Original article

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Peripheral blood circulating cytokeratin-19 mRNA-positive cells after the completion of adjuvant chemotherapy in patients with operable breast cancer

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Background: The purpose of this study was to evaluate the prognostic significance of the molecular detection of cytokeratin 19 (*CK-19*) mRNA-positive cells in the peripheral blood of women with operable breast cancer after the completion of adjuvant chemotherapy.

Patients and methods: Blood from 161 patients with stage I and II breast cancer, obtained after the completion of adjuvant chemotherapy, was tested by nested RT–PCR for *CK-19* mRNA detection. Using univariate and multivariate analyses possible interactions with other prognostic factors and association of *CK-19* mRNA detection with risk of relapse, disease-free interval (DFI) and overall survival were investigated.

Results: After completion of adjuvant chemotherapy, 27.3% of patients had peripheral blood *CK-19* mRNA-positive cells; there was no association of this finding with any other prognostic factors or the type of chemotherapy regimen used. For patients with less than four involved axillary lymph nodes the risk of relapse was 3.81 [95% confidence interval (CI) 1.06–13.71] times higher, and the DFI was significantly reduced ($P = 0.028$) if *CK-19* mRNA-positive cells were detectable in the blood after the completion of adjuvant chemotherapy. In contrast, for patients with four or more involved lymph nodes, the presence of *CK-19* mRNA-positive cells after adjuvant chemotherapy did not significantly affect the risk of relapse or DFI. Furthermore, the risk of relapse was higher (hazards ratio 3.70; 95% CI 1.09–13.89) and the DFI was reduced ($P = 0.022$) for patients with detectable *CK-19* mRNA-positive cells following adjuvant cyclophosphamide, methotrexate and 5-fluorouracil (CMF) as compared with epirubicin, cyclophosphamide and 5-fluorouracil (FEC) or sequential taxotere–epirubicin and cyclophosphamide (T/EC) chemotherapy.

Conclusions: The detection of *CK-19* mRNA-positive cells in the peripheral blood after adjuvant chemotherapy may be of clinical relevance for patients with early breast cancer and less than four involved axillary lymph nodes.

Key words: adjuvant chemotherapy, blood, breast cancer, *CK-19* mRNA

Introduction

Most patients with stage I or II breast cancer will be cured of their disease with surgery and adjuvant chemotherapy and hormone therapy; however, a significant proportion of patients with operable breast cancer may develop distant metastases.

The development of metastases is due to the migration of tumor cells from the original tumor to distant organs. This phenomenon probably occurs early during the evolution of the disease and even before the surgical excision of the primary tumor. In the last few years, several studies using either monoclonal antibodies against molecules expressed on epithelial cells, but not on mesenchymal cells, or molecular biology techniques permitted the detection of occult tumor cells in the bone marrow of patients with operable early stage I and II breast cancer [1–7]. Prospective studies have shown that the detection of occult tumor cells in the bone marrow aspirates of these patients is an independent adverse prognostic factor associated with decreased disease-free interval (DFI) and overall survival [2, 8–12].

Bone marrow occult tumor cells have been shown to present several aberrations in chromosomes 7, 8 and 18, as well as amplification of the *c-erbB2* gene, as detected by fluorescence *in situ* hybridisation [13, 14]. These findings strongly suggest that the occult epithelial bone marrow cells are most likely malignant in origin and may give rise to distant metastases. However, cell culture experiments have shown that these bone marrow occult tumor

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cells have a time-limited proliferative potential and thus may be dormant [15, 16]. The dormant status of the occult tumor cells may be the reason why adjuvant chemotherapy fails to prevent relapse in some patients with early breast cancer. Indeed, Braun et al. [17] used an immunohistochemical method to study 23 patients with inflammatory and 36 with operable breast cancer who had more than four involved axillary lymph nodes for the presence of occult bone marrow breast cancer cells before and after the completion of adjuvant chemotherapy; they found that 48.3% of patients who had cytokeratin (CK)-positive cells in the bone marrow before treatment became CK-negative post-treatment; multivariate analysis showed that the detection of CK-positive cells post-chemotherapy represents an independent poor prognostic factor for reduced overall survival [17].

