Flow Cytometry Immunophenotyping Evaluation in Acute Lymphoblastic Leukemia: Correlation to Factors Affecting Clinic Outcome

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The authors conducted a flow cytometry immunophenotyping study in patients with acute lymphoblastic leukemia (ALL) from Natal, Rio Grande do Norte, Brazil. The patients ($n = 126$) were newly diagnosed using a panel of monoclonal antibodies: CD1a, CD2, CD3, CD4, CD7, CD8, CD10, CD13, CD33, CD14, CD19, CD22, CD79a, CD117, CD34, anti-IgM, anti-TdT, anti-HLA-Dr, and anti-human kappa and lambda light chains. Additional data, such as patients' age and gender, clinical and laboratory findings such as presence of tumor masses, lymphadenopathy, hepatomegaly, splenomegaly, leukemic infiltration in the central nervous system (CNS) were also investigated. Results showed that 56.7% of the cases were B-lineage ALL and 55% were T-cell ALL. Also, we found that males were more affected by the disease, regardless of immunological classification. The correlation between age and immunological subtypes showed that the B-lineage

ALL occurred more frequently in patients aged under 15 while the T-cell ALL subtype was more frequent in adults. Immunophenotypic profiles and morphological subtypes showed a direct correlation between L3 subtype and B-lineage ALL, while L1 and L2 subtypes correlated more often with B-cell lineage and T-cell ALL, respectively. Correlation analysis between immunophenotypic and clinical profiles showed that T-cell ALL was more associated with a higher incidence of lymphadenopathy, hepatomegaly, splenomegaly and CNS leukemic infiltration, also showing a greater blast cell count in peripheral blood than the other subgroups. The presented data suggest that immunophenotyping is an important method in the diagnosis, monitoring and prognostic assessment in determining the pathological mechanisms of evolution of ALL. *J. Clin. Lab. Anal.* 26:431–440, 2012. © 2012 Wiley Periodicals, Inc.

Key words: flow cytometry; immunophenotyping; acute lymphoblastic leukemia

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignant clonal proliferation of lymphocytes expressing an immature phenotype. It is currently diagnosed and classified primarily according to cytomorphology, and immunophenotypic and genetic characteristics. For the diagnosis of ALL, a blast percentage of at least 20% in bone marrow (BM) or peripheral blood (PB) is required (1–4).

The primary purpose of the morphological classification is to separate the acute leukemias into acute myelogenous leukemia (AML) and ALL. The French–American–British (FAB) system, which was based primarily on the microscopic appearance of the leukemic cells as seen on Wright–Giemsa stained smears, classified ALL into three different morphologic subtypes: ALL-L1 (with small uniform cells), ALL-L2 (with large varied cells), and ALL-L3 (with large varied basophilic cells with vacuoles or Burkitt-like ALL) (4–6).

Flow cytometry immunophenotyping is a key factor in the diagnosis and prognosis of ALL, turning the morphological analysis method complete or the initial screening when combined with cytochemical staining methods (4). It is a more accurate method based on the principle of cell differentiation antigen expression (Cell Markers) determined by a panel of monoclonal antibodies (MoAb) in the maturation process of T and B lymphocytes (4, 7–9).

Based on the principle that during the lymphocyte maturation process, surface and cytoplasm antigens are acquired and lost, the European Group for the Immunological Characterization of Leukemias (EGIL) proposed a standardized immunological classification for acute leukemias according to antigen expression by detecting, in addition to cell line, the level of differentiation of the leukemic process (10). These expressions are to predict, for B or T phenotypes, the biological changes associated with therapeutic response, monitoring of minimal residual disease, and stratification of groups at higher or lower risk. Today, these expressions are known to be far more important than strictly morphological criteria (3, 9–11).

According to the cell line, ALL is classified into B-lineage ALL and T-cell ALL. Approximately, 20% of ALL cases are of T-cell lineage, 75% of B-cell precursors, and 5% of mature B cells (3, 4, 9–11).

