Expression of the human major vault protein LRP in human lung cancer samples and normal lung tissues

A.-M. C. Dingemans,1,2 J. van Ark-Otte,1 P. van der Valk,3 R. M. Apolinario,1 R. J. Scheper,3 P. E. Postmus2 & G. Giaccone1
Departments of 1Medical Oncology, 2Pulmonary Medicine and 3Pathology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands

Summary

Background: The recently discovered LRP protein has been shown to be involved in drug resistance and possibly in detoxification processes.

Materials and methods: To study the relation between LRP expression and exposure to cigarette smoke, LRP immunoreactivity was evaluated in 39 paraffin embedded normal lung tissues derived from patients operated on for pneumothorax, and related to amount of pack years smoked. We also studied the LRP protein expression in 36 non-small-cell lung cancer (NSCLC) samples and related the expression to patient characteristics and survival. Furthermore 17 lung tumor samples (10 NSCLC and 7 SCLC) derived from patients treated with chemotherapy were analysed in order to investigate the relation between LRP or MRP expression and the patient's response to chemotherapy.

Results: In the normal lung tissues, LRP intensity levels were not correlated to the amount of pack years smoked, although a trend was seen for higher LRP intensity levels in patients who smoked more than 10 pack years. LRP expression was significantly higher in NSCLC samples than in SCLC samples, and all SCLC samples displayed very low LRP expression. Within NSCLC, squamous cell and adenocarcinomas had higher LRP expression than large cell undifferentiated and mixed tumors. In NSCLC patients LRP expression was not a prognostic factor for survival. At initial analysis LRP expression levels did not predict for the response to chemotherapy. Only 3 out of 17 patients expressed MRP, and all SCLC samples were MRP negative.

Conclusions: Striking different expression levels were seen between NSCLC and SCLC for both LRP and MRP. In a preliminary analysis LRP expression was not predictive for response to chemotherapy in lung cancer patients. In pneumothorax patients LRP levels were not correlated with the amount of pack years smoked.

Key words: detoxification, immunohistochemistry, LRP, lung cancer, MRP, multidrug resistance

Abbreviations: SCLC - small-cell lung cancer; NSCLC - non-small-cell lung cancer; LRP - lung resistance-related protein; MRP - multidrug resistance-associated protein; MDR - multidrug resistance; MAb - monoclonal antibody; IHC - immunohistochemistry

Introduction

Drug resistance is common in lung cancer. Although small-cell lung cancer (SCLC) is initially very sensitive to chemotherapy, it recurs in most cases, which are then resistant to multiple drugs. Non-SCLC (NSCLC) is relatively resistant to chemotherapy from diagnosis. Among known mechanisms of drug resistance, overexpression of the MDR1 gene, which encodes for P-glycoprotein, does not seem to play an important role in lung cancer [1, 2].

Recently, a new resistance protein, called Lung Resistance-related Protein (LRP), has been identified in a lung cancer cell line selected for resistance to doxorubicin [3]. LRP is the major component of the human vaults [4, 5] and may be involved in transport mechanisms within the cell [6]. Overexpression of LRP was found in multi-drug resistant (MDR) cell lines not overexpressing the MDR1 gene [7]. LRP overexpression may involve cross-resistance to classical MDR drugs but also platinum agents [8]. LRP expression was found to have a predictive value for poor response to chemotherapy and adverse clinical outcome in advanced ovarian cancer and acute myeloid leukemia (AML) [8, 9]. The pattern of LRP expression in various normal tissues, similar to that of P-glycoprotein, suggests a role in detoxification processes [10].

The LRP gene maps to the short arm of chromosome 16 proximal to the MDR-associated Protein MRP [11]. Although LRP and MRP may be co-upregulated, they can also be switched on independently [7, 11]. Evidence exists that chromosome breakage plays a role in LRP or MRP amplification [11].

To elucidate the role of LRP in the detoxification process we estimated the LRP expression in lung tissues derived from non-cancer patients and related the expression to smoking of cigarettes. Furthermore the expression of LRP in lung tumor samples was evaluat-
ed and compared with patients' survival, tumor differentiation, TNM classification and histology. Finally, the LRP and MRP protein expression was preliminary assessed in tumor samples derived from lung cancer patients who underwent chemotherapy and related to the response to chemotherapy.

