Expression of the MRP and MDR1 Multidrug Resistance Genes in Small Cell Lung Cancer

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ABSTRACT

Acquired multidrug resistance is a major obstacle to a cure for small cell lung cancer (SCLC). Overexpression of the MDR1 gene occurs infrequently in multidrug-resistant SCLC cell lines. The multidrug resistance protein (MRP) can confer multidrug resistance, but its role in clinically acquired drug resistance is unknown. The purpose of this study was to measure expression of MRP and MDR1 mRNA in cell lines and clinical samples from SCLC patients and to correlate the results with drug sensitivity profiles. Twenty-three SCLC cell lines and 10 tumor samples from SCLC patients were examined. Samples expressing MRP and MDR1 were identified by reverse transcription-PCR, and levels of MRP mRNA in the cell lines were measured by quantitative reverse transcription-PCR. One of 23 cell lines (4%) expressed MDR1 mRNA, whereas MRP expression was detected in 19 of 23 cell lines (83%). There was a significant correlation between doxorubicin resistance and MRP expression levels (r = 0.422; P = 0.045). Of the 10 clinical samples, 3 expressed only MRP, 2 expressed only MDR1, and 4 expressed both drug resistance genes. In summary, MRP is frequently expressed in clinical samples and cell lines from SCLC patients, and the levels correlate with doxorubicin resistance in unselected SCLC cell lines. Expression of MDR1 can be detected in clinical samples of SCLC but is rarely found in cell lines from drug-resistant patients. These multidrug resistance proteins may contribute to the multifactorial problem of clinically acquired drug resistance in SCLC.

INTRODUCTION

Chemotherapy is the primary treatment modality for SCLC, but despite high initial response rates, most patients eventually die with drug-resistant tumors. In many in vitro systems and in some clinical settings, resistance to multiple chemotherapeutic agents is caused by overexpression of P-gp, a membrane transport protein encoded by the MDR1 gene, which actively effluxes many natural product-type drugs from cells. However, data from large panels of cell lines indicate that P-gp overexpression occurs infrequently in multidrug-resistant SCLC cell lines. Despite these findings, P-gp overexpression cannot be ruled out as a factor contributing to multidrug resistance in SCLC. Because most studies have used established cell lines rather than tumor samples, it is possible that P-gp expression may have been present in the original tumor but was lost during culture in vitro. Alternatively, other mechanisms of resistance may be more important in SCLC tumors. A novel transport protein, termed MRP, that is overexpressed in a doxorubicin-selected SCLC cell line has now been cloned. Like P-gp, MRP is a member of the ATP-binding cassette superfamily of transport proteins, and cDNA transfection studies demonstrate that MRP can confer multidrug resistance. It is not known whether this protein is involved in clinically acquired drug resistance in SCLC.

To investigate the role of MRP and MDR1 in SCLC, we examined 23 SCLC cell lines and 10 cryopreserved tumor samples obtained from SCLC patients at various stages of treatment. Six of these cryopreserved samples were the original specimens from which cell lines in this collection were derived. Expression of MRP and MDR1 mRNA was examined using RT-PCR, and levels of MRP mRNA were quantitated by Q-PCR. Results were correlated with the drug sensitivity profiles and the treatment histories of the patients from whom the cell lines were derived.

MATERIALS AND METHODS

Cell Lines. A collection of 23 unselected SCLC cell lines was established from patients at various stages of treatment. Table 1 summarizes the features of these cell lines, including the source of the tumor tissue from which the lines were derived, the treatment received by the patients at the time the line was established, as well as subsequent treatment and response. The conditions for establishing and culturing the cell lines have been

1 The abbreviations used are: SCLC, small cell lung cancer; P-gp, P-glycoprotein; MRP, multidrug resistance protein; RT-PCR, reverse transcription-PCR; Q-PCR, quantitative RT-PCR; VP-16, etoposide; TFRR, transferrin receptor; AUC, area under the curve.
Multidrug Resistance in Small Cell Lung Cancer

The cell lines were harvested in exponential growth phase. The tumor cells and the treatment history of the patient were obtained for diagnostic or therapeutic indications from SCLC patients treated at the Kingston Regional Cancer Centre from 1986 to 1994. With the patient’s informed consent, a needle aspirate was sent for research purposes as part of a study approved by the Research Ethics Board of Queen’s University.

