Expression of the Breast Cancer Resistance Protein in Breast Cancer

Ian F. Faneyte, Petra M. P. Kristel, Marc Maliepaard, George L. Scheffer, Rik J. Scheper, Jan H. M. Schellens, and Marc J. van de Vijver

Departments of Experimental Therapy [I. F. F., P. M. P. K., M. M., J. H. M. S.], Pathology [I. F. F., M. J. v. d. V.], and Medical Oncology [J. H. M. S.], The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, and Department of Pathology, Free University Hospital [G. L. S., R. J. S.], 1066 CX Amsterdam, the Netherlands

ABSTRACT

Purpose: The breast cancer resistance protein (BCRP) is involved in in vitro multidrug resistance and was first identified in the breast cancer cell line MCF7/AdrVp. The aim of this study was to investigate the role of BCRP in resistance of breast cancer to anthracycline treatment.

Experimental Design: BCRP mRNA was determined with real-time reverse transcription-PCR and immunostaining in nine breast cancer cell lines and in samples of 25 primary breast carcinomas and 27 patients who received preoperative anthracycline-based therapy. Tumor response to treatment and patient survival were recorded.

Results: In cell lines, only MCF7 and BT20 had BCRP mRNA levels coinciding with membrane-bound immunostaining. In clinical samples, BCRP expression varied widely (range, 0.01–0.86). With immunohistochemistry, BCRP was detected in vessels and normal breast epithelium but not in tumor cells. There was no difference in BCRP expression between anthracycline-naive and treated tumor samples. BCRP expression was not associated with decreased response or survival.

Conclusions: There is no indication that elevated BCRP expression in breast carcinomas confers resistance to anthracyclines. Expression was not detectable with immunohistochemistry.

INTRODUCTION

MDR frequently poses a problem in breast cancer patients with metastatic disease. Response rates of first-line chemotherapy in metastatic breast cancer range from 50 to 70%; the response rate to subsequent chemotherapy regimens is significantly less as a result of MDR (1). As yet no explanation for this MDR phenomenon in breast cancer has been found.

The BCRP (ABCG2) is a recently characterized xenobiotic half-transporter protein. It is a member of the ATP-binding cassette protein family and functions as an energy-dependent efflux pump (2, 3).

BCRP was first identified in the breast cancer cell line MCF7/AdrVp (2), which has a multidrug-resistant phenotype, notwithstanding the addition of a MDR1/P-glycoprotein blocking agent (verapamil). The parental cell line MCF7 also expresses BCRP but at much lower levels (4, 5). In vitro, elevated expression of BCRP causes resistance against at least two classes of drugs (anthracyclines and topoisomerase I inhibitors), which are used in the treatment of cancer patients (6–9).

Whether BCRP is also involved in clinical drug resistance is unknown. Studies published to date describing expression of BCRP in clinical material did not investigate such a role. Expression levels in blast cells of leukemia patients were found to be highly variable, but no correlation was described between BCRP expression and exposure to chemotherapy or sensitivity to cytoxic treatment (10). Antibody staining of a panel of 41 human solid tumor samples of mixed origin and 16 cytoxins of acute myeloblastic leukemia (11) showed no reactivity, except for weak staining in one of two small intestinal adenocarcinoma samples.

Recent studies have shown that BCRP activity can be effectively inhibited by several modulating agents (12–14). It is therefore of clinical interest to gain insight into the possible contribution of BCRP to drug resistance in breast cancer.

We have studied the expression of BCRP mRNA and of the BCRP protein in breast cancer cell lines and clinical breast carcinoma specimens. By analyzing both chemotherapy-naive and anthracycline-treated patients, we have aimed to detect a possible up-regulation of expression caused by chemotherapy. We have also studied the correlation of BCRP expression in tumor samples with response of the tumor to anthracyclines.

MATERIALS AND METHODS

Cell Lines. Cell line controls with a known expression of BCRP were the small cell lung carcinoma cell line GLC4/ADR (very low expression), the ovarian carcinoma cell line Iggro (low expression), its drug-selected variant Iggro/T8 (high expression), and the partially reverted subline Iggro/T8(p75)-40 (intermediate expression; Ref. 15). Breast cancer cell lines analyzed were 1.6.2.6, BT20, CAMA1, HBL100, MCF7, MPL13E, SKBR3, T47D, and ZR75-1. All cell lines were...
cultured in a 10% FCS-enriched medium with L-glutamine, penicillin, and streptomycin. The IGROV variants were grown in RPMI 1640 (Life Technologies, Inc., Paisley, Scotland); the other cell lines were grown in DMEM (Life Technologies, Inc.). To maintain the high expression of BCRP in IGROV/T8, these cells were exposed to topotecan at a 950 nM concentration for 1 h weekly. IGROV/T8(p75)-40 cells were cultured for 75 passages as IGROV/T8 and then for 40 passages in standard RPMI 1640 (no topotecan added). GLC4/ADR cells were cultured in 1120 nM doxorubicin permanently, until 4 days before harvesting for analysis.

