

Multidrug Resistance Proteins MRP3, MRP1, and MRP2 in Lung Cancer: Correlation of Protein Levels with Drug Response and Messenger RNA Levels¹

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ABSTRACT

Previously (L. C. Young *et al.*, *Clin. Cancer Res.*, 5: 673–680, 1999), we found, in a panel of 23 lung cancer cell lines that had not been selected for *in vitro* drug resistance, that the mRNA levels of MRP3 and MRP1, two members of the ATP-binding cassette superfamily of transport proteins, correlated with resistance to doxorubicin, vincristine, VP-16, and *cis*-diamminedichloroplatinum(II). To extend these studies, we measured multidrug resistance protein (MRP)1, MRP2, and MRP3 protein levels in a panel of 30 lung cancer cell lines that included the original 23 cell lines as well as an additional 7 unselected lung cancer cell lines. In the case of MRP3, a polyclonal antibody was developed that was found to be a sensitive reagent for the detection of MRP3 by Western blot analysis. We found good agreement in the original 23 cell lines between the cognate mRNA and protein levels for MRP1, MRP2, and, especially, MRP3 (r , 0.852), supporting the use of semiquantitative PCR to predict MRP1, MRP2, and MRP3 protein levels in patient samples. There were also strong correlations between the mRNA and protein levels of MRP3 and MRP1, which suggested that these genes might be expressed in a coordinate manner. MRP3, MRP1, and MRP2 protein levels were higher in the non-small cell lung cancer (NSCLC) than in the SCLC cell lines and, in addition, MRP3 and MRP2 were detected almost exclusively in the NSCLC cell lines. Finally, we found that both MRP3 and MRP1, but not MRP2, protein levels

correlated with decreased sensitivity of these lung cancer cell lines to doxorubicin, VCR, VP-16, and *cis*-diamminedichloroplatinum(II). These findings are consistent with our hypothesis that both MRP3 and MRP1 are components of the multifactorial multidrug resistance phenotype of lung cancer and that MRP3 contributes to the intrinsic resistance of NSCLC cells.

INTRODUCTION

Chemotherapy plays a major role in the treatment of lung cancer, the primary cause of cancer-related death in North America. However, resistance to multiple chemotherapy agents remains a major obstacle to the cure of many forms of cancer, including SCLC⁴ and NSCLC. Most SCLCs acquire multidrug resistance, whereas NSCLCs tend to be intrinsically resistant to chemotherapy. Some members of the ABC superfamily of transport proteins have been shown to confer drug resistance *in vitro*, including members from subfamilies B, C, and G (1). Subfamily B includes P-glycoprotein (MDR1, gene symbol ABCB1), the first human ABC transporter shown to confer multidrug resistance. Although P-glycoprotein is implicated in drug resistance in several tumor types, it is infrequently expressed in lung cancer (2, 3), and, in a panel of 23 lung cancer cell lines that had not been selected for drug resistance, we found that these low levels of expression did not correlate with drug response (4). Subfamily C includes the MRP1/ABCC1 as well as genes not associated with drug resistance, such as the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptors, SUR1/ABCC8 and SUR2/ABCC9 (recently reviewed in Ref. 1). MRP1, cloned from the doxorubicin-resistant H69AR SCLC cell line (5), is associated with drug resistance or poor patient outcomes in a variety of tumor types including lung carcinoma (6), breast carcinoma (7, 8), gastric carcinoma (9, 10), neuroblastoma (11, 12), retinoblastoma (13), endometrial carcinoma (14) and, possibly, acute myeloid leukemia (15). MRP3 (ABCC3) and MRP2 (ABCC2) are the ABC transporters that are most closely related to MRP1 with 58 and 49% amino acid identity, respectively (16).

