Positive Correlation between Cyclooxygenase 2 and the Expression of ABC Transporters in Non-small Cell Lung Cancer

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Abstract. *Background: The primary method of treatment of non-small cell lung cancer (NSCLC) in stage IIIB and IV is chemotherapy. Previous data suggested a correlation between cyclooxygenase-2 (COX-2) expression and the multidrug-resistant phenotype of cancer cells. Materials and Methods: In this prospective study, 32 patients with NSCLC in stage IIIB and IV from 1,078 patients were included. The expression of COX-2 as well as the expression of the ABC transporters MDR1/P-glycoprotein (MDR1/P-gp), BCRP and MRP1 were detected immunohistochemically. Results: Univariate and multivariate analyses demonstrated no prognostic or predictive significance of these proteins. It was merely demonstrated that complete or partial response are favourable factors for prediction of longer progression-free survival time. However, a strong positive correlation between the expression of COX-2, MDR1/P-gp and BCRP was found in NSCLC. Conclusion: These data suggest no clinical impact for the expression of MDR1/P-gp, MRP1, BCRP or COX-2 in NSCLC, but a putative coregulation of COX-2 and MDR1/P-gp and BCRP in NSCLC.*

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Non-small cell lung cancer (NSCLC) is responsible for around 80% of all lung cancer cases. Treatment of NSCLC, which is characterised by the low susceptibility to chemotherapy, is still problematic for investigation by clinical and basic researchers. The primary form of therapy is surgery but, unfortunately, surgery can merely be applied in 20-25% of the patients in stages I-IIIA as determined on the basis of the TNM classification. Patients diagnosed in stage IIIB make up a group of about 15% among all patients with NSCLC (1). The standard therapy in the case of most patients in stage IIIB, who do not qualify for the surgical treatment is chemoradiotherapy. Although chemoradiotherapy significantly increases the success of treatment, the 5-year survival of NSCLC patients is less than 10% (2). 5-Year survival in stage IV of NSCLC is less than 1%, and treatment with cytotoxic drugs has a rather palliative character.

The leading cause of the lack of the successful treatment of NSCLC in stage IIIB and IV is a therapy-resistant phenotype of the cancer cells. The cellular factors involved in therapy resistance, in particular drug resistance, can potentially act as targets in the development of new therapeutic strategies and may be useful for the prediction of resistance to individual drugs. This approach would help to avoid ineffective drugs in therapy and would allow the application of efficient drugs and effective therapeutic protocols. Drug resistance in NSCLC is commonly a multidrug resistance (MDR). This phenotype is characterised by simultaneous resistance against multiple antineoplastic

agents with different chemical structures and modes of action. MDR is often associated with the overexpression of energy-dependent transmembrane drug extrusion pumps, *i.e.* members of the family of adenosine triphosphate-binding cassette (ABC) transporters. The human genome project identified at least 48 human genes encoding different members of this protein family. Out of these proteins, MDR1/P-glycoprotein (MDR1/P-gp), multidrug resistance protein 1 (MRP1) and breast cancer-resistance protein (BCRP) were found to be the most important drug transporters associated with clinical drug resistance (3)

A biochemical study demonstrated that cyclooxygenase-2 (COX-2) can up-regulate the expression level of MDR1/P-gp *in vitro* (4). Furthermore, it was shown that there is a strong positive correlation between the expression of COX-2 and MDR1/P-gp in tissue specimens prepared from ovarian (5) and breast cancer (6). These data indicate that a potential application of COX-2 inhibitors as treatment parallel to anticancer chemotherapy may increase the susceptibility of cancer cells to the chemotherapeutic regimen.

The aim of this study was to analyse potential correlations between COX-2 expression and the expression of the clinically most important ABC transporters MDR1/P-gp, MRP1 and BCRP, as well as the prognostic and predictive significance of these transporter proteins in patients suffering from stage IIIB and IV NSCLC, ineligible for surgery and treated with chemotherapy.