The response of the patient to further chemotherapy (if given) after the cell line was established. CR, complete response: PR, partial response.

Table 1  SCLC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sourcea</th>
<th>Prior treatmentb</th>
<th>Subsequent treatmentc</th>
<th>Responsed</th>
<th>MDR1</th>
<th>MRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H69</td>
<td>Pleural effusion</td>
<td>CMC-VAP</td>
<td>Unknown</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NCI-H209</td>
<td>Bone marrow</td>
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<td>–</td>
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</tr>
<tr>
<td>SHP-77</td>
<td>Primary tumor</td>
<td>None</td>
<td>None</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AD-A</td>
<td>Subcutaneous lesion</td>
<td>CA, VP/CP, RT</td>
<td>Mitox</td>
<td>PD</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>BK-T</td>
<td>Primary tumor</td>
<td>None</td>
<td>CAV</td>
<td>NA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LG-T</td>
<td>Lymph node</td>
<td>None</td>
<td>CAV, VP/CP</td>
<td>CR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HG-E</td>
<td>Pleural effusion</td>
<td>None</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>JO-E</td>
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<td>CAV, VP/CP, RT</td>
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<td>–</td>
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<td>–</td>
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<td>JN-Mf</td>
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<td>CAV, VP/Carb</td>
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<tr>
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<td>Lymph nodef</td>
<td>CAV, VP/CP</td>
<td>CAV, VP/CP</td>
<td>PR</td>
<td>–</td>
<td>–</td>
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<td>MM-1</td>
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<td>CAV, VP/CP</td>
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<tr>
<td>LD-T</td>
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<td>CAV, VP/CP</td>
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<td>+</td>
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<td>–</td>
<td>–</td>
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<td>VP/CP</td>
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<td>+</td>
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<td>LV-E</td>
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<td>Oral VP</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>JS-E</td>
<td>Pericardial effusion</td>
<td>CAV, VP/CP</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GL-E</td>
<td>Pericardial effusion</td>
<td>CAV, VP/CP, RT</td>
<td>Oral VP</td>
<td>PD</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SM-E</td>
<td>Pleural effusion</td>
<td>CAV</td>
<td>VP/CP, CAV</td>
<td>PD</td>
<td>–</td>
<td>+</td>
</tr>
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<td>TY-E</td>
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<td>CAV, VP/CP</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>YR-A</td>
<td>Subcutaneous lesionf</td>
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<td>Oral VP</td>
<td>PD</td>
<td>–</td>
<td>+</td>
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<tr>
<td>HA-E</td>
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<td>CAV, VP/CP, RT, Oral VP</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

a Tumor cells from which the cell line was derived.
b Therapy that the patient had received at the time that the cell line was established. CMC-VAP, cyclophosphamide, methotrexate, CCNU, vincristine, Adriamycin, procarbazine; CAV, cyclophosphamide, Adriamycin, and vincristine; VP/CP, VP-16 and cisplatin; RT, radiotherapy; Carb, carboplatin; Mitox, mitoxantrone.
c Therapy that the patient received after the cell line was established.
d The response of the patient to further chemotherapy (if given) after the cell line was established. CR, complete response; PR, partial response.
e Needle aspirate.

Cryopreserved Tumor Samples. Tumor specimens were obtained for diagnostic or therapeutic indications from SCLC patients treated at the Kingston Regional Cancer Centre from 1986 to 1994. With the patient’s informed consent, a sample was sent for research purposes as part of a study approved by the Research Ethics Board of Queen’s University. The source of the tumor cells and the treatment history of the patients is indicated in Table 2. Effusion samples were centrifuged and washed in RPMI 1640 medium, and viable tumor cells were separated from RBCs and non-viable cells on a Ficoll-Hypaque density gradient. The proportion of tumor cells present was determined by examination of cytospin preparations. Only samples containing more than 90% tumor cells were cryopreserved. The cells were frozen in a viable state in 10% DMSO in RPMI 1640 medium with 20% fetal bovine serum and stored in liquid nitrogen. Cell lines were subsequently derived from six of these tumor samples, as indicated in Table 2. In addition, the cell line JN-M was established from a marrow sample taken prior to treatment from the same patient as tumor sample no. 4, which was a pleural effusion obtained at recurrence after chemotherapy treatment.

