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New ways to optimize breast cancer treatment

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Epithelial glycoprotein-2 as a marker for minimal residual disease in breast cancer patients

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Submitted

Abstract

Introduction Detection of single tumor cells may be helpful in breast cancer (BC) treatment. In this study, a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for the expression of membrane marker epithelial glycoprotein-2 (EGP-2) was evaluated for detecting BC cells in blood. **Materials and methods:** Sensitivity and specificity of the qRT-PCR was established in control nucleated blood cells, with or without EGP-2 positive MCF-7 tumor cells. The qRT-PCR was performed on breast tumors to determine a 'cut-off point' for EGP-2 expression in blood samples. Samples were also immunocyto(-histo)chemically (IC) stained with antibodies against EGP-2 (MOC31) and cytokeratin (CK) 19. **Results:** qRT-PCR sensitivity was 5-10 MCF-7 in 1.10^5 nucleated cells. Control samples without MCF-7 showed no (n=7) or low EGP-2 expression (n=3). EGP-2 expression varied 100-fold in breast tumors (n=12). IC sensitivity was 1 MCF-7 in 2.10^6 nucleated cells, and control samples were negative with MOC31. Aspecific staining was found with CK19. **Conclusions:** EGP-2 IC is more specific and sensitive than EGP-2 qRT-PCR or CK19 IC in this study. PCR methods for detecting BC cells in blood may be hampered by varying expression of tissue specific markers in BC tumors.

Introduction

Breast cancer patients without apparent distant metastases, who are treated with a curative intent, may later develop a relapse. Subgroups benefit from adjuvant chemotherapy treatment. The ability to detect very small numbers of tumor cells may provide the clinician with an important predictive tool with respect to recurrence and might help in a better selection for adjuvant therapy (33, 5). The detection of micrometastases in bone marrow was described to be of prognostic value in a number of solid tumors such as breast, gastric, colorectal and lung cancer (1, 3, 4, 7, 11, 23, 24, 29-31, 34). A particular challenge in solid tumors is to find a specific detection marker which is not shed into the circulation, which is not expressed by hematological cells and can therefore be used for tumor cell detection in blood, bone marrow or peripheral blood stem cells. For breast cancer, so far no tumor specific marker has been described. Many studies have been performed using tissue specific, epithelial markers (1, 3, 7, 11, 31). Sensitive detection methods such as RT-PCR, were found to detect low levels of background (or 'illegitimate') expression in non-epithelial tissue, including bone marrow and peripheral blood stem cells (21, 37) with these markers. To circumvent this problem, a quantitative RT-PCR (qRT-PCR) was developed in our institution for the expression of epithelial glycoprotein-2, EGP-2 (15). EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein, strongly expressed by most carcinomas and universally expressed in breast cancer specimens (18). As such, EGP-2 is a commonly used target antigen in many carcinoma-directed immunotherapeutical approaches (10, 18, 32). In this study, this qRT-PCR method was evaluated for detecting of breast cancer cells in blood. To this end, the sensitivity and specificity were studied, as well as the EGP-2 expression in breast samples. Results were compared to immunocytochemistry (IC) by means of antibodies against EGP-2 (MOC31) and cytokeratin (CK) 19, a tissue specific marker commonly used for this purpose (20, 22, 27).

Materials and methods

Isolation of nucleated cells from blood samples

Blood samples (of 40 mL each) were collected in tubes containing ethylenediaminetetraacetate (EDTA) as anti-coagulans, 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 NH₄Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium ethylene diamine tetra acetate, 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°C, until further processing.

The cells to be used for IC (1/4 of the total amount of cells) were allowed to sediment for

1 h onto slides, through a metal funnel-like device. This sedimentation method was found to introduce less mechanical damage for the cells than the standard cytopspin method. Also, it allows the analysis of $1 \cdot 10^6$ cells on one slide (instead of $5 \cdot 10^4$ on a cytopspin slide). Fixation of the cells was then performed for 10 min at -20°C, in a solution of 4% acetic acid in methanol, followed by 10 min at -20°C, in acetone. Because standard acetone fixated cytopspins were found to result in poor leukocyte morphology, and the morphological distinction between normal cells and tumor cells was thought to be important in this type of study, the above described alternative sedimentation- and fixation procedure was developed. Slides were maintained at -20°C until further processing.

