

## Predictive and Prognostic Value of Peripheral Blood Cytokeratin-19 mRNA-Positive Cells Detected by Real-Time Polymerase Chain Reaction in Node-Negative Breast Cancer Patients

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### A B S T R A C T

#### Purpose

To evaluate the predictive and prognostic value of peripheral blood cytokeratin-19 (CK-19) mRNA-positive cells in axillary lymph node-negative breast cancer patients.

#### Patients and Methods

Peripheral blood was obtained from 167 node-negative breast cancer patients before the initiation of any systemic adjuvant therapy, and was analyzed for the presence of CK-19 mRNA-positive cells using a real time polymerase chain reaction assay. The association with known prognostic factors and the effect of CK-19 mRNA-positive cells on patients' prognosis was investigated.

#### Results

CK-19 mRNA-positive cells were detected in the blood of 36 (21.6%) of the 167 patients. There was no correlation between the detection of CK-19 mRNA-positive cells in the peripheral blood and the various known pathologic and clinical prognostic factors; only overexpression of HER2 receptor (score 2+/3+) on the primary tumor was associated with a higher incidence of CK-19 mRNA-positive cell detection ( $P = .033$ ). Multivariate analysis revealed that detection of peripheral blood CK-19 mRNA-positive cells was associated with early clinical relapse ( $P < .00001$ ) and disease-related death ( $P = .008$ ).

#### Conclusion

Detection of peripheral-blood CK-19 mRNA-positive cells is an independent predictive and prognostic factor for reduced disease-free interval and overall survival, respectively, in node-negative breast cancer patients.

*J Clin Oncol* 24:3756-3762. © 2006 by American Society of Clinical Oncology

### INTRODUCTION

In the recent years, breast cancer is increasingly diagnosed at an early stage because of the massive use of screening mammography programs. Indeed, 56.2% of all breast cancer cases diagnosed in 1995 were stage 0 or 1 compared with 42.5% in 1985.<sup>1,2</sup> Adjuvant chemotherapy and hormone treatments have improved disease-free interval and overall survival.<sup>3</sup> Nevertheless, nearly 30% of patients with node-negative (N0) breast cancer will present distant recurrence and will die as a result of their disseminated disease,<sup>3</sup> whereas 40% of the patients with involved axillary lymph nodes will survive for 10 years without clinical recurrence.<sup>4</sup>

The concept of early tumor cell dissemination in patients with early-stage breast cancer was born about 100 years ago from Paget's "seed and soil" theory.<sup>5</sup> According to this hypothesis, metastasis is a result of rare cells migrating from the primary tumor through the lymphatic or hematogenous route. In breast cancer, tumor cell dissemination to the regional lymph nodes occurs through the lymphatic vasculature<sup>6</sup> but may also occur directly through the hematogenous route; it seems that different biologic mechanisms are required for the lymphatic or hematogenous dissemination of tumor cells.<sup>7,8</sup>

The detection of occult tumor cells in the bone marrow or peripheral blood of patients with early-stage breast cancer has been shown to be an independent predictive and prognostic factor for early

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Submitted October 14, 2005; accepted April 5, 2006; published online ahead of print at www.jco.org on June 12, 2006.

Supported in part by research grants from the Cretan Association of Biomedical Research (CABR). A.S and A.P were recipients of a CABR research fellowship.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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0732-183X/06/2423-3756/\$20.00

DOI: 10.1200/JCO.2005.04.5948

disease recurrence and decreased overall survival.<sup>9-13</sup> Recently, Braun et al<sup>4</sup> reported that the immunohistochemical detection of bone marrow–disseminated tumor cells (DTCs) in 4,703 patients with operable breast cancer was an independent prognostic parameter for early relapse and death during the first 5 years of follow-up.<sup>14</sup>

So far, the clinical relevance of occult tumor cells in breast cancer patients was studied in bone marrow aspirates using immunohistochemical assays.<sup>9-11,13</sup> However, bone marrow is not an easy source for monitoring occult tumor cells. Using a nested reverse-transcriptase (RT-) polymerase chain reaction (PCR) assay, we have previously shown in a cohort of 148 patients with stage I and II operable breast cancer that peripheral blood cytokeratin-19 (CK-19) mRNA-positive cells (circulating tumor cells [CTCs]) before the initiation of adjuvant treatment was an independent predictive and prognostic factor for early relapse and overall survival, respectively.<sup>12</sup>

Real-time PCR is an automated, rapid, versatile, and cost-effective technology capable of providing accurate and quantitative information about gene expression.<sup>15</sup> This method has been used for the detection of CTCs in the peripheral blood and lymph nodes of metastatic breast cancer patients through either differential<sup>16</sup> or multigene<sup>17</sup> expression.

