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Study of Multidrug Resistance Protein, Lung Resistance Protein, and Cyclin A2 in Adult Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Multidrug resistance agents: Multidrug resistance 1 (MDR1) gene and lung associated-resistance protein (LRP) are associated with unsuccessful treatment of acute lymphoblastic leukemia (ALL), however, their prognostic role is still largely unknown. Cyclin A2 is a member of the G2 cyclins that are involved in the cell cycle control and has been postulated to be associated with the chemosensitivity of leukemic blast cells. Its prognostic significance in adult ALL remains to be clarified.

Objective: Our aim in this study is to evaluate the frequencies of occurrence of multidrug resistance agents MDR1, and LRP, and cell proliferation marker cyclin A2 in Egyptian adult ALL patients, and to correlate them with disease prognosis.

Material and Methods: In this study, we measured the expression of MDR1 protein (P-gp), LRP, and cyclin A2 in 40 de novo adult ALL patients using flow cytometry.

Results: MDR1 protein was expressed in 20% of all cases and constituted 15% of complete remission (CR) cases, and 28.6% of non remission (NR) cases. LRP was positive in 32.5% of all cases, 23.1% of CR cases, and 50% of NR cases. Cyclin A2 was positive in 62.5% of all cases, 65.4% of CR cases, and 57.1% of NR cases. LRP showed significant correlation with cyclin A2 in all cases. There was also highly significant correlation between each of the 3 parameters with each other in NR cases. The 3 parameters showed no correlation with CR rate. None of the 3 parameters had any correlation with either of age, WBC count, Hemoglobin, BM blasts, or BM cellularity.

Conclusion: Our study revealed significant correlation between LRP and cyclin A2 in all adult ALL patients as well as highly significant correlation between the 3 parameters with each other in cases with no response to treatment. The exploration of such correlations could be further expanded with studies including more parameters.

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INTRODUCTION

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

Several molecular biological mechanisms have been identified as being associated with multidrug resistance **[2]**. P-glycoprotein (P-gp) is a product of the multidrug resistance1 gene (MDR1) and is an ATP-dependent pump capable of expelling drugs out of cancer cells **[3,4]**. P-gp is a transmembrane glycoprotein conferring cross-resistance to a variety of mechanistically and structurally unrelated cytotoxic drugs, such as anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins **[5]**. Another protein, the multidrug resistance related protein (MRP) is structurally similar to P-gp and belongs to the same transmembrane transporter superfamily **[6]**. In addition to these two proteins, a 110 kDa protein has been identified in a P-gp -negative multidrug resistant lung cancer cell line. This protein was termed the lung resistance protein (LRP) and acts as a major vault protein in humans **[7]**. The function of these vaults has been associated with nuclear-cytoplasmic transport **[8]**. More recently, the number of vaults was shown to be elevated in drug-resistant cell lines **[9]**.

Despite the identification of these proteins, the pathways that result in drug resistance in leukemic cells remain largely uncharacterized.

While drug resistance genes expression has been studied in acute leukemia **[10-13]**, the value of MDR1, MRP and LRP gene expression as independent predictors of treatment success is still controversial.

The regulation of the cell cycle is of particular importance for hemopoietic system. Critical components of the basic cell cycle regulations have been identified including cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CDKIs) **[14]**. Cyclin A2 is a member of the G2 cyclins that are involved in the control of the G2/M cell cycle transition and mitosis, as well as S-phase progression **[14,15]**. A previous study indicated that expression of cyclin A2 mRNA is a marker of cell proliferation in several hematological malignancies, and shows a highly significant correlation between expression of either cyclin A2 mRNA or protein and the cumulative percentage of cells in the S phase **[17]**. More recent studies show the correlation of lower levels of cyclin A2 with acute leukemia resistant to treatment **[18]**, and recurrent ALL **[19]**. High levels correlated positively with complete remission and high levels of topoisomerase II **[19]**.

Our aim in this study is to evaluate the frequencies of occurrence of multidrug resistance agents P-gp, and LRP, and cell proliferation marker cyclin A2 in Egyptian adult acute lymphoblastic leukemia patients, and to correlate them with disease prognosis and clinical and laboratory variables.

