Expression of programmed death ligand-1 in B-cell acute lymphoblastic leukemia

Amal M. Gaber, Hanan G.A. El Azeem, Mohammed A.Y.A. Malek

Department of Clinical Pathology, Assiut University, Assiut, Egypt

Correspondence to Amal M. Gaber, MSC Clinical Pathology, Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.
Postal Code: 71511;
Tel: +201091512969;
e-mail: aml.gaber57@yahoo.com

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Background
Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy in the world. Patients with good prognostic factors have excellent survival rates with further improvement over the last decades. However, refractory ALL and relapsed ALL after hematopoietic stem cell transplantation are still associated with a poor prognosis. Immune checkpoints have gained attention in recent years in the field of oncology as a mechanism of cancer to evade immunity, but their status in B-ALL has yet to be investigated.

Aim
The aim was to assess programmed death ligand-1 (PDL-1) expression in newly diagnosed B-cell acute lymphoblastic leukemia (B-ALL).

Materials and methods
This study was directed on 45 patients with newly diagnosed B-ALL. Surface expression of PDL-1 on the blast cells was evaluated by multicolor flow cytometry.

Results
The study participants exhibited a mean PDL-1 expression of 16.28%, ranging from 8.5 to 70.45%.

Conclusion
This study has revealed that patients with newly diagnosed B-ALL could express PDL-1 to allow cancer cells to evade the immune system, demonstrating PD-1/PDL-1 as a universal target for therapy.

Keywords:
programmed death ligand -1 , B-ALL patients, flow cytometry

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignant clonal proliferation of lymphoid progenitor cells, but most commonly of the B-cell lineage (B-ALL) [1].

Immunotherapy is a novel approach that has undergone implementation into treatment strategies in ALL [2].

Despite the encouraging results, it is unknown why T cells could attack malignant blasts in some cases or unable to attack them in others. There is emerging evidence that expression of co-inhibitory molecules and loss of co-stimulatory molecules have a pivotal role in immune escape of the tumor [3].

Supported inhibitory signaling mediated by expression of many co-signaling molecules on T cells such as programmed death-1 (PD-1) or cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) correlates with a stage of T-cell exhaustion, marked by a reduced T-cell effector function [4].

The main ligand for PD-1 is programmed death ligand-1 (PDL-1). This immune regulatory receptor and its ligand have been shown to have a critical role in autoimmunity, infectious immunity, transplantation immunity, and tumor immunotherapy [5].

PDL-1, which is known as B7-H1 (CD274), is a cell surface protein of B7 family member [6].

PD-L1 is expressed on all types of lympho-hematopoietic cells at variable levels and is constitutively expressed on T-cells, B-cells, macrophages, and dendritic cells [7].

Immune attack via interferon-γ release leads to inducible expression of PDL-1 by mucosa creating an "immune shield" to protect against autoimmune attack in the setting of chronic inflammation or infection [8].

Tumor cells have co-opted this PD-1/PDL-1 mechanism, designed to protect normal mucosa from autoimmune attack, and overexpress PDL-1 to avoid immunologic surveillance to facilitate the growth of cancer [9].

Immune checkpoint antibodies have been postulated to be a potential therapeutic option in many malignancies, and this is applied in many solid tumors such as malignant melanoma and many hematological malignancies [10].

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A higher expression of PDL-1 was found in the majority of different hematological malignant cells, including lymphoma, chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), and acute myeloid leukemia (AML) [11–14].

Little is known about expression of PDL-1 in B-cell acute lymphoblastic leukemia (B-ALL).

Materials and methods

This is a cross-sectional-study that was performed in the Flow Cytometry Unit, Clinical Pathology Department, Assiut University Hospital, Assiut, Egypt.

This study was conducted on 45 patients with B-ALL in the period from March 2018 to June 2019.

All cases were admitted to the Children's Hospital and Hematology Department at Assiut University Hospital. All of them are newly diagnosed, and none of them started treatment before sample collection, and it exclude patients in relapse and patients with past history of immune diseases and other types of acute leukemia.