The above observations indicate that the detection of disseminated occult tumor cells may be an important marker to follow in patients with early breast cancer, because it may identify a subgroup of patients who are at high risk of relapse. Since multiple bone marrow aspirations from the same patient during the follow-up time can not be easily tolerated, the peripheral blood would be a more convenient source of sampling for such longitudinal follow-up studies. Indeed, occult tumor cells have already been identified by nested reverse-transcription polymerase chain reaction (RT–PCR) in the peripheral blood [4, 5, 7, 18–20]. Our group has recently evaluated the presence of circulating tumor cells expressing *CK-19* mRNA by RT–PCR in the peripheral blood of patients with stage I and II breast cancer before the initiation of any adjuvant cytotoxic or hormone therapy; circulating CK-19 mRNA-positive cells could be detected in almost 30% of patients and multivariate analyses demonstrated that the presence of these cells was an independent prognostic factor for decreased DFI ($P = 0.0007$) and overall survival ($P = 0.01$) [21].

In the present study we investigated the effect of the detection of *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy on the risk of relapse, DFI and overall survival of patients with stage I and II breast cancer. Our data demonstrate that the presence of *CK-19* mRNA-positive cells in the blood after the completion of adjuvant chemotherapy is an independent prognostic factor associated with increased risk of relapse and decreased DFI for patients with three or less involved axillary lymph nodes. Therefore, the detection of *CK-19* mRNA-positive cells in the peripheral blood may be of clinical relevance during the follow-up of patients with operable breast cancer.

Patients and methods

Patients and clinical samples

Peripheral blood (10 ml in EDTA) was obtained from 161 patients with stage I and II breast cancer 4–8 weeks after the completion of adjuvant chemotherapy. The type of chemotherapy used in 87 patients was decided by the treating physician and included either the cyclophosphamide, methotrexate and 5-fluorouracil (CMF) or epirubicin, cyclophosphamide and 5-fluorouracil (FEC) regimen. In 59 patients with four or more positive axillary lymph nodes, adjuvant chemotherapy was based on a randomized study comparing four cycles of docetaxel 100 mg/m^2 followed by four cycles of EC (epirubicin 75 mg/m^2; cyclophosphamide 700 mg/m^2) to six cycles of FEC (epirubicin 50 mg/m^2; cyclophosphamide 700 mg/m^2; 5-FU 700 mg/m^2). In 15 patients who had no axillary lymph node involvement, chemotherapy was based on a randomized study comparing FEC with CMF in the context of an International Collaborative Cancer Group (ICCG) protocol. Patient characteristics are presented in Table 1. Patients were followed for a median of 29 months (range 7–55). All patients gave their informed consent for participation in the study, which was approved by the Ethics and Scientific Committees of our Institution.

In all cases peripheral blood samples were obtained at the middle of vein puncture after the first 5 ml of blood were discarded. Peripheral blood was diluted with an equal volume of phosphate-buffered saline (PBS; Gibco, UK) pH 7.3 and cells were dissociated by passing them through 25 guage 5/8 inch

Table 1. Patient characteristics

	No. of patients (%)
No. of patients enrolled	161
Median age, years (range)	54 (30–74)
Menstrual status	
Premenopausal	66 (41.0)
Postmenopausal	95 (59.0)
Stage	
I	51 (31.7)
II	110 (68.3)
Tumor size (cm)	
<1.0	4 (2.5)
1.1–3.9	113 (70.2)
≥4.0	44 (27.3)
Grade	
I/II	82 (50.9)
III	69 (42.9)
Unknown	10 (6.2)
Lymph nodes	
0	53 (32.9)
1–3	49 (30.4)
≥4	59 (36.6)
Hormone receptor status	
ER+	92 (57.1)
ER–	61 (37.9)
Unknown	8 (5.0)
PR+	46 (28.6)
PR–	106 (65.8)
Unknown	9 (5.6)
Adjuvant chemotherapy	
CMF	25 (15.5)
FEC	91 (56.5)
T/EC	45 (28.0)

CMF, cyclophosphamide, methotrexate and 5-fluorouracil; ER, estrogen receptor; FEC, epirubicin, cyclophosphamide and 5-fluorouracil; T/EC, taxotere, epirubicin and cyclophosphamide; PR, progesterone receptor.