In the present study, we investigated the presence of CK-19 mRNA-positive CTCs using a highly sensitive and specific real-time PCR assay<sup>18</sup> in a cohort of N0 breast cancer patients before the initiation of any adjuvant treatment. The data demonstrated that detection of CK-19 mRNA-positive CTCs in these patients was an independent predictive and prognostic factor for early recurrence and decreased overall survival, respectively.

## PATIENTS AND METHODS

### Patients and Clinical Samples

Peripheral blood (10 mL in EDTA) was obtained from 167 patients with N0 breast cancer on the day of initiation of adjuvant treatment (usually 3 to 4 weeks after primary surgery). All blood samples were obtained at the middle of vein puncture after the first 5 mL of blood were discarded. This precaution was undertaken to avoid contamination of the blood sample with epithelial cells from the skin during sample collection.

Before primary surgery, a complete baseline diagnostic evaluation for distant metastases was performed, including chest x-rays, mammography, ultrasound of the liver, and whole-body bone scan. Further imaging studies (computed tomography scans or magnetic resonance imaging) were performed if clinically indicated. No patient included in the present analysis showed any evidence of distant metastasis. Most of the patients received adjuvant chemotherapy in the context of research protocols of the Hellenic Oncology Research Group (HORG) especially designed for patients with high-or low-risk N0 breast cancer. Adjuvant chemotherapy consisted of either FEC (fluorouracil 700 mg/m<sup>2</sup> day 1 + epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for six cycles) or T/EC (epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for four cycles followed by docetaxel 100 mg/m<sup>2</sup> day 1 every 3 weeks for four additional cycles) as well as classical CMF (cyclophosphamide 100 mg/m<sup>2</sup> orally days 1-14; methotrexate 40 mg/m<sup>2</sup> days 1 and 8; fluorouracil 600 mg/m<sup>2</sup> days 1 and 8; every 4 weeks for six cycles). Tamoxifen (20 mg/d) for 5 years was administered to all patients with estrogen-or/and progesterone-positive tumors. Patients were followed with clinical examinations and laboratory and imaging studies every 3 months for the first 2 years, every 6 months for the next 3 years, and yearly thereafter. The study has been approved by the ethics and scientific committees of our institution, and all patients gave their informed consent in order to participate in the study.

### CK-19 Immunohistochemistry of Axillary Lymph Nodes

Paraffin-embedded level I and II axillary lymph nodes from patients without axillary lymph node involvement in hematoxylin and eosin staining were further evaluated by immunostaining using an anti-CK-19 (anti-CK-19) mouse antihuman monoclonal antibody (DAKO-CK19, Code#M888; DAKO A/S, Glostrup, Denmark). Anti-CK-19 moAb was used at a dilution 1/50 (vol/vol) for 60-minute, after unmasking by heating for 20 minutes, in citrate buffer, pH 6, in a microwave oven at 500 W. The immunostaining method applied was a polymer method based on the DAKO EnVision System, Alkaline Phosphatase, Universal (Code#K1396) with Fast Red substrate-chromogen (Roche Molecular Biochemistry, Mannheim, Germany), according to the manufacturer's instructions. Counterstaining was done with hematoxylin and eosin, and coverslipping with an aqueous-based mounting medium (Glycerol, Cook#C-0563, Carpinteria, CA). Known positive and negative controls were always used.

### RNA Extraction and Real-Time PCR Assay for CK-19 mRNA-Positive CTCs

Peripheral blood mononuclear cells were obtained by gradient density centrifugation using Ficoll-Hypaque (density 1,077 xg/mol; Sigma-Aldrich, St Louis, MO) at 1,200 g for 30 minutes at 4°C. The interface cells were removed, washed twice with 50 mL of sterile phosphate-buffered saline at 1,200 g for 10 minutes, pelleted, and stored at -80°C until used. Total RNA isolation was performed by using Trizol LS reagent (Gibco, Life Sciences, BRL, Grand Island, NY), according to the manufacturer's instructions. All RNA preparations and handling steps took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in diethylpyrocarbonate-treated water and stored at -80°C until used. RNA concentration was determined by absorbance reading at 260 nm with the Hitachi UV-VIS (U-2000) spectrophotometer (Tokyo, Japan). RNA integrity was tested by PCR amplification of the  $\beta$ -actin housekeeping gene. As positive and negative controls, RNA samples were prepared from the MCF-7 and ARH-77 breast cancer and leukemic cell lines, respectively.

