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## New ways to optimize breast cancer treatment

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# Detection of micrometastatic breast cancer by means of real time quantitative RT–PCR and immunostaining in perioperative blood samples and sentinel nodes

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## ABSTRACT

**Introduction:** the predictive value of conventional staging in breast cancer is limited, and more sensitive staging methods may be valuable in selecting patients for adjuvant systemic therapy. The aim of this study was to detect 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 58 breast cancer patients, 52 primary tumors, 75 sentinel nodes (SN) and 149 peripheral blood (PB) samples (from before, during and 4 days after operation) were examined. Immunostaining was performed with antibodies directed against EGP-2 and CK19. Detection limits were 1 MCF-7 breast cancer cell line cell/ $2 \cdot 10^6$  leukocytes (immunostaining) and 1 MCF-7 cell/ $10^6$  leukocytes (qRT-PCR). Control non-cancer lymph nodes (10) showed aspecific CK19 staining, but were qRT-PCR negative; control healthy volunteer PB (11) was always negative. **Results:** primary tumor samples, all positive with immunostaining, showed a wide variation of EGP-2 ( $>10^4$  fold) and CK19 mRNA expression ( $>10^3$  fold). SN (n=19) from 16 patients were tumor-positive with routine haematoxylin-eosin (H&E) and/or immunostaining. SN tumor presence was positively correlated to qRT-PCR expression, but 3 tumor-positive SN were false negative with qRT-PCR. Three SN were qRT-PCR positive, while tumor negative with H&E and/or immunostaining. No immunostaining positive PB was observed, but 19 patients (33%) had one or more qRT-PCR positive PB samples. **Conclusions:** Primary tumors have varying expressions of EGP-2 and CK19 mRNA. Both markers can be used in qRT-PCR to obtain adequate sensitivity for single tumor cell detection. In SN, immunostaining appears more sensitive/specific than H&E or qRT-PCR for tumor detection. In PB, no immunostaining positivity was found, while 33% of patients had qRT-PCR positive PB. The clinical implications of these findings will have to be clarified in large studies with long-term clinical follow-up data.

## INTRODUCTION

Staging of breast cancer patients, to determine prognosis and treatment, is largely based on assessment of tumor size and axillary node-status. However, the prognostic value of finding node-negative cancer based on conventional analysis is limited, and distant metastases develop in 20 to 30% of women with negative nodes (1). Although primary tumor characteristics may be helpful in selecting patients at risk of metastatic disease (2), the identification of new markers which predict the necessity of giving early adjuvant systemic treatment has gained much interest in the last decade. In view of this, the ability to detect micrometastases in lymph nodes, bone marrow and blood was examined in recent years (3-5). Careful evaluation of tumor cell presence in the sentinel lymph node, the first node in the lymphatic basin of the breast tumor, is particularly important. Removal of the sentinel node is increasingly used as an alternative staging procedure for total axillary lymph node dissection (6), in view of decreased morbidity in breast cancer patients. In addition to evaluation of the presence of lymph node micrometastases, it may be useful to examine early hematogenous spread of tumor cells. Recent immunocytochemical studies (7, 8) indicated that the ability of breast tumor cells to expand to the bone marrow hematogenously is independent of their ability to metastasize to axillary lymph nodes. Detection of micrometastatic disease by means of immunostaining or PCR based methods have been examined. The limitations of these methods have become clear over the last years. Conventional immunostaining techniques allow assessment of the actual nature of stained (tumor) cells, but appear to be very laborious (3-5). RT-PCR based methods, targeting tumor-associated, tissue specific antigens, were first considered to have a high sensitivity (9-11), but were found later to harbor the risk of giving false positive results (12-21).

The aim of the present study was to evaluate the use of two markers: epithelial glycoprotein-2 (EGP-2) as well as cytokeratin 19 (CK19) using immunostaining as well as a real time quantitative RT-PCR, and to relate this to standard morphology assessment, in the detection of micrometastatic breast cancer. 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 (22). It has been used as a target antigen in a number carcinoma-directed immunotherapeutical approaches (23-24), as well as for detection of single tumor cells (25). CK19 is an intracellularly located tissue-specific marker, commonly used for detection of micrometastatic disease in solid tumors (3-5). The analyses were performed on a unique series of corresponding primary breast tumors, sentinel nodes and sequential (perioperatively collected) peripheral blood samples. The parallel use of immunostaining as well as a qRT-PCR with two well known markers for cancer detection, on a large collection of patient samples has not been described before. This setting provides an opportunity to study the value of these methods in detecting micrometastases in breast cancer patients.