Materials and methods

Patients' material

Fifty peripheral lung tissue samples were analysed, derived from patients who underwent a bullectomy as treatment for pneumothorax. None of these patients had a history of cancer, and smoking habits of these patients were recorded. Pack years were calculated as the amount of cigarette packs smoked per day times the number of years smoked.

Tumor specimens of 36 patients with NSCLC were investigated; these patients were not treated with chemotherapy at any time and were operated on between 1987-1990. The median follow-up was 78 months (range 1–94 months).

Tumor samples of 17 patients (7 SCLC and 10 NSCLC) treated with chemotherapy were analysed for both LRP and MRP expression. These samples were derived from different tumor sites (primary tumor, mediastinal lymph nodes or supravacular lymph nodes). All these patients received chemotherapy before or after sampling the tumor tissues. Responses were evaluated by chest X-ray and/or CT-scan. Response was classified according to WHO criteria [12]. Expression of tumors of patients with a tumor reduction (i.e., minor, partial or complete response) after chemotherapy were compared to those that progressed.

All lung cancer samples were snap frozen in liquid nitrogen and stored at −80°C until use. The bullectomy samples were embedded in paraffin and stored at room temperature.

LRP immunostaining

Formalin-fixed paraffin embedded normal lung tissues were cut into sections of 4 μ thickness. Sections were deparaffinized and rehydrated and slides were microwaved in a citrate buffer at pH 6.0 for 15 minutes, in order to avoid background staining. Endogenous peroxidase activity was blocked with H2O2 in methanol, and all slides were incubated with normal rabbit serum (1:50). Controls were placed in phosphate buffered saline (PBS) or incubated with an irrelevant mouse myeloma IgG1 antibody. The remaining slides of normal lung tissue were incubated overnight with the mouse monoclonal anti-trypsin antibody (MAb) LRP-56 (IgG2b) (1:100) at 4°C. The avidin-biotin complex procedure was used with rabbit anti-mouse (1:500) and antirat immunoglobuline (1:25) + 2% human pool serum was added as described in the LRP section. Peroxidase conjugated rabbit-anti-rat immunoglobuline (1:25) + 2% human pool serum was added as described in the LRP section. Peroxidase conjugated rabbit-anti-rat immunoglobuline (1:25) + 2% human pool serum was added for 1 hour incubation with the rat MAb MRPrI [13]. Controls were treated as described in the LRP section. Peroxidase conjugated rabbit-anti-rat immunoglobuline (1:25) + 2% human pool serum was applied for 1 hour. The bound peroxidase was developed by DAB incubations, slides were counterstained with hematoxilin, dehydrated and mounted. As positive control the human SCLC cell line GLC4/ADR, known to overexpress MRP [14], was used. Tumor cells staining with the MRP MAb was recorded as the percentage of positive tumor cells, as described for the LRP staining of frozen sections.

Statistics

Overall survival and disease-free survival were calculated in months from surgery until relapse or death. As cut-off point for Kaplan-Meier curves [15], 10% LRP positive tumor cells was used. This cut-off point has been shown to efficiently distinguish between groups bearing different drug resistance profiles [10]. Comparisons of survival curves were performed by Mantel-Cox method [16]. Kruskal-Wallis one-way nonparametric analysis of variance and Mann-Whitney rank sum test were used to compare protein expression levels in various groups.

Results

LRP in normal lung tissue

Of the 50 bullectomy sections only 39 contained one or more bronchioli which were further analysed. Mean age of these 39 patients was 33 years (range 18–72), and most patients were male (30/39). Twenty-five patients were current smokers and 5 gave up smoking. Mean number of pack years was 9.9 (range 0–50) in the 29 cases in which information was available. None of these patients had an α1-anti-trypsinine deficiency, and 2 patients suffered from asthma.