Reverse transcription of RNA was carried out using the ThermoScript RT-PCR system (Invitrogen, Paisley, United Kingdom). cDNA was synthesized according to the manufacturer's instructions. The real-time RT-PCR assay for the detection of CK-19 mRNA-positive cells has already been described.<sup>18</sup> In brief, 2  $\mu$ L of cDNA were placed into a 18  $\mu$ L reaction volume containing 1  $\mu$ L of the sense primer CK-19 for (3 mmol/L), 1  $\mu$ L of the antisense primer CK-19-do (3 mmol/L), 2.4  $\mu$ L of the LightCycler Fast Start DNA Master Hybridization Probes reagent (10 $\times$  concentration), 1  $\mu$ L of the probe CK-19-FL (3 mmol/L), 1  $\mu$ L of the probe CK-19-LC (3 mmol/L). The primers used have been previously described.<sup>18</sup> PCR reaction was initiated with a 10-minute denaturation at 95°C and terminated with a 30-second cooling step at 40°C. The cycling protocol consisted of a denaturation step at 95°C, annealing at 60°C for 10 seconds, and extension at 72°C and repeated 50 times. Fluorescence detection was performed at the end of each annealing step for less than 1 second. Real-time PCR for the housekeeping gene *GAPDH* was performed in all of the clinical samples to evaluate the quality of the cDNAs used in the study. The lower detection limit of the assay was set at more than 0.6 MCF-7 cell equivalents/5  $\mu$ g RNA.<sup>18</sup> The within-run curve values (CVs) for MCF-7 cells as determined by the calibration curve ranged from 7.5% to 9.3%, while the corresponding crossing point (Cp) values from 0.9% to 1.5%; similarly the between-run CVs ranged from 10.7% to 16.0%, whereas the corresponding Cp values ranged from 2.2% to 3.2%.<sup>23</sup> It has been previously shown that this method was highly specific because only two (2.2%) out of 89 blood samples obtained from female blood donors were found to be positive.<sup>18</sup>

### Statistical Analysis

The main tools of analysis were logistic regression<sup>19,20</sup> and the Cox proportional hazards model<sup>21</sup> for outcomes related to point events and time variables, respectively. To select those factors with an independent significant influence on outcomes, both analyses were carried out in a stepwise (unconditional backward) fashion.<sup>20,21</sup> Before the application of these methods, univariate analyses were performed for a preliminary exploration of marked associations. Univariate analyses included contingency tables, *t* or Mann-Whitney U tests, log-rank tests, and simple Cox regression analyses.<sup>21-24</sup>

## RESULTS

**Patient Characteristics and Detection of CTCs**

Patient characteristics are shown in Table 1. The patients' median age was 49 years (range, 30 to 80 years), 58.1% were premenopausal or perimenopausal (within 12 months from the last menstrual cycle), and 77.8% had a lumpectomy; the primary tumor size was 2.0 cm or smaller in 37.6% of the patients, and 57.2% of the patients had estrogen receptor–negative tumors. A median number of 14 (range, five to 42) axillary level I and II lymph nodes were removed. Immunostaining of lymph node sections with the anti-CK-19 monoclonal antibody could not reveal positive cells, confirming that the study population was indeed N0. All patients received adjuvant chemotherapy (FEC, n = 75; T/EC, n = 51; CMF, n = 39; other miscellaneous regimens,

n = 2). Peripheral blood CK-19 mRNA-positive CTCs could be detected by real-time PCR in 36 patients (21.6%) before the initiation of any systemic adjuvant treatment. The median number of CK-19 mRNA-positive cells was 3.4 MCF-7 cell equivalents/5  $\mu$ g RNA (range, 0.6 to 1,105 equivalent). Detection of CK-19 mRNA-positive CTCs was significantly associated with HER2/*neu*-positivity (score 2+/3+;  $P = .033$ ) but not with other patients' or tumors' clinicopathologic characteristics (Table 1).