Patients and Methods

Patients. The studies were performed prospectively. Between January 2002 and December 2004, lung cancer was the reason for 1,078 thoracotomies at the Lower Silesian Centre of Lung Diseases, Thoracic Surgery Centre (Poland). The procedures included 933 (86.5%) anatomical resections (lobectomy, bilobectomy or pulmonectomy), 101 (9.4%) wedge resections, 15 (1.4%) exploratory thoracotomies and 29 (2.7%) diagnostic thoracotomies.

The clinical stage of tumour advancement (cTNM) was established by routine radiological examination (AP and lateral chest radiograms, computer-assisted tomography of the chest), bronchofiberoscopy, and ultrasonography of abdomen. When the presence of distant metastases outside of the chest was clinically suspected, computer-assisted tomography of abdomen, head or bone scintigraphy was additionally performed. Suspicion of metastases to mediastinal lymph nodes (their diameters exceeding 1 cm in radiological examination) provided indication to perform mediastinoscopy.

Before sampling of tumour tissue for further studies, none of the patients was subjected to chemotherapy or radiotherapy.

In line with the research program, data of 44 patients subjected to exploratory or diagnostic thoracotomy only were prospectively analysed. In neither of the procedures was the tumour removed, except for small samples taken for histopathological studies. In cases of macroscopically altered, enlarged lymph nodes in the mediastinum or hilus, sampling of the most representative lymph nodes for histopathological examination was conducted. When the primary lesion was accompanied by metastases in the same lobe (trait T4) or in another lobe (trait M1), samples of the lesions were taken for histopathological examination.

Twelve patients (27.3%) were excluded from further studies and observation: five were disqualified due to their poor general condition (grade of efficiency according to WHO >2, loss of body weight exceeding 10%); in 3 patients small cell cancer was diagnosed; 2 patients were subjected to radiotherapy only and 2 patients declined further treatment following the procedure. The study was approved by an Institutional Review Board and the patients gave their informed consent before their inclusion into the study.

Tumour samples were isolated from 32 patients (Table I) and subjected to immunohistochemical analysis. Histopathology confirmed in all cases the diagnosis of NSCLC and, in combination with pre-operative radiological diagnosis, established in all patients stage IIIB or IV advancement of the disease. The mean period of observation following surgery amounted to 14.4 months (ranging from 1.8 to 35.5 months). The clinical course of the disease was evaluated during visits before administration of consecutive chemotherapy cycles and then every three months, unless progression of the disease enforced earlier visits of the patients. Response to chemotherapy was monitored by review AP and lateral chest radiograms and using endoscopy after every cycle of chemotherapy and upon termination of the treatment (chest radiogram, chest tomography), which permitted the dimensions of the tumour to be measured. Abdominal ultrasonography and bronchofiberoscopy were also conducted. In cases of distant metastases to the brain, computer-assisted tomography of the head and, upon suspected metastases to bones, scintigraphy were also performed.

Before termination of the studies, 24 (75%) patients died due to progressive neoplastic disease. Thirty-two patients following exploratory or diagnostic thoracotomy were subjected to cisplatin-based chemotherapy. Individual therapeutic cycles were repeated every 21 days (Table I). In the study group, 6 patients following chemotherapy received palliative radiotherapy of Co-60, 20 Gy/5 fr: in 4 patients to the primary lesion (due to the lack of response to chemotherapy), in two patients to metastases to bones and brain. Standard criteria of the response to chemotherapy were applied (7).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was freshly cut (4 μm). The sections were mounted on Super frost slides (Menzel Gläser, Germany), dewaxed with xylene and gradually rehydrated. Activity of endogenous peroxidase was blocked by 5 minutes' exposure to 3% H_2O_2. All the studied sections were boiled in Antigen Retrieval Solution (DakoCytomation, Glostrup, Denmark), in the case of COX-2 for 10 minutes and in the case of MDR1/P-gp for 15 minutes. Immunohistochemical reactions were performed using monoclonal mouse antibodies against COX-2 (Cayman Chemical Company, Ann Arbor, MI, USA) at a dilution of 1:2,000; monoclonal mouse antibodies (clone C219) against MDR1/P-gp (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:100 and monoclonal mouse antibodies (clone JSB-1) against MDR1/P-gp (Roche Diagnostics, Mannheim, Germany) at a dilution of 1:100; monoclonal mouse antibodies (clone MRPr1) against MRP1 (Monosan, Holand) at a dilution of 1:100; and monoclonal mouse antibodies (clone BXP-21) against BCRP (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:100. The antibodies were diluted in Antibody Diluent with background reducing component (DakoCytomation). Tested sections were incubated with antibodies for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 minutes, room temperature) and streptavidin-biotinylated peroxidase complex (15 minutes, room

