Positive correlation between cyclooxygenase-2 and ABC-transporter expression in non-Hodgkin's lymphomas

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Abstract. One of the leading causes of chemotherapy failure in non-Hodgkin's lymphomas (NHLs) is multidrug resistance (MDR). MDR can be associated with expression of members of the family of ABC-transporters. Since a correlation between expression of cyclooxygenase-2 (COX-2) and MDR in various cancer cells was described, the expression of COX-2 and the ABC-transporters MDR1/P-glycoprotein (P-gp), MRP1, MRP2 and BCRP was examined in 56 previously non-treated patients by immunohistochemistry. The data show that: i) P-gp is not expressed in non-treated NHLs; ii) MRP2 can be localized in the nuclear membranes of NHL cells; iii) expression of MRP2 in the cytoplasm membrane correlates with clinical response; iv) elevated expression of BCRP is typical for the patients, who did not respond to primary chemotherapy and for cases with shorter progression-free survival time in a 30 months follow-up; and v) there is a strong correlation between COX-2 and MRP1, MRP2 and BCRP. It can be concluded that: i) BCRP may be a crucial factor involved in primary resistance of NHLs, thus it may be useful for prediction of chemotherapeutic treatment and risk of relapse; and ii) since there is strong correlation between COX-2 expression and MDR in NHLs, the application of COX-2 inhibitors may be considered for chemosensitization.

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Introduction

The primary method of treatment of the non-Hodgkin's lymphomas (NHLs) is chemotherapy. The method of the treatment is chosen on the basis of the histopathological type of the lymphoma and parallel assessment of the prognostic factors before the beginning of the treatment. Despite the increasing survival of patients with NHL by around 30% in the past 30 years, with most of the patients getting a primary response, the currently developed methods of treatment are not sufficient enough. Short-time relapse followed by the recurrence are the result of weaker or stronger response to the resistance of the following cycles of the chemotherapy (1). The main reason for the lack of the success in the treatment of the NHLs is the phenomenon of multidrug resistance (MDR). This phenotype is characterized by simultaneous resistance of tumor cells against various drugs of different chemical structure and modes of action. MDR can be associated with the expression of energy-dependent membrane drug extrusion pumps, i.e. members of the family of ABC (ATP-binding cassette)-transporters. The human genome project identified 48 human genes encoding ABC-transporters. Various studies showed that from those proteins, MDR1/P-glycoprotein (P-gp; or ABCB1), multidrug resistance proteins 1 and 2 (MRP1 and MRP2; or ABCC1 and ABCC2) and breast cancer resistance protein (BCRP, or ABCG2) can be associated with clinical drug resistance of malignant diseases (2).

In vitro studies of Patel *et al* (3) have demonstrated that cyclooxygenase-2 (COX-2) can up-regulate the expression of P-gp. In studies investigating samples of cancer patients, a strong positive correlation between expression of COX-2 and

Table I. Clinical data of studied patients and relationships between expression of studied proteins expression and clinical data.

Characteristics	No. (%)	COX-2 P-value	MRP1 P-value	MRP2n P-value	MRP2c P-value	BCRP P-value
All cases	56 (100)					
Age (mean ± SD)[b]	56.36±13.19					
≤60	32 (57)	0.8766	0.7182	0.6899	0.6233	0.8486
>60	24 (43)					
Sex[a]						
Female	24 (43)	0.6702	0.4163	0.5820	0.1535	0.5984
Male	32 (57)					
Ann Arbor stage[a]						
I/II	8 (16)	0.1655	0.6179	0.2868	0.2778	0.2064
III/IV	41 (84)					
Performance status[a]						
≥2	14 (56)	0.5359	0.8602	0.8187	0.7457	0.3022
<2	11 (44)					
Grade of malignancy[a]						
Low	26 (48)	0.5894	0.2413	0.3855	0.3805	0.4445
Moderate	27 (50)					
High	1 (2)					
Presence of systemic symptoms[a]						
Yes	34 (71)	0.9164	0.0873	0.7601	0.2372	0.3384
No	14 (29)					
IPI score[a]						
Low	24 (52)	0.3395	0.4884	0.4279	0.6713	0.0855
Medium/low	7 (15)					
Medium/high	8 (17)					
High	7 (15)					
Extranodal involvement[a]						
≤1	44 (86)	0.8277	0.9078	0.2292	0.2013	0.7306
>1	7 (14)					
BM/CNS involvement[a]						
Yes	17 (33)	0.8682	0.8874	0.8119	0.9449	0.4689
No	34 (67)					
LDH[c] level (befor Ch-th)[b]						
≤Norm	16 (62)	0.1876	0.4960	0.0839	0.9519	0.5039
>Norm	10 (38)					
LDH level (after Ch-th)[b]						
≤Norm	16 (53)	0.7132	0.7278	0.2505	0.4774	0.8587
>Norm	14 (47)					
$ß_2$-microglobulin[c] level[b]						
≤Norm	4 (33)	0.7426	0.9573	0.4279	0.8290	0.4902
>Norm	7 (67)					
Anemia[a]						
Yes	19 (38)	0.7461	0.8668	0.8632	0.0844	0.8521
No	31 (62)					
Infection of HCV[a]						
Yes	2 (4)	0.7203	0.0818	0.1096	0.4772	0.3497
No	52 (96)					

