

Distribution of multidrug resistance associated proteins in different histological subtypes and stages in operable non small cell lung cancer. A tissue microarray study

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Citation

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Abstract

The aim of this retrospective study was to comparatively investigate the expression of the three drug-resistance related proteins P-glycoprotein (P-gp), multidrug-resistance protein 1 (MRP1), and lung resistance protein (LRP), in non-small cell lung cancer (NSCLC) tissues using tissue microarray technology. Methods: Tumor specimens from 179 patients were analyzed by immunohistochemistry and data were statistically analyzed by SPSS. Results: The mean expression levels of tumor tissues in the case of P-gp and LRP did not exceed the one of normal epithelia, while MRP1 was significantly enhanced in NSCLC. No associations were observed between higher grading and P-glycoprotein expression ($p < 0.8$) as well as lower grading and MRP1 expression in the case of adenocarcinoma ($p < 0.05$). We also did not find any association between the immunohistochemical expression of MrP1, Lrp, Pgp, grading and staging. Conclusions: Our data point towards a major role of MRP1 in the intrinsic treatment resistance of NSCLC.

NOTE

The both first authors have an equal contribution.

INTRODUCTION

Almost all non-small cell lung cancer (NSCLC), representing approximately 85% of all lung cancers (Fry et al. 1996), display intrinsic multidrug resistance (MDR), generally limiting the chance of successful chemotherapy (Ihde and Minna 1991). This is one reason why lung cancer is currently a leading cause of cancer death worldwide. As the enhanced resistance of NSCLC cells affects a diverse range of antineoplastic drugs currently in clinical use, it is believed that several protection mechanisms are cooperatively active in NSCLC cells. This multi-factorial in vivo MDR phenotype is also reflected by a distinct chemoresistance of NSCLC cells in vitro (Scagliotti et al. 1999; Doyle 1993). Members of the ATP-binding cassette (ABC)-transporter family are able to confer MDR by transporting drugs in an energydependent manner out of the cell (Tan et al. 2000). P-glycoprotein (P-gp)—the archetype of an MDR molecule

(Lehne 2000)—has been suggested to be of minor

importance in the intrinsic chemoresistance of NSCLC cells (Doyle 1993). In contrast, a predictive value of P-gp for therapy response to paclitaxel in the case of advanced NSCLC has been suggested recently (Yeh et al. 2003). We and others have shown that the multidrug-resistance protein 1 (MRP1) is intrinsically expressed and functionally active in NSCLC cells and correlates inversely with chemosensitivity against diverse antineoplastic drugs (Young et al. 2001; Giaccone et al. 1996; Narasaki et al. 1997; Berger et al. 1997). Also, a correlation between MRP3 and MRP1, as well as of both transporter molecules with chemoresistance, has been described in unselected NSCLC cell lines (Zouny et al. 2001). The lung resistance-related protein (LRP), another marker for chemoresistance in vitro and in vivo, does not belong to the ABC-transporter family. It recently turned out to be the main component of the vault, the largest ribonucleoparticle known so far (Scheffer et al. 1995). Despite an overexpression in several drug-selected cell models (Kickhoefer et al. 1998), the precise cellular role of vaults is currently unclear. We have shown that LRP is differently expressed in NSCLC cell lines and correlates with resistance to cisplatin but not to several other drugs

(Berger et al. 2000). In order to further characterize the intrinsic, multifactorial MDR phenotype of NSCLC cells in vivo we comparatively investigated the expression of the MDR proteins P-gp, MRP1 and LRP and Topoisomerase II α with regards to the histological subtype of NSCLC, staging, and grading.

