

Prognostic Significance of Lung Resistance Protein (LRP) and Multidrug Resistance Protein (MRP1) in Patients with Diffuse Large B-Cell Lymphomas (DLBCL)

Wael H. ElSawy, M.D.¹; Fouad M. Abou Taleb, M.D.²; Mohamad Abdel Kader, M.D.³; Alaa A. Omeran, M.D.⁴ and Amal F. Gharib, M.D.⁵

The Departments of Clinical Oncology^{1,3}, Medical Oncology², Clinical Pathology⁴, Medical Biochemistry⁵, Faculty of Medicine, Zagazig University^{1,2,4,5}; Beni-Suef Branch of Cairo University³.

ABSTRACT

Aim of the work: Drug resistance of non-Hodgkin's lymphomas may involve mechanisms of the multidrug resistance phenotype including the lung resistance protein (LRP) and the multidrug resistance protein (MRP1). To determine the prognostic significance of these multidrug resistance factors, we studied LRP and MRP1 expression and their impact on clinical outcome in previously untreated 48 patients with diffuse large B-cell lymphomas.

Patients and methods: LRP and MRP1 expression were immunohistochemically assessed by means of the monoclonal antibodies LRP-56 and MRPr1, respectively.

Results: LRP was positive in 23% and MRP1 in 44% of the patients. LRP expression was associated with higher tumor stage ($p = 0.03$), elevated serum lactate dehydrogenase levels ($p = 0.01$) and the International Prognostic Index ($p = 0.0001$). LRP-positive patients had a lower complete response rate to chemotherapy than LRP-negative patients (18 versus 65%; $p = 0.006$) and a shorter overall survival (median of 0.9 years versus undetermined, $p = 0.001$). MRP1 expression was independent of clinical and laboratory parameters and had no impact on the outcome of chemotherapy or survival of the patients.

Conclusion: Our results suggest that LRP expression may be an important mechanism of drug resistance and is associated with a worse clinical outcome in previously untreated diffuse large B-cell lymphomas. Thus, the reversal of LRP-mediated drug resistance may improve clinical outcome in diffuse large B-cell lymphoma in the future.

Key Words: Non-Hodgkin's lymphoma - Chemotherapy - LRP - MRP1.

INTRODUCTION

Diffuse large B-cell lymphomas (DLBCL) can be effectively treated with conventional combination chemotherapy regimens with or without radiotherapy [13,31]. In addition, high-

risk patients may benefit from either high-dose consolidation treatment with hematopoietic stem cell support after having achieved complete response from initial chemotherapy [17] or initial high-dose induction chemotherapy with stem cell support [15]. Despite these improvements, 40-50% of the patients are not cured by chemotherapy because of drug-resistant disease [17].

Multidrug resistance (MDR) is an important type of drug resistance that is clinically relevant in leukemias [30,36] and several solid tumors [12]. Different mechanisms can contribute to MDR; some of them have already been studied in non-Hodgkin's lymphomas. MDR1/P-glycoprotein expression occurs with various frequencies in lymphomas and is associated with clinical drug resistance to various anticancer drugs including anthracyclines and Vinca alkaloids [12,41]. Clinical trials to overcome P-glycoprotein-mediated resistance in drug-refractory lymphoma by combining chemotherapy with resistance modifiers indicated that, at least in a subset of patients with drug-refractory lymphoma, modulation of P-glycoprotein function is feasible. This suggests that P-glycoprotein expression plays a role in the drug resistance of lymphomas [32,49,51]. MRP1, another important factor involved in MDR, is also expressed in lymphomas [52], but its impact on clinical outcome remains to be determined. Alterations in apoptosis and cell cycle regulation are also involved in drug resistance of lymphomas [22,33,39,50]. p53 mutations were associated

with poor outcome of chemotherapy and shorter survival in aggressive B-cell lymphomas [22] and in relapsed or drug-refractory non-Hodgkin's lymphomas [50]. The cyclin-dependent kinase inhibitor p27^{Kip1} has also been shown to be involved in drug resistance [45] and lack of its expression is associated with shorter disease-free survival and overall survival in patients with DLBCL [39].

