Expression of the multidrug resistance-associated protein 1 (MRP1) and the lung resistance-related protein (LRP) in human lung cancer*

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Fifty lung cancer samples (41 non-small cell lung cancer-NSCLC and 9 small cell lung cancer-SCLC) were immunohistochemically analyzed for lung resistance-related protein (LRP) and multidrug resistance-associated protein 1 (MRP1) expressions which were then correlated with histopathological subtype of the tumor. To detect these proteins, monoclonal antibodies LRP-56 and MRPm6 were used. NSCLC samples were divided into two groups, adenocarcinomas (17 samples) and squamous cell carcinomas (24 samples). Four categories of LRP and MRP1 quantity were distinguished: +++ = high level – 90–100% of positive cells, ++ = lower level – 10–90% of positive cells, + = low level – up to 10% of positive cells, – = negative cells – 0% of positive cells. Within the NSCLC group the most samples (36/41) had the similar level of LRP and MRP1. Significantly higher expression of both proteins was observed in the adenocarcinomas in comparison with squamous cell carcinomas. The lowest positive staining for LRP and MRP1 proteins has been found in SCLC. It is suggested that our finding can confirm the overall empirical clinical knowledge about much higher chemosensitivity of untreated SCLC comparing to NSCLC.

Key words: LRP, MRP1, immunohistochemistry, NSCLC, SCLC

Solid tumors usually consist of mixed populations of malignant cells, some of which are drug-sensitive, while the others are drug resistant. However, drug resistance can occur at multiple points between the administration of the drug to the patient and the definite site of molecular interaction with subsequent therapeutic effect. There exists a wide variety of inherent or acquired mechanisms of anticancer drug resistance on pharmacokinetic as well as pharmacodynamic levels. The ability of malignant tumor cells to exhibit simultaneous resistance conferred by different mechanisms to a number of structurally and functionally unrelated anticancer agents is indicated as multidrug resistance (MDR). In this broad spectrum of resistance mechanisms, which can be divided in non-cellular and cellular mechanisms [16], important role is ascribed to a large variety of cell proteins playing often important role in physiological cellular functions. It is believed today that overexpression of some of these membrane or intracellular proteins confers MDR phenotype to cancer cells.

The first transport-based classical MDR mechanism has been described after discovery of 170 kD membrane protein, called P-glycoprotein (Pgp) in Chinese hamster ovary cell lines [13]. Characterization of a lung cancer cell line that was resistant to doxorubicin and some other anticancer drugs showed that this cell line did not overexpress Pgp, but did express another 190 kD protein, namely multidrug-resistance associated protein (MRP) [5]. Pgp and MRP1 were the first human ABC superfamily members shown to confer resistance to multiple chemotherapeutic drugs. Like Pgp, MRP was also found to be a pump and a member of the ATP-binding cassette (ABC) transmembrane transporter superfamily. In humans, eight homologues of MRP1 are known to be expressed (MRP1 to MRP9) [11, 27]. In contrast to Pgp, efflux of drugs by

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MRP1 appears to occur as glutathion conjugates, glucuronides or sulfates [18]. However, most of the anticancer drugs to which MRP1 confers resistance are not conjugated to a significant extent *in vivo*. It appears that at least some of them are effluxed from cells by MRP1 via a co-transport mechanism. This provides an explanation how MRP1 confers resistance to some anticancer drugs that do not form conjugates *in vivo*. Overexpression of MRP1 can confer resistance to a broad range of natural anticancer drugs, including anthracyclines, vinca alcaloids, and epipodophyllotoxines [6].

A newly described protein related to MDR is lung resistance protein (LRP). Similarly to MRP it was first detected in a non-Pgp multidrug resistant lung cancer cell line [26]. LRP is a human major vault protein (MVP) and it is not a member of ABC superfamily [25]. Vaults are complex of ribonucleoprotein particles, which, in addition to the major vault protein, also contain several minor vault proteins and a small RNA. They are located in the cytoplasm and in part associated with the nuclear membrane. It is believed they function in nucleocytoplasmic transport [14]. Overexpression of LRP in tumor cells is associated with resistance to doxorubicin, vincristine, carboplatin, cisplatin and melphalan.

