MRP and MDR1 Gene Expression in Primary Breast Carcinomas

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ABSTRACT

To evaluate the clinically important mechanisms of drug resistance in breast cancer, the expression of the MRP gene and the corresponding one for the MDR1 gene were determined in primary breast carcinoma specimens by both reverse transcription-PCR (n = 134) and immunohistochemistry (n = 63). Expression of MRP RNA was observed in all breast carcinomas. MDR1 RNA was detected in 80 (60%) of the carcinomas. Staining with monoclonal antibodies QCRL-1 and QCRL-3, which both recognize MRP, was strong in 15 (24%) and weak in the remaining 48 specimens (76%). Staining with C219, which recognizes P-glycoprotein, was strong in 6 (9%), weak in 30 (48%), and negative in 27 (43%) of the samples. Strong MRP staining was more frequent in T3 and T4 tumors than in T1 and T2 tumors and in the primary tumors of patients with distant metastases but was independent of age, menopausal status, histology, histological grade, estrogen receptor, progesterone receptor, and lymph node involvement. No correlation between MRP staining and expression of MDR1 RNA or P-glycoprotein was observed. Thus, these results indicate expression of both the MRP gene and the MDR1 gene in primary breast carcinomas and suggest that clinical drug resistance in breast cancer is most likely multifactorial.

INTRODUCTION

Chemotherapy plays a major role in the treatment of patients with breast cancer, which in most countries is still the leading cause of cancer deaths among women. The efficacy of chemotherapy is limited by the intrinsic presence or development of drug-resistant tumor cells. Knowledge of the clinically active mechanisms of drug resistance might result in new treatment strategies either by overcoming drug resistance or by selection of drug-resistant patients for particular treatment modalities.

MDR1 has been recognized as an important type of drug resistance in tumor cell lines. Different mechanisms that can mediate multidrug resistance have been identified (1, 2). They include the expression of the MDR1 (3, 4), MRP (5, 6), and LRP (7) genes. The MDR1 gene codes for P-gp, which functions as a drug efflux pump (3, 4). MDR1 gene expression was observed in both solid tumors and leukemias (8–11). MRP is another recently characterized drug transporter belonging to the ATP-binding transporter gene superfamily (5, 12). Its clinical role in drug resistance is currently under investigation. Decreased activities of topoisomerase II and alterations in glutathione S-transferases as well as glutathione levels can also cause MDR (13–15). More recently, the process of programmed cell death (apoptosis) has been shown to be involved in MDR (16, 17). Inactivation of the mechanisms contributing to MDR could potentially improve the response of resistant tumors to anticancer drugs.

Because the MDR phenotype affects drugs that are widely used in the treatment of breast cancer (e.g., anthracyclines and Vinca alkaloids), we decided to study breast carcinoma specimens for the presence of the different mechanisms of MDR described above and to determine the relationship of these mechanisms to the clinical parameters of the patients. Previously, we found MDR1 RNA expression in 46% of the primary breast carcinomas but did not observe a significant association of this expression with clinical data, including prognostic parameters of the patients (18). Here, we report the results of our investigations on MRP gene expression and its relationship to both MDR1 gene expression of the tumors and clinical data of the patients.

MATERIALS AND METHODS

Tumor Specimens and Cell Lines. Breast carcinoma specimens from 173 patients were obtained by surgery, immediately frozen, and stored at −80°C until use. Some of the patients (n = 34) had been included in a previous study (18).

Peripheral blood mononuclear cells were obtained from healthy volunteers by Ficoll-Paque gradient centrifugation. Drug-sensitive KB-3–1 and multidrug-resistant KB-8–5 cells (provided by Drs. I. Pastan and M. Gottesman, National Cancer Institute, Bethesda, MD) were grown as described (9). Cyto- spins of C1 and T5 cells were kindly provided by Drs. S. P. C. Cole and R. G. Deele (Queen’s University).

