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New ways to optimize breast cancer treatment

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Real time quantitative RT–PCR and immunostaining for detection of circulating tumor cells in breast cancer patients

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ABSTRACT

Introduction: the predictive value of conventional staging in breast cancer is limited, and more sensitive staging methods may be useful clinically in selecting patients for adjuvant systemic therapy. The aim of this study was to evaluate detection methods for cells positive for epithelial glycoprotein-2 (EGP-2) and cytokeratin 19 (CK19), using immunostaining and real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). **Patients and methods:** from 59 breast cancer patients, randomized to receive standard- or high-dose chemotherapy and peripheral blood stem cell (PBSC) transplantation, peripheral blood samples (PB, collected prior to, during and after chemotherapy treatment, 145 samples) and PBSC samples (in the high-dose group, 29 samples) were collected. Immunostaining was performed using EGP-2 and CK19 antibodies. Controls were: 11 healthy volunteer PB samples, and 3 PBSC samples from patients with hematologic malignancies. Detection limits were 1 tumor cell of breast cancer cell line MCF-7 in $2 \cdot 10^6$ leukocytes for immunostaining, and 1 MCF-7 in 10^6 leukocytes for qRT-PCR. **Results:** Two PB samples from 2 patients (3%) were tumor positive with EGP-2 immunostaining. Expression was found of EGP-2 mRNA in twelve samples (5 PBSC, 7 PB) from 12 patients (20%), and for CK19 mRNA in one other PB sample (1 patient, 2%). One patient had one immunostaining and a qRT-PCR positive sample, but at different time-points. Controls were negative with both immunostaining and qRT-PCR. **Conclusions:** qRT-PCR and immunostaining with two markers were used for detection of micrometastatic breast cancer in sequential PB and PBSC samples. Of 59 patients, 3% had an immunostaining positive sample, and 22% had a sample positive for EGP-2 or CK19 mRNA expression. The clinical implications of these findings will have to be clarified in large studies with clinical follow-up data.

INTRODUCTION

Staging of breast cancer patients, to determine prognosis and treatment, is largely based on tumor size and axillary node-status. However, the prognostic value of node-positive cancer based on conventional analysis is limited, and 40% of women with tumor positive lymph nodes survive more than 10 years (1) without developing distant metastases. Only part of these women are likely to benefit from adjuvant systemic treatment, and more sensitive staging methods may facilitate the clinician to make a better selection of these patients (2, 3). Particularly in view of the use of adjuvant high-dose treatment, requiring haematopoietic stem cell transplantation to counteract profound bone marrow aplasia, this selection may have clinical impact. In the setting of early adjuvant systemic treatment, the ability to monitor the effects of treatment directly with a known substrate, might lead to improved treatment modalities. Detection of tumor cells in hematopoietic stem cells products may provide information on their clinical impact, in view of stem cell transplantations (3). Therefore, the detection of breast cancer micrometastases (by means of immunohistochemistry or the polymerase chain reaction technique) has gained interest in recent years (4-27).

Conventional immunostaining techniques were described to allow visual confirmation of the actual nature of the stained (tumor) cells, but they appear to be very laborious of nature (2-10). From RT-PCR based methods, targeting tumor-associated tissue-specific antigens, the high sensitivity was recognized early (15-17), but the risk of the risk of giving false positive results soon followed (18-23). In view of this, we examined the value of the detection of micrometastases by means of immunostaining as well as a real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) method in peripheral blood and peripheral blood stem cell samples of breast cancer patients. Two epithelial markers were

used: epithelial glycoprotein-2 (EGP-2) and cytokeratin 19 (CK19). EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein. As a pan-epithelial/carcinoma marker, EGP-2 is universally expressed in breast cancer specimens (28), and has been used as a target antigen in a number of carcinoma-directed immunotherapeutical approaches (29-31). Results were compared with tumor cell detection based on the intracellularly located tissue-specific cytokeratin CK19, commonly used for the detection of micrometastases in solid tumors (12, 13). The analyses were performed on a unique series of sequential peripheral blood and stem cell samples from breast cancer patients randomized to receive either standard- or high-dose treatment and stem cell transplantation. The parallel use of these detection methods with two well-known markers for cancer detection, on a large collection of blood and stem cell samples has not been described before. This setting provided the opportunity to study their value for detection of micrometastases in breast cancer patients, before and during standard- or high-dose adjuvant treatment.

