# Detection of Micrometastatic Disease and Monitoring of Perioperative Tumor Cell Dissemination in Primary Operable Breast Cancer Patients Using Real-Time Quantitative Reverse Transcription-PCR

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## ABSTRACT

***Purpose:*** **We previously found a statistically significant number of cytokeratin 19 (CK19)+ cells in peripheral blood (PB) of stage IV breast cancer (BC) patients compared with those of healthy volunteers, using a quantitative real-time reverse transcription-PCR. We aimed to apply the technique on bone marrow (BM) of primary operable BC patients. Pre- and postoperative PB samples of these patients were further analyzed to investigate possible shedding of CK19+ cells during the operation.**

***Experimental Design:*** **In 54 primary operable BC patients, we analyzed 50 BM samples taken preoperatively and 297 PB samples. PB samples were collected before surgery; immediately after surgery; on the first, second, and fifth day postoperatively; and one month postoperatively.**

***Results:*** **In BM of controls and BC patients, we detected a median of 28 and 568 CK19+ cells/5 × $10^6$ leukocytes, respectively ($P < 0.001$). In preoperative blood (B-1) samples, we measured a median of 109 CK19+ cells. Using the upper limit of 95% confidence interval of controls as cutoff, 74% and 52% of BM and (B-1), respectively were considered CK19+. There was no significant correlation between CK19+ cells in BM and (B-1) and classical prognostic factors. We found no significant difference between blood samples at different time points with respect to the average CK19+ cells.**

***Conclusions:*** **In primary BC patients, we detected high numbers of CK19+ cells in BM and PB (B-1) samples compared with controls. However, no significant correlation between the presence of CK19+ cells in BM and PB and classical prognostic factors was found. We detected no statistically significant influence of surgical manipulation on CK19+ cells.**

## INTRODUCTION

Breast cancer remains a common cause of death among women in industrialized countries (1). Although many cases of breast cancer are detected at an early stage, and standard investigations at diagnosis show no signs of disseminated disease, many patients will develop metastases after locoregional and/or systemic treatment. Whether this is a consequence of micrometastatic disease already present before surgery or dissemination of malignant cells during manipulation of the primary tumor is not clear.

Some studies have shown that tumors, whether manipulated or not, continuously shed malignant cells in the circulation. These studies have also shown that most circulating tumor cells do not survive, with as little as 0.1% being responsible for the formation of secondary deposits (2, 3). Evidence from animal studies has shown that malignant cells are shed into the blood stream during surgical manipulation of a primary tumor, leading to an increased incidence of distant metastases (4, 5). Preliminary investigations with small numbers of samples suggest that tumor manipulations during operation of primary breast (6, 7), colorectal (8), and prostatic cancers (9) induce tumor cell dissemination.

Currently, age, tumor size, nodal status, differentiation, and hormonal receptor status are the standard parameters to identify high-risk patients. In several studies (see Ref. 10 for review), the presence of micrometastases in BM, detected by immunohistochemical techniques, has been identified as an indicator of poor prognosis.

We previously developed a real-time quantitative reverse transcription (RT)-PCR technique to detect breast carcinoma cells in peripheral blood (PB). This technique is sensitive, accurate, and has a high reproducibility with many advantages over classic quantitative PCR methods. We detected significantly elevated cytokeratin 19 (CK19) transcript levels in PB of <10% of the volunteers, in ±30% of stage I-III patients, and in ±70% of stage IV breast cancer patients (11). The primary aim of our present work was to analyze CK19+ cells in BM samples