LRP is another protein that is associated with MDR. It was first detected in a non-P-glycoprotein-multidrug-resistant lung cancer cell line [43] and has been shown to be the human major vault protein [42]. Vaults are complex ribonucleoprotein particles that, in addition to the major vault protein, also contain several minor vault proteins and a small RNA [27]. Vaults are located mainly in the cytoplasm and to a smaller degree, also in the nuclear membrane. They are believed to mediate intracellular and in particular, nucleocytoplasmic transport [27]. LRP expression of tumor cell lines is associated with resistance to doxorubicin, vincristine, carboplatin, cisplatin and melphalan [24]. A recent report by Kitazono et al. [28] provides evidence that LRP expression is involved in resistance to doxorubicin, vincristine, etoposide, paclitaxel and gramicidin D and that LRP is associated with the transport of doxorubicin from the nucleus to the cytoplasm. LRP is physiologically overexpressed in colon tissue, lung tissue, renal proximal tubules, adrenal cortex and macrophages, but its physiological function remains to be evaluated [23].

Because LRP and MRP1 affect drugs commonly used in the treatment of DLBCL, expression of these proteins may affect response to chemotherapy and survival in DLBCL. To further address this possibility, we have studied LRP expression and MRP1 expression in non-Hodgkin's lymphoma and their association with both response to chemotherapy and survival of the patients.

PATIENTS AND METHODS

Forty-eight previously untreated patients (21 females, 27 males) with DLBCL, diagnosed between 1997 and 1999, were included in this study. All of the biopsy samples were classified according to the criteria provided in the Revised European-American Lymphoma (REAL) classification [1,18,19].

The clinical characteristics of the patients are summarized in Table (1). All patients were clinically examined and subjected to routine laboratory and radiological investigations for proper staging. All patients received standard therapy with CHOP protocol. Cycles were repeated every 21 days. Patients with stage I with bulky disease received 3-4 cycles of chemotherapy plus involved field radiotherapy (30 Gy, 2 Gy/fraction over 3 weeks).

Patients with stage II-IV disease received 6 cycles of chemotherapy. Two of them with bulky disease who achieved a complete response were treated with additional involved field radiotherapy after 6 cycles of chemotherapy. All of the patients were evaluable for response. Response to chemotherapy was assessed according to standard criteria [4,47]. Complete response was defined as the absence of clinical and radiological evidence of disease for a minimum of at least 2 months. Age, tumor stage, serum lactate dehydrogenase, performance status and the number of extranodal sites of the disease were used to determine the International Prognostic Index [46]. For statistical analysis, patients were grouped into low-risk (International Prognostic Index, 0-1), intermediate-risk (International Prognostic Index, 2-3) and high-risk (International Prognostic Index, 4-5) patients.

Immunohistochemical detection of LRP and MRP1:

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded lymphoma specimens. Paraffin sections were mounted on poly-L-lysine-coated glass microslides. Sections were deparaffinized and rehydrated by consecutive submersions in xylene (two changes, 10 min each), absolute ethanol (two changes, 5 min each), 70% ethanol (two changes, 5 min each) and distilled water (3 min). Endogenous peroxidase activity was blocked by incubation in 0.06% H₂O₂ for 10 min at room temperature and slides were washed in PBS. The tissues were preincubated for 20 min in normal serum (normal goat serum 1:50; Dako, Glostrup, Denmark) prior to incubation for 2 h with either the LRP-56 monoclonal antibody (Alexis, Läufelfingen, Switzerland) or the MRP1 monoclonal antibody (Alexis). Antibody binding was detected by the avidin-biotin-peroxidase method. Bound peroxidase was developed with 3,3'-diaminobenzidine (Dako). The slides were

counterstained with Mayer's Hämalaun and mounted with Aquatex (Merck, Darmstadt, Germany). All of the washes were performed in PBS.

Staining of lymphoma cells was examined. Specimens were scored for the percentage of lymphoma cells showing granular cytoplasmic staining in the case of LRP and cytoplasmic and/or membranous staining in the case of MRP1.

Statistical analysis:

Associations between LRP or MRP1 and clinical as well as laboratory parameters were assessed by χ^2 test, Fisher's exact test, or exact Mann-Witney test. Survival probabilities were calculated with the product limit method according to Kaplan-Meier. Overall survival time was defined as the period between the time of diagnosis and the time of relapse or death. Differences between survival curves were analyzed by means of the log-rank test. Logistic regression models and Cox proportional hazards regression models were used to assess the independent effects of covariables on survival. All of the p -values are results of two-sided tests. Data were entered, checked and analyzed using EPI-INFO (version 6.1) software package [5].

