Immunohistochemical Detection of Multidrug Resistance Protein in Human Lung Cancer and Normal Lung\textsuperscript{1}

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ABSTRACT

Monoclonal antibody QCRL-1 is highly specific for a defined linear epitope in a relatively poorly conserved region of the human multidrug resistance protein (MRP). We have used QCRL-1 to examine MRP expression in archival and fresh snap-frozen samples of untreated small cell (SC) and non-small cell (NSC) lung cancer (LCs), as well as normal lung. We found that the majority (87%) of all histological subtypes of NSCLC had detectable levels of MRP in most of the tumor mass. In a substantial proportion of adenocarcinomas (55%) and squamous cell carcinomas (28%), immunoreactivity approach the obtained with the highly multidrug resistant cell line H69AR from which the MRP was originally cloned. Both the level and frequency of MRP expression in untreated SCLC was significantly lower than in NSCLC. The MRP was detectable in only 56% of SCLC tumors and, in most cases, was expressed in small focal clusters of cells. Immunofluorescence studies of tumor tissue and normal lung confirmed the plasma membrane location of the MRP. However, in normal bronchial epithelium and seromucous glands, unlike in tumor cells, the MRP was detected only on basolateral membranes. In addition, strong MRP immunoreactivity was detected in reactive type II pneumocytes present in hyperplastic aleveoli, but not in normal type I and type II pneumocytes. No potentially founding correlation independent of its possible role in drug resistance was observed between MRP expression in untreated NSCLC and any clinicopathological parameter examined, including overall survival.

INTRODUCTION

Overall 5-year survival of patients with lung cancer is approximately 10%, and for both men and women, lung cancer is now the leading cause of death from malignant disease in North America (1). SCLC\textsuperscript{4} accounts for approximately 25% of all lung cancer cases. Although SCLC typically responds initially to chemotherapy, most patients relapse with a multidrug resistant form of the disease. NSCLC, which includes the major histological subtypes, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for the vast majority of other cases of lung cancer and is typically refractory to chemotherapy from the outset. It seems likely that the resistance observed is multifactorial in origin, because no single mechanism has been identified experimentally that can simultaneously confer resistance to the entire spectrum of drugs currently in clinical use (2). However, because the resistance profile includes many natural product-type drugs to which both P-gp and the more recently identified MRP confer resistance, increased expression of either or both of these proteins could potentially be involved (3–5).

In normal and neoplastic lung tissue, P-gp and/or its cognate mRNA are usually expressed at low or undetectable levels (6–10). Most clinical evidence does not support a major role for P-gp-mediated MDR in lung cancer (2, 6, 11, 12). In contrast, the MRP was originally identified and characterized in a multidrug resistant human SCLC cell line, H69AR (13). The MRP has subsequently been shown to be overexpressed in drug-selected NSCLC cell lines and to be readily detectable in clinical samples of NSCLC (14–23).

To facilitate studies of the clinical importance of the MRP, we have developed several MRP-specific MAbs (24). The epitope for one of them, QCRL-1, has been defined as a heptapeptide corresponding to amino acids 918–924 in one of the most evolutionarily variable regions of the protein. The epitope is not conserved in any MRP-related protein identified to date, nor in the murine ortholog of the MRP (25). Competition with the corresponding peptide permits validation of the specificity of QCRL-1 immunoreactivity. Here, we have used MAb QCRL-1 to examine MRP expression in different histological types of lung tumors as well as normal tissue. Using standard immunohistochemical methodology routinely used in hospital

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\textsuperscript{4}The abbreviations used are: SCLC, small cell lung cancer; MRP, multidrug resistance protein; MAb, monoclonal antibody; P-gp, P-glycoprotein; LSAB, horseradish peroxidase-labeled streptavidin-biotin; NSCLC, non-small cell lung cancer; MDR, multidrug resistance.
service laboratories, the prevalence and pattern of MRP expression was determined in archival formalin-fixed, paraffin-embedded tissue samples of lung cancers. In some cases, MRP levels in primary and metastatic tumor sites were also compared. These results were then correlated with clinicopathological parameters, including grade of differentiation, tumor size, presence of metastases, stage, and patient survival. Finally, using fresh snap-frozen tissue samples, the subcellular localization of the MRP in neoplastic and normal tissues was also determined.