All patients were subjected to the following investigations:

(1) Complete blood count.
(2) Bone marrow aspirate.
(3) Cytochemical examination.
(4) Flow cytometric immunophenotyping.

Sample collection, preparation, and staining

Bone marrow samples or 2 ml peripheral venous blood was withdrawn from patients and delivered into EDTA tube, for the assessment of the following**:**

(1) Primary panel of acute leukemia (as required).
(2) Secondary panel of acute leukemia (as required).
(3) CD 274 (PDL-1 marker): the fluorochrome-conjugated monoclonal antibody (McAb) used in this study is allophycocyanin (APC).

The EDTA anticoagulated blood was stored at room temperature (20–25°C), stained and examined within 24 h after blood collection.

The leukocytic count in the sample was adjusted to ~10 000 cells/ml with PBS (120 mmol/l NaCl, 2.7 mmol/l KCl, and 10 mmol/l phosphate buffer, with PH7.4). Overall, 5–20 µl of the fluorochrome-conjugated monoclonal antibody was added to the appropriate test tube, and then 50–100 µl of adjusted sample was added to the monoclonal antibody. Incubation of tubes for 15 min at room temperature was done and protected away from light. Thereafter, 3 ml 1×FACS Lysing solution (diluted 1: 10) was added to the sample tubes, and the tubes were inverted once. Tubes were vortexed and then analyzed using FACS Calibur flow cytometry and analyzed with the Cell Quest Software (San Diego, California, USA) (Becton Dickinson).

Acquisition and analysis of data on flow cytometry

Analysis of fluorescence data on a minimum of 15 000 cells was acquired on a FACS Calibur flow cytometry.

Using forward scatter (FSC) and right angle side scatter (SSC), a gate was created around the small mononuclear cells, which were predominantly lymphocytes. Gated cells were analyzed for the expression of PDL-1.

Principle

When the specimen is added to the reagent, the fluorochrome-labeled monoclonal antibodies in the reagent bind specifically to the leukocyte surface antigens. During acquisition, the cells travel through the laser beam and scatter the laser light. The stained cells fluoresce. These scatters and fluorescence signals, detected by the flow cytometry, provide information about the cell's size, internal complexity, and relative fluorescence intensity for the specific flourochrome.

Statistical analysis

Data were collected and analyzed those using SPSS (Statistical Package for the Social Sciences, version 20; IBM, Armonk, New York, USA). Continuous data were expressed in the form of mean or median (range) whereas nominal data were expressed in the form of frequency (percentage).

χ^2-Test was used to compare the nominal data of different groups in the study, whereas Mann–Whitney test was used to compare mean of different two groups, and Kruskal–Wallis test was done for more than two groups. Spearman's correlation was used to determine the correlation between serum PDL-1 and other continuous variables. Level of confidence was kept at 95% hence, *P* value was significant if less than 0.05.

Ethical consideration

Prescribed written consent was achieved from the patients. The Ethical Committee of the Faculty of Medicine, Assiut University, approved this study**.**

Results

This cross-sectional study was conducted on 45 newly diagnosed adult patients with B-ALL.

The study participants exhibited a mean age of 10.68 years, ranging from 3 to 50 years, and comprised 28 (62.2%) males and 17 (37.8%) females. Mean bone marrow blast percentage was 61%, ranging from 23 to 94%. Immunophenotyping divided patients into 28 (62.2%) cases with common B-ALL, 13 (28.9%) cases with pre-ALL, and four (8.9%) cases with pro-ALL.

Regarding the clinical data of the studied patients. The most frequent manifestations in those patients were bleeding tendency in 31 (68.9%) cases, fatigue 25 (55.6%) cases, followed by fever in 20 (44.4%) cases and hepatosplenomegaly (HSM) in 20 (44.4%) cases, and lymphadenopathy in 13 (28.9%) cases. It was noticed that extramedullary involvement presented in only five (11.1%) patients (Table 1).

