RNA Expression of Breast Cancer Resistance Protein, Lung Resistance-related Protein, Multidrug Resistance-associated Proteins 1 and 2, and Multidrug Resistance Gene 1 in Breast Cancer: Correlation with Chemotherapeutic Response

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ABSTRACT

Purpose: **The aim of this study was to investigate whether expression of particular drug resistance genes in primary operable breast cancer correlates with response to first-line chemotherapy in advanced disease.**

Experimental Design: **We determined mRNA levels of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1* in 59 primary breast tumor specimens of patients who received chemotherapy as first-line systemic treatment after diagnosis of advanced disease. The relative expression levels were measured by quantitative real-time reverse transcription-PCR and subsequently analyzed in relation to the type of response to chemotherapy, the length of progression-free survival (PFS), and post-relapse overall survival.**

Results: **For each of these drug resistance genes, a large variation in expression level was observed among the tumors of the different patients. When analyzing mRNA expression in relation to overall response, it was found that the median expression level of these five drug resistance genes in the responding tumors, as compared with nonresponding tumors, was markedly lower. Classification of tumors as high *versus* low with respect to the expression level of these genes showed that the overall response in the MDR1-high subset (17%), as compared with the MDR1-low subset (68%), was significantly lower ($P = 0.005$). Although similar differences in response rate were found for subsets of tumors stratified by the expression level of the other drug resistance genes, none of the observed differences were statistically significant. However, in the subgroup of patients treated with anthracycline-based chemotherapy (5-fluorouracil, Adriamycin/epirubicin, and cyclophosphamide), a correlation between response and the expression of *BCRP* and *MRP1* (only PFS) was found, whereas such an association was not present in the cyclophosphamide, methotrexate, and 5-fluorouracil-treated group of patients. Furthermore, high expression of *LRP* as well as *MDR1* was found to be significantly associated with a poor PFS ($P = 0.04$ and $P < 0.001$, respectively). For lung resistance-related protein, this association was limited to 5-fluorouracil, Adriamycin/epirubicin, and cyclophosphamide. Expression levels of *BCRP*, *MRP1*, or *MRP2* were not related with the length of PFS. Furthermore, no correlation between the expression level of these drug resistance genes and post-relapse overall survival was found.**

Conclusions: **In this pilot study, *MDR1* expression in primary breast tumors was inversely related with the efficacy of first-line chemotherapy, and high expression level was a significant predictor of poor prognosis for patients with advanced disease. Apart from *MDR1*, the expression levels of *BCRP*, *LRP*, and *MRP1* might have some additional predictive value for clinical outcome.**

INTRODUCTION

Chemotherapy is the treatment of choice for patients with ER[2]-negative and hormone-refractory breast cancer (reviewed in Refs. 1 and 2). Traditional chemotherapy regimens for the treatment of advanced breast cancer consisted of cyclophosphamide, MTX, 5-fluorouracil, prednisone, and vincristine combinations (3). Later on, anthracycline-based chemotherapy has

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[2] The abbreviations used are: ER, estrogen receptor; MDR, multidrug resistance; FAC/FEC, 5-fluorouracil, Adriamycin/epirubicin, and cyclophosphamide; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; RT-PCR, reverse transcription-PCR; PgR, progesterone receptor; MRP, multidrug resistance-associated protein; CI, confidence interval; PFS, progression-free survival; PR-OS, post-relapse overall survival; DEPC, diethyl pyrocarbonate; LRP, lung resistance-related protein; BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MTX, methotrexate; CR, complete response; SD, stable disease; PD, progressive disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBGD, porphobilinogen deaminase; C_T, threshold cycle; DFI, disease-free interval; RHR, relative hazard rate; AML, acute myeloid leukemia.

gradually become the standard in the treatment of advanced breast cancer. Anthracyclines such as doxorubicin (Adriamycin) and its analogue epirubicin are considered as highly active anticancer agents, which are commonly used in combinations with 5-fluorouracil and cyclophosphamide (FAC/FEC) in the treatment of breast cancer. Even though breast cancer is often considered as one of the more chemosensitive solid tumors, all initially responsive tumors relapse and develop resistance to a broad spectrum of drugs known as MDR. Consequently, metastatic breast cancer finally becomes refractory to cytotoxic drugs and is typically incurable by chemotherapy (3). Thus, intrinsic or acquired drug resistance is primarily responsible for the failure of current treatment regimens in metastatic breast cancer (4). Despite comprehensive knowledge on *in vitro* mechanisms of drug resistance, the precise nature of this *in vivo* MDR phenotype in breast cancer is still unclear.

One of the mechanisms of drug resistance in cancer cells is associated with altered anticancer drug transport, mediated by members of the ABC superfamily of transport proteins (5) such as MDR1 (6) and the MRPs (7). P-gp, the product of *MDR1*, was the first anticancer drug pump to be identified (8). The MDR phenotype conferred by overexpression of *MDR1* is characterized by resistance to structurally unrelated cytotoxic agents, including anthracyclines (doxorubicin and epirubicin are among the most effective anticancer drugs used in the treatment of breast cancer), epipodophyllotoxins, *Vinca* alkaloids, and taxanes (9). Thus, increased expression of *MDR1* is likely to contribute to clinical drug resistance in breast cancer.

Another ABC membrane transport protein implicated in clinical drug resistance and capable of actively decreasing the intracellular drug concentration in functional *in vitro* assays is MRP1 (10). Vesicular transport experiments have shown that the preferred substrates for MRP1 are drugs conjugated to glutathione, glucoronate, or sulfate and various other organic anions including MTX (reviewed in Refs. 7 and 11). Transfection studies established that MRP1 overexpression confers resistance to a wide variety of anticancer agents, including anthracyclines, vincristine, and epipodophyllotoxins. Thus, MRP1 expression may affect clinical outcome of chemotherapy in metastatic breast cancer. MRP2, originally known as the canalicular multispecific organic anion transporter (cMOAT), is another member of the MRP family that may play a role in the MDR phenotype of cancer cells (7). The whole spectrum of MRP2 substrates has not been completely defined, but it has been reported that MRP2 confers resistance to several anticancer agents, including MTX, cisplatin, vinblastine, and camptothecin derivatives (7). Thus, MRP2 may have a role in clinical drug resistance of breast cancer treated with MTX-containing chemotherapeutic regimens.

Recently, a novel ABC half transporter, BCRP, was identified as a drug efflux pump for anticancer drugs, including topoisomerase I and II inhibitors (12). To date, BCRP expression has hardly been studied in human cancers, and thus far no data are available correlating its expression with the efficacy of chemotherapy regimens in breast cancer. Because BCRP can confer cellular resistance to doxorubicin and epirubicin, it may play a role in the drug resistance phenotype of breast cancer.

In addition to an overall reduction of intracellular drug concentration, associated with overexpression of ABC transport proteins, a redistribution of the drug from the nucleus to the cytoplasm has also been implicated in MDR of cancer cells (13). It has been reported that LRP, as an integral part of the vault complex, is involved in the intracellular distribution of chemotherapeutic agents (14, 15). Moreover, LRP overexpression was found to be associated with redistribution of doxorubicin from the nucleus to the cytoplasm of colon carcinoma cells (16). Clinical data indicate that this LRP protein is often expressed in human malignancies and that its expression may be associated with poor response to chemotherapy in ovarian carcinoma and AML (14, 15). Although studies on LRP expression in breast cancer are limited, it has been reported that LRP protein is frequently expressed in primary breast carcinoma (17).

