Abstract. Determining an effective predictor of clinical drug resistance in small cell lung cancer (SCLC) is considered to be important. In this study, the relationship between the expression of P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and MRP2, which are the members of ATP-binding cassette superfamily transporter, and of the p53 tumor suppressor gene and the response to chemotherapy were analysed. The expression of P-gp, MRP1, MRP2, and p53 was determined by an immunohistochemical analysis of transbronchial biopsy (TBB) specimens from 61 SCLC patients. The relationship of such expression was also investigated regarding chemotherapy and clinicopathological factors. The response rate in the MRP2-negative group was significantly higher than that in the MRP2-positive group (88% versus 50%). The P-gp-negative group responded significantly better to chemotherapy than the P-gp-positive group, with a response rate of 81% versus 39%. No relationship could be found between the response to chemotherapy and immunostaining for MRP1 or p53. In 37 patients treated with platinum-based chemotherapy, the response rate of patients in the MRP2-negative group was significantly higher than that in the positive group (92% versus 50%). In a multiple logistic regression analysis, MRP2 as well as P-gp were shown to be statistically significant predictors of chemotherapy resistance. These results suggest that immunostaining of MRP2 for TBB specimens may help to predict clinical resistance to platinum agents. This is the first report which indicates that the immunohistochemical expression of MRP2 is positively related to a clinical resistance to platinum.

Chemotherapy plays a major role in the treatment of small cell lung cancer (SCLC). Although initial high response rates are seen in most cases, nearly 20% of all SCLC are refractory to chemotherapy, in most cases, a relapse is inevitable and long-term survival is rare (1).

Some members of the ATP-binding cassette (ABC) superfamily of transport proteins have been shown to confer drug resistance in vitro, but it is still unclear as to whether the expression of these molecules correlates with clinical drug resistance.

The ABC superfamily has an ATP-binding cassette in its molecule, while it also transports drugs at the expense of ATP hydrolysis and acts as a channel and a receptor protein. Among members of the ABC superfamily, P-glycoprotein/multidrug resistance (MDR)1 (encoded by MDR1) (2) and multidrug resistance protein (MRP)1 (encoded by MRP1) (3-5) have been shown to confer resistance to anticancer drugs. P-gp is a member of the ABC superfamily which was first reported by Juliano in 1976 (6), and the relationship between its expression and drug resistance has also been shown in several types of cancer, such as ovarian cancer and lung cancer (7, 8). We have previously reported that a P-gp negative group responded significantly better than the P-gp positive group in SCLC (8).

Like P-gp, MRP1 is overexpressed in numerous drug-selected cell lines and it has also been detected in a variety of tumor types (5, 9, 10). MRP1 expression has also been shown to be associated with drug resistance or poor patient outcomes in breast cancer (11), gastric cancer (12), neuroblastoma (13), retinoblastoma (14) and lung cancer (15, 16). Recognizing the potential importance of MRP-mediated multidrug resistance has led to a search for additional ABC superfamily members, and, recently, a number of MRP-related human gene products have been identified (17). Of these MRP family genes, MRP2 is also known to be a canalicular multispecific organic anion transporter. The tissue distribution of MRP2 mRNA includes the kidney, peripheral nerves, liver, ileum and...
The inactivation of the *p53* tumor suppressor gene through point mutations and/or a loss of heterozygosity is one of the most common genetic changes found in various human malignancies (21). It is thought that mutations in *p53* may lead to drug resistance (22, 23). We reported that the mutations in *p53* mRNA and protein levels in hepatoma cells has been reported to increase sensitivity to vincristine, cisplatin and doxorubicin, but did not increase sensitivity to VP-16 (20).

The immunohistochemical expression of *p53* significantly correlated with chemotherapeutic resistance in NSCLC (24, 25); our findings thus suggest the mutations in *p53* to be associated with drug resistance.

In this study, we immunohistochemically investigated the expression of *P-gp*, *MRP1*, *MRP2* and *p53* protein in tumor specimens obtained by TBB from patients with SCLC, and analyzed the relationship between their overexpression and patients’ clinicopathological factors, especially regarding their response to chemotherapy.

**Patients and Methods**

*Diagnosis of SCLC.* Sixty-one consecutive newly diagnosed patients with histopathologically confirmed SCLC who were also treated with chemotherapy at the Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, between 1985 and 2001 were included in this study. All patients underwent a series of staging procedures, which included fiberoptic bronchoscopy, chest plain radiographs and tomograms, computed tomography of chest, brain and upper abdomen, radionuclide bone scan and bone marrow biopsy. The clinical disease stage was defined using the current International Staging System.