MATERIALS AND METHODS

Cell Lines. The following cell lines were used as standards for determining relative levels of MRP expression and were cultured as described previously (26, 27): doxorubicin-selected, multidrug resistant H69AR (high-levels); MRP-transfected T14 HeLa (intermediate levels); the H69AR-revertant H69PR (low levels); and H69 and control-transfected C6 HeLa cells (expression undetectable). Cells were harvested after brief trypsin treatment, and cell pellets were fixed for 20 h in 10% neutral buffered formalin before embedding in paraffin.

Patient Materials. All tissues were obtained from the Department of Pathology at Kingston General Hospital (Queen’s University at Kingston). Using archival formalin-fixed paraffin-embedded tissue blocks, MRP levels were assessed in surgically resected lung tumors from 135 cases. None of the patients from whom the tumor samples were obtained had received chemotherapy or radiotherapy. The following number of tumors were evaluated: 53 adenocarcinomas, 47 squamous cell carcinomas, 7 large cell carcinomas, 2 adenosquamous carcinomas, 18 SCLCs, 8 mesotheliomas, 8 typical carcinoid tumors, and 2 carcinoma in situ. In addition, 31 nodal or distant metastases (from 9 adenocarcinomas, 19 squamous cell carcinomas, 1 large cell carcinoma, and 2 SCLCs) were compared with matching primary tumor specimens. Five fresh snap-frozen samples of NSCLC and normal lung, with matching formalin-fixed blocks, were analyzed to compare MAb QCRL-1-specific MRP staining on frozen and archival formalin-fixed material, as well as to examine the subcellular localization of the MRP in neoplastic and normal lung tissues.

For 102 patients with NSCLC, clinicopathological and follow-up data were obtained retrospectively from patient clinical charts and pathological records. All patients were staged (pTNM) at the time of surgery according to the revised International Staging System for NSCLC (28). The patients consisted of 60 males and 42 females, ranging in age from 38–90 years, with a mean age of 68 years. None of the patients had received prior chemotherapy, and all but seven were treated with surgery as their only initial therapy. Survival times were calculated from the date of surgery.

MRP-specific MAb QCRL-1. The MRP-specific murine MAb QCRL-1 (subclass IgG1) was generated as described (24). MAb QCRL-1 recognizes a linear intracellular epitope of human MRP (SSYSGDI) corresponding to amino acids 918–924 (25).

MRP Immunostaining. MRP levels and subcellular localization were assessed using LSAB and indirect immunofluorescence techniques, respectively. Briefly, for paraffin-embedded samples, 8-μm sections, mounted on silane-coated slides, were deparaffinized, rehydrated, and then pretreated using a modified version of the microwave epitope retrieval. Sections were immersed in preheated 10 mM citrate solution, pH 6.0, microwaved for 10 min at 80% power in a General Electric 650W microwave oven, and allowed to cool for 25 min. For fresh snap-frozen tissues, 8 μm cryostat sections, mounted on silane-coated slides, were allowed to air-dry for 5 min and fixed with either 4% paraformaldehyde in PBS at 4°C for 30 min or acetone at −20°C for 10 min. Unless otherwise stated, all subsequent washes were with PBS containing 0.1% Tween 20. Nonspecific staining was blocked with 5% goat serum in PBS containing 0.1% Tween 20. Formalin-fixed sections were incubated for 18 h at 4°C with MAb QCRL-1 (1.5 μg/ml) in a moist chamber, washed, and then incubated with a biotinylated goat antimouse IgG secondary antibody (1:300; Pierce Chemical Co., Rockford, IL) for 1 h at room temperature. For the LSAB technique, slides of formalin-fixed sections were incubated for 1 h with a streptavidin-peroxidase complex (1:300; DAKO Corp., Carpinteria, CA). The peroxidase reaction was visualized with filtered 0.06% 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) plus 0.03% H2O2 in 50 mM Tris-HCl pH 7.5 for 4 min. Sections were then counterstained with Harris’ hematoxylin and mounted with Clearene.

Both MRP-positive (H69AR, H69PR, and T14) and -negative (H69 and C6) control cells were stained concurrently with tissue sections. An irrelevant, isotype-matched murine IgG1 control antibody was included with each cell line and tissue section. An additional control for specificity was carried out by including a 600-fold molar excess of the peptide SSYSGDI corresponding to the MAb QCRL-1 epitope (25), or the irrelevant peptide SLNKEDTSEQ, during incubation with the MAb. For the LSAB technique, endogenous biotin was blocked using an avidin-biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA), and endogenous peroxidase activity was blocked by incubation with 3% H2O2 in PBS for 15 min at room temperature.