Table I. Continued.

Characteristics	No. (%)	COX-2 P-value	MRP1 P-value	MRP2n P-value	MRP2c P-value	BCRP P-value
All cases	56 (100)					
Infection of *H. pylori*[a]						
Yes	2 (4)	0.7203	0.9414	0.9005	0.3347	0.3629
No	52 (96)					
Clinical response[a]						
Complete response	10 (28)	1.000	0.4738	0.1880	**0.0469**	**0.0285**
Partial response	17 (49)					
Stable dissease	7 (20)					
Progressive dissease	1 (3)					
Relapse[a]						
Yes	7 (14)	0.3921	0.6054	0.9364	0.6539	0.4627
No	43 (86)					
Progression[a]						
Yes	31 (62)	0.8993	0.6933	0.7491	0.7756	0.9154
No	19 (38)					
Death[a]						
Yes	25 (50)	0.9906	0.2775	0.5708	0.3080	0.8928
No	25 (50)					

LDH, lactic dehydrogenase; HCV, hepatitis C type virus; *H. pylori*, *Helicobacter pylor*i. IPI, International Prognostic Index; Ch-th, chemotherapy. [a]ANOVA rank test of Kruskal-Wallis. [b]Spearmann's rank correlation; [c]Normal values for LDH, 200-480 U/l, for $ß_2$-microglobulin, 0.7-1.8 mg/l.

P-gp could be found in tumor tissues prepared from ovarian (4) and breast cancers (5). These and other experimental and clinical studies (6-9) suggest that the application of COX-2 inhibitors as supplementary cancer chemotherapy may increase the susceptibility of the cancer cells to the chemotherapy. Considering the fact that in the case of the NHL chemotherapy is the primary therapeutic strategy, potential alternative strategies for overcoming MDR in NHL is of major clinical interest.

Thus, the goal of this study was to analyze a potential correlation between the expression of COX-2 and clinical important ABC-transporters (P-gp, MRP1, MRP2 and BCRP) in patients suffering from NHL. We have also analyzed the prognostic value of the selected parameters and considered the subcellular localization of MRP2, which was found to be expressed not only in the cytoplasm membrane, but also in the nuclear membrane of ovarian cancer cells (10). In ovarian cancer, the nuclear localization correlated with disadvantageous prognosis of cytostatic drug treated patients.