Drug Sensitivity Testing. The sensitivity of each cell line was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (15). Four drugs, i.e., doxorubicin, vincristine, VP-16, and cisplatin were tested, usually within 3 weeks of mRNA isolation. The dose-response curves for the four drugs were summarized by calculating the area under the curve, using the trapezoidal method as described (15).

mRNA Isolation, cDNA Synthesis, and Oligonucleotide Synthesis. Polyadenylated mRNA was isolated from approximately 10⁷ cells using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). The cDNA synthesis reaction mixture consisted of 20 mM of each dinucleotide triphosphate (Pharmacia, Biotech, Inc., Baie d’Urfé, Quebec, Canada), 10 mM DTT, 5 ng/μl random hexanucleotide primers (Pharmacia Biotech, Inc.), 1.35 units/μl RNAguard (Pharmacia, Biotech, Inc.), 1 unit/μl avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL), and reverse transcriptase buffer (40 mM MgCl₂, 250 mM KCl, and 250 mM Tris-HCl, pH 8.3, at 42°C). mRNA (0.3 μg) was added to each 20 μl cDNA synthesis reaction. To minimize variation between samples, all cDNA synthesis reactions were carried out simultaneously under the same conditions. The cDNA synthesis reaction was incubated at 37°C for 1 h followed by enzyme...
Table 2 Clinical samples

The name of the corresponding cell line established from the same sample as in Table I is indicated. Cell line JN-M was established from a narrow sample taken prior to treatment from the same patient as tumor sample no. 4. The results of RT-PCR screening for MRP and MDR1 expression are shown.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sourcea</th>
<th>Prior treatmentb</th>
<th>Subsequent treatmentc</th>
<th>Responsec</th>
<th>Corresponding cell line</th>
<th>MDR1</th>
<th>MRP</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Pleural effusion</td>
<td>None</td>
<td>None</td>
<td></td>
<td>HG-E</td>
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<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Pleural effusion</td>
<td>None</td>
<td>RT, CAV</td>
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<td>3</td>
<td>Pleural effusion</td>
<td>VP/CP, CAV</td>
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<tr>
<td>4</td>
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<td>CAV, VP/CP</td>
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<td>JN-M*</td>
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<tr>
<td>5</td>
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<td>VP/CP</td>
<td>PD</td>
<td>SV-E</td>
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<td>+</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>Pleural effusion</td>
<td>CAV, VP/CP</td>
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<td></td>
<td>JS-E</td>
<td></td>
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<td>8</td>
<td>Pericardial effusion</td>
<td>VP/CP</td>
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<td>9</td>
<td>Pleural effusion</td>
<td>Oral VP</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Pericardial effusion</td>
<td>CAV, VP/CP, RT</td>
<td>Oral VP</td>
<td></td>
<td>GL-E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

aTumor cells from which the cell line was derived.
bTherapy that the patient had received at the time that the cell line was established. CMC-VAP, cyclophosphamide, methotrexate. CCNU, vincristine, Adriamycin, procarbazine; CAV, cyclophosphamide, Adriamycin, and vincristine; VP/CP, VP-16 and cisplatin; RT, radiotherapy; Carb, carboplatin; Mitox, mitoxantrone.
cThe response of the patient to further chemotherapy (if given) after the cell line was established. CR, complete response; PR, partial response; PD, progressive disease; NA, not assessable; -, patient was not treated or treatment status is unknown.

The cell line JN-M was established from a bone marrow aspirate taken prior to therapy. A clinical sample from the same patient (no. 4) was obtained at relapse following partial response to chemotherapy.

inactivation at 95°C for 3 min. The resulting cDNA was diluted 10-fold in sterile water.

The 25-mer primers specific for MRP, MDR1, and the human TFRR were synthesized on a Biosearch 8750 DNA synthesizer and purified by thin layer chromatography (Queen's University DNA Synthesis Laboratories, Kingston). The downstream (antisense) primers were biotinylated using biotin phosphoramidite (Prime Synthesis, Ashton, PA). The internal standard, TFRR, was used to control for variations in mRNA extraction and cDNA synthesis. TFRR was selected as an internal standard because it is expressed ubiquitously at relatively low levels (similar to that anticipated for MRP and MDR1), and expression levels are not cell cycle dependent.