## PATIENTS AND METHODS

### **Patients**

Between April 1997 until July 1999, patients undergoing primary breast tumor surgery and sentinel lymphadenectomy in the University Hospital Groningen were eligible for the present study, which was approved by the Medical Ethical Committee of our institution. Written informed consent for the collection of samples was obtained from all participating patients. The sentinel lymphadenectomy procedure (26, 27) is performed since October 1996 at the University Hospital Groningen, on patients with an operable breast tumor that appeared malignant on clinical examination, imaging (mammography, ultrasonography or both) and fine-needle aspiration cytology, without clinically suspect axillary lymph nodes (28). Staging of patients was performed according to the TNM system (Union Internationale Contre le Cancer, 1997).

### **Primary tumor samples**

Fresh tumor samples were placed on ice directly following surgery. One part of the tumor was subsequently snap frozen, and the other part was formalin fixed and paraffin embedded for standard H&E staining and histological examination, including determination of grading. The snap frozen sections were maintained at -80°C until further use. Prior to RNA isolation or immunostaining, 25 µm sections were prepared. The presence of tumor was verified using H&E stained sections on either side of the sample. Sections for RNA isolation (ten 25 µm sections per tumor sample of approximately the same size) were transferred immediately 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 homogenized through a 20-Gauge syringe. Sections elected for immunostaining were transferred onto slides.

### **Sentinel nodes**

The sentinel node was removed after localization of the node through intralesional injection of patent blue dye (Bleu Patenté V, Laboratoire Guerbet, Aulanay-sous-Bois, France) and/or radioactive tracer (Nanocoll; Sorin Biomedica Diagnostics, Sallugia, Italy), and subsequent detection using the hand-held  $\gamma$ -detection probe (Neoprobe, Neoprobe Corporation, Dublin, OH) (26). Fresh sentinel nodes were placed on ice directly following surgery. Each node was divided in two pieces. One piece was paraffin embedded for routine pathological examination, including step sections and immunostaining using the pan-keratin antibody CAM5.2 (Becton Dickinson Benelux, Balthoven, the Netherlands). The other piece was snap frozen. Sentinel nodes were further prepared and handled as described for the primary tumor. Control non-cancer lymph nodes (n=10) were processed and analyzed identically to the sentinel nodes.

### **Collection and isolation of nucleated cells from blood samples**

Blood samples were collected prior to surgery ( $t_0$ ), following tumor removal during surgery ( $t_1$ ) and four days after surgery ( $t_2$ ). Samples (40 mL) were collected in tubes containing ethylene diamine tetra acetate (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  $\text{NH}_4\text{Cl}$ , 10 mM potassium hydrogen carbonate, 0.1 mM sodium EDTA). This method was shown to preserve possible tumor cells in a superior fashion compared to the frequently used Ficoll method (29). The cells to be used for RNA analysis (3/4 of the total amount of cells) were transferred into GITC, and kept at  $-20^\circ\text{C}$ , until further processing. Control blood samples were collected from healthy volunteers, and processed in the same fashion as described above, to be used as negative controls in the qRT-PCR method 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 cells were added in various dilutions.

The cells to be used for immunostaining (1/4 of the total amount of cells) were allowed to sediment for 1 hour onto slides, through a metal funnel-like device, allowing the analysis of  $1.10^6$  cells on one slide. Cells were fixed at  $-20^{\circ}\text{C}$  for 10 minutes, in a solution of 4% acetic acid in methanol, followed by 10 minutes at  $-20^{\circ}\text{C}$ , in acetone. Slides were stored at  $-20^{\circ}\text{C}$  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 minutes to block endogenous peroxidase activity of nucleated cells, followed by rinsing with phosphate buffered saline solution (PBS, 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  $\text{KH}_2\text{PO}_4$ , pH 7.4). At least two of these slides (e.g.  $2 \times 1.10^6$  cells) were immunostained with each primary antibody. Samples were stained with monoclonal antibody directed against EGP-2 (MOC31), using an indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as secondary antibody (Dako, Glostrup, Denmark) and 3-amino-9-ethylcarbazole as substrate. Samples were routinely counterstained with haematoxylin. The antibody directed against cytokeratin19 was anti-CK19 (clone 170.2.14, Roche Diagnostics, Almere, The Netherlands), diluted 1:250 on slides and 1:500 on tumor samples in PBS, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands). Prior to staining with the CK19 antibody, slides were pre-treated with a 0.01% trypsin (Life Technologies, Breda, The Netherlands) solution in 0.1%  $\text{CaCl}_2$  in 0.1 M Tris (hydroxymethyl) aminomethane solution, pH 7.8, at  $37^{\circ}\text{C}$  for 5 minutes, 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, whereas slides prepared to contain MCF-7 cells were used as positive controls. For tumor samples, as positive control in each procedure, a sample of MOC31 and CK19 positive breast cancer tissue was used. Patient samples were examined independently for morphological malignant features by a technician as well as a pathologist.

