Expression of P-glycoprotein, Cyclin D1 and Ki-67 in Acute Lymphoblastic Leukemia: Relation with Induction Chemotherapy and Overall Survival

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Abstract Previous studies showed that non-cycling cells have a higher multidrug resistance (MDR) expression, which may be down-regulated by proliferation induction. Triggering these cells into proliferation down-regulates high MDR expression. The aim of this study was to determine the expression of P-glycoprotein (PGP) and cell cycle parameters (cyclin D1 and Ki-67) in acute lymphoblastic leukemia (ALL) at diagnosis, and to evaluate the correlation between the expressions of each marker, and the clinical significance of such expression with response to induction chemotherapy and overall survival. A total of 78 newly diagnosed ALL patients were enrolled in our study. PGP, cyclin D1 and Ki-67 were determined by flow cytometry. PGP expression was encountered in 10/78 (12.8%) of ALL cases. Cyclin D1 and Ki-67 were expressed in 16/77 (20.6%) and 27/76 (34.6%) of ALL cases, respectively. None of the parameters were associated with response to induction chemotherapy and overall survival. Based on the current analysis, we conclude that a joint immunophenotypic evaluation of PGP and cell cycle parameters like that adopted in this study is unlikely to reveal mechanisms of multidrug resistance associated with the clinical outcome.

Keywords P-gp · Cyclin D1 · Ki-67 · Acute lymphoblastic leukemia (ALL) · Multidrug resistance (MDR)

Introduction

Drug resistance is a major obstacle in the successful treatment and an important cause of death in acute leukemia (AL). Such resistance may be present before beginning the treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR) [1].

P-glycoprotein (PGP) also named P-170, is a product of the multidrug resistance one gene (MDR1) and is an ATP-dependant pump capable of expelling drugs out of cancer cells [2]. Earlier studies have suggested that, analysis of PGP and cell cycle parameters (Ki-67 and cyclin D1) could help to predict the clinical behavior of each acute non-lymphoblastic leukemia (ANLL) case at the time of diagnosis [3]. As yet, little is known about MDR expression in cycling and non-cycling acute lymphoblastic leukemic (ALL) cells.

Leukemic blasts from patients with untreated ANLL were studied for functional MDR expression in relation to cell cycle status and proliferation. Leukemic blast cells with a relatively high MDR expression appeared to be non-cycling cells compared with the more proliferating cells, with a relatively low MDR expression. This led to the hypothesis that non-cycling cells have a higher MDR expression, which may be down regulated by proliferation induction. Triggering these cells into proliferation down regulates high MDR expression [4].

At first presentation of childhood ALL, the prognostic value of the fraction of proliferating cells has remained

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controversial, and there is no clear evidence for an association with outcome. Previous studies correlated blast sensitivity to treatment with their rate of proliferation and apoptosis [5].

Lack of Ki-67 expression in children suffering from ALL may simply indicate a population of neoplastic cells in the G0 phase. The absence of this marker could be the evidence for the presence of blast cells with a lower proliferative rate and probably lower malignancy. On the other hand, it is possible that Ki-67-negative cells are not sensitive to chemotherapy [6].

Because cyclin D1 is a positive regulator of the cell cycle, and its expression increases upon stimulation of quiescent cells to enter the cell cycle, we would expect its high expression in leukemic cells, particularly those with positive Ki-67 expression. Therefore, therapy-resistant refractory ALL cells may persist not only by expression of MDR parameters, but also by escaping drug effect because they are resting G0 cells [7].

This close association between MDR and cell cycle parameters has encouraged us to determine the expression of PGP and cell cycle parameters (Ki-67 and cyclin D1) in ALL at diagnosis, to evaluate the correlation between the expressions of each marker, and the clinical significance of such expression with the response to induction chemotherapy and overall survival.

Patients

A total of 78 newly diagnosed ALL patients; 50 males and 28 females were enrolled in our study in a 6 month period from May to October 2007. Their median age was ten (0.08–56) years. They were 62 children with median age of 7.5 (0.08–15) years and 16 adults with median age of 20.5 (16–56) years. Diagnosis of ALL was based on morphologic evaluation, cytochemical stain, and immunophenotyping (IPT) following the European group for the immunological characterization of leukemia (EGIL) recommendations [8, 9]. The existence of hepatomegaly, splenomegaly, lymphadenopathy, infiltration to other organs, and the overall survival were checked to determine extent of disease and relation to the studied markers. Cytogenetic data were not available for correlation with the investigated parameters. This study was approved by the local ethics committee at the university.