The sequences of the primers used for PCR and Q-PCR are as follows: MRP, upstream primer (5'-AGTGCACCTCGGTCTCTAAAACAAGG-3') and downstream primer (5'-GAGGTAAGACCAAGATGCTTC-3'); MDR1, upstream primer (5'-ACACTCCGCTTACAGATGCTTC-3') and downstream primer (5'-CGAGATGGTAACCTAGAAGACAT-3'); TFRR, upstream primer (5'-GGATAAAGCGGTTCCTTTCACCAACGG-3') and downstream primer (5'-TGGAAATGACGGAGAAAGTGCCTTCC-3').

**RT-PCR and Q-PCR.** Each PCR reaction contained dinucleotide triphosphates (120 μM; Pharmacia), Vent NEBuffer (New England Biolabs, Mississauga, Ontario, Canada), upstream (sense) primer (0.2 pm), downstream (antisense) primer (0.2 pm), 0.005 units/μl Vent DNA polymerase (New England Biolabs), and cDNA (10 μl per 50 μl PCR reaction). The reaction mixture was overlaid with light mineral oil. RT-PCR screening of the cell lines and clinical samples was performed independently with the primers specific for MRP, MDR1, and TFRR.

The PCR was performed on a PHC-1 Dri-Block thermocycler (Technne, Cambridge, United Kingdom). Reactions were allowed to proceed through one cDNA second strand synthesis cycle of 95°C for 1.5 min, 58°C for 1 min, and 72°C for 3 min, followed by 27 PCR cycles of 95°C for 45 s, 58°C for 1 min, and 72°C for 1 min, with a final PCR cycle of 95°C for 45 s, 58°C for 1 min, and 72°C for 5 min. For reactions with the MRP-specific primers, the annealing temperature was 52°C. The expression of MRP and TFRR was quantified by Q-PCR using “mimic” standards in those cell lines which were shown to express MRP by RT-PCR. The “gene-mimic” is a competitive standard comprised of a heterologous BamHI/EcoRI DNA fragment of the human v-erbB gene (Clontech MIMIC Construction kit, Mississauga, Ontario, Canada) that is flanked on either side by the DNA sequence complementary to the gene-specific primer of interest. This allows both the target cDNA and the gene-mimic to be coamplified by the same primers. When the target DNA and the gene-specific mimic DNA are present in equal concentrations in the PCR reaction mixture, they compete equally for the primers and result in equal quantities of amplification product.

For each cDNA sample, five PCR reactions were performed using a 5-fold dilution series of the cDNA template. The reaction components for each sample were assembled in a master mixture, and a known amount of the gene-mimic standard was added, resulting in a series of competitive reactions with a constant concentration of the gene-mimic standard and variable concentrations of the target cDNA. For each sample, the cDNA concentration at which the PCR product of the target cDNA and the gene-mimic were present in equal amounts was determined by interpolation. The level of MRP mRNA was normalized to the level of TFRR expression in the same cell line and then expressed as a percentage of the MRP level in H69AR cells. The conditions for the Q-PCR were the same as for the RT-PCR except that 36 cycles of amplification were carried out.
Fig. 1 Chemosensitivity was measured using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The dose-response curves were summarized by calculating the AUC. The cell lines were ranked in order of AUC. Results for doxorubicin, vincristine, VP-16, and cisplatin are shown.