The lung tissue in general showed strong emphysematic changes and not much of the normal lung structure was left for histochemical assessment. The LRP intensity in the epithelial cells was low to moderate in most samples when compared to the intensity of adjacent alveolar macrophages: median LRP intensity was 1 (Figure 1). Number of pack years was not significantly different when 2 groups of patients were distinguished based on the median level of LRP intensity. Patients were further divided into three groups according to smoking history: non-smokers, 1–10 pack years, and more than 10 pack years. No difference in LRP intensity was found between these groups (Figure 2).

MRP immunostaining

Cryostat sections of tumor samples were acetone fixed, preincubated with normal rabbit serum (1:50) for 15 minutes, followed by a 1 hour incubation with the rat MAb MRPrI [13]. Controls were treated as described in the LRP section. Peroxidase conjugated rabbit-anti-rat immunoglobuline (1:25) + 2% human pool serum was applied for 1 hour. The bound peroxidase was developed by DAB incubations, slides were counterstained with hematoxilin, dehydrated and mounted. As positive control the human SCLC cell line GLC4/ADR, known to overexpress MRP [14], was used. Tumor cells staining with the MRP MAb was recorded as the percentage of positive tumor cells, as described for the LRP staining of frozen sections.

Formalin-fixed paraffin embedded normal lung tissues were cut into sections of 4 μ thickness. Sections were deparaffinized and rehydrated and slides were microwaved in a citrate buffer at pH 6.0 for 15 minutes, in order to avoid background staining. Endogenous peroxidase activity was blocked with H2O2 in methanol, and all slides were incubated with normal rabbit serum (1:50). Controls were placed in phosphate buffered saline (PBS) or incubated with an irrelevant mouse myeloma IgG1 antibody. The remaining slides of normal lung tissue were incubated overnight with the mouse monoclonal antibody (MAb) LRP-56 (IgG2b) (1:100) at 4°C. The avidin-biotin complex procedure was used with rabbit anti-mouse (1:500) and streptavidin-biotin horse radish peroxidase incubations. The slides were developed in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO), counterstained with hematoxilin, dehydrated and mounted with depex. As LRP is expressed in most samples when compared to the intensity of adjacent alveolar macrophages: median LRP intensity was 1 (Figure 1). Number of pack years was not significantly different when 2 groups of patients were distinguished based on the median level of LRP intensity. Patients were further divided into three groups according to smoking history: non-smokers, 1–10 pack years, and more than 10 pack years. No difference in LRP intensity was found between these groups (Figure 2).
Figure 1. Immunohistochemical staining of LRP and MRP proteins. A) LRP-56 immunoreactivity in a paraffin embedded normal lung tissue sample. Intense staining of the epithelial cells lining the bronchioli is visible. B) LRP positive tumor stained with the LRP-56 MAAb. Note the granular cytoplasmatic staining. C) MRP positive tumor displaying intense membranous and cytoplasmatic staining.

Table 1. Characteristics of 36 resected NSCLC patients.

<table>
<thead>
<tr>
<th>Sex (male/female)</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28/8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>44-77</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous</td>
<td>19</td>
<td>78 ± 20</td>
</tr>
<tr>
<td>Adeno</td>
<td>8</td>
<td>78 ± 17</td>
</tr>
<tr>
<td>Mixed adeno-squamous</td>
<td>2</td>
<td>15 ± 8</td>
</tr>
<tr>
<td>Large cell undifferentiated</td>
<td>7</td>
<td>31 ± 36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grading</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>2</td>
<td>35 ± 21</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>10</td>
<td>81 ± 27</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>17</td>
<td>74 ± 20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TNM staging</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>5</td>
<td>86 ± 15</td>
</tr>
<tr>
<td>T2</td>
<td>25</td>
<td>62 ± 34</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
<td>59 ± 29</td>
</tr>
<tr>
<td>T4</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>N0</td>
<td>21</td>
<td>66 ± 31</td>
</tr>
<tr>
<td>N1</td>
<td>14</td>
<td>62 ± 35</td>
</tr>
<tr>
<td>N2</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Stage I</td>
<td>17</td>
<td>69 ± 31</td>
</tr>
<tr>
<td>Stage II</td>
<td>12</td>
<td>59 ± 37</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>6</td>
<td>64 ± 29</td>
</tr>
<tr>
<td>Stage IIIb</td>
<td>1</td>
<td>90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of operation</th>
<th>Number of patients</th>
<th>LRP % pos. tumor cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobectomy</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Pneumonectomy</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Status (dead/alive)</td>
<td>24/12</td>
<td></td>
</tr>
<tr>
<td>Median survival (months)</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

