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Cytokeratin-19 mRNA-Positive Circulating Tumor Cells After Adjuvant Chemotherapy in Patients With Early Breast Cancer

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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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ABSTRACT

Purpose
To evaluate the prognostic significance of cytokeratin-19 (CK-19) mRNA-positive circulating tumor cells (CTCs) in peripheral blood of women with early-stage breast cancer after the completion of adjuvant chemotherapy.

Patients and Methods
Blood was obtained from 437 patients with early breast cancer before the start and after the completion of adjuvant chemotherapy, and the presence of CK-19 mRNA-positive CTCs was assessed by real-time reverse transcriptase polymerase chain reaction. Interaction with known prognostic factors and association of CTCs with clinical outcome were investigated.

Results
CK-19 mRNA-positive CTCs were detected before chemotherapy in 179 patients (41.0%). After adjuvant chemotherapy, a significant change in CK-19 status was observed, as status for 51% of patients with initially CK-19 mRNA-positive disease turned negative, and status for 22% of patients with initially CK-19 mRNA-negative disease became positive (McNemar test $P = .004$). The detection of CK-19 mRNA-positive CTCs postchemotherapy was associated with involvement of more than three axillary lymph nodes ($P = .026$). Clinical relapses and disease-related deaths were significantly increased in patients with detectable postchemotherapy CK-19 mRNA-positive CTCs (both $P < .001$, respectively). Disease-free and overall survival were significantly reduced in patients with detectable CK-19 mRNA-positive CTCs postchemotherapy ($P < .001$ and $P = .001$, respectively). In multivariate analysis, the detection of CK-19 mRNA-positive CTCs before and after adjuvant chemotherapy was an independent factor associated with reduced disease-free survival ($P < .001$) and overall survival ($P = .003$).

Conclusion
The detection of CK-19 mRNA-positive CTCs in the blood after adjuvant chemotherapy is an independent risk factor indicating the presence of chemotherapy-resistant residual disease.

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INTRODUCTION

Disseminated tumor cells (DTCs) in bone marrow[1,2] and circulating tumor cells (CTCs) in peripheral blood[3,4] of patients with early-stage breast cancer have been shown to be independent adverse prognostic factors for early disease recurrence and disease-related death. In 1869, Ashworth reported a patient with cancer in whom cells similar to those present in the tumor were found in the blood postmortem,[5] thus representing the first description of CTCs.

Immunocytochemistry using antibodies against proteins that are expressed on epithelial but not on mesenchymal cells is widely used for the detection of DTCs and CTCs; however, the detection of mRNA transcripts for such epithelial markers by using reverse transcriptase polymerase chain reaction (RT-PCR) and, more recently, the quantitative real-time RT-PCR seems to have higher diagnostic sensitivity.[6] The major advantage of RNA-based approaches is related to the rapid degradation of RNA released from cells in the blood by blood RNAses; therefore, the origin of detectable RNA transcripts is considered to be viable cells. Cytokeratin-19 (CK-19), a cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the

detection of breast cancer cells in mesenchymal tissues and seems to be the most sensitive and reliable tumor marker in both patients with early-stage and metastatic breast cancer.[7,8]

Recent studies have shown the prognostic significance of CK-19 mRNA-positive CTCs in patients with early-stage breast cancer.[3,4] However, most of these studies have investigated the prognostic value of CTCs/DTCs at the time of primary diagnosis, and only a few reports exist concerning their clinical relevance after the completion of adjuvant therapy.[9-11] Because DTCs and CTCs are the targets of adjuvant treatment, their fate after systemic therapy could be a potential marker permitting a direct and individualized assessment of treatment efficacy. Indeed, some small studies have shown that the detection of isolated tumor cells in bone marrow[11,12] and in peripheral blood[9] after the completion of adjuvant chemotherapy is associated with an unfavorable clinical outcome.

In the present study, we sought to evaluate the clinical relevance of CK-19 mRNA-positive CTCs after completion of adjuvant chemotherapy in patients with early-stage breast cancer, using a quantitative real-time RT-PCR assay.