Chemiluminescent Detection of PCR Products. The downstream primers contained a biotin group to allow for chemiluminescent detection of the PCR products. PCR-amplified samples were separated on a 2% agarose gel, stained with ethidium bromide, and photographed. The PCR products were then transferred to a Zetaprobe membrane (Bio-Rad, Mississauga, Ontario, Canada) by downward alkaline transfer (0.4 M NaOH, 0.6 M NaCl); the membrane was UV-irradiated and then agitated briefly in neutralizing buffer (1.5 M NaCl, 0.5 M Tris, pH 8.0). The membrane was blocked in SDS buffer (5% w/v SDS, 17 mM Na2HPO4, and 8 mM NaH2PO4) for 30 min, agitated in blocking buffer containing 4 units/100 ml streptavdin-alkaline phosphatase conjugate (Boehringer Mannheim) for 10 min, washed for 15 min in a 1:10 dilution of blocking buffer, and finally agitated in a wash buffer (1 mM MgCl2, 10 mM NaCl, and 10 mM Tris-HCl, pH 9.5) for 30 min. Lumi-Phos 530 (Boehringer Mannheim, Laval, Quebec, Canada) was applied, and the membrane was placed within plastic page protectors and allowed to incubate at 37°C for 1.5–2.5 h. X-OMAT film (Kodak) was exposed to the membrane for 30–60 s at room temperature. The intensity of the bands on the film was determined on a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA) using Image Quant 3.3 software.

Correlation Analysis. Statistical analyses of drug sensitivity, levels of MRP expression, and chemotherapy treatment histories were performed using the Systat software package (version 5.0 for DOS). The cell lines were classified as treated or untreated according to whether the patients from whom the cell lines were derived had received chemotherapy at the time the cell line was established (Table 1). The drug sensitivity data were normally distributed. In contrast, the MRP mRNA levels were highly skewed toward low values. Consequently, a logarithmic transformation of the MRP values was performed to approximate more closely a normal distribution. The cell line H69AR was not included in the correlation analysis, because unlike the other cell lines, it had undergone in vitro selection in...
doxorubicin. Furthermore, this cell line is highly drug resistant and has exceptionally high levels of MRP mRNA, resulting in an undue influence on the correlation analysis. Pearson correlation coefficients were calculated for all pairwise data combinations, including sensitivity to doxorubicin, vincristine, VP-16, and cisplatin, MRP mRNA levels, and treatment history.

RESULTS

Drug Sensitivity. The sensitivities of the 23 cell lines to doxorubicin, vincristine, VP-16, and cisplatin are shown in Fig. 1. The dose-response curves were summarized by calculating the AUC as described previously (15). The cell lines displayed a spectrum of drug responsiveness. One of the cell lines, SV-E, was highly resistant to the natural product drugs, doxorubicin, vincristine, and VP-16, but had an intermediate level of sensitivity to cisplatin. The cell line NCI-H69 (from which H69AR was derived) was among the most sensitive to doxorubicin, vincristine, and cisplatin but not to VP-16. The results for H69AR, which was the most resistant to doxorubicin and vincristine, are shown for comparative purposes only.

The correlation between response to each of the four drugs tested and response to the other three drugs is shown in Table 3. Doxorubicin, vincristine, and VP-16 are natural products, and resistance to these agents may be conferred by either MRP or P-gp. On the other hand, neither MRP nor P-gp would be expected to confer resistance to cisplatin. There was a highly significant correlation between responsiveness to doxorubicin and vincristine (r = 0.739; P = 0.0001). However, no significant correlation was observed between VP-16 and either doxorubicin (r = 0.360; P = 0.091) or vincristine (r = 0.347; P = 0.104). There was an unexpected correlation between responsiveness to cisplatin and the other drugs, including doxorubicin (r = 0.523; P = 0.010) and VP-16 (r = 0.539; P = 0.008). The association between cisplatin and vincristine was particularly striking (r = 0.624; P = 0.001).

Screening for MRP and MDR1 Expression in Cell Lines and Clinical Samples. MRP expression was detected in 20 of the 23 SCLC cell lines, whereas expression of MDR1 was detected in only 1 of 23 cell lines, i.e., SHP-77 (Table 1). Because of the low mRNA yield from the clinical specimens, 33 cycles of amplification were required to obtain levels of the control gene, TFRR, that were comparable to those obtained for the cell lines. The results of screening the 10 cryopreserved SCLC tumors for MRP and MDR1 expression are shown in Fig. 2 and summarized in Table 2. Seven of the 10 samples expressed MRP, and 6 expressed MDR1. Of the 10 clinical samples, 6 were the original specimens from which cell lines were derived. It is interesting to note that three of the clinical samples with detectable MDR1 expression (nos. 5, 8, and 9) corresponded to cell lines that had no MDR1 expression (SV-E, JS-E, and LV-E, respectively). Thus, it appears that the relative levels of MDR1 mRNA may have declined during in vitro culture. Sample no. 4 was derived from the same patient as cell line JN-M. The JN-M cell line (established from a bone marrow aspirate obtained prior to chemotherapy) was negative for MDR1 but positive for MRP expression, whereas the clinical sample (no. 4; a pleural effusion obtained from the same patient at recurrence following partial response to chemotherapy) was positive for both MRP and MDR1. Two of the clinical samples (nos. 8 and 10) had no detectable MRP expression, whereas MRP mRNA was detectable at very low levels in the corresponding cell lines (JS-E and GL-E). Because only 10 clinical samples were studied and only 3 of these samples were derived from patients who subsequently received chemotherapy, it was not possible to make any clinical correlations from this data.