Histopathology. Representative paraffin sections stained with H&E were used to assess histological tumor type and grade of differentiation. Two observers (S. R. W. and A. H. B.) independently scored the tissue sections for the level and pattern of MRP immunoreactivity without knowledge of the patient clinical data. The evaluations of the two observers were in concordance in 74% of cases. The other 26% of cases were reevaluated, and a consensus was reached in all cases.

MRP immunoreactivity was scored using a semiquantitative scale assessing both the proportion of positive tumor cells (as a percentage of the total tumor mass in the tissue section) and the intensity of staining. Intensity of staining was graded as follows: “negative” for no staining or the same as the H69 cell line and isotype controls; “low” for weak intracellular staining only slightly above background; “intermediate” for clearly pos-
itive staining with membranous and/or intracellular staining similar to the MRP-transfected T14 cell line and normal bronchial epithelium; and “high” for intensely positive membrane and intracellular staining similar to the H69AR cell line. An overall score was assigned for each tumor specimen by taking into consideration the levels of staining intensity and the proportions of tumor cells stained. A score of “+” was given to samples with no stained tumor cells above background. A score of “++” was assigned to samples with low levels of staining, and a score of “+++” was given to samples with intermediate or high staining in 50% or less of the total tumor mass. Intermediate or high staining in >50% of the tumor mass was scored as “++++.” Homogeneous staining was defined as 80% or more of tumor cells having the same intensity of staining. Conversely, heterogeneous staining was defined as <80% of the tumor cells displaying similar staining intensity. Focal staining was defined as small clusters of immunoreactive tumor cells.

Statistical Analyses. Dependence of MRP staining on histological type and clinicopathological parameters was assessed using the Pearson $\chi^2$ test or two-sided Fisher’s exact test, as appropriate. Survival curves were compiled by the Kaplan-Meier method and compared using the log-rank test (29). Statistical Analysis Software (SAS Institute Inc., Cary, NC) was used for the analyses.

RESULTS

Immunohistochemical Staining Characteristics of Cell Lines and Lung Tumors. Formalin-fixed, paraffin-embedded cell pellets were prepared from cell lines with known levels of the MRP and well characterized drug resistance phenotypes (H69AR, H69PR, H69, T14, and C6) in a manner as similar as possible to that used for formalin-fixed tissues. Sections from the cell pellets were then used as standards with which to compare MAB QCRL-1 staining intensity in tissue sections of normal or tumor tissue. High intensity staining was observed with multidrug resistant H69AR cells, in which the MRP comprises approximately 5% of total membrane protein (Fig. 1A). No staining was detected with the isotype control (Fig. 1B), and immunoreactivity could be completely blocked by the addition of peptide corresponding to the MAB QCRL-1 epitope (Fig. 1D), whereas an unrelated peptide had no effect (Fig. 1C). Intermediate intensity staining was observed in MRP-transfected T14 HeLa cells, which have a 3–5-fold lower level of the MRP than H69AR cells (Ref. 27; Fig. 1E). No staining was detected in control-transfected C6 HeLa cells (Fig. 1F). In both H69AR and T14 cells, the immunostaining was predominantly membranous; no nuclear staining was observed, although some diffuse intracellular staining was present. The partially revertant H69PR cell line, in which the level of the MRP is approximately 5% of that in H69AR cells, demonstrated low-intensity intracellular staining (Fig. 1G) that could not be consistently distinguished from that obtained with H69 cells in which the level of the MRP is several-fold lower (Fig. 1H). Thus, MRP expression in the H69PR cell line defined the lower limit of detection of the immunohistochemical technique.

More than 160 formalin-fixed, paraffin-embedded archival specimens of primary and metastatic lung tumors were stained for the MRP. The tumor cells demonstrated different intensities of staining with MAB QCRL-1, ranging from that observed in H69AR cells to that in H69PR cells, whereas the surrounding connective tissue was always negative for MRP expression. In formalin-fixed sections, positively staining tumor tissue from all histological types of lung cancer demonstrated at least one of three cellular patterns of immunoreactivity: mainly membranous, both membranous and intracellular, and mainly intracellular. Examples of frequently observed staining patterns obtained with adenocarcinoma (Fig. 2, A and B), squamous cell carcinoma (Fig. 2, C and D), and large cell carcinoma (Fig. 2, E and F) are shown. Within a tumor section, one or more of these staining patterns could be observed and could be blocked with the peptide corresponding to the epitope recognized by MAB QCRL-1, whereas an irrelevant peptide had no effect. In contrast to the membrane and/or intracellular staining patterns in archival tumor tissue, the five fresh snap-frozen lung tumor samples, fixed with cold paraformaldehyde or acetone and examined by immunofluorescence, demonstrated predominant membrane staining with minimal intracellular staining. An example of a strongly MRP-positive squamous cell carcinoma is shown in Fig. 2, G and H. The matching formalin-fixed, paraffin-embedded samples demonstrated strong intracellular staining in addition to membrane staining, suggesting a difference in MRP localization between fresh snap-frozen tissue and archival tissue.