Table I. *Patient and tumour characteristics*.

Characteristic	No. (%)
All patients	
32 (100)	
Age (years/mean 59.9)	
≤55	13 (40.6)
56-65	11 (34.4)
>65	8 (25)
Gender	
Male	21 (65.6)
Female	11 (34.4)
Stage	
IIIB	17
Infiltration of large vessels	13
Infiltration of spinal column	2
Infiltration of trachea	1
Infiltration of oesophagus	1
IV	15 (81.2)
cT	
T2/3	6 (18.8)
T4	26 (81.2)
cN	
N0	5 (15.6)
N1	3 (9.4)
N2	12 (37.5)
Nx	12 (37.5)
cM	
M0	17 (53.1)
M1	15 (46.9)
Location of the primary tumour	
Left	19 (59.4)
Right	13 (40.6)
Clinical response	
Complete response	1 (3.1)
Partial response	3 (9.4)
Stable disease	17 (53.1)
Progressive disease	11 (34.4)
Histology	
Planoepithelial	10 (31.2)
Adenocarcinoma	16 (50)
Giant cell tumour	4 (12.5)
Non-small cell, poorly differentiated	2 (6.3)
Chemotherapy (in total)	
Cisplatin/etoposide	18 (56.2)
Cisplatin/vinorelbine	7 (21.9)
Cisplatin/mitomycin/isophosphamide	4 (12.5)
Cisplatin/gemcitabine	3 (9.4)

temperature) using a LSAB+ HRP system (DakoCytomation). DAB+, Liquid (DakoCytomation) was used as a chromogen (7 minutes, room temperature). All the sections were counterstained with Meyer's hematoxylin. Each reaction was accompanied by the negative control in which the specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation).

Evaluation of reaction intensity. Intensity of immunohistochemical reactions was estimated independently by two pathologists. In doubtful cases, a re-evaluation was performed using a double-headed microscope and staining was discussed until a consensus was achieved. Intensity of immunohistochemical reactions was evaluated using a semi-quantitative immunoreactive score (IRS) scale (8) which took into account intensity of colour reaction and percentage of positive cells. The results represent the product of scores allocated for the evaluated traits and range between 0 and 12.

Control reactions. Sections of six formalin-fixed and paraffin-embedded normal human liver samples for each of the examined ABC transporters were used as positive control. To evaluate the specificity of COX-2 antibody (Cayman Chemical Company), blocking experiments using a COX-2 blocking peptide (Cayman Chemical Company) were performed as described elsewhere (6, 9).

Statistical analysis. Statistical analysis of the results took advantage of Statistica 98 PL software (Statsoft, Kraków, Poland). The employed tests included Spearman's rank correlation and Chi^2 tests. Kaplan-Meier's statistics, log-rank and Cox F tests were performed to estimate significance of differences in survival times.

Results

Expression of COX-2 and ABC transporters in NSCLC. As shown in Figure 1A, COX-2-specific staining reaction was detected in the cytoplasm of the cancer cells. The average intensity of the immunocytochemical reaction according to the IRS scale was 2.625±3.160 (min 0, max 12).