PATIENTS AND METHODS

Patients and Clinical Samples

From 1997 until 2004, a total of 437 consecutive patients who had received adjuvant chemotherapy for stage I to III breast cancer and had sufficient follow-up (at least 10 months) were included in this study. All patients belong to the same cohort of 444 patients for whom the clinical relevance of the detection of CK-19 mRNA-positive CTCs before the initiation of any systemic treatment has been recently reported.[13] All patients had a complete diagnostic evaluation to exclude the presence of distant metastases, consisting of chest x-rays, ultrasound of the liver, and a whole-body bone scan. Computed tomography scans and/or magnetic resonance imaging studies were performed if clinically indicated. All patients included in this study received adjuvant chemotherapy, and most were treated in the context of research protocols of the Hellenic Oncology Research Group; the chemotherapy regimens used in this cohort of patients have been previously reported in detail.[13] After completion of adjuvant chemotherapy, 358 patients received adjuvant radiotherapy according to their individual characteristics. All patients with estrogen receptor (ER) –positive and/or progesterone receptor (PR) –positive tumors received tamoxifen 20 mg daily for 5 years or tamoxifen for 2 to 3 years followed by aromatase inhibitors for an additional 2 to 3 years; premenopausal women also received luteinizing hormone-releasing hormone analogs for 2 years. There were no subgroups of patients who received hormone therapy only or no systemic therapy at all. Patients with HER-2/*neu*–positive tumors did not receive adjuvant trastuzumab, because all patients were enrolled before the positive results from the adjuvant trastuzumab trials were reported.[14,15] Patients' follow-up consisted of clinical examination with laboratory and imaging studies every 3 months for the first 2 years, every 6 months for the next 3 years, and yearly thereafter. The median follow-up period was 53.5 months (range, 10 to 106 months). All patients signed an informed consent to participate in the study, which was approved by the ethics and scientific committees of our institution.

Blood Samples and Real-Time RT-PCR Assay for CK-19 mRNA-Positive Cells

Peripheral blood (20 mL in EDTA) was obtained from every patient 3 to 4 weeks after primary surgery and before the initiation of adjuvant chemotherapy and within 3 to 4 weeks after the completion of adjuvant chemotherapy. To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood was discarded. The procedures of RNA extraction and cDNA synthesis as well as the real-time RT-PCR assay for CK-19 mRNA-positive CTCs and the primers used have already been described.[16] According to the analytic detection limit of our assay, the presence of $\geq$ 0.6 MCF-7 equivalents/5 μg of total RNA was considered a positive result. Using the above cutoff, only two of 89 female healthy donors were positive (2.2%). Furthermore, none of nine women with benign (fibroadenomas) breast disease had positive blood samples.[16]

Statistical Analysis

The time from study entry until the day of the first evidence of disease recurrence, either locoregional or distant (disease-free survival [DFS]), and the time from study entry to death (overall survival [OS]) were the main dependent variables of the study. DFS and OS Kaplan-Meier curves for subgroups of patients were compared using the log-rank test to provide a univariate assessment of the prognostic value of selected clinical risk factors. Clinicopathologic factors known to be associated with prognosis, such as menopausal status (premenopausal *v* postmenopausal), tumor size (T2-3 *v* T1), nodal infiltration (yes *v* no), histology grade (3 *v* 1 or 2), ER status (negative *v* positive), PR status (negative *v* positive), HER-2/*neu* status (positive *v* negative), and additionally, the detection of CK-19 mRNA-positive CTCs (yes *v* no), were tested in univariate analysis. Variables that were found to be significant at the univariate screen were then entered in a stepwise multivariate Cox proportional hazards regression model to identify those with independent prognostic information. Entry into and removal from the model were set at 5% and 10%, respectively. All statistical tests were performed at the 5% level of significance. SPSS version 13 (SPSS Inc, Chicago, IL) statistical software was used for the analysis. This report is written according to the reporting recommendations (reporting recommendations for tumor marker prognostic studies [REMARK] criteria) for tumor marker prognostic studies.[17]

RESULTS

Patient Characteristics

Median patient age was 54.0 years, and 189 patients (43.2%) were premenopausal (Table 1). The primary tumor was $\leq$ 2.0 cm in 154 patients (35.2%), whereas 188 patients (43.0%) had histologic grade 3 tumors, and 277 patients (63.4%) had one or more involved lymph nodes. Two-hundred fifty-six patients (58.6%) had ER-positive tumors, 200 patients (45.8%) had PR-positive tumors, and 87 patients (19.9%) had HER-2/*neu*–positive tumors (immunohistochemical score of 3+ or fluorescence in situ hybridization positive). CK-19 mRNA-positive CTCs were detected in 179 patients (41.0%) before the initiation of adjuvant chemotherapy. The median number of CK-19 mRNA-positive cells before adjuvant chemotherapy was 0.3 MCF-7 cell equivalents/5 μg RNA (range, 0.0 to 1,115 MCF-7 cell equivalents/5 μg RNA).