Quantitation of MRP Expression in Cell Lines. Levels of MRP mRNA expression were quantitated by Q-PCR in those cell lines that were positive by RT-PCR. Since MDR1 mRNA was detectable in only one cell line (SHP-77), no attempt was made to quantitate levels of expression of this gene. Because of the limited amount of material available from the clinical specimens, the levels of MRP and MDR1 were not quantitated in these samples.

An example of the Q-PCR for the cell line H69AR is shown in Fig. 3. Serial 5-fold dilutions of the cDNA, along with a constant amount of the “mimic” standard were amplified using TFRR- and MRP-specific primers. At high concentrations of MRP cDNA, the mimic standard is not visible due to competition for primers, whereas at low concentrations of the MRP cDNA, the “mimic” is able to compete for the primers and is, therefore, amplified.

The relative levels of MRP expression in the cell lines are shown in Fig. 4. The results are expressed as a percentage of the levels in H69AR cells. Five of the 24 cell lines had no detectable MRP expression, and in the remainder of the cell lines, the levels ranged from 0.1 to 17.5% of the levels detected in H69AR.

Correlation of MRP mRNA Expression and Drug Resistance. The results of the Pearson correlation analysis are shown in Table 3. The correlation between resistance to doxorubicin and MRP expression by Q-PCR (r = 0.422) was significant (P = 0.045). Although there was a significant correlation between response to doxorubicin and to vincristine (r = 0.739; P ≤ 0.001), the correlation between MRP mRNA levels and vincristine resistance, although positive, was not statistically significant (r = 0.336; P = 0.116). No correlation was found between MRP levels and sensitivity to either VP-16 or cisplatin.

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**Table 3 Pearson correlation analysis**

Pearson correlation matrix is shown. The correlation coefficients are indicated, and the P values indicating the statistical significance of the correlations are shown in parentheses below the correlation coefficients.

<table>
<thead>
<tr>
<th></th>
<th>Dox</th>
<th>Vinc</th>
<th>VP-16</th>
<th>CisPt</th>
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<tbody>
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<td>0.739</td>
<td>0.000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vinc</td>
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<td>0.347</td>
<td>0.624</td>
<td>0.539</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.091</td>
<td>0.104</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>CisPt</td>
<td>0.523</td>
<td>0.624</td>
<td>0.008</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*p Correlation between sensitivity to doxorubicin (Dox), vincristine (Vinc), VP-16, and cisplatin (CisPt).

*c Correlation between drug sensitivity and MRP levels.

**Correlation between drug sensitivity and MRP levels.**
There was no correlation between the chemotherapy treatment histories of the patients from whom the cell lines were derived and either MRP levels or response to doxorubicin, vincristine, VP-16, or cisplatin.

**DISCUSSION**

Ever since combination chemotherapy became the standard treatment for SCLC nearly two decades ago, there have been no major improvements in results of therapy of this disease. The acquisition of resistance to multiple chemotherapeutic agents continues to be the major impediment to cure (16). Despite the efforts of many groups, the molecular basis of clinically acquired resistance is not well understood. To identify mechanisms of resistance, we and others have studied cell lines that have been selected in vitro for drug resistance. Although a variety of alterations associated with resistance have been identified, it remains to be determined whether such alterations are present in patients with drug-resistant tumors and whether they are responsible for the drug resistance phenotype. In this study, we examined cell lines and clinical samples obtained directly from patients with a spectrum of clinically drug-sensitive and drug-resistant tumors (Tables 1 and 2). Such investigations may give a better indication of mechanisms that are involved in clinical drug resistance.