Like MRP1, MRP2 and MRP3 mRNA (17–19) and protein levels⁵ (17) are increased in some drug-selected cell lines. However, the reported drug resistance profiles conferred by MRP3 and MRP2 are variable. The reasons for this are unclear

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⁴ The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-SCLC; ABC, ATP-binding cassette; MRP, human multidrug resistance protein; MRP, gene encoding human MRP; DOX, doxorubicin; CDDP, *cis*-diamminedichloroplatinum(II); VCR, vincristine; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AUC, area under the dose response curve.

⁵ Unpublished observations.

but may be attributable to differences in cell culture conditions and to the choice of transfected host cell lines that affect both the expression level and the subcellular localization of these proteins (20–23). However, it seems clear that MRP3 and MRP2, like MRP1, can confer resistance to short-term methotrexate exposure (24, 25). Transfection of *MRP2* cDNA into drug-sensitive cells has been reported to confer low levels of resistance to CDDP, DOX, epirubicin, VCR, vinblastine, and, possibly, VP-16 (26–29). MRP3 may confer drug resistance (24, 30), but the resistance profile is different from those of MRP1 and MRP2. To date, the resistance conferred by MRP3-transfection has been difficult to evaluate because the protein expression and relative resistance levels have been low (24, 30).

Previously, we found that *MRP3* and *MRP1*, but not *MDR1*, *MRP2*, *MRP4*, or *MRP5*, mRNA levels correlated with the resistance of a panel of 23 unselected lung cancer cell lines to four chemotherapeutic agents (3, 4). Additionally, *MRP3* and *MRP1* mRNA levels were higher in the NSCLC than the SCLC cell lines (4). In the present study, we measured the protein levels of MRP3, MRP1, and MRP2 in an expanded panel of 30 unselected lung cancer cell lines. We examined the relationships between: (a) the protein levels of these genes; (b) the protein levels and their cognate mRNA levels; and (c) the protein levels and the drug sensitivity of the cell lines. We also determined the distribution of these protein levels in the NSCLC and SCLC cell lines.

MATERIALS AND METHODS

Cell Lines. The cell lines examined in the present study consisted of 30 lung cancer cell lines that had not been selected for drug resistance *in vitro*. This panel included the 23 lung cancer cell lines (13 NSCLC and 10 SCLC) used previously by us (4) and 7 additional SCLC cell lines (AD-A, GL-E, JS-E, LG-T, OS-A, SM-E, WL-E, and TY-E; Refs. 3 and 31). The conditions for establishing and culturing these cell lines have been described previously (31).

Cytotoxicity Testing. The toxicity of a 48-h exposure of the lung cancer cell lines to DOX, VCR, VP-16, and CDDP was measured using a modified MTT assay and was expressed as the AUC, as calculated by the trapezoidal method (32, 33).

Anti-MRP3 Antiserum. An anti-MRP3 polyclonal antiserum, designated LY1, was generated by immunizing rabbits with a synthetic 15-amino-acid peptide (₁₁₆₃AYNRSRDFEIS-DTK₁₁₇₇) in the form of a multiple antigen peptide (MAP). The sequence of the MRP3-specific MAP was based on regions of dissimilarity between the COOH terminus of MRP3 and the COOH termini of MRP1, MRP2, MRP4, and MRP5. The IgG fraction of the LY1 antiserum was purified on protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada) and was used at a dilution equivalent to 1:8000 of the original serum volume. Peptide synthesis and rabbit immunizations were performed by Research Genetics (Huntsville AL).

Preparation of Membrane-enriched Fractions. Membrane-enriched fractions were isolated from 60×10^6 cells as described previously (34). Protease inhibitors (Complete, Mini protease inhibitor cocktail tablets with 200 μ g/ml benzamidine; Roche Diagnostics, Laval, Québec, Canada) were included to minimize protein degradation and protein concentrations were

determined using the Bio-Rad Protein Assay Dye (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada).

PNGase F Treatment. N-linked carbohydrates were removed from membrane proteins with PNGase F (New England Biolabs, Ltd., Mississauga, Ontario, Canada). Digestion was performed according to the manufacturer's instructions except that samples were incubated for 30 min at 37°C in denaturing buffer instead of the recommended 10 min at 100°C. To minimize protein degradation, protease inhibitors were included in all of the steps, and digestion times were shortened to 45 min.