PATIENTS AND METHODS

Patients:

Forty patients with de novo acute lymphoblastic leukemia, who presented to the National Cancer Institute, Cairo University, in the period between September 2006 and March 2008, were included in this study, after an informed consent. They were 31 male and 9 female. Their ages ranged from 18 to 63 years, with a median of 29 years.

All patients were subjected to thorough history taking and full clinical examination. In addition radiological examination in the form of chest X-ray, abdominal ultrasound and CT scan whenever needed were performed.

Complete blood picture, bone marrow aspiration and morphological examination, liver and kidney function tests were also done. Acute lymphoblastic leukemia was diagnosed according to the criteria revised by the French-American-British (FAB) classification and immunological classification.

Immunophenotyping was done by flow cytometry (Partec III from DAKO cytomation), on marrow blast cells with a panel of monoclonal antibodies, purchased from DAKO (Denmark), including FITC and PE conjugated CD19 and CD20 for B ALL, and CD3 and CD7 for T ALL. Specific isotype control for FITC, PE conjugated monoclonal antibodies was used. Results were expressed as a percentage of cells showing positive expression. The monoclonal antibodies for P-gp, and cyclin A2 were also purchased from DAKO (Denmark), and that for LRP were purchased from Santa-Cruse biotechnology.

All patients were followed-up and classified according to treatment response, into complete remission (CR) group and no or incomplete remission (NR) group.

Complete remission in ALL was defined using the following criteria developed by an International Working Group **[20-22]** as follows:

- Normal values for absolute neutrophil count (>1000/μl) and platelet count (>100,000/μl), and independence from red cell transfusion.
- A bone marrow cellularity reveals normal maturation of all cellular components (i.e., erythrocytic, granulocytic, and megakaryocytic series).
- Less than 5 percent blast cells are present in the bone marrow.
- The absence of a previously detected clonal cytogenetic abnormality (i.e., complete cytogenetic remission, CRc) confirms the morphologic diagnosis of CR but not currently a required criterion.

Some patients may fulfill all of the above criteria for CR but may not recover peripheral blood counts to the required level. These are denoted as CRi, or CR with insufficient hematological recovery (platelets or neutrophils). CRp describes a subset of patients with CRi, where patients fulfill all criteria for CR except that platelet counts are <100,000/μl).

Patients who fail to achieve CR or CRi may experience a partial remission (PR), defined as a ≥50 percent decrease in bone marrow blasts with normalization of peripheral blood counts, or some other measure of hematologic improvement. A PR in ALL is generally expected to be of short duration, and in most circumstances, is unlikely to serve as a surrogate reasonably likely to predict for clinical benefit.

Methods:

Sampling: 5ml of peripheral blood were withdrawn under aseptic precautions, and were delivered into EDTA vacutainer tubes for complete hemogram, and flow cytometric analysis and for P-gp, LRP, and cyclin A2 measurement.

Surface study of MDR1 and LRP protein expression by flow cytometry:

A hundred µl of the heparinized blood were mixed with 10µl anti-MDR1 (monoclonal mouse antihuman MDR1 [SC-1313]) or anti-LRP (monoclonal mouse antihuman LRP [SC-18701]). An irrelevant monoclonal antibody of the same iso-type and protein concentration was used as a negative control. The tube was incubated at room temperature in the dark for 30min, washed twice with PBS; the supernatant was aspirated, leaving approximately 100µl fluid. Sheath liquid was added and analyzed by flow cytometry.

Cytoplasmic study for Cyclin A2 protein expression by flow cytometry:

Fifty µl of the diluted anticoagulated blood were added to 100µl intra-stain reagent A (fixation), vortexed gently, incubated at room temperature for 15 minutes, washed in PBS and then the supernatant aspirated, leaving approximately 50µl of fluid. A hundred µl DAKO intra-stain reagent B (permeabilization) (Fixative A and permeabilization B, DAKO Cytomation) were added, then 10µl PE conjugated monoclonal mouse antihuman cyclin A2 (SC-239) were also added. An irrelevant monoclonal antibody of the same iso-type and protein concentration was used as a negative control. The tubes were incubated in the dark at room temperature for 15 minutes, and washed twice by PBS. The pellets were resuspended in a sheath fluid for flow cytometric analysis.