For detection of MDR1/P-gp, two different antibodies were used, JSB-1 and C219. Both antibodies showed staining reactions in the membrane and the cytoplasm of the cancer cells. The average intensity of the immunocytochemical reaction was 1.437±1.625 (min 0, max 6) using JSB-1 (Figure 1A) and 1.475±1.694 (min 0, max 6) staining with C219 (Figure 1B).

Likewise, BCRP-specific staining could be detected in the membrane and the cytoplasm of the cancer cells (Figure 1C). The average intensity of the immunocytochemical reaction was 6.840±3.619 (min 0, max 12).

In the case of MRP1, the staining reaction was also detected in the membrane and the cytoplasm of the cancer cells (Figure 1D1), whereby the staining intensity in squamous cancer cells was higher than in cells with lower differentiation (Figure 1D2). The average intensity of the immunocytochemical reaction was 5.281±4.502 (min 0, max 12).

Correlation between expression of COX-2 and ABC transporters. The correlation between the expression of COX-2 and the expression of three different ABC transporters in NSCLC was determined. A statistically significant correlation was observed between expression the COX-2 and MDR1/P-gp using the JSB-1 antibody ($p<0.0001$) (Figure 2A) and antibody C219 antibody ($p<0.0001$) (Figure 2B), as well as between COX-2 and BCRP expression ($p=0.003$) (Figure 2C). The calculations showed a strong positive correlation between the different antibodies for detection of MDR1/P-gp, *i.e.* JSB-1 and C219 ($R=0.92$; $p<0.0001$). No statistically