B-lineage ALL was classified, according to the stages of normal B-cell progenitors differentiation in the BM, into Pro-B, Common Pre-B, Pre-B $\text{c}\mu+$, and B-cell ALL. Pro-B ALL represents 5% of pediatric cases and 10% of cases of ALL in adults (3, 4, 9–12). Leukemic cells express HLA-Dr, Terminal deoxynucleotidyl Transferase (TdT), CD34, and CD19 (3, 4, 9–12). Common Pre-B ALL expresses CD19, CD10, and also cytoplasmic CD22

(cCD22) and cCD79a (3, 4, 9–12). This leukemia represents 75% and 50% of pediatric and adult cases, respectively. Pre-B leukemia expresses cytoplasmic $\text{m}\mu$ chain ($\text{c}\mu+$) in addition to CD19, CD10, CD22, and cCD79a (3, 4, 9–12), representing approximately 15% and 10% of pediatric and adult cases of ALL, respectively (3, 4, 9–12). Lastly, mature B-cell ALL, representing 2–5% of pediatric and adult cases, presents an unusual phenotype characterized by expression of surface immunoglobulin IgM (sIgM) and either kappa (κ) or lambda (λ) immunoglobulin light chains on the membrane surface (3, 4, 9–13). The blasts present the same ALL-L3 morphological FAB-type and chromosomal translocations associated with malignant cell in Burkitt's lymphoma (3, 4, 9–12). This ALL type has poor prognosis because there is a high incidence of central nervous system (CNS) involvement, poor response to therapy, and shortened survival (3, 4, 9–13).

According to the differentiation antigens corresponding to normal levels of intrathymic differentiation, T-cell ALL is divided into pre-T, T-intermediate, and mature or medullar ALL (9, 11, 14). In pre-T ALL, blastic cells express cytoplasmic CD3 (cCD3) but not on the cell surface, characteristically expressing CD7, CD34, and high levels of TdT (9, 11, 14). In intermediate ALL, the cells begin to highly express CD7, cCD3, CD2, CD1a, and can co-express CD4 and CD8 simultaneously (cells double positive or $\text{CD4}+\backslash\text{CD8}+$) (9, 11, 14). In mature T-lineage ALL, medullar thymocytes express CD2, CD5, CD7, and surface CD3 (sCD3), leading to CD4 and CD8 dichotomy (9, 11, 14). T-cell ALL represents 25% of adult cases and 15% of pediatric cases of ALL, occurring most frequently in males and being associated with a high white blood cell (WBC) count at diagnosis, mediastinal mass and CNS involvement (9, 11, 14). The immunophenotypic profile of ALL is summarized in Table 1.

As a result of the lack of data on the immunophenotypic profile of acute leukemias and their correlation with demographic, clinical and laboratory aspects in Brazil, this study aimed to investigate this information in a group of patients with ALL in Natal, city from Northeastern Brazil.

METHODS

Sample Collection

PB and BM blast cells were collected from 126 newly diagnosed patients with ALL in the Department of Hematology of Hemocentro Dalton Cunha (HEMONORTE), Natal-city, Brazil. Demographic and clinical features of these patients are shown in Table 2. Diagnosis of ALL was initially based on clinical presentation, PB and BM smears analyzed according to the FAB criteria for morphological classification of ALL (5, 6)

TABLE 1. Immunophenotypic Subsets of ALL

Subset of ALL	Immunophenotyping
Pre-T ALL	CD7+; cCD3+/-; CD2-; CD1a-; CD34+; HLA-Dr+; TdT+
T-intermediate ALL	CD7+; cCD3+; CD2+; CD1a+; CD4+/CD8+; HLA-Dr+/-; TdT+
T-medullar ALL	CD7+; sCD3+; CD2+; CD1a-; CD4+ or CD8+; HLA-Dr-; TdT+/-
Pro-B ALL	HLA-Dr+; CD19+; cCD79a+; CD34+; CD10-; CD20-; cCD22-; cμ-; TdT+
Common ALL	HLA-Dr+; CD19+; cCD79a+; CD34+; CD10+; CD20+/-; cCD22+/-; cμ-; TdT+
Pre-B (cμ+) ALL	HLA-Dr+; CD19+; cCD79a+; CD34+/-; CD10+; CD20+; sCD22+; cμ+; TdT+/-
B-cell (sIgM+) ALL	HLA-Dr+; CD19+; cCD79a+; CD34-; CD10-; CD20+; sCD22+; sIgM+; TdT-

Note: cCD3, cytoplasmatic CD3; sCD3, surface CD3; cCD22, cytoplasmatic CD22; sCD22, surface CD22; cμ, cytoplasmic μ heavy chain of immunoglobulin; sIgM, surface IgM immunoglobulin; TdT, Terminal deoxynucleotidyl Transferase.

According to the modifications of Bachir et al. (9), Bene et al. (10), and Matutes (11).

TABLE 2. Subsets of Acute Lymphoblastic Leukemia in This Study

Immunological subsets of ALL	N° (%)
Total of T-cell ALL	55 (43.6%)
Pre-T ALL	14 (11.1%)
T-intermediate ALL	12 (9.5%)
T-medullar ALL	29 (25%)
Total of B-lineage ALL	71 (43.6%)
Pro-B ALL	06 (4.8%)
Common-ALL	48 (38.1%)
Pre-B cμ+ ALL	06 (4.8%)
B-cell ALL	11 (8.7%)
Total ALL	126 (100%)

and conventional immunophenotyping according to The World Health Organization Classification of Neoplasms of the Hematologic and Lymphoid Tissue (WHO classification) (9–11, 15–18). All patients provided a written informed consent according to the Declaration of Helsinki.

