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Cytologic Phenotypes of B-Cell Acute Lymphoblastic Leukemia- A Single Center Study

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid precursor cells, which could be classified according to morphological and cytochemical methods as well as immunophenotyping.

Twenty patients with ALL, who had been referred to the Children's Medical Center Hospital, during the year 2007, were enrolled in this study in order to evaluate the morphologic and immunophenotypic profile of these patients.

Cytologic analysis of blood and bone marrow samples revealed that the frequency of ALL-L1 was 70%, followed by ALL-L2 and ALL-L3. The onset age of the patients with ALL-L1 was significantly lower than the patients with L2/L3. Severe anemia was significantly detected more in L1 group. Flow cytometic study of bone marrow showed that 10 cases had Pre-B1 ALL and 7 cases had Pre-B2 ALL, while three cases had Pro-B ALL. Comparisons of the characteristics and clinical manifestations among these groups did not show any appreciable difference. There were an increase percentage of CD20+ cells and a decrease CD10+ cells in pre-B2 group in comparison with pre-B1 group. Fifteen patients were in standard risk and five were in high risk. Although standard risk patients were more common in the group of pre-B1, this was not significant.

Our results confirm the previous reports indicating heterogeneity of ALL. Immunophenotyping is not the only diagnostic test of importance, while morphological assessment still can be used in the diagnosis and classification of the disease.

Key words: Acute lymphoblastic leukemia; Immunophenotyping; Morphology

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a clonal malignant disorder of lymphoid precursor cells,

characterized by abnormal proliferation and accumulation of immature lymphoblasts arrested at various differentiation stages. This variability results in a diverse clinical behavior and different responses to therapy.[1] ALL represents the most common form of childhood malignancy and potentially a curable disorder in most cases. Following recent advances in chemotherapy regimens, the main research domain in this field is dedicated to classify patients into various risk groups based on known prognostic and predictive factors to apply less invasive strategies to low-risk groups.[2]

The diagnosis and classification of ALL was solely based on morphological and cytochemical methods before the availability of monoclonal antibodies.[3,4] In the recent years, immunophenotyping has provided significant new information on the heterogeneity of ALL and has shown to be very useful for the diagnosis, classification and prognostic evaluation of patients.[5] Especially, as the lineage of most cases of morphological and cytochemical is poorly differentiated, ALL can be accurately characterized by immunophenotyping.

A number of studies have evaluated the subtyping of leukemic cells from patients with ALL and its clinical implication to prognosis and outcome of the affected individuals.[2,6-13] In some studies, it has been shown that the immunophenotypic categories are particularly important because they identify distinctive treatment and prognostic groups.[2,6-10] In other studies the clinical significance of subtypes have remained unclear.[11-14] For example, a study conducted in United Kingdom failed to demonstrate any independent prognostic value in the B-cell ALL subgroups.[14]

Since it has still remained unclear whether classification of ALL patients by immunophenotyping will actually prove to be valuable in patients' management and prognosis, we were prompted to investigate the morphologic and immunophenotypic profile of Iranian patients with ALL and also to study their possible association with disease severity.

PATIENTS AND METHODS

Subjects

Twenty patients with ALL, who had been referred to the Children's Medical Center Hospital, the main referral center for pediatric leukemia in Iran, during January to December 2007, took part in this study. Diagnosis of ALL was based on morphologic and immunophenotypic criteria.[15,16] Clinical and laboratory data of the patients were documented. This study was approved by local ethics committee of the hospital. After taking informed consent from the patients, heparinized bone marrow and blood samples were collected prior to treatment.