In the 23 SCLC cell lines examined in this study, there was a close correlation between response to doxorubicin and to vincristine. This finding was not unexpected, because doxorubicin and vincristine are both natural product compounds that are included in the spectrum of cross-resistance that characterizes the multidrug resistance phenotype. VP-16 is also included in the multidrug resistance phenotype, and thus it is somewhat surprising that we detected no significant correlation between response to VP-16 and response to either doxorubicin or vincristine. On the other hand, there was also a close correlation between response to cisplatin and to doxorubicin, vincristine, and VP-16. Because cisplatin is not included in the multidrug resistance phenotype, this finding suggests the presence of resistance mechanisms other than MRP or P-gp in these unscreened SCLC cell lines.

Only one of the cell lines, i.e., SHP-77, was positive for MDR1 expression by RT-PCR. SHP-77 also expressed MRP and was one of the most resistant cell lines to both doxorubicin and vincristine but was not highly resistant to VP-16 or cisplatin (Fig. 1). In contrast to the cell lines, 6 of 10 clinical samples of SCLC expressed MDR1 (Fig. 2).

P-gp expression does not occur frequently in multidrug-resistant SCLC cell lines, although it has been detected (17). Lai et al. (4) measured expression of MDR1 in lung cancers of all major histological types as well as corresponding normal lung tissues and tumor cell lines. In most of these tumors, including the SCLC samples, the expression of MDR1 mRNA was low or undetectable. However, it is interesting to note that in three of the four SCLC samples in which MDR1 levels were measured in both tumor samples and corresponding cell lines, there was a decline in MDR1 expression in the derived cell line.

In contrast, two reports suggest that there may be a relation-
ship between clinical drug resistance and MDR1 levels in SCLC. Holzmayer et al. (18) found that the presence of even very low levels of MDR1 expression, as detected by PCR, correlated with lack of response to chemotherapy in seven tumor samples from SCLC patients. Poupon et al. (19) used Northern blot analysis to detect MDR1 mRNA in xenografts derived from seven SCLC patients. Expression of this gene was detected in all but two of the xenografts, and these two samples corresponded to the two patients in the series who were long-term survivors. They also noted that the levels of MDR1 mRNA were higher in tumor samples obtained directly from patients compared with those which had been passaged in nude mice. Although the results are suggestive, larger studies are required before firm conclusions can be drawn about the clinical relevance of MDR1 expression in SCLC.

In our study, MDR1 expression was detected more frequently in clinical samples than in cell lines established from SCLC patients. Furthermore, in some cases, MDR1 expression was detectable in the clinical sample, whereas the cell line established from the same material was negative. In human tumor cells, increased levels of P-gp are most often due to increased gene expression rather than gene amplification (16). This may explain the lack of persistence of P-gp overexpression when the tumor cells are propagated in vitro. Another possibility is that the cell types that become established as permanent cell lines may not reflect the heterogeneity of tumor cells present in the original sample. Furthermore, small numbers of contaminating nonmalignant cells in the clinical samples could lead to false-positive results. The frequent detection of P-gp in clinical samples of SCLC rather than established cell lines suggests that this transport protein could play a more significant role in clinically acquired multidrug resistance in SCLC than previously thought.

On initial screening, we detected MRP expression in 20 of our 23 cell lines (83%). It is interesting to note that the cell line with the highest level of MRP mRNA (SV-E) was also among the most resistant to doxorubicin, vincristine, and VP-16 but did not express MDR1 mRNA. However, despite the fact that the relative resistance of the SV-E cell line to these agents was comparable to that of H69AR, the level of MRP mRNA expression in this line was only 17.5% of that of H69AR, suggesting that MRP expression may not be the only factor accounting for the resistance of this cell line. In the panel of cell lines, there was a significant correlation between MRP mRNA levels and doxorubicin resistance. Although vincristine resistance correlated strongly with doxorubicin resistance, the correlation between vincristine resistance and MRP mRNA levels, although positive, was not statistically significant. Furthermore, there was no apparent correlation between levels of MRP mRNA and response to VP-16. Although VP-16 is one of the drugs to which MRP confers resistance, it is possible that other resistance mechanisms, such as altered topoisomerase II, may obscure significant correlations with MRP levels. MRP does not confer resistance to cisplatin (9, 10), and the lack of correlation between MRP mRNA levels and cisplatin resistance was not surprising.