The diagnosis of ALL was confirmed in patients with a blast percentage of at least 20% in BM, which was based on the FAB criteria by classifying into L1, L2, and L3 subtypes. The diagnosis was performed by using May–Grünwald-Giemsa (MGG) stained PB and BM smears and flow cytometry immunophenotyping.

Hematological Analysis From PB

Hematological analysis was performed with 10 ml of peripheral venous blood collected in blood collection tubes containing EDTA. WBC count, hemoglobin dosage, and platelet count were scored in the hematological analyzer (Cell-Dyn 3.000, Unipath Corp., Mountain View, CA). Cytomorphologic analysis was performed using MGG-stained blood films in which 100 leukocytes were counted and the result scored in percentage and cytomorphological alterations were also recorded.

BM Cytomorphology

BM aspirate smears were stained with MGG for morphological analysis. Additional cytochemical staining with Sudan Black (SB) was performed with the purpose of excluding cases of AML (3).

Flow Cytometry Immunophenotyping

Cells immunostaining

Immunophenotyping assays were performed by three-color flow cytometry analysis of PB and/or BM samples. Cytoplasmic and surface cell markers were identified using a panel of MoAbs based on the EGIL strategies (10).

The reactivity with fluorescent-conjugated MoAb was against T-cell-related antigens (CD1a, CD2, CD3, CD4, CD5, CD7, and CD8), B-cell differentiation markers (CD10, CD19, CD20, CD22, CD79a), myeloid cell antigens (CD11b, CD13, CD14, CD15, CD33, CD117, and MoAb antimyeloperoxidase [MPO]), nonlineage restrict molecules (nuclear enzyme TdT, CD45, HLA-Dr, CD34), and also MoAb against IgM heavy chains (μ chain) and light chains of immunoglobulins (κ and λ). All MoAbs were from Becton Dickinson Immunocytometry Systems (San Jose, CA).

For surface antigens, 100 μl of total PB and/or BM cells previously homogenized were incubated with 20 μl of MoAb for 30 min at room temperature in the dark. Furthermore, the cell suspension was increased to 1 ml of lysis solution (Becton Dickinson's FACS Lysing Solution), previously prepared in distilled water (1:10; v:v), and then incubated for 10 min at room temperature in the dark. Then, the cell suspension was centrifuged for 5 min at 600 × g, the supernatant fluid was discarded and the cell pellet was resuspended in cold PBS (pH 7.2) and centrifuged again. The last step was repeated. Finally, the cell pellet was resuspended in 1 ml of 0.5% formaldehyde

Fig. 1. Immunophenotyping profile of one case of Common ALL. (A) FSC and SSC, morphologic characteristic of blood cells in especially blastic cells in the recessed area (gate); (B) CD19 and CD3 expressed in 80% and 10% of blastic cells, respectively; (C) CD10/CD22 co-expression in 68% of blastic cells; (D) negative expression of CD7 antigen; (E) cytoplasmic CD79a expression in 70% and cytoplasmic CD13 in blastic cells; (F) MoAb against Terminal deoxynucleotidyl Transferase (TdT) expression in 80% of blastic cells and negative expression of myeloperoxidase antigen (MPO).

in PBS, and cell suspension was kept in the dark at 4°C until flow cytometry analysis.

In the intracytoplasmic cell markers staining assay (TdT, CD3, CD13, CD22, CD79a, μ chain, and MPO), cells samples were first incubated for 10 min at room temperature with Becton Dickinson's FACS Lysing Solution. Then, cell suspensions were centrifuged for 5 min at 600 $\times$ g. The supernatant was discarded and the cell pellet was resuspended in cold PBS, and washed twice by centrifugation for 5 min at 600 $\times$ g. The cell pellet was then resuspended in 1 ml of cold PBS containing 1% formaldehyde, and kept in the dark at 4°C until flow cytometry analysis (19, 20). For each test (surface and cytoplasmic analysis), an isotype-matched MoAb was used as negative control.

Flow Cytometry Analysis

A total of 20,000 events per tube were acquired with Fluorescence Activated Cell Analyzer (FACScan, San Jose, CA) with Cell Quest software (Cell Quest™ Software, Becton Dickinson Immunocytometry Systems). The following parameters were considered: forward scatter (FSC) to evaluate cellular size, side scatter (SSC) to evaluate cellular complexity, and analysis of cell marker expression with fluorescence analysis by FL1 (green), FL2

(orange), and FL3 (red) in logarithmic scale, which represents the reaction antigen–antibody conjugated to fluorescein isothiocyanate (FICT), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP), respectively.

