Circulating Tumor Cell Detection Predicts Early Metastatic Relapse After Neoadjuvant Chemotherapy in Large Operable and Locally Advanced Breast Cancer in a Phase II Randomized Trial

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Abstract

Purpose: Circulating tumor cells in blood from metastatic breast cancer patients have been reported as a surrogate marker for tumor response and shorter survival. The aim of this study was to determine whether circulating tumor cells are present in the blood of patients with large operable or locally advanced breast cancer before neoadjuvant chemotherapy and after neoadjuvant chemotherapy before surgery.

Experimental Design: Blood samples of 7.5 mL were obtained on CellSave tubes from patients included in a phase II trial (REMAGUS 02). Circulating tumor cells were immunomagnetically separated and fluorescently stained by the CellSearch system. Blood from 20 metastatic breast cancer patients was used as a positive control.

Results: From October 2004 to July 2006, preneoadjuvant chemotherapy and/or postneoadjuvant chemotherapy blood samples were obtained from 118 patients. At least 1 circulating tumor cell was detected in 22 of 97 patients with preneoadjuvant chemotherapy samples (23%; 95% confidence interval, 15-31%; median, 2 cells; range, 1-17 cells). Circulating tumor cell positivity rates were 17% in 86 postneoadjuvant chemotherapy samples and 27% in all 118 patients. Persistence of circulating tumor cells at the end of neoadjuvant chemotherapy was not correlated with treatment response. After a short median follow-up of 18 months, the presence of circulating tumor cells ($P = 0.017$), hormone receptor negativity, and large tumor size were independent prognostic factors for shorter distant metastasis – free survival.

Conclusion: Circulating tumor cells can be detected by the CellSearch system at a low cutoff of 1 cell in 27% of patients receiving neoadjuvant chemotherapy. Circulating tumor cell detection was not correlated to the primary tumor response but is an independent prognostic factor for early relapse.

Primary, preoperative, or neoadjuvant chemotherapy was introduced in the early 1970s as part of an integrated therapeutic approach to treat inoperable locally advanced breast cancer. Preoperative chemotherapy was then gradually extended to include patients with large but operable early-stage breast cancer, as it sometimes achieved downstaging of the primary tumor, thereby avoiding mastectomy and allowing breast-conserving surgery (1). Preoperative systemic therapy is now an established part of the management of large, potentially operable, and locally advanced breast cancers (2). Neoadjuvant therapy seems to be equivalent to adjuvant therapy in terms of survival and overall disease progression (3). This preoperative approach allows the tumor to be used as an *in vivo* measure of treatment response. Clinical, pathologic, and molecular end points may reflect tumor sensitivity to chemotherapy and could be used as surrogate markers to predict long-term outcome in the adjuvant setting (4, 5). The achievement of pathologic complete response, including nodal involvement, is currently the main end point reported by neoadjuvant chemotherapy protocols (6, 7). Clinical trials in this setting therefore require fewer patients and can be completed more rapidly. However, not all patients achieving pathologic complete response will be cured; other surrogate markers must therefore be identified (8, 9).

The effect of neoadjuvant or adjuvant chemotherapy has been attributed to its ability to eradicate microdisseminated tumor cells that could potentially develop into distant metastases. Minimal residual disease in breast cancer can be assessed in bone marrow, and patients with positive

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Received 1/4/08; revised 4/6/08; accepted 4/16/08.

Grant support: French Programme Hospitalier de Recherche Clinique ISRCTN10059974, PHRC: AOM 02 11 and research grant from Veridex.