Table 1 Demographic and laboratory characteristics of the studied patients

Variables	*n*=45
Age (years)	10.68 (3-50)
Sex	
Male	28 (62.2)
Female	17 (37.8)
Hemoglobin (mg/dl)	7.78 (3-10.8)
Total leukocytic count (×10^3/ml)	4.2 (1.50-25)
Platelets count (×10^3/ml)	55.4 (7-109)
Peripheral blast count	18 (5-90)
Bone marrow blast	61 (23-94)

Data were expressed as frequency (percentage) and mean (range).

Table 2 Flow cytometry in the studied patients

	n=45
PDL-1	16.28 (8.5-70.45)
Positive PDL-1 (>20%)	11 (24.44)
CD34	53.38 (13.45-91)
Positive CD34 (>20%)	43 (95.6)
CD10	75 (11-96)
Positive CD10 (>20%)	41 (91.1)
HLA-DR	49.11 (22-83)
Positive HLA-DR (>20%)	45 (100)
CD19	64.30 (26-92)
Positive CD19 (>20)	45 (100)
Cyto CD79a	54.92 (14.79-96)
Positive Cyto CD79a (>20%)	22 (48.9)
Cyto CD22	56.27 (25-99)
Positive Cyto CD22 (>20%)	45 (100)
Cyto IgM	17.40 (10.11-99)
Positive Cyto IgM (>20%)	13 (28.9)
Anti-TdT	78.78 (57-95)
Positive anti-TdT (>20%)	45 (100)

Data were expressed as frequency (percentage) (represents the number of patients) and mean (range) (represents the value of reaction of blast cells to a given monoclonal antibody). Ig, immunoglobulin; PDL-1, programmed death ligand-1.

Flow cytometry in the studied patients

Table 2 shows flow cytometry in the studied patients. It was noticed that mean PDL-1 was 16.28%, with a range between 8.5 and 70.45%. Only 11 (24.44%) patients had positive PDL-1 (>20%). All patients had positive HLA-DR, CD19, Cyto CD22, and anti-TdT (>20%). Positive CD34, Cyto CD79a, CD10, and Cyto IgM (>20%) presented in 43 (95.6%), 22 (48.9%), 41 (91.1%), and 13 (28.9%) patients, respectively (Fig. 1).

Correlation between PDL-1 expression and different parameters of the studied patients

It was noticed that PDL-1 had an insignificant correlation with the most of the different studied parameters ($P > 0.05$).

There was no statistically significant association between PDL-1 expression and age, with P value of 0.83. It was noticed that the mean level of PDL-1 was insignificantly lower in male patients in comparison with female patients (15.74 vs 17.71%; $P = 0.78$).

Correlating PDL-1 expression to different laboratory quantitative variables, including total leukocyte count, hemoglobin, platelet, BM blast percentages, and peripheral blood blast percentages, failed to prove an association between any of these parameters and PDL-1 expression, with P values of 0.48, 0.85, 0.09, 0.91, and 0.47, respectively (Table 3).

There was no association that could be delineated between PDL-1 expression and status of different cluster of differentiation, with P values of greater than 0.05 (Table 4).

A comparison was held between different immunophenotypes of B-ALL in terms of their PDL-1 expression, and it was noticed that level of mean of PDL-1 was insignificantly higher in pre-ALL (18.88%) in comparison with common ALL (15.74%) and pro-ALL (11.69%), with P value of 0.72 (Table 5).