Prevalence of MRP Expression in NSCLC. The pattern and frequency of immunoreactivity with MAB QCRL-1 in the different histological types of lung tumors is summarized in Table 1. Of the 109 NSCLC samples studied, 13 (12%) had no staining (-), 16 (15%) had only low intensity staining (+), 36 (33%) had intermediate and high intensity staining in <50% of the tumor mass (++), and 44 (40%) had intermediate and high intensity staining in >50% of the tumor mass (+++). Some level of positive staining was observed in 96 (88%) of 109 NSCLC tumor samples, with intermediate and high intensity staining observed in 80 (73%) samples. We also noted that in 29 (55%) of 53 adenocarcinomas, intermediate and high intensity staining was observed in >50% of the tumor mass, as compared with 13 (28%) of 47 squamous cell carcinomas and 1 (14%) of 7 large cell carcinomas. As shown in Table 2, a statistically significant difference was found between adenocarcinoma and squamous cell carcinoma histological subtypes and MRP staining score ($P = 0.037$). Adenocarcinomas were found to express intermediate and high levels of the MRP more often than squamous cell carcinomas ($P = 0.029$), and in a greater proportion of the tumor mass ($P = 0.0062$). Moreover, in the two cases of adenosquamous carcinomas analyzed for MRP expression, the adenocarcinoma cells were stained more strongly than the squamous cell carcinoma cells. Although the number of large cell carcinomas examined was small, the proportion of this histological class of NSCLC with no detectable MRP expression seemed to be greater than either the adenocarcinomas or squamous cell carcinomas ($P = 0.075$).

Pattern of MRP Expression in Lung Tumors Other Than NSCLCs. In contrast to NSCLCs, the MRP-positive SCLC samples tended to have small focal clusters of cells with

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high levels of MRP staining adjacent to tumor cells in which MRP levels were low or undetectable (Fig. 3, A and B). Furthermore, of 103 samples of NSCLC that included the tumor margin, 20 (19%) samples demonstrated moderate or strong MRP staining preferentially at the leading margins of the tumor. In contrast, no preferential margin staining was observed in any of the SCLC samples.

Among the 18 SCLC samples analyzed, 8 (44%) cases were negative and 2 cases (11%) displayed equivocal, low intensity staining with MAb QCRL-1. Intermediate and high levels of staining in <50% of the tumor mass were detected in five (28%) cases, and three (17%) cases demonstrated intermediate or high levels of staining in >50% of the tumor mass (Table 1). In contrast to NSCLC, where 88% of cases exhibited some level of positive staining, only 56% of SCLC cases were immunopositive; this difference was highly significant \( P = 0.00058 \). Moreover, moderate and strong staining was observed in only 44% of SCLC cases versus 73% for NSCLC tumors \( P = 0.0014 \), with the moderate and strong staining tending to occur in <50% of the tumor mass \( P = 0.054 \). These results indicate that both levels of the MRP and the number of MRP-positive tumor cells are lower in SCLC than in NSCLC.

Although NSCLCs and SCLCs typically displayed markedly heterogeneous staining, a more homogeneous staining pattern was found in lung carcinoid tumors and mesotheliomas. Of the eight cases of typical carcinoid tumors stained with MAb
Fig. 2 Detection of the MRP in NSCLC samples. Examples of frequently observed patterns of MRP immunoreactivity are shown for adenocarcinoma (A), squamous cell (C and G), and large cell carcinomas (E). A, C, and E illustrate MRP immunoreactivity detected with MAb QCRL-1 in sections of formalin-fixed, paraffin-embedded archival tumor samples, using the horseradish peroxidase streptavidin coupled detection method described in the legend to Fig. 1. B, D, and F are irrelevant antibody sections adjacent to those shown in A, C, and E, respectively, stained with an isotype-matched control in place of MAb QCRL-1. G, a section of a fresh-frozen squamous cell tumor in which the MRP has been visualized by indirect immunofluorescence using MAb QCRL-1 and fluorescein-conjugated goat antimouse IgG secondary antibody, as described in “Materials and Methods.” H, the isotype matched control.