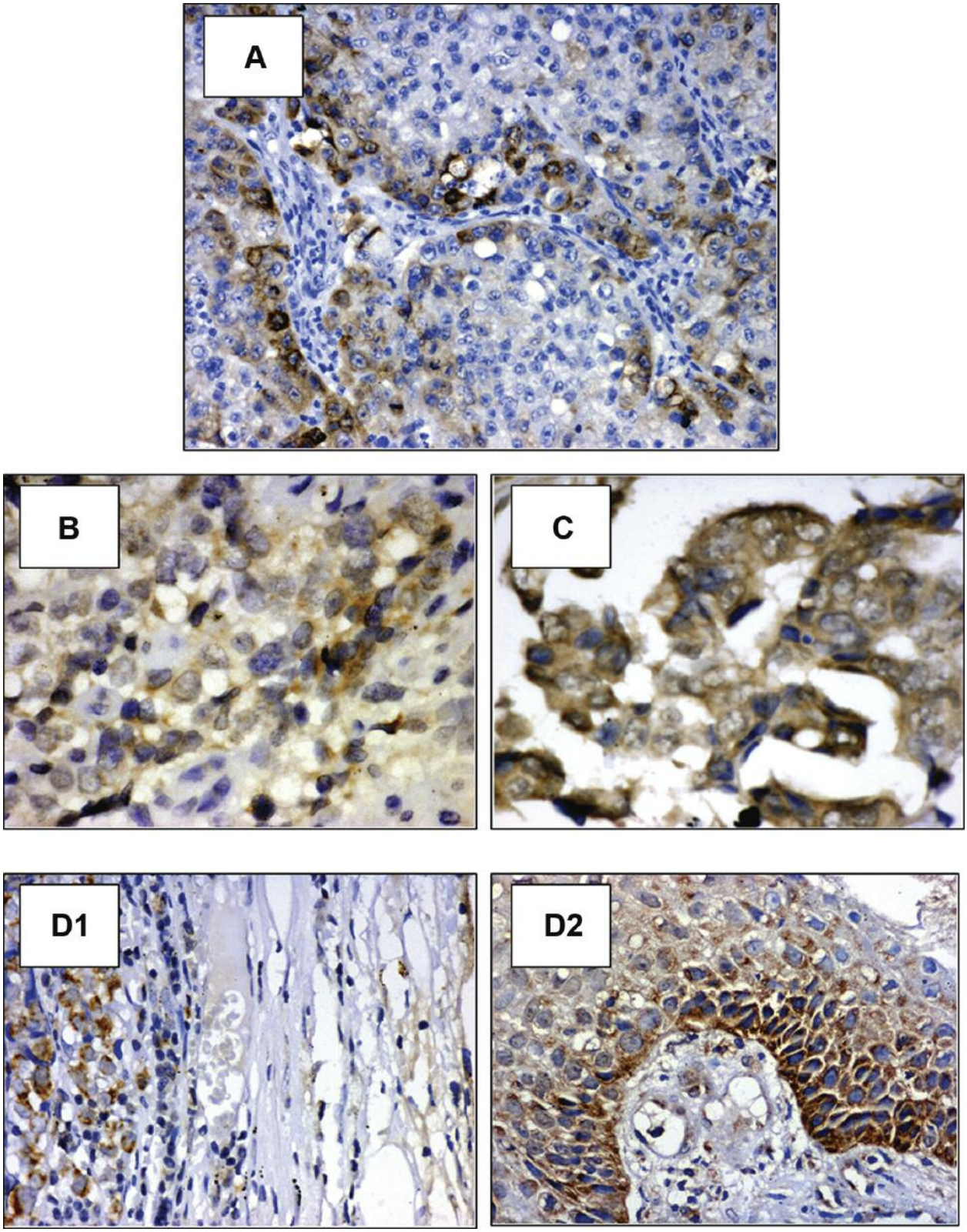

Figure 1. *Immunohistochemical localization of protein expression in NSCLC sections. A, Expression of COX-2 (hematoxylin, ×200). B, Expression of MDR1/P-gp (C219) (hematoxylin, ×400). C, Expression of BCRP (hematoxylin, ×400). D1-2, Expression of MRP1 (hematoxylin, ×400).*

