ferrofluid coated with buffered epithelial cell-specific EpCAM (epithelial cell adhesion molecule) antibodies. The isolated cells were fluorescence stained with the nucleic acid dye 4',6-diaminodino-2-phenylindole (DAPI) labeled with monoclonal antibodies specific for leukocytes (CD45) and epithelial cells (cytokeratin 8, 18, 19) (CK-PE) and assayed with a Cellspotter Analyzer (Veridex™, LLC). The analysis showed images of cells which, to be defined as CTC, needed to comply with the following criteria: round to oval morphology with a diameter >4 µm, cytoplasm positively stained for CK-PE and negative for CD45 and >50% of the nucleus stained with DAPI. The results are expressed as number of CTC cells/7.5 ml of blood. The interpretations of the results were independently confirmed by four trained specialists (MLMC, MV, SV, VO) who were blinded with respect to the provenance of the samples.

Statistical analyses. Since this was a pilot study and no prior information existed on potential circadian differences in CTC numbers, an empirical sample size of 50 patients was established. The data were analyzed using the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA). Gaussian distributions could not be assumed and, therefore, the Wilcoxon matched-pair test was applied to compare the a.m. vs. the p.m. sample from each individual. The value for statistical significance was set at $p<0.05$ (two-sided).

Results

Between November 2005 and June 2006, 58 patients and 58 age-matched healthy controls were studied. All the patients had been hospitalized because of complications of their disease (including pain, dyspnea, central nervous system metastases, medullary compression). In all, 116 samples from patients (58 samples taken at 8:00 a.m. and 58 samples at 8:00 p.m.) and 58 samples from healthy volunteers (all taken at 8:00 a.m.) were collected for analysis. The results from 7 cancer patients were rejected due to sample processing errors. The characteristics of the remaining 51 evaluable patients and their previous therapies are shown in Table I. The vast majority of the patients had widespread disease (median disease sites, 3), visceral involvement (two thirds of the patients) and extensive prior therapy (median number of hormonal and chemotherapy lines, 1 and 2, respectively). With the exception of three patients who were receiving tamoxifen therapy and four patients who were scheduled to start chemotherapy, all the patients were receiving palliative symptom treatment (essentially analgesics, radiation and corticosteroids).

Table I. Characteristics of the patients and their previous therapies.

Characteristic	N (%)
Evaluable patients	51
Median age, years	59 (range: 36-78)
Hormone receptor status ¹	
Positive	29 (57)
Negative	18 (35)
Not assessed	4 (8)
Her2/neu (c-erbB2)	
Amplified ²	14 (28)
Normal	25 (49)
Not assessed	12 (24)
Site of disease ³	
Liver	24 (47)
Bone	29 (57)
Soft tissue	27 (53)
Lung	30 (59)
Central nervous system	15 (29)
Plural effusion	23 (45)
Disease sites	
1	7 (14)
2	7 (14)
3	12 (24)
4 or more	25 (49)
Previous chemotherapy lines ⁴	
0	3 (6)
1	9 (18)
2	14 (28)
3	7 (14)
4 or more	18 (35)
Previous hormone therapy lines ⁵	
0	17 (33)
1	19 (37)
2 or more	15 (30)

¹Positive: either estrogen receptor or progesterone receptor positive; negative: both estrogen receptor and progesterone receptor negative. ²By fluorescent *in situ* hybridization. ³Most patients had more than one site of disease. ⁴Including adjuvant chemotherapy and chemotherapy for metastatic disease. ⁵Including adjuvant hormones and hormones for metastatic disease.

No CTCs were isolated from the samples of any of the healthy control individuals. The numbers of CTCs isolated at 8:00 a.m. and at 8:00 p.m. in all the patients are shown in Figure 1. No statistically significant differences between day-time and night-time CTC values were observed ($p=0.8427$, Wilcoxon matched pair test). There were 15 patients (29%) with 0 CTC in the 8:00 a.m. determination, 8 patients (16%) with 2 to 4 CTCs and 28 patients (55%) with 5 or more CTCs (range 5-2987). A cut-off of 5 CTCs/7.5ml of whole blood has been previously established as classifying patients into two prognostic groups *i.e.* patients with 5 or more CTCs have a shorter median PFS and OS (poorer prognosis) than patients with <5 CTCs (2). According to this classification, only two of our patients (one

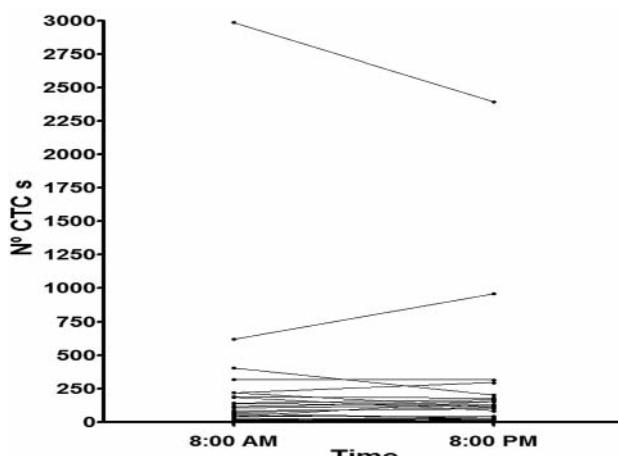


Figure 1. CTC values at 8:00 a.m. and at 8:00 p.m. in the 51 evaluable patients.

who had 0 CTCs at 8:00 a.m. and 10 cells at 8:00 p.m. and another who had 8 cells at 8:00 a.m. and 3 cells at 8:00 p.m.) could be classified in different prognostic categories depending on the time of CTC determination. The prognostic classification of the remaining 49 patients was unchanged irrespective of the blood sample timing. The results seen between patients on chronic treatment with corticoids were similar to those of the remaining patients (no differences between day-time and night-time determinations).

Discussion

To our knowledge, this is the first prospective study assessing circadian variations of CTC numbers in patients with metastatic breast cancer. No significant differences between day-time and night-time numbers of circulating tumor cells were found in the study. Circadian rhythm is an approximately 24 h cycle in the physiological and biological processes of most organisms. Several *in vivo* studies have indicated that circadian rhythm plays a role in cell-cycle rates (7). However, the present study did not demonstrate a circadian CTC rhythm. This is fortunate since the findings indicated that CTCs can be determined at any time without fear of bias and, as such, were valuable from a practical point of view.

The results also showed that the CTC numbers were similar in two determinations carried out for the same patient. This was reassuring in terms of the reproducibility of the technique. Recent data suggested that the cell enrichment technique used by the CellSearch System could lead to a considerable numerical loss of epithelial tumor cells in the course of the measurement procedure (8, 9). The present results showing a lack of significant intra-patient variability in CTCs in two consecutive determinations would not support this hypothesis.

However, this study did have some limitations. The population sample was composed, mainly, of breast cancer patients with very advanced disease, and with several disease sites. As such, it is unclear whether these results could be extrapolated to a different population of breast cancer patients.

In summary, there is no significant circadian variation in the number of circulating tumor cells in peripheral blood in patients with advanced metastatic breast cancer.

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Conflict of Interest

No author has any involvement that can be construed as a conflict of interest

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