Lung cancer is divided in two major subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The differences between SCLC and NSCLC result in different treatment modalities. With respect to the rate of growth, spread in the organism and responsiveness to non-surgical therapies, chemotherapy is the mainstay of treatment for SCLC and is always a part of any treatment plan. Although SCLC is initially sensitive to chemotherapy, it recurs very often in most cases, which are then resistant to chemotherapy to multiple anticancer drugs. In contrast, NSCLC is relatively resistant to anticancer chemotherapeutics from diagnosis. Chemotherapy is not standard treatment for stage I and II NSCLC and may be used as induction, combination or palliative therapy in stages II to IV NSCLC. Therefore effective chemotherapy in the mangement of NSCLC is the last modality to be introduced [9].

In the present study, the immunohistochemical expression of the drug resistance proteins MRP1 and LRP was determined in lung carcinomas and related to the histopathological type of malignant disease.

Material and methods

Tissues and sections. Formalin fixed, paraffin wax embedded tissue blocks of 50 cases of lung cancers (SCLC and NSCLC) were examined. We have obtained them from Department of Pathology of the National Institute of Tuberculosis and Respiratory Diseases, Bratislava-Podunajské Biskupice, and from the Department of Pathology, Faculty of Medicine, Šafarik University, Košice.

Immunohistochemistry. Indirect enzymatic immunohistochemical method was used to detect LRP and MRP1. Paraffin tissue sections (4–5 μm) were deparaffinized, rehydrated and washed with phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tw), pH 7.6. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 30 minutes at room temperature. Sections were pretreated in citrate buffer solution in the microwave oven differently. The slides staining for LRP were pretreated in the microwave 2x5 minutes, MRP1 slides for 20 minutes.

LRP staining procedure continued by blocking of nonspecific staining with blocking serum (prediluted normal horse serum – Vector Laboratories, USA) for 90 minutes in humidified chamber at room temperature. Blocking serum was removed from slides by flipping the slide without rinsing and the primary antibody LRP-56 (BD Transduction Laboratories, USA) was applied overnight in humidified chamber at 4 °C.

Sections for MRP1 staining were immediately exposed to the primary antibody MRPm6 (kind gift from Prof. Rik Scheper, Amsterdam), overnight in humidified chamber at 4 °C.

All sections were properly washed in PBS-Tw 3x5 minutes and subsequently incubated with the secondary antibody (prediluted biotinylated horse antibody – Vector Laboratories, USA) for 30 minutes. After rinsing the streptavidine-peroxidase (prediluted R.T.U. Vectastain – Vector Laboratories, USA) was applied for 30 minutes. The slides were then incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) at a concentration of 0.5 mg/ml in Tris buffer, pH 7.6 and 0.015% H_2O_2. Sections were streamrinsed with tap water, counterstained with hematoxylin for 2 minutes, washed in tap water, dried, mounted and coverslipped. Negative control included PBS-Tw alone in place of the primary antibody.

The evaluation of immunostaining results was scored independently by three investigators and agreement was reached in all cases, in difficult cases after discussion.

Four categories of LRP and MRP1 quantity were distinguished: +++ = high level – 90–100% of positive cells, ++ = lower level – 10–90% of positive cells, + = low level – up to 10% of positive cells, – = negative cells – 0% of positive cells. For statistical analysis, tumors scored as +++ or ++ were considered positive, and tumors scored as + or - were considered negative [12].

Statistical analysis. Statistical evaluation was performed using non-parametric two sided Mann-Whitney test analysis. $p<0.05$ was considered to be significant.

Results

Screening for LRP and MRP expression in clinical samples. Fifty lung cancer samples (41 NSCLC and 9 SCLC)

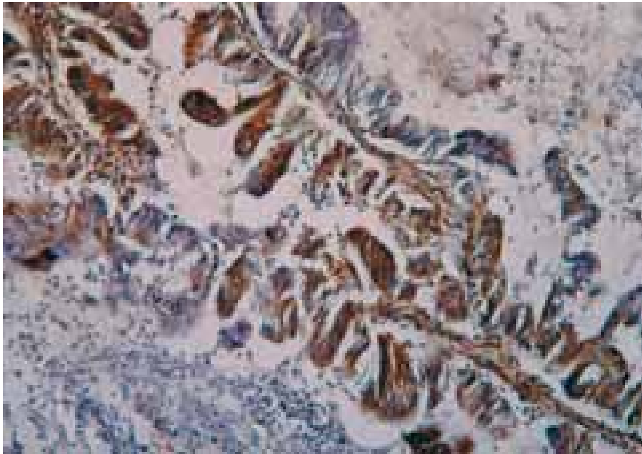

Figure 1. Expression of LRP in lung adenocarcinoma as immunohistochemically detected with the monoclonal antibody LRP-56. LRP positive cells show strong brown staining in contrast to adjacent cells (5x20).