QRCL-1, two exhibited intermediate or high intensity staining in <50% of tumor cells, whereas in the remaining six, >50% of the tumor mass stained at intermediate or high intensity (Fig. 3, C and D). Thus, all of the carcinoid tumors were positive for the MRP and none displayed low intensity staining (Table 1). The two examples of squamous cell carcinoma in situ that were examined were also strongly positive for the MRP (Fig. 3, E and F). In contrast, only three of eight mesotheliomas were positive and these displayed only low or moderate staining (Fig. 3, G and H).

MRP Expression in Local and Distant Metastases. In total, the MRP positivity between primary and metastatic tumors was in agreement in 27 of 31 (87%) matched cases (Table 3). In the other four cases, the primary tumor alone was positive in two (6%) cases, and similarly, the metastases were positive in two (6%) cases. As with the primary tumor sites, heterogeneous staining was observed in metastatic tumor sites, with no propensity for the metastases to be MRP positive as compared with the primary tumor. Although the level of staining was comparable between primary and metastatic tumors in 87% of cases, only 18 (72%) of the 25 matching positive cases had both equivalent intensity of staining and proportion of positive-staining tumor cells. Therefore, although the overall MRP positivity tends to agree between the majority of primary and metastatic tumor sites, there is some discordance, particularly in the proportions of stained tumor cells. However, there was no signifi-
MRP Expression in Normal Lung Tissue. In formalin-fixed material, intermediate levels of staining intensity were observed in normal bronchial and bronchiolar epithelium, as well as mixed seromucous glands. In airway epithelium, membrane staining as well as diffuse intracellular staining from the ciliated apical membrane to the basement membrane was present (Fig. 4A). Similar to formalin-fixed airway epithelium, mixed seromucous glands demonstrated both membranous and intracellular staining (Fig. 4C). However, in serous glands, large secretory vacuoles did not stain with MAb QCRL-1. In contrast to the diffuse MRP staining observed in formalin-fixed normal tissues, airway epithelium and sero-mucous glands in fresh snap-frozen tissue demonstrated predominantly basolateral membrane staining with minimal intracellular staining (Fig. 4B and D). In bronchial/bronchiolar epithelium, no apical or perinuclear staining was observed and staining of cilia was minimal or absent. Normal alveolar tissue demonstrated minimal or no staining with MAb QCRL-1 in either type I or type II pneumocytes (Fig. 4, E and F). In contrast, type II pneumocytes in areas of reactive or atypical alveolar hyperplasia were strongly positive for the MRP (Fig. 4, G and H). High levels of peroxidase activity in alveolar macrophages could not be completely blocked precluding a reliable estimate of the level of the MRP present in this cell type. In all samples analyzed, the MRP was not detectable in blood vessel endothelium.

Correlation of MRP Expression in NSCLC with Clinicopathological Parameters. The relationship of MRP expression in NSCLC tumors with various clinicopathological parameters is summarized in Table 4. When all subtypes of NSCLCs were analyzed together, no significant association was found between the MRP score and patient age, gender, tumor size, presence of metastases, stage, or grade of differentiation (Table 5). This finding held true regardless of staining score or clinicopathological groupings. However, when similar analyses were performed for each histological subtype of NSCLC, a significant correlation was found in the adenocarcinoma cases between the MRP staining score and grade of differentiation. A higher proportion of well-differentiated adenocarcinomas stained strongly for MRP in the majority of the tumor mass when compared with moderately or poorly differentiated tumors (P = 0.028). No significant correlation between grade and MRP expression was found in squamous cell carcinoma (Table 5). However, the number of well-differentiated squamous cell carcinomas analyzed was relatively small. A significant difference was found between the MRP staining score of squamous versus non-squamous cell carcinomas, with a higher proportion of non-squamous tumors expressing intermediate and high levels of the MRP in a greater proportion of the tumor mass than squamous cell carcinomas (P = 0.032). When only samples from patients with stage I or II NSCLC were included in the analyses, the above finding remained statistically significant (P = 0.040).