In slides containing leukocytes from 11 healthy controls, 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 a total of  $2 \cdot 10^6$  leukocytes (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, 350  $\mu$ L of cell lysate (or a quantity corresponding with  $5 \cdot 10^6$  nucleated cells, in the case of the blood samples) 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 seconds. 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 minutes, and maintained in 50  $\mu$ 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.

### **Real time quantitative RT-PCR (qRT-PCR) for assessment of 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 µL total RNA in 20 µL 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: TGTCTGCAGATCGACAACGCCC-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 hybridisation step of 25 minutes at 55°C, followed by a denaturation step of 30 seconds at 95°C, then amplification in a three-step cycle procedure (denaturation 95°C, ramp rate

20°C/second; annealing 56°C, 15 seconds, ramp rate 20°C/second; and extension 72°C, 20 seconds, ramp rate 2°C/second) for 45 cycles. Finally, a melting three step cycle was performed (95°C, ramp rate 20°C/second; 60°C, 10 seconds, ramp rate 20°C/second; 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; 1:10<sup>2</sup>; 1:10<sup>3</sup>; 1:10<sup>4</sup>; 1:10<sup>5</sup>; 1:10<sup>6</sup> respectively), with a total of 1.10<sup>6</sup> cells. All results were always expressed in relation to the standard curve, e.g. the number of MCF-7 cells in a total number of 1.10<sup>6</sup> cells (leukocytes). Samples of primary tumor and sentinel nodes were checked for the presence of tumor, and corrections were made for the RNA expression of  $\beta$ -2 microglobulin. The sequence of the  $\beta$ -2 microglobulin specific primers were:  $\beta$ -2 microglobulin F: CCAGCAGAGAATGGAAAGTC (corresponding with bases 33 to 52) and  $\beta$ -2 microglobulin R: GATGCTGCTTACATGTCTCG (corresponding with bases 301-282). The sequences of the  $\beta$ -2 microglobulin specific probes were:  $\beta$ -2 microglobulin-FL: TTCTTCAGTAAGTCAACTTCAATGTCGGA-x (corresponding to bases 118-90) and  $\beta$ -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<sup>6</sup> healthy donor leukocytes (n=3 separate experiments). In leukocyte samples from n=11 healthy donors, the EGP-2 or CK19 signal remained below the detection limit.

## **Statistics**

Statistical analyses were performed by means of the SPSS statistical software package. The relation of morphology H&E, immunostaining and qRT-PCR data (primary tumor, sentinel nodes and peripheral blood) and tumor size, grading and disease stage was analyzed by means of the Pearson correlation test. Comparisons of PCR expression data between the group with or without tumor (primary tumor, sentinel nodes and peripheral blood) were performed by the Mann-Whitney test. Only p-values <0.05 were considered significant.

## RESULTS

### **Patients**

From April 1997 until July 1999, 58 patients participated in this study. Patients' characteristics are given in table 1.

### **Primary tumors**

From 57 patients, the primary tumor could be obtained; in one case, no primary tumor was removed. From 3 patients, 2 different tumor lesions were removed in the same surgical procedure (2 patients had a tumor in both left and right breast; 1 patient had two separate lesions in the same breast). From the total of 60 removed tumors (tumor characteristics shown in table 1), 52 contained sufficient material to allow snap-freezing, in addition to the material needed for establishing a pathological diagnosis.

**Immunostaining:** Tumor content of the frozen samples was verified using H&E sections; in 46 samples carcinoma was detected. In the other 6 samples, H&E examination showed in situ carcinoma in 2 lesions, whereas in 4 lesions no residual carcinoma could be detected. Immunostaining using the MOC31 or anti-CK19 antibody was positive in all frozen sections.