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doi:10.1158/1078-0432.CCR-08-0030

Translational Relevance

Circulating tumor cells can be detected by the CellSearch system at a low cutoff of 1 cell in the peripheral blood of 27% of patients receiving neoadjuvant chemotherapy for large operable and locally advanced breast. Circulating tumor cell detection was not correlated to the primary tumor response but is an independent prognostic factor for early relapse.

disseminated tumor cell detection have a poorer prognosis (10, 11). Monitoring disseminated tumor cells in bone marrow after adjuvant therapy may present a major clinical value (12, 13), but repeated bone marrow aspiration can be difficult to do under local anesthesia. Blood sampling would therefore be a more convenient procedure. Circulating tumor cell detection by an immunostaining method has been proven to be a strong prognostic factor at the metastatic stage (14, 15). Only one group, however, has shown a prognostic value of circulating tumor cell detection on the adjuvant setting, using a reverse transcription-PCR technique (16, 17), although this technique, as other methods, can be associated with false positive results (illegitimate transcript, skin cell, etc.; refs. 18, 19). Immunostaining methods have the advantage of allowing morphologic verification of detected cells (20–22).

The Cellsearch technique (Veridex) is currently the only Food and Drug Administration approved, fully automated method to detect breast cancer circulating tumor cells (23–25). The prognostic significance of circulating tumor cell detection has been shown in metastatic breast cancer, but not yet at the early stage of this disease (26). The aim of this study was to determine whether circulating tumor cell are present in the blood of patients before and after neoadjuvant chemotherapy, but before surgery, for large operable and locally advanced breast cancer and to correlate circulating tumor cell detection with tumor response and prognosis.

Patients and Methods

Patients and chemotherapy regimen. Preneoadjuvant chemotherapy and/or postneoadjuvant chemotherapy blood samples were obtained in a subgroup of the patients included in the phase II randomized REMAGUS 02 trial from four different institutions. Circulating tumor cell detection was done whenever possible, and no attempt was made in this study to define a target statistical power. All samples were obtained with the patient's written informed consent, after approval by the regional ethics committee. Neither patients nor clinicians were informed of the results of circulating tumor cell analysis. Eligibility criteria for the study were female patients ages ≥18 and <65 y with histologically proven nonmetastatic invasive breast carcinoma (stages II and III), with tumors ineligible for breast-conserving surgery (diameter > 3 cm, central, multifocal) or with risk factors making neoadjuvant chemotherapy the preferred treatment (i.e., ipsilateral lymph node involvement, rapid growth rate). Eligible patients had no history of previous malignancy other than being treated *in situ* for carcinoma of the cervix or nonmelanoma skin cancer, no bilateral breast cancer, and no distant metastasis. The routine diagnostic work-up included mammography, breast magnetic resonance imaging, tumor biopsy with frozen sample, chest X-rays, abdominal ultrasound, bone scan, blood sampling, and clinical examination. HER2 status was considered positive if the Herceptest result was 3+. In doubtful cases (2+), a fluorescence *in situ* hybridization analysis was done. The cutoff used to define hormone receptor positivity was 10% of stained cells.

All patients received four cycles of epirubicin-cyclophosphamide every 3 wk followed by four cycles of docetaxel with or without trastuzumab, according to randomization, for HER2-positive tumor patients and with or without celecoxib for HER2-negative tumor patients. Surgery was followed by local and regional radiotherapy when indicated. All patients with HER2-positive tumors received adjuvant trastuzumab for 1 y, whereas all patients with hormone receptor–positive tumors received adjuvant tamoxifen or aromatase inhibitors. Pathologic tumor response (primary tumor and axillary nodal status) was evaluated according to Chevallier criteria; a pathologic complete response was defined by Chevallier stage 1 or 2 criteria (27).

Circulating tumor cell detection. Samples were maintained at room temperature and processed within 72 h after collection. All evaluations were done with no knowledge of the patient's clinical status. The standardized Cellsearch technique has been reported previously (28). Briefly, 7.5 mL of blood is drawn on CellSave tubes. The CellSearch System is used to isolate and count circulating tumor cells. It consists of a semiautomated sample preparation system and is used with the CellSearch Epithelial Cell Kit. The procedure enriches the sample for cells expressing the epithelial cell adhesion molecule with antibody-coated magnetic beads, and labels the cells with the fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride. Fluorescently labeled monoclonal antibodies specific for leukocytes (CD45-allophycocyan) and epithelial cells (cytokeratin 8,18,19-phycoerythrin) are used to distinguish epithelial cells from leukocytes. Identification and counting of circulating tumor cells are done with the CellSpotter Analyzer, a semiautomated fluorescence-based microscopy system that allows computer-generated reconstruction of cellular images. Circulating tumor cells are defined as nucleated cells lacking CD45 and expressing cytokeratin.