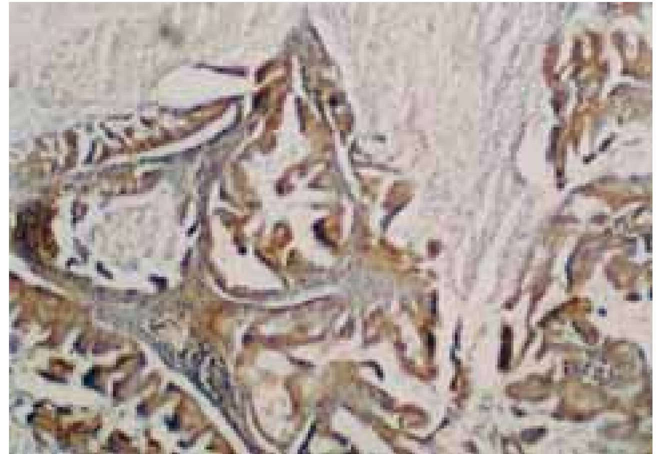

Figure 3. Lung adenocarcinoma-demonstration of MRP1 expression. Parrafin sections were immunohistochemically stained with monoclonal antibody MRPm6. Brown staining cells prove the presence of MRP1 (5x10).

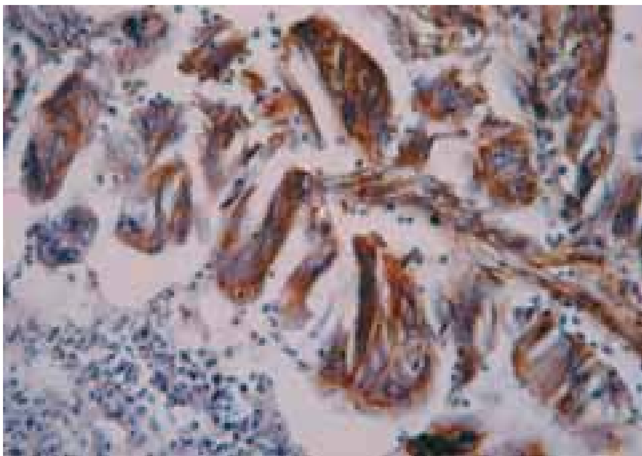

Figure 2. Expression of LRP in lung adenocarcinoma as immunohistochemically detected with the monoclonal antibody LRP-56. Large magnification (5x40) clearly indicates that LRP positive cells show strong brown cytoplasmic staining.

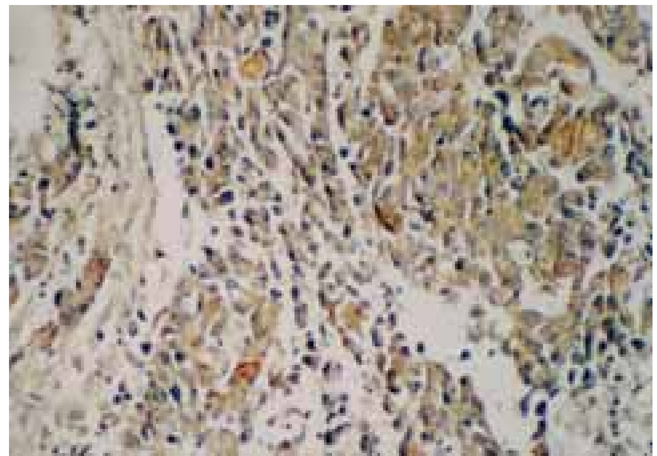

Figure 4. MRP1 staining of the tumorous cells of the lung squamous cell carcinoma. Notice the brown staining which indicates the positivity of cells for MRP1 (5x10).

were immunohistochemically analyzed for both LRP and MRP1 expression. NSCLC samples were divided into two groups, adenocarcinomas (17 samples) and squamous cell carcinomas (24 samples).

LRP positive NSCLC (Fig. 1, 2) as well as SCLC (not shown) tumor cells showed the granular cytoplasmic staining. MRP1 positive cells in both groups of lung carcinomas displayed intense membranous and cytoplasmic staining. The examples of MRP1 positive adenocarcinoma and squamous cell carcinoma cells are shown in Figures 3, 4.