Nucleated cells from blood samples were used from healthy volunteers for spiking experiments with breast cancer cell line MCF-7 in order to determine the sensitivity of the qRT-PCR and IC methods.

The specificity of the methods was evaluated in samples from healthy volunteers used as a negative control.

Primary breast tumor samples

As a part of this study, fresh tumor samples from primary surgery in breast cancer patients were snap frozen and maintained at -80°C . Prior to RNA isolation or IC staining, 25 μ slices were prepared. Tumor content was verified with hematoxylin-eosin (HE) staining of slices on either side of the sample, and examination of these slides by a technician as well as a pathologist. Slices for RNA isolation were transferred immediately into GITC and homogenized through a 20-Gauge syringe; slices for staining were transferred onto slides. Benign breast samples were obtained from patients undergoing prophylactic mastectomy procedures, and were processed in the same way as the tumor samples. The collection of patient samples from breast cancer patients for studying circulating tumor cells was approved by the Medical Ethical Committee of our institution.

The expression of EGP-2 in primary breast samples was analyzed by means of the qRT-PCR, with the aim to use this information for establishing a cut-off point for the expression of EGP-2 in blood samples.

RNA isolation

RNA isolation was performed by means of chloroform/isopropanol extraction (6). Samples were maintained in 500 μL GITC, at -20°C . After thawing, 50 μL sodium acetate (3M, pH 5.0), 500 μL water saturated phenol and 100 μL chloroform/isoamyl alcohol (49:1 vol:vol) were added and mixed by vortexing. The mixture was left on ice

for 10 min, after which it was centrifuged, 15,000 g, at 4°C. The supernatant was transferred into a fresh microfuge tube, and RNA was induced to precipitate by adding an equal volume of isopropanol and placing it at -20°C for 1 h. RNA was pelleted by centrifugation, 15,000 g, at 4°C, and re-precipitated in 150 µL GITC and isopropanol. RNA was pelleted by centrifugation as described above, and washed with 70% ethanol. The pellet was dried in a vacuum exicator and dissolved in 30 µL diethylpyrocarbonate (DEPC, Sigma, St. Louis, MO) treated water. The integrity of the RNA samples was assessed on formaldehyde-containing (2.2 M) agarose gels. RNA samples with visible and discrete 28S and 18S ribosomal RNA bands were used for the RT-PCR experiments.

Quantitative RT-PCR on EGP-2:

The qRT-PCR on EGP-2 was performed according to Helfrich et. al (15). The EGP-2 specific primers were synthesized on an oligonucleotide synthesizer (Pharmacia Biotech Europe, Brussels, Belgium), using the phosphite triester method. The sequences of the EGP-2 specific primers are: EGP2 FW: 5'-GAACAATGATGGGCTTTATG-3' (corresponding to bases 374 to 394 of the EGP-2 cDNA) and EGP2 REV: 5'-TGAGAATTCAGGTGCTTTTT-3' (bases 868 to 888). Amplification of cDNA with these primers gives rise to a 513 bp fragment. No signal can be obtained with genomic EGP-2 with these primers. For quantifying the EGP-2 signal, a deletion construct of recombinant EGP-2 RNA was used. This construct yields a 315 bp fragment when subjected to PCR amplification with EGP-2 specific primers, which can be readily distinguished from the 513 bp fragment generated by genuine EGP-2 cDNA on an 1.5 % agarose gel. A series of recrRNA solutions, decreasing in concentration, was mixed with a fixed amount of cellular RNA, containing the EGP-2 RNA to be quantified. The mixture of recrRNA and cellular RNA was converted into cDNA by reverse transcription using the EGP-2 REV primer. cDNA was then subjected to 30 PCR cycles consisting of 30 sec denaturation at 94 °C, 60 sec