Detection of CK-19 mRNA-Positive Cells After Chemotherapy

Circulating CK-19 mRNA-positive cells could be detected in 143 patients (32.7%) after the completion of adjuvant chemotherapy (Table 1). There was no association between the detection of CK-19 mRNA-positive CTCs and the various patient- or tumor-related clinicopathologic characteristics, except for the case of four or more involved axillary lymph nodes compared with the group of patients with three or fewer involved axillary lymph nodes (Table 1; $P = .026$). Of the 437 patients, 271 patients (62%) had 0.0 MCF-7 cell equivalents/5 μg RNA detected after chemotherapy, and therefore the median number of CK-19 mRNA-positive cells in the entire cohort was 0.0 MCF-7 cell equivalents/5 μg RNA (range, 0 to 1,000 MCF-7 cell equivalents/5 μg RNA); this was significantly lower than the median number of circulating CK-19 mRNA-positive cells detected before the initiation of adjuvant chemotherapy ($P = .003$). Fifty-six patients (21.7%) who

Table 1. Patient Characteristics and Detection of CK-19 mRNA-Positive Cells After Chemotherapy

Characteristic	All Patients		CK-19 Negative		CK-19 Positive		*P*
	No.	%	No.	%	No.	%	
Patients enrolled	437	100	294	67.3	143	32.7	
Age, years							
Median	54.0		55.0		54.0		
Range	26-78		30-78		26-76		
Menopausal status							.538
Premenopausal	189	43.2	124	42.2	65	34.4	
Postmenopausal	248	56.8	170	57.89	78	31.5	
Tumor size, cm							.277
T1 (≤ 2.0)	154	35.2	111	72.1	43	27.9	
T2 (2.1-5.0)	247	56.5	159	64.4	88	35.6	
T3 (> 5.0)	36	8.3	24	66.7	12	33.3	
Histology grade							.102
1/2	200	45.8	145	72.5	55	27.5	
3	188	43.0	118	62.8	70	37.2	
Lobular	49	11.2	31	63.3	18	36.7	
Lymph nodes							.061
N0	160	36.6	111	69.4	49	30.6	.526*
N1-N3	120	27.5	88	73.3	32	26.7	.026†
≥ N4	157	35.9	95	60.5	62	39.5	
ER							.463
Negative	172	39.4	112	65.1	60	34.9	
Positive	256	58.6	176	68.8	80	31.3	
Unknown	9	2.0	6	66.7	3	33.3	
PR							.063
Negative	228	52.2	144	63.2	84	36.8	
Positive	200	45.8	144	72.0	56	28.0	
Unknown	9	2.0	6	66.7	3	33.3	
HER-2/*neu*							.199
Negative (0/1+/2+)	337	77.1	234	69.4	103	30.6	
Positive (3+)	87	19.9	54	62.1	33	37.9	
Unknown	13	3.0	6	46.2	7	53.8	
Surgery							.437
Lumpectomy	305	69.8	209	68.5	96	31.5	
Mastectomy	132	30.2	85	64.4	47	35.6	
Chemotherapy							.644
CMF	42	9.6	29	69.0	13	31.0	
FEC	206	47.1	134	65.0	72	35.0	
T/EC	189	43.2	131	69.3	58	30.7	

Abbreviations: CK-19, cytokeratin-19; ER, estrogen receptor; PR, progesterone receptor; CMF, cyclophosphamide, methotrexate, fluorouracil; FEC, fluorouracil, epirubicin, and cyclophosphamide; T/EC, docetaxel followed by epirubicin/cyclophosphamide.
*Node-negative versus node-positive patients.
†Patients with 0 to 3 positive axillary nodes versus patients with four or more positive axillary nodes.

tested negative for CK-19 mRNA-positive cells before chemotherapy tested positive afterward, whereas 92 patients (51.4%) who initially tested positive for CK-19mRNA-positive cells tested negative after completion of adjuvant chemotherapy (Table 2; McNemar test $P = .004$).

Detection of CK-19 mRNA-Positive Cells and Clinical Outcome

Disease recurrence. After a median follow-up period of 53.5 months (range, 10 to 106 months), 95 patients (21.7%) developed a distant (n = 76; 80%) and/or a locoregional (n = 19; 20%) recurrence. Clinical recurrence was significantly more frequent in patients with CK-19 mRNA-positive CTCs postchemotherapy (n = 46; 32.2%) than in patients without (n = 49; 16.7%; Fisher's exact test, $P < .001$; Table 3). The median number of CK-19 mRNA-positive cells after chemotherapy was significantly higher in patients experiencing relapse than in patients who did not experience relapse patients (median, 0.3 MCF-7 cell equivalents/5 μg RNA [range, 0 to 25.6 MCF-7 cell equivalents/5 μg RNA]; and median, 0.0 MCF-7 cell equivalents/5 μg RNA [range, 0 to 1,000 MCF-7 cell equivalents/5 μg RNA], respectively; Mann-Whitney *U* test: $P = .002$).