In the present study, we investigated the expression of a number of drug resistance genes in breast cancer and examined whether the expression levels correlated with clinical outcome. Therefore, mRNA levels of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1* were determined in primary operable breast tumor tissues of patients who, upon relapse, were treated with systemic first-line chemotherapy. The level of mRNA expression was determined by quantitative real-time RT-PCR and subsequently analyzed in relation to the type of response on chemotherapy, the length of PFS, and PR-OS.

MATERIALS AND METHODS

Patients and Tumors. A total of 59 patients with primary operable breast cancer who underwent resection of their primary tumors between 1985 and 1995 were included in this study. The resected primary tumor tissues were kept in liquid nitrogen. The patient and tumor characteristics, gathered from the pathology reports, are listed in Table 1 (information on histological grade was missing for some patients). ER and PgR levels were determined in primary tumor cytosols as described previously (18). The cutoff point used to classify tumors as ER and PgR positive was 10 fmol receptor protein/mg cytosol protein.

Of the 59 patients, 30 (51%) received some form of adjuvant therapy (18 received endocrine therapy, 11 received chemotherapy, and 1 received combination chemo-endocrine therapy). At the start of chemotherapy for advanced disease, the median age of the patients was 49 years (range, 28–74 years). Of the 59 patients with advanced disease, 28 were treated with CMF, whereas the other 31 patients received anthracycline-based chemotherapy (FAC/FEC). Type of response to systemic treatment was defined by the standard Union International Contre Cancer criteria as described previously (19). Accordingly, objective response is CR plus partial response. Patients with no change for more than 6 months were defined as having prolonged SD, whereas patients with PD or with SD with progression within 6 months (SD $<$ 6 months) were classified as nonresponders. Hence, 22 of the 59 patients (37%) showed an objective response [CR, $n = 3$; PR, $n = 19$), and 12 patients (20%) had prolonged SD (no change for $>$6 months), whereas 25 patients (42%) did not respond (PD, $n = 14$; SD with progression within 6 months, $n = 11$) to the chemotherapeutic treatment. Thus, the overall response rate, defined as objective response ($n = 22$) plus prolonged SD ($n = 12$), was found to be 58% (34 of 59 patients). At the time of analysis, 51 patients

Table 1 Patient and tumor characteristics[a]

Characteristic	No. (%)
All patients	59
Age at start of therapy (yrs)	
≤40	12 (20)
>40–55	28 (47)
>55–70	17 (29)
>70	2 (3)
Menopausal status at start of therapy	
Premenopausal	31 (53)
Postmenopausal	28 (47)
T status	
pT_1	12 (20)
pT_2	39 (66)
$pT_{3/4}$	8 (14)
Nodal status	
N_0	18 (31)
$N_{1–3}$	14 (24)
$N_{>3}$	26 (44)
Unknown[b]	1 (2)
Histological grade	
Poor	37 (63)
Good/moderate	3 (5)
Unknown[b]	19 (32)
ER status[c]	
Negative	30 (51)
Positive	29 (49)
PgR status	
Negative	31 (53)
Positive	28 (47)
DFI	
≤12 mo	26 (44)
>12 mo	33 (56)
First site of relapse	
Soft tissue	7 (12)
Skeletal	16 (27)
Visceral	36 (61)
Adjuvant treatment	
No	29 (49)
Yes	30 (51)

[a] Patients and tumor characteristics are detailed in "Materials and Methods."

[b] Information on nodal status and histological was not available for some patients.

[c] Cut point used: 10 fmol/mg protein.

(86%) had died, whereas 8 patients were still alive with a median follow-up of 64 months (range, 13–105 months). During follow-up after start of chemotherapy, 55 (93%) of the patients had disease progression, with a median time to progression of 6 months (range, 1–26 months). Of these patients, 25 were eventually treated with endocrine therapy immediately after progression on first-line chemotherapy, and 10 of these patients received endocrine therapy after one or two additional chemotherapy regimens.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from three 15-μm cryostat sections of frozen breast cancer biopsies using the RNazol B extraction method (Campro Scientific, Veenendaal, the Netherlands) according to the manufacturer's instructions (TEL-Test Bulletin No. 2). An additional cryostat section was used to determine the content of tumor cells. For all breast cancer samples included in the analyses, the number of tumor cells represented at least 50% of total nucleated cells (range, 50–90%; median, 70%), as judged by H&E staining of the sections. Tumor sample RNA was diluted in DEPC-treated RNase-free ultra-pure water (DEPC; Sigma-Aldrich, Zwijndrecht, the Netherlands) and stored at −80°C. Total RNA (3 μg) was reverse transcribed for 1 h at 37°C in a 50-μl volume of 1× first strand buffer containing 250 units of Superscript II RNase H^- reverse transcriptase, 15 units of RNAseOUT recombinant RNase inhibitor, and 1 mM DTT (all from Life Technologies, Inc.), further supplemented with deoxynucleoside triphosphates (1 mM each), 1.5 μg of $pd(T)_{12–18}$ oligo(dT) primer, and 1.5 μg of $pd(N)_6$ random hexamer (all from Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands). The resulting cDNA was diluted in ultra-pure water and aliquoted at −80°C. Forty ng of cDNA diluted in a 10-μl volume of DEPC-treated RNase-free ultra-pure water were used in each real-time amplification reaction.

Quantitative Real-time RT-PCR. The mRNA levels of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1* were measured by real-time RT-PCR based on TaqMan chemistry and quantitated using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). In addition, the mRNA levels of two internal control genes, *i.e.*, *GAPDH* and *PBGD*, were measured and used to normalize the mRNA levels of the drug resistance genes. PCR products were detected using a dual-fluorescent nonextendable probe containing a 5′ FAM (6-carboxy-fluorescein) reporter dye and a 3′ TAMRA (6 carboxy-tetramethylrhodamine) quencher dye for all reactions, except for the GAPDH reaction, in which FAM was substituted by VIC. The *GAPDH* mRNA levels were measured using the Pre-developed TaqMan Assay Reagents for human *GAPDH* (Applied Biosystems). All other primer pairs, in conjunction with their appropriate fluorescent hybridization probes (sequences are given in Table 2), were designed by Oligo 6.0 primer analysis software (Medprobe, Oslo, Norway) and purchased by Oswel Research Products (Eurogentec, Seraing, Belgium). Notably, the primers and probe sets were designed to amplify across an intron/exon boundary, thereby preventing amplification of residual genomic DNA. Real-time PCR was performed in duplicate reactions of 50 μl of 1× TaqMan buffer A (Applied Biosystems) containing 40 ng of tumor sample cDNA as a template. PCR was performed in the presence of 300 nM forward and reverse primers, 200 nM probe, 250 μM deoxynucleoside triphosphates, 1.25 units of AmpliTaq Gold DNA polymerase, and 4 mM $MgCl_2$. Samples were heated for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of amplification for 15 s at 95°C and 1 min at 60°C. Under these assay conditions, a linear relation was noted for log amount of cDNA from MCF-7, an established breast cancer cell line. Furthermore, serial diluted cDNA prepared from pooled RNA of four leukemia cell lines (CEM, K562, and two EBV-induced lymphoblastoid B-cell lines) was used as an additional positive control. The PCR efficiencies of the five target genes and the two endogenous reference genes were comparable (≥95%).

To compare the expression levels among different tumor samples, the relative expression level of the resistance genes was calculated using the comparative C_T method and compared with a calibrator (20, 21). The comparative C_T method eliminates the use of standard curves for relative quantitation as long as the PCR efficiency of target and reference gene is similar. Briefly, reactions are characterized by comparing C_T values.