SDS-PAGE and Western Blot Analysis. SDS-PAGE was performed by the Laemmli method with a 4% stacking gel and a 7.5% separating gel. Prior to loading, samples were diluted in solubilizing buffer [final concentration, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol] and incubated for 30 min at 37°C. The separated proteins were electrotransferred onto Immobilon-P membranes (Millipore Ltd, Etobicoke, Ontario, Canada) in Tris-glycine-SDS transfer buffer, and blots were blocked in 4% (w/v) dry milk, Tris-buffered saline, and 0.05% Tween 20. Primary antibodies used were: LY1, a rabbit anti-MRP3 polyclonal antibody (IgG fraction, described above, 1:8,000); QCRL-1, a mouse anti-MRP1 mAb (1:10,000; Ref. 34, 35); MRPr1, a rat anti-MRP1 mAb (1:2,000; Signet Laboratories, Inc., Dedham, MA; Ref. 36); EAG15, a rabbit anti-MRP2 polyclonal antibody (1:20,000; a generous gift of Dr. D. Keppler, Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Heidelberg, Germany; Refs. 37 and 38); mouse monoclonal anti-MRP2 M₂III-6 (1:250; Alexis Biochemicals Corp, San Diego, CA); and M₃II-9, a mouse anti-MRP3 mAb (1:40; Kamiya Biomedical Company, Seattle, WA). Antibody binding was visualized using the appropriate horseradish peroxidase-conjugated secondary antibody and the Renaissance-enhanced luminol reagent (NEN Life Science, Mandel Scientific Co., Ltd. Guelph, Ontario, Canada) on X-OMAT Blue film. X-ray films were scanned and analyzed using ImageQuantNT densitometry software v 4.2.a (Molecular Dynamics, Sunnyvale, CA).

Statistics. Statistical analyses were performed using the Systat software package, version 7 (SPSS Inc., Chicago, IL). Natural logarithmic (Ln) transformations of MRP1, MRP2, and MRP3 protein levels were performed so that these data would more closely approximate a normal distribution. The Pearson correlation coefficients (*r*) and associated probabilities (*P*) were calculated for the following combinations of data sets: MRP1–3 protein levels and cognate mRNA levels; MRP1, MRP2, and MRP3 protein levels; and MRP1–3 protein levels and AUC values. Values for mRNA levels (*n* = 23) are from our previous study (4). Descriptive terms for the strength of the correlations were defined as follows: strong, $|r| \geq 0.7$; moderate, $|r| \geq 0.5$; weak, $|r| \geq 0.3$; and none (no appreciable correlation), $|r| < 0.3$.

RESULTS

Characterization of the LY1 Anti-MRP3 Polyclonal Antiserum. We found that the LY1 antiserum (IgG fraction), raised against a 15-amino-acid MRP3-peptide, was a sensitive reagent for the detection of MRP3 by Western blot analysis. LY1 detected a *M_r* 200,000 integral membrane protein with properties consistent with those of MRP3 (Fig. 1). Digesting the