The 61 patients included 54 males and 7 females, ranging in age from 43 to 82 years old (median 65 years), the performance status (PS) of all patients was 1 or less. Based on the staging work-up, 17 patients were classified as having limited disease (LD) and 44 patients as extensive disease (ED). The characteristics of the patients are shown in Table I.

**Chemotherapy and response criteria.** The chemotherapy regimens are summarized in Table II. Twenty-four patients received a combination of cyclophosphamide, doxorubicin and vincristine (CAV). Thirty-seven patients received platinum-based chemotherapy: VP-16 plus platinum (cisplatin and/or carboplatin) in 35 patients; cisplatin plus CPT-11 in 2 patients. All patients received two or more courses of chemotherapy with 3–4 week intervals. The patient responses were evaluated according to the RECIST criteria after every course by repeating appropriate radiographic studies (27). The response rate was defined as the number of cases having a complete response plus those having a partial response divided by the total number of patients.

**Immunohistochemical staining.** Sections 4 µm-thick were cut from the formalin-fixed paraffin-embedded TBB samples and collected on poly-L-lysine-coated glass slides. The slides were allowed to dry overnight in an incubator at 37°C and were then stored at room temperature until use. All staining procedures were performed according to the avidin-biotin complex method using a Histofine kit (Nichirei, Tokyo, Japan). Briefly, samples were deparaffinized in xylene and absolute alcohol, then autoclaved at 121°C with distilled water for 20 min. After cooling, 10% rabbit serum was placed on the slides to reduce any background staining. The slides were then incubated overnight with the primary antibody at 4°C in a moist chamber. The following antibodies were used: JSB-1 for *P-gp* (Nichirei, Tokyo, Japan), diluted 1:200 (0.75 µg/ml); MRPM6 for *MRP1* (Kamiya Biomedical, Seattle, WA, USA), diluted 1:50 (5 µg/ml); M21-4 for *MRP2* (Alexis, San Diego, CA, USA), diluted 1:50 (5 µg/ml); and DO-1 for *p53* (Oncogene Science, New York, NY, USA), diluted 1:200 (0.5 µg/ml). These antibodies were diluted in 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). After washing with PBS, the slides were incubated with the secondary antibody for 30 min (biotinylated anti-mouse IgG; Nichirei). After washing with PBS, the slides were then incubated with streptavidin-peroxidase reagent (Nichirei) for 30 min. After another PBS wash, the antigen-antibody complex was...
Figure 1. Expression of P-gp, MRP1, MRP2 and p53 in tumor tissue from small cell lung cancer patients detected by immunohistochemical staining using the relevant antibody. Each protein expression was visualized by avidin-biotin complex method described in Materials and Methods section. (A) Tumor tissue with positive P-gp expression in the cell membrane. (B) Tumor tissue with strong membranous MRP1 expression. (C) Tumor tissue with strong MRP2 expression with a cytoplasmic and membranous pattern. (D) Tumor tissue with strong p53 expression in the nuclei. Magnification x400 for all images.
visualized using a 0.05% solution of diaminobenzidine tetrahydrochloride in distilled water containing 0.013% H₂O₂ for approximately 5 min at room temperature. Thereafter, for P-gp, MRP1 and MRP2, the slides were counterstained with Mayer’s hematoxylin (Muto pure chemicals, Tokyo, Japan). As control tissues, normal human adrenal gland was used for P-gp (28), lung for MRP1 (29) and liver for MRP2 (30). P-gp in the epithelial cells of the adrenal cortex showed strong ring-shaped staining. MRP1 immunostaining was strong granular cytoplasmic and membranous in the bronchial epithelium, whereas MRP2 immunostaining was apical membranous in the hepatocytes lining the liver canaliculi.

**Scoring of immunostaining results.** The evaluation of immunostaining results was made comparing immunostained samples with haematoxylin-eosin slides. For P-gp, MRP1, MRP2 and p53, samples were divided into two groups, positive or negative, with a cut-off value of 10% positive tumor cells, which was based on findings of previous reports for P-gp in lung cancer by Beer et al. (31), for P-gp, MRP1 and MRP2 in ovarian cancer by Arts et al. (29), for MRP1 and p53 in lung cancer by Oshika et al. (32), and for p53 in SCLC by Gemba et al. (33).