Table 2 Sequences of primer and probes set

BCRP	235-bp amplicon
Forward primer	5′-TGGCTGTCATGGCTTCAGTA-3′
Reverse primer	5′-GCCACGTGATTCTTCCACAA-3′
Probe	5′-AGCAGGGCATCGATCTCTCACCCTG-3′
LRP	68-bp amplicon
Forward primer	5′-CAGCTGGCCATCGAGATCA-3′
Reverse primer	5′-TCCAGTCTCTGAGCCTCATGC-3′
Probe	5′-CAACTCCCAGGAAGCGGCGGC-3′
MRP1	65-bp amplicon
Forward primer	5′-CAATGCTGTGATGGCGATG-3′
Reverse primer	5′-GATCCGATTGTCTTTGCTCTTCA-3′
Probe	5′-AGACCAAGACGTATCAGGTGGCCCAC-3′
MRP2	219-bp amplicon
Forward primer	5′-ATGCTTCCTGGGGATAAT-3′
Reverse primer	5′-TCAAAGGCACGGATAACT-3′
Probe	5′-TGTATCTGTTCAGATGTTTTATGTGTCTACCT-3′
MDR1	195-bp amplicon
Forward primer	5′-GGAAGCCAATGCCTATGACTTTA-3′
Reverse primer	5′-GAACCACTGCTTCGCTTTCTG-3′
Probe	5′-TGAAACTGCCTCATAAATTTGACACCCTGG-3′
PBGD	98-bp amplicon
Forward primer	5′-CTGCACGATCCCGAGACTCT-3′
Reverse primer	5′-GCTGTATGCACGGCTACTGG-3′
Probe	5′-CTGAGGCACCTGGAAGGAGGCTG-3′
GAPDH[a]	

[a] The GAPDH primer set and the corresponding fluorogenic probe were designed by and purchased from Applied Biosystems.

The C_T value is defined as the fractional cycle number at which the emitted sample fluorescence passes a fixed threshold above the baseline. The ΔC_T value is defined as the difference in C_T value for the target and reference gene(s). Accordingly, ΔC_T = (mean target gene C_T) – [(the mean of duplicate C_T values for GAPDH + the mean of duplicate C_T values for PBGD)/2]. The relative gene expression in a particular sample, normalized to *GAPDH* and *PBGD* and compared with the expression of that target gene in a calibrator, is then given by the following: relative amount of target = $2^{-\Delta\Delta CT\ value}$. The $\Delta\Delta C_T$ value in this formula is defined as the ΔC_T value of the target gene for a particular sample less the ΔC_T value of that target gene for a calibrator ($\Delta\Delta C_T = \Delta C_{T(target)} - \Delta C_{T(calibrator)}$). In this study, the calibrator was a cDNA pool from normal peripheral WBCs. We have used the average C_T value of two endogenous reference genes instead of a single one to correct for tumor sample-specific variation in housekeeping gene expression. Notably, tumor samples with an aberrant GAPDH:PBGD ratio were omitted from the analysis.

Statistical Analysis. The associations between expression levels of *BCRP*, *MRP1*, *MRP2*, *LRP*, and *MDR1* and their relationship with age and PgR and ER levels were studied with Spearman rank correlation (r_s). The strength of the association between the expression levels of the drug resistance genes (treated as continuous variables) and other patient and tumor characteristics and DFI (treated as grouping variables) was tested with the Mann-Whitney U test or the Kruskal-Wallis test. In an exploratory analysis to search for optimized cut points to classify tumors as high *versus* low for the respective factor, isotonic regression analysis (22) was used with the overall response rate as end point. PFS and PR-OS probabilities were calculated by the actuarial method of Kaplan and Meier (23). Response was analyzed with logistic regression. PFS and PR-OS were analyzed using the Cox proportional hazards model. The length of PFS was defined as the time from the start of treatment of advanced disease until the time of PD or intercurrent death, and differences in PFS were assessed by the log-rank test. All statistical analyses were performed with Stata Statistical Software (release 7.0; Stata Corp., College Station, TX).

RESULTS

Expression of Drug Resistance Genes in Breast Cancer. The mRNA expression levels of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1*, estimated by real-time RT-PCR, were expressed in arbitrary units for all 59 individual primary tumor samples (Fig. 1, *A-E*). A marked variation in expression level was found for each of these genes, and the estimated relative mRNA expression levels showed an approximately log-normal distribution. The median relative expression levels were 177 (range, 24–2065) for BCRP, 111 (range, 6–729) for LRP, 85 (range, 11–784) for MRP1, 59 (range, 0.5–1123) for MRP2, and 8.8 (range, 0.5–84.8) for MDR1.

Subsequent analysis with regard to potential interrelationships of these drug resistance genes revealed that the mRNA levels of *BCRP* were positively related with those of *LRP* ($r_s = 0.42$; $P = 0.001$), *MRP1* ($r_s = 0.37$; $P = 0.005$), and *MDR1* ($r_s = 0.69$; $P < 0.0001$). In addition, the levels of *LRP* were positively related with those of *MRP1* ($r_s = 0.46$; $P < 0.001$), *MRP2* ($r_s = 0.36$; $P = 0.007$), and *MDR1* ($r_s = 0.36$, $P = 0.006$). Furthermore, *MRP1* expression levels were positively related with those of *MRP2* ($r_s = 0.30$; $P = 0.024$) and *MDR1* ($r_s = 0.51$; $P < 0.0001$). Next we investigated the mRNA expression of these genes in relation to traditional clinical prognostic factors as listed in Table 1. Only weak correlations were found with ER, PgR, and DFI. The expres-

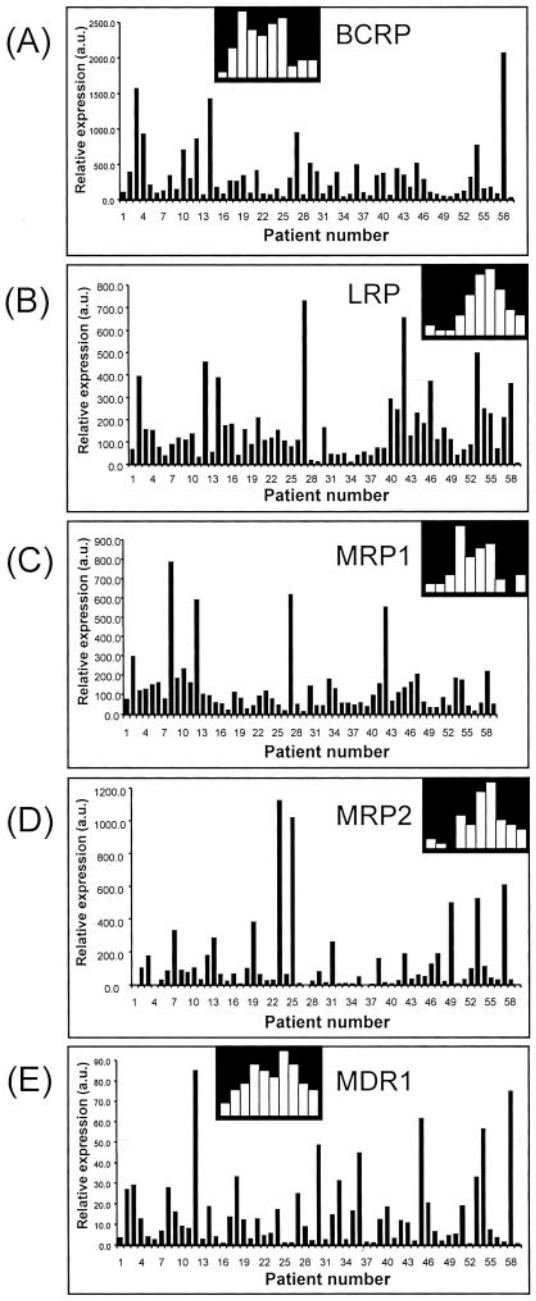

Fig. 1 Relative mRNA expression levels of *BCRP* (*A*), *LRP* (*B*), *MRP1* (*C*), *MRP2* (*D*), and *MDR1* (*E*), as estimated by real-time RT-PCR and expressed in arbitrary units (*a.u.*), are shown for all individual primary breast tumor samples. Expression data were available for all 59 tumor samples, except for MRP2, where expression data of patients 1, 4, and 27 are missing. *Insets* show the log-normal distribution.