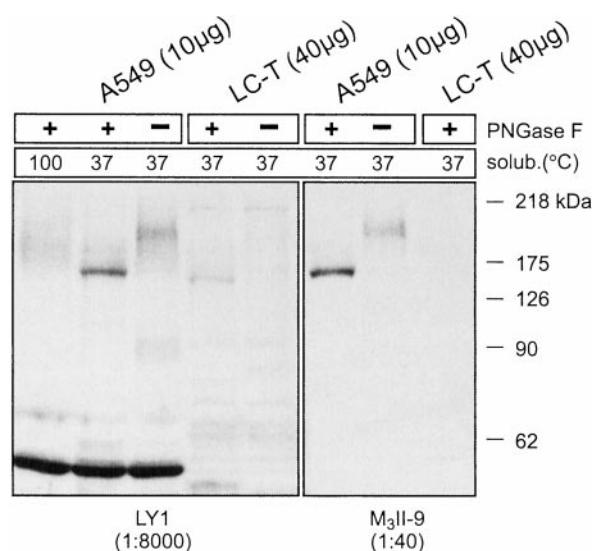


Fig. 1 LY1 polyclonal antiserum (IgG) is a sensitive reagent for the detection of MRP3. Immunoblots of A549 (10 µg) and LC-T (40 µg) NSCLC cell line membrane proteins as detected by LY1 polyclonal and M₃II-9 mAb. Before separation by 7.5% SDS-PAGE, membrane proteins were either incubated with PNGase F (+) or a glycerol control (-). LY1 detected a M_r 200,000 membrane protein that, on deglycosylation, migrated with an apparent molecular weight of M_r 170,000 (Lanes 2 and 3). Similar results were obtained with the M₃II-9 mAb (Lanes 6 and 7). However, in cell lines with low MRP3 levels (e.g., the LC-T NSCLC cell line), the LY1 polyclonal antiserum (1:8000) was at least as sensitive in detecting the M_r 200,000 band as the M₃II-9 mAb (1:40; Lanes 4, 5, and 8). Solubilization of the membrane proteins before deglycosylation was performed at 37°C because heating to 100°C greatly reduced the detection of MRP3 by LY1 (Lane 1) or M₃II-9 (data not shown).

membrane proteins with PNGase F caused the broad M_r 200,000 band to migrate as a focused M_r 170,000 band. This electrophoretic mobility is consistent with the molecular weight for MRP3 predicted by analysis of the amino acid sequence. Detection of this protein was diminished greatly if the membrane samples were heated to 100°C (Fig. 1). (For this reason, the denaturing of samples prior to PNGase F treatment and solubilizing of samples in SDS-PAGE loading buffer were performed at 37°C.) Similar results were observed with the previously characterized anti-MRP3 M₃II-9 mAb, and LY1 appears to be at least as sensitive as M₃II-9. The prominent M_r 50,000 band (Fig. 1) was observed only in the A549 membrane preparations, and its origin is unknown. The LY1 antiserum immunoprecipitates MRP3 but not MRP1 or MRP2, and the binding in immunoblots can be eliminated in a dose-responsive manner by competition with the LY1 peptide (data not shown).

MRP1, MRP2, and MRP3 Protein Levels. For each of the 30 lung cancer cell lines, 40 µg of PNGase F treated and 40 µg control membrane proteins were separated by SDS-PAGE and MRP3, MRP1, and MRP2 were detected by Western blot analysis with LY1, QCRL-1, and M₂III-6, respectively. We found that deglycosylation of the membrane proteins increased the ability to detect and quantitate low levels of glycoproteins (Fig. 2). MRP3 was detected in most of the NSCLC cell lines but was not detected in any of the SCLC cell lines (Fig. 3).

Similarly, MRP2 was detected in most of the NSCLC cell lines but in only one SCLC cell line, SHP-77. However, relative MRP3 levels tended to be higher than MRP2 levels. MRP1 was detected in all of the 30 unselected lung cancer cell lines and, like MRP3 and MRP2, median levels were higher in the NSCLC than in the SCLC cell lines.

Correlation Analyses. Pearson correlation coefficients (r) and associated probabilities (P) were calculated to determine whether (a) MRP1-3 protein levels correlated with the mRNA levels, (b) MRP1-3 protein levels were correlated with each other, and (c) MRP1-3 protein levels correlated with the resistance of the cell lines to DOX, VCR, VP-16, or CDDP.

Previously, we determined *MRP1*, *MRP2*, and *MRP3* mRNA levels by semiquantitative PCR for 23 of the 30 cell lines (4). Using these values, we analyzed the correlations between the protein and mRNA levels of MRP3, MRP1, and MRP2 (Table 1). The high MRP2 values for the A549 NSCLC cell line were omitted from this calculation because they exerted undue influence on the correlation. The correlations between the protein levels and their cognate mRNAs levels were strong for both MRP3 and MRP2, and moderate for MRP1 (Table 1).