Statistical methods. Distant metastasis–free survival was calculated from the time of initial diagnosis to the time of distant metastatic relapse. Differences between categorical variables were analyzed by χ^2 tests or Fisher's exact test. Survival curves were plotted according to the Kaplan-Meier method. Statistical significance between survival curves was assessed using the log-rank test. Multivariate analysis was done by the Cox proportional hazards model with prognostic factors with P value under 0.10 at univariate analysis. For all analyses, a P value of <0.05 was considered to be statistically significant. This report was written in accordance with the REporting of tumor MARKer Studies guidelines (29).

Results

From October 2004 to July 2006, preneoadjuvant chemotherapy and/or postneoadjuvant chemotherapy blood samples were obtained from 118 patients. Patient and tumor characteristics are summarized in Table 1. Median age was 47 years (range, 29-65 years). Preneoadjuvant chemotherapy samples were available for 97 patients. One or more circulating tumor cells were detected in 22 patients (detection rate, 23%; 95% confidence interval, 15-31%; median, 2 cells; range, 1-17 cells). Twelve patients were classified as positive with a cutoff of 2 or more cells (detection rate, 12%; 95% confidence interval, 6-18%), whereas only 4 patients had >5 cells per sample. Postneoadjuvant chemotherapy samples were available for 86 patients and were positive for at least 1 cell in 15 patients (detection rate, 17%; median, 1 cell; range, 1-10 cells). By combining the samples available before or after neoadjuvant chemotherapy, circulating tumor cell detection could be done for 118 patients, of which 32 were positive for circulating tumor cells (detection rate, 27%) on at least one sample. Blood

Table 1. Patient characteristics and correlation with CTC detection before and/or after neoadjuvant chemotherapy

	PreCT (n = 97 patients)			PostCT (n = 86 patients)			PreCT and/or postCT (n = 118 patients)		
	n (%)	CTC positive (n = 22)	P (χ^2)	n (%)	CTC positive (n = 15)	P (χ^2)	n (%)	CTC positive (n = 32)	P (χ^2)
Age									
≤50	62 (64%)	18		56 (65%)	12		76 (64%)	27 (36%)	
>50	35 (36%)	4	0.08	30 (35%)	3	0.30	42 (36%)	5 (12%)	0.005
Tumor size									
T_1 and T_2	53 (55%)	12		47 (55%)	7		65 (55%)	16 (25%)	
T_3 and T_4	44 (45%)	10	0.99	39 (45%)	8	0.49	53 (45%)	16 (30%)	0.54
Node									
N_0	37 (38%)	8		29 (38%)	5		43 (36%)	11 (26%)	
N_1 and N_2	60 (72%)	14	0.84	57 (72%)	10	0.97	75 (64%)	21 (28%)	0.83
Histology									
Ductal	87 (88%)	19		77 (88%)	13		104 (88%)	28 (27%)	
Lobular	6 (7%)	1		8 (7%)	2		9 (8%)	2 (22%)	
Other	4 (5%)	2	0.45	1 (5%)	0	0.76	5 (4%)	2 (40%)	0.77
Tumor grade									
1 and 2	51 (53%)	12		45 (52%)	7		61 (52%)	16 (26%)	
3	46 (47%)	10	0.83	41 (48%)	8	0.63	57 (48%)	16 (28%)	0.83
Hormone receptors									
ER or PgR positive	58 (60%)	12		54 (60%)	9		71 (60%)	16 (22%)	
ER and PgR negative	39 (40%)	10	0.12	32 (40%)	6	0.80	47 (40%)	16 (34%)	0.20
HER2									
Positive	24 (25%)	7		26 (30%)	5		35 (30%)	11 (31%)	
Negative	73 (75%)	15	0.39	69 (70%)	10	0.77	83 (70%)	21 (25%)	0.50
Pathologic response									
pCR	19 (20%)	4		17 (20%)	2		23 (19%)	6 (23%)	
Non-pCR	78 (80%)	18	0.84	69 (80%)	13	0.47	95 (81%)	26 (27%)	0.90