Immunophenotyping results were considered positive when at least 25% of cells were positive for all MoAb. Figures 1, 2, and 3 show histograms in percentage of the cellular population with positive or negative reactions and fluorescence intensity.

RESULTS

Immunophenotyping Findings

From the 126 patients with ALL analyzed in this study, 55 cases were T-cell ALL while 71 were B-lineage ALL (Table 2).

T-cell ALL diagnosis was based on T-cell reactivity to various MoAbs anti-T antigens (Table 3). All diagnosed T-cell ALL had surface CD7 and cCD3 antigens. CD5 antigen was found in 50% of T-intermediate ALL and in all T-medullary ALL. In the latter, 14 cases (11.1%) had a characteristic phenotype of Pre-T ALL with CD7+, cCD3+, HLA-Dr+, CD34+, and high levels of TdT+. T-intermediate ALL comprised 12 cases (9.5%), whose phenotypes were characterized by a positive reaction to CD1a, CD2, and also double-positive

Fig. 2. Immunophenotyping profile of one case of Pre-B $c\mu+$ ALL. (A) FSC and SSC, morphologic characteristic of blood cells in especially blastic cells in the recessed area (gate); (B) CD19 and CD3 expressed in 70% and 1% of blastic cells, respectively; (C) CD10 expression in 30% of blastic cells and negative in CD20; (D) negative expression of cytoplasmic CD3 antigen and cytoplasmic expression of μ chain of immunoglobulin in 85% of blastic cells; (E) negative expression of CD41 and CD33 antigens; (F) negative expression of MoAb against myeloperoxidase antigen (MPO) and positive expression of cytoplasmic expression of CD22 in 90% of blastic cells.

cells (CD4+/CD8+), maintaining an expression of CD7, cCD3, CD34+, TdT+, and HLA-Dr+ in most cases. T-medullar ALL included 29 cases (25%), whose main features were the selective expression of CD4+ or CD8+ similarly to mature T lymphocytes and the expression of surface CD3 (sCD3) and negative expression of CD1a, TdT, HLA-Dr, and CD34 antigens. In this group of ALL, CD10 was found in 17 cases (31%).

B-lineage ALL was diagnosed when more than 25% of leukemic cells were expressing pan-B cells markers CD19 and cCD79a. In this group, six cases (4.8%) had a phenotype characteristic of Pro-B ALL, expressing only CD19, HLA-Dr, CD34, and TdT. Common ALL accounted for 48 cases (38.1%), whose phenotypes showed the acquisition of CD10, as well as expression of CD19, cCD22, and HLA-Dr in most cases. Pre-B $c\mu+$ ALL represented a total of six cases (4.8%), which characteristically expressed $c\mu+$ in addition to CD19, HLA-Dr, CD10, and cCD22. Finally, 11 cases (8.7%) were B-cell ALL blast cells, which presented the complete expression of surface IgM immunoglobulin (sIgM+), also expressing either the κ or λ immunoglobulin light chains.

Aberrant expression of myeloid antigens such as CD13 and CD33 was also observed, where CD13 expression was more frequent than CD33. The first was found in five cases of pre-B-lineage ALL and T-cell ALL, while the latter was found in two cases of pre-B ALL and in one case of T-cell ALL. MoAbs against MPO and CD117 were negative in all cases analyzed.

Clinical and Demographic Findings

Table 4 summarizes all data on general groups, including age, gender, and some clinical characteristics of patients at diagnosis. There was a predominance of males regardless of the immunophenotypic profile: Pro-B ALL (83.3% males, 16.7% females), Common ALL (56.2% males, 43.8% females), Pre-B $c\mu+$ ALL (66.7% males, 33.3% females), and B-cell ALL (72.7% males, 27.3% females). In T-cell ALL, we found the same trend, where of 55 patients studied, 39 (70.9%) were males and 16 (29.1%) females.

The study group age ranged from 1 to 63 years: Pro-B ALL (4–40 years, median = 15 years), Common ALL (4–25 years, median = 8), Pre-B $c\mu+$ ALL (1–25 years,

Fig. 3. Immunophenotyping profile of one case of T-cell ALL. (A) FSC and SSC, morphologic characteristic of blood cells in especially blastic cells in the recessed area (gate); (B) CD19 and surface CD3 expressed in 1% and 96% of blastic cells, respectively; (C) CD10 and CD22 negative; (D) CD22 and CD20 expression in 1% of blastic cells; (E) Terminal deoxynucleotidyl Transferase (TdT) expression in 90% of blastic cells; (F) cytoplasmic expression of CD3 antigen in 99% of blastic cells and negative expression of cytoplasmatic μ chain of immunoglobulin.

median = 18), B-cell ALL (4–21 years, median = 6), and T-cell ALL (1–63 years, median = 32).

