Immunophenotypic Subtyping of Leukemic Cells from Iranian Patients with Acute Lymphoblastic Leukaemia: Association to Disease Outcome

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ABSTRACT

Background: Immunophenotypic characterization of the leukemic cells has been widely used as a tool for diagnosis, classification, stratification and prognosis of leukaemia. **Objective:** To investigate the immunophenotypic subtype profiles of Iranian patients with acute lymphoblastic leukemia (ALL) and its association to disease outcome. **Methods:** In this study, a total of 60 Iranian patients with ALL were immunophenotyped by flow cytometry using a panel of monoclonal antibodies specific for CD2, CD3, CD5, CD10, CD13, CD14, CD19, CD20, CD33, CD34, CD45, HLA-DR and TdT molecules. **Results:** The samples were initially categorized into T-ALL (n=9), B-ALL (n=50) and mixed lineage (n=1) based on the expression patterns of CD3 and CD19 molecules. B-ALL patients could further be classified into four subtypes, including Pro-B (n=7, 11.7%), Pre-B I (n=28, 46.7%), Pre-B II (n=13, 21.7%) and immature/mature B cells (n=2, 3.3%) on the basis of expression of CD10, CD19, CD20, HLA-DR and TdT. Clinical manifestations and laboratory findings of the patients did not reveal association with immunophenotypic subtypes of ALL, with the exception of mediastinal mass and WBC count at the time of diagnosis which were found to be significantly higher in patients with T-ALL compared with B-ALL (p=0.001 and 0.014), respectively. **Conclusion:** Our results indicate that overall the immunophenotypic profile of Iranian ALL patients is similar to previous reports and it might be used for monitoring of minimal residual disease and prognosis.

Keywords: Immunophenotyping, ALL, Pro-B, Pre-B, Flow cytometry

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a biologically and clinically heterogeneous group of diseases characterized by abnormal proliferation and accumulation of immature lymphoblasts within the bone marrow (BM), peripheral blood (PB) and lymphoid tissues (1). ALL represents the most common form of cancer in children and its incidence is five times greater than that of acute non-lymphoblastic leukemias. In adults the disease accounts for 15% of acute leukemias (2).

Before identification of monoclonal antibodies (mAbs) by Kohler and Millstein (3), morphological and cytochemical methods were the main tools for diagnosis and classification of acute leukemias. French American British (FAB) co-operative group has introduced a reference method for classification of leukemias (4). According to their criteria, the ALL patients were subdivided in 3 subsets, L1, L2 and L3. There is no correlation between L1 morphology and immunophenotype, but some studies have shown a higher percentage of L2 blasts in T-ALL (5). Furthermore the L3 subtype was found to be closely associated with the mature B-cell phenotype (6, 7). It has been shown that immature cells, despite their morphologic similarities, belonged to various differentiation stages with consequent implications for primary diagnosis and treatment schedule (8). The immunologic characteristics of leukemic cells have been extensively investigated using flow cytometric analysis of various surface and cytoplasmic antigens that correspond to various developmental stages and lineages of malignant cells (9). Based on the currently available extensive antibody panels, lineage determination is possible in almost all acute leukemias, with the exception of less than 1% of cases (10).

Since most cell differentiation antigens do not belong to specific lineage, immunologic classification of acute leukemias was performed with a panel of lineage associated mAbs rather than the presence or absence of a single antigen (11).

Despite many reports about immunophenotypic subsets of ALL patients in Asian countries such as India (12), Saudi Arabia (13), Thailand (14), China (15), Japan (16), Oman (17), United Arab Emirates (18), Malaysia (19), and Hong Kong (20), there is no report about immunophenotypic subsets of Iranian ALL patients.

This together with the high incidence of ALL in Iranian population prompted us to investigate the immunophenotypic profile of our ALL patients and also to study its possible association with disease outcome.

MATERIALS AND METHODS

Patients. From November 2003 to June 2005, 60 newly diagnosed Iranian patients (43 children and 17 adults) with ALL were studied. Diagnosis of ALL was based on morphologic and immunophenotypic criteria (4, 21). Heparinized bone marrow (BM) or peripheral blood (PB) samples were collected prior to treatment. All patients were consented to the routine laboratory and hospital procedures including blood and bone marrow studies. Clinical and hematological parameters were determined. FAB classification was performed as previously described (4). Major clinical and laboratory findings are listed in Table 1.