**Detection of CK-19 mRNA-Positive CTCs and Clinical Outcome**

**Disease recurrence.** The median follow-up period was 32 months (range, 3 to 88 months). Twenty patients (12%) had a median follow-up time of 12 months or less. During the follow-up period, 20

**Table 1.** Patient Characteristics

	All Patients		CK-19 mRNA Positive		CK-19 mRNA Negative		P
	No	%	No	%	No	%	
Patients enrolled	167		36	21.6	131	78.4	—
Age, years							.333
Median	49		51.5		48.0		
Range	30-80		36-77		30-80		
Menopausal status							.46
Premenopausal	97	58.1	19	52.8	78	59.5	
Postmenopausal	70	41.9	17	47.2	53	40.5	
Surgery							.643
Lumpectomy	130	77.8	27	75.0	103	78.6	
Mastectomy	37	22.2	9	25.0	28	21.4	
HER2							.033
Negative (0/1+)	135	80.8	22	61.1	101	77.1	
Positive (2+/3+)	26	15.6	13	36.1	25	19.1	
Unknown	6	3.6	1	2.8	5	3.8	
Tumor size, cm							.675
0.0-1.9	64	38.3	11	30.5	53	40.5	
2.0-3.9	84	50.3	20	55.6	64	48.9	
$\geq 4.0$	19	11.4	5	13.8	14	10.6	
Histology grade							.206
I/II	81	48.5	14	38.9	67	51.1	
III	70	41.9	18	50.0	52	39.7	
Unknown	16	9.6	4	11.1	12	9.2	
ER							.094
Negative	95	56.9	25	69.4	70	53.4	
Positive	71	42.5	11	30.6	60	45.8	
Unknown	1	0.6	—	—	1	0.8	
PR							.521
Negative	102	61.1	20	55.6	82	62.6	
Positive	63	37.7	15	41.7	48	36.6	
Unknown	2	1.2	1	2.7	1	0.8	
Adjuvant chemotherapy							.685
FEC	75	44.9	16	44.4	59	45.0	
T/EC	51	30.5	13	36.1	38	29.0	
CMF	39	23.4	7	19.5	32	24.5	
Other	2	1.2	—	—	2	1.5	
Recurrences							.197
None	147	88.2	20	55.6	127	97	
Distant	15	8.9	11	30.5	4	3.0	
Local	5	2.9	5	13.9	0	0.0	

Abbreviations: CK-19, cytokeratin-19; ER, estrogen receptor; PR, progesterone receptor; FEC, fluorouracil 700 mg/m<sup>2</sup> day 1 + epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for six cycles; T/EC, epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for four cycles followed by docetaxel 100 mg/m<sup>2</sup> day 1 every 3 weeks for four additional cycles; CMF, cyclophosphamide, methotrexate, fluorouracil.

patients (12%) presented a distant (n = 15; 75%) or/and locoregional (n = 5; 25%) recurrence. Clinical recurrence was significantly more frequent in patients with (44.4%) than without (3%) detectable CK-19 mRNA-positive CTCs ( $P < .000001$ ; Table 2). The median number of CK-19 mRNA-positive CTCs was 0.8 MCF-7 cell equivalents/5  $\mu$ g RNA (range, 0 to 14) and 0.0 MCF-7 cell equivalents/5  $\mu$ g RNA (range, 0 to 1,105 equivalent) in relapsed and nonrelapsed patients, respectively (Mann-Whitney test,  $P < .00001$ ). There was no significant correlation between the site of relapse (distant v locoregional) and the detection of CK-19 mRNA-positive CTCs ( $P > .1$ ; Table 1). The median disease-free interval (DFI) for patients with detectable CK-19 mRNA-positive CTCs was 55 months (range, 4 to 74 months), whereas DFI has not yet been reached for patients without CK-19 mRNA-positive CTCs (log-rank  $P < .00005$ ; Fig 1). There was no correlation between the DFI and the number of CK-19 mRNA-positive CTCs (nonparametric Spearman's analysis,  $P = .349$ ). Patients with estrogen receptor-negative tumors had a significantly lower DFI compared with patients with estrogen receptor-positive tumors (mean DFI,  $66.5 \pm 3.5$  months [range, 3 to 83 months] and  $82.7 \pm 2.5$  months [range, 4 to 88 months], respectively; log-rank  $P < .007$ ). There was no association of DFI with other clinicopathologic parameters such as histopathologic grade (Scarff, Bloom and Richardson [SBR] classification), tumor size, *HER2* overexpression, or administration of various chemotherapy regimens.