MATERIALS AND METHODS

Study population and tumor samples. 179 consecutive patients diagnosed with early stage and operable NSCLC between years 1996 and 2001 were enrolled in this study. Out of these 58 were adenocarcinomas (20 GI, 38 GII), 106 were squamous cell carcinomas (35 GI, 50 G2, 21 G3) and 15 large cell carcinomas (G3). The patient population consisted of 150 males and 29 females. The distribution of patients within stages, at the time of diagnosis, was the following: 104 patients were in stage IIIa, 20 patients were in stage IIIb, 34 patients in stage I and II, and 21 patients in stage IV.

TISSUE MICROARRAY CONSTRUCTION

Tumor tissue microarrays were constructed using 179 formalin-fixed primary lung cancers, as stated above. The tissue area for sampling was selected based on visual alignment with the corresponding H&E-stained section on a slide. Three-five tissue cores (2 mm in diameter, 3-4 mm in height) taken from a donor tumor block were placed into a recipient paraffin block with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was also punched from each case in parallel. Four μ m sections of the microarray block were used for immunohistochemical analysis.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded samples were cut in 8 μ m sections, mounted on silane-coated slides, deparaffinized, rehydrated and pretreated in a microwave at 650 W for 30 min in citrate buffer pH 6.0. Nonspecific binding was blocked with 5% swine or rabbit serum, respectively, in phosphate buffer solution (PBS). Sections were incubated in the primary antibodies at the given working dilutions overnight at 4°C. Antibody binding was visualized by use of the streptavidin biotinylated alkaline phosphatase system from DAKO (Copenhagen, Denmark). As a second antibody, a biotinylated multilink swine anti-goat, mouse, rabbit immunoglobulin (Ig) biotin and a biotinylated rat Ig at dilutions of 1:50 were applied for 30 min at room temperature. Diamino benzidine substrate was used as a chromogen, according to the manufacturer's suggestions.

Sections were counterstained with haematoxylin. Staining was evaluated independently by two of the authors (J.S., T.T.). Results were scored in a qualitative and a quantitative way. Qualitative score (QLS) was always evaluated relative to the bronchial epithelium, which stained positive in all cases analyzed for all three markers investigated, and graded: 0=below the normal epithelium or negative; 1=resembling the normal epithelium with weak cytoplasmic staining; 2=distinctly enhanced cytoplasmic staining; 3=strong staining in cytoplasm and cell membrane. When different subgroups of tumor cells gave different scores, the percentages of the cells/group were counted and the QLS calculated by multiplying the grading (1–3) with the percentage of cells/100. Thus, QLS levels were continuously distributed from 0 to 3. The quantitative analysis determined the percentage of cells staining positively and was expressed as quantitative score (QNS). At least 1,000 cells/sample in four different parts of the tumor were analyzed and scored: 0=0–5% positive; 1=6–25% positive; 2=26–50% positive; 3=51–100% positive. For statistical analysis the expression index (EI) for each sample was calculated from both scores by the equation: $QLS \cdot QNS$ and thus gave values from 0 to 9.

STATISTICS

Analysis of data was performed using the computer software SPSS for Windows (version 10.0). Nonparametric comparison between independent groups was done using the Kruskal-Wallis test. Doplní Lenka Radová.

RESULTS AND DISCUSSION

EXPRESSION OF P-GP, MRP1, AND LRP IN NORMAL AND TUMOR TISSUE

In the non-malignant superficial bronchial epithelium of lung cancer patients P-gp was generally found to be expressed at low levels. Also, immunoreactivity in the malignant tissue was relatively weak and comparable with the normal bronchial epithelium in 60/179 of

NSCLC patients. In 24/179 samples the tumor tissue was, in contrast to the normal bronchial epithelium, decreased or completely negative for P-gp immunostaining, indicating a loss of P-gp during malignant progression. In 27/179 patients a distinct up-regulation of P-gp in >10% of tumor cells was detectable. In some other cases (9/179), including both adenocarcinomas (AC) and squamous cell carcinomas (SCC), a small subgroup of tumor cells (3–6%) distinctly overexpressed P-gp (Fig. 1A). When scored for an expression index (EI) (as described above) the mean

expression level for P-gp in tumor tissue did not significantly differ from the normal bronchial epithelium. In normal as well as malignant cells, P-gp was detected as diffuse cytoplasmic staining and only in strongly positive cells concentrated at the plasma membrane (Picture. 1).