of primer annealing at 54 °C, and elongation at 72 °C for 90 sec. The samples were subjected to an initial denaturation for 180 sec. The final elongation step was extended by 10 min. Reactions were performed using the DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT), with supertaq (0.125 U) (HT Biotechnology LTD), in the reaction buffer supplied by the manufacturer, supplemented with MgCl₂ (1.75 μM), 200 μM dNTP's, and 300 ng of both EGP2 FW and EGP2 REV. The MCF-7 breast cancer cell line was used as positive control. A sample to which all the above mentioned components were added, without RNA, was used as a negative control. The reaction products were analyzed by gel electrophoresis, and the weight ratios per lane were determined by densitometry. The lane in which the weight ratio was closest to the ratio of the respective RNAs was used to quantify the amount of EGP-2 mRNA. On all samples, RT-PCR was also performed to show glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) mRNA, with primers GAPDH FW: 5'-CCACCCATGGCAAATTCCATGGCA-3' and GAPDH REV: 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (25 cycles). EGP-2 expression was normalized to expression of the house-keeping gene GAPDH, and expressed relative to GAPDH expression.

Immunocyto (-histo) chemical (IC) staining

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 Na₂HPO₄·2H₂O, 1.5 mM KH₂PO₄, pH 7.4). With each antibody, at least two of these slides (e.g. 2x 1.10⁶ cells) were stained. Tumor samples in paraffin were pre-treated with protease (type XXIV, Sigma, St. Louis, MO), 0.1% in PBS pH 7.4, at 37°C, for 30 min, prior to staining. Samples were stained with monoclonal antibody MOC31, directed against EGP-2, using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a second antibody (Dako, Glostrup, Danmark) and AEC

as a substrate. Samples were routinely counterstained with HE. The antibody directed against cytokeratin 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 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. Also for the CK19 antibody, the indirect immunoperoxidase staining procedure was used as described above.

Isotype specific controls for MOC31 and CK19 were performed with primary antibody mouse IgG1 (X0943, Dako, Glostrup, Danmark). In each staining procedure, a negative control was included using PBS 1% BSA without the primary antibody. Slides prepared from MCF-7 breast cancer cells (staining positively for CK19 and MOC31) were used as positive controls in each procedure for staining slides. For tumor samples, as positive control a sample of breast cancer tissue was used, that was found to stain positive with MOC31 and CK19 antibodies on previous occasions.

Patient samples were independently examined for morphological features by a technician as well as a pathologist.

The classical immunocyto (-histo) chemistry method was used as comparison with the (less established) qRT-PCR method.

Results

qRT-PCR for EGP-2 expression

Spiking experiments (sensitivity)

MCF-7 breast cancer cells were used for spiking in nucleated cell samples from healthy volunteers. Consistently, 5 to 10 MCF-7 cells in $1 \cdot 10^5$ nucleated cells could be detected by means of the qRT-PCR.

Healthy (negative) controls (specificity)

The specificity of the assays was examined using nucleated cells from healthy volunteers (n=10). The qRT-PCR showed no (n=7) or low expression (0-1 relative to GAPDH expression, n=3) of EGP-2. These results are in line with those described by de Graaf et al. (9).

Malignant and benign breast samples (cut-off point EGP-2 expression)

In primary tumor samples (n=12), a range of EGP-2 expression was found: mean 3.4, range 0.1- 11.5 relative to GAPDH expression. A representative picture of a gel reflecting a qRT-PCR of two tumor samples is shown in figure 1. Benign breast samples (n=3), all showed low EGP-2 expression of 0-1 relative to GAPDH expression.

IC with antibodies against EGP-2 (MOC31) and CK19

Spiking experiment (sensitivity)

In the same samples as used for qRT-PCR analysis, 1 MCF-7 tumor cell could be detected in the amount of nucleated cells screened, e.g. $2 \cdot 10^6$ total, with IC staining with the MOC31 antibody directed against EGP-2, as well as the anti-CK 19 antibody. In figure 2, an example is shown of MCF-7 tumor cells added to leukocytes, stained with MOC31.

Healthy (negative) controls (specificity)

IC with MOC31 staining was consistently negative in these samples (n=10). However, in some samples, slightly positive cells were detected with the anti-CK 19 antibody. These cells were judged to be segmented granulocytes.

Malignant and benign breast samples

Primary tumor samples (n=12), found morphologically malignant by regular HE staining, all stained positive with MOC31 and anti-CK 19 antibody. Epithelial tissue of benign breast samples (n=3) stained positive with the MOC31 antibody against EGP-2 and the anti-CK 19 antibody.