Survival Analysis. Clinical follow-up was available on 93 patients with stage I-IIIA NSCLC who underwent surgical resection with curative intent (49 adenocarcinomas, 39 squamous cell carcinomas, and 5 large cell carcinomas). As shown in Fig. 5, there was no difference in the overall survival of patients with tumors that stained strongly for MRP compared with those whose tumors had lower levels of MRP staining (P = 0.46 by log rank test). These data suggest that the level of MRP immunoreactivity is not by itself a significant prognostic factor in NSCLC. Because none of these patients had received chemotherapy before surgery and only three (3.2%) were treated with chemotherapy at recurrence, it was not possible to determine whether MRP levels correlated with lack of response to treatment.

DISCUSSION

In this study, the well characterized, highly specific MAb QCRL-1 was used to examine the patterns and level of MRP expression in lung tumors and in normal lung. Immunohistochemistry of archival and fresh frozen tissue revealed substantial levels of the MRP in a variety of different histological samples.
subtypes of lung tumors, with marked heterogeneity of MRP expression both within and between tumor types. In previous studies, P-gp-positive tumors have been found to arise with high frequency from tissues that normally express the protein, such as colonic epithelium (8, 9). Consistent with this observation, relatively high levels of the MRP were detected in normal lung epithelium, seromucous glands, and hyperplastic alveolar epithelium.

We detected the MRP in 85% of NSCLCs ranging from low levels characteristic of revertant H69PR cells, to intermediate and high levels comparable with those present in MRP transfectants and drug-selected H69AR cells, respectively. This overall frequency of MRP positivity is comparable with that reported by Nooter et al. (20) in a study of 35 samples of adenocarcinoma and squamous cell carcinoma using a different MRP MAb. A large proportion (73%) of NSCLCs had intermediate and high levels of MRP expression, with 44% of cases having intermediate or high MRP expression in a majority of the tumor mass. Although the MRP is commonly expressed in NSCLC, and typically in a large proportion of the tumor mass, 12% of cases had no detectable MRP expression. NSCLCs generally respond poorly to combination chemotherapy from the outset, although a minority demonstrate complete or partial responses (30, 31). Lung carcinoid tumors are also typically inherently multidrug resistant and generally expressed high, relatively uniform levels of the MRP throughout the tumor mass (32). Thus, the pattern of

Fig. 3 MRP expression in lung tumors other than NSCLC. Examples are shown of formalin-fixed, paraffin-embedded sections of SCLC (A and B), a carcinoid tumor (C and D), carcinoma in situ (E and F), and a mesothelioma (G and H). The sections shown (A, C, E, and F) were stained with MAb QCRL-l as in Fig. 1. Corresponding adjacent sections stained with an isotype control are shown (B, D, F, and H).
MRP expression is consistent with the possibility that the protein may contribute to the inherent MDR of both NSCLC and carcinoid tumors.

In contrast to NSCLC and carcinoid tumors, most SCLC cases respond initially to chemotherapy, although the majority subsequently relapse (33). A statistically significant difference was observed between NSCLC and SCLC with respect to MRP expression ($P < 0.00058$). In 15 of the 18 cases of SCLC examined, MRP levels were either low or undetectable with infrequent expression of high levels in small focal clusters of

**Table 3** Comparison of MRP expression in matched primary tumors and metastases

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>n</th>
<th>Both primary and metastases positive* or negative, n (%)</th>
<th>Positive* primary and negative metastases, n (%)</th>
<th>Positive* metastases and negative primary, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>9</td>
<td>8 (89)</td>
<td>1 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>19</td>
<td>16 (84)</td>
<td>1 (5)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SCLC</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>27 (87)</td>
<td>2 (6)</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>

* Positive means +, ++, or +++.
tumor cells. This profile of MRP expression is consistent with the typical initial response of SCLC to chemotherapy and its subsequent recurrence as a multidrug resistant disease. However, in the present study, serial tumor samples from SCLC patients before and after relapse were not available and, consequently, levels of the MRP in drug-resistant SCLC could not be determined.

Statistically significant differences in the levels and pattern of MRP expression were found between the major histological subtypes of NSCLC \( (P = 0.037) \). Marked heterogeneity of MRP expression was found in all subtypes of NSCLC, and a majority of each subtype had substantial levels of MRP expression. However, we found that squamous cell carcinomas and large cell carcinomas were more likely to be MRP negative than adenocarcinomas, and a greater proportion of adenocarcinomas had intermediate or high levels of the MRP than did squamous cell carcinomas. These observations are in general agreement with those of Sugawara et al. (22) but contrast with those of Ota et al. (18), who observed a higher frequency of MRP expression in squamous cell carcinoma.