Abbreviations: CTC, circulating tumor cells; pCR, pathologic complete response; preCT, preneoadjuvant chemotherapy; postCT, postneoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor.

from 20 metastatic breast cancer patients, collected before initiation of a new chemotherapy (first-line or subsequent) was used as a positive control. Of the 20 patients, 13 (65%) had ≥1 circulating tumor cell per sample, and 9 of 19 patients (47%) had ≥2 circulating tumor cells, with a median of 6 cells (range, 1-750) per sample. Blood from 15 healthy volunteers was analyzed under the same conditions. None of these samples was positive with a threshold of 1 circulating tumor cell. Circulating tumor cell detection before chemotherapy (n = 97 patients) or at any time before or after neoadjuvant chemotherapy (n = 118) was not correlated with clinical or pathologic parameters except for young age (<50 years; Table 1).

Preneoadjuvant chemotherapy and postneoadjuvant chemotherapy blood samples were both available for 65 patients. Forty-four patients were circulating tumor cell negative before and after neoadjuvant chemotherapy, and four patients were circulating tumor cell positive before and after neoadjuvant chemotherapy; nine patients who were initially circulating tumor cell positive became circulating tumor cell negative, and eight patients who were initially circulating tumor cell negative became circulating tumor cell positive after neoadjuvant chemotherapy (Fig. 1).

Of these 118 patients, 23 (19%) obtained pathologic complete response. Pathologic complete response was correlated with small tumor size, high tumor grade, hormone receptor negativity, and HER2 positivity. Neither circulating tumor cell detection before or after neoadjuvant chemotherapy nor changes in circulating tumor cell count during neoadjuvant chemotherapy was predictive of pathologic complete response (Table 2). Nevertheless, circulating tumor cell detection before and/or after neoadjuvant chemotherapy was significantly associated with early metastatic relapse (Fig. 2; P = 0.013; median follow-up, 18 months). The other prognostic factors for a shorter distant metastasis–free survival on univariate analysis were hormone receptor negativity and large tumor size (Table 3). In the subgroup of 97 patients with only a preneoadjuvant chemotherapy sample, the correlation between circulating tumor cell detection and shorter metastasis-free survival did not reach statistical significance (P = 0.07). On multivariate analysis, circulating tumor cell detection before and/or after neoadjuvant chemotherapy remained an independent prognostic factor (Table 3). Changes in circulating tumor cell count during neoadjuvant chemotherapy were not significantly correlated with distant metastasis–free survival in the 65 patients for which the two samples were available. However, the only patient with a marked increase in circulating tumor cell count during neoadjuvant chemotherapy (preneoadjuvant chemotherapy, 0 cells; postneoadjuvant chemotherapy, 10 cells) developed multimetastatic relapse only 1 month after surgery and died within 6 months.

Discussion

This is the first study to show that circulating tumor cell detection, assessed by the Food and Drug Administration–approved CellSearch system, has a prognostic value in nonmetastatic breast cancer patients, indicating that this