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*Table 1* Clinical characteristics of the studied 54 operable breast cancer patients

| Clinical data | No. of patients (%) |
|---|---|
| Age | |
| ≤35 years | 0 (0) |
| >35 but ≤60 years | 27 (50) |
| >60 years | 27 (50) |
| Menopausal status | |
| Premenopausal | 17 (31.4) |
| Postmenopausal | 37 (68.6) |
| Family history of breast cancer | |
| Positive | 16 (29.6) |
| Negative | 34 (63) |
| Unknown | 4 (7.4) |
| Grade | |
| I | 7 (13) |
| II | 24 (44.4) |
| III | 23 (42.6) |
| Pathological tumor size | |
| ≤2 cm | 26 (48.1) |
| >2 cm but ≤5 cm | 22 (40.8) |
| >5 cm | 6 (11.1) |
| Pathological stage | |
| I | 18 (33.3) |
| II | 28 (51.9) |
| III | 8 (14.8) |
| Nodal status | |
| Negative | 31 (57.4) |
| 1–3 positive lymph nodes | 14 (26) |
| >3 positive lymph nodes | 9 (16.6) |
| Estrogen receptors | |
| Positive | 37 (68.6) |
| Negative | 13 (24) |
| Unknown | 4 (7.4) |
| Progesterone receptors | |
| Positive | 36 (66.6) |
| Negative | 14 (26) |
| Unknown | 4 (7.4) |
| Surgical procedure | |
| Mastectomy & axillary evacuation | 22 (40.7) |
| Tumorectomy & axillary evacuation | 32 (59.3) |

to investigate the possible presence of micrometastatic disease preoperatively. A second aim was to evaluate the PB shedding of CK19+ cells in the perioperative period.

## PATIENTS, MATERIALS, AND METHODS

**Patients and Samples.** According to the rules of the local ethical committee, we collected 53 BM (BM) and 299 PB samples from 54 patients with stage I-III operable breast cancer, who were from 37 to 86 years of age (median age, 62 years). Patient characteristics are described in Table 1. PB samples were collected by peripheral venipuncture from each patient just before surgery (B-1), immediately after surgery (B0), on the first (B +1), second (B +2), fifth (B +5) days postoperatively, and one month (B +1m) postoperatively. To avoid epithelial contamination from venipuncture, the first 10 ml of blood were discarded. All samples were taken on 10 ml EDTA tubes (Becton Dickinson, Vacutainer Systems Europe). BM aspirates from punctures of both anterior iliac crests and the sternum were obtained just before initiating surgery. These samples were collected in lithium heparin tubes. In our previous work (11), only the detection of CK19 positivity in PB was investigated. Using similar criteria, we aimed to establish criteria for quantification of CK19 positivity in BM. The ideal control group would be BM obtained from women undergoing breast surgery for nonmalignant diseases such as fibroadenomas. Because the access to BM samples from nonmalignant patients or healthy volunteers is very much restricted, a control population was established by collecting archived cDNA samples from BM of 22 patients with hematological malignancies in complete clinical remission and without known gene rearrangements.

**Sample Processing, RNA Extraction, cDNA, and PCR.** We previously described these procedures and their reproducibility extensively (11). Briefly, starting from RNAzol lysates, total RNA was extracted using chloroform, precipitated with isopropyl alcohol, and washed with 70% ethanol. The resulting pellet was redissolved in nuclease-free water. RNA concentration was measured using a spectrophotometer (260 nm/280 nm). After heating at 65°C for 5 min to denature RNA and to inactivate RNases, 1 μg of total RNA was subjected to reverse transcription using 300 units M-MLV Reverse Transcriptase (Life Technologies, Inc.), 30 units RNasin RNase inhibitor, 25 μM random hexamer primers, and reverse transcription buffer containing 250 mM Tris HCl (pH 8.3), 375 mM KCl and 15 mM $Mg^{2+}$ in a total volume of 40 μl at 37°C for 2 h. The reaction was terminated by heating at 65°C for 10 min. For each PCR reaction, 6 μl of 3-fold-diluted cDNA, corresponding to 50 ng of total RNA, 25 μl of Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM probe, and nuclease-free water were added to a final volume of 50 μl. Amplification and detection were performed with the ABI Prism 7700 sequence detection system (Applied Biosystems). With regard to the day-to-day precision, several samples were run at least twice on separate plates and on different days and similar values were obtained in each case. If the variation between the duplicates for either CK19 or glyceraldehydes 3-phosphate dehydrogenase exceeded twice the cycle threshold or one of the duplicates was bad, we ran the sample again.