PATIENTS AND METHODS

Patients

Patients included in this study participated in a national randomized adjuvant breast carcinoma study (32). Chemotherapy naive breast cancer patients with four or more tumor-involved axillary lymph nodes (stage II and III, according to the TNM system of the Union Internationale Contre le Cancer, 1997), ≤ 55 years of age with negative chest X-ray, liver ultrasound and bone scan, were randomized to receive 5 courses of standard-dose chemotherapy followed by radiotherapy, or 4 courses of the same combination chemotherapy followed by high-dose chemotherapy, peripheral blood stem cell (PBSC) transplantation and radiotherapy. From now on, these groups will be referred to as the standard-dose group, and the high-dose group, respectively. The combination chemotherapy consisted of 5-fluorouracil (500 mg/m²), epirubicin (90 mg/m²) and cyclophosphamide (500 mg/m²), administered intravenously 1x/3 weeks. For the high-dose group, PBSC were mobilized following the third or last course of FEC with daily subcutaneous recombinant human granulocyte-colony stimulating growth factor (rhG-CSF, 263 μ g), from day 2 of the course. Leucapheresis was performed from day 9 of this course by means of continuous flow cell separation, until $\geq 5.10^6$ CD34+ cells/kg body weight (as determined by flow cytometric analysis with the fluorescein isothiocyanate-labelled anti-CD34 antibody directed against the HPCA-2 epitope on CD34+ cells, Becton Dickinson, Leiden, the Netherlands) were obtained. High-dose chemotherapy consisted of cyclophosphamide (1,500 mg/m²), thiotepa (120 mg/m²) and carboplatin (400 mg/m²) on days -6, -5, -4 and -3, followed by reinfusion of PBSC on day 0. After reinfusion, daily subcutaneous rhG-CSF was administered until the leukocyte count exceeded 3.10^9 /L. Locoregional radiotherapy (50 Gy in 25 fractions) was administered after completion of the chemotherapy scheme with sufficient bone

marrow recovery (defined as platelets $>100.10^9/L$). Oral tamoxifen 40 mg daily was administered after platelet recovery for at least two years, in both groups. The study, and the collection of blood or PBSC samples as described, was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients, enrolled in the University Hospital Groningen for the national randomized study, were approached to participate for collection of blood samples (and PBSC samples, in the high-dose group) from March 1997 until May 1999. All participating patients gave informed consent.

Sampling times

Sampling times were: t_0 : directly prior to start of chemotherapy (peripheral blood); t_1 : day 9, 10 or 11 after the third or fourth course of FEC (peripheral blood in the standard-dose group or PBSC material in the high-dose group); t_2 : 6 to 8 weeks after completion of chemo- and radiotherapy.

Collection and isolation of nucleated cells from patient samples

Peripheral blood samples (of 40 mL each), and PBSC samples (5 mL) were collected in tubes containing ethylenediamine tetraacetate (EDTA) as anti-coagulant, after prior collection of a waste amount of sample (2 mL). Samples were put on ice immediately. Erylysis was performed with an ammonium chloride solution (155 mM NH_4Cl , 10 mM potassium hydrogen carbonate, 0.1 mM sodium EDTA). The cells to be used for RNA analysis (3/4 of the total amount of cells) were transferred into a guanidinium-isothiocyanate solution (GITC solution: 4 M guanidinium-isothiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium-citrate pH 7.0 and 0.1M β -mercaptoethanol) and kept at $-20^\circ C$, until further processing. Control blood samples were collected from healthy volunteers (laboratory co-workers, after consent), and processed in the same fashion as described above, to be used as negative controls in the qRT-PCR and