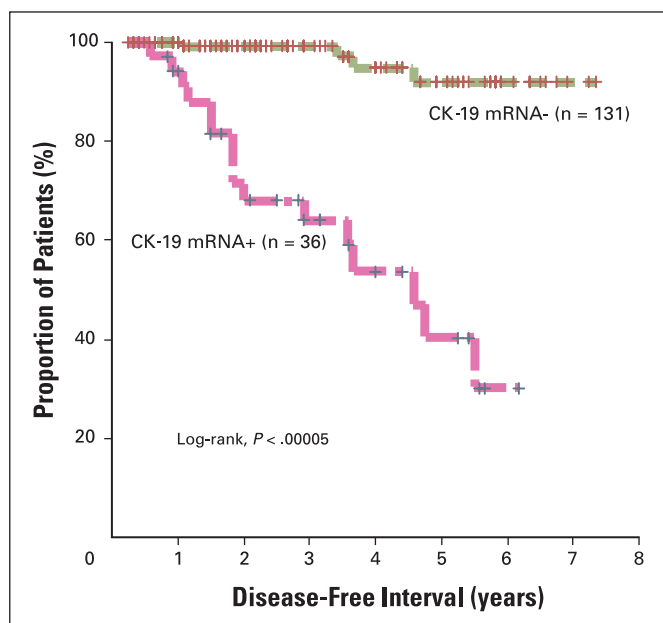
**Survival.** During the follow-up period, eight patients (4.8%) died as a result of disease progression. Seven (87.5%) of these patients had detectable CK-19 mRNA-positive CTCs compared with only one death observed in the group of patients without detectable CK-19 mRNA-positive CTCs (Pearson's  $\chi^2$  test,  $P < .00005$ ; Table 2). The median overall survival has not yet been reached (mean,  $83.2 \pm 1.6$  months); however, patients with detectable CTCs had a significantly shorter overall survival compared with patients with undetectable CTCs (log-rank  $P = .00005$ ; Fig 2) (mean,  $64.6 \pm 4.1$  months [range, 4 to 77 months] and  $87.3 \pm 0.7$  months [range, 3 to 88 months], respectively). Median overall survival was not associated with established clinicopathologic parameters (tumor size, histopathologic grade, estrogen receptor expression, *HER2* overexpression or administration of various chemotherapy regimens).

**Univariate and Multivariate Analysis**

Peripheral blood CK-19 mRNA cell status, menopausal status, tumor size, histopathologic grade, estrogen and progesterone receptor status, *HER2* status and chemotherapy regimen were all tested in univariate analysis for association with DFI (Table 3). Detection of CK-19 mRNA-positive CTCs was significantly associated with reduced DFI (hazard ratio [HR], 15.174; 95% CI, 3.068 to 45.431;  $P < .00005$ ); in addition, estrogen receptor-negative tumors and the

Group	Clinical Relapses			Deaths		
	No.	%	<i>P</i>	No.	%	<i>P</i>
CK-19 mRNA positive (n = 36)	16	44.4	.000001	7	19.4	.00005
CK-19 mRNA negative (n = 131)	4	3.0		1	0.8	

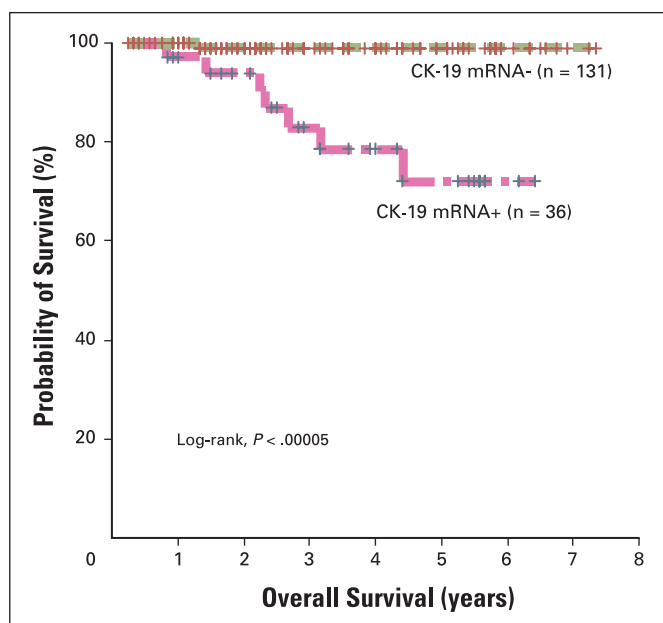
Abbreviations: CK-19, cytokeratin-19.