According to the detection of circulating CK-19 mRNA-positive cells before and after chemotherapy, four groups of patients could be identified: prechemotherapy-negative/postchemotherapy-negative, prechemotherapy-negative/postchemotherapy-positive, prechemotherapy-positive/postchemotherapy-negative, and prechemotherapy-positive/postchemotherapy-positive (Table 3). The incidence of clinical recurrences was 13.9% in patients without CK-19m RNA-positive cells both before and after chemotherapy versus 39.1% in patients with CK-19m RNA-positive cells at both time

Table 2. Effect of Chemotherapy on the Detection of CK-19 mRNA-Positive Circulating Tumor Cells

CK-19 mRNA Status Before Chemotherapy	CK-19 mRNA Status After Chemotherapy			
	Positive		Negative	
	No.	%	No.	%
Positive, n = 179	87	48.6	92	51.4
Negative, n = 258	56	21.7	202	78.3

Abbreviation: CK-19, cytokeratin-19.

points (Fisher's exact test, $P < .001$; Table 3). Table 4 presents the effect of adjuvant chemotherapy on the median number of CK-19 mRNA-positive CTCs for the different groups of patients.

The 3-year and 5-year DFS rates were 78% versus 92% and 68% versus 84% for CK-19m RNA-positive versus -negative patients postchemotherapy, respectively. As shown in Figure 1A, patients with CK-19 mRNA-positive CTCs after the completion of adjuvant chemotherapy had a significantly shorter DFS than that of patients without detectable CTCs ($P = .0004$). Moreover, there was a progressive decrease in the DFS of the four groups of patients according to the detection of CK-19 mRNA-positive CTCs before and after the completion of adjuvant chemotherapy (Fig 1B).

Survival. During the follow-up period, 42 patients (9.6%) died as a result of disease progression. Twenty-four (16.8%) and 18 (6.1%) of these deaths occurred in 143 patients with and 294 patients without detectable CK-19 mRNA-positive CTCs after the completion of adjuvant chemotherapy, respectively (Fisher's exact test; $P < .001$; Table 3). In addition, the incidence of deaths was significantly higher in patients with CK-19 mRNA-positive CTCs both before and after the completion of adjuvant chemotherapy than in those without detectable CTCs at the same time periods (19.5% and 4.0%, respectively; Fisher's exact test, $P < .001$; Table 3). The median number of CK-19 mRNA-positive CTCs after adjuvant chemotherapy in patients who died was significantly higher than that of patients who were alive: median, 0.65 MCF-7 cell equivalents/5 μg RNA (range, 0 to 13 MCF-7 cell equivalents/5 μg RNA) and median, 0.0 (range, 0 to 1,000 MCF-7 cell equivalents/5 μg RNA), respectively (Mann-Whitney U test $P = .004$).

The 3-year and 5-year OS rates were 91% versus 97% and 82% versus 93% for CK-19m RNA-positive versus -negative patients postchemotherapy, respectively. As shown in Figure 1C, patients with CK-19 mRNA-positive CTCs after completion of adjuvant chemotherapy had a significantly shorter OS compared with patients without such cells ($P = .001$). In addition, there seemed to be a progressive decrease in the OS of the four groups of patients according to the detection of CK-19 mRNA-positive CTCs before and after chemotherapy (Fig 1D).

Univariate and Multivariate Analysis

Detection of CK-19 mRNA-positive CTCs before the initiation or after the completion of adjuvant chemotherapy or persistent positivity both before and after chemotherapy, tumor size more than 2.0 cm, more than three involved axillary lymph nodes, histologic grade 3 tumors, and ER-negative status were significantly associated with reduced DFS and OS in univariate analysis (Appendix Table A1, online only). Multivariate analysis revealed that detection of CK-19m RNA-positive CTCs before and after adjuvant chemotherapy, ER-negative status, tumor size more than 2.0 cm, and more than three involved axillary lymph nodes were independent prognostic factors for DFS. The same parameters except tumor size were also independent prognostic factors for OS (Table 5).