needles. Peripheral blood mononuclear cells (PBMC) were obtained by gradient density centrifugation using Ficoll-Hypaque 1077 (Sigma, USA) at 1200 *g* for 30 min at 4°C. The interface cells were removed, washed twice with 50 ml sterile PBS (pH 7.3), pelleted and resuspended in 1 ml PBS. The cells were pelleted again at 1200 *g* for 2 min. Cell pellets were kept at –80°C until RNA extraction. Total RNA isolation was performed by using Trizol LS reagent (Gibco, BRL) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a laminar flow hood under RNAse-free conditions. The isolated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at –80°C until use. RNA concentration was determined by absorbance readings at 260 nm with the Hitachi UV-VIS (U-2000) spectrophotometer. RNA integrity was tested by PCR amplification of the β-actin housekeeping gene. RNA samples were also prepared from the human tumor cell lines MCF-7 and ARH-77 as positive and negative controls, respectively.

RT–PCR

Reverse transcription of RNA was carried out with the Thermoscript RT–PCR system (Gibco, BRL). c-DNA was synthesized according to the manufacturer's instructions. Two different PCR reactions, with the respective negative controls, were performed with each sample in order to amplify fragments of *CK-19* and β-*actin*. The sequences of primers utilized (synthesized by Genset, Paris, France) were as follows: for CK-19, AAGCTAACCATGCAGAACCTCAACGACCGC (forward; P1); TTATTGGAGGTCAGGAGAAGAGCC (reverse; P2); TCCCGCGACTACAGCCACTACTACACGACC (forward; P3); CGCGACTTGATGTCCATGAGCCGCTGGTAC (reverse; P4); and for β-actin, CATCCTGTCGGCAATGCCAGG (forward; A1); CTTCTTGGGCATGGAGTCCTG (reverse; A2). The corresponding sizes of the PCR products were 745 and 154 base pairs, respectively. These primers extend across at least one intron; thus, the eventual DNA contamination would not pose a significant problem. *CK-19* gene expression was evaluated by nested PCR as described by Datta et al. [4]. The conditions for β-actin PCR were one cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 4 min. Ten microliters of all PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide.

Statistical analysis

The main methods of analysis were logistic regression [22, 23] and the Cox proportional hazards model [24] for outcomes related to point events and time variables, respectively. To select those factors with independent significant influence on outcomes, both analyses were carried out in a stepwise (unconditional backward) fashion [23, 24]. Prior to the application of these methods, univariate analyses were performed for a preliminary exploration of marked associations. Univariate analyses included contingency tables (Pearson's χ^2-test and its partition to components, or Fisher's test where appropriate, relative risks and/or odds ratios) and Kaplan–Meier estimates of survival curves (log-rank and Wilcoxon tests) [24–26].

Results

Detection of peripheral blood *CK-19* mRNA-positive cells after the completion of adjuvant chemotherapy

Forty-four (27.3%) of 161 patients with operable breast cancer had detectable *CK-19* mRNA-positive cells in peripheral blood after the completion of adjuvant chemotherapy. Table 2 indicates that the presence of *CK-19* mRNA-positive cells was not associated with any of the commonly studied clinicopathological prognostic factors (menopausal status, stage, tumor size, histological grade, number of involved axillary lymph nodes or estrogen receptor expression). There was, however, a marked but not significant (P = 0.068) association with the expression of progesterone receptors (PR). Indeed, 18 (39.1%) of 46 patients who had PR+ tumors had detectable *CK-19* mRNA-positive cells after the completion of adjuvant chemotherapy as compared with 26 (24.5%) of 106 patients who had PR– tumors (all nine patients with unknown PR expression had no detectable *CK-19* mRNA-positive cells). Regarding the used adjuvant chemotherapy regimens, 25 patients received CMF, 91 FEC and 45 taxotere followed by EC (T/EC). The corresponding numbers of patients with detectable *CK-19* mRNA-positive cells after the completion of adjuvant chemotherapy were nine (36%), 23 (25.3%) and 12 (26.7%), respectively (P = 0.563).