NSCLC samples showed generally significantly higher LRP ($p<0.0858$) and MRP1 ($p<0.0029$) expression than SCLC samples. Within the NSCLC group we have seen close relation between LRP and MRP1 protein expression levels. The most of samples (36) showed the same levels, only 5 of them had different levels of examined proteins (Tab. 1).

Comparing adenocarcinomas with squamous cell carcinomas within NSCLC group, significantly higher levels of LRP ($p<0.0024$) and MRP1 ($p<0.0313$) proteins were expressed in adenocarcinomas (Tab. 1).

NSCLC

Adenocarcinomas – 17 samples: Twelve of the 17 samples expressed LRP and MRP1 at very high concentrations (+++), in 4/17 samples lower expression levels were detected (++), only 1/17 sample had low expression (+ – considered as negative), and no one sample was LRP and

Table 1. Expression of LRP and MRP1 according to tumor type

Tumor type	LRP				MRP				LRP+MRP			
	–	+	++	+++	–	+	++	+++	–	+	++	+++
NSCLC (n=41)	3	10	11	17	1	7	12	21	1	7	11	17
Adenocarcinoma (n=17)	0	1	4	12	0	1	4	12	0	1	4	12
Squamous cell carcinoma (n=24)	3	9	7	5	1	6	8	9	1	6	7	5
SCLC (n=9)	2	3	3	1	3	3	3	0	2	3	3	0

Majority of NSCLC samples expressed much higher levels of both LRP and MRP1 proteins as compared with SCLC. The difference can be seen also between adenocarcinomas and squamous cell carcinomas. NSCLC – non small-cell lung cancer, SCLC – small cell lung cancer, LRP – lung resistance-related protein, MRP1 – multidrug resistance-associated protein, LRP+MRP-the number of double positive samples for LRP and concomitant MRP1 immunoreactivity.

MRP1 negative (–). When comparing LRP and MRP1 expression, all samples had the same levels of both proteins examined.

Squamous cell carcinomas – 24 samples: Some differences were found in LRP and MRP1 expressions in squamous cell carcinomas. Nighteen/24 samples showed the same levels of proteins, different levels were obtain in 5/ 24 samples.

Five/24 samples were highly LRP and 9/24 samples highly MRP1 positive (+++), 7/24 samples had lower expression of LRP (++) and 8/24 samples had the same level of MRP1 (++). Low levels of LRP (+ – considered as negative) were detected in 7/24 samples and 6/24 samples of MRP1 (+). Two/24 samples were LRP negative (–) but MRP1 positive (++, +++), and one sample was both LRP and MRP1 negative (–).

SCLC

LRP and MRP1 expression was detected in 9 samples. Eight of them showed the same levels of both proteins. Three/9 samples expressed the lower levels of LRP and MRP1 (++), 3/9 samples had low expression (+ – considered as negative), whereas 2/9 samples were LRP and 3/9 MRP1 negative (–). Different levels were seen in 1/9 samples.

Expression levels of proteins in both NSCLC and SCLC tumor types were not different with regard to different tumor differentiation grades.

Discussion

MRP1 is overexpressed in many non-Pgp MDR cell lines. Similarly to this, it has been detected in many types of human malignancies, e. g. ovarian carcinomas [1] and acute myeloid leukemia [17]. In normal human lungs MRP1 is expressed in bronchial and bronchiolar epithelium, seromucinous glands and in alveolar macrophages [24]. The expression of MRP1 in human lung carcinomas has also been observed [22, 29]. In SCLC and NSCLC cell lines the correlation of MRP mRNA levels and resistance to doxorubicine was described [3, 30].

In the present study, immunostaining for MRP1 was observed in 83% of untreated NSCLC (in 33 of 41 samples). In patients with adenocarcinoma 94% of 17 NSCLC samples and in patients with squamous cell carcinoma 71% of 24 NSCLC samples were identified as positively staining for MRP1. Our results are in quite good agreement with the findings of WRIGHT et al [29] who found that the majority (87%) of different histological subtypes of NSCLC had detectable levels of MRP in most of the tumor mass.

In contrast to NSCLC, the expression of MRP1 in SCLC was found to be elevated only in 33% of 9 tumor samples. Similarly to this, significantly lower SCLC MRP expression in comparison to NSCLC has been detected by others. The immunoreactivity for MRP in different sets of SCLC samples started from totally negative samples [7] or approached 31% [15], 34% [12] and 56% [29].