**Tissue Samples.** All materials were obtained from the Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital Tissue Bank (fresh-frozen in liquid nitrogen upon surgery and preserved at −70°C), where they were originally stored between 1984 and 1999. Normal human liver was used in both RNA analyses and immunohistochemistry as a positive control for BCRP expression.

**RNA Isolation and Analysis.** RNA from 52 breast cancer specimens was isolated and analyzed. Twenty-five patients were chemotherapy-naïve: 18 patients had primary operable tumors (n = 13) or residual tumor (n = 5) after radiotherapy for locally advanced T3 (>5 cm) breast cancer; 7 specimens were biopsies from a primary tumor (n = 2) or subcutaneous lymph node with metastasis (n = 5) of patients entered in a clinical trial, described below.

Twenty-seven patients had been treated previously with anthracycline-based chemotherapy (2 also with radiotherapy): 23 patients who took part in a clinical trial, described below, and who received neoadjuvant FEC treatment; and 4 patients with locally advanced breast cancer that had initially responded to anthracycline-based chemotherapy [up to seven cycles of FEC (60 mg/m² of epirubicin) and have become progressive.

Immunohistochemistry was performed with breast cancer specimens from: 20 patients who had received no prior treatment (the RNA isolated from two corresponding frozen tissue samples was also analyzed); 4 patients who took part in the trial described below and who received neoadjuvant FEC treatment (the RNA isolated from all of the corresponding frozen-tissue samples was also analyzed); and 3 patients with progressive locally advanced breast cancer that had initially responded to anthracycline-based chemotherapy (the RNA isolated from all 3 corresponding frozen tissue samples was also analyzed).

In all patients treated with preoperative chemotherapy, surgery was performed within 8 weeks of the last administration of chemotherapy.

**Histology.** For all patients, paraffin slides were used for typing and grading. Histological grading was performed according to criteria described by Elston and Ellis (16).

**Patients from High-Dose versus Normal-Dose Chemotherapy Trials.** We assessed 30 tumor samples with RT-PCR from a group of patients, treated previously in a Phase II prospective clinical trial comparing high-dose versus normal-dose chemotherapy (17). In this trial, 97 patients with operable breast cancer and extensive axillary lymph node involvement were entered. Patients received three cycles of epirubicin (120 mg/m²), 5-fluorouracil (500 mg/m²), and cyclophosphamide (500 mg/m²) every 3 weeks before surgery. After surgery (modified radical mastectomy or wide local excision and axillary lymph node dissection) and radiotherapy, the patients were randomized to receive either one more course of FEC or FEC followed by a high-dose regimen with peripheral blood progenitor cell support. All patients received additional treatment with tamoxifen.

Tumor samples were taken before treatment (diagnostic biopsies) and after three cycles of FEC (surgical specimens) given as a neoadjuvant. We quantified the response to neoadjuvant treatment clinically, according to criteria of the Union International Contre Cancer (18). In addition, we studied the surgical specimens for histopathological evidence of response.

**Survival.** We determined both overall survival and disease-free survival from the start of neoadjuvant treatment. End points were death for overall survival and death or any recurrence of breast cancer for disease-free survival. Survival comparisons between the two randomly assigned treatment groups have been published (17). The outcome after 6 years of follow-up was identical for both treatment groups. For the analysis, we have therefore used trial-patient data, regardless of the treatment arm they were assigned to. For the other patients, the survival data were available in The Netherlands Cancer Institute’s patient database.

**Northern Blot Analysis.** For total RNA isolation from either cells or tissue specimens, the RNAzol B kit (Tel-Test, Inc., Friendswood, TX) was used according to the manufacturer’s protocol. RNA was isolated from approximately 7.5 × 10⁶ cells taken from culture after trypsinization or from 10 to 20 30-μm slices of the cryopreserved tissue samples. Staining of both ribosomal bands on a 1% agarose gel with ethidium bromide was used to judge the quality of the isolated RNA.