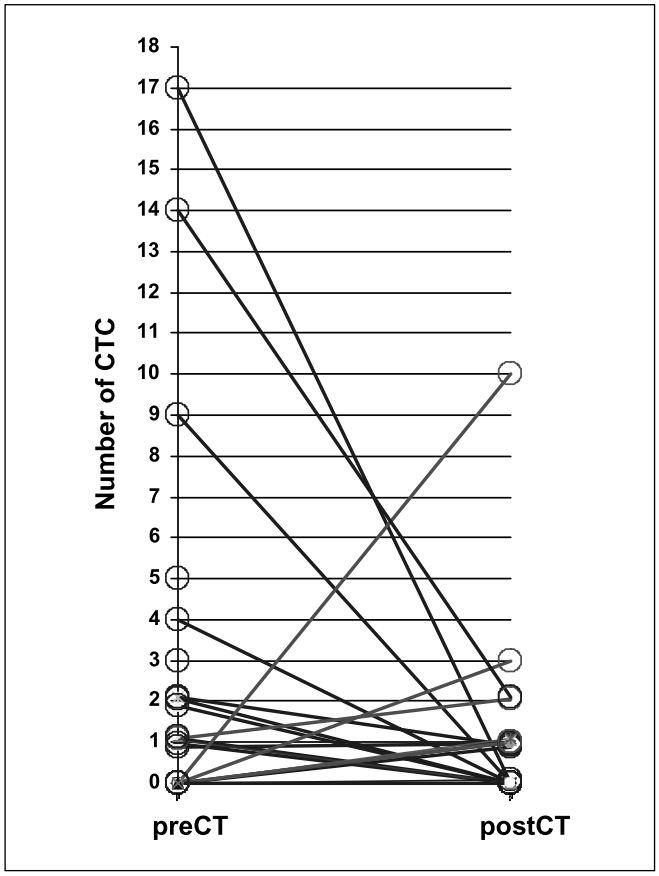

Fig. 1. Circulating tumor cell detection before and after neoadjuvant chemotherapy. $n = 65$ patients.

immunocytofluorescence-based technique is an accurate and highly sensitive method for the detection of epithelial cell adhesion molecule–bearing circulating tumor cells (30). Using the minimum cutoff of 1 circulating tumor cell per sample (7.5 mL of blood), a circulating tumor cell detection rate of 23% was observed in this patient series before chemotherapy. Using the same detection method in metastatic breast cancer patients, Cristofanilli reported a detection rate of 50%, with a cutoff of ≥ 5 circulating tumor cells per sample (31). If the cutoff defined by Cristofanilli had been used in the present study, only 4 of 97 patients (4%) would have been positive before chemotherapy. This suggests that circulating tumor cell detection is a rare event in early breast cancer and obeys a Poisson's law (or law of small probabilities) in each patient. This statistical distribution is associated with a high rate of false negative results (i.e., no circulating tumor cell detected in patients with circulating tumor cells), but the robustness of circulating tumor cell detection in a given patient was not tested here. On the other hand, the use of a lower cutoff could result in false-positive cases, and Allard et al. reported a rate of 5.5% of samples with 1 circulating tumor cell per 7.5 mL of blood in 145 healthy women and none contained more than 1 circulating tumor cell (28). This issue of the appropriate cutoff was discussed in a recent study that showed that a cutoff of 1 circulating tumor cell per 7.5 mL of blood is theoretically reliable, provided the sources of errors, mostly due to interreader variability, are eliminated (32). In the light of these technical issues (low detection rate and minimum cutoff of 1 circulating tumor cell per sample), more sensitive detection protocols (which would increase the mean number of circulating tumor cells detected) would be clinically valuable in the early breast cancer setting. A higher sensitivity could be achieved by a more reliable method of analysis, but also by repeating circulating tumor cell counts with the CellSearch system, to increase the total amount of blood tested. We did not try to use larger blood volume as suggested by other authors. A recently published article by Lang et al. reported a positivity rate of 38% analyzing 30 mL of blood with a threshold of 1 circulating tumor cell in a series of 92 patients with operable breast cancer (33). Rack et al. observed, however, that only 10% of patients had more than 1 circulating tumor cell in 23 mL of blood (three tubes instead of one) in a very large series of 1,767 patients after surgery for primary breast cancer (34). In nonmetastatic patients, it is not shown that the number of circulating tumor cells detected has any prognostic significance.