sion levels of BCRP and LRP were found to be positively correlated with those of ER [$r_s = 0.32$ ($P = 0.014$) and $r_s = 0.38$ ($P = 0.003$), respectively] and PgR [$r_s = 0.30$ ($P = 0.021$) and $r_s = 0.31$ ($P = 0.017$), respectively]. In addition, low *MDR1* and *MRP1* mRNA levels were both associated with a prolonged DFI with respective *P*s of 0.036 and 0.018.

Response to Chemotherapy and Correlation with Expression. The objective response rate of the patients included in these analyses was 37% (22 of 59 patients), and the overall response rate was 58% (34 of 59 patients). Within this cohort of 59 patients, the type of response to chemotherapy was not significantly related to any of the clinicopathological patient and tumor parameters listed in Table 1.

When analyzing BCRP, LRP, MRP1, MRP2, and MDR1 as log-transformed continuous variables, there were no statistically significant associations with the overall response rate. However, comparing the tumors of responding *versus* nonresponding patients with respect to the relative expression levels of these genes showed that tumors of responding patients had the lowest median expression levels for *BCRP* (174 *versus* 255), *LRP* (108 *versus* 119), *MRP1* (83 *versus* 95), *MRP2* (32 *versus* 79), and *MDR1* (7 *versus* 17). Therefore, in an exploratory analysis, we used isotonic regression analysis (22) with the overall response rate as end point to search for optimized cut points to classify tumors as high *versus* low for the respective factor. The resulting cut points chosen were BCRP $>$ 360, LRP $>$ 152, MRP1 $>$ 85, MRP2 $>$ 59, and MDR1 $>$ 26.

The overall response rates for the subgroups of patients classified as high *versus* low for the different drug resistance genes are shown in Table 3. Although tumors with high mRNA expression levels of *BCRP*, *LRP*, *MRP1*, or *MRP2* showed a lower overall response rate as compared with tumors with low mRNA levels, none of the observed differences was statistically significant. In contrast, for *MDR1*, a significant inverse correlation was found between expression level and overall response rate. Only 2 of 12 patients (17%) with a high *MDR1* expression responded, compared with 32 of 47 patients (68%) with a low *MDR1* level (odds ratio, 0.09; 95% CI, 0.02–0.48; $P = 0.005$).

Expression Levels in Relation to PFS and PR-OS. The expression levels of the drug resistance genes were further analyzed in relation to the length of PFS and PR-OS. Kaplan-Meier curves, stratified by expression of the respective drug resistance genes, revealed that BCRP (Fig. 2*A*), MRP1 (Fig. 2*C*), or MRP2 (Fig. 2*D*) status was not significantly related with the length of PFS. However, high expression levels of *LRP* or *MDR1* were found to be significantly associated with a poor PFS (Fig. 2, *B* and *E*, respectively). The RHR for high (as compared with low) *LRP* expression was 1.85 (95% CI, 1.04–3.30; $P = 0.04$), and for high *MDR1* expression, the RHR was 4.22 (95% CI, 1.95–9.15; $P < 0.001$). A significant correlation between the level of mRNA expression and the length of PR-OS was not found for any of the investigated drug resistance genes (data not shown).

Anthracycline-based Chemotherapy (FAC/FEC) *versus* CMF. The breast cancer patients included in this study received either anthracycline-based chemotherapy (FAC/FEC) or CMF as first-line treatment for metastatic disease. Therefore, we further analyzed mRNA expression of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1* in relation to clinical outcome for these distinct regimens. Furthermore, no significant difference was seen in the overall response rates between CMF-treated (54%) and FAC/FEC-treated patients (61%). However, classification of tumors into high and low with respect to expression of the drug resistance genes showed different response rates for CMF and FAC/FEC (Table 3). For all drug resistance genes except

Table 3 Expression in relation to overall response to chemotherapy and for FAC/FEC and CMF separately[a]

	Chemotherapy (all)	CMF	FAC/FEC
Subset of patients[b]	R/N[c] (%) [OR; 95% CI; *P*]	R/N (%) [*P*]	R/N (%) [*P*]
All patients ($n = 59$)	34/59 (58)	15/28 (54)	19/31 (61) [$P = 0.55$]
BCRP-low	27/42 (64)	11/20 (55)	16/22 (73)
BCRP-high	7/17 (41) [0.39; 0.12–1.23; $P = 0.11$]	4/8 (50) [$P = 1.0$]	3/9 (33) [$P = 0.056$]
LRP-low	24/37 (65)	10/17 (59)	14/20 (70)
LRP-high	10/22 (45) [0.45; 0.15–1.33; $P = 0.15$]	5/11 (45) [$P = 0.70$]	5/11 (45) [$P = 0.26$]
MRP1-low	18/30 (60)	8/16 (50)	10/14 (71)
MRP1-high	16/29 (55) [0.82; 0.29–2.31; $P = 0.71$]	7/12 (58) [$P = 0.66$]	9/17 (53) [$P = 0.30$]
MRP2-low	18/28 (64)	5/8 (63)	13/20 (65)
MRP2-high	13/28 (46) [0.48; 0.16–1.41; $P = 0.18$]	8/18 (44) [$P = 0.40$]	5/10 (50) [$P = 0.43$]
MDR1-low	32/47 (68)	13/22 (59)	19/25 (76)
MDR1-high	2/12 (17) [0.09; 0.02–0.48; $P = 0.005$]	2/6 (33) [$P = 0.37$]	0/6 (0) [$P < 0.001$]

[a] The overall response rate was defined as objective response (CR + PR) plus SD (= prolonged ''no change').
[b] Cut points used were as described in Fig. 2. Three MRP2 data points were missing for analysis because the expression levels of the respective tumors were not available.
[c] R/N, number of responders/total number of patients.

MRP2, we found a trend that higher levels of mRNA expression were related with poor response in FAC/FEC-treated patients, whereas no such relation was seen in the subgroup of CMF-treated patients. The difference in response rate between high and low expression for tumors that received anthracycline-based chemotherapy was found to be statistically significant for MDR1 ($P < 0.001$) and borderline significant for BCRP ($P = 0.056$). Notably, in the FAC/FEC subset, 0 of 6 MDR1-high tumors responded, whereas 19 of 25 (76%) MDR1-low tumors responded to this type of chemotherapy. Unlike the FAC/FEC-treated tumors, the level of *MDR1* expression was not related with the rate of response in the CMF-treated subgroup of patients.