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**TaqMan Quantitative RT-PCR.** In a cDNA reaction (100 μl), 2.5 μg of total RNA were used as template. Quantification of the message was performed with a TaqMan real-time RT-PCR assay (P.E. Applied Biosystems, Foster City, CA), according to the manufacturers’ protocol.
The housekeeping gene PBGD served as the internal reference and was amplified parallel to BCRP for every sample, in separate vessels. The oligonucleotide primers used to amplify the PBGD gene (National Center for Biotechnology Institute GenBank no. NM000190; Ref. 19) were amplified were 5′-AGG ATC CCG AGA CTC TGC TTC-3′ (forward) and 5′-GCA CGG CTA CTG GCA CAC T-3′ (reverse), with an expected product length of 87 bp. The VIC-labeled probe (P.E. Applied Biosystems) used for PBGD detection was 5′-CAG CCT CCT TCC AGG TGC CTC AGG-3′. Primers for BCRP amplification were 5′-CAC AAC CAT TGC ATC TTG GC-3′ (forward) and 5′-GCT GCA AAG CCG TAA ATC CA-3′ (reverse), with an expected product length of 74 bp. The 6-carboxyfluorescein-labeled (reverse), with an expected product length of 74 bp. The 6-carboxyfluorescein-labeled product of 87 bp. The VIC-labeled probe (P.E. Applied Biosystems) was amplified parallel to PBGD using 5′-TCA TGG CTT CAG TAC TTC AGC ATT CCA CG-3′. The PBGD amplicon spans an exon in the genomic DNA sequence.

The constituents of each PCR (25 μl) were 2.5 μl of cDNA (or dH2O), 2X (forward and reverse) 0.5 μl of primer 15 pmol/μl each (Isogen Bionics BV, Maarssen, the Netherlands), 2.5 μl of fluorescent-labeled probe 1 μmol/μl (P.E. Applied Biosystems), 6.5 μl of dH2O, and 12.5 μl of TaqMan Universal PCR Mastermix (P.E. Applied Biosystems).

Product amplification was performed up to 50 PCR cycles, after uracil removal (2 min at 50°C) and polymerase activation (10 min at 95°C). Each two-step PCR cycle comprised denaturing (15 s at 95°C), annealing and extending (1 min at 60°C).

A positive control (liver) and negative controls with no template and genomic DNA as template were added in every experiment. All assays were run in triplicate.

**Immunohistochemistry.** Immunohistochemistry was performed on cytopsins and on 5-μm sections of frozen tissue according to a protocol published previously (20). We used two mouse monoclonal antibodies against BCRP, BXP34 (11) and BXP21 (21).

Cytopsins and tissue slides were air-dried overnight and subsequently fixed in acetone (10 min), blocked with normal goat serum (5%; 30 min), incubated with the primary antibody (1:50; 60 min), and quenched in 0.15% H2O2 containing PBS (10 min).

The monoclonal antibody was detected with horseradish peroxidase-labeled goat antimouse IgG (1:100; 60 min; DAKO A/S, Glostrup, Denmark), FITC-conjugated tyramine (1:500; 10 min; NEN Life Science Products, Inc., Boston, MA), and horseradish peroxidase-labeled rabbit IgG anti-FITC (1:100; 60 min; DAKO A/S). Bound peroxidase was developed with 2.5% 3-amino-9-ethyl-carbazole and 0.02% H2O2 in sodium acetate (pH 5.0).

All reagents were diluted in a 1% bovine albumin solution in PBS except for FITC-tyramine, which is diluted in the supplier’s amplification fluid. Antimouse IgG solutions were admixed with 10% normal human serum to prevent nonspecific binding. The primary antibody was replaced with 1% bovine albumin solution in PBS as negative control for every cell line and tissue sample tested.

**Quantification of Results.** For quantification of the RT-PCR results, fluorescent signal intensities were plotted against the number of PCR cycles on a semilogarithmic scale. The amplification cycle, at which the first significant increase of fluorescence occurred, was designated the threshold cycle (Ct). This was done for all samples to be tested parallel with a standardization series, with known concentrations of Igrov/T8(p75)-40 cDNA as template. The Ct of each sample was then compared with those in the standardization series, and the corresponding concentration of Igrov/T8(p75)-40 cDNA was read out as the expression level of the tested gene. This process is fully automated and carried out by the TaqMan with the manufacturers’ software.