Materials and methods

Patients. Immunohistochemical analysis was conducted retrospectively in samples of tumors sent for routine diagnostic studies to the Department of Pathology, University School of Medicine in Wroclaw, from 1994-2003, obtained from patients treated in the Department of Hematology, Blood Neoplasia and Bone Marrow Transplantation, University School of Medicine in Wroclaw (Poland). A group of 56 previously non-treated patients with NHL (Table I) qualified for these studies, including 52 cases of lymph node tumors and 4 cases of extra nodular tumors. In 49 cases, B-cell lymphomas were diagnosed, in 5 cases the lymphomas originated from T-cells. Clinical advancement of the disease was classified according to the Ann Arbor scale. The group of lymphomas manifesting low malignancy included 26 cases (14 women and 12 men), the group of aggressive lymphomas included 27 cases (9 women and 18 men) and in one case a very aggressive lymphoma was diagnosed. In two cases documentation on the course of the disease was incomplete. The study was approved by an Institutional Review Board and the patients gave their informed consent before their inclusion into the study. All the patients were subjected to the standard protocols of chemotherapy and radiotherapy and subsequently were monitored by periodic medical check-ups. In the study period 7 patients (14%) demonstrated relapse, 31 patients (62%) showed progression and 25 patients deceased (50%). The mean progression-free survival amounted to 32 months (ranging from 1 to 102 months), while mean duration of relapse-free survival was 40 months (ranging from 1 to 102 months). In the statistical analysis, the cases were allocated

into four groups according to the International prognostic index (IPI) including patients of low risk, those of moderately low risk, those of moderately high risk and patients of high risk. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. In each case, hematoxylin and eosin stained preparations were subjected to histopathological evaluation by two pathologists.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene, and gradually hydrated. Activity of endogenous peroxidase was blocked by 30-min exposure to 1% H_2O_2. All the studied sections were boiled for 20 min at 500 W in the Antigen Retrieval Solution (DakoCytomation, Poland). Immunohistochemical reactions were performed using monoclonal mouse antibodies against COX-2 (Cayman Chemical Co., Ann Arbor, MI, USA) at dilution of 1:2000, monoclonal mouse antibodies (clone C219) against P-gp (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:100, monoclonal mouse antibodies (clone MRPr1) against MRP1 (Monosan, The Netherlands) at a dilution of 1:100, monoclonal mouse antibodies (clone M2I-4) against MRP2 (Monosan, The Netherlands) at a dilution of 1:100 and monoclonal mouse antibodies (clone BXP-21) against BCRP (Alexis Biochemicals) at a dilution of 1:100. The antibodies were diluted in Antibody Diluent with background reducing component (DakoCytomation). Tested sections were incubated with antibodies against COX-2 for 1 h at room temperature (RT) and with the other antibodies overnight at 4˚C. Subsequent incubations involved biotinylated antibodies (20 min, RT) and streptavidin-biotinylated peroxidase complex (20 min, RT) using an LSAB$^+$ HRP system (DakoCytomation). DAB$^+$, Liquid (DakoCytomation) was used as a chromogen (7 min, RT). All sections were counterstained with Meyer's hematoxylin (20 sec).

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 were evaluated using a semi-quantitative IRS (ImmunoReactive Score) scale (11), which took into account intensity of color reaction and percentage of positive cells. The result represented product of scores allocated for the evaluated traits and it ranged between 0 and 12.

Control reactions. In each case, negative controls were performed as described above but without the monoclonal antibody, which was substituted by PBS. 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 specificity of COX-2 antibodies, we (5) and other investigators (12) performed blocking experiments using a COX-2 blocking peptide (Cayman Chemical Co.) according to the manufacturer's instructions. In the case of the MRP2, the analysis of the subcellular expression using immunocytochemistry, immunohistochemistry and ultra-immunocytochemistry, and immunohistochemical analysis of the expression in the normal human cells (together with normal lymphatic nodes) were described in our previous work (10).

Statistical analysis. The obtained results were subjected to statistical analysis using Statistica 97 PL software (Statsoft, Poland). In order to examine the relationship between expression of the studied proteins on one hand and age of patients, serum protein levels (LDH, ß$_2$-microglobulin) on the other Spearmann's rank correlation was used. The relationship between protein expression intensity and clinical and pathological variables Kruskal-Wallis rank test (ANOVA) was used. Evaluation of the relationship between expression of the protein on one hand and total survival and progression-free survival on the other took advantage of Kaplan-Meier analysis using SPSS software (version 10.0, SPSS Inc., Chicago, IL, USA). The survival time was defined as the period between establishing the diagnosis and death or time of last observation while the relapse-free survival time was defined as the period between establishing the diagnosis and manifestation of progression or relapse. Value of $P<0.05$ was assumed to indicate statistical significance.