Table 1. Clinical features and laboratory findings in Iranian ALL patients

		Age group		Immunophenotypic subtypes					
		Adults	**Children**	**T-ALL**	**Total B Lineage**	**Pro-B**	**Pre-B I**	**Pre-B II**	**Immature/ mature B**
Total Frequency %		28.3	71.7	15	83.4	11.7	46.7	21.7	3.3
Children Frequency %		-	-	11.6	86.1	9.3	51.2	23.2	2.3
Adults Frequency %		-	-	23.5	76.5	17.6	35.3	17.6	5.9
Mean age (year)		22.5 ±6.8	6.0 ±3.9	12.9 ±8.7	10.3 ±9.1	12.1 ±8.2	9.7 ±8.7	10.7 ±11.1	10.5 ±7.8
Mean WBC Count * 10^9/L		12.7 ±14.3	46.3 ±86.2	64.3 ±75.2	23 +37.7	52.8 ±86.9	21.3 ±23.4	12.3 ±10.6	11.5 ±12.7
Mean RBC count * 10^{12}/L		3.8 ±0.8	3.2 ±0.8	3.6 ±0.9	3.2 ±0.8	2.8 ±0.8	3.4 ±0.8	3.2 ±0.8	3.3 ±0.6
WBC count >50000 * 10^9/L		0/17 (0)	9/43 (21)	3/9 (33.3)	5/50 (10)	1/7 (14.3)	4/28 (14.3)	0/13 (0)	0/2 (0)
FAB classification	L1	7/14 (50)	33/41 (80.5)	2/7 (28.6)	38/48 (79.1)	5/7 (71.4)	23/28 (82.1)	10/11 (90.9)	0/2 (0)
	L2	6/14 (42.9)	6/41 (14.6)	5/7 (71.4)	7/48 (14.5)	2/7 (28.6)	4/28 (14.3)	1/11 (9.1)	0/2 (0)
	L3	1/14 (7.1)	2/41 (4.9)	0/7 (0)	3/48 (6.2)	0/7 (0)	1/28 (3.6)	0/11 (0)	2/2 (100)
Hepatomegaly		4/17 (23.5)	32/43 (74.4)	5/9 (55.6)	30/50 (60)	5/7 (71.4)	17/28 (60.7)	7/13 (53.8)	1/2 (50)
Splenomegaly		8/17 (47)	29/43 (67.4)	4/9 (44.4)	32/50 (64)	5/7 (71.4)	17/28 (60.7)	8/13 (61.5)	2/2 (100)
Lymphadenopathy		3/17 (17.6)	10/43 (23.2)	4/9 (44.4)	8/50 (16)	0/7 (0)	6/28 (21.4)	2/13 (15.4)	0/2 (0)
Mediastinal mass		3/17 (17.6)	6/43 (14)	6/9 (66.7)	2/50 (4)	0/7 (0)	2/28 (7.1)	0/13 (0)	0/2 (0)
Relapse		6/17 (35.3)	4/43 (9.3)	4/9 (44.4)	6/50 (12)	1/7 (14.3)	4/28 (14.3)	1/13 (7.7)	0/2 (0)

Data presented in parentheses represents percent of corresponding parameters. WBC: White Blood Cell, RBC: Red Blood Cell, FAB: French-American-British staging system

Cell Preparation. Peripheral blood or bone marrow mononuclear cells were separated using Histopaque (Sigma, USA) density- gradient centrifugation, as described (22). Isolated cells were washed twice with RPMI 1640 medium (Sigma, USA) prior to immunophenotyping.