immunostaining methods described below. Also, the sensitivity of this qRT-PCR method was determined in control healthy donor leukocytes in which (EGP-2 and CK19 positive) breast cancer cell line MCF-7 tumor cells were spiked in various dilutions. Control PBSC material was obtained from 3 non-breast cancer patients (with a haematological malignancy, after informed consent), and processed in a similar fashion.

The cells to be used for immunostaining (1/4 of the total amount of cells) were allowed to sediment for 1 h onto slides, through a metal funnel-like device, allowing the analysis of $1 \cdot 10^6$ cells on one slide. Cells were then fixed for 10 min at -20°C , in a solution of 4% acetic acid in methanol, followed by 10 min at -20°C , in acetone. Slides were stored at -20°C until further processing. Of each patient sample, one slide was used for Giemsa staining to assess morphology.

Immunostaining

Nucleated cell slides from blood samples were pre-treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity of nucleated cells, followed by rinsing with phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 mM KH_2PO_4 , pH 7.4). At least two of these slides (e.g. $2 \times 1 \cdot 10^6$ cells) were immunostained with each primary antibody. Samples were stained with the monoclonal antibody directed against EGP-2 (MOC31), using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a secondary antibody (Dako, Glostrup, Denmark) and 3-amino-9-ethylcarbazole as substrate. Samples were counterstained with hematoxylin. The antibody directed against cytokeratin was anti-CK19 (clone 170.2.14, Roche Diagnostics, Almere, The Netherlands), diluted 1:250 on slides in PBS, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands). Prior to staining with the CK19 antibody, slides were treated with a 0.01% trypsin (Life Technologies,

Breda, The Netherlands) solution in 0.1% CaCl₂ in 0.1 M Tris (hydroxymethyl) aminomethane solution, pH 7.8, at 37°C for 5 min, and the indirect immunoperoxidase staining procedure was used as described above.

Isotype specific controls for MOC31 and CK19 were performed using primary antibody mouse IgG1 (X0943, Dako, Glostrup, Denmark). In each staining procedure, a negative control was included using PBS, 1% BSA without the primary antibody, and a positive control prepared from MCF-7 cells on slides. Patient samples were independently examined for morphological features by a technician as well as a pathologist.

In slides containing leukocytes from healthy controls (n=11), no CK19 or MOC31 positive cells were observed. In spiking experiments of MCF-7 cells, diluted in leukocytes, 1 tumor cell could be detected in $2 \cdot 10^6$ leukocytes total, e.g. the total number of cells analyzed.

RNA isolation

RNA isolation from all samples was performed by means of the Rneasy Mini Kit (Qiagen, Westburg b.v., Leusden, The Netherlands), according to the manufacturer's instructions. Briefly, a quantity of cell lysate corresponding with $5 \cdot 10^6$ nucleated cells was obtained. One volume of 70% ethanol was added to the lysate, and mixed well by pipetting. The mixture was applied to a spin column, to allow adsorption of RNA to the membrane, and the column was subsequently centrifuged at 8,000 g, for 30 sec. Columns were washed and the RNA sample was eluted from the column by means of RNase free water, after centrifugation at 8,000 g for 1 min, and maintained in 50 μ L Rnase free water.

The integrity of the RNA samples was assessed on formaldehyde-containing (2.2 M) agarose gels. Only RNA samples with visible and discrete 28S and 18S ribosomal RNA bands were used for the RT-PCR experiments.