Results

Expression of COX-2 and ABC-transporters in non-Hodgkin lymphomas. Immunohistochemical reactions were carried out on the samples of the 56 patients with NHL. In the evaluation of the expression of the proteins data from three patients were omitted because of damage of the investigated material.

Expression of COX-2 was found in 42 cases (79%). Immunohistochemical staining was localized in the cytosplasm and revealed different stage of the saturation in individual cases (Fig. 1A). Average intensity of the reaction in the IRS scale was 3.82±3.53 SD.

We did not observe expression of the P-gp in any of the NHL cases (Fig. 1B). As positive controls, sections originating from normal human liver were used. As expected, clear staining signals in the apical part of the cytoplasm membrane of the hepatocytes could be detected (Fig. 1B).

Expression of MRP1 was observed in 43 cases (81%) of NHL. Staining reaction was localized in the cytoplasm membrane and in selected cytoplasm granulations with different saturation in individual cases (Fig. 1C). Average intensity of the reaction in the IRS scale was 4.08±3.76 SD. In the positive control, a staining effect was observed in the membrane and in the cytoplasm granulations of hepatocytes (Fig. 1C).

Expression of BCRP was observed in 51 cases (96%) of NHL. The localization of the staining reaction was predominantly distributed to the cytoplasm membrane with different saturation in individual cases (Fig. 1D). Average intensity of the reaction in the IRS scale was 4.87±3.04 SD. In the positive control, a staining effect was observed in the apical part of the cytoplasm membrane and in the cytoplasm part of hepatocytes (Fig. 1D).

Expression of MRP2 was observed in 38 cases (72%). Staining reaction was localized in the cytoplasm membrane and within the cytoplasm (MRP2c) (Fig. 1F), and in 48 cases (91%) MRP2 was localized in the nuclear membrane