As a measure for the intensity of staining, the mean fluorescence index (MFI) was used, which represents the ratio between the mean fluorescence intensity of cells stained with the specific antibody and that of cells stained with the isotype-matched control antibody, the case was considered over expressing or positive for P-gp at a ratio of ≥1.1 **[23]**, and was considered positive for LRP when the ratio exceeds 0.3 **[24]** and for cyclin A2 when the ratio exceeds 0.2 **[25]**.

Statistical analysis:

The data were coded entered and processed on an IBM-PC compatible computer using SPSS (version 15).

Student's *t*-test was used to assess the statistical significance of the difference between two population means in a study involving independent samples.

Correlation analysis: Assessing the strength of association between two variables. The correlation coefficient denoted symbolically *r*, defines the strength and direction of the linear relationship between two variables. The level $p<0.05$ was considered the cut-off value for significance.

RESULTS

Forty patients with de novo acute lymphoblastic leukemia were included in this study. They were 31 male and 9 female. There ages ranged from 18 to 63 years, with a median of 26 years, and a mean of 31.95±12.5 years.

Their WBC count was 82.6±85.7 $x10^9$/L, and ranged between 11.2 and 315.4 $x10^9$/L. Their hemoglobin level was 8.5±1.84g/L and ranged between 5.3-11.4g/L. Their BM blasts was 73.2%±14.32 with a range of 35-91%. Thirty three (82.5%) of them had hypercellular marrow and 7 (17.5%) had normocellular marrow.

After induction therapy 26 (65%) patients achieved complete remission (CR group), and 14 (35%) did not achieve it (NR group).

The mean fluorescent intensity (MFI) of P-gp in all ALL cases was 16.28±38.46, that of LRP was 8.62±22.95, and that of cyclin A2 was 3.20±6.28. The case was considered over expressing or positive for P-gp at a ratio of ≥1.1, and was considered positive for LRP when the ratio exceeds 0.3, and for cyclin A2 when the ratio exceeds 0.2.

The cellular expression of P-gp, LRP, and cyclin A2 proteins in all patients group, CR group, and NR group is shown in Table (1).

As regards the correlation between P-gp, LRP, and cyclin A2 in all cases, LRP showed significant correlation with Cyclin A, (r=0.67, p=0.001), and non significant correlation with MDR1, (r=0.37, p=0.10). MDR showed no significant correlation with cyclin A2 (r=0.36, p=0.1).

The correlations between cyclin A2, P-gp, and LRP in cases with complete remission, were not statistically significant. However, the correlations between each of P-gp and LRP and cyclin A2, were highly significant in cases with no response to treatment (all r=0.99, p<0.0001).

We found no significant difference in MFI of P-gp, LRP, and cyclin A2 in patients with CR, and those with NR (p=0.94, 0.36, 0.72 respectively) (Table 2).

Tables (3) describes correlation studies between each of cyclin A2, P-gp, and LRP, respectively, and age, TLC, Hb, and BM blasts. They revealed no correlation.

As well, when comparing the levels of cyclin A2, P-gp, and LRP in relation to BM cellularity, no statistically significant difference was found between the levels in normocellular, and hypercellular marrows, (p>0.05) (Mann-Whitney test) Table (4).

Table (1): Expression of Pgp, LRP, cyclin A2 in all, CR, and NR cases in adult ALL.

Group	All cases	CR cases	NR cases
P-gp	8/40 (20%)	4/26 (15.4%)	4/14 (28.6%)
LRP	13/40 (32.5%)	6/26 (23.1%)	7/14 (50%)
Cyclin A2	25/40 (62.5%)	17/26 (65.1%)	8/14 (57.1%)
P-gp & LRP	5/40 (12.5%)	3/26 (11.5%)	2/14 (14.3%)

Table (2): Correlation between levels of P-gp, LRP, Cyclin A2 in NR and CR groups in adult ALL.

