Multidrug resistance in small cell lung cancer: Expression of P-glycoprotein, multidrug resistance protein 1 and lung resistance protein in chemo-naive patients and in relapsed disease

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Summary The aim of this study was to investigate the expression of multidrug resistance-associated proteins in metastatic small cell lung cancer (SCLC) cells correlated to cisplatin/etoposide chemotherapy response and the level of those proteins in relapsed disease. Samples were obtained by transbronchial fine needle aspiration biopsy (TBNA) of enlarged mediastinal lymph nodes in 17 patients. After cytological confirmation of SCLC, cells were stained by a panel of mAbs against internal epitopes of P-gp (JSB-1), MRP1 (MRPr1), LRP (LRP-56) and cytokeratin (MNF116) and analyzed by flow cytometry. We observed a significant negative correlation for better response rate to chemotherapy with individual expression of P-gp ($r = -0.93$, $P < 0.0001$; Pearson correlation) and MRP1 ($r = -0.78$, $P = 0.0002$; Pearson correlation) in chemo-naive SCLC cells and a non-significant correlation for LRP expression. P-gp and MRP1 expression was markedly increased in metastatic cells in four out of five patients with relapsed disease (4–12 months after starting chemotherapy), in comparison to their chemo-naive values. In conclusion, the results suggest that P-gp and MRP1 might be associated with SCLC cell survival during metastasis and chemotherapy, and that overexpression of those transporters in relapsed disease could assist short-term chemotherapy efficiency.

1. Introduction

Drug resistance is one of the most important causes of unsuccessful lung cancer chemotherapy. Some tumours are initially resistant and never respond to cytostatic drug treatment; whereas others become resistant after a good initial response [1]. Multidrug resistance is cross-resistance to some structurally and functionally unrelated naturally derived drugs, such as epipodophyllotoxins, vinka alkaloids, antracyclines, taxanes, colchicines and others, and is mostly caused by overexpression of P-gp and MRP [2,3].

In small cell lung cancer (SCLC), resistance is usually associated with the emergence of drug-resistant cell clones dur-
ing chemotherapy [4]. Studies of cell culture systems have established that tumour cells exposed to only a single drug expressed cross-resistance to a broad range of structurally and functionally dissimilar drugs. While various mechanisms may contribute to chemotherapy resistance, the primary ones are the so-called "pump" and "non-pump" forms of resistance [5,6]. The basic mechanism of non-pump resistance is activation of cellular antiapoptotic defence. Pump resistance or transport-mediated resistance is due to the decreased concentration of the active drug in target cells because of decreased drug uptake or increased drug efflux across tumour cell membranes, or to increased drug efflux through cytoplasmic vesicles. Certain proteins are able to transport toxic materials and drugs across cellular membranes, against a concentration gradient, decreasing their intracellular concentration [7]. A large subclass of these proteins is ATP-binding cassette (ABC) proteins, which are expressed in both normal and malignant cells. The main ABC transporters are P-gp and MRP1. The 170 kDa MDR1 P glycoprotein (P-gp) was the first human ABC transporter to be identified, and therefore most of our knowledge regarding these transporters is based on studies of P-gp. The 190 kDa multidrug resistance protein (MRP1) is probably involved in an antioxidant defence mechanism. Most drug-resistant lung cancers overexpress both P-glycoprotein and MRP1 [1,8,9].

While the lung resistance protein (LRP) is not an ABC transporter, it is a major vault protein, and is found in the cytoplasm and nuclear membrane. LRP is responsible for the uptake of drugs in cytoplasmic vesicles that are then probably extruded from the cell by exocytosis. Some investigators have reported that overexpression of LRP correlates with resistance to cisplatin [10].

The aim of our study was to investigate the expression levels of P-gp, MRP1 and LRP at diagnosis in chemo-naive patients and to determine if any correlation exists between expression levels and the overall response to chemotherapy. We also investigated the levels of those proteins in patients with relapsed disease.

2. Patients and methods

2.1. Patient selection

We initiated a prospective study after it was approved by the state ethical committee and once patients had given informed consent. The study focused on patients with ECOG performance status 0 or 1 who had clearly positive and morphologically evident SCLC in samples obtained from mediastinal lymph nodes and who had no prior chemotherapy or radiotherapy. We excluded all patients with SCLC whose mediastinal lymph nodes were not involved with SCLC tumour cells or from whom samples from enlarged lymph nodes were not diagnostic. Samples for cytological examination and flow cytometric (FACS) analysis were obtained by transbronchial fine needle aspiration biopsy (TBNA) of mediastinal lymph nodes under endobronchial ultrasound (EBUS) guidance. After the diagnosis of SCLC was confirmed, all patients received cisplatin/etoposide chemotherapy. The response rate to chemotherapy was evaluated after completion of the fourth cycle of chemotherapy by response evaluation criteria in solid tumours: RECIST criteria [8]. If the disease had relapsed in a follow-up period, we again collected samples from the involved lymph nodes. We compared the levels of P-gp, MRP1 and LRP expression before chemotherapy and after treatment in patients with recurrent disease.