DISCUSSION

A recent meta-analysis has clearly demonstrated that the detection of cytokeratin-positive cells in bone marrow aspirates of patients with stage I to III breast cancer is an independent prognostic factor associated with an unfavorable clinical outcome.[1] In addition, it has been previously reported that adjuvant chemotherapy could not, in many

Table 3. Incidence of Clinical Recurrence and Death According to the Detection of CK-19 mRNA-Positive Circulating Tumor Cells Before and After the Completion of Adjuvant Chemotherapy

CK-19 mRNA	No. of Patients	Relapses		Deaths	
		No.	%	No.	%
CK-19 mRNA status after chemotherapy					
Negative	294	49*	16.7	18†	6.1
Positive	143	46	32.2	24	16.8
CK-19 mRNA status prechemotherapy/ postchemotherapy					
Negative/negative	202	28	13.9	8	4.0
Negative/positive	56	12‡	21.4	7§	12.5
Positive/negative	92	21‖	22.8	10¶	10.9
Positive/positive	87	34#	39.1	17**	19.5

Abbreviation: CK-19, cytokeratin-19.
*$P < .001$ for relapses of CK-19 mRNA positive versus negative after chemotherapy.
†$P < .001$ for deaths of CK-19 mRNA positive versus negative after chemotherapy.
‡$P = .209$ for relapses of CK-19 mRNA negative prechemotherapy/positive postchemotherapy versus negative prechemotherapy/negative postchemotherapy.
§$P = .024$ for deaths of CK-19 mRNA negative prechemotherapy/positive postchemotherapy versus negative prechemotherapy/negative postchemotherapy.
‖$P = .064$ for relapses of CK-19 mRNA positive prechemotherapy/negative postchemotherapy versus negative prechemotherapy/negative postchemotherapy.
¶$P = .033$ for deaths of CK-19 mRNA positive prechemotherapy/negative postchemotherapy versus negative prechemotherapy/negative postchemotherapy.
#$P < .001$ for relapses of CK-19 mRNA positive prechemotherapy/positive postchemotherapy versus negative prechemotherapy/negative postchemotherapy.
**$P < .001$ for deaths of CK-19 mRNA positive prechemotherapy/positive postchemotherapy versus negative prechemotherapy/negative postchemotherapy.

Table 4. Effect of Adjuvant Chemotherapy on Median Number of CTCs

Group	No. of CTCs Before Chemotherapy (MCF-7 cell equivalents/5 μg RNA)		No. of CTCs After Chemotherapy (MCF-7 cell equivalents/5 μg RNA)		*P*
	Median	Range	Median	Range	
All patients, n = 437	0.3	0-1,115	0.0	0-1,000	.002
CK-19 mRNA negative/CK-19 mRNA positive, n = 56	0.0	0.0-0.5	1.7	0.6-178	< .001
CK-19 mRNA positive/CK-19 mRNA negative, n = 92	1.7	0.6-896	0.0	0.0-0.5	< .001
CK-19 mRNA positive/CK-19 mRNA positive, n = 87	2.8	0.6-1,115	2.2	0.6-1,000	.826

Abbreviations: CTCs, circulating tumor cells; CK-19, cytokeratin-19.

cases, eliminate bone marrow CK-19–positive DTCs and that their detection after adjuvant chemotherapy was an independent predictor for reduced OS.[11,12,18] However, data regarding the prognostic value of CTCs in patients with early-stage breast cancer are limited. Our group has previously reported that the detection of circulating CK-19 mRNA-positive cells in patients with node-negative breast cancer before the initiation of any systemic treatment was an independent prognostic factor associated with an increased risk of disease recurrence.[4] More recently, we reported that although the prognostic value of the detection of circulating CK-19 mRNA-positive cells before the initiation of adjuvant chemotherapy was an independent factor both for patients with or without axillary lymph node involvement, the prognostic implication after 5 years of follow-up was significant for patients with ER-negative, triple-negative, or HER-2/*neu*–positive