Next we analyzed the expression levels of the drug resistance genes in relation to the length of PFS for these two distinct chemotherapeutic regimens (Fig. 3). The length of PFS in relation to type of chemotherapy (CMF *versus* FAC/FEC) was identical ($P = 1.0$; Kaplan-Meier curves not shown). However, a clear difference in the length of PFS between high- and low-expressing tumors was seen for BCRP (Fig. 3*B*; not significant), LRP (Fig. 3*D*, $P = 0.04$), and MRP1 (Fig. 3*F*, $P = 0.04$) in the FAC/FEC-treated tumors, but not in those treated with CMF (Fig. 3, *A*, *C*, and *E*). No difference in PFS was observed for these two treatment modalities after stratification for *MRP2* expression (data not shown). With respect to MDR1, we found that in both the FAC/FEC-treated subgroup (Fig. 3*H*) and the CMF-treated subgroup of patients (Fig. 3*G*), high levels of expression were significantly correlated with a shorter PFS. However, it should be noted that the RHR of the MDR1-high subgroup compared with the MDR1-low subgroup is higher in the FAC/FEC-treated patients (Fig. 3*H*) as compared with the CMF-treated subgroup (Fig. 3*G*).

DISCUSSION

Chemotherapy resistance is a major problem in the management of patients with breast cancer. In general, breast cancer is considered to be one of the more chemosensitive solid tumors, and major response rates (20–80%) in patients with metastatic disease have been reported (2). However, CRs are rare, and most of the initially responsive tumors relapse and develop resistance to multiple anticancer agents of different structure and function, a phenomenon known as MDR. Eventually, metastatic breast cancer becomes refractory to standard combination chemotherapy (CMF or FAC/FEC). The nature of this chemotherapy resistance in breast cancer and the potential role of drug resistance genes involved in transport or sequestration of anticancer agents are still unclear (4). A better understanding of the mechanisms(s) of chemotherapy resistance in breast cancer and further knowledge about the genes whose expression affects the outcome of chemotherapy in advanced disease may open the way for specific pharmacological intervention with reversal agents to circumvent therapy resistance (24). The aim of the present study was to investigate whether the expression of *BCRP*, *LRP*, *MRP1*, *MRP2*, and *MDR1*, genes potentially involved in transport or redistribution of anticancer agents, is correlated with clinical outcome.

We have developed and validated real-time RT-PCRs for these five genes to quantitate their mRNA levels in primary breast tumors. Whereas commonly used methods such as RNase protection and, in particular, immunohistochemistry fail to detect low-abundance expression, the detection threshold of fluorescence-based kinetic RT-PCR enables us to detect and distinguish very low-level mRNA expression (25). Here, we showed that (low abundance) mRNA expression of these five genes was readily detected and that small differences in mRNA levels obtained from limited frozen tissue could be reproducibly quantitated. Significant positive correlations between the mRNA levels of drug resistance genes were found, particularly for MDR1/BCRP, MDR1/MRP1 and LRP/MRP1, and may indicate that the expression of these genes is likely to be coordinately regulated. Similar correlations between the expression of these genes were observed previously (26, 27). Moreover, MDR1, MRP1, and LRP were significantly correlated with the protein kinase Cη isozyme (26), which in turn has been reported to transcriptionally regulate the *MDR1* promoter (28). Thus, protein kinase Cη could represent a key regulator factor for up-regulation of various MDR-associated genes.

To investigate the clinical significance of BCRP in breast

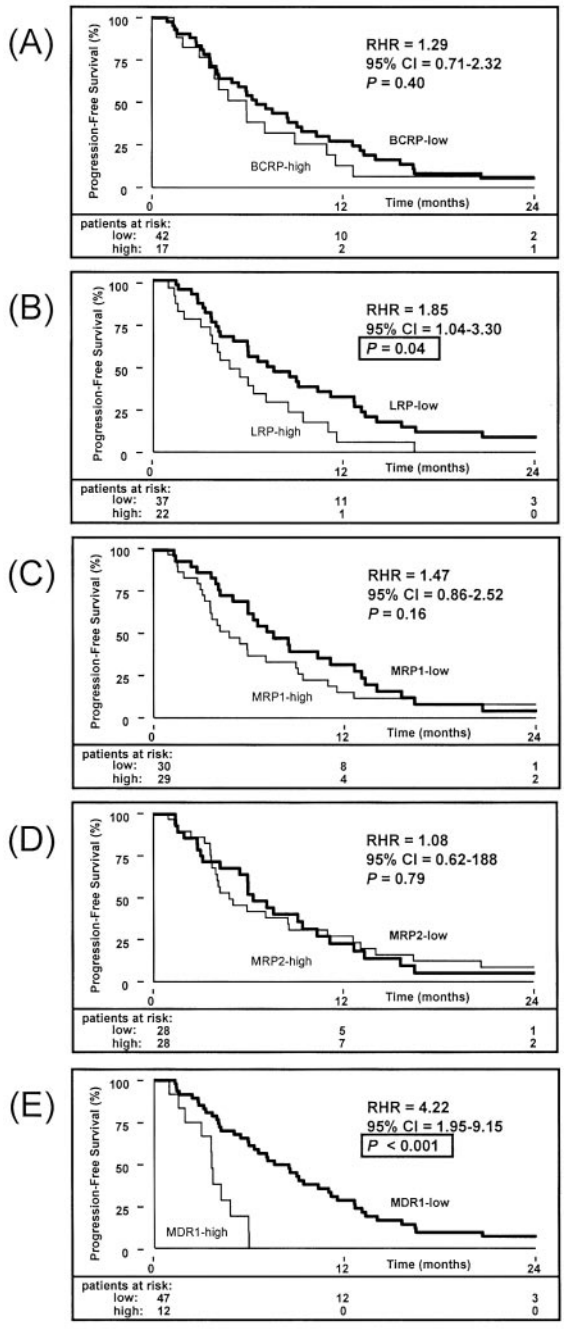

Fig. 2 Kaplan-Meier curves for PFS as a function of the relative mRNA levels of *BCRP* (*A*), *LRP* (*B*), *MRP1* (*C*), *MRP2* (*D*), and *MDR1* (*E*). The optimized cut points to classify tumors as high *versus* low for BCRP, LRP, MRP1, MRP2, and MDR1 were >360, >152, >85, >59, and >26, respectively. The number of patients at risk in each group at different time points is shown.

cancer, we compared *BCRP* mRNA levels with those of *LRP*, *MRP1*, *MRP2*, and *MDR1*. Although advanced breast cancer patients with BCRP-high primary tumors had a lower response rate to first-line chemotherapy (Table 3) together with a trend toward a poor PFS in patients with BCRP-high tumors as compared with BCRP-low tumors (Fig. 2*A*), these differences were not statistically significant. From these data, we concluded that BCRP expression had no or only limited power to predict response to first-line chemotherapy in our studied cohort of advanced breast cancer patients. To date, BCRP expression has hardly been studied in human cancers, and thus far, only two exploratory studies in AML have been conducted to evaluate the clinical significance of BCRP expression (29, 30). No clear correlation between BCRP expression and response to subsequent treatment was found in AML, and, similar to our results, BCRP expression was correlated with that of MDR1. Based on the substrate specificity of BCRP, a more prominent correlation between expression and clinical outcome could be expected in the subgroup of patients with advanced disease who received anthracycline-containing regimens. Indeed, we found that the negative correlation between *BCRP* mRNA expression and response rate (Table 3) as well as PFS (Fig. 3, *A* and *B*) was stronger in the FAC/FEC-treated subgroup, as compared with CMF-treated patients. This might suggest that anthracyclines are actively exported from breast cancer cells by BCRP and that its expression in primary breast tumors has some predictive value in relation to clinical outcome of anthracycline-based chemotherapy. Hence, the putative predictive value of BCRP expression seems to be exclusive for anthracycline-based chemotherapy. In contrast, two studies showed a lack of correlation between BCRP mRNA expression and response to anthracycline-based chemotherapy (27, 31). Moreover, Kanzaki *et al.* (27) reported that BCRP mRNA levels, as determined by semiquantitative RT-PCR, were very low and hardly varied among individual tumors. This discrepancy may be explained by differences in detection threshold of the used methodologies (*i.e.*, highly sensitive and quantitative TaqMan chemistry *versus* semiquantitative RT-PCR). In accordance with our study, Faneyte *et al.* (31) showed that BCRP expression in clinical breast cancer samples, which was not detectable with immunohistochemistry, was clearly detected by real-time RT-PCR and varied widely. Also in that particular study, there was no clear indication that elevated BCRP expression in breast carcinomas conferred resistance to anthracyclines. The results of our present study warrant more extensive investigations on the role of BCRP in chemotherapy resistance of breast cancer.