Effect of the detection of *CK-19* mRNA-positive cells in the peripheral blood post-adjuvant chemotherapy on relapse and DFI

Relapse. Five (11.4%) of 44 patients who had detectable *CK-19* mRNA-positive cells in peripheral blood after the completion of adjuvant chemotherapy developed distant relapse during the follow-up period compared with 11 (9.4%) of 117 patients who had no such cells (P = 0.711). Moreover, none of the known clinicopathological prognostic factors showed any significant association with relapse; the only marked, but not significant (P = 0.086), association with relapse was the number of involved axillary nodes (if greater than three).

DFI. Kaplan–Meier estimates of the cumulative DFI rates showed no significant difference between patients with detectable *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy and those patients with no such cells (P = 0.664). After a median follow-up of 29 months (range 7–55), the median times to tumor progression have not yet been attained in either group.

The proportional hazards model revealed that age, menopausal status, tumor size, stage, histological grade and the presence of ER or PR did not have any influence on DFI, irrespective of the detection of *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy. Only the number of involved axillary lymph nodes had a significant effect on DFI (P = 0.018); the hazard of relapse for patients with more than three involved lymph nodes was 4.2 times (95% CI 1.27–13.56) higher than that of patients with three or less involved lymph nodes. Moreover, the presence of *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy had a significant effect on DFI, which was dependent upon the number of involved lymph nodes. More specifically, the hazard of relapse for patients with three or less involved lymph nodes and detectable *CK-19* mRNA-positive cells after adjuvant chemotherapy was 3.81 (95% CI 1.06–13.71) times higher than that of patients with *CK-19* mRNA-negative cells. In contrast, for patients with more than three involved lymph nodes, the presence of *CK-19* mRNA-positive cells post-adjuvant chemotherapy did not affect significantly the risk of relapse (P = 0.509).

Table 2. Patient clinicopathological prognostic characteristics and frequency of occult *CK-19* mRNA-positive cells in peripheral blood after adjuvant chemotherapy

Variables	*CK-19* mRNA+		*CK-19* mRNA–		*P* value
	n	%	*n*	%	
Total	44	27.3	117	72.7	
Menstrual status					
Premenopausal	18	27.3	48	72.7	
Postmenopausal	26	27.4	69	72.6	0.989
Stage					
I	15	29.4	36	70.6	
II	29	26.4	81	73.6	0.686
Tumor size (cm)					
<1.0	1	25.0	3	75.0	
1.1–3.9	31	27.4	82	72.6	
≥4.0	12	27.3	32	72.7	0.994
Grade					
I/II	24	29.3	58	70.7	
III	18	26.1	51	73.9	0.664
Unknown[a]	2	20.0	8	80.0	
Lymph nodes					
0	15	28.3	38	71.7	
1–3	13	26.5	36	73.5	
≥4	16	27.1	43	72.9	0.979
Hormone receptor					
ER+	24	26.1	68	73.9	
ER–	20	32.8	41	67.2	0.370
Unknown[a]	0	0.0	8	100.0	
PR+	18	39.1	28	60.9	
PR–	26	24.5	80	75.5	0.068
Unknown[a]	0	0.0	9	100.0	
Adjuvant chemotherapy					
CMF	9	36.0	16	64.0	
FEC	23	25.3	68	74.7	
T/EC	12	26.7	33	73.3	0.563

[a]Category excluded from the calculation of the associated *P* value.
CMF, cyclophosphamide, methotrexate and 5-fluorouracil; CK, cytokeratin; ER, estrogen receptor; FEC, epirubicin, cyclophosphamide and 5-fluorouracil; T/EC, taxotere, epirubicin and cyclophosphamide; PR, progesterone receptor.

The above results were further illustrated by Kaplan–Meier analysis (Figure 1A and B). Patients with three or less involved lymph nodes who had detectable *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy had a significantly decreased DFI (log-rank test; $P = 0.028$) compared with that of patients without such cells in the peripheral blood (Figure 1A). However, for patients with more than three involved lymph nodes, the difference in the DFI rates according to the presence or absence of CK-19 mRNA-positive cells was not significant (log-rank test; $P = 0.501$; Figure 1B).