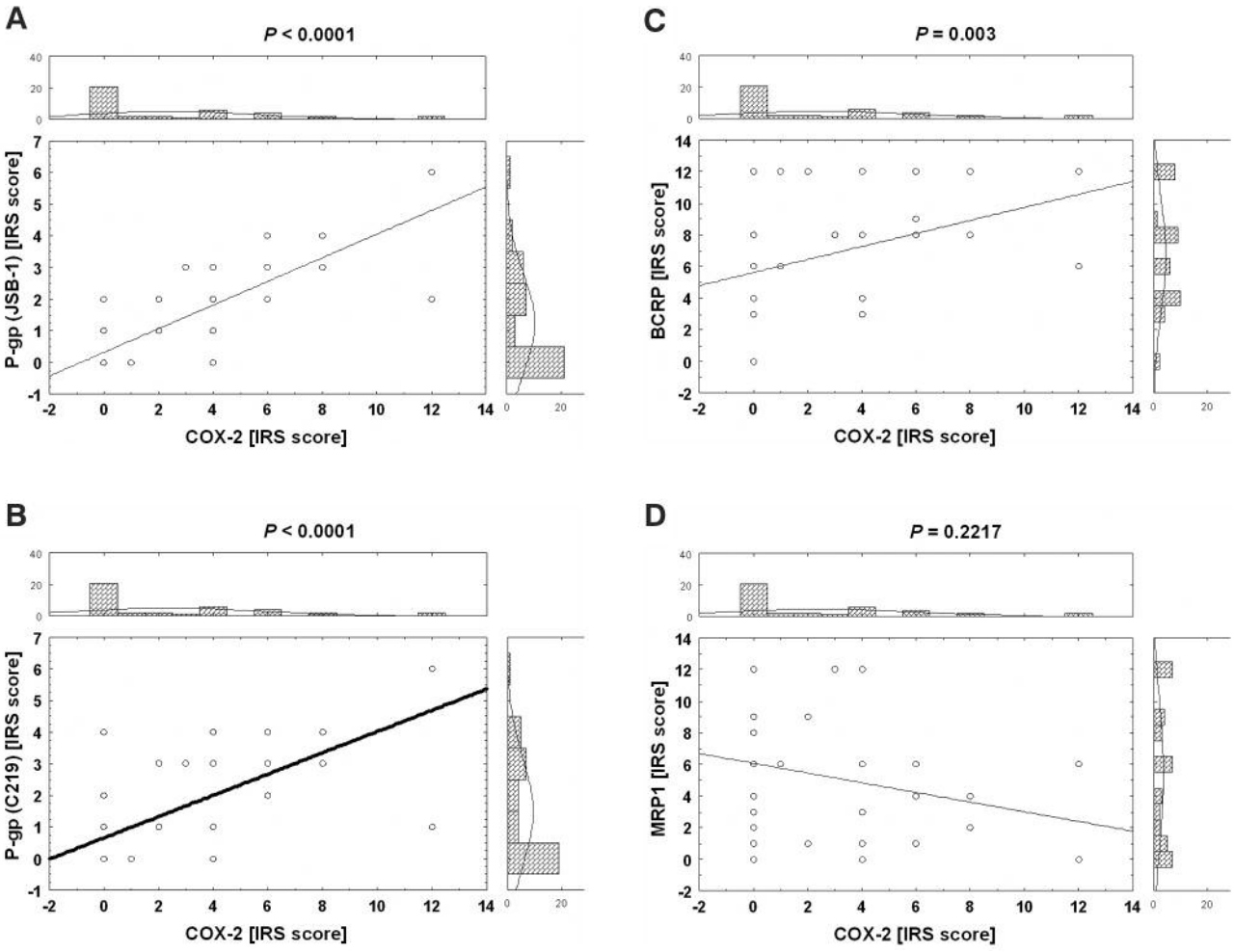

Figure 2. *Relationships between COX-2 expression and the expression of: A, MDR1/P-gp using the antibody JSB-1; B MDR1/P-gp using the antibody C219; C, BCRP and D, MRP1 (Spearman's rank correlation).*

significant correlation was observed between COX-2 and MRP1 expression (p=0.2217) (Figure 2D).

Relationships between expression of studied proteins and clinical/pathological data. Analysis of the dependence between the expression of the investigated proteins and clinical and pathological data of the patients revealed merely two dependences: (i) increased (above average) expression of MDR1/P-gp was characteristic for patients older than 65 years (Table II), and (ii) increased (above average) expression of BCRP in NSCLC was characteristic for patients reporting a higher number of toxic symptoms of chemotherapy (according to WHO) (Table II). Table II summarises the data of all calculations.

Patient survival. The period of survival free of progression of the neoplastic disease amounted in the study group to a mean of 9.2 months (median: 5 months), with patients at IIIB stage surviving for a mean of 9 months (median: 4 months) and patients at IV stage surviving a mean of 9.4 months (median: 5 months). The mean period of survival of patients in the study group amounted to 14.6 months (median: 12.3 months; range: 1.8 to 35.9 months). Patients at stage IIIB survived an average of 14.3 months (median: 11.7 months; range: 2.2 to 35.9 months). At this stage, 41.2% patients survived a period of one year and 11.8 % patients survived two years. At stage IV, the mean survival time was 15.1 months (median: 14.9 months; range: 1.8 to 32 months) with 53.3% patients surviving a period of one year and 20% patients surviving two years.

Univariate survival analysis. Log-rank test and Kaplan-Meier analyses did not show any dependence between the expression of the studied proteins, *i.e.* neither COX-2, MDR1/P-gp (JSB-1 and C219), BCRP, MRP1, stage, clinical response and progression-free or overall survival (Table III).

Table II. *Relationships between protein expression and various clinicopathological factors.*