Discussion

In this study, we evaluated the use of a qRT-PCR for the expression of EGP-2 for detecting minimal amounts of breast cancer tumor cells in blood samples. The sensitivity as well as the specificity of the method were studied, and the expression of EGP-2 in breast samples was evaluated. Classical immunocyto (-histo) chemistry with antibodies against EGP-2 (MOC31) and CK19, was used as comparison with the molecular biological qRT-PCR method.

For the preparation of blood samples, the nucleated cell fraction was isolated by means of erythrocyte lysis. This method was recently shown in blood, bone marrow and leukapheresis products to preserve tumor cells in a superior way compared to the frequently used Ficoll isolation (19). To establish the sensitivity of the detection methods, spiking experiments with MCF-7 in nucleated cells from healthy volunteer blood samples were performed. With IC, a detection level with IC of 1 MCF-7 tumor cell in $2 \cdot 10^6$ nucleated cells was found, using the MOC31 antibody directed against EGP-2 or the anti-CK 19 antibody. The qRT-PCR resulted in a detection level of only 5 to 10 MCF-7 cells in $1 \cdot 10^5$ nucleated cells (in line with ref. 15): a 100-200 fold difference in sensitivity. In determining the specificity of the methods, the control samples of nucleated cells from healthy volunteers were found positive in 3 out of 10 cases, with a low EGP-2 expression. None of these samples stained positive with IC for MOC31.

It can be suggested, with the techniques used here, that this EGP-2 based qRT-PCR method may be less sensitive and specific than IC for MOC31, based on these results. Although early reports have suggested a high sensitivity (up to 1 tumor cell in $1 \cdot 10^7$ nucleated cells) of PCR based methods for detecting tissue specific expression of single solid tumor cells in blood of bone marrow samples (8, 12, 35), more recent reports indicate that these methods harbor the risk of false positive results (2, 9, 13,

16, 21, 25, 37). This may be due to detection of so called 'illegitimate transcription' of apparent tissue specific markers by non-epithelial cells, or due to the sensitivity of this method (particularly the nested RT-PCR) (36) to contamination. It is also clear that analytical variables of the RT-PCR methodology may have a profound impact on the obtained results (38). In an effort to circumvent the problem of false positive results with the regular RT-PCR, the qRT-PCR method for EGP-2 was designed in our institution (15). It was presumed that quantifying the signal of EGP-2 would allow the definition of a cut-off point from low-level or insignificant expression. With this method, it was found that within tumor cell lines, positive with IC for MOC31, a wide variation (of 100-fold) in EGP-2 expression could be detected (15). This is in line with EGP-2 expression results in tumor cell lines described earlier (9). If EGP-2 expression were to vary in primary tumor samples as well, establishing a cut-off point for EGP-2 expression, as well as relating EGP-2 expression to tumor load, would be difficult. A low expression of EGP-2 in blood or bone marrow might then reflect a relevant tumor load in one patient, but not in the other. Indeed, also in primary breast tumors we observed a 100-fold difference in expression of EGP-2, normalized for GAPDH expression. Thus, based on these results, we suggest that with this qRT-PCR for EGP-2, the definition of a generally applicable cut-off point for EGP-2 expression seems not feasible. Unless the expression of EGP-2 in primary tumor tissue and blood samples are related for each individual, it appears difficult to exclude false positive results with this method. Recently, the same problem of varying expression was described for primary colorectal tumors (26). Immunocytochemical detection methods, have the advantage of providing a visual evaluation of stained (tumor) cells, thus decreasing the risk of false positive results. In fact, it was recently suggested that recruitment of tumor cells into peripheral blood cannot be confirmed by RT-PCR alone, and that IC should be performed as validation (14). It can be argued that the IC method is considerably laborious and potentially sensitive to inter-observer variation in the

judgement of tumor cells. However, based on the results presented here, we currently prefer the use of the anti-EGP-2 antibody MOC31 for detection of single tumor cells in breast cancer patients over this qRT-PCR.