We divided the expression values of BCRP mRNA by those of PBGD mRNA. This returned the expression ratio in each tested sample of the BCRP versus PBGD mRNA, as a measure of the relative expression level. The expression level of BCRP mRNA in a tested sample was thus defined as the ratio of BCRP mRNA and PBGD mRNA expression in that sample relative to the ratio of BCRP mRNA and PBGD mRNA in Igrov/T8(p75)-40.

Two independent observers (M. V. and I. F.) judged the immunohistochemical staining as: +++, 100% membrane-bound staining; +, membrane-bound staining in up to 75% of cells; +/-, cytoplasmic staining in up to 25% of cells; and –, no staining. The two observers scored all completely negative and strongly positive staining results identically. Discordance only occurred assessing the cell lines HBL100, 1.6.2.6, and CAMA1. Because both observers did score positive staining to some extent in these cells but not as clearly specific as in the positive samples, we agreed to define a third category for immunohistochemical staining, +/-.

**Statistical Analyses.** All data were analyzed with SPSS 10.0.7 for Windows. For correlation between BCRP expression levels and tumor parameters, we used the two-sided Spearman’s test for nonparametric correlation. Differences of expression levels between groups of samples were assessed with the two-sided Mann-Whitney U test (nonnormal distribution). BCRP expression was tested for association with outcome in a Cox regression model.

**RESULTS**

BCRP mRNA and protein expression were assessed in a panel of cell lines. The results of these analyses are summarized in Table 1.

With TaqMan analysis, an increased BCRP expression compared with Igrov/T8(p75)-40 (level 1.0) was found in Igrov/T8 cells (level 4.04) and liver (level 1.26). Low expression was detected in the parental Igrov cell line (level 0.02) and in GLC4/ADR (level 0.0008). Northern blot hybridization and immunostaining detected BCRP in Igrov/T8 and in liver. Positive BCRP Northern blot hybridization of Igrov/T8(p75)-40 was found previously by Maliepaard et al. (21). Staining of Igrov/T8 was bound to the membrane and occurred in all cells. In Igrov/T8 (p75)-40 cytopsins, membrane-bound staining occurred in 50–75% of cells. A bile canalicular staining pattern was seen in 50–75% of cells in liver sections.

Expression of BCRP mRNA in breast cancer cell lines varied widely (median, 0.06; range, levels 0.0001–0.48). In two breast cancer cell lines with the highest mRNA levels (MCF7, level 0.48; BT20, level 0.18), BCRP could be detected by Northern blot hybridization. In these cells, BCRP expression...
was also detected immunohistochemically in the cell membrane. None of the cell lines with expression levels below BT20 had detectable BCRP on Northern blot or membrane-bound immunostaining. Cytoplasmic staining did occur in three breast cancer cell lines with lower BCRP levels (HBL100, level 0.14; 1.6.2.6, level 0.11; CAMA1, level 0.06). A threshold for the detection of membrane-bound staining with both antibodies appears to lie at a level 0.11; CAMA1, level 0.06). None of the cell lines with expression levels below BT20 had a partial remission, and 8 had stable disease. No correlation was found between BCRP mRNA expression levels and tumor response to therapy. Patients with stable disease did not have an increased BCRP levels (0.23) compared with responsive patients (0.18; \( P = 0.54 \)). Overall survival and disease-free survival were not significantly associated with BCRP mRNA expression in a Cox regression model.

High tumor malignancy grade was associated with a decreased BCRP mRNA expression (\( P = -0.33; \ P = 0.02 \)). Grades 1 and 2 tumors (\( n = 28 \)) had a mean BCRP mRNA expression of 0.23 versus grade 3 tumors with 0.14 (\( P = 0.03 \)). With two monoclonal antibodies against BCRP, we stained frozen sections of 9 breast cancer samples in which we had detected relatively high BCRP expression levels. In none of the samples could specific tumor cell staining be observed. Additionally we stained tumor sections of 18 breast carcinoma specimens with unknown expression of BCRP. Again, no specific staining of tumor cells was seen.

Positive staining of vascular endothelium and of a part of the normal epithelium in large mammary ducts did occur with both antibodies. This pattern has been described previously by Maliepaard et al. (21) and served as an internal positive control. No staining of lymphoid cells was seen, and the expression of BCRP mRNA was not correlated with the percentage of cancer cells or with the amount of lymphoid infiltrate present in the sample.