**QRT-PCR:** the mean mRNA expression of EGP-2 in malignant tumors was equivalent to the expression of 17,693 (SEM 4,995) MCF-7/10<sup>6</sup> leukocytes; the mean mRNA expression of CK19 was 12,382 (SEM 2,058) MCF-7/10<sup>6</sup> leukocytes. EGP-2 mRNA expression correlated with CK19 mRNA expression ( $r=0.382$ ,  $p=0.006$ ) (figure 1). In figure 1, also the wide range of both EGP-2 and CK19 mRNA expression in tumors is illustrated. The mRNA expression of either EGP-2 or CK19 was not related to primary tumor size, tumor grading, disease stage or the presence of carcinoma in the frozen samples.

### **Sentinel nodes**

From 50 out of 58 patients, apparent sentinel nodes were detected and surgically retrieved. This retrieval rate is in line with the surgical learning-curve, of the period during which samples were collected for the present study (28). A total of 94 nodes was obtained from these patients. From 75 nodes of 44 patients, sufficient frozen material was available for qRT-PCR.

**Immunostaining:** Examination of the above 75 nodes for tumor contamination by means of H&E staining yielded 17 tumor-positive nodes in 14 patients. Immunostaining for EGP-2 and CK19 indicated the presence of tumor in two nodes from two additional patients. Thus, a total of 16 out of 44 patients had tumor-positive sentinel nodes, as indicated either by H&E or immunostaining (19 nodes). One node was tumor-negative with immunostaining, while positive for tumor in one adjacent H&E examined slide. The H&E slide on the opposite side of the part of the node, examined with immunostaining and qRT-PCR, was negative for tumor. This indicates that apparently no residual tumor tissue was present in the part of the node examined with immunostaining and qRT-PCR.

In all 10 control non-cancer lymph nodes, EGP-2 staining was negative, while 9 out of 10 nodes showed occasional CK19 positive dendritic reticulum cells.

**QRT-PCR:** similar as in the primary tumors, in the sentinel nodes a correlation was found between the mRNA expression levels of EGP-2 and CK19 ( $r=0.301$ ,  $p=0.047$ ). The mRNA expression of both EGP-2 and CK19 was related to the presence of tumor by means of H&E and/or immunostaining: the mean EGP-2 mRNA expression was equivalent to 24,010 (SEM 12,734) MCF-7/ $10^6$  leukocytes in tumor-positive sentinel nodes, and 69 (SEM 34) MCF-7/ $10^6$  leukocytes in tumor-negative nodes ( $r=0.361$ ,  $p=0.016$ ). The mean CK19 mRNA expression was equivalent to 15,704 (SEM 7,669) MCF-7/ $10^6$  leukocytes in tumor-positive sentinel nodes, and 124 (SEM 70) MCF-7/ $10^6$

leukocytes in tumor-negative nodes ( $r=0.385$ ,  $p=0.01$ ). Tumor-positive nodes had a higher mRNA expression of EGP-2 ( $p<0.001$ ) and CK19 ( $p=0.004$ ) than tumor-negative nodes. The above findings are shown in figure 2.

Three of the 19 sentinel nodes demonstrated to be tumor-positive with H&E and/or immunostaining, showed no/very low EGP-2 or CK19 mRNA expression. One of these nodes was tumor-positive with H&E (on one side) but not with immunostaining; as described above, apparently no residual tumor was left in the part of the node, examined with immunostaining and qRT-PCR. In the other 2 nodes, this may be caused by sampling errors as well, as only very few tumor cells were detected by H&E and immunostaining. The other 16 of the 19 sentinel nodes, demonstrated to be tumor-positive with H&E and/or immunostaining, were all positive with qRT-PCR. In these 16 nodes, tumor presence was observed on both sides of the part examined by immunostaining and qRT-PCR. Numbers are also given in table 2.

Of the remaining sentinel nodes, found tumor-negative with H&E and/or immunostaining, 3 nodes were shown to have mRNA expression of either EGP-2 or CK19 ( $>2x$  SD above the mean). All of these 3 nodes contained dendritic reticulum cells, staining positively for CK19 (example shown in figure 3), but no tumor cells.

In none of the control non-breast cancer lymph nodes, EGP-2 or CK19 mRNA expression was detected.

### **Peripheral blood samples**

From 58 patients, 149 blood samples were obtained. At  $t_0$  55, at  $t_1$  54 and at  $t_2$  40 samples were drawn. When  $t_2$  blood samples were not obtained, this was due to the fact that patients were already dismissed from the hospital.