Parameter	*P* value (Chi-quare test)				
	COX-2	MDR1/P-gp (JSB1)	MDR1/P-gp (C219)	BCRP	MRP1
Age	0.9650	**0.0490**	**0.0480**	0.1540	0.1540
Gender	0.3730	0.5292	0.5284	0.5292	0.9072
Smoking	0.9570	0.8379	0.8383	0.8379	0.8379
Degree of fitness	0.6807	0.8379	0.8379	0.8379	0.8379
Stage	0.7547	0.4916	0.5231	0.4916	0.1493
Histology	0.0679	0.5993	0.5993	0.3158	0.3158
Location of the primary tumour (left/right)	0.6179	0.4302	0.4315	0.9461	0.4302
Size of the tumour before chemotherapy	1.0000	0.7232	0.7232	0.0765	0.1541
Size of the tumour after chemotherapy	0.8881	0.5292	0.5485	0.9072	0.1691
Change of tumour size after chemotherapy	0.5820	0.5820	0.5820	0.1990	0.7136
pT (T2/3 *vs*. T4)	0.7321	0.8649	0.8543	0.2811	0.8649
pT4 feature (scatter in a flap *vs*. local infiltration)	0.5077	0.6637	0.6644	0.7685	0.4726
pN (pN2 *vs*. pN0 / pN1)	0.0679	0.0679	0.0723	0.8522	0.3613
M	0.7547	0.4916	0.4920	0.4916	0.1493
Chemotherapy toxicity	0.4575	0.8632	0.8632	**0.0112**	0.8632
Clinical response to the chemotherapy	0.4190	0.3486	0.3486	0.2282	0.8935

Table III. *Univariate survival analysis of relationships between clinical data, protein expression, progression-free and overall survival time.*

Studied parameter	*P* Value log-rank test	
	Progression-free survival	Overall survival
COX-2	0.9036	0.7284
MDR1/P-gp (JSB-1)	0.5625	0.8437
MDR1/P-gp (C219)	0.5626	0.8441
BCRP	0.4982	0.8049
MRP1	0.9603	0.9494
Stage	0.8753	0.9206
Clinical response to chemotherapy	0.8142	0.7971

Table IV. *Multivariate survival analysis of relationships between clinical data, protein expression, progression-free and overall survival time.*

Studied parameter	*P* Value (Cox F test)	
	Progression-free survival	Overall survival
COX-2	0.543	0.656
MDR1/P-gp (JSB-1)	0.597	0.847
MDR1/P-gp (C219)	0.597	0.847
BCRP	0.536	0.808
MRP1	0.963	0.949
Stage	0.554	0.987
Clinical response to chemotherapy	**0.049**	0.082

Multivariate survival analysis. The calculations using Cox F test demonstrated that patients who reached chemotherapy-associated complete or partial response had a statistically significantly longer progression-free survival as compared to those who achieved stabilization or progression of the disease (p=0.049) (Table IV). No correlation between clinical response and overall survival time was observed. In the case of the other factors, *i.e.* COX-2, MDR1/P-gp (JSB-1 and C219), BCRP, MRP1 and stage, no correlation was found with progression-free or overall survival time (Table IV).

Discussion

The chemotherapeutic treatment of NSCLC, usually with different drug-resistant phenotypes, is a major problem in clinics, particularly in the case of advanced stages IIIB and IV, where successful treatment with surgery is impossible. Resistance against antineoplastic drugs is responsible for the poor prognosis in the advanced stages of NSCLC, in which chemotherapy is the primary form of treatment (13). Investigations of chemoresistance in NSCLC are obscure and many of them describe very differentiated and small groups of

patients. One of the reasons for the small number of investigated patients is the problem with obtaining sufficient amounts of material and of good quality for the investigations, especially in the advanced stages. In some earlier evaluated clinical data, patients were in different stages and with different prognosis of NSCLC (from I to IV). These discrepancies may influence the overall score of the prognostic factor of the investigated proteins in cancer tissue (11-13).

The expression and potential correlations between COX-2 and MDR-associated ABC transporters in NSCLC of stage IIIB and IV were analysed here. In addition the correlation between the expression of these proteins and clinical and pathological factors were analysed. The data demonstrated that neither COX-2 nor any of the investigated ABC transporters has any influence on the length of the progression-free or overall survival. A strong positive correlation between staining data of MDR1/P-gp using two different antibodies, JSB-1 and C219, was shown. Multivariate survival analysis demonstrated that patients with a complete or partial response had a significantly longer progression-free survival as compared to those who achieved stabilisation or progression of the disease.