RNA Isolation, First-Strand cDNA Synthesis, and Amplification by PCR. Total cellular RNA was isolated from tumor specimens by means of RNAzol (Cinna Scientific, Inc., Cincinnati, OH) according to the manufacturer’s manual. The

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The abbreviations used are: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; B2-m, B2-microglobulin; ER, estrogen receptor; PR, progesterone receptor; RT, reverse transcription; NS, not significant.
Intactness of RNA was assessed by formaldehyde agarose gel electrophoresis.

cDNA was synthesized from 1 μg total cellular RNA in the presence of 1× Moloney murine leukemia virus reverse transcriptase buffer (Promega, Madison, WI), 20 units RNAsin (Promega), 250 μM of each deoxynucleotide triphosphate, 1 μg random hexanucleotide primers (Boehringer Mannheim GmbH, Mannheim, Germany) and 200 units Moloney murine leukemia virus reverse transcriptase (Promega) in a total volume of 50 μl. After 1 h of incubation at 37°C, 5 min at 95°C, and a quick chill to 4°C, cDNA was stored at −20°C until use.

The cDNA reaction mixture (5–10 μl) was used for amplification of specific DNA sequences in the presence of 1× Taq DNA polymerase buffer (Promega), 1.5 mM MgCl₂, 250 μM of each deoxynucleotide triphosphate together with 37.5 pmol of each primer, and 2 units Taq DNA polymerase (Promega) in a total volume of 50 μl. Thirty to 35 cycles at 95°C for 25 s, 57°C for 30 s, 73°C for 1 min, and a quick chill to 4°C in a 9600 thermocycler (Perkin Elmer, Emeryville, CA) were done. All oligonucleotides used as primers were synthesized by Fa. Biomedica GmbH (Vienna, Austria). The primers were as follows: 5′-TGAAGGCTTCGTCAGCC-3′ (forward primer, residues 4419–4438) and 5′-GTCCATGATGGTTGTTG-3′ (reverse primer, residues 4656–4675) of the MRP gene (19); 5′-ACCCCCACTGAAAAAGATGA-3′ (forward primer, residues 1544–1563) and 5′-ATCTTCAAACCTCCATG-3′ (reverse primer, residues 2596–2615) and 5′-GT-TCAAACCTCTGCTTCA-3′ (reverse primer, residues 2733–2752) of the MDR1 gene (20). All primers span an intron of the β2-m gene and the MDRI gene products (23) and cross-react with myosin (24), was used for the detection of P-gp. Immunohistochemistry was performed as described above, with C219 (dilution, 1:10) replacing the anti-MRP antibodies. In selected cases, immunohistochemistry by means of the MRK16 antibody was performed after fixation in paraformaldehyde. KB-3-1 and KB-8–5 cells were chosen as negative and positive controls for P-gp expression, respectively.

Immunostained slides were examined independently by two observers (M. F. and G. D.), who were blinded to clinical outcome. MRP and P-gp immunostaining were evaluated and scored separately.

**Statistical Analysis.** Frequencies were tested by χ² tests. In addition, Kruskal-Wallis tests were performed.

**RESULTS**

Primary breast carcinoma specimens were studied for the expression of the MRP gene at both the RNA and the protein level. Results were compared with MDR1 gene expression and clinical data of the patients. Initially, the expression of MRP RNA and the corresponding one for MDR1 RNA were determined by RT-PCR (Fig. 1). Peripheral blood mononuclear cells were used as positive controls for MRP gene expression. With regard to MDR1 gene expression, drug-sensitive KB-3–1 and multidrug-resistant KB-8–5 cells served as negative and positive controls, respectively (3, 8, 9). For both assays, β2-m was used as an internal control. Isolation of sufficient amounts of intact RNA was possible in 134 of 173 breast carcinoma specimens. In the remaining 39 samples, the isolated RNA was degraded and, therefore, unsuitable for further analysis. MRP RNA was expressed in all 134 evaluable specimens (100%), and MDR1 RNA was detected in 80 (60%) of the specimens (Table 1).

To determine the expression of both MRP and P-gp on individual cells and, thereby, to detect any potential heterogeneity of expression, immunohistochemical assays on frozen sections were established. In the case of MRP, the immunohistochemical assay was particularly required, because RT-PCR revealed positive results in all specimens. MRP was detected by means of monoclonal antibodies QCRL-1 and QCRL-3 (21). P-gp expression was assessed by means of the C219 antibody. No staining with either irrelevant isotype-matched antibodies was performed as a negative control in selected cases. C1 and T5 cells were used as negative and positive controls for MRP expression, respectively (22).