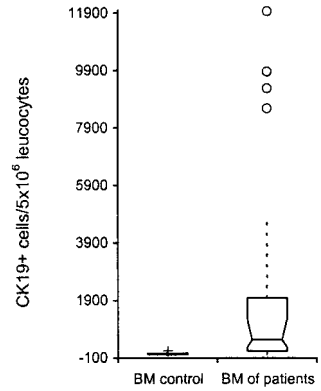


*Fig. 1* Box-Whisker comparative plots of the number of cytokeratin 19 (CK19)+ cells/5 × $10^6$ leukocytes in bone marrow (BM) samples of the control group ($n$ = 22) and breast cancer (BC) patients ($n$ = 50).

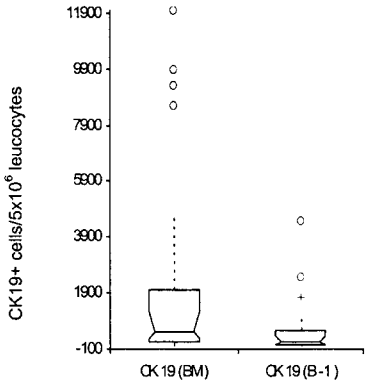


*Fig. 2* Box-Whisker comparative plots of the number of cytokeratin 19 (CK19)+ cells/5 × $10^6$ leukocytes in the bone marrow (BM; $n = 50$) and preoperative peripheral blood [PB (B-1); $n = 50$] samples of breast cancer (BC) patients.

*Table 2* Results of the real-time quantitative PCR in BM and PB (B-1) of BC patients

| BM[a] | PB (B-1) | No. (%) |
|---|---|---|
| + | + | 22/47 (47) |
| + | − | 14/47 (30) |
| − | + | 3/47 (6) |
| − | − | 8/47 (17) |
| Concordance | | 30/47 (64) |

[a] BM, bone marrow; PB (B-1), peripheral blood before surgery; BC, breast cancer; + and − indicate values above or below the cutoff, respectively (see text).

**Statistics.** To compare the BM samples of the patients with the control population, a Mann-Whitney test was used. The relationship between the number of CK19+ cells in BM and PB samples of 54 operable breast cancer patients and classical prognostic factors such as the pathological tumor size, grade of differentiation, axillary nodal involvement, hormonal receptor status, and clinical stage has been investigated using ANOVA and linear regression models. To satisfy the normality assumption, both responses have been transformed logarithmically.

The pattern of evolution of the number of CK19+ cells in time, which was recorded for the different blood samples of each patient to evaluate the possible shedding of CK19+ cells during operation, was studied using a linear mixed model that takes into account the fact that multiple measurements within subjects may be correlated.

The level of significance was taken as equal to 5%. Data analysis was carried out with the SAS and S-Plus statistical packages (Department of Biostatistics, Katholicke Universiteit, Leuven, Belgium).

## RESULTS

We analyzed 50 BM and 297 blood samples from 54 operable breast cancer patients (5 samples were excluded because of technical problems; 3 BM and 2 PB samples).

In the BM of the control population and breast cancer patients, we detected a median of 28 [95% confidence interval (CI), 16–67] and 568 (95% CI, 266-1573) CK19+ cells/5 × $10^6$ leukocytes, respectively ($P < 0.001$; Fig. 1). Using the upper limit of the 95% CI of the control group as a cutoff, 74% of BM samples (37/50) were considered CK19+.

In PB (B-1) samples, we measured a median of 109 (95% CI, 58–298) CK19+ cells/5 × $10^6$ leukocytes. Using the upper limit of the 95% CI of the control group (11) as a cutoff, 52% of PB (B-1) samples (26/50) were considered CK19+.