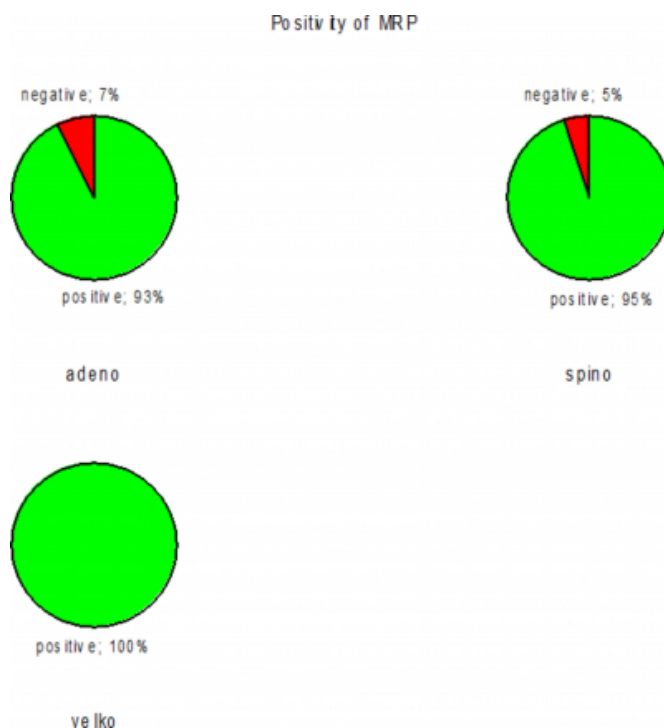
Figure 1

Figure 1: These graphs show the percentage of Pgp (P-glycoprotein positive and negative patients with regards to three basic histological subtypes of NSCLC (Non small cell lung cancer). No differences between the expression have been observed.



Figure 2

Figure 2: These graphs show the percentage of Mrp-1 (Multidrug resistance related protein-1 with regards to three basic histological subtypes of NSCLC (Non small cell lung cancer). No differences between the expression within basic histological subtypes have been observed.



With regard to the histological subtypes (Fig. 1,2), no significant association of P-gp and MRP expression could be detected. Only in the few bronchioloalveolar carcinoma (BAC) samples analyzed, P-gp levels were generally lower as compared with the respective normal bronchial epithelia (Picture 1). The expression level of MRP1 appeared to be independent of the histological subtype in the case of NSCLC (Fig. 2).

With regard to tumor-node-metastasis (TNM) staging, while neither one of the proteins was related to staging. (Fig. 3,4) With regard to grading, P-gp-expression tended to be enhanced in highly dedifferentiated tumors ($p=0.08$), while neither MRP1 nor LRP displayed a significant relation to grading. However, when AC were analyzed separately, MRP1 expression correlated positively with the grade of differentiation ($p<0.05$).

Figure 3

Figure 3: These graphs show the percentage of Pgp (P-glycoprotein positive and negative patients with regards to stages of NSCLC (Non small cell lung cancer). No significant differences between the expression have been observed.