Children received the standard pediatric ALL chemotherapy protocol applied at the National Cancer Institute (NCI), Cairo University. The induction phase included the following drugs vincristine, IV, 1.5 mg/m², daunorubicin, IV, 25 mg/m², prednisone, 40 mg/m² and L-asparaginase, IM, 6,000 IU/m² with triple intrathecal therapy (methotrexate, cytarabine and hydrocortisone).

Adult ALL patients received VCR, 2 mg total, doxorubicin, 45 mg/m², dexamethasone, 10 mg/m², and L-asparaginase, 10,000 unit. Intrathecal was given twice weekly if cerebrospinal examination CSF examination was positive and once if CSF was negative.

Methods

Immunophenotyping

Immunophenotyping (IPT) of leukemic cells was performed on EDTA anticoagulated bone marrow using a panel of monoclonal antibodies (moAb) as previously described [10]. Samples were analyzed by flow cytometry (FACSan, Becton-Dickinson, San Jose, CA, USA).

PGP Expression

PGP expression was detected using unconjugated 4E3 moAb (DAKO, M3523, FITC). Expression of antigen was quantified as mean fluorescence index (MFI = the ratio between the mean fluorescence intensity of the cells incubated with 4E3 and the mean fluorescence intensity of the respective isotype control). Blasts were defined as low expression for PGP (PGP negative) when their 4E3 MFI was <6 (Fig. 1) [3, 11].

Cell Cycle Parameter Expression

The monoclonal antibodies (Dako cat # M 7155, FITC) cyclin D1 and (BD cat # 556026, FITC) Ki-67 were used for assessment of cell cycle parameters. A MFI value of 6.8 was defined as the cutoff point for cyclin D1 positivity [12]. Samples were considered positive for Ki-67 if the antigen was expressed on more than or equal ($\geq 5\%$) of the leukemic cells [13]. Since it may be a large difference if Ki-67 equals 5% or Ki-67 equals 50%, expression of Ki-67 was represented as a continuous variable and dichotomized as positive and negative variable as well.

Statistical Methods

Data were analyzed using SPSS statistical package version 12. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For comparing quantitative data between the two groups, Mann-Whitney test was done. Kaplan-Meier method was used for survival analysis, with the log-rank test to compare survival curves. A *P* value less than 0.05 were considered significant. All tests were two-tailed.

Fig. 1 Flow cytometric (FCM) detection of MDR-1/PGP expression. FCM dot blots of a representative ALL sample, forward scatter/side scatter ungated cells (a). Gating on dim CD45 positive cells, CD45/

side scatter (b). Histogram showing PGP expression with x-axis is fluorescent intensity and y-axis is cell number (c). Histogram made to compare fluorescence of IgG2 isotype control with the 4E3 moAb (d)

Results

Immunophenotyping results were available for 73 patients and are shown in Table 1.

PGP expression was encountered in 10/78 (12.8%) of ALL cases. PGP expression did not differ with age, gender, presence of hepatomegaly, splenomegaly or lymphadenopathy, IPT, initial white blood cell count (WBC), platelet count (PLT), and hemoglobin level (HGB), (Table 2).

Cyclin D1 and Ki-67 were expressed in 16/77 (20.6%) and 27/76 (34.6%) of ALL cases, respectively. When the expression of cell cycle parameters compared with clinical manifestations, a higher frequency of Ki-67 positive-ALL cases were presenting with hepatomegaly, splenomegaly and lymphadenopathy and, a higher frequency of cyclin D1 positive-ALL were presenting with hepatomegaly and lymphadenopathy although no statistical significant difference

was reached because of the small number in each group, (Table 2).

A weak positive correlation was found between PGP expression and cyclin D1 ($r = 0.36$, $P = 0.001$) while no correlation with Ki-67 ($r = 0.17$, $P = 0.14$) was found. No correlation was found between cyclin D1 and Ki-67 ($r = 0.17$, $P = 0.14$). When considered as continuous variable, Ki-67 levels were higher [4.45 (0.4–45)] in the PGP positive group, when compared to the PGP negative group [3 (0–31)] with no difference between the two groups ($P = 0.29$). Also, higher levels of Ki-67 [6.25 (0.1–23)] were detected in the cyclin D1 positive group compared to the cyclin D1 negative group [3 (0–45)] with no difference between the two groups ($P = 0.14$).