With respect to LRP, we found in our study a statistically significant correlation between the expression level and length of PFS (Fig. 2*B*). Because this correlation was not found for the subgroup treated with CMF (Fig. 3*C*), our results suggest that LRP may play a role in anthracycline resistance. It has been reported that enhanced LRP protein expression in colon carcinoma cells was associated with redistribution of doxorubicin from the nucleus to the cytoplasm (16). With regard to the clinical significance of LRP expression in breast carcinoma, it has been reported that LRP protein expression in locally advanced breast cancer was found to be associated with the presence of axillary nodal metastasis after induction chemotherapy, suggesting that LRP may play a role in the resistance phenotype of intrinsically resistant clones that persist after induction chemotherapy (32). On the other hand, several other breast cancer studies reported a lack of correlation between LRP expression and clinical outcome (33–35). It should be noted that in these latter (negative) studies, LRP expression was determined by

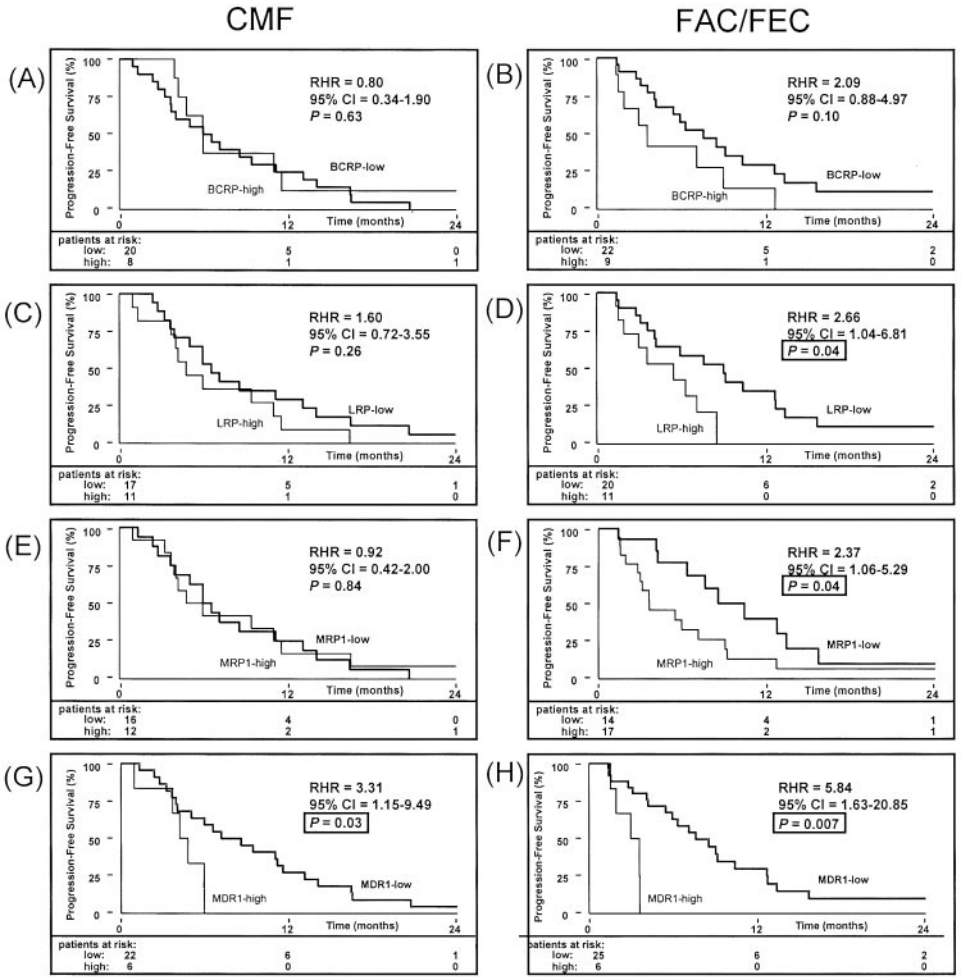

Fig. 3 PFS as a function of the relative mRNA levels of *BCRP* (*A* and *B*), *LRP* (*C* and *D*), *MRP1* (*E* and *F*), and *MDR1* (*G* and *H*) in CMF- and FAC/FEC-treated patients. For cut points, see the Fig. 2 legend.

immunohistochemistry, which has a rather low detection threshold as compared with the TaqMan technology. Although compelling evidence has not been provided yet, our exploratory study indicates that LRP expression in primary breast cancer may have some predictive power with respect to clinical outcome.

It has been reported that MRP2 confers *in vitro* resistance to MTX, which is used in the CMF treatment of advanced breast cancer (7). However, we showed that the level of *MRP2* expression had no relationship whatsoever with the efficacy of CMF treatment in advanced breast cancer, suggesting that MRP2 has no major role in clinical drug resistance of breast cancer. With respect to MRP1, we found that the level of expression was only related to clinical outcome in the subgroup of patients treated with anthracycline-based chemotherapy, and not in the CMF subgroup. Although hyperexpression of MRP1 has been observed in several human cancers, including non-small cell lung cancer, esophagus squamous cell carcinoma, and several types of leukemia (36–38), comprehensive evidence about the significance of MRP1 expression in clinical resistance of these human cancers is still lacking. Previously, we reported that MRP1 might play a role in clinical chemotherapy resistance in breast cancer (39, 40). There, it was concluded that MRP1 protein expression is a predictor of poor prognosis in breast cancer patients who received chemotherapy as first-line systemic treatment for recurrence (39). Furthermore, MRP1 might be of prognostic significance in the subgroups of patients with a more favorable prognosis, *i.e.*, node-negative patients with relatively small tumors, as well as in the setting of adjuvant systemic chemotherapy (40). The data as presented here suggest that the clinical role of MRP1 in chemotherapy resistance of breast cancer might be restricted to anthracycline resistance because MRP1 expression had no effect on clinical outcome of CMF-treated patients (Fig. 3, *E* and *F*).