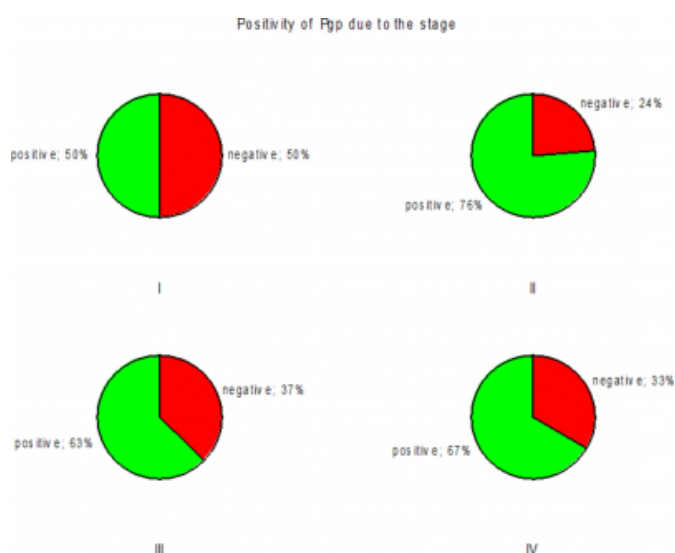
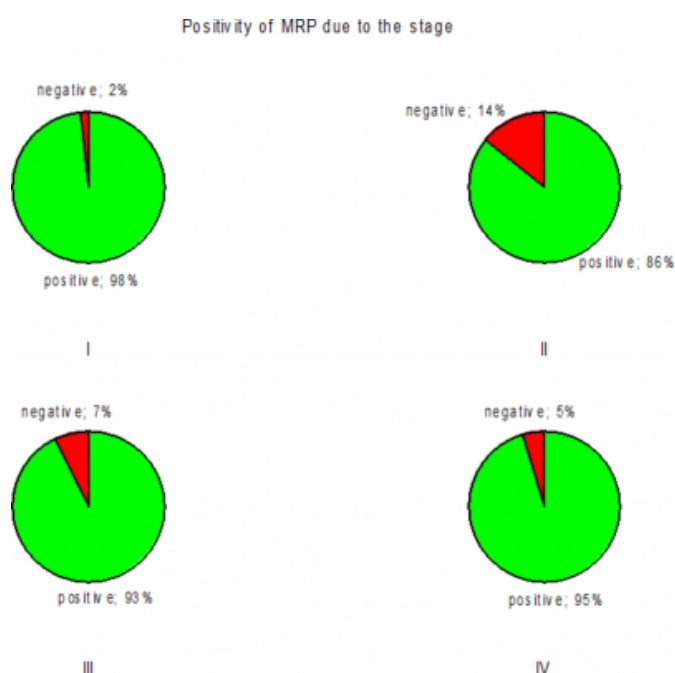


Figure 4

Figure 4: These graphs show the percentage of patients expressing Mrp-1 (Multidrug resistance associated protein) with regards to stages of NSCLC (Non small cell lung cancer). No significant differences in the expression between individual groups have been observed.



DISCUSSION

Chemotherapy resistance is a major problem in the clinical

management of lung cancer. While SCLC initially responds readily to several chemotherapeutic regimens, NSCLC is generally characterized by intrinsic resistance to many antineoplastic drugs (Scagliotti et al. 1999). This distinct resistance to treatment is one reason for the persisting unfavorable prognosis of NSCLC patients.