The distribution of CK19+ cells found in BM and PB (B-1) is illustrated in Fig. 2. Either BM or PB (B-1) was missing for three patients, and among the remaining 47 patients, we

*Table 3* The relationship between the classical prognostic factors and amount of CK19+[a] cells in BM and PB (B-1) samples of BC patients

| | | Log CK19 (BM) | | Log CK19 [PB (B-1)] | |
|---|---|---|---|---|---|
| Effect | Number of cases | Mean (SD) | *P* value | Mean (SD) | *P* value |
| Pathological stage | | | 0.8858 | | 0.8180 |
| I | 18 | 6.42 (2.08) | | 5.33 (1.61) | |
| IIA | 18 | 6.42 (1.86) | | 4.62 (2.26) | |
| IIB | 10 | 5.76 (1.23) | | 4.60 (1.44) | |
| IIIA | 6 | 6.00 (1.44) | | 5.07 (2.08) | |
| IIIB | 2 | 6.58 (0.65) | | 5.22 (1.65) | |
| Grade of differentiation | | | 0.5939 | | 0.7532 |
| I | 7 | 6.80 (1.72) | | 4.45 (1.72) | |
| II | 24 | 6.03 (1.79) | | 5.05 (1.87) | |
| III | 23 | 6.31 (1.72) | | 4.99 (1.93) | |
| Positive nodes | | | 0.0904 | | 0.8798 |
| 0 | 31 | 6.65 (1.88) | | 5.04 (2.05) | |
| 1–3 | 14 | 5.35 (1.41) | | 4.78 (1.46) | |
| >3 | 9 | 6.20 (1.19) | | 4.74 (1.70) | |
| Estrogen receptors | | | 0.2776 | | 0.7052 |
| Positive | 37 | 6.07 (1.79) | | 4.87 (1.93) | |
| Negative | 13 | 6.65 (1.60) | | 5.09 (1.70) | |
| Progesterone receptors | | | 0.5230 | | 0.8376 |
| Positive | 36 | 6.37 (1.66) | | 4.90 (1.84) | |
| Negative | 14 | 6.04 (1.89) | | 5.02 (1.94) | |

[a] CK19, cytokeratin 19; BM, bone marrow; PB (B-1), peripheral blood before surgery; BC, breast cancer.

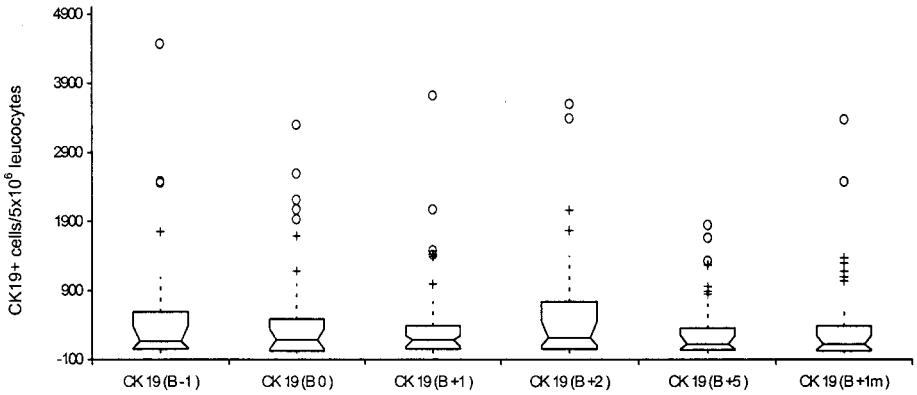


*Fig. 3* Box-Whisker plot comparative plots of the number of cytokeratin 19 (CK19)+ cells/5 × $10^6$ leukocytes in peripheral blood (PB) samples of breast cancer (BC) patients at different time points in relation to surgery: preoperative blood (B-1; $n = 50$); immediately after surgery (B0; $n = 53$); first day postoperatively (B +1; $n = 52$); second day postoperatively (B +2; $n = 50$); fifth day postoperatively (B +5; $n = 46$); and one month postoperatively (B +1m; $n = 46$), respectively.

found a concordance of positivity or negativity in 64% (Table 2).

There was no significant correlation between the presence of CK19+ cells in BM or PB (B-1) and the classical prognostic factors such as pathological tumor size, nodal involvement, stage, differentiation grade, and hormonal receptor status (Table 3).