In our previous study, we found moderate-to-weak correlations between the mRNA levels of *MRP3*, *MRP1*, and *MRP2* (r , 0.570–0.495) in the 23 cell lines (4). In comparing the protein levels in the expanded panel of 30 cell lines, we found an even stronger correlation between MRP3 and MRP1 levels (r , 0.716), whereas the correlation between MRP3 and MRP2 levels remained weak (r , 0.407), and there was no correlation between MRP1 and MRP2 levels (r , 0.131; Table 2).

Previously, when examining the correlations of *MRP1* and *MRP3* mRNA levels with the resistance of the 23 cell lines to DOX, VCR, VP-16, and CDDP, we found a strong correlation between *MRP3* mRNA levels and DOX resistance and a weak correlation between *MRP1* mRNA levels and CDDP resistance; all of the remaining correlations with these mRNAs were moderate (4). Also, we found no correlation between the *MRP2*, *MRP4*, or *MRP5* mRNA levels and resistance to these drugs. The patterns of the correlation between the protein levels and chemosensitivity in this study (Table 3) were in agreement with those using mRNA levels (4) but generally were not as strong. One notable exception was the correlation between MRP1 and DOX resistance. The correlation coefficient of DOX resistance with MRP1 protein levels was 0.741 (Table 2) versus 0.519 with *MRP1* mRNA levels (4). MRP3 protein levels also demonstrated a strong correlation with the resistance of the panel of cell lines to DOX (Table 3). The correlation between MRP1 protein levels and resistance of the cell lines to CDDP was moderate (Table 3). The correlations of the remaining MRP3 or MRP1 protein levels with the AUC data were weak (r , 0.423–0.477; Table 3). MRP2 protein levels did not correlate with resistance to any of the four drugs tested (Table 3).

DISCUSSION

MRP1 has been shown to confer multidrug resistance *in vitro* (39–42) and to be associated with poor patient outcomes for several types of cancer (7–13), including lung cancer (6). The response of lung cancer to chemotherapy treatment is characterized by multifactorial multidrug drug resistance that

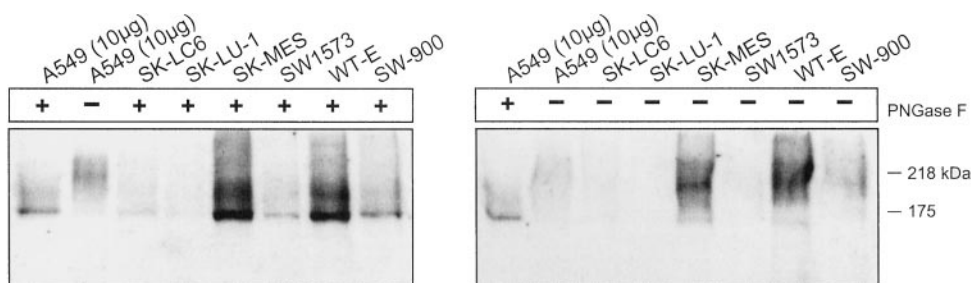


Fig. 2 An example of MRP3 detection in NSCLC cell lines. For each cell line, 40 μg of both PNGase F treated (+) and glycerol control (-) membrane proteins were separated by 7.5% SDS-PAGE and analyzed by Western blotting. PNGase F digestion of the membrane proteins concentrated the MRP3 band, which allowed for more sensitive detection and more accurate quantitation of low levels of MRP3. Also, 10 μg of A549 NSCLC cell line membrane protein were included on each blot to serve as a positive control and to allow for comparison between blots.