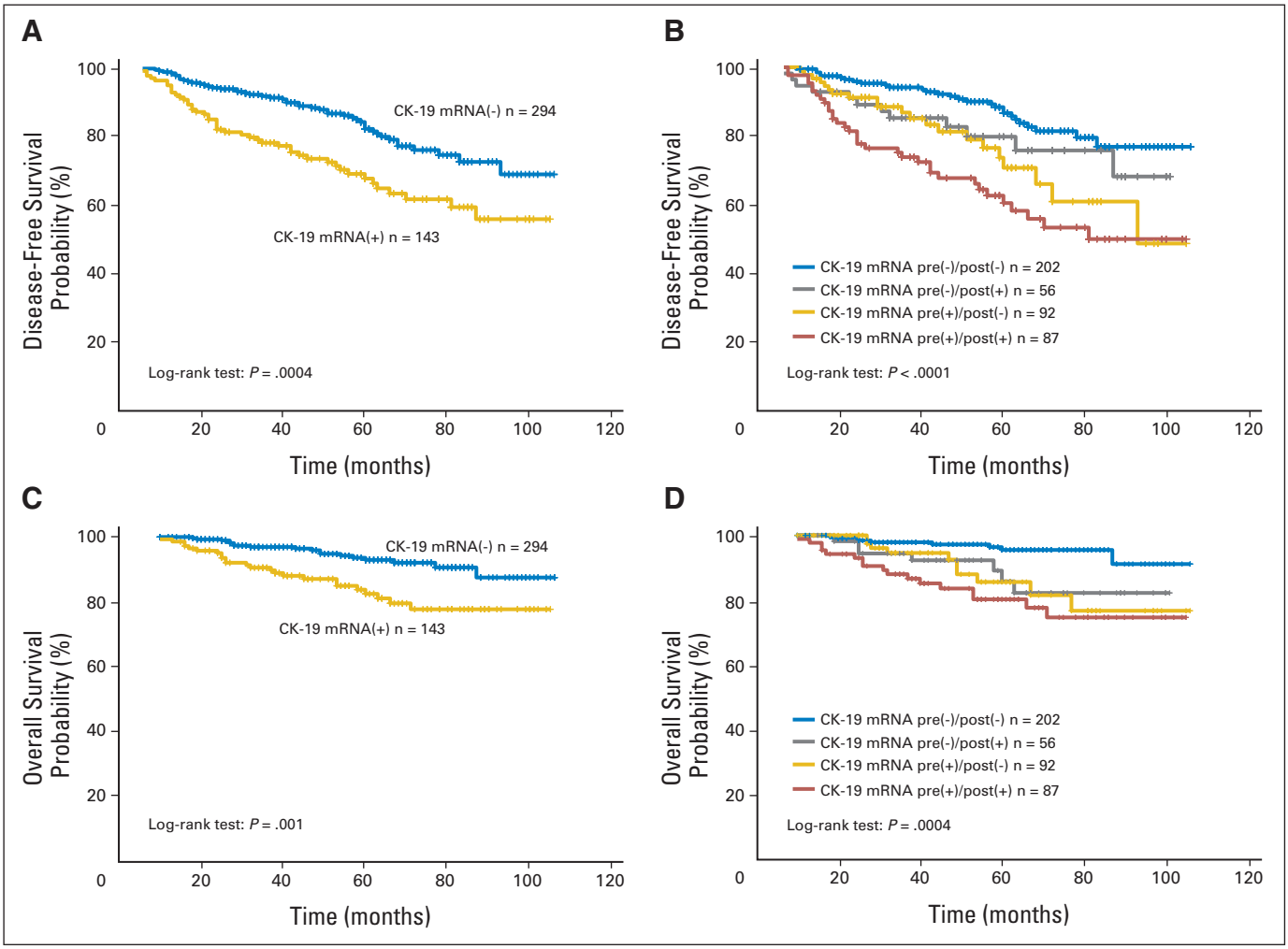

Fig 1. (A) Disease-free survival (DFS) of patients with or without detectable cytokeratin-19 (CK-19) mRNA-positive circulating tumor cells (CTCs) after the completion of adjuvant chemotherapy. (B) DFS according to the detection of CK-19 mRNA-positive CTCs both before the initiation and after the completion of adjuvant chemotherapy. (C) Overall survival of patients with or without detectable CK-19 mRNA-positive CTCs after the completion of adjuvant chemotherapy. (D) Overall survival according to the detection of CK-19 mRNA-positive CTCs both before the initiation and after the completion of adjuvant chemotherapy.

Table 5. Independent Prognostic Factors by Multivariate Analysis for Disease-Free and Overall Survival of Patients With Early-Stage Breast Cancer

Survival Measure	Hazard Ratio	95% CI	*P*
DFS			
Tumor size, T2, 3 *v* T1	2.058	1.198 to 3.535	.009
Lymph nodes, ≥ N4 *v* N0-N3	1.676	1.101 to 2.550	.016
Estrogen receptor, negative *v* positive	1.821	1.206 to 2.751	.004
CK-19 mRNA status prechemotherapy/postchemotherapy			
CK-19 negative/CK-19 negative, reference			
CK-19 negative/CK-19 positive	1.944	0.980 to 3.858	
CK-19 positive/CK-19 negative	2.390	1.346 to 4.243	
CK-19 positive/CK-19 positive	3.239	1.937 to 5.415	
OS			
Lymph nodes, ≥ N4 *v* N0-N3	2.487	1.325 to 4.666	.005
Estrogen receptor, negative *v* positive	2.617	1.391 to 4.924	.003
CK-19 mRNA status prechemotherapy/postchemotherapy			
CK-19 negative/CK-19 negative, reference			.003
CK-19 negative/CK-19 positive	3.631	1.309 to 10.069	.013
CK-19 positive/CK-19 negative	3.756	1.476 to 9.559	.005
CK-19 positive/CK-19 positive	4.893	2.086 to 11.479	< .001

Abbreviations: DFS, disease-free survival; CK-19, cytokeratin-19; OS, overall survival.

tumors.[13] In the present study, the prognostic value of the detection of circulating CK-19 mRNA-positive cells after the completion of adjuvant chemotherapy in patients with early-stage breast cancer was investigated.