**Immunostaining:** None of the 149 blood samples, were found to contain tumor cells in the slides for morphology assessment (Giemsa). Immunostaining with antibodies MOC31 and anti-CK19, showed that none of these samples contained EGP-2- or

CK19-positive tumor cells. However, in some samples (n=2), EGP-2-and CK19-positive cell fragments were observed: these were found not to represent (tumor) cells.

**qRT-PCR:** in a total of 31 samples from 19 patients, EGP-2 or CK19 mRNA expression was found (range EGP-2 expression 1-447 MCF-7/10<sup>6</sup> leukocytes in 16 samples; range CK19 expression 2-1,945 MCF-7/10<sup>6</sup> leukocytes in 15 samples). This is shown in figure 4. At t<sub>0</sub>, 13 samples were positive (7 for EGP-2 mRNA expression, and 6 for CK19 mRNA expression), at t<sub>1</sub>, 10 samples were positive (5 for EGP-2 and 5 for CK19), and at t<sub>2</sub>, 8 samples were positive (4 for EGP-2 and 4 for CK19).

The presence of positive samples for either EGP-2 or CK19 mRNA expression was correlated to CK19 mRNA expression in the sentinel node (r=0.363, p=0.015) and grading (r=0.329, p=0.014) of the primary tumor. In 7 patients, more than one consecutive peripheral blood sample was found positive for either EGP-2 or CK19 mRNA expression. The occurrence of more than one qRT-PCR positive blood sample was correlated to sentinel node expression of EGP-2 (r=0.425, p=0.004) and CK19 mRNA (r=0.330, p=0.029).

In 4 patients, 1 sample was found with mRNA expression of both EGP-2 and CK19. The occurrence of mRNA expression of both markers in one peripheral blood sample, was again correlated to sentinel node expression of EGP-2 (r=0.533, p<0.001) and CK19 mRNA (r=0.406, p=0.006). As in primary tumors and sentinel nodes, the mRNA expression of EGP-2 and CK19 was correlated in these peripheral blood samples (r=0.794, p<0.001).

## DISCUSSION

The possibility to detect micrometastatic disease in breast cancer, is still hampered by the fact that the optimal detection method including a clear marker for detection of minimal amounts of tumor cells, is still lacking. We applied two target markers, and two detection methods, to a series of matching tumor, sentinel node and blood samples of breast cancer patients.

The target markers were EGP-2 and CK19. Both these markers have been described to give false positive results in peripheral blood samples (12, 13, 15). The quantitative RT-PCR method was therefore used to establish a 'cut-off' point to exclude low-level or insignificant EGP-2 or CK19 mRNA expression. With the qRT-PCR, no false- positive results for either marker were observed in control peripheral blood or control lymph nodes 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 this qRT-PCR method, in which RNA is directly used for the analysis. This may reduce possible contamination with exogenous epithelial targets, leading to false positive results (20). 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 older RT-PCR methods, that measure at an end-point (9-11, 13). With all samples, the results on mRNA expression of either EGP-2 or CK19 were related to the standard of MCF7 tumor cells diluted in control leukocytes. This was done to allow comparisons of all the analyzed material, and not for speculation on the amount of tumor cells present in the patient sample -as tumor cell lines likely have different (i.e. higher) expression levels than patient tumor cells (20).

In primary tumors, EGP-2 as well as CK19 mRNA expression varied widely ( $>10^4$  fold, and  $>10^3$  fold respectively). Although the mRNA expression of EGP-2 and CK19 correlated in primary tumors, clearly there are certain tumors that do not have

a simultaneously high or low expression of both markers. Varying mRNA expression levels of tissue specific markers in breast cancers have not been described before. This finding has a number of implications. First, it may be difficult to quantify tumor load by assessing mRNA expression of tumor-associated tissue specific markers, even when mRNA expression levels are quantified. This was already suggested based on varying expressions in cell lines (20), and it can be concluded that the data from the present study support this idea. Second, this finding may question the use of multiple target markers for the detection of micrometastatic breast cancer. Multiple targets were suggested to improve the specificity of PCR based detection (30), compared to the single target mRNA detection methods (traditionally CK19, 9-11, 31-34). However, when not all tumors express multiple targets at similar levels, this approach may actually lead to false-negative results. Therefore, based on the results presented here, we suggest that the sensitivity of multiple-marker assays would likely benefit when based on expression of either one of the targets.