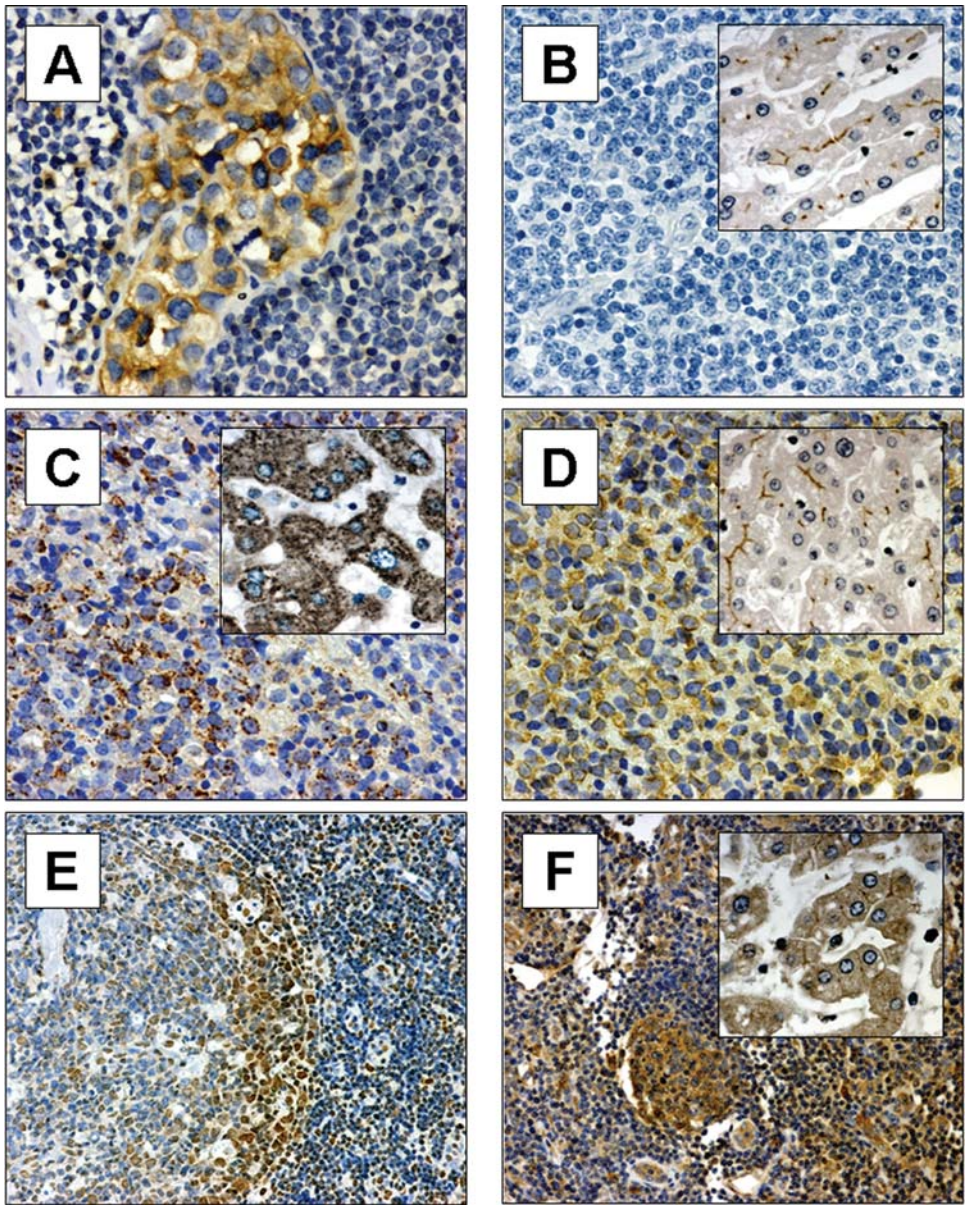

Figure 1. Immunohistochemical localization of (A) COX-2, (B) P-glycoprotein, (C) BCRP, (D) MRP1, (E) nuclear MRP2 and (F) cytoplasmic/membranous MRP2 expression in the sections originating from NHLs. Inserts, control reactions in the healthy human liver samples.

(MRP2n) (Fig. 1E). Average intensity of the reaction in the IRS scale for MRP2c was 2.61±2.58 SD, and for MRP2n was 4.96±3.54 SD. In the positive control, staining effect was observed in the apical part of the cytoplasm membrane of hepatocytes (Fig. 1F).

Relationships between COX-2 and ABC-transporter expression. We have investigated potential correlations between expression of COX-2 and expression of different ABC-transporters in NHLs. We found significant statistical correlation between expression of COX-2 and MRP1 (P=0.0002) (Fig. 2A), MRP2n (P=0.027) (Fig. 2B), MRP2c (P=0.027) (Fig. 2C) and BCRP (P=0.006) (Fig. 2D). Furthermore, the calculations demonstrate that there is a positive correlation between expression of BCRP and MRP1 (P=0.026) and MRP2c (P=0.011) as well as between expression of MRP1 and MRP2c (P=0.032).

Relationships between expression of studied proteins and clinico-pathological data. The clinico-pathological parameters of the NHL cases are summarized in Table I. We did not find any statistically significant correlation between expression of COX-2, MRP1 and MRP2n and clinico-pathological data from patients (P>0.05; Table I). However, expression of MRP2c and BCRP correlated with clinical response (P=0.0469 and P=0.0285, respectively), but we did not observe a correlation between the expression of these proteins with the data obtained from patients (P>0.05; Table I).

Expression of studied proteins versus total survival time and relapse-free survival time. Survival time was defined as a time from the diagnosis to death or last observation, and free from progression time was defined as a time between diagnosis to the observed progression or recurrence.