Here, we demonstrated the clinical significance of MDR1 expression in primary breast tumors. Evaluation of the literature with respect to MDR1 expression in breast cancer revealed that, despite numerous reports, there is no consensus about the actual level of expression and the clinical significance of MDR1 hyperexpression. It is therefore not clear whether MDR1 expression affects the efficacy of chemotherapy in breast cancer. The inconsistency about this subject in the literature is likely due to the lack of a sensitive and highly quantitative methodology to assess the MDR1 expression levels. MDR1 was mostly deter-

mined by immunohistochemistry (reviewed in Ref. 41). Furthermore, *MDR1* mRNA is generally expressed at very low levels in breast carcinoma cells, and consequently, P-gp expression is too low to be accurately quantitated by rather insensitive immunohistochemical methods or semiquantitative RT-PCR (41–43). Although the *MDR1* expression in the present study was also low, we showed by real-time RT-PCR that *MDR1* mRNA levels of individual tumors varied markedly (100-fold range). Furthermore, we demonstrated that *MDR1* expression was a statistically significant predictor for the type of response to chemotherapy and the length of PFS in our cohort of advanced breast cancer patients. Taken together, our results suggest that MDR1 expression in primary breast tissue is an important predictor of clinical outcome for patients with advanced disease receiving chemotherapy as first-line treatment. Notably, in a multivariable analysis adjusted for adjuvant therapy, dominant site of relapse, and relapse-free interval, MDR1 expression was found to be an independent predictor for response. This conclusion is in line with the outcome of a combined review and large meta-analysis of data from MDR1 assessment of more than 1200 breast tumors (44). Although the individual studies showed considerable heterogeneity, the final conclusion of this meta-analysis was that it seems likely that MDR1 expression in breast cancer is associated with a poor response rate to chemotherapy.

Our results suggest that in addition to *MDR1*, the drug resistance-associated genes *BCRP*, *LRP*, and *MRP1*, but not *MRP2*, may also play a role in the chemotherapy resistance of breast cancer. Interestingly, we noted that this association between expression and clinical outcome was stronger in (MDR1), limited to (LRP), or solely present in (BCRP and MRP1) the subgroup of FAC/FEC-treated patients. Therefore, elevated expression of *BCRP*, *LRP*, *MRP1*, and *MDR1* in breast tumor tissue might be associated with anthracycline resistance.

It has been suggested that MDR1 expression is merely a measure of malignancy or advanced disease, rather than an indicator of chemotherapy resistance. Pursuing this thought further, it is reasonable to expect that MDR1 expression has an apparent effect on clinical outcome of both FAC/FEC- and CMF-treated tumors, reflecting respectively the functional drug efflux pump activity of P-gp and its function as a surrogate marker for a more aggressive tumor cell behavior. Furthermore, we found that a high MDR1 expression was correlated with a shorter DFI interval, indicating that P-gp expression may be linked to a more aggressive and malignant phenotype. Such a correlation between MDR1 expression and tumor invasiveness has been documented for breast cancer (45). In one study, it was suggested that MDR1 expression in breast carcinoma cells serves mainly as a surrogate marker for drug resistance (46). This postulation was based on an observed absence of functional P-gp-mediated pump activity as measured by the rhodamine 123 efflux assay in immunohistochemically P-gp-positive tumor cells. Another study reported that MDR1, in addition to its role in drug transport, might play a drug efflux-independent antiapoptotic role through modulation of the sphingomyelin-ceramide apoptotic pathway (47). More compelling evidence for an antiapoptotic role for MDR1 has been provided by a study showing that MDR1 hyperexpression may affect ceramide production and subsequently affect the expression of multiple downstream target genes of ceramide (48). In conclusion, MDR1 is likely to be involved in anthracycline efflux-mediated resistance and may be involved in apoptosis-associated MDR as well.

In summary, our study suggests that MDR1 is an important predictor of poor prognosis in breast cancer patients receiving chemotherapy as first-line treatment for recurrent disease. Furthermore, high MDR1 expression in primary breast tumors may be related to altered biological behavior of the tumor cells, including a more aggressive phenotype resulting in resistance to first-line chemotherapy of advanced metastatic disease. Apart from *MDR1*, expression of the drug resistance genes *BCRP*, *LRP*, and *MRP1* may have some additional predictive value for clinical outcome in breast cancer. Clearly, further investigations are needed to confirm the conclusions from this exploratory study.

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REFERENCES

1. Esteva, F. J., Valero, V., Pusztai, L., Boehnke-Michaud, L., Buzdar, A. U., and Hortobagyi, N. Chemotherapy of metastatic breast cancer: what to expect in 2001 and beyond. Oncologist, *6:* 133–146, 2001.

2. Hortobagyi, G. N. Treatment of breast cancer. N. Engl. J. Med., *339:* 974–984, 1998.

3. Harris, J. R., Lippman, M. E., Morrow, M., and Osborne, C. K. Treatment of metastatic breast cancer. *In:* J. R. Harris, M. E. Lippmann, M. Morrow, *et al.* (eds.) , Diseases of the Breast, p. 749. Philadelphia: J. B. Lippincott Williams & Wilkens, 2000.

4. Kaye, S. B. Multidrug resistance in breast cancer: is the jury in yet? J. Natl. Cancer Inst. (Bethesda), *89:* 902–903, 1997.

5. Klein, I., Sarkadi, B., and Varadi, A. An inventory of the human ABC proteins. Biochim. Biophys. Acta, *1461:* 237–262, 1999.

6. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant cells. Cell, *47:* 381–389, 1986.

7. Borst, P., Evers, R., Kool, M., and Wijnholds, J. A family of drug transporters: the mutidrug-resistance-associated proteins. J. Natl. Cancer Inst. (Bethesda), *92:* 1295–1302, 2000.

8. Juliano, R. L., and Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta, *455:* 152–162, 1976.

9. Nooter, K., and Herweijer, H. Multidrug resistance (mdr) genes in human cancer. Br. J. Cancer, *63:* 663–669, 1991.

10. Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science (Wash. DC), *258:* 1650–1654, 1992.

11. Hipfner, D. R., Deeley, R. G., and Cole, S. P. C. Structural, mechanistic and clinical aspects of MRP1. Biochim. Biophys. Acta, *1461:* 359–376, 1999.

12. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc. Natl. Acad. Sci. USA, *95:* 15665–15670, 1998.

13. Larsen, A. K., Escargueil, A. E., and Skladanowski, A. Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol. Ther., *85:* 217–229, 2000.

14. Dalton, W. S., and Scheper, R. J. Lung resistance-related protein: determining its role in multidrug resistance. J. Natl. Cancer Inst. (Bethesda), *91:* 1604–1605, 1999.

15. Scheffer, G. L., Schroeijers, A. B., Izquierdo, M. A., Wiemer, E. A., and Scheper, R. J. Lung resistance-related protein/major vault protein and vaults in multidrug-resistant cancer. Curr. Opin. Oncol., *12:* 550–556, 2000.

16. Kitazono, M., Sumizawa, T., Takebayashi, Y., Chen, Z. S., Furukawa, T., Nagayama, S., Tani, A., Takao, S., Aikou, T., and Akiyama, S. Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. J. Natl. Cancer Inst. (Bethesda), *91:* 1647–1653, 1999.

17. Pohl, G., Filipits, M., Suchomel, R. W., Stranzl, T., Depisch, D., and Pirker, R. Expression of the lung resistance protein (LRP) in primary breast cancer. Anticancer Res., *19:* 5051–5055, 1999.

18. Foekens, J. A., Portengen, H., van Putten, W. L., Peters, H. A., Krijnen, H. L., Alexieva-Figusch, J., and Klijn, J. G. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer Res., *49:* 5823–5828, 1989.