**Fig 1.** Disease-free interval in cytokeratin-19 (CK-19) mRNA-positive and CK-19 mRNA-negative patients.

administration of CMF compared with T/EC were significantly associated with reduced DFI (HR, 4.033; 95% CI, 1.342 to 12.120;  $P = .0070$ ; and HR, 3.638, 95% CI, 0.999 to 13.246;  $P = .0434$ , respectively).

Peripheral blood CK-19 mRNA cell status, menopausal status, tumor size, histopathologic grade, estrogen and progesterone receptor status, *HER-2* status, and chemotherapy regimen were also tested in univariate analysis for overall survival (Table 4). Only the detection of CK-19 mRNA-positive CTCs (HR, 20.943; 95% CI, 2.575 to 17.035;  $P < .00005$ ) and the administration of CMF compared with T/EC



**Fig 2.** Overall survival of cytokeratin-19 (CK-19) mRNA-positive and CK-19 mRNA-negative patients.

**Table 3.** Univariate Analysis (unadjusted relative risks) for Disease-Free Interval of Patients With Early Breast Cancer

Parameter	Hazard Ratio	95% CI	P
Age ( $\leq 50$ v $> 50$ years)	1.010	0.970 to 1.052	.05
Menopausal status (pre v post)	2.342	0.932 to 5.886	.0612
CK-19 mRNA (pos v neg)	15.174	3.068 to 45.431	< .00005
Tumor size ( $> 2.1$ v $\leq 2.0$ cm)	1.605	1.176 to 2.190	.042
Histologic grade (III v I/II)	1.536	0.636 to 3.709	.3336
ER (neg v pos)	4.033	1.342 to 12.120	.0070
PR (neg v pos)	1.226	0.465 to 3.231	.6785
HER2 (post v neg)	1.377	0.523 to 3.628	.5138
CMF v FEC	1.311	0.404 to 4.260	.6589
FEC v T/EC	1.311	0.404 to 4.260	.659

Abbreviations: ER, estrogen receptor; neg, negative; pos, positive; PR, progesterone receptor; CK-19, cytokeratin-19; CMF, cyclophosphamide, methotrexate, fluorouracil; FEC, fluorouracil 700 mg/m<sup>2</sup> day 1 + epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for six cycles; T/EC, epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for four cycles followed by docetaxel 100 mg/m<sup>2</sup> day 1 every 3 weeks for four additional cycles.

(HR, 8.065; 95% CI, 0.854 to 76.131;  $P = .0079$ ) were associated with a decreased overall survival.

Subsequently, multivariate analysis was performed for DFI and overall survival (Table 5). Detection of CK-19 mRNA-positive CTCs, as well as estrogen receptor–negative tumors histopathologic grade 3, premenopausal status and the administration of CMF were all found to be independent predictive factors for early relapse. Similarly, Cox regression analysis revealed that detection of CK-19 mRNA-positive CTCs remained the only independent prognostic factor for overall survival (Table 5).

## DISCUSSION

The TNM system is incapable of identifying a subgroup of women who, although they have an early-stage breast cancer, may be at high

**Table 4.** Univariate Analysis (unadjusted relative risks) for Overall Survival of Patients With Early Breast Cancer

Parameter	Hazard Ratio	95% CI	P
Age ( $\leq 50$ v $> 50$ years)	1.019	0.956 to 1.087	.05
Menopausal status (pre v post)	2.038	0.486 to 8.556	.3206
Tumor size ( $> 2.1$ v $\leq 2.0$ cm)	1.439	0.907 to 2.281	.1134
Histopathologic grade (III v I/II)	3.964	0.800 to 19.645	.0686
ER (neg v pos)	2.755	0.556 to 13.858	.1945
PR (neg v pos)	1.539	0.299 to 7.939	.6034
HER2 (pos v neg)	1.269	0.246 to 6.545	.7757
CK-19 mRNA (pos v neg)	20.943	2.575 to 17.035	< .00005
CMF v FEC	1.828	0.190 to 17.591	.6037
FEC v T/EC	1.429	0.453 to 7.243	.2856