Our data demonstrate that CK-19 mRNA-positive CTCs could be detected in 32.7% of patients after completion of adjuvant chemotherapy, and their detection was significantly associated with an increased risk of disease recurrence and death owing to disease progression. Moreover, the detection of CK-19 mRNA-positive CTCs after chemotherapy was significantly associated with the extent of axillary lymph node involvement, suggesting a possible relationship with tumor load. A similar observation concerning the detection of DTCs before any systemic treatment has also been reported by Braun et al[1] in the pooled analysis of 4,703 patients.

Adjuvant chemotherapy significantly decreased the number of detectable CK-19 mRNA-positive cells in the entire cohort of patients. However, CK-19 mRNA-positive cells remained detectable in 48.6% of patients, whereas in an additional 21.7% of patients, CK-19 mRNA-positive CTCs became detectable after the end of adjuvant treatment. Similar data have also been reported for bone marrow cytokeratin-positive DTCs.[18] This observation, which is compatible with the hypothesis that CK-19 mRNA-positive cells represent a heterogeneous cell population with different sensitivities to various chemotherapy regimens, is in agreement with prior studies evaluating the proliferation potential[19] and the genomic analysis[20] of micrometastatic cells. Because DTCs and CTCs are the targets of adjuvant systemic treatment in patients with early-stage breast cancer, these data raise the question whether further adjuvant treatment of chemotherapy-resistant occult tumor cells by either hormone therapy[21] or other targeted agents should be initiated.[22] Indeed, previous studies conducted by our group have demonstrated that chemotherapy-resistant CTCs can be effectively eliminated, at least in part, by tamoxifen administration[21] or the anti-HER-2 monoclonal antibody trastuzumab.[22]

In the current study, a significant association between the detection of CK-19 mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy with reduced DFS and OS was observed. Similar results were also reported by Braun et al[18] for cytokeratin-positive cells in the bone marrow after adjuvant chemotherapy in a group of patients with relatively more advanced tumors. Interestingly, the subgroup analysis revealed that the incidence of relapse and death was significantly higher in patients who had CK-19 mRNA-positive CTCs both before the initiation and after the completion of adjuvant chemotherapy (Table 3); similarly, DFS and OS were significantly decreased in this particular subgroup of patients, as already has been reported by others.[23] This observation implies that these patients have a large micrometastatic tumor load that adjuvant chemotherapy fails to eliminate or reduce, thus leading to early disease relapse and death. On the contrary, the two intermediate subgroups, namely women whose disease was CK-19 mRNA positive before but negative after or those whose disease was negative before but positive after chemotherapy had similar outcomes (Fig 1). This could be due to the detection limit of our assay, which does not allow clear discrimination between the two groups. Alternatively, the micrometastatic tumor load in both intermediate subgroups could be similar, despite the detected differences in the circulating tumor cells.

The multivariate analysis clearly demonstrated that the detection of CK-19 mRNA-positive CTCs before and after adjuvant chemotherapy is an independent prognostic factor associated with an increased risk of early disease recurrence and death owing to disease progression. These data support the notion that detection of CK-19 mRNA-positive CTCs by real-time RT-PCR could be used as a tool to monitor the efficacy of adjuvant chemotherapy. Similar conclusions have also been reached by other investigators using different techniques, such as laser scanning cytometry[23] or immunocytochemistry,[24] to monitor the response of CTCs to adjuvant systemic therapy.

An important question concerns the viability of circulating CK-19 mRNA-positive cells, because it is reported that after successful chemotherapy, a substantial number of disseminated tumor cells in bone marrow are apoptotic.[25] Nevertheless, the clear association of the detection of CK-19 mRNA-positive cells after the completion of adjuvant chemotherapy with an increased risk of relapse and death suggests that at least some of these cells are viable and capable of generating metastases.