The possibility that the differences in MRP expression observed in various NSCLC subtypes could correlate with tumor behavior, independent of their MDR characteristics, was also examined. However, no dependence of MRP expression on patient survival independent of treatment was observed in this study (Fig. 5). MRP levels in untreated NSCLCs were also independent of all other clinicopathological factors examined, including patient age, gender, presence of metastases, stage of disease, tumor grade, and size (Table 5). Furthermore, the presence of invasive MRP-positive cells at the leading tumor margin of some NSCLCs (18%) was not associated with the presence of metastases (data not shown). Thus, in untreated NSCLC, the MRP does not seem to be a significant marker of tumor aggressiveness or to affect patient survival. Consequently, studies of the correlation between the MRP status and patient response to chemotherapy in NSCLC should not be confounded by the association between MRP expression and clinical parameters other than drug resistance. No association was found between tumor grade and levels of the MRP when all forms of NSCLC were grouped. However, there was a significant correlation between grade and MRP positivity in adenocarcinoma, with well-differentiated tumors having a majority of the tumor mass strongly expressing MRP \( (P = 0.028) \) (Table 5). This observation combined with the detection of high levels of the MRP in carcinoma \( \text{in situ} \) and hyperplastic alveolae, suggests that the MRP could be an early

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### Table 4 Patient clinicopathological parameters and MRP expression in NSCLC

| Variable                  | MRP positivity, \( n (%)) \n|---------------------------|-------------------------------|
|---------------------------|-------------------------------|
| Age                       | \( n \) |  | + | ++ | +++ | ++++ |
| \( \leq 65 \) yrs          | 50 | 6 | 8 | 17 | 19 |
| >65 yrs                   | 52 | 6 | 7 | 15 | 24 |
| Gender                    |     |     |     |     |     |
| Male                      | 60 | 8 | 9 | 16 | 27 |
| Female                    | 42 | 4 | 6 | 16 | 16 |
| Histological subtype      |     |     |     |     |     |
| Squamous cell             | 45 | 7 | 10 | 16 | 12 |
| Nonsquamous cell          | 55 | 5 | 5 | 15 | 30 |
| Tumor                     |     |     |     |     |     |
| \( T_1 \)                  | 56 | 5 | 8 | 18 | 25 |
| \( T_2 \)                  | 36 | 7 | 4 | 11 | 14 |
| \( T_3 \)                  | 4  | 0 | 3 | 1  | 0  |
| \( T_4 \)                  | 6  | 0 | 0 | 2  | 4  |
| Nodes and distant sites\( a \)|     |     |     |     |     |
| Negative                  | 63 | 8 | 9 | 21 | 25 |
| Positive                  | 39 | 4 | 6 | 11 | 18 |
| Stage\( b \)              |     |     |     |     |     |
| I                         | 59 | 8 | 6 | 20 | 25 |
| II                        | 20 | 2 | 4 | 10 | 10 |
| IIIa                      | 11 | 2 | 3 | 5  | 1  |
| IIIb                      | 5  | 0 | 2 | 3  | 3  |
| IV                        | 7  | 0 | 2 | 1  | 4  |
| Grade\( b \)              |     |     |     |     |     |
| Well                      | 12 | 1 | 1 | 2  | 8  |
| Moderate                  | 55 | 6 | 7 | 18 | 24 |
| Poor                      | 30 | 3 | 7 | 10 | 10 |

\( a \) At time of surgery with pathological confirmation.

\( b \) Not including large cell carcinomas.

### Table 5 Univariate comparisons of MRP protein expression with clinicopathological parameters in NSCLC

<table>
<thead>
<tr>
<th>Variable</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ( \leq 65 ) vs. ( &gt;65 )^( b )</td>
<td>0.86</td>
</tr>
<tr>
<td>Gender (male vs. female)^( b )</td>
<td>0.65</td>
</tr>
<tr>
<td>Histological subtype (squamous vs. nonsquamous)</td>
<td>0.032</td>
</tr>
<tr>
<td>Grade (well vs. moderate vs. poor)^( c )</td>
<td>0.014</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0.028</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0.36</td>
</tr>
<tr>
<td>Tumor ( T_1 ), ( T_2 ), and ( T_4 )^( b )</td>
<td>0.50</td>
</tr>
<tr>
<td>Metastases (present vs. absent)^( b )</td>
<td>0.90</td>
</tr>
<tr>
<td>Stage (I and II vs. IIIa, IIIb, and IV)^( b )</td>
<td>0.63</td>
</tr>
</tbody>
</table>

\( * \) Pearson \( \chi^2 \) test.

\( ^ a \) Histological subgroup analysis not significant.