Immuunophenotyping

Isolated cells from bone marrow were washed twice with RPMI 1640 medium (Sigma, USA) prior to immunophenotyping. Using a panel of antibodies against leukocytes antigens, we analyzed the immunophenotype of the ALL patients by flow cytometry to determine the immunologic classification based on the B-phenotype. After separation, the mononuclear cells were stained with a panel of florescent-conjugated monoclonal antibodies (mAbs) (DAKO, Denmark) specific for B cell lineage. These mAbs consisted of the following: [CD10+IgM+IgD (clone SS2/36), CD19 (clone HD37) and CD20+CD22 (clone B-Ly1)], T cell lineage [CD3 (clone UCHT1)] and non specific lineage [CD34 (clone QBEnd10), HLA-DR (clone AB3) and Terminal Deoxy nucleotidyl Transferase, TdT (clone HT-6)].

For surface staining, cells were washed twice with phosphate buffer saline (PBS) and after incubation of 10^6 cells with 10 µl of mAb at 4ºC for 30 minutes, cells were washed twice with phosphate buffered saline (PBS 0.15M, pH=7.2) followed by scanning by flow cytometer (Partec, Germany).

In addition, staining for TdT and IgM were also performed at the cytoplasmic level. The same method was used for intracytoplasmic staining, but before the addition of mAb, cells were made permeable using permeabilizing solution (DAKO, Denmark) and then were washed with PBS. Forward and side-scatter gates were used for analysis of leukemic antigenic expression. Sample analysis and data acquisition were performed by Flomax flow cytometry analysis software (Partec, Germany). The criterion for surface marker was expressed positively by at least 20% of the leukemic blast cell population after subtraction of background staining with isotype-matched conjugated mAbs of irrelevant specificity.

Based on the expression pattern of CD19, CD10, CD20, HLA-DR and TdT, we classified our B-ALL patients into three subtypes including Pro-B, Pre-BI, and Pre-BII (Table 1).[17-22]

Statistical Analysis

Data analysis was performed using SPSS statistical software package (version 14.0). Patients aged 1 to 9 years with the white blood cell count less than $50*10^9$/L, with no mediastinal mass, and no leukemic infiltration of the central nervous system were considered to have standard risk. All others were considered to have high risk. Statistical differences of various clinical and laboratory parameters between groups were evaluated by Chi-square or the Fisher's exact tests. Independent-samples T test was performed to compare the means between the groups.

RESULTS

Characteristics of the Patients

Twenty patients (13 males and 7 females), with the age range of 2 to 14 years, who were referred to the Children's Medical Center Hospital during 2007, were enrolled in this study (Table 1). The median onset age of the patients was 68 (range 5-157) months, while the median diagnostic age was 69 (range 27-159) months with median diagnosis delay of 1 month (range 0-57 months).

Presenting Manifestations

The main presentation symptoms of the patients were fever (13 cases), malaise (11 cases), bone pain (9 cases), pallor (7 cases), abdominal pain (4 cases), and weight loss (4 cases). Some patients had been presented with overlapping of foregoing signs and symptoms. Echymosis, purpura, or melena were the first findings in three cases. Diarrhea was also the first presenting manifestation in two cases, while two patients were referred with upper respiratory tract infections. One case was presented with lymphadenopathy and fever, whereas another case was referred with growth retardation, malaise and pallor.

Table 1. Immunophenotype characteristics of B-cell ALL subtypes.

Subtype	CD19	CD10	CD20	HLA-DR	TDT	Cytoplasmic IgM	Surface IgM	Surface IgD
Pro-B	+	-	-	+	+	-	-	-
Pre-B1	+	+	-	+	+	-	-	-
Pre-B2	+	+	+	+	±	±	-	-

Table 2. Characteristics of the ALL patients.