The underlying cellular mechanisms are still quite unclear, but intrinsic resistance is believed to be due to multifactorial causes (Volm et al. 2002). In the present study we comparatively investigated the *in vivo* expression of three well-known drug-resistance proteins, i.e., P-gp, MRP1, and LRP, which all have been associated with the drug insensitivity of NSCLC cells *in vitro* (Abe et al. 1994; Berger et al. 1997; Berger et al. 2000; Doyle 1993). We demonstrate that all these resistance proteins are expressed in normal lung epithelium at detectable levels. In contrast, intrinsic MRP1 overexpression in the tumor tissue was an almost general feature of NSCLC. In summary our data suggest that MRP1 is a major cause of the intrinsic drug resistance of NSCLC cells. P-gp, the archetype of a drug-resistance ABC-transporter encoded by the MDR1 gene, causes chemoresistance of diverse cancer types (Gottesman et al. 2002). In our series of lung cancer tissues, we only scarcely found P-gp to be overexpressed as compared with the normal bronchial epithelium. This relative limited expression of P-gp especially in chemo-naïve patients supports previous studies suggesting that P-gp overexpression is of minor relevance for intrinsic therapy resistance of NSCLC (Doyle 1993; Scagliotti et al. 1999). In contrast, in recent reports a predictive value of P-gp expression for the therapy with taxanes has been demonstrated (Chiou et al. 2003; Yeh et al. 2003), implying that the role of P-gp in NSCLC chemoresistance might have been underestimated. Correspondingly, an up-regulation of P-gp expression by chemotherapy in NSCLC xenografts has been shown (Abe et al. 1996). A major drawback of the present study is that, based on the clinical practice, only a limited number of treated patients (N = 36) could be regimens. Thus, further studies monitoring P-gp expression during application of different forms of chemotherapy in lung cancer are warranted. When comparing the expression levels of P-gp with the other investigated drug-resistance proteins no association with other drug resistance proteins has been found. While recently LRP was reported as a predictive marker for treatment response in NSCLC (Harada et al. 2003), another study failed to detect any such association (Dingemans et al. 1996). In our investigation LRP

expression was relatively low and comparable with that of the normal bronchial epithelium in most patients. MRP1 was first cloned from a drug-selected SCLC cell line (Cole and Deeley 1993). In the case of NSCLC, MRP1 overexpression was not only detected in cells with acquired MDR but has also been found in untreated NSCLC cell cultures (Berger et al. 1997; Chuman et al. 1996; Young et al. 2001).

Expression of MRP1 in vitro correlated with enhanced resistance to a broad spectrum of MRP1 substrate drugs (Berger et al. 1997; Zouny et al. 2001). Blockade of MRP1 by pharmacological agents including probenecid and benzobromarone upregulated drug sensitivity of unselected NSCLC cells (Berger et al. 1997; Giaccone et al. 1996). These in vitro data suggested a substantial contribution of MRP1 to the intrinsic MDR phenotype of NSCLC cells in vitro. In the present investigation we detected an almost general overexpression of MRP1 in NSCLC tissues in vivo. Also, SCLC and carcinoids expressed MRP1 (data not shown), however, at lower levels as compared with NSCLC. A prevalence of MRP1 overexpression in NSCLC has been found in other studies as well (Nooter et al. 1996; Sugawara et al. 1995; Wright et al. 1998), while lower MRP1 expression in NSCLC were also reported (Dingemans et al. 1996; Ota et al. 1995). These differences might be explained by the detection methods used (such as mRNA vs protein assays) and the investigated patient collective (early vs advanced stage NSCLC). The patients included in our investigation mainly suffered from early stage and operable NSCLC. On contrary to other reports, in this report, MRP1 overexpression did not correlate with a higher grade of differentiation, especially in AC (Nooter et al. 1996; Sugawara et al. 1995; Wright et al. 1998). With regard to histology, both a predominant expression of MRP1 in SCC (Chuman et al. 1996; Ota et al. 1995) and in AC (Sugawara et al. 1995; Wright et al. 1998) have been demonstrated. In our series of patients no significant differences of MRP1 expression between different histological subtypes could be detected.

However, especially in the case of SCC, a distinct overexpression of MRP1 was present at the outer cell layer of the tumor nodules in contact with stromal cells and small vessels (Picture 1a). A comparable MRP1 overexpression at the tumor borders was also observed by others (Nooter et al. 1996; Thomas et al. 1994) and suggested that MRP1 expression might be up-regulated due to paracrine signals derived from the surrounding normal tissues. The enhanced expression of MRP1 in those tumor cells located in close

proximity to microvessels additionally suggests that MRP1 represents major intrinsic defense mechanisms of NSCLC tissues against poisons delivered by blood circulation. Summarizing our study further supports a central role of ABC-transporter proteins in the chemotherapy resistance of NSCLC and places emphasis on the necessity for new treatment modalities that are not limited by these drug-resistance mechanisms.

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