The use of circulating tumor cell detection to monitor response to neoadjuvant chemotherapy has been previously reported only in a smaller series of 58 patients (35). In this study, we showed that the circulating tumor cell response during the first cycles of neoadjuvant treatment was predictive of the final tumor response. Circulating epithelial tumor cells were detected in almost all peripheral blood samples before the first cycle of chemotherapy. Initial pretreatment circulating epithelial tumor cell counts varied considerably between patients from the highest value of 273,150 to the lowest value below the limit of detection, with a mean of 26,079 cells per mL. The number of circulating tumor cells detected in this study was several logs higher than the values reported by other groups, raising a doubt concerning the specificity of the technique used (Laser Scanning Cytometer and antihuman epithelial antibody). However, this technique seems also promising to monitor response to adjuvant chemotherapy (36).

In the present study, the lack of correlation (without any trend) between changes in circulating tumor cell count and pathologic complete response is a challenging observation, as a decrease in circulating tumor cell count has been reported to be a strong predictive biomarker of response to treatment in metastatic breast cancer (31, 37). Becker et al., in a series of 120 patients undergoing primary systemic therapy, also reported that bone marrow disseminated tumor cells were not completely eradicated in patients with complete pathologic response (38). In another series of 154 patients, viable disseminated tumor cells were still present in the bone marrow of 10 of 24 patients with pathologic complete response (39).

Table 2. Changes in CTC detection during chemotherapy, pCR, and metastatic outcome in the 65 patients assessed before and after chemotherapy

CTC preCT-postCT	*n*	pCR (*n*)	Metastatic relapse (*n*)
Positive-positive	4	0	1
Positive-negative	9	2	2
Negative-positive	8	2	1
Negative-negative	44	9	3

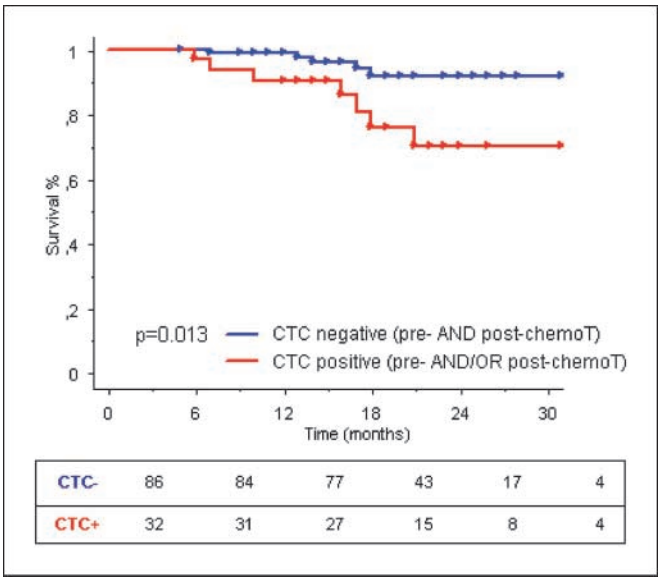

Fig. 2. Distant metastasis – free survival on univariate analysis in 118 patients with preneoadjuvant and/or postneoadjuvant chemotherapy samples according to the presence of circulating tumor cells.

Our results are concordant with these studies and suggest that circulating tumor cells do not display the same chemosensitivity as the breast primary tumor. It should be noted that a dissociated response to chemotherapy between two metastatic sites is a rare event in stage IV breast cancer patients. Biologically, the metastatic process is generally considered to be a selective multistep process (40), and cancer cells invading axillary lymph nodes may be derived from just a few tumor subclones (41). This selection of metastatic cancer cells may be even more marked in the context of hematogenous spread, explaining why circulating tumor cells may show a different pattern of chemosensitivity to that of the primary tumor. As an example, it has been previously reported that HER2 status may change between primary tumors and circulating tumor cells: circulating tumor cells may express HER2 although their primary tumor was considered as HER2 negative (42). This could be associated with a worst prognosis (43). Finally, another explanation of the discrepancy observed between primary tumor and circulating tumor cell chemosensitivity is that circulating tumor cells have been described as Ki-67 negative, i.e., nonproliferating "dormant" cells, a state associated with a higher resistance to chemotherapy agents (44, 45).