In the sentinel nodes, two extra nodes were found tumor-positive with immunostaining, compared to examination after routine H&E staining. This is in line with conversion rates reported in other studies (35-37). The specificity of qRT-PCR on sentinel nodes was impaired by the observation of 3 false positive nodes. These nodes were found to contain dendritic reticulum cells. These cells have been described to express cytokeratins, and are considered a pitfall in detection of micrometastases using immunostaining (38). Immunostaining allows visual distinction of these cells from tumor cells, but the qRT-PCR method does not. We also observed dendritic reticulum cells in control lymph nodes by CK19 immunostaining, but these few cells were not CK19 positive with qRT-PCR. Furthermore, 3 of 19 nodes (positive for tumor with H&E or immunostaining) were found to be false-negative by qRT-PCR. One node was exceptional because apparently no residual tumor was present (also immunostaining was negative). The other two nodes contained few tumor cells,

detectable with immunostaining, but not by qRT-PCR. The reason is probably that the sections of lymph nodes contained so many 'diluting' lymphocytes that the expression in single tumor cells was below the detection level. This problem could possibly be solved by analyzing each section of the lymph nodes separately, thus minimizing the quantity of analyzed tissue. This is hardly compatible with the purpose of a sensitive, yet practically feasible detection method for micrometastases. Furthermore, these problems are not encountered with the conventional immunostaining. For the sentinel nodes therefore, it may be suggested that a qRT-PCR method such as described here, particularly when using tumor-associated tissue-specific markers, are not specific and sensitive enough for detecting micrometastases. In spite of earlier reports in favor of PCR based detection methods (39), immunostaining appears more suitable for detection of micrometastases in lymphoid tissue in particular (35). This is in line with previous reports by Bostick et al. (12), showing that tumor-associated tissue-specific marker expression in lymph nodes does not necessarily imply the presence of tumor. In view of this, recent reports on the clinical significance of PCR detection of micrometastases from solid tumors in lymph nodes without immunostaining confirmation should be regarded with caution (40, 41), especially with the use of markers with a high propensity of inducing aspecific results (12).

In peripheral blood samples of 33% of patients, EGP-2 or CK19 mRNA expression was observed. Seven patients had more than one positive blood sample, and 4 patients showed a blood sample with simultaneous expression of both EGP-2 and CK19. None of these samples contained immunostaining positive tumor cells. Only a few immunostaining positive cell fragments were found in these samples, although we have previously observed occasional aspecific CK19 staining of segmented granulocytes in peripheral blood samples (unpublished data). Several studies have used only a PCR based method for detection of circulating tumor cells in breast cancer patients (9, 11, 32, 34), but it has been suggested that immunostaining may be

performed as validation for PCR findings in peripheral blood (20). However, a realistic risk of sampling error between PCR and immunostaining samples remains in the 'diluted' setting of single circulating tumor cells, which implies that tumor cells are not necessarily present in both samples. Furthermore, tumor cells with high expressions may still be detected by the qRT-PCR assay, while their number is below the detection limit of the immunostaining method. On the other hand, mRNA expression of tumor-associated tissue-specific markers in peripheral blood does not necessarily imply the presence of tumor (42). Early reports indicated that breast cancer surgery may induce shedding of tumor cells into peripheral blood during operation (43, 44). These data have not yet been confirmed in larger studies. Our present data appear not to support these results, as the incidence of qRT-PCR positive samples was not increased during operation. Our data are roughly in line with other studies combining immunostaining and (q)RT-PCR, in which an incidence of patients with positive peripheral blood samples of 5 to 9% with immunostaining, and 13 to 36% with (q)RT-PCR was reported (11, 32). In metastatic breast cancer patients, the reported incidence of blood samples positive for CK19 mRNA expression by qRT-PCR was up to 50%, and decreased with disease response (33). No previously published data are available on EGP-2 mRNA expression in peripheral blood patient samples.

In conclusion, using a combination of two targets and two techniques in relation to the standard H&E examination, we evaluated the possibilities to detect breast cancer micrometastases. In primary tumors, EGP-2 and CK19 expression was found with a wide variation in expression levels. So, both markers may be used in qRT-PCR for adequate sensitivity of single tumor cell detection. In sentinel nodes, immunostaining appeared more sensitive and specific than H&E staining or qRT-PCR. In peripheral blood, no immunostaining positive samples were found, while many proved to be positive by qRT-PCR. The clinical implications of these findings will have to be clarified in large studies with long-term clinical follow-up data.