Number	Age (months)	Sex	FAB	Immunophenotype	Risk
P1	27	Male	L1	Pre-B1 ALL	Standard
P2	28	Female	L1	Pre-B2 ALL	Standard
P3	31	Male	L1	Pre-B1 ALL	Standard
P4	33	Male	L1	Pre-B1 ALL	Standard
P5	42	Male	L1	Pre-B1 ALL	Standard
P6	43	Male	L1	Pre-B2 ALL	Standard
P7	44	Female	L1	Pre-B2 ALL	Standard
P8	58	Male	L1	Pre-B2 ALL	High
P9	62	Female	L1	Pre-B2 ALL	Standard
P10	63	Male	L2	Pro-B ALL	Standard
P11	75	Male	L2	Pre-B1 ALL	Standard
P12	84	Female	L1	Pre-B1 ALL	Standard
P13	99	Female	L1	Pre-B1 ALL	Standard
P14	110	Female	L1	Pre-B2 ALL	Standard
P15	127	Male	L2	Pre-B1 ALL	Standard
P16	132	Male	L1	Pro-B ALL	High
P17	135	Male	L3	Pro-B ALL	High
P18	146	Female	L3	Pre-B2 ALL	High
P19	151	Male	L1	Pre-B1 ALL	High
P20	159	Male	L2	Pre-B1 ALL	Standard

Clinical Manifestations during the Course of Disease

During chemotherapy and follow-up, the following signs and symptoms were detected in these patients: fever (18 cases), petechia or purpura (9 cases), and bone pain (10 cases). Thirteen patients developed splenomegaly, whereas 11 cases had lymphadenopathy and 11 cases also had hepatomegaly.

Central nervous system evaluation showed the lymphoblast infiltration in four patients (P2, P6, P7, P20).

Cytologic Morphology Phenotype

Cytologic analysis of blood and bone marrow samples revealed that the frequency of ALL-L1 was 70%, followed by ALL-L2 (4 cases) and ALL-L3 (2 cases) (Table 2).

In order to analyze data among the groups, we compared the clinical findings between two groups of L1 (14 cases) and L2 or L3 (6 cases) Table 3. The onset age of the patients with ALL-L1 was significantly lower than the patients with L2/L3 (P-value= 0.02) (Table 3).

Consequently, diagnosis was made often earlier in L1 group (P-value= 0.02) (Table 3). Comparison of clinical manifestations among these groups indicates that the number of L1 patients who had lymphadenopathy was significantly more than the number of L2/L3 with such finding. Severe anemia (Hb <7 mg/dl) was also more often detected in L1 group (Table 3).

Immunophenotype

Flow cytometric study of bone marrow showed that 10 cases had Pre-B1 ALL and 7 cases had Pre-B2 ALL, while three cases had Pro-B ALL (Table 4). Comparisons of the characteristics and clinical manifestations among these groups did not show any significant difference (Table 3). Severe anemia (Hb <7 mg/dl) was less common in Pre-B2 ALL (14.3% in pre-B2 vs. 50% in pre-B1 and 66.7% in pro-B, P-value= 0.15).

Although we did not find either any case with ALL-L3 in pre-B1 group or ALL-L2 in pre-B2 group, it was not statistically significant (Table 4).

Flow cytometric analysis of bone marrow revealed a significant increase in the percentage of CD20+ cells in group of pre-B2 group, while the patients in pre-B2 had significantly lower number of CD10+ cells in comparison with pre-B1 group (Table 4).

Risk Factors

Considering the associated risk factors, 15 patients were in standard risk and five were in high risk. The onset age and diagnosis age of the patients with standard risk were significantly lower than the patients with high risk (63.60±42.78 vs. 123.40±37.71 months, P-value= 0.019 and 68.46±40.17 vs. 124.40±37.92 months, P-value= 0.025, respectively). There was not any significant difference between cytologic morphology phenotype and the risks of disease (Table 3). Although standard risk patients were more common in the group of pre-B1, it was not statistically significant (Table 4).

Table 3. Characteristics of the patients based on cytologic morphology phenotype.