	Response to treatment		
	NR (14)	CR (26)	p
	Median	Median	
Cyclin A2 MFI	1.20	1.15	0.72
MDR MFI	1.29	1.25	0.94
LRP MFI	1.00	1.10	0.36

Table (3): Correlation of P-gp, LRP and Cyclin A2 with clinical and laboratory data in 40 adult ALL patients.

	MFI					
	MDR		LRP		Cyclin A2	
	r	p	r	p	r	p
Age	–0.33	0.15	–0.30	0.18	0.10	0.67
TLC	0.49	0.49	0.19	0.41	0.31	0.17
Hb	–0.11	0.63	–0.23	0.31	0.00	1.00
BM blasts	0.30	0.21	0.23	0.35	0.22	0.37

Table (4): Correlation between levels of P-gp, LRP, Cyclin A2 and BM cellularity in 40 adult ALL patients.

	BM Cellularity		
	Normocellular	Hypercellular	p
	(3)	(19)	
Cyclin A2 MFI	1.46	1.15	1.4
MDR MFI	1.33	1.25	1.38
LRP MFI	1.40	1.10	1.05

DISCUSSION

Studies on the treatment of adult ALL have shown only modest improvements over the last 2 decades, with the actual cure rate still ranging between 15% and 40%. The resistance of tumor cells to chemotherapeutic drugs is a major limitation in cancer treatment. Multidrug resistance phenotype is the most frequently studied mechanism for intrinsic drug resistance, yet the prognostic role of P-gp and other multidrug resistance-associated proteins in adult ALL is still largely unknown **[26]**.

On the other hand deregulation of the cell cycle is a prerequisite for the formation of most if not all malignant tumors. When tumor cells proliferate abnormally, cyclins, including cyclin A2, may be expressed abnormally **[23]**. Several reports show direct correlation between the expression of cyclins and better prognosis **[18,19, 22,23]**. Some authors **[29]** remarked that these results are counterintuitive considering that these cyclins may enhance cellular proliferation by accelerating entry into S phase. However, they postulated that the detailed implications of overexpression of cyclin A2 in leukemic cells are still unknown. They proposed that CDK2-cyclin A2 complexes may exhibit negative regulation for S phase progression, or, alternatively,

cyclin A2 overexpression may contribute to the increased chemosensitivity of leukemic cells by stimulating these cells into S phase of the cell cycle. It has been also postulated that cyclin A2 may be associated with the chemosensitivity of leukemic blast cells for the following reasons: (1) Expression levels of cyclin A2 mRNA have a tendency to decrease after relapse compared with the primary leukemia; (2) Expression levels of cyclin A2 have a positive correlation with topoisomerase II mRNA; (3) Expression levels of cyclin A2 have an inverse correlation with multidrug resistance 1 (mdr1) RNA expression, and elevated levels of the latter are characteristic of refractory acute leukemia cells **[31]**.

Our aim in this study was to evaluate the multidrug resistance effect of MDR agents versus the postulated chemosensivity effect of cyclin A2, through studying the frequencies of occurrence of MDR1 protein, LRP, and cyclin A2 in Egyptian adult acute lymphoblastic leukemia patients, and their correlation with disease prognosis and clinical and laboratory variables. Our patients were grouped into 2 groups according to response to induction therapy: Those who achieved complete remission (CR group), and those who did not achieve it (NR group).

P-gp expression was found positive in 8/40 patients (20% of all ALL cases), of them, 4/26 achieved complete remission (CR) which makes 15% of CR cases, and 4/14 cases with no response to treatment (NR) which makes 28% of NR cases.

A multicenter study, on adult ALL cases at diagnosis, found 21.7% of patients positive for P-gp, with lower CR rate among positive cases (53.5%) than among negative cases (79.6%) **[25]**.

Some authors, in another study on adult ALL cases at diagnosis, found 47% of patients positive for P-gp, with similar CR rates in positive and negative cases **[27]**.

While other authors studied MDR1 mRNA expression by RT-PCR in adult and childhood acute leukemia. They found that MDR1 mRNA was expressed in 25% of cases at diagnosis. Of the positive cases, 71% achieved CR, compared to 78% of the negative cases. They stated that MDR1 expression appeared to have no statistically significant effect on patient outcome following induction chemotherapy **[28]**.