LRP in tumor samples

Characteristics of the 36 resected NSCLC patients are described in Table 1. In normal tissue, adjacent to the tumor, alveolar macrophages, epithelial cells lining the bronchus, and connective tissue also stained with the LRP antibody. LRP staining gave raise to a coarsely granular cytoplasmatic staining, as previously described (Figure 1) [3]. IHC of the tumor sections showed different expression of LRP in the different histological subtypes of lung cancer (Figure 3): NSCLC samples had a significant ($P < 0.0001$) higher LRP expression than 7 SCLC samples. Within the NSCLC group squamous cell carcinomas and adenocarcinomas showed a significantly ($P < 0.05$) higher expression than the large cell undifferentiated carcinomas, suggesting a relation between LRP and tumor differentiation. Two patients had a mixed adeno-squamous tumor, large parts of these tumors being undifferentiated; with regard to LRP expression these tumors were comparable to the large cell undifferentiated tumors and, no difference was seen in these tumors between LRP expression in the differentiated and the undifferentiated areas. Expression levels were not significantly different in different
stages or in different tumor differentiation grades. No relation was seen between LRP expression and the survival of the patients (Figure 4); however, patients with squamous cell carcinoma had a significant better survival than patients with adeno or mixed carcinomas ($P < 0.05$).

**Expression of LRP and MRP and response to chemotherapy**

Seventeen samples derived from patients treated with chemotherapy were analysed. All 7 SCLC patients were treated with combination chemotherapy including cyclophosphamide, doxorubicin and etoposide. The 10 NSCLC patients all received cisplatin-containing chemotherapy combined with either paclitaxel, etoposide or teniposide. Two patients who had a stage I SCLC were operated on and received adjuvant chemotherapy; these patients were in remission at last follow-up and were excluded from response analysis. In addition one NSCLC patient who died after receiving 1 cycle of chemotherapy due to toxicity was also excluded from response analysis. Four out of 14 patients included in the response analysis had a progressive disease, and the other 10 patients had a response to chemotherapy; no patient had stable disease.

LRP levels in the 7 SCLC samples were low ($\leq 10\%$ positive cells). None of the SCLC samples showed immunoreactivity with the MRP MAb. Although all NSCLC samples showed a high percentage of tumor cells staining with the LRP MAb (mean 75%, range 30%--100%) only 3 NSCLC samples expressed MRP. Out of these 3 samples only one tumor sample displayed the typical intense membranous MRP staining (Figure 1), whereas the other 2 had a less intense staining and more diffuse to the cytoplasm. No relation was seen between MRP and LRP protein levels. In this small and heterogeneous group of patients LRP and MRP levels in patients with a response to chemotherapy were not different from protein levels in patients with progressive disease.

**Discussion**

The recently discovered vaults are highly conserved organelles among different species, suggesting an essential role in the cell physiology [17]. The finding that part of the vault ribonucleoprotein particles specifically localise to the nuclear pore complex, suggests that LRP may be responsible for bidirectional nucleocytoplasmic transport [18]. Vaults were first discovered in cytoplasmic vesicles fractions, which is in agreement with the punctuate cytoplasmic staining pattern of LRP-56, and there is evidence that cytostatic drugs can be trapped into vesicles and transported through the cytoplasm, resulting in a reduced nuclear accumulation of the drug [19]. In human normal lung tissue high LRP expression is present in bronchial epithelial cells and in alveolar macrophages [10].

Because the LRP distribution among normal tissues suggests a role in the detoxification process [10, 17] we investigated its expression in normal lung tissues. In this study we showed immunoreactivity with the LRP-56 MAb in the vast majority of normal lung tissues tested (34/39). The intensity of the LRP immunoreactivity varied among these samples but most samples expressed a lower amount of LRP in the epithelial cells lining the bronchioli than in the alveolar macrophages. No relation was seen between cigarette smoke
and LRP expression, but there was a trend for higher expression levels in patients who had smoked more than 10 pack years compared with the patients who never smoked. However, the pneumothorax patients are not age-matched with the lung cancer patients, who are older, have a higher frequency of smoking history, and have a longer exposure to smoke. In our series, from 19 of 36 NSCLC patients from whom data were available on smoking history, the mean number of pack years smoked was 38 (range 0–100), and only one patient had never smoked cigarettes.