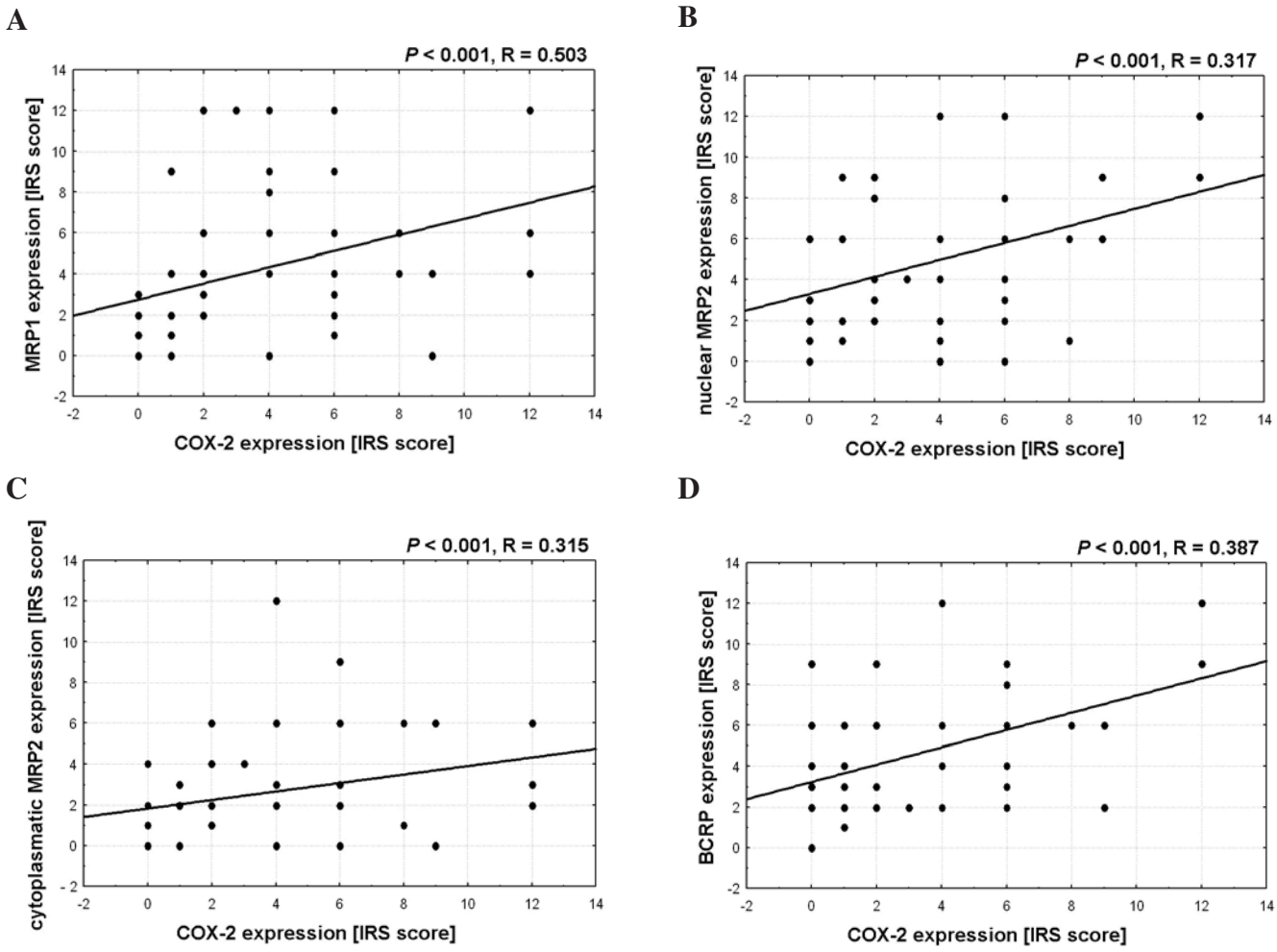

Figure 2. Positive correlation between COX-2 expression and the expression of (A) MRP1, (B) nuclear MRP2, (C) cytoplasmic MRP2 , and (D) BCRP.

To find potential correlations between the expression of COX-2, ABC-transporters and survival time or progression-free survival time, Kaplan-Meier analyses were performed. Survival or progression-free survival time was comparable between groups with different levels of the expression of the investigated proteins. We have separated the following groups: group with the lover expression level of COX-2 (IRS 0-2), MRP1 (IRS 0-3), MRP2n (IRS 0-2), MRP2c (IRS 0-2) and BCRP (IRS 0-4) and the group with the higher expression levels of COX-2 (IRS 3-12), MRP1 (IRS 4-12), MRP2n (IRS 3-12), MRP2c (IRS 3-12) and BCRP (IRS 5-12). No correlation between expression of the investigated proteins and total observation time or progression-free survival time could be found (P>0.05; Table II).