**Statistical analysis.** The Chi-squared test or Fisher’s exact test were used to evaluate the association between immunohistochemical expression and the clinical variables. A multivariate analysis was performed with the Stat View 5.0I statistical software package (SAS Institute Inc., Cary, NC). A logistic regression analysis was used to control any possible confounding factors and to estimate the odds ratio. All reported P values are two-sided. A level of \( p < 0.05 \) was considered to be statistically significant.

**Results**

**Response to systemic chemotherapy.** Twenty-four patients received the CAV regimen, while 37 patients received platinum-based chemotherapy. The overall response for 61 patients with SCLC was 69% (42/61): 63% (15/24) for CAV and 73% (27/37) for platinum-based chemotherapy.

**Expression of P-glycoprotein, MRP1, MRP2 and p53 in tumor specimens.** The results of immunohistochemistry are summarized in Table III. Eighteen (30%) of 61 tumor samples stained positive for P-gp; positive membranous staining was observed as shown in Figure 1A. MRP1 immunostaining was found in 35/55 tumor samples (64%) with predominantly diffuse, partly granular cytoplasmic staining in >10% of tumor cells. In some tumors with strong MRP1 staining, sporadic membranous staining was observed (Figure 1B). Immunostaining for MRP2 was positive with a cytoplasmic and membranous pattern in >10% of tumor cells in 30/54 (56%) tumor samples (Figure 1C). p53 immunostaining was found in 46/59 (76%) tumor samples, and positively p53 overexpression was detected only in the nuclei (Figure 1D).

The MRP2 expression was positively related both to MRP1 (\( p=0.0014 \)) and to P-gp (\( p=0.0089 \)) expression. In contrast, the p53 expression showed no correlation with P-gp (\( p=0.5163 \)), MRP1 (\( p=0.5371 \)) or MRP2 (\( p=0.8415 \)) expression (Table IV).
The relationship between expression of P-gp, MRP1, MRP2, and p53 and clinicopathological characteristics. The relationship of these expressions in SCLC tumors with various clinicopathological parameters is summarized in Table III. No significant association was found between immunostaining for P-gp, MRP1, MRP2 or p53 and any other clinicopathological characteristic (age, gender, extent of disease, serum neuron specific enolase (NSE) level) or chemotherapy regimen except between MRP1 and NSE.

The relationship between expression of P-gp, MRP1, MRP2, and p53 and response to chemotherapy. The MRP2-negative group responded to chemotherapy significantly better than the MRP2 positive-group, with a response rate of 88% versus 50% (p=0.0043). The response rate of patients in the P-gp-negative group (81%) was significantly higher than that in the P-gp-positive group (39%, p=0.0030). No relationship, however, could be established between the response to chemotherapy and the immunostaining findings for MRP1 or p53 (Table V).

Since MRP2 is reported to be involved in platinum resistance, we analyzed the relationship between MRP2 expression and resistance to chemotherapy with or without platinum agents. In 37 patients who were treated with platinum-based chemotherapy, the response rate of patients in the MRP2-negative or P-gp-negative group was significantly higher than that in the respective positive groups (Table VI). No relationship, however could be found between the response to chemotherapy and immunostaining of any of these four factors in 24 patients treated with CAV regimens (Table VI). In a multiple logistic regression analysis according to chemotherapy resistance, MRP2 (Odds ratio (OR): 6.238, 95% C.I.: 1.041-37.395) and P-gp (OR: 4.767, 95% C.I.: 1.085-20.938) were both found to be independent predictors of the chemotherapy response (Table VII).

### Table IV. P value regarding the relationship of positive staining for two factors.

<table>
<thead>
<tr>
<th>Factor</th>
<th>MRP1</th>
<th>MRP2</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>0.0719</td>
<td>0.0089*</td>
<td>0.5163</td>
</tr>
<tr>
<td>MRP1</td>
<td>–</td>
<td>0.0014*</td>
<td>0.5371</td>
</tr>
<tr>
<td>MRP2</td>
<td>–</td>
<td>–</td>
<td>0.8415</td>
</tr>
</tbody>
</table>

*Statistically significant, Fisher's exact test.