Immunophenotyping. After separation, the mononuclear cells were stained with a panel of florescent-conjugated mAbs (DAKO, Denmark) specific for myeloid lineage [CD13 (clone WM-47), CD14 (clone TUK4) and CD33 (clone WM-54)], B cell lineage [CD10 (clone SS2/36), CD19 (clone HD37) and CD20 (clone B-Ly1)], T cell lineage [CD2 (clone MT910), CD3 (clone UCHT1) and CD5 (clone DK23)] and non specific lineage [CD34 (clone QBEnd10), CD45 (clone T29/33), HLA-DR (clone AB3) and TdT (clone HT-6)]. For most markers except for CD10 – CD19 and TdT- CD3 where dual staining was performed, single color staining was used. For surface staining, cells were washed twice with RPMI medium 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) before scanning by flow cytometer (Partec, Germany). In addition, staining for TdT and CD3 were performed at the cytoplasmic level. The same method was used for intra-cytoplasmic staining, but before addition of mAb, cells were made permeable using permeabilizing solution (Becton Dickinson, USA) and then were washed with washing buffer (PBS, 0.15M; BSA, 0.2% ; EDTA, 0.5mM ; sodium azide, 0.1% and saponin , 1%). Forward and side-scatter gates were used for analysis of leukemic antigenic expression (23). 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 (12).

Statistical Analysis. Statistical analysis was done using SPSS ver. 11.5 software. Statistical differences of various clinical and laboratory parameters between groups were evaluated by Chi-Square or the Fisher's Exact tests. To compare the mean of two groups, the two independent sample t-test was used. Correlation of immunophentypic results between paired BM and PB samples was determined by the Spearman Ranks Correlation Test. P-values of less than 0.05 were considered significant.

RESULTS

Of 60 Iranian ALL patients, 50 (83.4%) were identified as B-ALL, 9 (15%) as T-ALL and one patient (1.7%) as mixed lineage (Table 1). Based on the expression pattern of CD19, CD10, TdT, HLA-DR and CD20, we classified our B-ALL patients into four subtypes including Pro-B, Pre-BI, Pre-BII and immature-mature B (Table 2) (24, 25 and 26). Anti IgD Ab is essential for discrimination of mature and immature B-cells which was not available in this study. Representative flow cytometry results for Pro-B, Pre-BI and Pre-BII subtypes are illustrated in figure 1. The cumulative data obtained for all patients is given in figure 2 presented as a dot histogram.

Table 2. Classification basis of B-ALL patients

Subtype	HLA-DR	TdT	CD19	CD10	CD20
Pro-B	+	+	+	-	-
Pre-B I	+	±	+	+	-
Pre-B II	+	-	+	+	+
Immature / mature B	+	-	+	-	+