Clinical and tumor characteristics were indicated by the presence of lymphadenopathy, hepatomegaly, splenomegaly, tumor mass, and leukemic infiltration of the CNS. Lymphadenopathy was observed in most cases of ALL: Pro-B ALL (four cases or 66.6%), Common ALL (25 cases or 52.1%), Pre-B μ + ALL (three cases or 50.0%), B-cell ALL (nine cases or 81.8%), and T-cell ALL (30 cases or 54.5%). Splenomegaly and hepatomegaly were also observed in most cases. For splenomegaly: Pro-B ALL (three cases or 50%), Common ALL (24 cases or 41.7%), Pre-B μ + ALL (four cases or 66.7%), B-cell ALL (ten cases or 90%), and T-cell ALL (31 cases or 56.4%). For hepatomegaly: Pro-B ALL (three cases or 50%), Common ALL (24 cases or 41.7%), Pre-B μ + ALL (four cases or 66.7%), B-cell ALL (four cases or 36.4%), and T-cell ALL (27 cases or 49.1%). Tumor mass was observed only in B-cell ALL and T-cell ALL. Abdominal mass was detected in ten patients (90.9%) with B-cell ALL and in one case of T-cell ALL (0.02%). Mediastinal mass was observed in 50 cases (90.9%) of T-cell ALL. CNS involvement at diagnosis was observed in eight cases (11.7%) of the Common ALL, in two cases

(18.2%) of B-cell ALL, and in 11 cases (20%) of T-cell ALL.

Laboratorial Findings

WBC, blast cell count (BCC) in PB and BM cytomorphologic analysis are shown in Table 5. WBC ranged from less than $5 \times 10^9/l$ to more than $100 \times 10^9/l$. Values below $5 \times 10^9/l$ were observed in a small number of cases in all groups except T-cell ALL: Pro-B ALL (one case or 16.7%), Common ALL (four cases or 8.3%), Pre-B μ + ALL (one case or 16.7%), and B-cell ALL (two cases or 18.2%). Samples with WBC between $10 \times 10^9/l$ and $50 \times 10^9/l$ were observed in three cases (50%) of the Pro-B ALL, 12 cases (25%) of Common ALL, three cases (50%) of Pre-B ALL μ +, four cases (36.4%) of B-cell ALL, and 12 cases (21.8%) of T-cell ALL. WBC between $50 \times 10^9/l$ and $100 \times 10^9/l$ was observed in two cases (33.3%) of Pro-B ALL, nine cases (18.8%) of the Common ALL, one case (9.0%) of B-cell ALL, and 14 cases (25.4%) of T-cell ALL. Finally, WBC higher than $100 \times 10^9/l$ was observed in 12 cases (25%) of Common ALL, two cases (33.3%) of Pre-B μ + ALL, two cases (18.2%) of B-cell ALL, and 20 cases (36.4%) of T-cell ALL.