**DISCUSSION**

In this study, we investigated whether the expression of BCRP in breast cancer may play a role in resistance to epirubicin-based chemotherapy. The substrate specificity of BCRP was determined for 18 breast cancer cell lines and the BCRP levels needed for positive immunostaining, we analyzed clinical breast carcinoma samples and breast cancer cell lines, with TaqMan real-time PCR, Northern blotting, and immunohistochemistry with two monoclonal antibodies. Two monoclonal antibodies against BCRP, we stained breast cancer cell lines and the BCRP levels needed for positive immunostaining, we analyzed clinical breast carcinoma samples and breast cancer cell lines, with TaqMan real-time PCR, Northern blotting, and immunohistochemistry with two monoclonal antibodies.

**Table 1** BCRP expression measured in controls and breast cancer cell lines, with TaqMan real-time PCR, Northern blotting, and immunohistochemistry with two monoclonal antibodies

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<tr>
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<th>TaqMan real-time PCR</th>
<th>Northern blot</th>
<th>Immuno BXP21</th>
<th>Immuno BXP34</th>
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<tr>
<td><strong>Controls</strong></td>
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<tr>
<td>Igrov/T8</td>
<td>4.0</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<td>+,d</td>
<td>+</td>
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<td>Igrov</td>
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<tr>
<td>Liver</td>
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<td>+</td>
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<td>GLC4/ADR</td>
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<td>−</td>
<td>ND,d</td>
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<td><strong>Breast cancer cell lines</strong></td>
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<tr>
<td>MCF7</td>
<td>0.48</td>
<td>+</td>
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<td>BT20</td>
<td>0.18</td>
<td>+</td>
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<td>SKBR3</td>
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<tr>
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*a* Ratio of BCRP and PBGD in samples versus in Igrov/T8(p75)-40.

*b* Autoradiogram after hybridization with BCRP-specific probe; +, signal at 2.4 kb; −, no signal.

*c* Results of immunostaining with BXP21 and BXP34 mouse monoclonal antibodies against BCRP; ++, 100% membrane-bound staining; +, membrane-bound staining in up to 75% of cells; +/−, cytoplasmic staining in up to 25% of cells; −, no staining.

*d* ND, not done.
has become an issue of debate recently because various mutated forms of the protein were shown to behave differently (22). However, the ability of BCRP, in wild type or mutated form, to transport anthracyclines still stands. More specifically, Litman et al. (8) reported that epirubicin is a substrate of BCRP. In a panel of controls with a known expression of BCRP, we first showed that there is a good correlation of all three methods of detecting BCRP (real-time RT-PCR, Northern blotting, and immunohistochemistry). From this we conclude that immunostaining of the cell membrane with both monoclonal antibodies against BCRP (BXP21 and BXP34) is associated with marked expression of BCRP mRNA. We defined a threshold mRNA level for the immunohistochemical detection of 0.15 relative to the expression in Igrov/T8(p75)-40 cells. Basal expression of BCRP in most breast carcinoma cell lines was below the threshold of immunohistochemical detection.

With immunohistochemistry, we did not detect BCRP in tumor cells, whereas staining was positive in surrounding vessels and normal ducts. This indicates that there is a marked contribution of non-tumor cells to the BCRP mRNA measured in tumor specimens.

Because tumor cells in clinical samples did not stain with the BCRP antibodies, our results do not support the hypothesis of singular carcinoma cells in a breast tumor with an increased expression of BCRP, which can survive chemotherapy and become drug-resistant subclones by selection.

Clinical MDR development in cancer cells could also be caused by increased expression of intrinsically present BCRP levels after treatment with cytotoxic agents. We found no evidence to support that putative mechanism. We did not detect increased expression levels of BCRP mRNA in treated versus untreated carcinoma samples. There was even a trend toward more BCRP expression in untreated samples. As we have shown, BCRP detected in tumor samples largely originates in the vascular endothelium. It may well be that one effect of chemotherapy exposure is a decreased vascular density in the tumor. BCRP levels also did not exceed the immunohistochemistry detection threshold after cytotoxic treatment, as should be
We conclude that basal levels of BCRP in breast cancer cells are low and cannot be detected with immunohistochemistry. There is no apparent effect of in vivo anthracycline treatment on BCRP expression in breast cancer. Furthermore, BCRP expression in breast carcinoma does not seem to negatively influence the response of the tumor to anthracycline-based treatment, nor does it impair overall or disease-free survival of patients. BCRP is therefore unlikely to be responsible for clinical drug resistance as seen in breast cancer patients. Decreased BCRP expression may be a marker of loss of differentiation in epithelial breast tissue.

REFERENCES