These results show that circulating tumor cell count has a significant effect on distant metastasis survival. Interestingly, Table 2 shows that changes in circulating tumor cell count during neoadjuvant chemotherapy seem to correlate with metastatic relapse. This observation is not statistically significant due to the small number of patients in each group, but should be addressed in further studies. If this result is confirmed, circulating tumor cell changes would be clinically relevant in the neoadjuvant setting, together with pathologic complete response. The use of circulating tumor

Table 3. Patient characteristics and correlation with pCR, and DMFS on univariate and multivariate analyses

	All patients (n = 118)	pCR (n = 23)	P (χ^2)	DMFS univariate P*	DMFS multivariate RR (95 CI) †	P
Age						
≤50	76 (64%)	12 (16%)				
>50	42 (36%)	11 (26%)	0.22	0.50		
Tumor size						
T_1 and T_2	65 (55%)	19 (29%)			1	
T_3 and T_4	53 (45%)	4 (8%)	0.004	0.078	3.80 (1.10-12.98)	0.034
Node						
N_0	43 (36%)	6 (14%)				
N_1 and N_2	75 (64%)	17 (23%)	0.33	0.61		
Histology						
Ductal	104 (88%)	21 (20%)				
Lobular	9 (8%)	1 (11%)				
Other	5 (4%)	1 (20%)	0.80	0.40		
Tumor grade						
1 and 2	61 (52%)	4 (7%)				
3	57 (48%)	19 (33%)	0.0003	0.17		
Hormone receptors						
ER or PgR positive	71 (60%)	6 (8%)			1	
ER and PgR negative	47 (40%)	17 (36%)	0.0003	0.007	5.97 (1.57-22.70)	0.0086
HER2						
Positive	35 (30%)	11 (31%)				
Negative	83 (70%)	12 (14%)	0.044	0.33		
Pathologic response						
pCR	23 (19%)					
Non-pCR	95 (81%)			0.28		
CTC pre and/or postCT						
Positive	32 (27%)	6 (19%)			1	
Negative	86 (73%)	17 (20%)	0.99	0.013	4.15 (1.29-13.3)	0.017

Abbreviations: DMFS, distant metastasis – free survival; RR, relative risk; 95% CI, 95% confidence interval.
*Log-rank test.
† Cox model.

cell monitoring would be of critical importance as pathologic complete response does not have a strong prognostic value. The addition of taxanes or trastuzumab to neoadjuvant regimens has increased the frequency of pathologic complete response (46), but higher pathologic complete response rates with taxanes did not result in an improvement in overall survival (46); and only a small series with short follow-up seems to indicate an improved outcome after trastuzumab, but no correlation with pathologic complete response was shown (47). The value of circulating tumor cells in the neoadjuvant setting is also reinforced by the potential use of circulating tumor cell gene expression profiles or circulating tumor cell whole genomic investigation, which might lead to individualized targeted therapy and a better understanding of the metastatic process (48–50).

The results of this study show that circulating tumor cells can be detected with an immunomagnetic and fluorescent technique at a low cutoff of 1 cell in about one quarter of patients receiving neoadjuvant chemotherapy for operable or locally advanced breast cancer. Pathologic complete response remains the gold standard to assess response to treatment and we were not able to show that circulating tumor cells, detected by this technique, can be used to monitor the efficacy of neoadjuvant chemotherapy. This should be assessed on a larger series of patients. However, circulating tumor cells are an independent prognostic factor for early relapse. If circulating tumor cells are considered to correspond to the early systemic spread of cancer cells, neoadjuvant chemotherapy protocols using sensitive techniques should focus on eradicating circulating tumor cells after treatment to reduce early relapse rates.

Disclosure of Potential Conflicts of Interest

J.Y. Pierga has received a research grant from Veridex and is on the speakers' bureau of Roche.

Acknowledgments

We thank Mustapha Khazour for technical support, Jocelyne Goubet for data management, and Dr. Bernard Asselain (Biostatistics Department, Institut Curie).

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