Characteristics	ALL-L1	ALL-L2 or ALL-L3	P-value
Number	14	6	-
Sex (Male/Female)	8/6	5/1	0.35
Onset age (months)	62.36±43.72	116.33±39.09	0.020
Diagnosis age (months)	67.42±40.99	117.50±39.26	0.028
CNS involvement	3 (21.4%)	1 (16.7%)	0.65
Fever	12 (85.7%)	6 (100%)	0.47
Petechia or purpura	6 (42.9%)	3 (50%)	0.57
Bone pain	6 (42.9%)	2 (33.3%)	0.54
Hepatomegaly	8 (57.1%)	3 (50%)	0.57
Splenomegaly	9 (64.3%)	4 (66.7%)	0.66
Lymphadenopathy	10 (71.4%)	1 (16.7%)	0.049
Anemia (<7 mg/dl)	11 (78.6%)	1 (16.7%)	0.018
Standard/high risk*	11/3	4/2	0.48

* Patients aged 1 to 9 years with the white blood cell count less than $50*10^9$/L , and no mediastinal mass, no leukemic infiltration of the central nervous system were considered to have standard risk. All others were considered to have high risk.

Table 4. Characteristics of the patients according to immunophenotype.

Characteristics	Pre-B1	Pre-B2	Pro-B	P-value
Number	10	7	3	-
Sex (Male/Female)	8/2	2/5	3/0	0.058*
Onset age (months)	81.50±49.52	61.00±48.47	109.67±41.31	0.35**
Diagnosis age (months)	82.80±50.19	70.14±42.33	110.00±40.73	0.48**
Cytologic morphology phenotype				
L1	7 (70%)	6 (85.7%)	1 (33.3%)	0.44*
L2	3 (30%)	0 (0%)	1 (33.3%)	0.17*
L3	0 (0%)	1 (14.3%)	1 (33.3%)	0.64*
Clinical Manifestations				
CNS involvement	1 (10%)	3 (42.9%)	1 (33.3%)	0.16*
Fever	8 (80%)	7 (100%)	3 (100%)	0.33*
Petechia or purpura	4 (40%)	4 (57.1%)	1 (33.3%)	0.41*
Bone pain	4 (40%)	4 (57.1%)	2 (66.7%)	0.41*
Hepatomegaly	5 (50%)	5 (71.4%)	1 (33.3%)	0.35*
Splenomegaly	7 (70%)	5 (71.4%)	1 (33.3%)	0.68*
Lymphadenopathy	6 (60%)	4 (57.1%)	1 (33.3%)	0.65*
Bone Marrow Flow Cytometry				
CD3 (%)	12.17±9.75	10.52±8.22	-	0.78*
CD19 (%)	64.29±23.22	54.41±30.41	49.83±36.62	0.48*
CD20 (%)	8.81±5.36	45.88±25.96	8.83±10.56	0.009*
CD22 (%)	6.65±4.72	30.07±27.30	13.33±19.65	0.06*
CD34 (%)	18.80±10.85	26.32±30.48	22.25±25.10	0.55*
CD10 (%)	71.12±22.99	40.08±23.60	2.23±1.50	0.018*
HLA-DR (%)	54.01±25.26	51.10±26.83	33.93±36.71	0.82*
Cytoplasmic IgM (%)	12.07±12.88	24.28±13.36	10.66±6.02	0.09*
Surface IgM (%)	3.41±3.89	5.18±3.93	8.00±6.55	0.04*
Surface IgD (%)	3.54±3.59	4.85±2.82	8.56±8.03	0.43*
TDT (%)	28.90±20.27	26.22±31.26	20.66±9.60	0.84*
Risks***				
Standard Risk	9 (90%)	5 (71.4%)	1 (33.3%)	0.36*
High Risk	1 (10%)	2 (28.6%)	2 (66.7%)	0.36*

* P-value was calculated for the comparison between two groups of Pre-B1 and Pre-B2 ALL.

** P-value was calculated by One-Way ANOVA for the comparison of means among three groups.

*** Patients aged 1 to 9 years with the white blood cell count less than $50*10^9$/L , with no mediastinal mass, and no leukemic infiltration of the central nervous system were considered to have standard risk. All others were considered to have high risk.