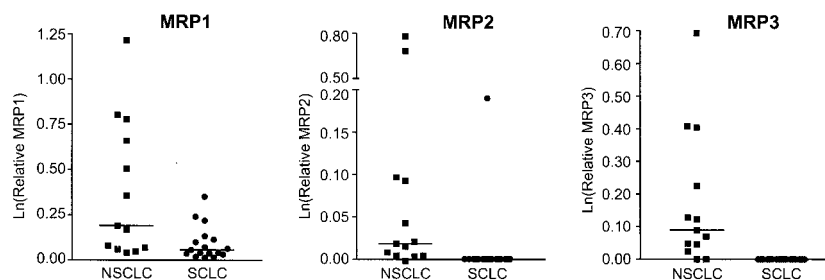


Fig. 3 A columnar scatter plot of MRP1–3 protein levels in 30 lung cancer cell lines. MRP1, MRP2, and MRP3 protein levels were measured by immunoblot analysis. MRP1 was detected in all of the 30 lung cancer cell lines. The levels of MRP2 and MRP3 proteins were below detection in the SCLC cell lines with the exception of one cell line in which MRP2 was detected. Protein levels were normalized to those found in the A549 NSCLC cell line and then Ln-transformed for statistical analyses. *Horizontal line*, the median protein level for each data set.

Table 1 Pearson correlation coefficients (r) and associated probabilities (P) for the correlation of mRNA and protein levels for MRP3, MRP1, and MRP2 in a panel of 23 unselected lung cancer cell lines

	MRP3 ^a	MRP1 ^a	MRP2 ^b
r	0.852	0.519	0.787
P	<0.001	0.011	<0.001

^a $n = 23$.

^b $n = 22$.

Table 2 Pearson correlation coefficients (r) and associated probabilities (P) for the correlation of MRP3, MRP1, and MRP2 protein levels in a panel of 30 unselected lung cancer cell lines ($n = 30$)

	MRP3	MRP1
MRP1		
r	0.716	
P	<0.001	
MRP2		
r	0.407	0.131
P	0.028	0.439

may be either acquired (SCLC) or intrinsic (NSCLC) and results in treatment failure and death within 2 years of diagnosis in the majority of cases. Previously, we found that both *MRP1* and *MRP3* mRNA levels correlated with decreased chemosensitivity and that median mRNA levels were higher in NSCLC than in SCLC cell lines (3, 4). In the present investigation, we have analyzed the protein levels of MRP3, MRP1, and MRP2 in a panel of lung cancer cell lines comprised of 23 cell lines from our previous study plus an additional 7 cell lines that also had not been selected for drug resistance *in vitro*.

We found that the protein levels of MRP3, MRP1, and MRP2 were higher in the NSCLC cell lines than in the SCLC cell lines (Fig. 3). These results are in agreement with semiquantitative PCR of *MRP1* mRNA levels by us (4) and by Narasaki *et al.* (43), and with our immunohistochemical investigation of untreated NSCLC and SCLC tumors (44). Unlike

MRP1, which was present in all 30 of the SCLC and NSCLC cell lines, we detected MRP3 and MRP2 only in the NSCLC cell lines (with the exception of MRP2 in the SHP-77 SCLC cell line; Fig. 3). We found moderate-to-strong correlations between the levels of MRP1–3 and their cognate mRNAs (Table 1), thus supporting the use of semiquantitative PCR to predict the levels of MRP3, MRP1, and MRP2 proteins in patient samples in which material is limited.

When one considers that only 3 of 13 NSCLC cell lines were established from chemotherapy-treated patients, compared with 10 of 17 SCLC cell lines, it is interesting that MRP3 and MRP2 are readily detected in the NSCLC cell lines but generally below detection in the SCLC cell lines (Fig. 3). This pattern indicates that MRP3 protein is present prior to chemotherapy in the NSCLC cells. In agreement with these observations, we

Table 3 Pearson correlation coefficients (*r*) and associated probabilities (*P*) for the correlation of MRP1-3 protein levels and AUC values in a panel of 30 unselected lung cancer cell lines (*n* = 30)

	DOX	VCR	VP-16	CDDP
MRP3				
<i>r</i>	0.709	0.467	0.423	0.436
<i>P</i>	<0.001	0.009	0.020	0.016
MRP1				
<i>r</i>	0.741	0.477	0.438	0.585
<i>P</i>	<0.001	0.008	0.015	0.001
MRP2				
<i>r</i>	0.056	0.291	0.255	0.194
<i>P</i>	0.769	0.119	0.174	0.304

found previously that *MRP3* mRNA levels were significantly higher in samples from NSCLC patients than in samples from SCLC (4). These data indicate that MRP3 protein, like MRP1, may have a role in the drug resistance of lung cancer and that the role of MRP3 may be specific to the intrinsic resistance of NSCLC cells.