Brooks *et al*. (14) reported that in a group of NSCLC patients treated with vinorelbine and cisplatin in stage III, no correlation between expression of MDR1/P-gp and total survival time could be found, but they also did not find influence of MDR1/P-gp expression on the progression-free survival. In contrast, Yoh *et al*. (15) did not find any difference in the survival time with overexpression of MDR1/P-gp or benefits in progression-free survival. Further studies, analysing MDR1/P-gp as a potential predictive factor towards treatment with cytotoxic drugs did not find significant differences in the survival time of the patients with high or low expression of MDR1/P-gp (11, 16). However, an increased 5-year survival rate of NSCLC patients with lower expression of MDR1/P-gp following surgery and adjuvant chemotherapy using cisplatin was reported by Yokoyama *et al*. (12). According to some studies, higher levels of MDR1/P-gp expression can be observed in patients treated with neoadjuvant chemotherapy. This observation may suggest a potential role of this ABC transporter in drug resistance triggered by treatment with cytotoxic drugs (11).

In this study, we did not find any prognostic value for MRP1. These data are in agreement with those obtained by Yoh *et al*. (15). Interestingly, Berger *et al*. (11) found a higher average survival in patients with overexpression of MRP1. Unfortunately, in the multivariate analysis, they could not confirm the role of MRP1 as an independent prognostic factor of patient survival. In *in vitro* investigations, there was a correlation between expression of MRP1 and a broad spectrum of the drugs which are substrates for this protein, including cisplatin (17, 18).

Likewise, no impact of BCRP expression on NSCLC patient outcome could be found in this study. This observation is contrary to the conclusions of Yoh *et al*. (15) in which positive immunostaining for BCRP appeared to be a predictor of survival in patients with advanced NSCLC. However, a final assessment of a potential clinical impact of BCRP for NSCLC is currently not possible.

In recent years, considerable interest has arisen in the potential role of COX-2 as a prognostic factor for malignant diseases. Some studies are available that report a prognostic value of COX-2 expression in NSCLC (20-22). These correlations could not be confirmed in the group of patients investigated here. Thus, there is further need for clarification of the potential clinical impact of COX-2 in NSCLC.

The fact that in this study no correlation between expression of ABC transporters and clinical response of NSCLC patients could be found is in line with the findings of others (15, 16, 22-24). The absence of a positive correlation between the expression of the investigated ABC transporters, clinical response and survival rates may reflect the more sophisticated mechanisms of drug resistance in clinical settings than under *in vitro* conditions. However, a contribution of these transporters to a complex, multi-modal mechanism of clinical drug resistance of NSCLC cannot be excluded.

Studies are available that suggest a role of COX-2 in the regulation of the expression of MDR1/P-gp (4, 25). Strong positive correlation between the expression of COX-2 and MDR1/P-gp was observed in hepatocellular carcinoma (26), breast cancer (6) and ovarian cancer (5). Furthermore, it was reported that COX-2 inhibitors can modulate MRP1-dependent drug resistance (27). In this study, we show strong positive correlation between COX-2 expression and the levels of MDR1/P-gp and BCRP expression. Therewith, this is the first report that indicates a potential role of COX-2 for the regulation of BCRP. Taking all these observations into consideration, COX-2 may be a factor that can regulate the expression of all three ABC transporters, MDR1/P-gp, MRP1 and BCRP. Thus, COX-2 inhibitors should be considered as potential agents for the modulation of clinical drug resistance of NSCLC. However, further studies are necessary to substantiate this.

In conclusion, the data of this study did not show a prognostic or predictive value of COX-2, MDR1/P-gp, MRP1 or BCRP1 in NSCLC. However, it was found that clinical response to chemotherapy is a prognostic factor in a longer progression-free survival and COX-2 may be involved in the regulation of MDR1/P-gp and BCRP. These correlations should be analysed in more detail to demonstrate any potential application of COX-2 inhibitors for the supporting chemotherapeutic treatment of advanced stages of NSCLC.

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