TABLE 3. Diagnosis of ALL Based On Reactivity With Various Monoclonal Antibodies

Cell markers	B-cell lineage ALL				T-cell ALL		
	Pro-B NT\+ (%)	Common NT\+ (%)	Pre-B cμ+ NT\+ (%)	B-Cell NT\+ (%)	Pre-T NT\+ (%)	T-int. NT\+ (%)	T-med. NT\+ (%)
CD10	06\00 (–)	48\48 (100)	06\06 (100)	11\01 (9.1)	14\02 (14.3)	12\00 (–)	29\15 (51.7)
CD19	06\06 (100)	48\48 (100)	06\06 (100)	11\11 (100)	14\00 (–)	12\00 (–)	29\00 (–)
cCD22	06\00 (–)	48\32 (66.7)	06\06 (100)	11\11 (100)	14\00 (–)	12\00 (–)	29\00 (–)
sCD22	06\00 (–)	48\00 (–)	06\06 (100)	11\11 (100)	14\00 (–)	12\00 (–)	29\00 (–)
cCD79a	06\06 (100)	48\48 (100)	06\06 (100)	11\11 (100)	14\00 (–)	12\00 (–)	29\00 (–)
cμ	06\00 (–)	48\00 (–)	06\06 (100)	NA	14\00 (–)	12\00 (–)	29\00 (–)
sIgM	06\00 (–)	48\00 (–)	06\00 (–)	11\11 (100)	14\00 (–)	12\00 (–)	29\00 (–)
anti-κ	NA	NA	NA	11\06 (54.5)	NA	NA	NA
anti-λ	NA	NA	NA	11\05 (45.4)	NA	NA	NA
CD1a	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\12 (100)	29\00 (–)
CD2	06\06 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\12 (100)	29\29 (100)
cCD3	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\08 (57.1)	12\12 (100)	29\26 (89.7)
sCD3	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\00 (–)	29\29 (100)
CD4 [–] /CD8 [–]	06\06 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\14 (100)	12\00 (–)	29\00 (–)
CD4 ⁺ /CD8 ⁺	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\12 (100)	29\00 (–)
CD4 ⁺ /CD8 [–]	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\00 (–)	29\18 (62.1)
CD4 [–] /CD8 ⁺	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\00 (–)	29\13 (44.8)
CD5	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\06 (50.0)	29\29 (100)
CD7	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\14 (100)	12\12 (100)	29\29 (100)
CD13	06\02 (33.3)	48\02 (4.16)	06\01 (16.6)	11\00 (–)	14\05 (35.7)	12\00 (–)	29\00 (–)
CD33	06\01 (16.6)	48\00 (–)	06\01 (16.6)	11\00 (–)	14\01 (4.14)	12\00 (–)	29\00 (–)
CD117	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\00 (–)	29\00 (–)
anti-MPO	06\00 (–)	48\00 (–)	06\00 (–)	11\00 (–)	14\00 (–)	12\00 (–)	29\00 (–)
HLA-Dr	06\05 (83.3)	48\44 (91.7)	06\06 (100)	11\10 (90.9)	14\11 (78.6)	12\09 (75.0)	29\08 (27.6)
CD34	06\04 (66.6)	48\17 (35.4)	06\02 (33.3)	11\00 (–)	14\10 (71.4)	12\08 (66.6)	29\00 (–)
TdT	06\06 (100)	48\46 (95.8)	06\04 (66.6)	11\00 (–)	08\07 (87.5)	11\09 (81.8)	25\02 (08.0)

Note: cCD3, cytoplasmatic CD3; sCD3, surface CD3; cCD22, cytoplasmatic CD22; sCD22, surface CD22; cμ, cytoplasmic μ heavy chain of immunoglobulin; MPO, myeloperoxidase; sIgM, surface IgM immunoglobulin; TdT, Terminal deoxynucleotidyl Transferase; cCD79a, cytoplasmic CD79a; anti-κ, anti-light chain *kappa* of immunoglobulin; anti-λ, anti-light chain *lambda* of immunoglobulin; NT, number of cases tested; +, number of cases positives; NA, not applicable; T-int., T-intermediate ALL; T-med., T-medullar ALL.

BCC in PB ranged from 10% to 100% in most patients. BCC less than 20% was observed in six cases (12.5%) of Common ALL, one case of Pre-B cμ+ ALL (16.7%) and B-cell ALL (9.1%), and four cases (7.3%) of T-cell ALL. BCC ranging from 21% to 30% was found in two cases (4.2%) of Common ALL, one case (16.7%) of Pre-

B cμ+ ALL, one case of B-cell ALL (9.1%), and four cases (7.3%) of T-cell ALL. Values from 31% to 60% were observed in two cases (33.3%) of Pro-B ALL and Pre-B cμ+ ALL, five cases (10.4%) of Common ALL, four cases (36.4%) of B-cell ALL, and 11 cases (20%) of T-cell ALL. Finally, values higher than 60% were observed

TABLE 4. Clinical and Demographic Features of the Subjects in 126 ALL Patients

	Pro-B ALL <i>n</i> = 6	Common ALL <i>n</i> = 48	Pre-B cμ+ ALL <i>n</i> = 6	B-cell I ALL <i>n</i> = 1	T-cell ALL <i>n</i> = 55
Age (median)	4–40 (15)	4–21(8)	1–25 (18)	4–21 (6)	2–63 (32)
Male	5 (83.3%)	27 (56.2%)	4 (66.7%)	8 (72.7%)	39 (70.9%)
Female	1 (16.7%)	21 (43.8%)	2 (33.3%)	3 (27.3%)	16 (29.1%)
Lymphadenopathy	04 (66.6%)	25 (52.1%)	03 (50.0%)	09 (81.8%)	30 (54.5%)
Splenomegaly	03 (50.0%)	24 (50.0%)	04 (66.7%)	11 (90.0%)	31 (56.4%)
Hepatomegaly	03 (50.0%)	20 (41.7%)	04 (66.7%)	04 (36.4%)	27 (49.1%)
Abdominal mass	0 (–)	0 (–)	0 (–)	10 (90.9%)	01 (1.81%)
Mediastinal mass	0 (–)	0 (–)	0 (–)	0 (–)	50 (90.9%)
CNS involvement	0 (–)	08 (16.7%)	0 (–)	02 (18.2%)	11 (20.0%)

Note: CNS, central nervous system.