Quantitative RT-PCR (qRT-PCR) for assessing EGP-2 and CK19 expression

The qRT-PCR was performed by means of the LightCycler (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The LightCycler system is based on the continuous monitoring of the formation of PCR product by measuring the amount of hybridization probes annealing to the target sequence in every cycle. Hybridization probes emit a fluorescent signal, that correlates to the concentration of target. The sequence of specific primers and probes was checked prior to use to avoid amplification of genomic DNA or

pseudogenes (CK19), by means of software packages SeqMan 3.57 and PrimerSelect 3.10 (DNASTAR Ltd., London, U.K.). A volume of 5 μ L total RNA in 20 μ L master mix (Roche Diagnostics, Mannheim, Germany), 3 pmol of the specific primers and 2 pmol of the specific hybridization probes was added to each glass capillary. The sequences of the EGP-2 specific primers were: EX3F: 5'-GAACAATGATGGGCTTTATG-3' (corresponding to bases 374 to 394 of the EGP-2 cDNA) and EX7R: 5'-TGAGAATTCAGGTGCTTTTT-3' (bases 868 to 888). The sequences of the EGP-2 specific probes were: ALL-FL: ATCCAGTTGATAACGCGTTGTGAT-x and ALL-NEW: Red 640-TCCTTCTGAAGTGCAGTCCGCAA-p. The sequence of the CK19 specific primers were: CK19 F: 5'-ACTACAGCCACTACTACACGAC-3' (corresponding to bases 409-430), and CK19 R: CAGAGCCTGTTCCGTCTCAAAC (corresponding to bases 557-536). The sequences of the CK19 specific probes were: CK19-FL: TGTCCCTGCAGATCGACAACGCCC-x (corresponding to bases 485-507), and CK19-705: Red 705-TCTGGCTGCAGATGACTTCCGAACCA-p (corresponding to bases 509-534). The detection of EGP-2 or CK19 target was performed in separate glass capillaries.

Samples were submitted to an initial reverse transcriptase hybridization step of 25 min at 55°C, followed by a denaturation step of 30s at 95°C, then amplification in a three-step cycle procedure (denaturation 95°C, ramp rate 20°C/s; annealing 56°C, 15s, ramp rate 20°C/s; and extension 72°C, 20s, ramp rate 2°C/s) for 45 cycles. Finally, a melting three step cycle was performed (95°C, ramp rate 20°C/s; 60°C, 10s, ramp rate 20°C/s; 95°C, ramp rate 0.2°C). On several occasions, after the LightCycler procedure, the contents of the glass capillaries were also checked on gel, always conveying the expected PCR product for the specific primers in tumor positive samples. In all runs, a standard curve was obtained using the same samples of RNA from dilutions of MCF-7 cells in healthy donor leukocytes (1:10; 1:10²; 1:10³; 1:10⁴; 1:10⁵; 1:10⁶ respectively), with a total of 1.10⁶ cells. All

results were always expressed in relation to the standard curve. Corrections were made for the expression of β -2 microglobulin. The sequence of the β -2 microglobulin specific primers were: β -2 microglobulin F: CCAGCAGAGAATGGAAAGTC (corresponding with bases 33 to 52) and β -2 microglobulin R: GATGCTGCTTACATGTCTCG (corresponding with bases 301-282). The sequences of the β -2 microglobulin specific probes were: β -2 microglobulin-FL: TTCTTCAGTAAGTCAACTTCAATGTCGGA-x (corresponding to bases 118-90) and β -2 microglobulin-705: LC Red 705-ATGAAACCCAGACACATAGCAATTCAG -p (corresponding to bases 86-60). As a positive control, the RNA from undiluted MCF-7 cells was used; as a negative control, a sample was used to which no RNA at all was added. Also in each experiment, a sample containing healthy donor leukocytes without tumor cells was included as a negative control.

With this method, a sensitivity was obtained of 1 spiked MCF-7 cell, detected in 1.10^6 healthy donor leukocytes (n=3 separate experiments). In leukocyte samples from n=11 healthy donors, the EGP-2 or CK19 signal remained below this detection limit.