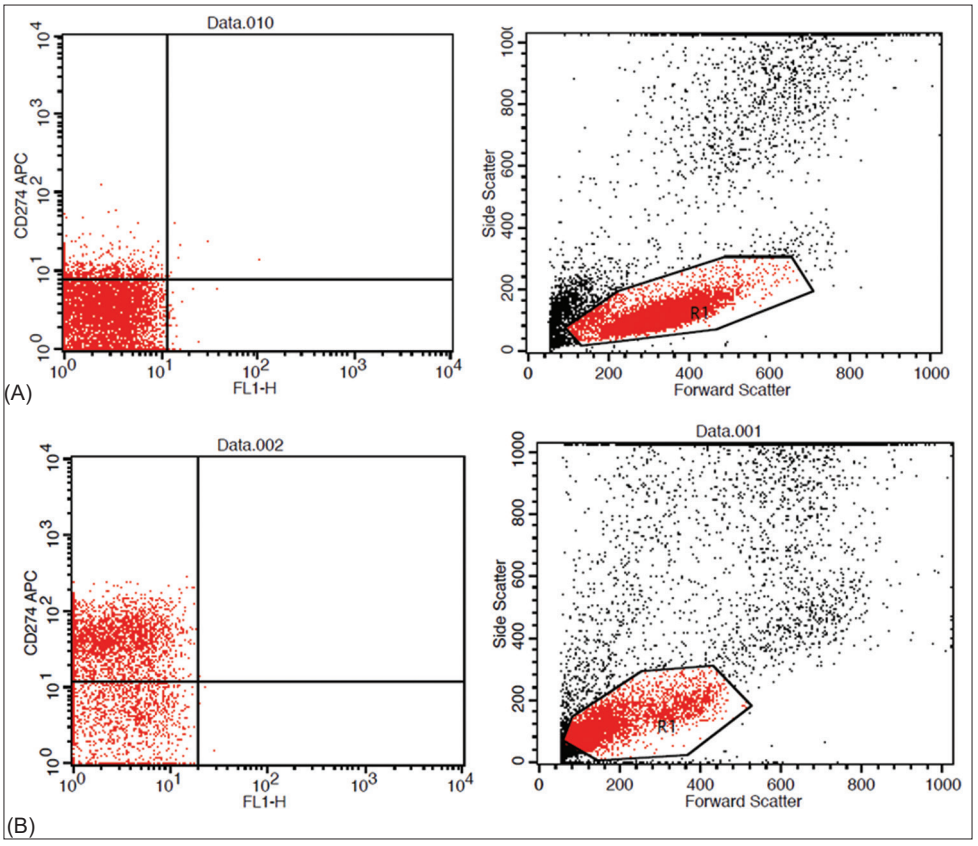
Patients with extramedullary involvement, which is considered as one of the aggressiveness criteria denoting hostile tumor behavior, exhibited significantly higher level of mean of PDL-1 in comparison with patients without extramedullary involvement (16.88 vs 11.49%; $P = 0.01$) (Table 6).

Table 7 illustrates that patients with HSM had a significantly higher level of mean of PDL-1 in comparison with those without HSM (16.41 vs 11.56; $P = 0.03$).

Patients with lymphadenopathy had a significantly higher level of mean of PDL-1 in comparison with those without lymphadenopathy (18.69 vs 10.36; $P = 0.01$).

Patients with bleeding tendency had a significantly higher level of mean PDL-1 in comparison with those without bleeding tendency (17.91 vs 12.68; $P = 0.01$).

Figure 1



Programmed death ligand-1 (CD 274) expression levels in the studied patients , for example (A= 8.5% , B=70.45%), respectively, in the studied patients. Using forward scatter and right angle side scatter, a gate was created around the small mononuclear cells, which were predominantly lymphocytes. Gated cells were analyzed for the expression of programmed death ligand-1.

Discussion

The main purpose of this study was to assess PDL-1 expression status in patients with newly diagnosed B-ALL and to relate it to patients and disease characteristics.

The present study included 45 patients who are newly diagnosed as having B-ALL.

Upon the assessment of PDL-1 expression in our study, the study participants exhibited a mean PDL-1 expression of 16.28, ranging from 8.5 to 70.45%.

These results showed concordance with the findings highlighted in the study conducted by Feucht *et al.* [15], which included two groups of patients with B-ALL (eight primary diagnosed patients and 11 relapsed patients) who were tested for their PDL-1 expressions using flow cytometry. The study reported that median PD-L1 surface expression was higher on patients' ALL blasts at relapse as compared with patients with primary diagnosis (median PD-L1 expression was 9.5 vs 1.1%).