Of the 72 patients that could be followed, 59 (75.6%) patients achieved complete remission (CR) within 6 weeks after induction chemotherapy and 13 (16.7%) patients did

Table 1 Initial patient characteristics

Variable	Number (% or range)
No. of patients in study	78
Gender	
Males	50 (64.1)
Females	28 (35.9)
Age (median)	
Adults	20.5 (16–56)
Children	7.5 (0.08–15)
Hepatomegaly	42/70 (53.8)
Splenomegaly	41/70 (52.6)
Lymphadenopathy	42/70 (53.8)
WBC $\times 10^9/l$ (median)	16.9 (0.40–614)
Hemoglobin (g/dl) (median)	7.2 (1.6–14)
Platelets $\times 10^9/l$ (median)	38.5 (4–232)
BM blasts (median)	89 (29–98)
Immunophenotyping ($n = 73$)	
B-cell phenotype	59 (75.6)
Pro B	1
Common B	24
Pre-B	31
Mature B	3
T-cell phenotype	13 (16.7)
Early	11
Intermediate	1
Late	1
Biphenotypic (B/T)	1
Response to induction chemotherapy	
Complete remission (CR)	59 (75.6)
Resistant	13 (16.7)

not achieve CR. There was no significant relation between CR rates and expression of PGP, Ki-67 or cyclin D1, (Table 2).

The cumulative overall survival was 80.3% for the 78 patients in the present study at a median follow-up period of 15.6 (0.03–38) months. The OS for patients positive and negative for the analyzed parameters is shown in Table 3.

Discussion

In accordance with other studies, we found only weak PGP expression in ALL samples [14]. This weak PGP expression level detected by the 4E3 moAb could be related to the concentration of the antibody used. This fact explained the discrepancies between reported PGP expression levels in AL cell samples detected by the same PGP moAb in different laboratories and studies [14]. Higher fluorescence intensity could have been achieved by conjugation of the PGP moAb by the fluorochrome phycoerythrin (PE) instead of with

FITC. Obviously, the use of other antibodies specific for PGP, such as C219 and MRK-16 plus at least one functional study would have supported our results.

Based on the multifactorial phenomenon of drug resistance, one protein can not explain resistance to a variety of drugs in all patients, especially with a heterogeneous group in our study. In this respect, the effect of other drug efflux pumps, such as MDR-associated protein (MRP) and the major vault protein/lung resistance protein (LRP) should be considered.

An important risk factor that can not be neglected in the current work is the cytogenetic data of the examined patients. ALL is a genetically heterogeneous disease and different genotypes imply different responses to chemotherapy.

Although some studies showed that PGP expression may be related to poor prognosis [15, 16], we and others [17, 18] give no indication that PGP is clinically important in ALL (relatively young population). This discrepancy may be explained by the relatively short time (15.6 months) for which our patients were observed considering the long term possibility of the occurrence of a relapse in ALL.

Data on cell cycle parameter expression and relation to prognosis in ALL patients are still scarce and not yet clear. In agreement with previous studies, [13, 19–21], we found over expression of cyclin D1 and Ki-67 in ALL patients. On the other hand, [22] we did not detect any elevation of cyclin D1 level among their ALL patient group (0/24).

Although Ki-67 and cyclin D1 are expressed in cycling cell, when we correlated Ki-67 expression with the expression of cyclin D1, no correlation was found between the two proliferation markers ($r = 0.17$, $P = 0.14$). However, when Ki-67 was considered as a continuous variable, higher levels of Ki-67 were detected in the cyclin D1 positive group when compared to cyclin D1 negative group with no difference between the two groups ($P = 0.14$). High levels of cyclin D1 are not necessary connected with high rates of cell proliferation. The positive role of cyclin D1 in the cell cycle did not correlate with growth properties in ALL [19] and cells of human renal cell carcinoma [23]. In addition, a distinct population of Ki-67 negative cells was found not only in G1 phase, but also in the proliferative compartments of acute leukemia patients [24]. Our results suggest that, Ki-67 and cyclin D1 moAb should be combined with flow cytometric DNA analysis for better assessment of the proliferative activity of leukemic cells (Fig. 2).

On the other hand, cyclin D1 negative samples does not necessary mean that no cyclin D1 mRNA is present in that sample. The quantitative real time RT-PCR technique appears to be the most suitable method for that purpose [22].