Taking into consideration the fact that the investigated group was treated intensively by chemotherapy, which should significantly influence the biology of the cancer cells, we have investigated correlation between expression of COX-2, ABC-transporters and progression-free time and overall survival time analyzing short-term follow-ups. Calculations were performed by the shortening the observation time to 50 and 30 months. Kaplan-Meier analysis demonstrated that progression-free time was much shorter in the group with the higher expression of the BCRP during the first 30 months of observation (P=0.0378; Table II; Fig. 3). No influence of BCRP expression on the survival time during the 30th and 50th month and the progression-free time during the 50-month observation could be found (P>0.05; Table II; Fig. 3). The analyses demonstrate that expression of COX-2, MRP1 and MRP2 does not influence survival and progression-free time in the shortened observation time (P>0.05; Table II).

Discussion

We have investigated the expression of COX-2 and different ABC-transporters in patients with NHL and analyzed the potential correlation between the expression of these factors and clinico-pathological parameters. Expression of COX-2 was detected in 79% of the cases; expression of MRP1 was found in 81% of NHLs; cytoplasm membrane localized MRP2 was stained in 72% of the patient samples whereas 91% were positive for MRP2 in the nuclear membrane; and 96% of the NHL expressed BCRP. All NHL cases were completely negative for P-gp expression.

Revealing anti-apoptotic and pro-angiogenetic effects, over-expression of COX-2 promotes development and progression of the multiple cancers, e.g. colon, oesophagus, stomach, lung cancer, mammary gland, ovarian, skin, prostate (12-16). The presence of COX-2 in 79% of the NHL cases suggests a putative participation of this enzyme in the pathogenesis of

Table II. Relationships between expression of studied proteins on one hand and duration of survival and of progression-free survival measured in months on the other (Kaplan-Meier analysis).

Studied protein (IRS score)	No. of cases	Total observation time		50-month observation		30-month observation	
		OST[a] P-value	PFS[b] P-value	OST[a] P-value	PFS[b] P-value	OST[a] P-value	PFS[b] P-value
COX-2							
0-2	19						
3-12	22	0.3323	0.5542	0.2490	0.4555	0.0611	0.1687
MRP1							
0-3	20						
4-12	22	0.7932	0.4925	0.5988	0.3761	0.7875	0.8159
MRP2n							
0-2	14						
3-12	28	0.7628	0.3900	0.9367	0.4638	0.7535	0.3906
MRP2c							
0-2	25						
3-12	17	0.4239	0.5936	0.5325	0.4836	0.5288	0.2900
BCRP							
0-4	23						
5-12	19	0.3568	0.1402	0.2965	0.1045	0.1183	**0.0378**

[a]OST, overall survival; [b]PFS, progression-free survival.

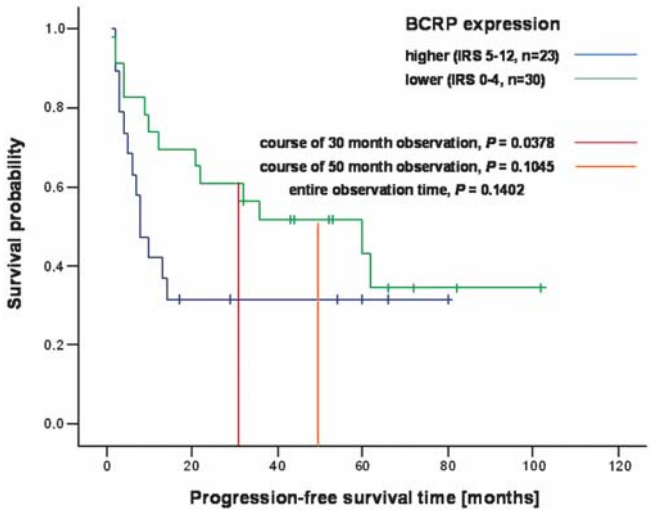

Figure 3. Kaplan-Meier's progression-free survival curves in the studied group of NHLs of lower and of higher expression of BCRP in the course of 30, 50 and 102 months. After 30 months of observation patients with higher BCRP expression manifested significantly shorter progression-free survival.