In the same study, PD-L1 expression was significantly higher on patients' blasts of non-responders to blinatumomab as compared with responders (median PD-L1 expression was 14.6 vs 5.0%) [15].

Table 3 Correlation between programmed death ligand-1 expression and demographic and laboratory data

	r	*P*
Age (years)	−0.04	0.83
Hemoglobin (mg/dl)	0.02	0.85
Total leukocytic count (×10³/ml)	−0.11	0.48
Platelets count (×10³/ml)	0.25	0.09
Peripheral blast count	0.11	0.47
Bone marrow blast	−0.01	0.91

Data were expressed as *r* value (indicates the strength of correlation) and *P* value (indicates significant of correlation).

Table 4 Correlation between programmed death ligand-1 and the expression of different cluster of differentiation markers

	r	*P*
CD34	−0.08	0.57
HLA-DR	0.20	0.18
CD19	0.16	0.29
CD10	0.11	0.33
Cyto CD79a	−0.21	0.31
Cyto CD22	−0.38	0.15
Cyto IgM	0.28	0.05
Anti-TdT	0.23	0.45

Data were expressed as *r* value (indicates the strength of correlation) and *P* value (indicates significant of correlation). Ig, immunoglobulin.

This study confirmed the expression of PDL-1 in B-ALL depending on a comparison between primary diagnosed and relapsed patients and between responders to blinatumomab and nonresponders.

The bispecific T-cell engager (blinatumomab) has shown encouraging clinical activity in B-ALL and was approved for the treatment of relapsed or refractory B-ALL by the FDA. However, about half of relapsed/refractory patients do not respond to therapy, being resistant to treatment with blinatumomab [16].

Köhnke *et al.* [16] assessed PDL-1 expression in the bone marrow of patients with refractory B-ALL and showed marked increase of PDL-1 positivity (2% at baseline vs 40% after blinatumomab treatment). This study was on only a refractory B-ALL case and assessed the increase of PD-L1 after blinatumomab treatment to report the resistance of immunotherapy in B-ALL and its cause.

In the present study, assessments of the relationship of PDL-1 expression and the different demographic as well as disease parameters have been performed. It is clear that there was no significant association between PDL-1 expression and age or sex, with *P* values greater than 0.05.

Upon correlating PDL-1 expression with laboratory quantitative variables, PDL-1 showed no significant correlation with total leukocyte count, HB, platelet, peripheral blood, or BM blast percentage.

Table 5 Level of programmed death ligand-1 in studied patients based on immunophenotypes of B-cell acute lymphoblastic leukemia

Immunophenotyping	Mean (range) of PDL-1
Common B-ALL	15.74 (8.5-62.96)
Pre-ALL	18.88 (10.45-70.45)
Pro-ALL	11.69 (8.5-26.87)
P	0.72

ALL, acute lymphoblastic leukemia; PDL-1, programmed death ligand-1.

Table 6 Level of programmed death ligand-1 in studied patients based on extramedullary involvement

Extramedullary involvement	Mean (range) of PDL-1
Yes	16.88 (8.5-70.45)
No	11.49 (8.5-16.73)
P	0.01

PDL-1, programmed death ligand-1.

Comparison of PDL-1 expressions in different B-ALL immunophenotypes failed to prove any significant association of certain immunophenotype with higher PDL-1 expression.

Different immunophenotypes exhibited variable mean of PDL-1 expression with the highest of all being for pre-ALL at 18.88%, whereas the lowest expression for expression was for pro-ALL at 11.69%. However, these discrepancies were not of statistical significance, with *P* values of greater than 0.05.

Ma and Li [17] also assessed the expression of PDL-1 in 60 patients diagnosed as having primary acute leukemia using flow cytometry and found that expression rate of PDL-1 in patients with ALL was not significantly different from that in AML and also reported no significance of age, sex, HB, WBCS, PLTS, and blast ratio in positive or negative PDL-1 in primary acute leukemia.