Effect of type of adjuvant chemotherapy on DFI

The analysis by the proportional hazards model indicated significant interaction effects between types of regimen and presence of *CK-19* mRNA-positive cells after the completion of adjuvant treatment. More specifically, for patients without detectable *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant treatment, the type of chemotherapy regimen had no effect on DFI (Figure 2A). In contrast, the type of regimen had a significant effect on the DFI of patients with detectable *CK-19* mRNA-positive cells after adjuvant therapy. For these patients,

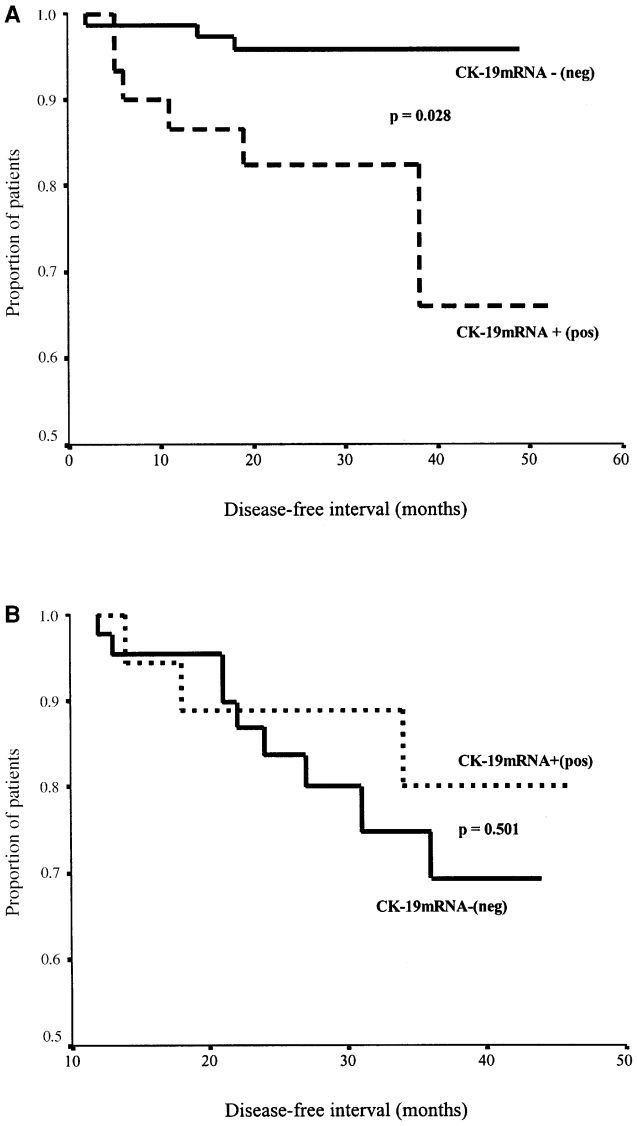

Figure 1. Kaplan–Meier estimates of disease-free interval (DFI) rates according to the presence or absence of cytokeratin 19 (*CK-19*) mRNA-positive cells after adjuvant chemotherapy. (**A**) Three or less involved axillary lymph nodes; (**B**) greater than three involved axillary lymph nodes.