It is possible that the relationship between drug response and MRP expression may be stronger than the correlation analysis would seem to indicate. As noted, there are other drug resistance mechanisms that may be involved in SCLC, and these could obscure significant relationships with drug response. For example, our analyses showed strong correlations between cisplatin resistance and resistance to doxorubicin, vincristine, and VP-16, suggesting the presence of other mechanisms that confer resistance to all four of these drugs. The correlation coefficients were similar when only those cell lines derived from untreated patients were included in the analysis, indicating that these relationships do not result from prior treatment with these four drugs. Because cisplatin resistance is not conferred by MRP (9, 10) (and does not correlate with MRP levels in this study), the correlations of doxorubicin, vincristine, and VP-16 with MRP mRNA levels could be obscured.

Although increased MRP and MDR1 expression have been clearly implicated in the resistance of certain tumors, they are not the only factors that may result in multidrug resistance. For example, resistance to multiple chemotherapeutic agents has been associated with increased drug detoxification by glutathione and its associated enzymes (20). Although alterations of glutathione and related enzymes have been detected in multidrug-resistant SCLC cell lines, the functional significance of these changes remains to be determined (21, 22). In a study using many of these same unselected SCLC cell lines, we found no significant correlation between drug response and levels of GSH and associated enzymes (12).

Increasing evidence has indicated that reduced levels or function of topoisomerase II are important factors in drug resistance in SCLC as well as other cancers. It is now recognized that topoisomerase II is the common intracellular target for several of the natural product drugs that are also part of the classical “multidrug resistance phenotype.” In this collection of SCLC cell lines, we have shown that reduced levels of topoisomerase II correlate with resistance to a variety of agents, including some drugs that are not known to exert their cytotoxicity through this target (23). Other studies using unselected lung cancer cell lines have also shown an inverse correlation between topoisomerase II levels and drug resistance (24, 25).

In summary, the data presented here indicate that drug resistance in SCLC is complex and unlikely to be explained by a single resistance mechanism. Our results further emphasize the importance of examining clinical samples directly to identify clinically important resistance mechanisms. Results obtained with established cell lines may be misleading, in view of the discordance that we observed in MDR1 expression in tumor samples and cell lines established from these samples. However, the analysis of clinical samples is technically demanding and results may be difficult to interpret. Often the diagnosis of SCLC is made on very small amounts of tumor tissue, which may be partially necrotic. Furthermore, tumor heterogeneity and infiltration with nonmalignant cells may pose significant problems.

The ideal method for measuring levels of drug resistance genes or proteins in clinical samples should be sensitive, reproducible, applicable to small samples, and capable of distinguishing between nonmalignant infiltrating cells and tumor cells. The quantitative PCR technique used in this study fulfills some of these criteria but requires a homogeneous population of tumor cells. Normal bronchial epithelium is known to express MRP, and this could complicate the analysis of MRP expression in lung tumor samples. In some cases, measurement of protein levels may be more informative than gene expression levels.
However, the quantitation of MRP protein may be complicated by its relatively high sensitivity to proteolytic degradation. The use of immunohistochemistry for detecting multidrug resistance proteins at the cellular level has a number of advantages but may not necessarily reflect levels of functional protein.

Despite the infrequent expression of P-gp in SCLC cell lines, this transport protein appears to be more frequently detectable in clinical samples of these tumors. Thus, it is possible that P-gp may play a significant role in clinical drug resistance in SCLC. The multidrug resistance protein MRP is detectable in a majority of cell lines as well as clinical samples of SCLC and correlates with resistance to doxorubicin in SCLC cell lines. The difference in expression of these multidrug resistance genes between cell lines and clinical samples emphasizes the importance of studying clinical material and correlating results with drug responsiveness and clinical outcome. An understanding of clinically significant mechanisms of resistance in SCLC may lead to effective strategies to overcome this important clinical problem.

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