2.2. Antibodies

For immunophenotypical evaluation of multidrug resistance (MDR1), we assessed P-gp, MRP1 and LRP expression using PE conjugated mAbs: anti-P-gp mouse mAbs JSB-1 (IQ Products, Groningen, The Netherlands) that recognized the internal epitope, anti-P-gp mAbs 15D3 (BD Biosciences, San Jose, CA, USA) directed against the external epitope, anti-MRP1 rat mAbs MRP-r1 and anti-LRP mouse mAb LRP-56 that both recognized internal epitopes (IQ Products) and FITC conjugated anti-cytokeratin mouse mAbs MNF116 (cytokeratins 5/6/8/17/19; Dako, Glostrup, Denmark). Negative isotype-matched control PE conjugated mAbs (BD Biosciences) were included in every experiment.

2.3. Flow cytometric analysis

Cells were washed in PBS supplemented with 0.2% BSA (PBS/BSA), lysed for 10 min in 2 ml of 1× FACS lysing solution (BD Biosciences), and then centrifuged and permeabilized for 10 min in 0.5 ml of FACS 1× permeabilizing solution 2 (BD Biosciences). Cells were then washed with PBS/BSA and incubated for 30 min with JSB-1, MRP-r1, LRP-56 mAbs or isotype-matched control mAbs and MNF116 mAbs, then washed in PBS/BSA and fixed in FACS 1× cell-fix (BD Biosciences). Data were collected and analysed by FACSCalibur (BD Biosciences) using Cell Quest software. SCLC cells were distinguished and on the basis of forward/side scatters and cytokeratin staining (Fig. 2A). A similar technique of flow cytometric detection of cytokeratin-positive cancer cells was evaluated in breast cancer cells [11,12], in small cell carcinoma [13] and for detection of lymph node metastasis in non-small cell lung carcinoma [14]. The expression of P-gp, MRP1 or LRP was quantified by the percentage of positive cells according to isotype-matched control staining, and the results were scored as the frequency of positive carcinoma cells [15].

To further validate the clinical assessment of P-gp expression using the JSB-1 mAbs, other P-gp specific mAbs, namely 15D3, which recognizes the separate external epitope (surface 15D3 versus intracellular JSB-1 staining), was tested on selected clinical samples [16]. In those cases, cells were also incubated for 20 min with 15D3 mAbs or isotype-matched control after the first PBS/BSA wash, and then processed by the same protocol as described above, using two-colour flow cytometric analysis in which cells were stained with MNF116. We detected a very strong correlation between JSB-1 and 15D3 staining (Fig. 1).

2.4. Statistical analyses

The distribution of data was recalculated by the Shapiro-Wilk test. The strength of association between P-gp, MRP1 and LRP expression and the response rate to chemotherapy was obtained by the Pearson (P-gp and MRP1) or Spear...
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3. Results

A total of 17 patients (7 women, 10 men; median age 68 years [range: 40—72]) were included in the present analysis. The results of P-gp (JSB-1), MRP1 and LRP expression in metastatic SCLC cells in chemo-naive patients in comparison to chemotherapy response according to RECIST criteria are summarized in Figs. 3 and 4. The response rate to chemotherapy showed progressive disease in two patients, stable disease in three patients, partial response in four patients and complete response in nine patients. P-gp and MRP1 expression was detected in all cases, with frequencies ranging from 2.51 to 93.78% (mean, 36.74%) of P-gp (JSB-1) positive SCLC cells and 1.61—100% (mean, 34%) of MRP1 positive SCLC cells (Figs. 2 and 3). There was a significant negative correlation for better response rate to chemotherapy with individual P-gp (JSB-1; \( r = -0.93, P < 0.0001 \); Pearson correlation) and MRP1 (\( r = -0.78, P = 0.0002 \); Pearson correlation) expression. The overall LRP expression was much lower (median (range); 0.88% (0—79)): the majority of patients showed around 1% of LRP positive metastatic SCLC cells (Fig. 3) and there was also no significant correlation between the response rate to chemotherapy and LRP expression (\( r = -0.37, P = 0.1476 \); Spearman correlation).