Statistics

Statistical analyses were performed by means of the SPSS statistical software package. The relations between PCR or immunostaining and tumor size, number of tumor positive lymph nodes, the presence of tumor in the apical lymph node, and tumor characteristics were analyzed by means of the Pearson correlation test. Only p-values <0.05 were considered significant.

RESULTS

Patients

A total of 59 breast cancer patients participated (of n=60 eligible patients) in this study from March 1997 until May 1999. The standard-dose group consisted of 29 patients, and the high-dose group of 30 patients. Mean age at the start of treatment was 45.1 years (range 28-56 years) and 45.7 years (range 33-54 years) in these groups respectively (N.S.). Patient characteristics are reflected in table 1. A total of 174 samples were collected (59 at t_0 , 57 at t_1 of which 29 PBSC samples, and 58 at t_2).

Immunostaining

None of the 174 blood samples, were found to contain tumor cells in the nucleated cell slides for morphology assessment (Giemsa). With immunostaining, nucleated cell slides from blood samples were found to contain a cell staining positive with MOC31, identified as being a tumor cell, in two cases: one patient of the standard-dose group at t_1 , and one patient of the high-dose group at t_1 (PBSC material). Therefore, 2 patients out of 59 total (3%) had a tumor positive sample by immunostaining. In none of the other samples immunostaining positive (tumor) cells or cell fragments were identified, neither with MOC31 or the antiCK19 antibody.

Real time qRT-PCR for the expression of EGP-2 and CK19

An overview of samples positive for immunostaining or qRT-PCR is given in table 2. In a total of 12 patient samples of 12 patients, EGP-2 mRNA expression was found: in the standard-dose group, at t_0 (1 peripheral blood sample: expression equivalent to 28 MCF-7/10⁶ leukocytes) and t_2 (3 peripheral blood samples: expressions equivalent to 15 to 17 MCF-7/10⁶ leukocytes). In the high-dose group, EGP-2

mRNA expression was found at t_1 (5 PBSC samples: expressions equivalent from 15 to 29 MCF-7/ 10^6 leukocytes), and at t_2 (3 peripheral blood samples: expressions equivalent from 14 to 29 MCF-7/ 10^6 leukocytes). In one peripheral blood sample, CK19 mRNA expression was found: in the standard-dose group, at t_0 . The expression in this sample was equivalent to 10 MCF-7/ 10^6 leukocytes of the standard. Thus, of 29 PBSC samples, 5 were qRT-PCR positive and 1 was immunostaining positive, while of 145 peripheral blood samples, 8 were positive with qRT-PCR and 1 with immunostaining.

The peripheral blood sample positive for CK19 mRNA expression was not found positive for EGP-2 expression. One peripheral blood sample found positive for EGP-2 mRNA expression (standard-dose group, t_0), belonged to a patient with a peripheral blood sample at t_1 , containing an EGP-2 positive tumor cell with immunostaining (figure 1). Thus, a total of 13 patients out of 59 (22%) had a sample positive for either EGP-2 or CK19 mRNA expression. However, none of the samples found positive for EGP-2 or CK19 mRNA expression were positive with immunostaining.

No relations between the mRNA expression of EGP-2 or CK19 and tumor size or number of tumor positive lymph nodes was found, except that EGP-2 mRNA expression at t_2 was positively correlated to primary tumor size ($r=0.409$, $p=0.001$). The follow-up ranging from 40 to 12 months after finishing chemotherapy, was considered too modest to allow relating follow-up data to immunostaining or qRT-PCR data.