19. Foekens, J. A., Look, M. P., Peters, H. A., van Putten, W. L., Portengen, H., and Klijn, J. G. Urokinase-type plasminogen activator and its inhibitor PAI-1: predictors of poor response to tamoxifen therapy in recurrent breast cancer. J. Natl. Cancer Inst. (Bethesda), *87:* 751–756, 1995.

20. User Bulletin No. 2. ABI PRISM 7700 Sequence Detection System, Revision A, pp. 11–18. Foster City, CA: Applied Biosystems, 1997.

21. Livak, K. J., Flood, S. J. A., Marmaro, J., Giusti, W., and Deetz, K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Applications, *4:* 357–362, 1995.

22. Barlow, R. E., Bartholomew, D. J., Bremner, J. M., and Brunk, H. D. Statistical Inference Under Order Restrictions. New York: John Wiley & Sons, Inc., 1972.

23. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.

24. Van Zuylen, L., Nooter, K., Sparreboom, A., and Verweij, J. Development of multidrug-resistance convertors: sense or nonsense? Invest. New Drugs, *18:* 205–220, 2000.

25. Bustin, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol., *25:* 169–193, 2000.

26. Beck, J., Bohnet, B., Brugger, D., Bader, P., Dietl, J., Scheper, R. J., Kandolf, R., Liu, C., Niethammer, D., and Gekeler, V. Multiple gene expression analysis reveals distinct differences between G2 and G3 stage breast cancers, and correlations of PKCη with MDR1, MRP and LRP gene expression. Br. J. Cancer, *77:* 87–91, 1998.

27. Kanzaki, A., Toi, M., Nakayama, K., Bando, H., Mutoh, M., Uchida, T., Fukumoto, M., and Takebayashi, Y. Expression of multidrug resistance-related transporters in human breast carcinoma. Jpn. J. Cancer Res., *92:* 452–458, 2001.

28. Gill, P. K., Gescher, A., and Gant, T. W. Regulation of MDR1 promoter activity in human breast carcinoma cells by protein kinase C isozymes α and θ. Eur. J. Biochem., *268:* 4151–4157, 2001.

29. Ross, D. D., Karp, J. E., Chen, T. T., and Doyle, L. A. Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. Blood, *96:* 365–368, 2000.

30. Sargent, J. M., Williamson, C. J., Maliepaard, M., Elgie, A. W., Scheper, R. J., and Taylor, C. G. Breast cancer resistance protein expression and resistance to daunorubicin in blast cells from patients with acute myeloid leukaemia. Br. J. Haematol., *115:* 257–262, 2001.

31. Faneyte, I. F., Kristel, P. M., Maliepaard, M., Scheffer, G. L., Scheper, R. J., Schellens, J. H. M., and Van De Vijver, M. J. Expression of the breast cancer resistance protein in breast cancer. Clin. Cancer Res., *8:* 1068–1074, 2002.

32. Schneider, J., Gonzalez-Roces, S., Pollan, M., Lucas, R., Tejerina, A., Martin, M., and Alba, A. Expression of LRP and MDR1 in locally advanced breast cancer predicts axillary node invasion at the time of rescue mastectomy after induction chemotherapy. Breast Cancer Res., *3:* 183–191, 2001.

33. Schneider, J., Lucas, R., Sanchez, J., Ruibal, A., Tejerina, A., and Martin, M. Modulation of molecular marker expression by induction chemotherapy in locally advanced breast cancer: correlation with the response to therapy and the expression of MDR1 and LRP. Anticancer Res., *20:* 4373–4377, 2000.

34. Linn, S. C., Pinedo, H. M., van Ark-Otte, J., van der Valk, P., Hoekman, K., Honkoop, A. H., Vermorken, J. B., and Giaccone, G. Expression of drug resistance proteins in breast cancer, in relation to chemotherapy. Int. J. Cancer, *71:* 787–795, 1997.

35. Pohl, G., Filipits, M., Suchomel, R. W., Stranzl, T., Depisch, D., and Pirker, R. Expression of the lung resistance protein (LRP) in primary breast cancer. Anticancer Res., *19:* 5051–5055, 1999.

36. Nooter, K., Westerman, A. M., Flens, M. J., Zaman, G. J., Scheper, R. J., van Wingerden, K. E., Burger, H., Oostrum, R., Boersma, T., Sonneveld, P., Gratama, J. W., Kok, T., Eggermont, M. M., Bosman, F. T., and Stoter, G. Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. Clin. Cancer Res., *1:* 1301–1310, 1995.

37. Burger, H., Nooter, K., Sonneveld, P., Van Wingerden, K. E., Zaman, G. J., and Stoter, G. High expression of the multidrug resistance-associated protein (MRP) in chronic and prolymphocytic leukaemia. Br. J. Haematol., *88:* 348–356, 1994.

38. Burger, H., Nooter, K., Zaman, G. J., Sonneveld, P., van Wingerden, K. E., Oostrum, R. G., and Stoter, G. Expression of the multidrug resistance-associated protein (MRP) in acute and chronic leukemias. Leukemia (Baltimore), *8:* 990–997, 1994.

39. Nooter, K., Brutel de la Riviere, G., Klijn, J., Stoter, G., and Foekens, J. Multidrug resistance protein in recurrent breast cancer. Lancet, *349:* 1885–1886, 1997.

40. Nooter, K., Brutel de la Riviere, G., Look, M. P., van Wingerden, K. E., Henzen-Logmans, S. C., Scheper, R. J., Flens, M. J., Klijn, J. G., Stoter, G., and Foekens, J. A. The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. Br. J. Cancer, *76:* 486–493, 1997.

41. Faneyte, I. F., Kristel, P. M., and van de Vijver, M. J. Determining MDR1/P-glycoprotein expression in breast cancer. Int. J. Cancer, *93:* 114–122, 2001.

42. Yang, X., Uziely, B., Groshen, S., Lukas, J., Israel, V., Russell, C., Dunnington, G., Formenti, S., Muggia, F., and Press, M. F. MDR1 gene expression in primary and advanced breast cancer. Lab. Investig., *79:* 271–280, 1999.

43. Dexter, D. W., Reddy, R. K., Geles, K. G., Bansal, S., Myint, M. A., Rogakto, A., Leighton, J. C., and Goldstein, L. J. Quantitative reverse transcriptase-polymerase chain reaction measured expression of MDR1 and MRP in primary breast carcinoma. Clin. Cancer Res., *4:* 1533–1542, 1998.

44. Trock, B. J., Leonessa, F., and Clarke, R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. J. Natl. Cancer Inst. (Bethesda), *89:* 917–931, 1997.

45. Linn, S. C., Giaccone, G., van Diest, P. J., Blokhuis, W. M., van der Valk, P., van Kalken, C. K., Kuiper, C. M., Pinedo, H. M., and Baak, J. P. Prognostic relevance of P-glycoprotein expression in breast cancer. Ann. Oncol., *6:* 679–685, 1995.

46. Hegewisch-Becker, S., Staib, F., Loning, T., Pichlmeier, U., Kroger, N., Reymann, A., and Hossfeld, D. K. No evidence of significant activity of the multidrug resistance gene product in primary human breast cancer. Ann. Oncol., *9:* 85–93, 1998.

47. Pallis, M., and Russell, N. P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. Blood, *95:* 2897–2904, 2000.

48. Watts, G. S., Futscher, B. W., Isett, R., Gleason-Guzman, M., Kunkel, M. W., and Salmon, S. E. cDNA microarray analysis of multidrug resistance: doxorubicin selection produces multiple defects in apoptosis signaling pathways. J. Pharmacol. Exp. Ther., *299:* 434–441, 2001.