TABLE 5. Laboratorial Finding of the Subjects in 126 ALL Patients

Laboratorial finding	Pro-B ALL <i>n</i> = 6	Common ALL <i>n</i> = 48	Pre-B cμ+ ALL <i>n</i> = 6	B-cell ALL <i>n</i> = 11	T-cell ALL <i>n</i> = 55
WBC ($\times 10^9/l$)	3.6–448.0	1.0–400.0	40.6–600.0	1.5–600.0	10.5–720.0
<5.0	01 (16.7%)	04 (8.3%)	01 (16.7%)	02 (18.2%)	00 (–)
5.0–10.0	00 (–)	11 (22.9%)	00 (–)	02 (18.2%)	09 (16.4%)
10.0–50.0	03 (50.0%)	12 (25.0%)	03 (50.0%)	04 (36.3%)	12 (21.8%)
50.0–100.0	02 (33.3%)	09 (18.8%)	00 (–)	01 (9.1%)	14 (25.4%)
>100.0	00 (–)	12 (25.0%)	02 (33.3%)	02 (18.2%)	20 (36.4%)
% BCC (median)	60–100 (80)	10–100 (60)	20–100 (60)	20–100 (80)	10–100 (80)
10–20	00 (–)	06 (12.7%)	01 (16.7%)	01 (9.1%)	04 (7.3%)
21–30	00 (–)	02 (4.2%)	01 (16.7%)	01 (9.1%)	04 (7.3%)
31–60	02 (33.3%)	05 (10.4%)	02 (33.3%)	04 (36.4%)	11 (20.0%)
61–100	04 (66.7%)	35 (72.9%)	02 (33.3%)	05 (45.4%)	36 (65.4%)
French American British (FAB) morphologic classification					
ALL-L1	05 (83.3%)	30 (62.5%)	05 (83.3%)	0 (–)	17 (30.9%)
ALL-L2	01 (16.7%)	18 (37.5%)	01 (16.7%)	0 (–)	38 (69.1%)
ALL-L3	0 (–)	0 (–)	0 (–)	11 (100%)	0 (–)

Note: CNS, central nervous system; PB, peripheral blood; WBC, white blood cell.

in four cases (66.7%) of Pro-B ALL, 35 cases (72.9%) of Common ALL, two cases (3.3%) of Pre-B cμ+ ALL, five cases (45.5%) of B-cell ALL, and 36 cases (65.4%) of T-cell ALL.

BM cytomorphologic analysis showed 57 cases characterized as ALL-L1, 58 ALL-L2, and 11 ALL-L3, with a majority of ALL-L1 in B progenitors cells and ALL-L2 in T-cell ALL. All ALL-L3 cases were classified as B-cell ALL, with eight males and three females aged from 4 to 21. Blast cells were positive to CD19, cCD79a, sCD22, HLA-Dr, and sIgM, and negative to CD34 and TdT in all cases with a low expression of CD10 in one case. Immunoglobulin light chains κ and λ were expressed in six and five cases, respectively.

DISCUSSION

In the present study, we described an important topic for diagnosis of ALL, a neoplastic proliferation of immature lymphocytes. The results presented here support the validity of using an immunophenotypic profile for diagnosis, monitoring, and prognostic assessment of ALL, which may be useful as an additional procedure to confirm the diagnosis or develop a treatment plan. Our data support that diagnosis, prognosis, and clinical course of patients with ALL depend on a number of factors, including age, gender, initial WBC, cytogenetic abnormalities, immunophenotypic profile, and response to treatment.

Among the factors considered in this study, the following suggest a favorable clinical course: 2–10 years age group, females, a WBC below $20 \times 10^9/l$, absence of organomegaly, a Pre-B Common ALL immunopheno-

typic profile, morphological subtype ALL-L1, and low BCC in BM (3, 4, 9, 14, 21). On the other hand, the 1–10 years range group, males, a WBC greater than $50 \times 10^9/l$, presence of organomegaly, Pro-B ALL, B-cell ALL and T-cell ALL immunophenotypic profiles, morphological subtypes ALL-L2 and ALL-L3, and a high BCC in BM and PB were all unfavorable factors (3, 4, 9, 14).