RESULTS

LRP and MRP1 expression in DLBCL at diagnosis:

LRP expression of previously untreated patients was immunohistochemically determined by means of the monoclonal antibody LRP-56. LRP staining was detected as characteristic granular cytoplasmic staining and ranged from 0 to 60%. In the case of positive staining, at least 10% of the lymphoma cells were stained. LRP expression was scored positive if any of the lymphoma cells showed brown cytoplasmic staining (Fig. 1A and B). LRP was positive in 11 (23%) of 48 lymphoma specimens at diagnosis (Table 1). MRP1 staining ranged from 0 to 30% of the lymphoma cells. Any brown staining of lymphoma cells either cytoplasmic or membranous was scored as positive expression (Fig. 2); MRP1 expression was positive in 21 (44%) of 48 samples (Table 2).

Correlation of LRP and MRP1 with clinical and laboratory parameters:

The major clinical and laboratory findings of the patients are summarized in Table (1). There

was no significant association between LRP expression and age, sex, or β_2 -microglobulin (Table 1). However, LRP expression was more frequently observed in patients with stage III and IV disease and in patients with elevated serum lactate dehydrogenase (> 240 units/liter; Table 1). Whereas, 24 of 25 low-risk patients (International Prognostic Index, 0-1) were LRP-negative, 6 of 18 intermediate-risk (International Prognostic Index, 2-3) and 4 of 5 high-risk patients (International Prognostic Index, 4-5) were LRP-positive ($p = 0.0001$; Table 1).

MRP1 expression was independent of age, sex, β_2 -microglobulin, serum lactate dehydrogenase and the International Prognostic Index (Table 2). In addition, no correlation between MRP1 and LRP expression was observed.

LRP and MRP1 expression and response to chemotherapy:

All of the patients were evaluable for response to chemotherapy. The complete response rate was 54%. Partial responses and no responses were seen in 21 and 25% of the patients, respectively. The complete response rate was 65% for patients without LRP expression but only 18% for patients with LRP expression ($p = 0.006$; Table 3). Partial responses and no responses occurred in 8 (22%) and 5 (13%) of LRP-negative patients but in 2 (18%) and 7 (64%) of LRP-positive patients. With regard to MRP1, the complete response rate was 56% for MRP1-negative patients and 52% for MRP1-positive patients ($p = 0.8$; Table 3). Tumor stage ($p = 0.001$), serum lactate dehydrogenase ($p = 0.01$) and the International Prognostic Index (0.0001) were also significantly associated with complete response Table (3).

A logistic regression analysis that included LRP and the International Prognostic Index was performed. In the univariate analysis, the odds ratios for no complete response were 8.3 for LRP ($p = 0.006$) and 9.5 for the International Prognostic Index ($p = 0.0001$; Table 4). In the multivariate analysis, the odds ratios for no complete response were 2.3 for LRP ($p = 0.4$) and 7.6 for the International Prognostic Index ($p = 0.004$; Table 4).

LRP and MRP1 expression and survival:

Overall survival was estimated according to Kaplan-Meier. Fifteen patients died (7 LRP-negative patients, 8 LRP-positive patients).

Overall survival was significantly shorter in patients with LRP expression (Fig. 3). At a median follow-up of 2.1 years, median overall survival of all of the patients was not reached. Median overall survival was 0.9 years for LRP-positive patients and was not reached for LRP-negative patients ($p = 0.001$). As regard MRP1, 8 MRP1-negative patients and 7 MRP1-positive patients died. Median overall survival was not different between patients with MRP1 expression and those without MRP1 expression ($p = 0.9$) (Fig. 4). In patients with stage II-IV disease ($n = 37$), overall survival remained significantly

shorter in LRP-positive patients than in LRP-negative patients (median 0.9 years versus median not reached; $p = 0.03$).

