Original article

Acute lymphoblastic leukemia in northern India

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Abstract:

Objective: To study the morphology, analyse the immunophenotype of acute lymphoblastic leukemias by flowcytometry and compare the morphology with immunophenotypic profile.

Materials and methods: The present study was conducted in the Department of Pathology at Rajiv Gandhi Cancer Institute to evaluate and analyze morphology and immunophenotype of acute lymphoblastic leukemias. A total of 75 cases that presented to the Hemato-Oncology OPD, and were diagnosed and documented as Acute Leukemias, prospectively during the period January 2009 to December 2011, were included in the present study. Only newly diagnosed untreated acute leukemia patients of all age groups and both sexes were included in the study. A written consent was taken from patients or parents as appropriate. The diagnosis of acute leukemia was established based on peripheral blood and bone marrow morphology and cytochemistry. Immunophenotyping was done in all cases.

Results: Out of the 75 cases included in the study period, Acute myeloid leukemia comprised 31 of 75 cases (41.3%), acute lymphoblastic leukemia comprised of 40 of 75 cases (53.3%) and biphenotypic leukemias comprised the rest of 4 cases (5.3%). Out of total 40 cases of acute lymphoblastic leukemais,72%(29) cases had B cell phenotype while 28% (11) cases had T cell phenotype. B cell ALL was predominantly seen in children constituting 75.8 % while T cell ALL was predominantly seen in adults (72.7 %). Immunophenotypic analysis showed that the pro B-cell phenotype was encountered in 22 (53.6 %), mature B-cell in 7 (1.7%) and T-cell in 11 (26.8 %) cases. CD10 positivity was found in 27 cases of B cell ALL (93.1%) and 5 cases of the T-ALL (45.4%).

Conclusion: The diagnostic accuracy and reproducibility of morphological diagnosis and cytochemistry varies between 70-90% and this is further increased to greater than 95% by the incorporation of the flowcytometry in a resource poor country like ours where molecular and cytogenetic studies are available in limited centres.

Keywords: acute lymphoblastic leukemia, morphology, flowcytometry.

Introduction:

Leukemias are the neoplastic proliferations of hematopoietic cells. Leukemias are classified into two major groups-Acute and chronic. Acute leukemia (AL) results from clonal proliferation of immature hematopoietic cells “frozen” at an early stage of differentiation[1] including primitive cells with multilineage potential[2]. The first published description of a case of leukemia in medical literature dates to 1827, when French physician Alfred-Armand-Louis-Marie Velpeau[3] described that the blood of this patient had a consistency "like

gruel", and speculated that the appearance of the blood was due to white corpuscles[3][1] It was Vircho4[4] who used the term "weiss blutt" to describe the predominance of white cells in the blood and later, in 1847, proposed the term "Leukemia", derived from two Greek words *leukos*, meaning "white," and *haima*, meaning "blood ."

Although acute leukemias account for less than 3 % of all cancers, these diseases are the leading cause of death due to cancer in persons younger than 35 years of age. The median age at diagnosis for acute lymphocytic leukemia is 13 years and for acute myeloid leukemia is 67 years of age[5]. ALL is predominant in childhood and AML in adults, but the age profiles overlap[6]. At birth there is a mild predominance of ALL in females versus males, but this is replaced marginally by mild male predominance during the peak in incidence in the first 5 years of life, with a mild male predominance persisting throughout life[6]. The small second bump in ALL incidences in the second decade occurs only in males.

The cause of ALL is largely unknown[7]. Inherited factors and genetic predisposition syndromes are implicated in ALL. In adults, ALL is associated with occupational exposure to low-dose ionizing radiation and chemical environmental exposure (benzene exposure). Secondary acute leukemias occurring after exposure to chemotherapeutic agents are usually myeloid, although rare cases of therapy-related ALL have been observed[7].

AML and ALL differ substantially in response to therapy and course, and accurate differentiation of the two is fundamental to therapeutic decisions. So, the diseases must be described, defined and named before they can be diagnosed, treated and studied.

Classification

In the absence of consensus on nomenclature, a good deal of confusion prevailed in the literature until the 1970s, and therapeutic studies were generally based on only two groups of AL, namely lymphoblastic (ALL) and nonlymphoblastic (ANLL). In 1976 a group of French, American, and British hematologists (the FAB group)[8] proposed a simplified nomenclature based on the cell line (or lines) involved in the malignant proliferation and on the degree of maturation of the malignant cells. Classification hinged solely on the cytologic features of blood and bone marrow smears after panoptic staining (May-Grunwald Giemsa or Wright), and on the results of certain cytochemical reactions (myeloperoxidase or Sudan black and monocytic esterases). FAB system[8] of classification was subsequently revised at various times to improve concordance[9,10,11]. This system provided structured criteria for the diagnosis of various sub types of AML and is based mainly on morphological and cytochemical features; for some of the categories, immunophenotyping is necessary[2]. Since then, it has been widely adopted nationally and internationally for classification of acute leukemias, although ambiguities still give rise to confusion and it has been the subject of recent criticism[12]. So, in 1997 a revised WHO classification of AML was published which included cytogenetic studies as well. The clinical and biological significance is also claimed for this system which accounts for distinct prognostic differences for various subtypes and their close association with chromosomal abnormalities.