### Table V. Response to chemotherapy according to immunostaining.

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>CR + PR</th>
<th>SD + PD</th>
<th>Response rate</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp +</td>
<td>7</td>
<td>11</td>
<td>39% (7/18)</td>
<td>0.0030</td>
</tr>
<tr>
<td>P-gp –</td>
<td>35</td>
<td>8</td>
<td>81% (35/43)</td>
<td>0.3905</td>
</tr>
<tr>
<td>MRP1 +</td>
<td>22</td>
<td>13</td>
<td>63% (22/35)</td>
<td>0.0043</td>
</tr>
<tr>
<td>MRP1 –</td>
<td>15</td>
<td>5</td>
<td>75% (15/20)</td>
<td></td>
</tr>
<tr>
<td>MRP2 +</td>
<td>15</td>
<td>15</td>
<td>50% (15/30)</td>
<td></td>
</tr>
<tr>
<td>MRP2 –</td>
<td>21</td>
<td>3</td>
<td>88% (21/24)</td>
<td></td>
</tr>
<tr>
<td>p53 +</td>
<td>30</td>
<td>15</td>
<td>67% (30/45)</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>p53 –</td>
<td>10</td>
<td>4</td>
<td>71% (10/14)</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test; LD: limited disease; ED: extensive disease; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Response rate</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV P-gp +</td>
<td>38% (3/8)</td>
<td>0.0994</td>
</tr>
<tr>
<td>P-gp –</td>
<td>75% (12/16)</td>
<td>0.6214</td>
</tr>
<tr>
<td>MRP1 +</td>
<td>61% (11/18)</td>
<td>0.1930</td>
</tr>
<tr>
<td>MRP1 –</td>
<td>80% (4/5)</td>
<td>0.0200</td>
</tr>
<tr>
<td>MRP2 +</td>
<td>50% (6/12)</td>
<td>0.7120</td>
</tr>
<tr>
<td>MRP2 –</td>
<td>82% (9/11)</td>
<td>0.0200</td>
</tr>
<tr>
<td>p53 +</td>
<td>53% (8/15)</td>
<td>0.3998</td>
</tr>
<tr>
<td>p53 –</td>
<td>75% (6/8)</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test.

### Table VII. Multiple logistic regression analysis for chemotherapy response.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>70&lt; versus ≥70</td>
<td>0.662 (0.153-2.864)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>female versus male</td>
<td>0.358 (0.031-4.178)</td>
</tr>
<tr>
<td>Extent of disease</td>
<td></td>
</tr>
<tr>
<td>LD versus ED</td>
<td>0.314 (0.057-1.722)</td>
</tr>
<tr>
<td>P-gp + versus –</td>
<td>4.767 (1.085-20.938)*</td>
</tr>
<tr>
<td>MRP1 + versus –</td>
<td>0.763 (0.139-4.205)</td>
</tr>
<tr>
<td>MRP2 + versus –</td>
<td>6.238 (1.041-37.395)*</td>
</tr>
<tr>
<td>p53 + versus –</td>
<td>0.936 (0.159-5.499)</td>
</tr>
</tbody>
</table>

95% CI, 95% confidence interval. *p<0.05.
Discussion

Drug resistance in SCLC is reported to be mediated by the overexpression of drug efflux pumps such as P-gp and these of the MRP family, tumor suppressor gene p53. Some previous studies reported that the gene expression of P-gp and MRP1 correlated with the multidrug resistance phenomenon of SCLC (16, 34), but it is still unknown as to whether or not the expression of these molecules at the protein level correlates with clinical drug resistance (35).

Although chemotherapy plays a major role in the treatment of SCLC and high response rates are seen in most cases, some patients with SCLC are nevertheless refractory to chemotherapy. Since the treatment in SCLC depends on chemotherapy and resistance to chemotherapy remains a major problem, selection of the effective drug is clinically important.

Evaluation of TBB specimens seems to be best suited for the prediction of chemosensitivity considering its usefulness and convenience, and we previously reported on the response to chemotherapy in NSCLC and SCLC (36, 8, 24, 25). In a previous study, we reported that the P-gp expression was related to a poor response to chemotherapy in SCLC. In the present study, we evaluated the other pumps at play such as P-gp, and we investigated the importance of the ABC superfamily and discussed which factor was the most important for the prediction of clinical drug resistance.

In 1976, P-gp was first reported by Juliano et al. (6), and the association between its expression and drug resistance has since been discussed in many types of cancer. Some reports have shown that P-gp mediates the drug resistance to vinca alkaloids, anthracyclines and paclitaxel. In previous studies on the expression of MDR1, Holzmayer et al. reported that the expression of the MDR1 gene was related to resistance to chemotherapy in SCLC patients (8). Savaraj et al. showed the MDR1 expression to correlate with chemoresistance in tumor tissues obtained from 35 patients of SCLC, using reverse transcription-polymerase chain reaction (RT-PCR) and a northern blot analysis (34). Our results were consistent with their reports, and together these findings suggest that P-gp may be one of the most useful predictive markers for clinical drug resistance in SCLC.