CD: Cluster of Differentiation, TdT: Terminal Deoxy nucleotidyl Transferase

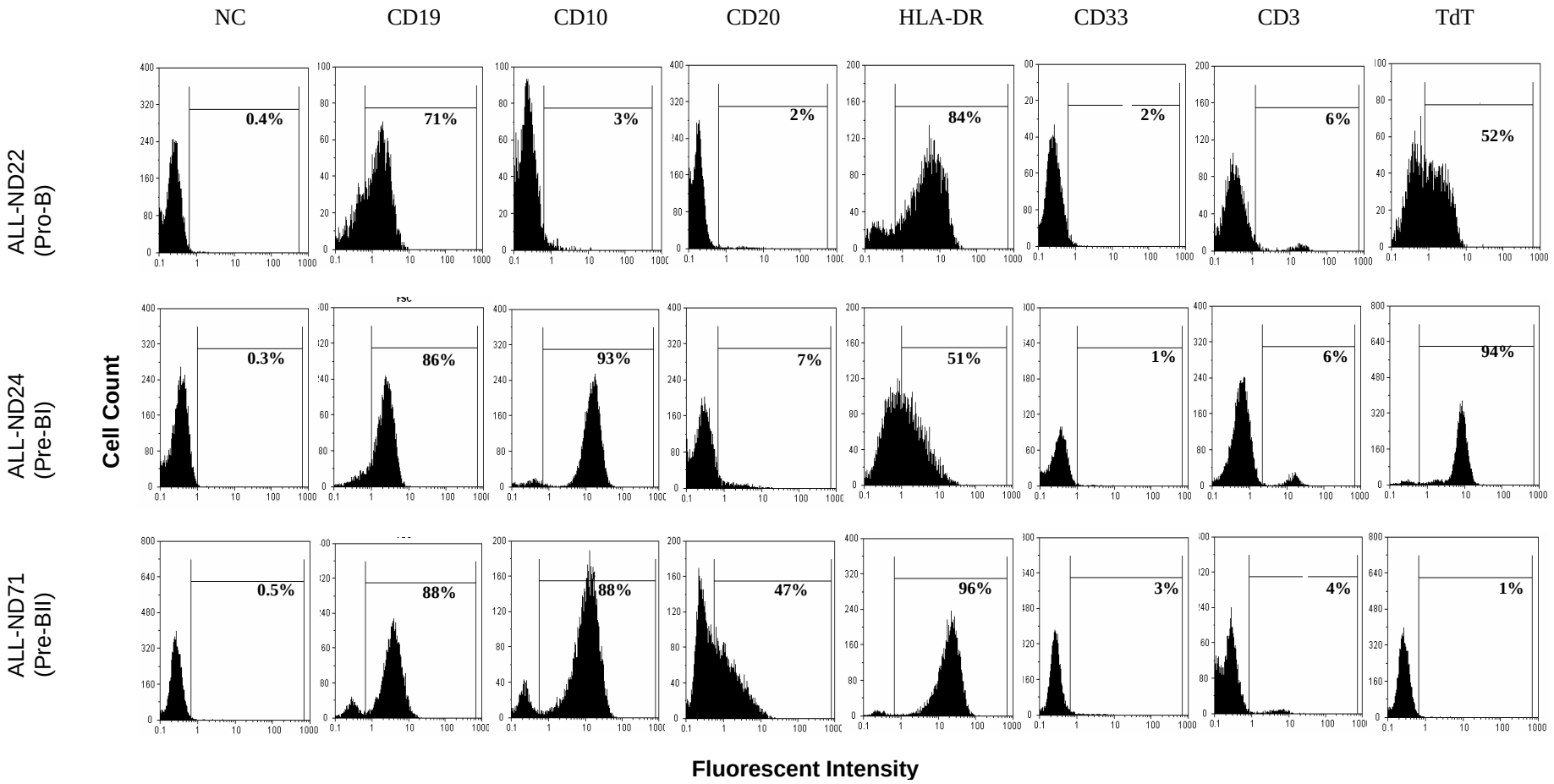

Figure 1. Representative immunophenotypic results obtained for three B-ALL patients (Pro-B, Pre-BI and Pre-BII) by flow cytometry. NC: Negative control, CD: Cluster of differentiation, TdT: Terminal deoxynucleotidyl transferase, ALL-ND: ALL newly diagnosed (patient designation). Percentages presented in each diagram represent proportion of positively stained cells.