Abbreviations: ER, estrogen receptor; neg, negative; pos, positive; PR, progesterone receptor; CK-19, cytokeratin-19; CMF, cyclophosphamide, methotrexate, fluorouracil; FEC, fluorouracil 700 mg/m<sup>2</sup> day 1 + epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for six cycles; T/EC, epirubicin 75 mg/m<sup>2</sup> day 1 + cyclophosphamide 700 mg/m<sup>2</sup> day 1 every 3 weeks for four cycles followed by docetaxel 100 mg/m<sup>2</sup> day 1 every 3 weeks for four additional cycles.

**Table 5.** Independent Predictive and Prognostic Factors by Multivariate Analysis for DFS and OS of patients with early breast cancer

Parameter	Hazard Ratio	95% CI	P
DFS			
CK-19 (positive)	26.319	6.425 to 107.806	< .00001
ER (negative)	5.541	1.353 to 22.692	.017
Histopathologic grade (III)	3.795	1.037 to 13.878	.044
Premenopausal status	4.644	1.280 to 16.845	.019
CMF regimen	8.175	1.242 to 53.795	.029
OS			
CK-19 (positive)	17.936	2.157 to 149.157	.008

Abbreviations: DFS, disease-free survival; OS, overall survival; CK-19, cytokeratin-19; ER, estrogen receptor; CMF, cyclophosphamide, methotrexate, fluorouracil.

risk of relapse and death.<sup>24</sup> This is especially true for patients with small and N0 tumors; therefore, it is important to develop new predictive and prognostic markers. The results presented in the current study demonstrate, for the first time, that the detection of CTCs in the peripheral blood of patients with N0 breast cancer, using a sensitive and specific real-time PCR assay, was associated with a high incidence of systemic relapse and death resulting from breast cancer; moreover, the detection of CTCs was revealed in the multivariate analysis to be an independent predictive and prognostic factor for early relapse and disease-specific death, respectively. However, the wide range of 95% CIs in the multivariate analysis of CK-19 mRNA-positive cells for overall survival, which is due to the small number of events, requires further confirmation and should be interpreted with caution.

These findings are in agreement with previous studies supporting the prognostic value of occult micrometastatic cells in patients with early-stage (N0 and N1) breast cancer.<sup>10-14,25,26</sup> However, in most of these studies, occult tumor cells were identified in the bone marrow aspirates using an immunohistochemical assay and their clinical relevance was determined in both N0 and node-positive (N1) tumors. Braun et al<sup>11</sup> reported that the incidence of both clinical recurrence and disease-related deaths in N0 patients who did not receive systemic adjuvant therapy was higher in the group of patients with detectable DTCs compared with those without detectable DTCs. It is interesting to note that patients with N0 tumors and detectable DTCs had a practically similar median overall survival with that of patients with N1 tumors who did not have detectable DTCs.<sup>11</sup> A recent meta-analysis study further confirmed the above prognostic value of detectable CK-19-positive DTCs; indeed, in 1,036 patients with small (T1) and N0 tumors, both the DFI and the overall survival were significantly worse in patients with detectable DTCs compared with those without detectable DTCs.<sup>14</sup> Conversely, Gebauer et al,<sup>26</sup> evaluating 194 patients with N0 tumors, could not demonstrate any difference in the incidence of distant recurrences and the rate of cancer-related deaths between the patients with or without detectable DTCs. In addition, Wiedswang et al<sup>13</sup> reported that among the N0 patients, systemic relapse as well as overall survival did not differ whether or not DTCs were detected; these findings were in overt contrast to those observed in the group of patients with N1 tumors. The reasons for the discrepancies concerning the prognostic and predictive value of DTCs in N0 breast cancer patients are not obvious; we cannot exclude that methodologic reasons that may influence the detection limits of the used assay may account for this phenomenon.