The C219 antibody (Centocor, Inc., Malvern, PA), which recognizes the MDR1 and MDR2 gene products (23) and cross-reacts with myosin (24), was used for the detection of P-gp. Immunohistochemistry was performed as described above, with C219 (dilution, 1:10) replacing the anti-MRP antibodies. In selected cases, immunohistochemistry by means of the MRK16 antibody was performed after fixation in paraformaldehyde. KB-3-1 and KB-8–5 cells were chosen as negative and positive controls for P-gp expression, respectively.
regard to the degree of MRP expression, patients were divided according to the intensity of staining into a group with strong staining and a second group with only weak staining. Strong staining was observed in the positive control cell line T5. A typical example of strong staining of a breast carcinoma specimen is shown in Fig. 2. Staining with anti-MRP antibodies was strong in 17 specimens (24%) and only weak in the remaining 48 samples (76%; Table 1). In the case of strong staining, the majority of tumor cells within the specimens were affected. Strong staining was only observed in tumor cells but not in surrounding normal tissues. Completely negative MRP staining was not seen in any of the carcinoma specimens.

With regard to P-gp expression, 27 specimens (43%) were classified as P-gp negative, and 36 (57%) were classified as P-gp positive, with strong staining in 6 specimens (9%) and weak staining in 30 specimens (48%). Both plasma membrane and cytoplasmic staining patterns were observed. P-gp staining strongly correlated with MDR1 RNA expression, because P-gp was detected in 17% of the MDR1 RNA-negative but in 73% of the MDR1 RNA-positive tumors ($P < 0.001$; Table 2). In selected cases ($n = 33$), P-gp expression was also studied by means of the monoclonal antibody MRK16 and was found to be positive in 39% of the tumor specimens (data not shown).

Next, the relationship between the MRP and MDR1 genes was studied (Table 3). Strong MRP staining was seen in 22% of the MDR1 RNA-negative tumors and 19% of the P-gp-negative tumors and in 24% of the MDR1 RNA-positive tumors and 28% of the P-gp-positive tumors ($P = \text{NS}$).

Finally, the relationship between MRP expression at the protein level and clinical data of the patients ($n = 63$) was determined. As shown in Table 3, strong MRP staining was independent of ages and menopausal status of the patients, histology and ER and PR status of the tumors, and axillary lymph node involvement. However, MRP expression was dependent on local tumor size, because strong MRP staining was seen in only 19% of patients with $T_1$ and $T_2$ tumors but in 67% of patients with $T_3$ and $T_4$ tumors ($P = 0.01$). In addition, strong MRP staining was detected in 75% of the primary tumors of patients with distant metastases but only in 21% of the patients without distant metastases ($P = 0.015$).

**DISCUSSION**

In the present study, primary breast carcinoma specimens were studied for the expression of the MRP and MDR1 genes, and the relationship of the expression of these genes with clinical data of the patients was determined. MRP expression as assessed by immunohistochemistry was found to be strong in 24% and weak in 76% of the specimens. Consistent with positive MRP staining in all samples studied, the sensitive RT-PCR yielded MRP RNA expression in all samples. The percentage of strong staining in our study is similar to the percentage recently reported by Nooter et al. (25), who found MRP expression in 2 (18%) of 11 breast carcinoma specimens. This similarity is also interesting because different monoclonal antibodies were used in both studies, QCRL-1 and QCRL-3 in the study by Nooter et al. (25). Recently, MRP expression was also observed in other cancers, including lung cancer, neuroblastoma, anaplastic thyroid carcinoma, bladder cancer, and leukemias (25–31). In non-small cell lung carcinomas, high MRP RNA levels were seen in 18% of the specimens (26).
In comparison to the \textit{MRP} gene, \textit{MDRI} gene expression was completely negative in 43\% of the specimens, and the frequencies of both weak and strong P-gp staining were lower than the corresponding ones for MRP (Table 1). The frequency of \textit{MDRI} gene expression confirms our previous findings on \textit{MDRI} RNA expression obtained by slot blot analysis (18) and is consistent with reports by other investigators (32, 33). Nevertheless, \textit{MDRI} gene expression reported in the various studies ranged from 0 to 85\% of the breast carcinomas (18, 32–41). This wide range of positivity might be explained by differences in methods of detection, cutoff levels, antibodies, sample size, and other factors among the various studies.