DISCUSSION

In this study, the detection of micrometastatic breast cancer was explored, by means of a qRT-PCR method, as well as immunostaining. Two markers were used: EGP-2 and CK19. The analyses were performed on a series of sequential samples of blood as well as stem cell material, of breast cancer patients receiving either adjuvant standard- or high-dose chemotherapy including haematopoietic stem cell transplantation. Detection of circulating tumor cells with two markers, by means of immunostaining and qRT-PCR, in this setting has not been described before. In many studies, techniques for detection of micrometastatic disease have been examined (4-27), but as yet in solid tumors, no optimal technique is available. Conventional immunostaining techniques are now acknowledged for allowing visual confirmation of the actual nature of the stained (tumor) cell, but they may be susceptible to inter-observer differences (33). PCR based methods are found to harbor the risk of false positive results with the use of tumor-associated, tissue-specific markers in solid tumors (18-23). In view of this, we chose to combine these two methods in this study. For the detection of circulating tumor cells, in some studies used only a PCR based method was used (15, 21, 25, 27), while this methods was combined with immunostaining in other studies (16, 23, 24, 26),

In addition, we evaluated the presence of both EGP-2 and CK19 positive cells. CK19 is the most commonly used marker for the detection of micrometastatic breast cancer (12, 13), and as such it was used to compare with EGP-2. Both markers can give false positive results, as described in the evaluation of peripheral blood (19, 21, 23). Therefore, we used the qRT-PCR method to quantify expression levels, in order to establish a 'cut-off' point to exclude low-level or insignificant EGP-2 or CK19 mRNA expression. No positive results for either marker were observed in peripheral blood from healthy controls in the present study. This important finding

is in contrast to the earlier studies and may be explained by the fact that no separate cDNA processing steps were used with the present qRT-PCR method, in which RNA is directly used for the analysis. This may reduce the risk of contamination with external epithelial targets. This contamination is known to induce false positive results in the setting of micrometastases detection in breast cancer (33, 34). Furthermore, it is clear that the real time evaluation allows the detection of a signal at the earliest stage, providing a more accurate indication of minimal amounts of tumor cells than the older RT-PCR methods, that measure at an end-point (15-17, 19). With all samples, the results on mRNA expression of either EGP-2 or CK19 were related to the standard of MCF7 cells diluted in control leukocytes. This was done, not for speculation on the amount of tumor cells present in the patient sample (as tumor cell lines likely have higher expression levels than patient tumor cells (33), but solely for comparisons of all the analyzed materials. The isolation of the nucleated cell fraction was performed by means of lysis of erythrocytes. This method was described to preserve tumor cells in a fashion superior compared to the frequently used Ficoll isolation (35).

Most studies on molecular detection of micrometastases have relied on a single mRNA marker, usually CK19 (15-17, 24-27). The use of multiple-marker assays for solid tumors has been described before (36, 37) including breast cancer micrometastases (38, 39). One advantage of the use of more than one marker may be the improved specificity of molecular detection methods. The expression of more than one target gene is usually not found in control tissue (39), and may be related to poor prognostic clinico-pathologic factors (38). In view of the fact that, similar as in tumor cell lines (11), the expression of tumor-associated tissue specific markers in primary tumors can vary considerably (unpublished data), it can be postulated that not all tumors are likely to express all the selected markers. It may therefore be considered preferable to use multiple-marker assays to improve detection sensitivity