In light of the IC detection of single tumor cells, we also compared the EGP-2 to the commonly used marker CK19 (20, 22, 27). Aspecific staining of granulocytes was found in some samples from healthy volunteers. This is in line with a recent study performed by Lambrechts et al. (21), who found as much as 75% false positivity in breast cancer patient samples as well as samples from healthy volunteers, with the CK19 antibody. The fact that the cytokeratin epitope is found not only on all epithelia, but also on endothelia, mesothelia, skin and mucosa (17) may explain this higher rate of false positive results with CK19 than MOC31, as EGP-2 is only expressed on non-squamous epithelia (28).

Concluding, we suggest that the current classic molecular methods for detecting single breast cancer tumor cells are impeded by the lack of a tumor-specific marker for breast cancer cells. The use of fluorescent probes in quantitative RT-PCR may enhance tumor cell detection to the level of IC; this method is possibly the least laborious method available. Currently, research is in progress to evaluate this issue. For now, IC based on tissue-specific markers, has the advantage of allowing an additional visual evaluation of the stained (tumor) cells. When using IC staining, the EGP-2 marker yields more specific results than CK19.

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M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

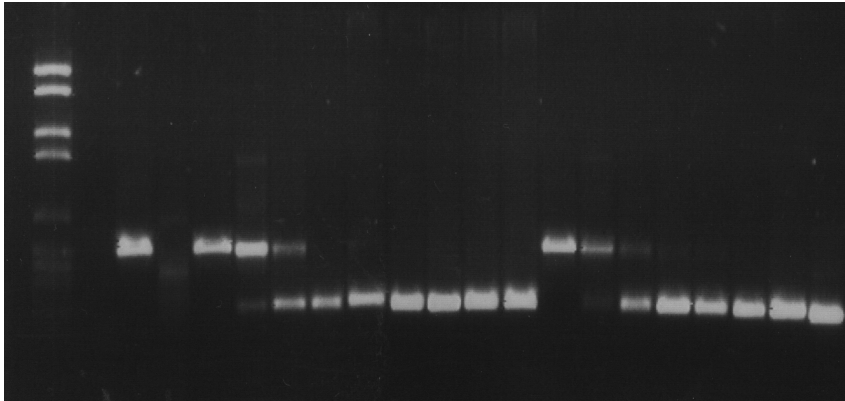


Figure 1:

A representative gel reflecting the qRT-PCR of two malignant breast tumors. M: marker; 1: negative control without RNA; 2: positive control of MCF-7 tumor cells; 3: negative control of GLC4 tumor cells; lanes 4-12 and lanes 13-20: two separate tumor samples to which dilutions of recombinant RNA was added (ranging from 0 to 1000 pg: lanes 4 as well as 13, and lanes 12 as well as 20 respectively).

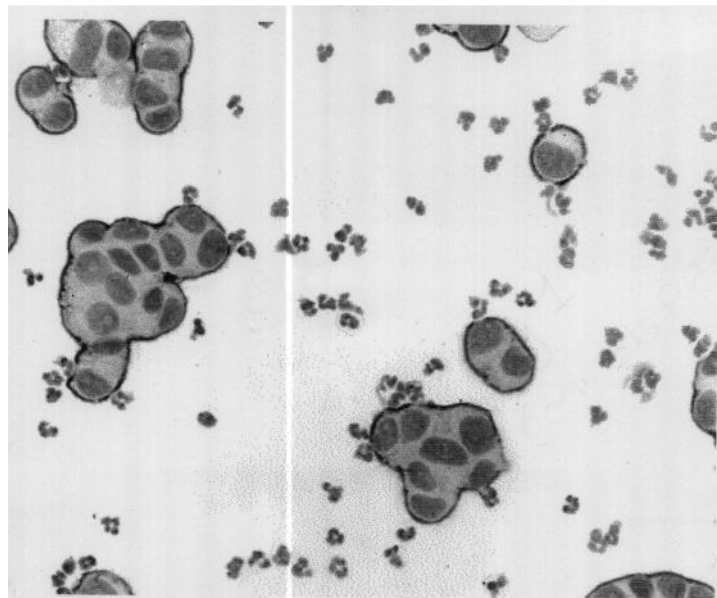


Figure 2:

Representative picture of MCF-7 tumor cells added to leukocytes for spiking purposes, stained with MOC31 antibody. Magnification 40x10.

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