For the various time points that were analyzed, we found the following median number of CK19+ cells/5 × $10^6$ leukocytes in PB: in (B0) 179 (95% CI, 48–341); in (B +1) 179 (95% CI, 86–243); in (B +2) 213 (95% CI, 98–451); in (B +5) 123 (95% CI, 49–252); and in (B +1m) 117 (95% CI, 43–244), respectively. Using the upper limit of the 95% CI of the control group (11) as a cutoff, 56, 57, 54, 48, and 48% of these samples, respectively, were considered CK19+ (Fig. 3). CK19 negativity was observed in preoperative blood (B-1) samples of some patients, whereas postoperative samples of the same patients were positive (Table 4).

There was no statistically significant difference between the different time points with respect to the average CK19+ cells ($P = 0.32$).

With a median follow-up of 56 months (range 45–63 months), seven of the analyzed 54 patients developed distant metastases. All except one patient with bone metastases had high levels of CK19+ cells in BM and/or PB (B-1) at diagnosis (Table 5).

*Table 4* Overview of CK19[a] positivity in peripheral blood before and after surgery in 41 patients

| Before surgery | Immediately after surgery | One month after surgery | Number of patients | % |
|---|---|---|---|---|
| + | + | + | 13 | 31.7 |
| + | + | − | 2 | 4.9 |
| + | − | + | 1 | 2.4 |
| + | − | − | 4 | 9.8 |
| − | + | + | 3 | 7.3 |
| − | + | − | 6 | 14.7 |
| − | − | + | 3 | 7.3 |
| − | − | − | 9 | 21.9 |

[a] CK19, cytokeratin 19; + and − indicate values above or below the cutoff, respectively (see text).

## DISCUSSION

The quantitative RT-PCR assay we developed also proved to be highly sensitive for detecting CK19+ cells in the BM of primary operable breast cancer patients as shown previously for PB (11). This is in agreement with the results published by other investigators (12, 13). The quantitative RT-PCR assay was reported to be a more sensitive technique than immunohistochemistry in detecting micrometastases in BM and PB of breast cancer patients (12–14).

Our study shows that BM is more likely to be positive than PB in patients with primary operable breast cancer [37 of 50 BM compared with 26 of 50 PB (B-1) samples], which is also consistent with findings of other studies (12, 14, 15).

When considering the positive and negative results, the concordance between preoperative blood and BM samples is high (64%), which is in agreement with the results of Schoenfeld *et al.* (14). But, when considering the positive results only, we detected much higher concordance (47%), compared with 27% obtained by Schoenfeld *et al.* (14).

We did not find any statistically significant correlation between the detected BM positivity for CK19 and classical prognostic factors such as tumor size, grade of differentiation, stage, nodal status, or hormonal receptor status. These results are consistent with two previous studies (16, 17), but in contrast to the work of Ikeda *et al.* (13), who found that positivity of axillary lymph node metastases and lymphatic vessel invasion were significantly higher ($P < 0.05$) in the CK19 PCR-positive patients than the negative patients.

We also found no correlation between the detection of CK19+ cells in preoperative blood samples (B-1) and the aforementioned classical prognostic factors. This issue has not yet been investigated in other studies.

It has long been thought that manipulation of malignant tumors encourages tumor cell dissemination. The evidence for this phenomenon in animal models and human malignant tumors is open to criticism because of the poor sensitivity of techniques used. Several studies have attempted to detect circulating tumor cells in patients with malignant disease, but the results have been unreliable because of problems associated with the isolation and identification of a minor subpopulation of tumor cells in blood (18, 19). Recent advances in molecular

*Table 5* Overview of the 7 patients who developed metastases

| Patient | Stage | BM[a] | PB (B-1) | Site of metastases | DFS (months) | Survival |
|---|---|---|---|---|---|---|
| 1 | I | + | − | Lung | 7 | Died at 15 months |
| 2 | I | − | + | Liver | 30 | Died at 49 months |
| 3 | II | + | + | Liver and bone | 48 | Alive at 62 months |
| 4 | II | − | − | Bone | 43 | Alive at 59 months |
| 5 | II | + | − | Bone | 43 | Alive at 59 months |
| 6 | III | + | − | Liver | 20 | Died at 33 months |
| 7 | III | + | + | Liver and bone | 13 | Died at 23 months |