rather than specificity, by selecting for expression of at least one marker (40). The clinical justification for either one of these approaches remains to be examined. In this study, a total of 15 samples of 14 patients had evidence for the presence of EGP-2 or CK19 positive cells. Two samples were positive with immunostaining, and 13 samples showed either EGP-2 or CK19 mRNA expression. None of the samples showed expression of both markers at the same time, or were positive for mRNA expression as well as immunostaining. In this case therefore, if the aim of using the two markers would be to improve specificity of the qRT-PCR, none of the samples could be regarded as positive. With the opposite approach, to improve sensitivity, 13 samples would be regarded as positive. It has been suggested that recruitment of tumor cells into peripheral blood cannot be confirmed by RT-PCR alone, and that immunostaining should be performed as validation (33). In our study, the application of this suggestion implies that again, none of the 13 samples, positive for mRNA expression, would be considered tumor positive. While this approach is likely valuable in detecting micrometastases in lymph nodes, the setting of tumor cells in the circulation harbors the realistic risk of sampling error between PCR and immunostaining samples, which implies that tumor cells are not necessarily present in both samples. Of 59 patients in this study, 3% had a tumor positive sample by immunostaining (both for EGP-2), and 22% had a sample positive for EGP-2 or CK19 mRNA expression (2% for CK19, and 20% for EGP-2). Most EGP-2 mRNA positive samples were observed at t_1 , in the PBSC samples of the high-dose group. In an early report by Brügger et al. (3), it was suggested that tumor cells, detected by CK19 immunostaining, were possibly mobilized into the peripheral blood simultaneously with haematopoietic stem cells. In this study, one PBSC sample was found positive for tumor cell presence with immunostaining for EGP-2, but not for CK19. The EGP-2 mRNA expression in the PBSC samples of 5 patients (a considerable part of patients in the high-dose group: 17%) may originate from the

presence of tumor cells. The high incidence of qRT-PCR positivity in PBSC compared to peripheral blood samples, may in part be explained by the fact that PBSC material already contained relatively more mononuclear cells compared to peripheral blood, due to the leucapheresis selection procedure (31). Whether haematopoietic growth factor G-CSF, used for obtaining PBSC, may induce EGP-2 mRNA expression (as described for tissue specific marker CEA, 41), is as yet unknown.

The patients in this study all had node positive breast cancer, but no distant metastases. In other studies with breast cancer patients of comparable disease stages, the incidence of positive peripheral blood samples varied with immunostaining from 5 to 9% (16, 26) and 13 to 36% with (q)RT-PCR (16, 25-27). In a majority of metastatic breast cancer patients, Smith et al. (24) could monitor disease response using a qRT-PCR for CK19 expression. No previously published data are available on EGP-2 mRNA expression in peripheral blood patient samples. Notwithstanding differences in techniques, our results appear to be in line with those previously published. Like others, we find a lower incidence of tumor cell positive samples with immunostaining than with the qRT-PCR. The clinical implications of this however, are not yet clear.

In conclusion, the use of qRT-PCR and immunostaining for detection of micrometastatic breast cancer disease was evaluated in a series of sequential peripheral blood samples and peripheral blood stem cell material. Of 59 patients, 3% had an immunostaining positive sample, and 22% had a sample positive for EGP-2 or CK19 mRNA expression. The clinical implications of these findings will have to be clarified in larger studies with long-term clinical follow-up data.

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TABLE 1: Patient characteristics

		<u>Standard-dose (n=29)</u>	<u>High-dose (n=30)</u>
<u>Age</u> (years)		45.1 (28-56)	45.7 (33-54)
<u>Stage</u>	IIA	9	5
	IIB	18	18
	IIIA	2	7
<u>LN*</u>	< 10	19	19
	≥ 10	10	11
<u>Tumor size</u> (cm)		2.7 (SD 1.4)	3.9 (SD 2.9)

* lymph nodes containing tumor as assessed by standard morphological examination

TABLE 2: positive samples with immunostaining or qRT-PCR

	Immunostaining		QRT-PCR	
	<u>EGP-2</u>	<u>CK19</u>	<u>EGP-2</u>	<u>CK19</u>
T₀ <u>standard-dose</u>	0	0	1 *	1
<u>high-dose</u>	0	0	0	0
T₁ <u>standard-dose</u>	1 *	0	0	0
<u>high-dose</u>	1 +	0	5 +	0
T₂ <u>standard-dose</u>	0	0	3	0
<u>high-dose</u>	0	0	3	0

* one patient from the standard-dose group had a blood sample positive at t₀ for EGP-2 mRNA expression, as well as an immunostaining positive sample at t₁.

+ The samples of the high-dose group at t₁ were PBSC samples

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