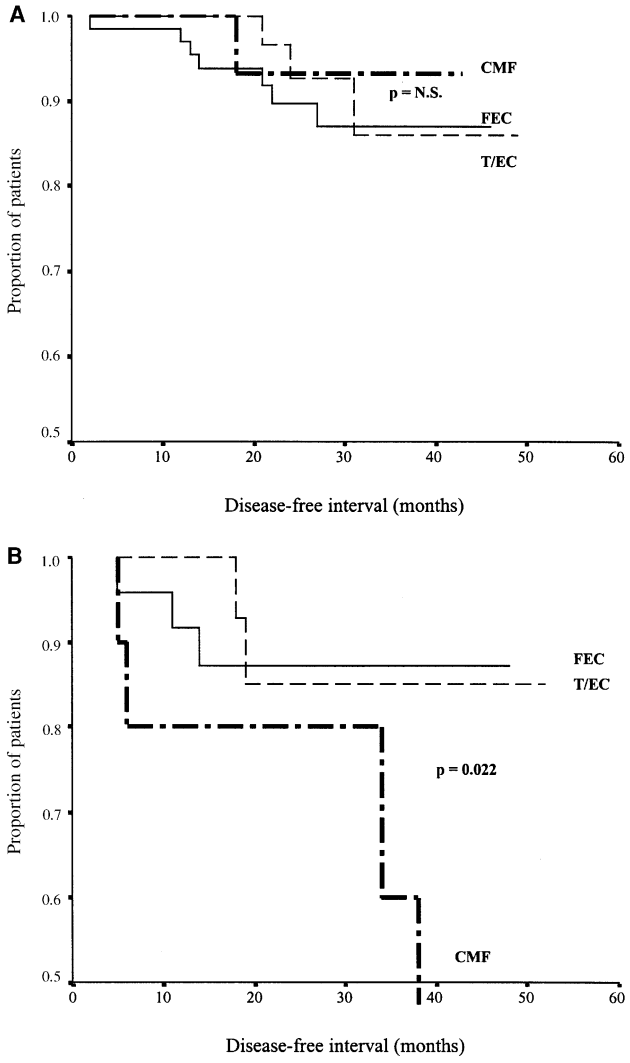

Figure 2. Cumulative disease-free interval (DFI) rates according to the type of adjuvant chemotherapy regimen used. (**A**) Cytokeratin 19 (*CK-19*) mRNA-negative status after adjuvant therapy; (**B**) *CK-19* mRNA-positive status after adjuvant therapy.

the hazard of relapse associated with the CMF regimen was significantly higher ($P = 0.022$) than that of patients who were treated with either FEC or T/EC (hazards ratio 3.70; 95% CI 1.09–13.89) (Figure 2B). Patients treated with CMF had a worse DFI with a median of 38 months compared with the other two groups where the median DFIs have not yet been reached.

Overall survival

Three (6.8%) of 44 patients with detectable peripheral blood *CK-19* mRNA-positive cells after the completion of adjuvant chemotherapy had died during the follow-up period compared with six (5.1%) of 117 patients with no such cells in the peripheral blood. The difference was not statistically significant (Fisher's exact test: $P = 0.707$). The very small number of observed deaths during follow-up did not allow the use of multivariate analyses; instead only univariate analyses were carried out. The results indicated that neither any of the known prognostic factors nor the presence or absence of *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy had any significant association with overall survival.

Discussion

Although the prognostic significance of the detection of CK-positive cells in the bone marrow of patients with early breast cancer at initial diagnosis is well documented [2, 8, 10], very few data exist on the prognostic value of the detection of such cells circulating in the peripheral blood. We have recently evaluated the detection of *CK-19* mRNA-positive cells by RT–PCR in the peripheral blood of 148 patients with stage I and II breast cancer before the initiation of any adjuvant therapy and we found that the

presence of such cells was an independent adverse prognostic factor associated with decreased DFI and overall survival [21]. In the present study we investigated the effect of the detection of *CK-19* mRNA-positive cells in the peripheral blood after the completion of adjuvant chemotherapy in 161 patients with stage I and II breast cancer in association with the risk of relapse, DFI and overall survival.

We found that in 27.3% of patients *CK-19* mRNA-positive cells were detectable in the blood after the completion of adjuvant chemotherapy. This relative lack of efficacy of adjuvant chemotherapy to kill disseminated occult tumor cells has also been demonstrated by others [17] and may be due to the dormant nature of such cells [9, 15, 16], thus reducing their sensitivity to chemotherapy. Alternative targeted therapies, such as monoclonal antibodies, that are able to eliminate resting cells may be more effective in eliminating disseminated occult tumor cells [27, 28].

Overall there was no significant association of *CK-19* mRNA-positive cells in peripheral blood after the completion of adjuvant chemotherapy with the risk of distant relapse, DFI or overall survival. This is in contrast with the findings of Braun et al. [17], where the presence of CK-positive cells in the bone marrow after adjuvant chemotherapy was an independent predictor for reduced overall survival (relative risk = 5.0; $P = 0.016$). However, there are significant differences between the two studies that may account for this discrepancy. In the study by Braun et al. [17], only patients with inflammatory or advanced (greater than four nodes involved) disease who were at high risk of relapse were included. Furthermore, a different methodology was used (immunohistochemistry instead of RT–PCR) to detect occult tumor cells.