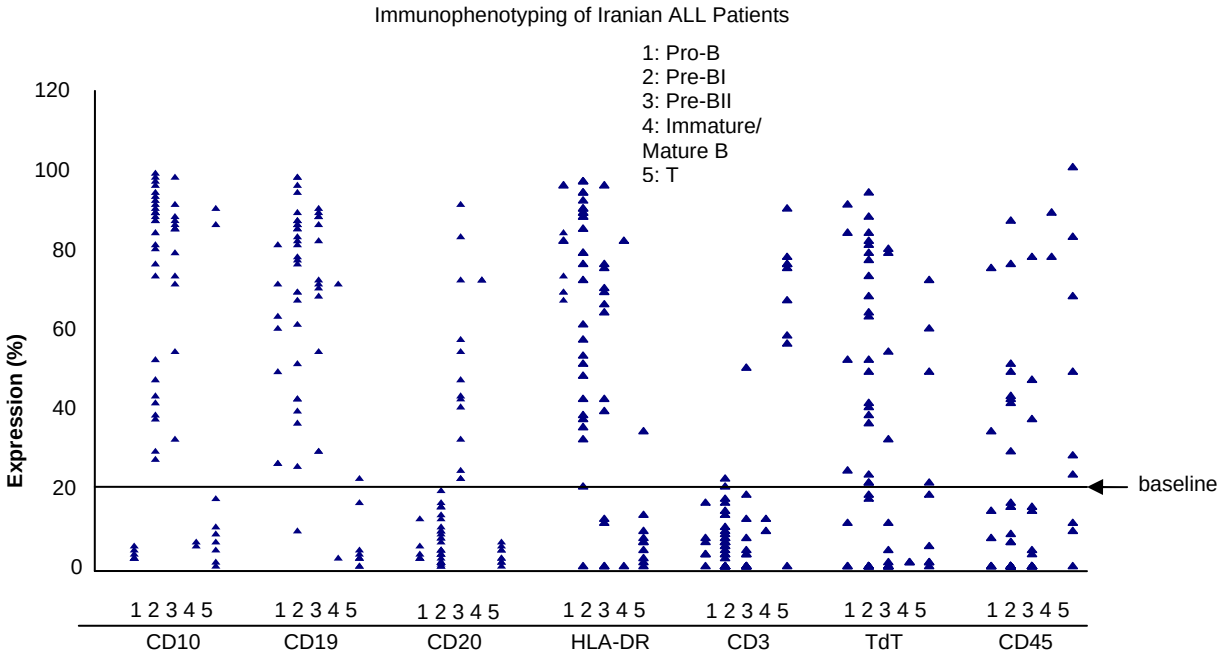

Figure 2. Expression pattern of major immunophenotypic markers in different subtypes of Iranian ALL patients. Baseline denotes a cut off expression value (20%) arbitrary assigned to differentiate positive and negative results.

Comparison of immunophenotypic subtypes with FAB classification revealed a higher frequency of L2 in T-ALL than in B-ALL types (p=0.03, Table 1). Since both of L3 cases in this study belonged to the immature/mature B subtype, a high association between these two phenotypes are suggested; however, the small sample size does not give a clear cut conclusion. The L1 morphology was evenly distributed in Pro-B, Pre-BI and Pre-BII subtypes (Table 1).

Frequency analysis of the clinical and laboratory findings in different B-ALL subtypes failed to establish significant association for any of the subtypes. However, increased mediastinal mass and mean WBC count ($>50\times10^9$/L) were found to be correlated with T-ALL, but not B-ALL types (p=0.001 and p=0.01, respectively) (Table 1). The frequency of relapse was also higher in T-ALL compared to B-ALL cases, though the association was not significant (p=0.064). The relapse rate was also more predominant in adults than children (35% vs. 9%). None of the other parameters were observed to be significantly associated with the T- or B-ALL subtypes.

Paired BM and PB samples were available from 29 patients. Mononuclear cells were immunophenotyped for each patient. Representative results are illustrated in Figure 3. Concordant profiles for CD10, CD19, CD20, HLA-DR and TdT molecules were obtained for all paired samples (data not presented).