In the univariate Cox regression analysis, the relative risk for death was 4.9 for LRP ($p = 0.001$) and 4.6 for the International Prognostic Index ($p = 0.0001$; Table 5). In the multivariate Cox regression analysis that included LRP and the International Prognostic Index, the relative risk for death was 1.4 for LRP ($p = 0.6$) and 4.0 for the International Prognostic Index ($p = 0.005$; Table 5).

Table (1): Correlation of LRP expression and characteristics of patients with DLBCL.

	Total	LRP-negative patients n (%)	LRP-positive patients n (%)	<i>p</i>
Number of patients	48	37 (100)	11 (100)	
<i>Age:</i>				
≤ 60 years	29	24 (65)	5 (45)	0.3 ^a
> 60 years	19	13 (35)	6 (55)	19
<i>Sex:</i>				
Male	27	20 (54)	7 (64)	0.7 ^a
Female	21	17 (46)	4 (36)	
<i>Stage:</i>				
I + II	23	21 (57)	2 (18)	0.03 ^b
III + IV	25	16 (43)	9 (82)	
<i>Lactate dehydrogenase:</i>				
Normal (≤ 240 units/liter)	20	19 (51)	1 (9)	0.02 ^a
Elevated (> 240 units/liter)	28	18 (49)	10 (91)	
<i>β₂-microglobulin:</i>				
Normal (≤ 3 mg/liter)	31	24 (77)	7 (78)	1.0 ^a
Elevated (> 3 mg/liter)	9	7 (23)	2 (22)	
<i>International prognostic index:</i>				
0+1	25	24 (65)	1 (9)	0.0001 ^c
2+3	18	12 (32)	6 (55)	
4+5	5	1 (3)	4 (36)	

^a *p* of Fisher's exact test.

^b *p* of χ^2 test.

^c Exact Mann-whitney test.

Table (2): Relationship of MRP1 and characteristics of patients with DLBCL.

	Total	MRP1-negative patients n (%)	MRP1-positive patients n (%)	<i>p</i>
Number of patients	48	27 (100)	21 (100)	
<i>Age:</i>				
≤ 60 years	29	15 (56)	14 (67)	0.4 ^a
> 60 years	19	12 (44)	7 (33)	
<i>Sex:</i>				
Male	27	15 (56)	12 (57)	0.9 ^a
Female	21	12 (44)	9 (43)	
<i>Stage:</i>				
I + II	23	13 (48)	10 (48)	0.97 ^a
III + IV	25	14 (52)	11 (52)	
<i>Lactate dehydrogenase:</i>				
Normal (≤ 240 units/liter)	20	12 (44)	8 (38)	0.7 ^a
Elevated (> 240 units/liter)	28	15 (56)	13 (62)	
<i>β₂-microglobulin:</i>				
Normal (≤ 3 mg/liter)	31	17 (77)	14 (78)	1.0 ^b
Elevated (> 3 mg/liter)	9	5 (23)	4 (22)	
<i>International prognostic index:</i>				
0+1	25	15 (56)	10 (48)	0.5 ^c
2+3	18	10 (37)	8 (38)	
4+5	5	2 (7)	3 (14)	

p of x² test. ^b *p* of Fisher's exact test. ^c Exact Mann-whitney test.

Table (3): Relationship of various predictors and outcome to chemotherapy.

	Total	Complete response n (%)	No complete response n (%)	<i>p</i>
<i>LRP:</i>				
Negative	37	24 (65)	13 (35)	0.006 ^a
Positive	11	2 (18)	9 (82)	
<i>MRP1:</i>				
Negative	27	15 (56)	12 (44)	0.8 ^a
Positive	21	11 (52)	10 (48)	
<i>International prognostic index:</i>				
0+1	25	20 (80)	5 (20)	0.0001 ^b
2+3	18	6 (33)	12 (67)	
4+5	5	0 (0)	5 (100)	
<i>Age:</i>				
≤ 60 years	29	18 (62)	11 (38)	0.2 ^a
> 60 years	19	8 (42)	11 (58)	
<i>Sex:</i>				
Male	27	12 (44)	15 (56)	0.1 ^a
Female	21	14 (67)	7 (33)	
<i>Stage:</i>				
I + II	23	18 (78)	5 (22)	0.001 ^a
III + IV	25	8 (32)	17 (68)	
<i>Lactate dehydrogenase:</i>				
Normal (≤ 240 units/liter)	20	15 (75)	5 (25)	0.01 ^a
Elevated (> 240 units/liter)	28	11 (39)	17 (61)	
<i>β₂-microglobulin:</i>				
Normal (≤ 3 mg/liter)	31	21 (68)	10 (32)	0.02 ^c
Elevated (> 3 mg/liter)	9	2 (22)	7 (78)	

p of x² test. ^b *p* of Fisher's exact test. ^c Exact Mann-whitney test.