Experiments in cell lines selected for resistance to several drugs have shown LRP expression in low level of drug resistance [3], as an early event in the selection for resistance to several MDR related drugs. LRP expression level drops in cells with a higher resistance level where Pgp expression emerges. High expression levels of LRP were seen in our study in NSCLC, as only 5 out of 36 NSCLC samples were negative, independent of TNM-classification or tumor differentiation. This was in contrast to 100% negativity in 7 SCLC samples, independent of pretreatment or response to chemotherapy. It is known that highly chemosensitive tumors (i.e., testicular tumor, Wilm’s tumor, leukemias and SCLC) show low LRP expression [10].

LRP and MRP expression was found in 87% and 78% of unselected cell lines respectively [7], suggesting that both proteins can be involved in the intrinsic drug resistance and not only in the acquired drug resistance as a response to previous chemotherapy. In contrast to studies in advanced ovarian cancer and AML [8, 9], in 36 NSCLC with long term follow-up the level of LRP expression was not a prognostic factor for survival. As the prognostic of AML and advanced ovarian cancer is tightly linked to the response to chemotherapy, it is likely that LRP influences response to chemotherapy in AML and ovarian cancer, and secondarily survival. Whether LRP as P-glycoprotein could also be a sign of tumor aggressiveness is still unclear [20], but it is not likely to be the case in our NSCLC series.

In our study no relation was seen between LRP expression and response to chemotherapy. Expression levels in all SCLC samples were very low, independent of response and previous exposure to chemotherapy. Although all 3 NSCLC with progressive disease displayed more than 85% LRP expression, expression levels were not significantly different from the 6 NSCLC patients who responded to chemotherapy. A study in melanoma showed a significant correlation between increase in LRP expression levels and pretreatment with chemotherapy [21]. Our results, however, should still be considered preliminary, because of the relatively small number of patients.

In a recent study MRP mRNA expression assessed by RNase protection assay was detectable in all NSCLC tumor samples and adjacent normal lung tissues tested [22]. Moreover, Ota et al. evaluated the MRP and MDR1 transcript in 104 NSCLC samples by Northern blot analysis and semi-quantitative reverse-transcriptase polymerase chain reaction [23]. Patients with a high MRP expressing tumor showed a significantly worse prognosis than patients with a no or low MRP expressing tumor. However, MRP protein expression is present in non-neoplastic cells infiltrating the tumor [22], making unreliable the results obtained by bulk techniques. In our series only 3 samples out of 17 samples tested were positive for MRP by IHC, 2 of which were derived from pretreated patients. Nevertheless, all three patients with MRP-overexpressing tumors achieved a response to cisplatin-etoposide-containing chemotherapy. In unselected human lung cancer cell lines only a modest association was observed between MRP mRNA levels and chemosensitivity to doxorubicin and etoposide, but not to cisplatin [22].

Clinical drug resistance is likely to be multifactorial: in addition to drug transport mechanisms DNA topoisomerases and the glutathione system may be involved in drug resistance [24–26].

In conclusion, although we did not find a relation between LRP intensity and the level of smoke exposure, higher LRP intensity levels tended to be related with an increased load of cigarette smoke as was observed in lung cancer patients. LRP expression in SCLC was significantly lower than in NSCLC samples, and did not seem to play a role in drug resistance in a relatively small group of lung cancer patients treated with chemotherapy.

Acknowledgements

This work was supported by grant VU94-776 from the Dutch Cancer Society (A.-M.C. Dingemans). We thank R. Stoffelsens and F. Schramel for participation in the normal lung tissue study.

References


Received 6 April 1996; accepted 12 June 1996.

Correspondence to:
Giuseppe Giaccone, MD
Department of Oncology
University Hospital Vrije Universiteit
P.O. Box 7057, 1007 MB Amsterdam
The Netherlands