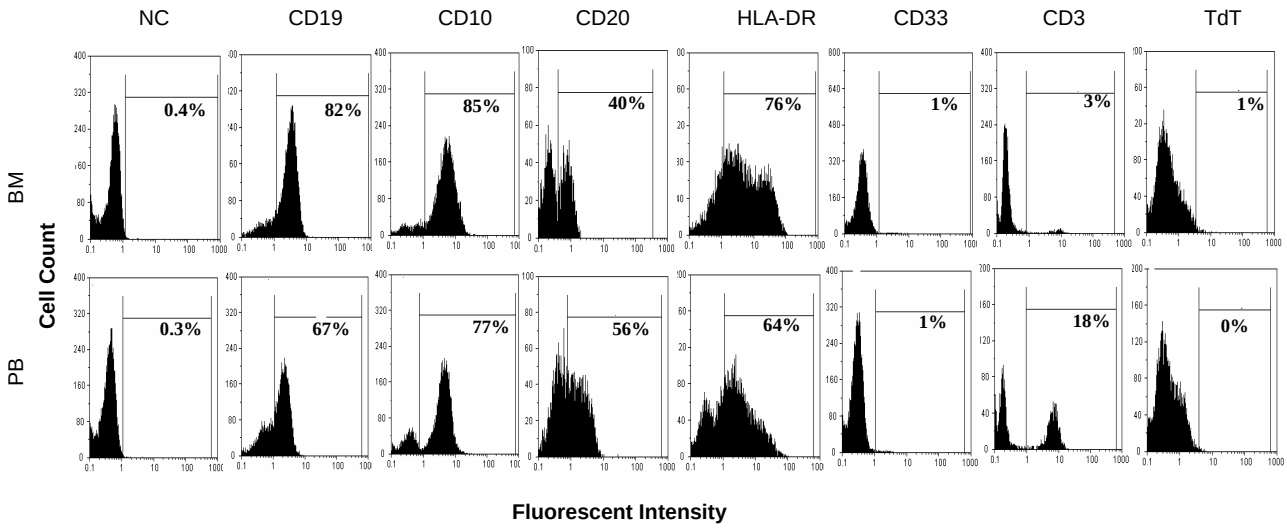

Figure 3. Representative flow cytometry results of a paired BM and PB sample obtained from an ALL patient with Pre-BII immunophenotype. See footnote to figure 1.

DISCUSSION

Immunophenotyping is a crucial component of the initial diagnosis of acute leukemias and a valuable tool for monitoring minimal residual disease (MRD) after therapy (1). Several hundred mAbs have been assigned against cluster of differentiation (CD) antigens for diagnosis of different types of leukemia, lymphoma and other malignancies (27). Immunophenotypic characterization of leukemic cells has several goals including lineage assignment, evaluation of cell maturation and assessment of phenotypic aberrations (1).

In the present study, we employed a panel of mAbs against CD2, CD3, CD5, CD10, CD13, CD14, CD19, CD20, CD33, CD34, CD45, HLA-DR and TdT to determine the phenotype of the leukemic cells. Initial screening with CD3 and CD19 molecules enabled broad classification of the leukemic cells into T- and B-cells. Based on the expression of CD10, CD19, TdT, HLA class II and CD20, we classified our B-ALL cases into four subtypes. In some studies cytoplasmic and surface IgM expression together with CD19 and CD10 analysis were used to identify and classify the B-ALL cells (11, 27). Detection of membrane CD20 has been employed by many investigators as an alternative to IgM for classification of B-ALL (24, 25 and 28). Although European Group for Immunophenotypical Characterization of Leukemias (EGIL) (21) has proposed the use of IgM expression for B-ALL subtype classification, comparison of published results reveals no obvious difference between the frequency of B-ALL subtypes using either IgM or CD20 expression for B-ALL sub-classification (references presented in Table 3).

Table 3. Frequency of ALL immunophenotypic subsets in other studies

No	Year	Sample size	Age group	Country	T-ALL %	Total B lineage %	Pro B %	Common B		Immature/ mature B %	Reference
								Pre-B I %	Pre-B II %		
1	1990	39	Adult	USA	20.5	74.4		66.7 *		7.7	32
2	1992	2404	Pediatric	USA	15.1	80.9		78.9 *		2	33
3	1985	140	Pediatric	Italy	11.4	88.6	9.3	79.3 **		0	34
4	1985	50	Adult	Italy	26	74	10	64 **		0	34
5	1990	578	Pediatric	Germany	13.5	86	5.3	77.9 **		2.8	35
6	1994	526	Adult	France	28.5	68.5	8.7	30.4	26.2	3.2	28
7	1988	54	Pediatric	Croatia	11.1	86.2	7.4	75.9 **		1.9	36
8	1995	171	Pediatric	Bulgaria	28	72	13	42	11	1	37
9	1982	49	Pediatric & Adult	Israel	32.7	38.8	10.2	28.6 **		0	38
10	1988	152	Pediatric & Adult	India	37.7	54.6	33.1	21.5 **		0	39
11	1994	125	Pediatric & Adult	India	53.6	37.2	6.4	26.4 **		4	12
12	1990	163	Pediatric	Saudi Arabia	12.3	87	21.5	57 **		8.6	40
13	2003	65	Pediatric & Adult	Oman	21.5	77.1	7.8	35.4	18.5	15.4	17
14	1992	28	Pediatric	Thai	7	93	18	71.5 **		3.5	41
15	1999	38	Pediatric	Thai	18.4	81.6	0	65.8 **		15.8	14
16	2005	263	Pediatric & Adult	Thai	19.4	80.6	3.8	26.6	34.2	15.9	8
17	1998	36	Pediatric	Malaysia	22	75		75 *		3	19
18	1999	402	Pediatric	Mexico	9.4	90.6	5	82.1 **		3.5	42
19	2001	81	Pediatric	Mexico	8.6	91.4	7.4	77.8	6.2	0	11
20	1996	153	Pediatric	Brazil	16.4	81.7	6.5	72.6 **		2.6	43
21	1996	72	Adult	Brazil	26.4	73.6	1.4	56.9 **		15.3	43
22	1996	500	Pediatric	Chile	10	90	14	74 **		2	44
23	1996	113	Adult	Chile	15	85	11	72 **		2	44
24	1989	186	Pediatric & Adult	Egypt	50	47.2	4.8	39.2 **		3.2	45
25	1992	64	Pediatric & Adult	Zimbabwe	25	50	18.7	31.3 **		0	46
26	2006	60	Pediatric & Adult	Iran	15	83.4	11.7	46.7	21.7	3.3	present study