Table (4): Logistic regression analysis of no complete response.

	Univariate			Multivariate		
	Odds ratio	95% CI ^a	<i>p</i>	Odds ratio	95% CI	<i>p</i>
LRP	8.3	1.6-44.3	0.006	2.3	0.3-16.5	0.4
International Prognostic index	9.5	2.7-33.7	0.0001 ^b	7.6	2.0-29.6	0.004 ^b

^a CI, confidence interval.

^b Test for trend.

For this analysis, the International Prognostic Index was grouped into low risk (0-1), intermediate risk (2-3) and high risk (4-5).

Table (5): Cox regression analysis of overall survival.

	Univariate			Multivariate		
	Odds ratio	95% CI ^a	<i>p</i>	Odds ratio	95% CI	<i>p</i>
LRP	4.9	1.7-14.3	0.001	1.4	0.4-5.5	0.6
International Prognostic index	4.6	2.2-9.9	0.0001 ^b	4.0	1.5-10.6	0.005 ^b

^a CI, confidence interval.

^b Test for trend.

For this analysis, the International Prognostic Index was grouped into low risk (0-1), intermediate risk (2-3) and high risk (4-5).

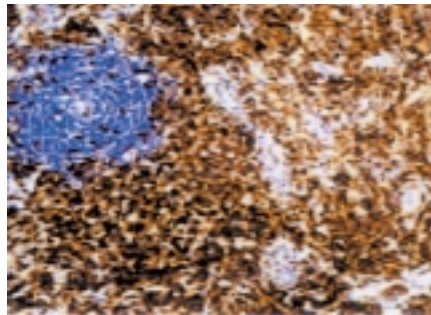


Fig. (1-A)

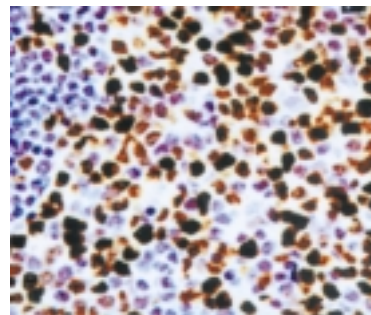


Fig. (1-B)

Fig. (1): LRP expression by lymphoma cells LRP-56 monoclonal antibody, A) x 100 (low-power) and B) x 400 (high-power).

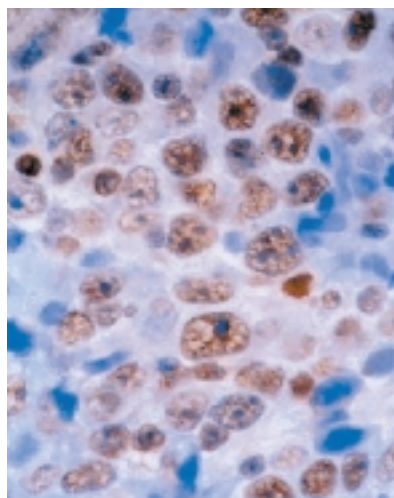


Fig. (2): MRP1 expression by lymphoma cells using MRP1 monoclonal antibody (x 400).

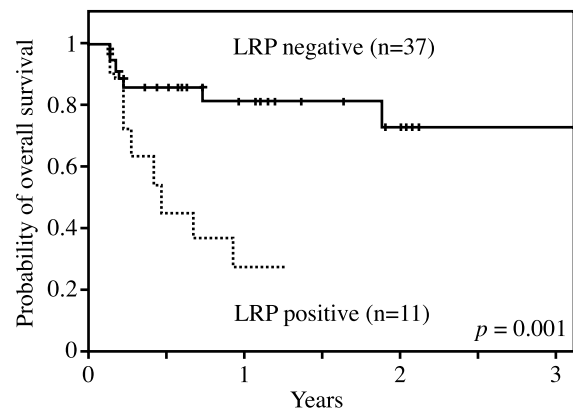


Fig. (3): LRP and overall survival estimated according to Kaplan-Meier in 48 patients. Survival data based on LRP expression are shown. Statistical comparison between survival curves was done by the log-rank test.