In accordance with previous results, [19–21, 25], the frequency of cell cycle parameters (Ki-67 and cyclin D1)-

Table 2 Relation between the studied parameters and different variables

Variable	p-gp			Cyclin D1			Ki-67		
	Negative N = 68	Positive N = 10	P value	Negative N = 57	Positive N = 16	P value	Negative N = 49	Positive N = 27	P value
Gender									
Males	45(90)	5(10)	0.32	40(81.6)	9(18.4)	0.49	33(68.8)	15(31.3)	0.30
Females	23(82.1)	5(17.9)		21(75.0)	7(25.0)		16(57.1)	12(42.9)	
Hepatomegaly									
Yes	40(95.2)	2(4.8)	0.10	34(81.0)	8(19.0)	0.65	28(66.7)	14(33.3)	0.82
No	23(82.1)	5 (17.9)		23(85.2)	4(14.8)		18(69.2)	8(30.8)	
Splenomegaly									
Yes	38(92.7)	3(7.3)	0.43	34(85.0)	6(15.0)	0.53	27(67.5)	13(32.5)	0.97
No	25(86.2)	4(13.8)		23(79.3)	6(20.7)		19(67.9)	9(32.1)	
Lymphadenopathy									
Yes	37(88.1)	5(11.9)	0.69	34(81.0)	8(19.0)	0.65	27(65.9)	14(34.1)	0.69
No	26(92.9)	2(7.1)		23(85.2)	4(14.8)		19(70.4)	8(29.6)	
Response to induction									
CR	53(89.8)	6(10.2)	0.20	47(79.7)	12(20.3)	0.71	38(65.5)	20(34.5)	0.78
Resistance	10(76.9)	3(23.1)		9(75.0)	3 (25.0)		8(61.5)	5(38.5)	
IPT									
B-ALL	53(89.8)	6(10.2)	0.58	48(82.8)	10(17.2)	0.62	38(65.5)	20(34.5)	0.63
T-ALL	11(84.6)	2(15.4)		10(76.9)	3(23.1)		7(58.3)	5(41.7)	
Age									
<16 year	55(88.7)	7(11.3)	0.42	50(82.0)	11(18.0)	0.24	39(65.0)	21(35.0)	0.85
≥16 year	13(81.3)	3(18.8)		11(68.8)	5(31.3)		10(62.5)	6(37.5)	
Hb									
<10 g/dl	55(93.2)	4(6.8)	0.07	50(86.2)	8(13.8)	0.008	37(64.9)	20(35.1)	0.40
≥10 g/dl	10(76.9)	3(23.1)		7(53.8)	6(46.2)		10(76.9)	3(23.1)	
WBC									
<100 × 10 ⁹ /l	54(91.5)	5(8.5)	0.44	47(79.7)	12(20.3)	0.77	39(68.4)	18(31.6)	0.63
≥100 × 10 ⁹ /l	11(84.6)	2(15.4)		10(83.3)	2(16.7)		8(61.5)	5(38.5)	
PLT									
<30 × 10 ⁹ /l	22(100)	0	0.065	19(86.4)	3(13.6)	0.38	13(59.1)	9(40.9)	0.33
≥30 × 10 ⁹ /l	43(86.0)	7(14.0)		38(77.6)	11(22.4)		34(70.8)	14(29.2)	

CR complete remission, IPT immunophenotyping, Hb hemoglobin, TLC total leucocytic count, PLT platelet count

Table 3 Cumulative survival (%) at 18 months for lymphoblastic leukemia patients in relation to expression of p-gp, ki-67, and cyclin D1 genes

Variable	Expression	Cumulative survival (%)	P value
p-gp	Negative (n = 68)	80.7	(0.715)
	Positive (n = 10)	77.7	
Cyclin D1	Negative (n = 57)	83.8	(0.162)
	Positive (n = 16)	65.6	
Ki-67	Negative (n = 49)	85.1	(0.157)
	Positive (n = 27)	68.8	

positive ALL was higher in patients presenting with organomegaly and lymphadenopathy although not statistically significant. Cyclin D1 overexpression might have a role in blast mobilization from the bone marrow to lymph

nodes [21] and Ki-67 expression was also related to organomegaly and extramedullary infiltration in ANLL with some reports concerning specific organ infiltrates [20, 26].

Fig. 2 FCM detection of cyclin D1 expression. FCM dot blots of a representative ALL sample, forward scatter/side scatter ungated cells (a). Gating on dim CD45 positive cells, CD45/side scatter (b).

Histogram showing cyclin D1 expression with x-axis is fluorescent intensity and y-axis is cell number (c). Histogram made to compare fluorescence of IgG2 isotype control with the cyclin D1 moAb (d)

The aim of this study was to correlate PGP expression with cell cycle parameters expression, however, we could not draw a firm conclusion if PGP positive cells are cycling or not cycling because of the small number (ten patients) of PGP positive patients.

Based on the current analysis, we conclude that a joint immunophenotypic evaluation of PGP and cell cycle parameters like that adopted in this study is unlikely to reveal mechanisms of multidrug resistance associated with the clinical outcome.

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