P-gp expression significantly correlated with \textit{MDRI} RNA expression in our study. A similar correlation was recently found by Charpin \textit{et al.} (32). These correlations are noteworthy with regard to the ongoing discussions about the optimal method for the clinical determination of the expression of drug resistance genes. “Bulk” methods are often criticized because their results might be affected by contamination with nonmalignant tissues. Because results at the RNA level were similar to those at the protein level in both studies, RNA analysis and immunohistochemistry are suitable for clinical applications with regard to the \textit{MDRI} gene. Contamination with normal breast tissue, which to some extent also expresses P-gp, does not appear to play a major role, albeit this possibility is often raised.

Although strong MRP staining was slightly more frequent in P-gp-positive tumors than in P-gp negative tumors (Table 3), no statistically significant correlation between MRP staining and \textit{MDRI} RNA or P-gp expression was observed. In etoposide-selected, drug-resistant H69 small cell lung cancer cell lines, however, a sequential coexpression of the \textit{MRP} gene and the \textit{MDRI} gene with MRP preceding \textit{MDRI} expression was described (42). Strong MRP staining was somewhat more frequent in ER- and PR-negative carcinomas than in their positive counterparts. Thus, further studies on a larger sample size are warranted to prove or exclude a potential association of MRP with either \textit{MDRI} gene expression or receptor status.

Interestingly, strong MRP staining was seen more frequently in the primary tumors of patients with metastatic disease and in locally advanced T1 and T2 tumors compared with smaller T1 and T2 tumors. These findings suggest that MRP might be associated with advanced disease and/or might be a marker of biological tumor behavior. The latter possibility was recently raised for P-gp in sarcomas, in which P-gp expression of the tumors was associated with shorter survival of the patients but was not predictive of tumor necrosis induced by anticancer drugs (43). Recently, \textit{MRP} RNA levels of bladder carcinomas were found to be reduced more frequently in G3 than in G1 and G2 tumors (30). P-gp expression was also suggested to be associated with a more malignant phenotype in colorectal carcinomas, because it was predominantly expressed in invasively growing tumor cells (44).

No data on the prognostic or predictive significance of MRP in solid tumors except lung cancer and neuroblastoma exist to date. In patients with non-small cell lung cancer, MRP expression did predict a worse outcome of vindesine and etoposide chemotherapy (26). In patients with neuroblastomas, high levels of MRP expression were strongly associated with reductions in both survival and event-free survival (28). Thus, further studies are required to determine whether MRP expression of primary breast carcinomas affects dis-

\begin{table}[h]
\centering
\caption{Correlation between \textit{MDRI} RNA and P-gp}
\label{tab:correlation}
\begin{tabular}{lcc}
\hline
 & \textit{MDRI} RNA & P-gp \\
\hline
Negative & 15 (83)\textsuperscript{a} & 3 (17) \\
Positive & 12 (27) & 33 (73) \\
\hline
\end{tabular}
\textsuperscript{a}Numbers in parentheses are percentages. \\
\textsuperscript{b}X\textsuperscript{2} test.
\end{table}
Table 3  MRP expression of breast carcinomas and clinical data of the patients

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>All patients (n = 63)</th>
<th>Patients with strong MRP staining (n = 15)</th>
<th>Patients with weak MRP staining (n = 48)</th>
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<td>27</td>
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* Kruskal-Wallis or χ² test.

Numbers in parentheses are percentages.

ease-free survival and/or the response to adjuvant chemotherapy with MDR drugs. Similar studies are also necessary with regard to the MDR1 gene, although P-gp expression was recently reported to be associated with shorter durations of overall survival of breast cancer patients (41). Previously, MDR1 gene expression was shown to be associated with poor outcome in acute myeloid leukemia (9, 11, 45), osteosarcoma (43), and childhood soft-tissue sarcoma (46). However, conflicting results have been reported for neuroblastoma. Whereas P-gp expression did predict poor outcome in a previous study (47), MDR1 RNA expression was not predictive of survival or event-free survival in a recent study (28). Furthermore, P-gp expression did not indicate prognosis in colon cancer (10).

Both the MRP and the MDR1 genes are expressed to various extents in primary breast carcinomas and are most likely involved in the clinical drug resistance of these carcinomas. These findings, together with previous reports of elevated glutathione S-transferase π expression (15, 33), support the assumption of a multifactorial nature of clinical drug resistance in breast cancer. This should be considered with regard to both the development of strategies to over-
come clinical drug resistance and, in particular, the planning of future trials with resistance modifiers (48–52).

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