Although clinically there appears to be large differences among pre-T, T-intermediate, and T-medullar ALL, some authors define the early Pre-T ALL subtype (cCD3+, CD7+, CD4–/CD8–) as with lower survival and resistance to treatment (22, 23). However, other authors correlate the last stage of intrathymic differentiation with T-cell ALL (T-medullar ALL with sCD3+), whose blast cells express the transferrin receptor (CD71), as a worse clinical outcome (24).

An interesting outcome of this study was the association between the predominance of the ALL-L1 type with B-cell precursor ALL. On the other hand, T-cell ALL correlated to L2 lymphoblasts on 69.1% of the cases analyzed, while B-cell ALL presented ALL-L3 in all cases. The latter results are in accordance with previous studies, where B-cell ALL was associated with L3 type (3, 4, 9–11). The other immunological subtypes (T or pre-B ALL), however, may present blasts with L1 or L2 morphology. Blast cells with L1 morphology are most frequent in Common ALL while those with L2 morphology are found in T ALL and in a more immature B-lineage ALL such as pro-B ALL (3, 4, 9, 14).

The clinical results were relevant in the characterization of different treatment groups. We have evidence that patients with ALL had a higher incidence of tumor infiltration in T-cell ALL and B-cell ALL. In some cases,

resulting in large tumor masses (abdominal and mediastinal), leukemic infiltration in the CNS, as well as disseminated lymphadenopathy and massive hepatosplenomegaly.

In the clinical manifestation of ALL, hepatomegaly and lymphadenopathy were usually seen in most cases of ALL when compared to testicular infiltration, neurologic manifestations due to leukemic infiltration in the CNS, as well as the presence of tumor masses (3, 4, 9, 14). Lymphadenopathy and splenomegaly were observed in most cases of Pro-B (66.6%), Common (52.1%), and Pre-B μ + ALL (50%). CNS leukemic involvement was seen in eight cases (16.7%) of Common ALL (Table 4). In the latter, patients had a high BCC in PB.

Reports in the literature have associated CNS leukemic involvement directly to the size of the tumor mass in the PB (high BCC in PB) (25, 26). Some studies, however, reported that leukemic cells migrate directly from the BM microenvironment within the CNS, moving through the adventitia layer of the vessels and the perineurium following the path of the nerves that run through the space into the subdural space in the meninges (27). Another hypothesis is that these cells penetrate the CNS due to bleeding caused by thrombocytopenia, which is commonly observed in patients with acute leukemias (25, 26). Recent studies have shown that this manifestation was in fact a result of biological properties of leukemic cells regardless of the type and immune leukocytes. These properties were being translated by the aberrant expression of adhesion molecules such as CD44 and the integrin family of molecules such as VLA-4 and LFA-1 (27–32).

It is known that the interaction between the CNS and the immune system is regulated by the blood–brain barrier, which has specialized endothelial cells that regulate the passage of blood cells to the CNS in order to protect the brain tissue of the possible attacks from the immune system. It is also known that inflammatory processes affecting the CNS may allow the passage of a greater number of leukocytes across the blood–brain barrier as a result of increased permeability and increased expression of adhesion molecules on their surface, as well as in lymphocytes, as has been observed in multiple sclerosis, viral encephalitis, and meningitis. With this assumption, it is expected that in leukemias and other neoplastic processes, the leukemic cells would settle into the endothelial cells through the blood–brain barrier within the CNS through mechanisms of adhesion (28).

Finally, it is interesting to note that the expression of CD34 and TdT was more detected in acute leukemias with little cellular differentiation. CD34 is more expressed in blast cells with little or no differentiation, as in acute undifferentiated leukemia (3, 4, 7–12). In this study, the expression of this antigen was further restricted to Pro-B ALL and Pre-T ALL (Table 3).

TdT is a nuclear enzyme expressed in B- and T-lineage progenitor cells and plays the same role as the recombinase genes in the rearrangement of the T-cell receptor (TCR) and immunoglobulins genes, contributing to the genetic diversity of these cells (3). In this study, we analyzed TdT expression in 42 cases of T-cell ALL, where the 27 positive cases were more frequent in Pre-T and T-medullar ALL. In B-cell lineage ALL, TdT was expressed in Pro-B and Common ALL and was negatively expressed in all patients with B-cell ALL.

In conclusion, it is important to emphasize the importance of immunophenotypic profiles in the diagnosis of ALL, relating them to physiopathological events. Thus, the results showed here suggest that immunophenotyping is pivotal for research and monitoring of the evolution and prognostic factors in ALL. The present study is the first description of clinical cases of patients with ALL in Natal, Northeastern Brazil, using immunophenotyping and clinical findings.

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