Interestingly, in subgroup analysis of the present study, the risk of relapse for patients with three or less involved axillary lymph nodes and detectable *CK-19* mRNA-positive cells after adjuvant chemotherapy was almost four times higher and DFI was significantly decreased compared with that of patients without *CK-19* mRNA-positive cells. There was no significant association between the presence of *CK-19* mRNA-positive cells after adjuvant therapy and the risk of relapse in patients with more than three involved axillary lymph nodes. This difference in the prognostic value of *CK-19* mRNA detection after adjuvant therapy in association with the number of involved axillary lymph nodes may be due to the different micrometastatic tumor load and the associated risk of relapse. It is well known that patients with more than three involved axillary lymph nodes have a higher risk of relapse compared with patients with three or less positive nodes [29]. Therefore, it is possible that the qualitative nested RT–PCR assay of *CK-19* mRNA was able to discriminate the prognostic risk only in patients with a relatively low micrometastatic tumor load. Conversely, patients with a high micrometastatic tumor load, such as patients with more than three positive axillary lymph nodes, may require quantitative assessment of the micrometastatic disease for the accurate prediction of prognostic risk [19, 20].

Another interesting finding of this study was the effect of the type of regimen used on DFI of patients with detectable *CK-19* mRNA-positive cells in the peripheral blood after adjuvant treatment. The risk of relapse was significantly higher and DFI was reduced for patients receiving CMF as compared with either FEC or T/EC. Since all of these patients had circulating tumor cells following the completion of adjuvant therapy, the difference in the risk of relapse may be related to the different number of circulating tumor cells (tumor load) which may be affected by the type of adjuvant chemotherapy used. In order to demonstrate such an association, a quantitative assessment of circulating tumor cells is necessary using quantitative RT–PCR methodology [19, 20].

The detection of minimal numbers of circulating breast cancer cells in the blood by RT–PCR of *CK-19* mRNA has certain limitations, which have been well described [7]. False positive results may be due to the detection of *CK-19* pseudogenes a and b [30] or even due to sample contamination with epithelial cells of skin during vein puncture [31]. Although the primers used in this study do not rule out amplification of these *CK-19* pseudogenes, all blood samples were obtained at the middle of vein puncture in order to avoid blood contamination with epithelial cells. Moreover, because the RT–PCR assay detects minimal amounts of specific mRNA amongst a plethora of other RNA, the risk of illegitimate transcription of the *CK-19* gene is increased. This may involve the ectopic transcription of the *CK-19* gene in hematopoietic cells [32] or the expression of any gene in any cell type [33]. In a previous report [21] we have presented that by using this assay *CK-19* mRNA-positive cells were detected in the blood of two of 54 (3.7%) healthy female blood donors and four of 28 (14.3%) patients with hematological malignancies. These findings may be explained by the aforementioned limitations of the assay. Furthermore, the administration of chemotherapy may adversely affect the sensitivity and specificity of the detection in many different ways. Chemotherapy could induce the secretion of pro-inflammatory cytokines, which could modify gene expression [34]. Cytotoxic agents may also cause mucositis and thus introduce normal epithelial cells into the circulation, which could be detected as *CK-19* mRNA-positive cells. Alternatively, chemotherapy may induce apoptotic cancer cells in the blood, which could be detected by the molecular method.

In this study, we have demonstrated that the molecular detection of *CK-19* mRNA-positive cells in the peripheral blood following adjuvant chemotherapy was an independent adverse prognostic factor for patients with three or less involved axillary lymph nodes, associated with increased risk of relapse and reduced DFI. Furthermore, for patients with *CK-19* mRNA-positive cells in the blood after adjuvant therapy, the type of chemotherapy used had prognostic implications. Therefore, the detection of *CK-19* mRNA-positive cells in the blood after the completion of adjuvant chemotherapy may be a useful surrogate test to assess the risk of relapse in patients with stage I and II breast cancer.

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