MRP family members have been recently characterized as major contributors to multidrug resistance phenotypes which were not mediated by P-gp (4), and seven MRP family members (MRP1-7) have so far been identified (37). However, the functions of many of them in cancer patients have yet to be elucidated (38). In this study we analyzed the expression of two members of the MRP family, MRP1 and MRP2, which are considered to correlate with chemotherapy resistance. An expression of MRP1 was observed in 35 of 55 (64%), but no significant relationship could be found between response to chemotherapy and immunostaining for MRP1, even though the MRP1 negative group appeared to respond to chemotherapy better than did the positive group. MRP1 transports MDR phenotype substrates such as doxorubicin and vincristine (6), and we supposed that MRP1 expression was related to chemotherapy resistance in patients treated with CAV regimens, but no correlation between MRP1 expression and drug resistance was observed.

Allen et al. showed that cell lines lacking P-gp were markedly more sensitive to paclitaxel, anthracyclines and vinca alkaloids, and that lines lacking both P-gp and MRP1 were also more sensitive to epipodophyllotoxins (etoposide, teniposide), anthracyclines, camptothecins, arsenite and vinca alkaloids (39). Johnson et al. reported that mice deficient in the three genes, mdr1a/1b and mrp1, exhibited an increased toxicity to vincristine and etoposide (40). As a result, it seemed that P-gp and MRP1 play important roles in resistance to etoposide. In this study, we found a correlation between P-gp expression and chemoresistance in platinum-based chemotherapy. One of the reasons why P-gp plays an important role in resistance in platinum-based chemotherapy may depend on the regimen containing etoposide, since most patients (95%) treated with platinum-based chemotherapy were given etoposide in this study. Conversely, overexpression of MRP1 and P-gp was reported to increase the sensitivity to gemcitabine in a recent paper (41).

MRP2 shows 49% of its amino acid identity with MRP1 (17) and it seems to have a similar substrate specificity to MRP1. In contrast to MRP1, however, the expression of MRP2 was found to be elevated in a number of cell lines selected for cisplatin resistance (17, 42, 43). In some previous studies, a correlation between MRP2 and cisplatin resistance has been suggested in vitro (17, 19, 20), and Hinoshita et al. reported that the MRP2 mRNA expression was correlated with cisplatin resistance in tissues obtained from 45 patients with colorectal cancer (44). However, so far there has been no report about the relationship between protein expression of MRP2 and clinical drug resistance. Regarding chemotherapy regimens such as CAV or platinum-based chemotherapy, we only found a correlation between the response to chemotherapy and the MRP2 expression in platinum-based regimens, not in CAV. This result was also consistent with previous reports showing MRP2 protein expression to correlate with cisplatin resistance in vitro using a few cell lines resistance (45), and that MRP2 mRNA expression may be a useful predictive marker for clinical cisplatin resistance.

A mutation of the p53 gene is one of the most common abnormalities found in many malignancies, and Lohmann et al. reported that mutant p53 genes were detected in 61% of SCLC patients (46). Gemba et al. reported that the expression of mutant p53 protein identified immunohistochemically was significantly associated with the treatment outcome, using TBB specimens of 103 patients with SCLC (33). However, in the present study no correlation was observed between p53 expression and
chemotherapy resistance, thus suggesting that the ABC superfamily plays a more important role than p53 mutation in drug resistance in SCLC, while, in addition, the expression of p53 does not necessarily correspond with p53 mutation. In other mechanism of cisplatin resistance, altered potassium ion fluxes was proposed previously. However, recent paper reported that the potassium ion homeostasis is not critical factor in cisplatin resistance (47).

Conclusion

In the present study, P-gp and MRP2 were found to be useful predictors for clinical chemotherapy resistance using a multiple logistic regression analysis. In platinum-based chemotherapy the expression of P-gp and MRP2 correlated with chemoresistance and our findings suggest that the immunohistochemical expression of MRP2 may be a useful predictor in the clinical resistance to cisplatin. Platinum, such as cisplatin and carboplatin, is a key drug in the treatment of SCLC, and to predict such resistance to this drug is indeed clinically useful. Therefore, investigating the expression of MRP2 in TBB specimens of untreated SCLC patients is considered to be a good modality for determining chemotherapy resistance. This is the first report to indicate a positive correlation between immunohistochemical MRP2 expression and clinical resistance to platinum.

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References


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