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**TABLE 1: Patient and tumor characteristics**

**Patients:**

Total no.		58
Mean age (years)		60 (range 35-89)
Disease stage (no. of patients):	I	21
	II a	21
	II b	12
	III a	3
	IV	1

**Tumors:**

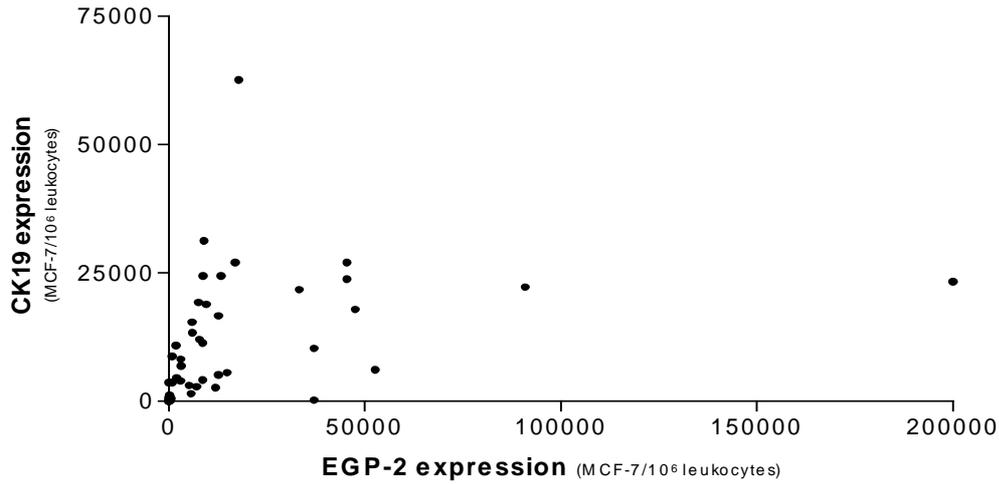
Total no.		60 (of 57 patients)
Mean size (cm)		2.2 (range 0.8-6)
Histological tumor type:	ductal carcinoma	53
	lobular carcinoma	2
	DCIS	2
	benign	2
	undifferentiated	1
Differentiation grade	I	16
	II	23
	III	15
	not assessable	2
	DCIS (III)	2
	benign	2

TABLE 2: detection of EGP-2 or CK19 positive cells

	H&E *	Immunostaining		QRT-PCR	
		EGP-2	CK19	EGP-2	CK19
Primary tumors (n=52)	46	52	52	52	52
Sentinel nodes (n=75)	17	18 **	18	18	16
Peripheral blood (n=149)	0	0	0	16	15