Ma *et al.* [18] assessed the expression of PDL-1 in 84 patients of acute leukemia with difference types and diseases stages and 40 controls using flow cytometry showed and also reported that the expression rate of PDL-1 in patients with newly diagnosed ALL was not significantly different from that in newly diagnosed AML.

The previous two studies were on both types of acute leukemia, not especially for B-ALL, but reported no difference in the expression of PDL-1 between ALL and AML.

Mostafa *et al.* [14] reported in a most recent study on 40 patients with newly diagnosed AML who were recruited for PDL-1 assessment by flow cytometry that PDL-1 expression had a mean expression of 43.01 ± 24.72 (range: 1.52–88.1%) and also reported a correlation of PDL-1 expression with laboratory quantitative variables, where PDL-1 showed no significant correlation with HB, WBCS, platelet, peripheral blood, or BM blast percentage. However, this was reported in only AML.

In the present study, another comparison was held between different disease parameters on one side and PDL-1 expression on the other side. Patients with

Table 7 Level of programmed death ligand-1 in studied patients based on clinical manifestations

Clinical manifestations	Yes	No	*P*
Fever	16.39 (8.5-63.94)	17.20 (8.5-70.45)	*0.98*
Hepatosplenomegaly	16.41 (8.5-63.94)	11.56 (9.45-16.95)	*0.03*
Lymphadenopathy	18.69 (11.45-70.45)	10.36 (8.5-20.80)	*0.01*
Bleeding tendency	17.91 (10.09-70.45)	12.68 (8.5-32.70)	*0.01*
Fatigue and anemic manifestations	14.44 (11.09-70.45)	13.30 (8.5-63.94)	*0.08*
Weight loss	12.89 (8.5-63.94)	14.01 (11.45-70.45)	0.13
Bone pain	11.16 (11.45-63.94	12.55 (8.5-70.45)	0.09

extramedullary involvement had significantly higher PDL-1 expression in relation to those free from extramedullary disease (11.49 vs 16.88, P = 0.01), which is considered as one of the aggressiveness criteria of the tumor.

Mostafa *et al.* [14] assessed that patients with extramedullary involvement had higher PDL-1 expression in relation to those free from extramedullary disease, but this was not statistically significant. However, this was reported in AML.

In the present study, patients with HSM had a significantly higher level of PDL-1 in comparison with those without HSM (16.41 vs 11.56, with P value of 0.03).

Patients with lymphadenopathy had a significantly higher level of PDL-1 in comparison with those without lymphadenopathy (18.69 vs 10.63, with P value of 0.01).

Patients with bleeding tendency had significantly higher level of PDL-1 in comparison with those without bleeding tendency (17.91 vs 12.68, with P value of 0.01).

Extramedullary involvement, HSM, and lymphadenopathy may be signs of dissemination or severity of the disease, underscoring the importance of this mechanism for cancer progression and metastasis.

No previous studies confirmed the relationship between PDL-1 expression and HSM or lymphadenopathy in acute leukemia.

Ma H reported that the complete remission rate in PDL-1-positive patients was lower than that of the negative patients after chemotherapy; the difference was statistically significant. The death rate of PDL-1-positive patients was higher than that PDL-1 negative [17].

Ma *et al.* [18] also found the expression level of PDL-1 positive rate in remission group to be less than newly diagnosed and the expression level of PDL-1-positive rate in relapse to be more than newly diagnosed ones and in remission group. Our study did not follow up the patients because of the short period.

Across all tumor types, the use of antibodies that block the PD-1/PDL-1 pathway results in response rates of 0–17% in patients with PDL-1-negative tumors, but response rates in patients with PDL-1-positive tumors range from 36 to 100% [9].

So, PDL-1 antibodies have gained interest in recent years in the field of oncology as an effective mechanism to overcome cancer, but their status in ALL has to be investigated.

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Conflicts of interest
There are no conflicts of interest.

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