* The results refer to the cumulative frequency of Pro-B, Pre-B I and Pre-B II subtypes
* * The results represent the cumulative frequency of Pre-B I and Pre-B II subtypes

Analysis of the association between clinical and laboratory findings with immunophenotypic subtypes of ALL revealed close association between disease relapse and T-ALL, although the difference was not significant due to the small sample size of T-ALL in our patients (Table 1). Similar findings have also been reported by many other investigators (29, 30). A significantly higher WBC count and higher frequency of mediastinal mass were also observed in T-ALL patients. Such findings have also been reported by other investigators (28, 31). Despite methodological differences, our results were reasonably in concordance with reports of other investigators from different countries with dissimilar ethnic populations (Table 3). These studies have been conducted in countries from North and Latin America, Europe, Africa and Asia. There have been only three published reports from the Middle East with substantial differences in the frequency of T and Pro-B subtypes between these studies (17, 38, 40). Our study is the first detailed immunophenotypic study conducted on Iranian ALL patients. Our results on the frequency of B-ALL and T-ALL subtypes are similar to the results of the study performed in Oman (17).

As a general finding from all published studies summarized in table 3, the frequency of T-ALL has always been substantially lower than B-ALL with few exceptions. T-ALL constituted almost 50% of all immunophenotyped ALL patients from India and Egypt (12, 45). High frequencies of T-ALL (more than 25%) have also been reported in ALL patients from Israel (38), Bulgaria (37), France (28), Italy (34) and adult Brazilian patients (43) (Table 3).

Comparison of different subtypes of B-ALL among published reports has demonstrated a lower frequency of immature/mature B subtype followed by Pro B subtype, with the exception of Omani (17) and adult Brazilian patients (43). The common B-ALL subtype which constitutes the Pre-B I and Pre-B II subtypes is the dominant subtype in all studies. In many of the previous publications sub-classification of the common B type ALL has not been possible due to lack of appropriate mAbs in the immunophenotypic panel employed in those studies (see Table 3). The dissimilarities observed among various studies could largely be due to the small sample size of the patients in many studies, methodological differences in classification of ALL (47, 48) as well as ethnicity (49) and age (50). It has already been shown that certain subtypes of ALL may be prevalent in some regions of the world (49).The prevalence of T and Pro-B ALL subtypes were higher in our adults, which was similar to the previous report in a large number of German patients (50).

We used FAB and immunophenotyping methods for classification of ALL patients. The results indicated that most patients with B subtype in our study had L1 morphology with the exception of patients with late stage B-ALL. The latter subtype with only 2 cases was entirely restricted to the L3 morphology (Table 1). Association between immature/mature B-ALL with the FAB L3 morphology has already been demonstrated (6, 7). The L2 morphology was mostly confined to the T-ALL cases, a finding also reported by others (5). No other significant associations were observed for the remaining clinical and laboratory findings with immunophenotypic subtypes of our ALL patients (Table 1).

In conclusion, our results confirm and extend previous reports indicating heterogeneity of ALL and the significance of immunophenotyping of the leukemic cells for monitoring of disease outcome and prognosis.

ACKNOWLEDGEMENTS

This study was partly supported by a grant from Medical Sciences/University of Tehran.

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