[a] BM, bone marrow; PB (B-1), peripheral blood before surgery; DFS, disease free survival; + and − indicate values above or below the cutoff, respectively (see text).

biology have developed more sensitive techniques allowing re-examination of this important issue. Our study used the highly sensitive technique of real-time quantitative RT-PCR to detect CK19+ cells in PB of patients undergoing surgery for breast cancer.

Our results do not confirm the data of other studies, which suggested that tumor manipulations during operation for primary breast cancer induce tumor cell dissemination (6, 7). However, our results are consistent with two other studies where intraoperative sampling of effluent venous blood from colon (19) and of renal cancers (20) was investigated. Neither of these studies showed an effect of surgery on the rate of tumor cell shedding.

In our study, we found some patients whose blood samples were negative for CK19 preoperatively and turned positive postoperatively, which is in accordance with other studies (8, 9). These studies concluded that surgical manipulations of primary colorectal (8) and prostatic cancer (9) induce tumor cell dissemination. However, our study differs in two respects. First, positivity was detected in 52% of preoperative blood samples *versus* 26% in the study of Weitz *et al.* (8) *versus* 21% in the study of Eschwege *et al.* (9). This high positivity in preoperative blood samples in our study suggests that breast cancers continuously shed malignant cells in the circulation, even before surgery. This favors the belief that breast cancer is a systemic disease. Second, the overall statistical analysis failed to yield a significant difference between the different time points with respect to the average number of CK19+ cells in our study ($P = 0.32$). Therefore, surgery may have no obvious effect on the shedding of tumor cells.

It is as yet unclear whether intraoperative tumor cell shedding represents a significant event in the development of metastatic disease. The observation that some patients in our study showed CK19+ cells in the preoperative blood sample and subsequently turned negative postoperatively, may be consistent with studies of Fidler *et al.* (2).

The presence of tumor cells in the blood stream does not necessarily indicate that metastases will develop (21). Conditions that allow growth of epithelial cells at metastatic sites are largely unknown, but undoubtedly include the appropriate microenvironment for tumor cell growth (*e.g.*, hormonal milieu, oxygenation, nutrients, or growth factors), and an environment for the formation of new blood vessels (angiogenesis). The factors determining the length of the period from the dissemination of tumor cells to the appearance of clinically manifest metastases are unclear (22).

Interestingly, after a median follow-up of 12 years, Mansi *et al.* (23) found that 22 of 89 patients who had micrometastases in BM, detected by immunohistochemistry at presentation, remained alive with no relapse, and an additional eight patients with micrometastases at presentation died from a cause unrelated to breast cancer with no known relapse before death. An additional nine patients had relapsed only locally, seven of whom remained alive, and the other two died from causes unrelated to breast cancer.

Worthy of note, only one of the patients found to be CK19 negative by our technique relapsed after a median follow-up of 56 months, whereas six of the CK19+ patients developed distant relapse. Nevertheless, this follow-up is short, and too few events have occurred to draw reliable conclusions concerning the prognostic value of assaying CK19 cells in blood and BM.

In conclusion, the real-time quantitative RT-PCR is highly sensitive in detecting CK19+ cells considered as surrogate markers for the presence of epithelial neoplastic cells in PB and BM, but with higher sensitivity in the latter. The assay used showed no correlation with the established classical prognostic factors. Preliminary results show that surgical manipulations do not obviously influence the shedding of CK19+ cells in the bloodstream. Larger studies with longer follow-up periods are now required to evaluate the prognostic value of detecting circulating tumor cells in PB and BM, and their clinical implications. Future work at our laboratory will attempt to develop techniques to understand the exact nature of the detected CK19+ cells in breast cancer patients and to determine their clonogenic ability to grow and develop metastases.

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