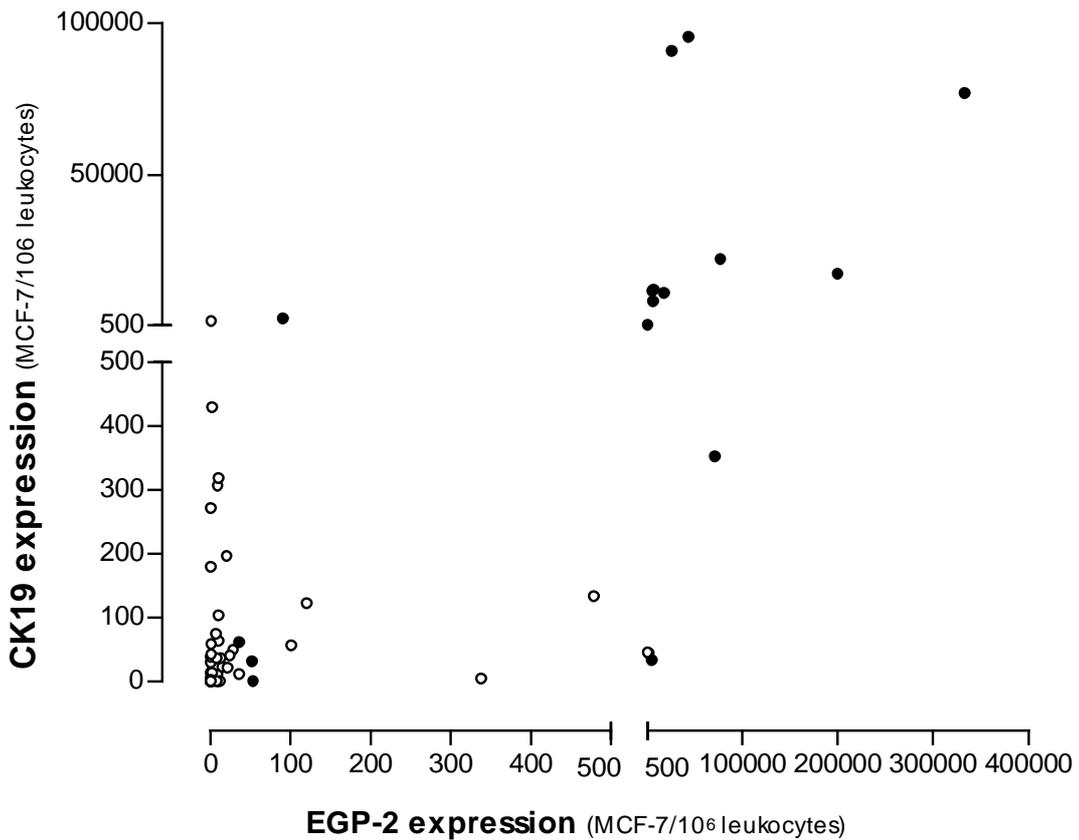
\* positive for malignant tumor cells

- \*\*
- 1 sample was found tumor-positive with H&E, but negative for immunostaining and/or qRT-PCR: apparently no residual tumor
  - 2 nodes were found tumor-positive with immunostaining additional to evaluation after routine H&E: in total 19 SN were tumor-positive by means of H&E or immunostaining
  - of these 19 SN, 3 showed no (1x EGP-2, 3x CK19) or low (2x EGP-2) expression with qRT-PCR



**FIGURE 1: Expression of EGP-2 and CK19 mRNA in primary tumors**

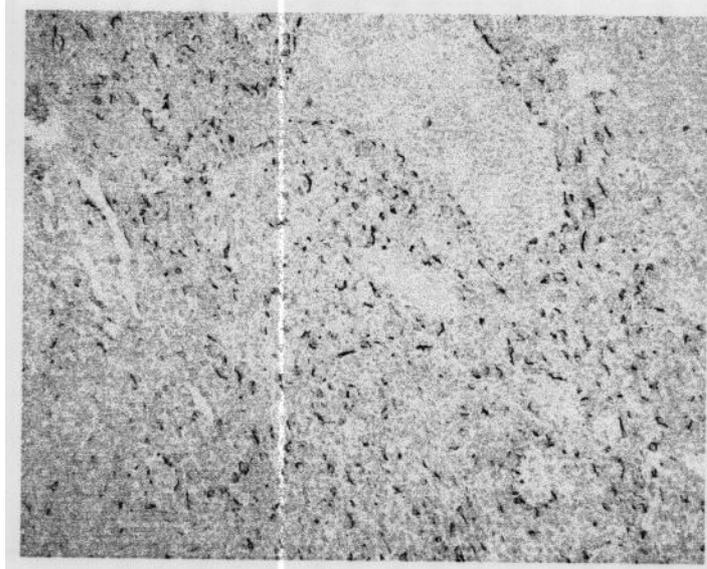
X-axis: EGP-2 mRNA expression (related to the standard curve of MCF-7/10<sup>6</sup> leukocytes). Y-axis: CK19 mRNA expression (related to the standard curve of MCF-7/10<sup>6</sup> leukocytes).



**FIGURE 2: Expression of EGP-2 and CK19 mRNA in sentinel nodes**

X-axis: EGP-2 mRNA expression (related to the standard curve of MCF-7/10<sup>6</sup> leukocytes). Y-axis: CK19 mRNA expression (related to the standard curve of MCF-7/10<sup>6</sup> leukocytes).

7/10<sup>6</sup> leukocytes). The open dots reflect the H&E/immunohistochemical tumor-negative sentinel nodes; the closed dots reflect the H&E/immunohistochemical tumor-positive sentinel nodes. The figure shows the relation between EGP-2 and CK19 expression in sentinel nodes, and the relation between this expression and the presence of tumor in the sentinel nodes.



**FIGURE 3:**

Dendritic reticulum cells in lymph node tissue staining positive for CK19

CK19 positive dendritic reticulum cells in a sentinel node, magnification 100x (reflected as black staining in a furthermore negative lymph node). This sentinel node belonged to a patient who also had a blood sample positive for CK19 mRNA expression.







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