A different group, studied P-gp expression by flow cytometry on childhood ALL cases and found 26% of cases positive at diagnosis, with no difference between positive and negative cases in CR **[23]**.

The percentage of expression in all of the above studies is widely variable. This conclusion was also described by two groups of authors who attributed its causes to the use of different techniques and methods, different cut-off values and pooling of heterogenous groups of patients such as AML and ALL, initial and relapse samples, and adult and pediatric cases **[32,33]**.

Our study showed that LRP was expressed in 13 (32.5%) patients, of them, 6/26 had CR (46.8% of all CR cases), and 7/14 had NR (53.2% of all NR cases). A multicenter study, found that 60.5% of cases were positive for LRP, and that LRP expression had no influence on CR **[26]**. Some authors reported the frequency of LRP expression 18% of adult ALL cases. They stated that the positive cases were too few to make statistical comparison between CR and NR cases **[27]**. A different group, in a study on acute leukemia patients, found 15% of cases expressing LRP, making 33% of CR cases, and the LRP negative cases made 84% of NR cases **[29]**.

Dual expression of P-gp and LRP was detected in our study in 5 cases (12.5% of all cases), two of them were in CR (2/26 of all CR cases, 7.7%), and three cases were in NR (3/14 of all NR cases, 21.4%). Although the result is suggestive of significant difference, the sample size does not support statistical confirmation.

Some authors reported 21.1% coexpression of MDR1 and LRP with no significant influence on CR rate **[26]**. However, two different groups concluded from their studies that coexpression of LRP and MDR1 might result in worse prognosis **[29,37]**.

We had 25/40 cases expressing cyclin A2 most of them had CR (17/26, 66% of CR group), and 15/40 negative for cyclin A2 (8/14, 57.1% of NR group).

Another group found 50/75 of AL cases expressing cyclin A2. They also found CR rate

87.9% in cases with over expression of cyclin A2, and 38% of the negative cases had CR **[19]**.

Our study revealed significant correlation between LRP and cyclin A2 (p=0.001) in all ALL cases, while P-gp correlation with cyclin A2 was not significant. Both P-gp and LRP did not have any significant correlation with cyclin A2 in the CR group. However both had a highly significant correlation with cyclin A2 in NR group.

Some authors discovered a negative correlation between the gene expression levels of MDR1 and cyclin A2 (r=–0.37, p=0.029) in NR group. Their study was conducted on AL patients using RT-PCR **[19]**.

Another group in a study on ALL patients revealed no significant correlation between MDR1 and cyclin A2 **[35]**.

Our results showed no significant difference between CR group and NR group in the expression of P-gp, LRP, and cyclin A2.

In agreement with our results, a study group stated that CR rates were similar in MDR1 positive and negative patients **[27]**. Also another group found the difference in CR rates between LRP positive and negative patients was not significant, (p>0.05) **[36]**. Cyclin A2 expression rates showed no difference between newly diagnosed AL patients and patients in CR, in the study conducted by a different group **[30]**.

However, two different groups disagree with the previous results stating in their studies that patients with negative MDR1 expression had a significantly higher CR rate than patients with positive ones **[26,29]**. Same conclusion was deduced by a third group regarding LRP who reported that CR rate in LRP positive was lower than LRP negative patients **[37]**. Finally, some authors found that LRP expression was associated with lower CR rate, while MDR1 appeared to have statistically no significant effect on CR **[28]**.

No correlation was detected between P-gp, LRP, and cyclin A2 and known prognostic markers such as age and WBCs count, which represent tumor cell mass, as well as hemoglobin and BM blasts.

Our results are in accordance with two different groups who revealed no relation between each of P-gp and LRP with either age or WBCs count **[32,33]**.

Conclusion:

The present study demonstrates a significant correlation between LRP and cyclin A2 in adult ALL patients, as well as highly significant correlation between the 3 parameters with each other in cases with no response to treatment. Significance of correlation of dual expression of MDR1, and LRP on leukemic cells was not supported statistically due to small sample size.

For better understanding of the impact of the factors involved in multidrug resistance we recommend, in a future study, involving MRP with MDR1 and LRP.

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