In conclusion, the present study demonstrated that the presence of CK19 mRNA-positive CTCs after chemotherapy in patients with early breast cancer is an independent unfavorable prognostic factor for reduced DFS and OS. Moreover, the detection of these cells after therapy could be considered as indirect evidence of chemotherapy resistance, suggesting that CK-19 mRNA-positive CTCs could be a potential surrogate marker for the efficacy of systemic adjuvant treatment. Therefore, monitoring of CTCs during the administration of adjuvant treatment could permit tailoring of the treatment to the risk of each individual patient. In addition, detection of CTCs during patient follow-up would offer the opportunity for early intervention, perhaps making eradication of cancer cells more feasible, when the tumor burden is still low and before the appearance of clinically overt metastases. Because occult tumor cells are often chemotherapy- and hormonotherapy-resistant, novel agents with potential antitumor activity could be investigated for their elimination. These hypotheses should be tested in well-designed, adequately powered, prospective, randomized clinical studies. Such a prospective, randomized clinical trial should be designed so that treatment decisions in the experimental arm are based on CTC detection. In this way, definitive proof will be provided that the monitoring of CTCs can be used to improve clinical outcome in patients with breast cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

Table A1. Univariate Analysis (unadjusted relative risks) for Disease-Free and Overall Survival of Patients With Early-Stage Breast Cancer

Parameter	DFS				OS			
	Hazard Ratio	*P*	95% CI		Hazard Ratio	*P*	95% CI	
Menopausal status, pre- *v* postmenopausal	1.405	.113	0.923	2.137	1.118	.722	0.604	2.072
Tumor size, T2, 3 *v* T1	2.241	.002	1.356	3.706	2.231	.041	1.032	4.819
Lymph node involvement								
Node-positive *v* node-negative	1.309	.226	0.846	2.027	1.434	.292	0.734	2.801
N1-N3 *v* N0	0.772	.388	0.428	1.390	0.655	.397	0.246	1.745
≥ N4 *v* N0-N3	1.399	.001	1.143	1.711	1.549	.005	1.141	2.104
Histology grade, 3 *v* 1/2/lobular	1.279	.017	1.045	1.565	1.518	.009	1.112	2.073
ER status, negative *v* positive	1.738	.008	1.157	2.612	2.387	.007	1.274	4.475
PR status, negative *v* positive	1.539	.051	0.999	2.731	1.790	.090	0.912	3.513
HER2/*neu* status, positive *v* negative	1.187	.479	0.739	1.906	1.182	.648	0.576	2.428
CK-19 mRNA cells before chemotherapy, positive *v* negative	2.431	< .001	1.615	3.659	2.984	.001	1.585	5.615
CK-19 mRNA cells after chemotherapy, positive *v* negative	2.038	< .001	1.363	3.049	2.698	.001	1.464	4.973
CK-19 mRNA pre/postchemotherapy								
CK-19 negative/CK-19 negative, reference	1.650	< .001	0.839	3.247	3.193	.002	1.157	8.810
CK-19 negative/CK-19 positive	2.137	.147	1.211	3.773	3.403	.025	1.340	8.642
CK-19 positive/CK-19 negative	3.349	.009	2.030	5.527	5.288	.010	2.281	12.257
CK-19 positive/CK-19 positive		< .001				< .001		
Persistent positivity prechemotherapy/ postchemotherapy, CK-19 positive/CK-19 positive *v* CK-19 positive or CK-19 negative/CK-19 positive or CK-19 negative	2.474	< .001	1.626	3.765	2.777	.001	1.499	5.143

Abbreviations: DFS, disease-free survival; OS, overall survival; CK-19, cytokeratin-19.

Glossary Terms

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.

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.

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.

HER-2/neu (human epithelial growth factor receptor-2): Also called ErbB2, HER-2/neu belongs to the EGFR family and is overexpressed in several solid tumors. Like EGFR, it is a tyrosine kinase receptor whose activation leads to proliferative signals within the cells. On activation, the HER family of receptors are known to form homodimers and heterodimers, each with a distinct signaling activity. Because HER-2 is the preferred dimerization partner when heterodimers are formed, it is important for signaling through ligands specific for any members of the family. It is typically overexpressed in several epithelial tumors.

ER (estrogen receptor): Belonging to the class of nuclear receptors, estrogen receptors are ligand-activated nuclear proteins present in many breast cancer cells that are important in the progression of hormone-dependent cancers. After binding, the receptor-ligand complex activates gene transcription. There are two types of estrogen receptors (α and β). ERα is one of the most important proteins controlling breast cancer function. ERβ is present in much lower levels in breast cancer and its function is uncertain. Estrogen-receptor status guides therapeutic decisions in breast cancer.

FISH (fluorescence in situ hybridization): In situ hybridization is a sensitive method that is generally used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. FISH uses a fluorescence probe to increase the sensitivity of in situ hybridization.