LRP expression can be detected in several human malignancies. It is associated with resistance to various anticancer drugs including melphalan, which increases the interest for clinical outcome in patients with multiple myeloma [8]. Previously, the expression of LRP in normal as well as in human lung cancer samples has been studied [7]. The expression of LRP in normal human lungs is strong in the cytoplasm of the bronchial and bronchiolar epithelial layers and in a lesser extent in the alveolar macrophages [24]. It was found that in smoking people the expression of LRP in normal lung tissue was not correlated to the amount of pack years smoked. In lung cancer samples the expression of LRP was significantly higher in NSCLC samples than in SCLC samples [7]. The analysis of LRP expression in our set of human lung tumor samples revealed 70% positivity in unclassified NSCLC. When subdivided, 94% of adenocarcinomas and 50% of squamous cell carcinomas were positive for LRP. The present study also confirms significantly lower expression of LRP in SCLC, where only 44% of 9 SCLC were positively identified samples. It is currently believed that overexpression of MRP1 and/or LRP participates in multidrug resistance of different malignant diseases in humans. However, there exist highly variable findings, when MRP1 and/or LRP expression and drug response in cancer cells is correlated. For example, MRP expression and its correlation with

chemoresistance to daunomycin, doxorubicin, etoposide and vinblastin was shown in 15 unselected NSCLC cell lines [2]. When NSCLC were subdivided in adenocarcinomas and squamous cell carcinomas, the expression of MRP was significantly higher in adenocarcinomas and negatively correlated with 5 years survival of patients [23]. Negative correlation has also been found in NSCLC patients with coexpression of MRP and mutant p53 and response to postoperative chemotherapy [22].

From 23 SCLC cell lines 19 (83%) and from 10 SCLC clinical samples 7 (70%) expressed MRP with a significant correlation between doxorubicin resistance and MRP expresssion levels [3]. In 19 from 23 poor response patients with SCLC the effect was associated with the expression of MRP and/or Pgp. Only 4 patients from this group and all 27 SCLC patients with good response had negative MRP and Pgp expressions [12]. On the other hand, in the panel of 17 SCLC xenografted into nude mice from treated and untreated patients 71% were found to be positive for MRP with no relationship between the response to the treatment and MRP expression [4]. Similarly, no potentially confounding correlation was observed between MRP expression in untreated NSCLC and any clinicopathological parameter [29], as well as between the expression of MRP and the pathology and the stage of human lung cancer [10]. Additionally, in two human cell types derived from untreated tumors, MCF-7 breast cancer and A549 NSCLC, the cytotoxic assays showed that A549 cells were less sensitive to doxorubicin treatment than MCF-7 cells. The analysis of Pgp and MRP expression did not show significant differences between the two cell lines, while a high expression of LRP was detected in A549 cells, indicating the importance of only the last protein in lower drug sensitivity [20]. Supporting the function of LRP in anticancer drug resistance, significant correlation between LRP expression and tumor resistance to doxorubicin or the efficacy and prognosis of chemotherapy has been observed in human NSCLC samples [28, 19]. To study and to confirm a direct relation between LRP and MDR a LRP knockout mouse model has been generated. However, contrary to all previously mentioned studies LRP wild-type and deficient mice treated with doxorubicin responded similarly to this treatment. It was suggested that LRP is not directly involved in the resistance to cytostatic agents [21].

We had a relatively small number of samples to extract a valid information from the correlation of the expression of the MDR proteins and the stage of the disease. However, the preliminary checking of the results did not show such a correlation between MRP1 and LRP expression and the stage of disease in NSCLC patients. Because of the small number of patients treated with chemotherapeutic agents it was not possible to correlate the clinical outcome of chemotherapy and MDR proteins expression. However, our results indicate that in NSCL adenocarcinomas the expression of MRP1 is strongly correlated with the immunostaining for LRP, which achieved the same level. In contrast to NSCL adenocarcinomas, lower immunostaining for MRP1 as well as for LRP was observed in NSCL squamous cell carcinomas. The lowest positive immunostaining for both MDR proteins has been found in SCLC. The expression of both MRP1 and LRP strongly correlated in this type of human lung cancer similarly to the situation in NSCLC.

To summarize, MRP1 and LRP coexpression highly correlated in NSCL adenocarcinomas and in SCLC. The total number of positively staining tumors and the intensity of staining were much lower for both MRP1 and LRP in SCLC in comparison with NSCLC. It is suggested that this finding can confirm the overall empirical clinical knowledge about much higher chemosensitivity of untreated SCLC comparing to NSCLC. The possible role of MRP1 and LRP in chemosensitivity differences between SCLC and NSCLC is therefore also suggested.

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