DISCUSSION

ALL is a malignant disorder of lymphoid precursor cells, which could be classified according to morphological and cytochemical methods and also immunophenotyping.[1,3-5] Prior to the emergence of immunophenotyping, morphologic studies were the mainstay of the practicing hematologists and seemed to be sufficient to establish a diagnosis of hematologic malignancies. However, because of the subjectivity of morphologic analysis even among experts, this system has not been proved useful in the clinical management of ALL.[10] Therefore immunophenotyping has become an increasingly relevant approach for the diagnosis and classification of hematologic malignancies.[23]

Prevalence of the male sex in our ALL has been shown to reach 65%, which was similar to previous reports in which 65-70% of the total ALL patients were

males.[24,25] In this study, 70% of the patients were diagnosed morphologically as L1, followed by L2 and L3. Higher frequency of L1 morphology was also reported in the previous studies from Morocco and Oman.[24,26] It has been previously reported that the L2 morphology is mostly confined to the T-ALL cases and is less frequent in the B-ALL subtypes.[14] The L3 morphology was also the least subgroup observed among our patients which is in concordance with other reports.[14,24,27] The onset age and diagnosis age of the patients with L1 were significantly lower than other groups, which was similar to previous study in Morocco.[24] This finding also supported previous study in the UK, which revealed that ALL patients with L1 morphology had a significant lower age in comparison with other groups.[14] Severe anemia was more common in L1 group. The large previous study in the UK also indicated that mean hemoglobulin level was lower in the L1 group.[14] Although presence of lymphadenopathy in L1 group was much higher than other groups, we did not find any significant difference in hepatos-plenomegaly and other clinical findings among the groups. Mediastinal masses were not detected in our patients, which was similar to the report on B-cell lineage in Omani patients;[26] however, such finding was observed in 71% of cases in the T-cell ALL and in 29% of the B-cell ALL in Morocco.[24]

In the present study, the analysis by flow cytometry of the samples from Iranian patients with ALL of B origin showed that the Pre-B1 ALL stage is the most represented B-ALL phenotype with a frequency of 50%. This value is similar to the results of other studies,[24,26,28-31] while Pre-B2 ALL was the most common subtype in Thailand.[32] The Pre-B2 ALL stage was the second most prevalent phenotype, which was similar to previous reports.[24,26,28-31] Although association of CD markers with some characteristics of the patients has been reported in a few studies, it has not been further supported. Although the associations of CD10 with lower white cell count at diagnosis, younger age and L1 morphological subtype were reported in the UK study,[14] it has not been confirmed in our study. CD10 is expressed in 80% of our patients, which is very similar to previous studies.[8,9,24] Frequency analysis of the clinical features in different B-ALL subtypes, based on immunophenotyping, failed to establish significant association for any of the subtypes. Although in a study, presence of lymphadenopathy differed among the immunophenotyping subtypes,[26] such difference was not observed among our patients. However, according to the FAB classification, the rate of lymphadenopathy was significantly higher in the L1 morphology. Severe anemia was also more common in the L1 morphology. This finding is in concordance with other studies in which only minor differences were observed among B-cell and T-cell ALL patients, but not among B-cell subgroups.[24,29,33] These data showed that the classification based on immunophenotype can not predict the clinical manifestations in B-ALL subtypes, while FAB classification has somewhat more benefits in this regard. However, the small size of population, studied in this report, is one of the limitations of this study; therefore, more studies in the different geographical regions on more patients are necessary to evaluate these classification systems.

In conclusion, our results confirmed and extended previous reports indicating heterogeneity of ALL and that immunophenotyping is not the only diagnostic criterion of importance, and should not be taken in isolation because morphological assessment remains crucial both for diagnosis and the prediction of clinical manifestations.

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