To determine whether the expression levels of P-gp, MRP1 or LRP in SCLC cells was associated with acquired drug resistance; we also correlated the level of those proteins in a follow-up period. In five patients with relapsed disease, 4—12 months after starting chemotherapy, we again collected samples from the involved lymph nodes. In four of five patients we detected highly increased P-gp expression (up to 60—80% of P-gp positive metastatic SCLC cells) in comparison to their chemo-naive values (Fig. 4). Similarly, in two of four patients, MRP1 expression was also markedly increased (up to 40—80% of MRP1 positive cells) (Fig. 4). Among the four patients, LRP expression was increased in two and decreased in one (Fig. 4).
Fig. 3 The proportion of P-gp (JSB-1), MRP1 and LRP positive metastatic SCLC cells in 17 chemo-naive patients and correlation to chemotherapy response according to RECIST criteria after completion of the fourth cycle of chemotherapy. PD: progressive disease, SD: stable disease, PR: partial response, CR: complete response.

Fig. 4 The frequency of metastatic SCLC positive for P-gp (JSB-1), MRP1 or LRP in five chemo-naive patients and after disease relapse, 5—12 months after the start of chemotherapy. ND: not defined.

4. Discussion

The aim of the present study was to investigate P-gp, MRP1 and LRP expression in SCLC cells. To our knowledge, this is the first report comparing the levels of transporter expression in metastatic SCLC cells with chemotherapy response and their levels in relapsed disease.

Our results clearly indicate that chemotherapy response is strongly associated with the level of P-gp expression in all 17 chemo-naive SCLC patients. A low level of P-gp expression was associated with a good chemotherapy response; whereas higher expression predicted a worse outcome.

Several previous studies also indicated that overexpression of P-gp most frequently predicts multidrug resistance [17]. For example, Leith et al. reported that leukemic cells exhibiting a lower expression of P-gp (17%) responded better to chemotherapy than leukemic cells with a higher (39%) expression rate [18].

The structural similarities between MRP1 and P-gp are paralleled by some overlap in their resistance spectra [19]. The expression of both proteins correlated to poor response to chemotherapy [20,21,22], a result that was also confirmed in our study.

We determined a significant negative correlation for response rate to chemotherapy with the individual expression of P-gp and MRP1; moreover, in one chemo-naive patient who presented with over-expression of all three MDR proteins (P-gp, MRP1 and LRP), we observed no response to chemotherapy.
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Jensen et al. [23] observed that some small cell lung cancer cells overexpressing P-gp were more sensitive to taxotere, taxol, topotecan and gemcitabine. Bergman et al. [24] also confirmed the increased sensitivity to gemcitabine in MRP-1 and P-gp over-expressing human cancer cell lines. It is thus reasonable to hypothesize that the detection of ABC transporter over-expression may represent another approach for SCLC treatment.

Chemo-naive SCLC usually expresses lower levels of transporters at baseline; after exposure to chemotherapy expression levels are upregulated. Whether this represents selection and repopulation of primary resistant clones, or upregulation due to cytotoxic therapy exposure is still unclear [20]. It is frustrating that SCLC cells, which are exquisitely chemoradiosensitive on presentation, invariably relapse and become resistant to therapy over a short period of time. In almost all of our patients with relapsed disease, we observed higher levels of transporters; in most of these patients, the disease relapsed after less than a year. A breast cancer meta-analysis also concluded that P-gp expression increases after chemotherapy [25].

P-gp and MRPI were also found in normal bronchial and bronchiolar epithelial layers, as well as in seromucinous glands and in alveolar macrophages, where they prevent the accumulation of toxic substances by removing them into lumina or into interstitial fluid [26]. It has been reported that ABC transporters are expressed at low levels in healthy tissues [17]. Despite these data, some errors due to normal tissue contamination of tumour tissue can occur. To minimize contamination with normal tissue in the present study, we collected tumour cells from mediastinal lymph nodes with TBNA, where contamination with epithelial cells is probably substantially lower compared to bronchial biopsy specimens.

In normal human lung tissue, LRP is primarily expressed in the cytoplasm of bronchial and bronchiolar cells [26]. Overexpression of LRP is much higher in NSCLC compared with SCLC [27]. The present study confirmed the low expression of LRP in almost all chemo-naive SCLC cells, with the exception of primary chemo-resistant tumours. Positive LRP and P-gp at diagnosis is associated with a poor clinical outcome [28], a result that was also confirmed in the present study.

5. Conclusion

Our results confirm that the expression level of P-gp strongly correlates with the degree of response to chemotherapy and that the levels of P-gp, MRPI and LRP usually increase in relapsed disease. Advances in diagnostic techniques and the possibility of accurate selection of patients with tumours that overexpress the aforementioned transporters should result in more successful treatment of SCLC with drugs that are not dependent on the ABC transport system, with new targeted therapies, or with additional ABC inhibitors therapies.

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