\( ^ c \) +++ versus ++, +, and −; not including undifferentiated large cell.

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**Fig. 5** Overall survival and MRP status. Overall survival from time of surgery is shown for 93 patients with stage I-III NSCLC (49 adenocarcinomas, 39 squamous cell carcinomas, and 5 large cell carcinomas) who underwent surgical resection with curative intent. Patients were grouped according to MRP status (+++ versus ++, +, or +). There was no significant difference in survival between the two groups \( (P = 0.46 \) by log rank test). \( \text{a) , ++} +; \text{b) ,} + , \text{or +} + \).
mechanism of resistance that may be augmented, or replaced by other mechanisms during tumor progression. As observed in primary NSCLCs and SCLCs, the pattern and level of the MRP in local or distant metastases was heterogeneous. However, MRP positivity was concordant in the majority (87%) of matching primary and metastatic samples. Although relatively infrequent, the discordance of MRP expression in the remaining cases could be important if MRP status of the primary tumor was used to predict the response of metastatic lesions to chemotherapy.

The subcellular localization of the MRP in neoplastic and normal lung structures was also investigated using fresh snap-frozen tissue samples. Unlike formalin-fixed samples in fresh snap-frozen samples of NSCLC, the MRP was detected predominantly on the plasma membrane with little cytoplasmic staining. In normal bronchial and bronchiolar epithelium, prominent staining of the plasma membrane was also observed that was restricted to basolateral membranes with no evidence of cytoplasmic staining. A similar basolateral membrane localization of the MRP was observed in mixed seromucous glands. This is consistent with in vitro studies using a polarized, MRP-transfected kidney cell line in which the MRP was detected exclusively on basolateral plasma membranes (34). However, our results differ from those obtained previously using different MABs to examine MRP expression in normal human tissues. In the previous study, no membrane staining was observed in normal tissues and immunoreactivity was detected only in the cytoplasm (35). This observation is difficult to reconcile with what is known from in vitro studies of the structure and function of the MRP. On the basis of the differences in subcellular distribution of MRP immunoreactivity that we have observed when comparing formalin-fixed and snap-frozen tissues, it is possible that redistribution of the MRP may have occurred during sample processing, although differences in the specificities of the MABs used cannot be excluded.

The physiological function of the MRP in the lung is presently unknown. In vitro studies have firmly established that the potent bronchoconstrictor, leukotriene C4 is a high-affinity substrate for the MRP (36–38). Gene knock-out mice that are nullizygous for the mdr allele also have a compromised response to some leukotriene C4-mediated inflammatory stimuli (39). Whether the expression of the MRP in bronchial and bronchiolar epithelium indicates an involvement in the regulation of airway muscle tone remains to be established. Given the range of anionic conjugates that can act as MRP substrates in vitro, it is also possible that the protein is involved in antioxidant defense mechanisms in the lung and/or defense against airborne exposure to xenobiotics. For example, glutathione disulfide has been shown to be transported by the MRP, as well as aflatoxin B1, and its glutathione conjugate (40, 41). The observation that hyperplastic reactive type II pneumocytes express relatively high levels of the MRP whereas normal type I and type II alveolar pneumocytes do not, may be related to such functions. These cells are the reparative precursors to type I pneumocytes and their proliferation is triggered by exposure to a wide variety of insults to the lung including chemicals and particulates, as well as hyperoxia (42). In addition, the persistence of type II pneumocyte-specific markers such as surfactant proteins in some forms of NSCLC, notably adenocarcinomas, has prompted the suggestion that the alveolar type II cell could be the progenitor cell in this type of tumor (43, 44). If correct, this possibility is also consistent with the observed high frequency and levels of the MRP we and others have detected, particularly in well-differentiated adenocarcinomas (22).

Overall, these data support a possible role for the MRP in the inherent drug resistance of NSCLC and carcinoid tumors. Immunohistochemistry with MRP-specific MABs such as QCRL-1 may be useful as a means of identifying the minority of NSCLC patients whose tumors do not express the protein. Given the frequent lack of initial response to chemotherapy and the high morbidity associated with NSCLC, it seems likely that definitive evidence implicating the MRP in drug resistance in this disease will require clinical intervention with MRP inhibitors when effective compounds become available. To further assess the role of the MRP in acquired drug resistance in SCLC, longitudinal studies that compare tumor MRP status before chemotherapy and at relapse are needed.

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Immunohistochemical detection of multidrug resistance protein in human lung cancer and normal lung.

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