Some of these problems may be eliminated by the development of standardized assays that are sensitive enough to detect a low load of occult tumor cells, thus facilitating the evaluation of the clinical significance of occult tumor cells in N0 breast cancer patients. The high sensitivity and reproducibility of the used real-time PCR assay (0.6 MCF-7 cell equivalents/5  $\mu$ g RNA) could perhaps explain the highly significant association observed between the detection of CTCs and the risk for clinical recurrence and disease-related death in the present study. Wiedswang et al<sup>13</sup> used a negatively-selected bone marrow cell population in order to increase the sensitivity of his DTCs detection assay; however, their methodology could not reveal significant differences in patients with N0 tumors. Nevertheless, the superiority of the real-time PCR assay over other assays for the detection of DTCs or/and CTCs should be demonstrated in a prospective comparative study.

Most of the patients enrolled in the studies evaluating the clinical relevance of DTCs mentioned herein<sup>11,13,26</sup> had not received adjuvant chemotherapy. Conversely, patients included in the current study received adjuvant chemotherapy and those with estrogen receptor- or/and progesterone receptor-positive tumors also received tamoxifen. It is interesting to note that the association of the increased risk of relapse as well as of disease-related death and the detection of CTCs before the initiation of any adjuvant treatment was independent of the administered chemotherapy regimen. This observation further supports the hypothesis that detection of either DTCs or CTCs represented a specific biologic characteristic of the tumor, which could be independent of the possibility of some chemotherapy regimens to eliminate bone marrow<sup>27</sup> or peripheral blood<sup>28</sup> occult tumor cells.

However, the administration of CMF was also an independent predictive factor for early relapse when it was compared with the administration of the more intense T/EC regimen. It could be argued that T/EC eliminates CTCs in a significantly higher proportion of patients compared with CMF, thus resulting in a lower incidence of clinical recurrences. These findings should be interpreted with caution until their confirmation with a prospective and well designed study of longitudinal detection of CTCs during the different phases of systemic adjuvant treatment.

Our findings were obtained using RNA extracted from peripheral blood mononuclear cells; therefore, blood could be a valuable source for monitoring CTCs. This opens the way to further investigate important questions such as whether the detection of CTCs should be performed in all patients at the time of primary diagnosis to identify high-risk patients or whether CTCs detection at diagnosis should modify the adjuvant therapeutic strategy, or, finally, whether the detection of CTCs during the administration of adjuvant treatment would allow the development of secondary adjuvant therapeutic strategies. Indeed, several studies have shown the expression of various markers on occult tumor cells, such as uPA/uPAR complex, c-erbB2/*neu*, and p53, which could serve as targets for tailored treatments.<sup>25-31</sup> We have recently demonstrated that trastuzumab, a humanized monoclonal antibody against the HER2/*neu* molecule, could eliminate chemotherapy-resistant CTCs and DTCs in 20 of 30 patients with breast cancer.<sup>32</sup> Therefore, the study and evaluation of the clinical relevance of CTCs opens the possibility of early and tailored treatment interventions for patients with persisting occult tumor cells after systemic adjuvant treatment.

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## Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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## Glossary

**CK-19 (cytokeratin-19):** CK-19 belongs to the intermediate filaments, which create a cytoskeleton in almost all cells. CK-19 is normally not expressed in the hematopoietic cells, although it is commonly expressed in epithelial cells such as mammary cells, either normal or neoplastic.

**CT scan (computed tomography scan):** A series of pictures created by a computer linked to an x-ray machine taken of the inside of the body from different angles.

**CTC (circulating tumor cell):** Demonstration of isolated tumor cell circulation/ dissemination in the peripheral blood.

**DTC (disseminated tumor cell):** Demonstration of isolated tumor cells disseminated in the bone marrow.

**MRI (magnetic resonance imaging):** A procedure in which radio waves and a powerful magnet linked to a computer are used to create detailed pictures of areas inside the body. These pictures can show the difference between normal and diseased tissue.

**PBMC (peripheral blood mononuclear cell):** A single-nucleus cell found circulating in the bloodstream (normally includes lymphocytes and monocytes).

**RT-PCR (reverse-transcriptase polymerase chain reaction):** PCR is a method that allows logarithmic amplification of short DNA sequences within a longer, double-stranded DNA molecule. Gene expression can be measured after extraction of total RNA and preparation of cDNA by a reverse-transcription step. Thus, RT-PCR enables the detection of PCR products on a real-time basis, making it a sensitive technique for quantitating changes in gene expression.