NHL, similar to the development of other malignant diseases. In this study, no correlation was found between elevated expression levels of COX-2 in the neoplastic cells and clinical and/or pathological data of the NHL patients. Likewise, no correlation between COX-2 and survival time or progression-free time could be observed.

Expression of the multiple ABC-transporters in most cases with non-treated NHL may suggest a role in the development of the drug resistance in this type of lymphoma. Our studies have shown the positive correlation between expression of BCRP and cytoplasmic expression of MRP2 and clinical response. We have also shown that patients with elevated expression levels of BCRP have shorter progression-free time in the short-term 30-month observation period. The high degree of differentiation in the investigated NHL cases indicates that BCRP may play an important role in primary resistance of NHLs to chemotherapy. The fact that NHL patients were subjected to intensive therapy regimens using several drugs and chemotherapy cycles, the lack between expression of BCRP and the absence of BCRP-dependent impact on patient survival during longer observation time is obvious. Thus, NHL cells exposed to anticancer drugs may develop additional resistance mechanisms.

In this study, for the first time expression of MRP2 in the nuclear membrane of NHL cells was demonstrated. The observation that MRP2 can be localized on nuclear membranes of NHL cells is in line with previous observations (10). It was demonstrated that nuclear membraneous expression of MRP2 is typical for the cells with lower differentiation level in multiple normal tissues. Furthermore, nuclear membraneous MRP2 expression correlated with shorter survival time of ovarian cancer resistant to cisplatin. However, in this study no correlation between nuclear membraneous MRP2 and clinical and/or pathological data could be found. In the previous study (10), a nuclear membrane expression of MRP2 was found in normal lymphocytes of lymphatic nodes. Similar observations

were made in the case of lymphocytes in the spleen. These observations indicate that nuclear membraneous expression of MRP2 in NHL cells may be a consequence of their origin and not the result of their lower differentiation.

Expression of the MRP1 does not influence the response after the treatment and survival time in the NHLs. Similar results were described earlier for the lymphomas or patients with AML (17-19). Contradictory results were obtained by Ohsawa *et al* (20), who found that expression of the MRP1 is significantly correlated with poor response to chemotherapy in B-cell lymphomas.

Several reports suggest cross dependence between expression of the COX-2 and P-gp (3,8). Strong positive correlation between expression of the COX-2 and P-gp was found in hepatocellular cancer cells (21), breast cancer cells (5) and ovarian cancer cells (4). Some reports show also inhibiting effect of COX-2 inhibitors on MRP1-dependent drug resistance (17). This effect may be explained by a similar regulatory mechanism, in which expression of COX-2 and MRP1 is induced by the tumor necrosis factor (TNF) lipopolysaccharides (LPS) (22). It is also well known that products of lipooxygenase and cyclooxygenase proinflammatory factors, such as leukotrienes (LTC_4) and prostaglandins (PGA_1, PGA_2) are substrates of MRP1 and MRP2. Kang *et al* (23) showed in lung cancer cells that increased expression of COX-2 was associated with elevated expression level of MRP1. Treatment of these cells with the COX-2 inhibitor celecoxib decreased the expression of that drug extrusion pump.

In conclusion, expression of COX-2, MRP1 and nuclear membraneous MRP2 have no prognostic nor predictive value in NHLs. Subcellular localization of MRP2 in the nuclear envelope of NHL cells was desribed for the first time in this study. Expression of MRP2 in the cytoplasm membrane may be useful for the prediction of the clinical response to primary chemotherapy. The strong positive correlation between expression of COX-2 and the ABC-transporters MRP1, MRP2, and BCRP may represent the basis for further clinical investigations of potential therapeutic applications of COX-2 inhibitors for chemosensitization in NHLs. The application of the relatively inexpensive COX-2 inhibitors may significantly increase the susceptibility of NHL cells to chemotherapy. Since NHL is a deadly disease, the risk of cardiovascular side-effects of COX-2 inhibitors may be reasonable. Since expression of BCRP is correlated with clinical response and shorter pro-gression-free survival time, this ABC-transporter appears to be involved in the primary resistance of NHL cells to chemotherapy. BCRP may be useful as predictive factor for chemotherapy response and risk of relapse of NHL.

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