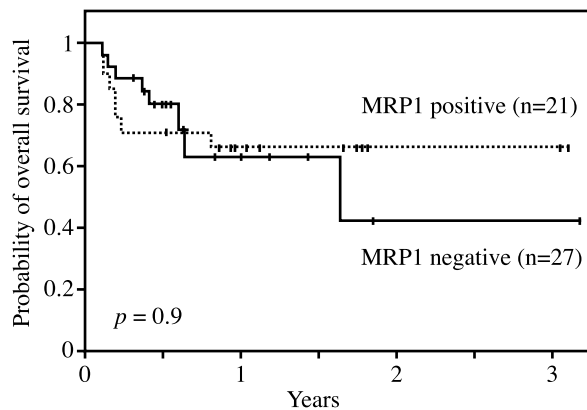


Fig. (4): MRP1 and overall survival estimated according to Kaplan-Meier in 48 patients. Survival data based on MRP1 expression are shown. Statistical comparison between survival curves was done by the log-rank test.

DISCUSSION

In the present study, we determined the expression of LRP and MRP1 in DLBCL and compared their expression with clinical and laboratory parameters of the patients. LRP was positive in 23% and MRP1 in 44% of newly diagnosed DLBCL. LRP expression was associated with poor response to chemotherapy and shorter overall survival, suggesting that LRP is a clinically relevant drug-resistance factor in DLBCL. A similar predictive and prognostic value of LRP expression was previously reported for acute myeloid leukemia [2,9,10,29], acute lymphoblastic leukemia [6], multiple myeloma [9,37] and advanced ovarian cancer [25].

In the present study, LRP strongly correlated with the International Prognostic Index. Meanwhile, MRP1 expression had no impact on the outcome of chemotherapy or survival of the patients. Similar results were previously reported in refractory lymphoma patients in which MRP expression was determined by means of a quantitative PCR assay both before and after chemotherapy [52]. MRP1 levels were not different between the pre-and post-chemotherapy groups, which suggesting that MRP1 overexpression is not responsible for non-P-glycoprotein-mediated drug resistance in these patients. Previous studies in acute myeloid leukemia [2,10,11], acute lymphoblastic leukemia [6] and advanced ovarian cancer [25] also failed to demonstrate a predictive or prognostic significance of MRP1 expression.

MDR1/P-glycoprotein expression of lym-

phomas has been examined in previous studies. Immunohistochemical studies reported P-glycoprotein expression that ranged from 0 to 49% of samples from untreated patients [3,7,16,6,32,34,35,38,44]. Conflicting results with regard to the clinical importance of MDR1/P-glycoprotein in lymphomas have been reported. MDR1/P-glycoprotein predicted a poor response to induction chemotherapy in two studies [3,35] but not in other studies [34,38]. In pretreated lymphomas, MDR1/P-glycoprotein expression was increased because of induction or selection of P-glycoprotein expressing clones [26,32].

Mutations or overexpression of the p53 gene have been described as predictors of poor response to chemotherapy and shorter survival of lymphoma patients [22,33,50]. In aggressive B-cell lymphomas, patients with p53 mutations had a lower complete response rate and a shorter overall survival as compared with patients with wild-type p53 [22]. A multivariate analysis that included p53 and factors of the International Prognostic Index demonstrated that mutant p53 was an independent predictive and prognostic factor [22].

Overexpression of bcl-2 confers drug resistance in vitro by inhibiting apoptosis [48]. Although an association between bcl-2 and response to chemotherapy could not be demonstrated [14,20,21,40,50], bcl-2 expression correlated with a higher relapse-rate [21], shorter disease-free survival [14,20,40] and shorter overall survival [14].

In conclusion, LRP expression is associated with poor response to chemotherapy and with shorter survival of the patients with DLBCL with statistical significance in univariate analysis and therefore, may prove to be an important mechanism of drug resistance in this disease if large sample was examined. Thus, the development of strategies to clinically overcome LRP-mediated drug resistance may be attempted and might improve clinical outcome in DLBCL in the future.

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