In our panel of lung cancer cell lines, we found that there was a strong correlation between MRP3 and MRP1 protein levels, a weak correlation between MRP3 and MRP2 protein levels, and no significant correlation between MRP1 and MRP2 protein levels (Table 2). These data confirm our previous observation that *MRP1* and *MRP3* mRNA are positively correlated (4) and, together, suggest that there is a relationship between the expression of MRP1 and MRP3.

We found that MRP3 and MRP1, but not MRP2, levels correlated with the resistance of the lung cancer cell lines to DOX, VCR, VP-16, and CDDP (Table 3). The multidrug resistance of lung cancer is considered to be multifactorial, and these correlation analyses do not presume to separate this resistance into its components or establish "cause and effect" relationships. These analyses show that cell lines with higher levels of MRP3 and MRP1 also tend to exhibit higher levels of drug resistance. For example, the correlation of MRP1 levels with CDDP-resistance is surprising because MRP1, unlike MRP2 (27, 28, 38), has not been shown to confer CDDP-resistance in transfected cell lines (40, 41), and MRP1 protein levels correlated with the resistance of 15 unselected lung cancer cell lines to DOX and VP-16, but not to CDDP (45). However, some studies have found associations between MRP1 and CDDP response (6, 46). It is possible that the correlation between MRP1 and CDDP resistance could be a reflection of an association between MRP1 and another protein capable of conferring CDDP-resistance. Human MRP3 has been reported to confer resistance to methotrexate, as well as conferring low levels of resistance to VP-16, teniposide, and, possibly, VCR, but not to DOX or CDDP (24, 30). However, our results suggest that MRP3 may also be involved in DOX and CDDP resistance.

Human MRP2 has been shown to confer resistance to, or to transport, CDDP, DOX, VCR, and, possibly, VP-16 in some cell types (26-29, 38, 47) and, therefore, the MRP2 levels may have been expected to correlate with the resistance of the cell lines to these anticancer agents. *MRP1*, but not *MRP2*, mRNA levels were increased in lung tumors from patients who had received

CDDP-containing combination chemotherapy (48). In colorectal tumors that were surgically resected from untreated patients, MRP2 levels were increased and correlated with the *in vitro* resistance of the colorectal cells to CDDP (49). We showed no correlation between MRP2 protein levels and drug resistance, which indicated that, although MRP2 is expressed, it does not contribute to the multidrug resistance of these cell lines.

In conclusion, we measured MRP3, MRP1, and MRP2 in a panel of 30 lung cancer cell lines that had not been selected for *in vitro* drug resistance, and we found that there was good agreement between the protein levels of MRP1-3 and their cognate mRNAs, particularly for MRP3 (*r*, 0.852). Our data support the use of semiquantitative PCR to estimate the levels of MRP1, MRP2, and MRP3 proteins in samples in which material is limited. Also, the MRP3-specific LY1 polyclonal antibody that we developed was a sensitive reagent for the measurement of MRP3 by Western blot analysis. Correlation analyses indicated a relationship between the expression of MRP3 and MRP1 and, in agreement with our mRNA studies (3, 4), both the MRP3 and the MRP1 protein levels correlated with the drug response of these lung cancer cell lines. Our data are consistent with the idea that MRP3, like MRP1, is a component of the multifactorial multidrug resistance phenotype of lung cancer cells and that MRP3 contributes in particular to the intrinsic multidrug resistance of NSCLC.

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