The French-American-British (FAB) group[8] has proposed a widely used classification of eight different types of AMLs (M0 to M7) and three types of ALLs (L1 to L3) based on morphology and cytochemistry; monoclonal antibody immunophenotype is also used in undifferentiated cases in which morphology and cytochemistry are inconclusive. Because the treatments of ALL and

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AML differ significantly, the most important first step in the diagnostic assignment is to distinguish myeloid and lymphoid lineages to assign therapy. Lymphoid blasts are typically small with more regular nuclei, clumped chromatin, and scant agranular cytoplasm. B-lineage leukemic blasts are not distinguishable from T-lineage blasts based on morphology alone, except if they are mature B-cell (Burkitt's type, FAB L3) blasts, which have characteristic voluminous, vacuolated, deeply basophilic cytoplasm(table 1).

In classic cases , L1 ALL blasts can be confused with normal lymphocytes (in poor preparations), L2 cells resemble AML (FAB M0 or M1) blasts and L3 morphology suggests either Burkitt's or occasionally other types of non –Hodgkin lymphomas. In practice, however, concordance rates in classification of L1 compared with L2 ALL for most studies have been in range of 70 % if the criteria already discussed have been applied. Because of this, in 1981, the FAB group proposed a scoring system for the distinction of L1 and L2 ALL (Table 2). Scoring blasts based on features of N: C ratio, prominence of nucleoli, nuclear membrane irregularity and cell size, concordance rates improved to close to 90%[13,14].

Cytochemistry

Myeloperoxidase (MPO), Sudan Black B (SBB), Non-specific esterase (NSE), Periodic Acid Schiff (PAS) and Acid phosphatase stains are used to characterise the blast cells in acute leukemias[15]. MPO positivity rules out acute lymphoblastic leukemia and confirms myeloid lineage. Presence of Auer rods is diagnostic of myeloid lineage. The most important stains for determining lineage initially include myeloperoxidase, which can be positive (golden brown), even in the absence of visible primary azurophilic granules, and Sudan Black B, which stains primary and secondary granules membrane lipids black. Myeloid differentiation is inferred if either of these stains is positive in 3% or more blasts(figure 1). Acid phosphatase is most useful in T-cell ALL, where it stains as a block or patch. In B-cell ALL, Periodic Acid Schiff stain shows block positivity. In L3-ALL, the vacuoles stain with Oil red O.

Materials and methods:

The present study was conducted in the Department of Pathology at our hospital to evaluate and analyze morphology and immunophenotype of acute lymphoblastic leukemias. A total of 75 cases that presented to the Hemato-Oncology OPD, and were diagnosed and documented as Acute Leukemias, prospectively during the period January 2009 to December 2011, were included in the present study. Only newly diagnosed untreated acute leukemia patients of all age groups and both sexes were included in the study. Patients who had been treated and were at presentation in remission were not included in the study. A written consent was taken from patients or parents as appropriate. The study was duly approved by Hospital Ethical Committee. The diagnosis of acute leukemia was established based on peripheral blood and bone marrow morphology (figure 2) and cytochemistry. Immunophenotyping was done in all cases. The study was duly approved by the Hospital Scientific Review Committee.

Morphology and cytochemistry

For morphological classification, all peripheral blood smears (PBS)/ bone marrow aspirates before initiation of definitive therapy were air dried and subsequently stained with Leishman's stain. Differential count was done on 200 cells for peripheral blood and 500 cells for bone marrow aspirates. Only cytochemical stain used was myeloperoxidase (MPO) and was done in all cases. MPO positivity was taken only when more than 3% blasts took the stain. Myeloperoxidase staining was done using 3, 3'- Diaminobenzidine (DAB) as

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chromogen with subsequent counterstaining using Harris's hematoxyllin stain. All bone marrow trephine biopsies were fixed in 10% buffered formalin, decalcified using 10% formic acid, routinely processed and embedded in paraffin. Blocks were sectioned at 4 micron thickness. Sections were stained with hematoxylin and eosin (H&E), and a morphology based histopathologic diagnosis was made.

Immunophenotyping

The immunophenotyping was performed on heparinised bone marrow aspirates or peripheral blood using a four-colour FACSCallibur flow cytometer (Fluorescence-Activated Cell Sorting Becton Dickinson Immunocytometer Systems). Commercially available monoclonal antibodies were obtained from BD, Pharmingen, Dako. The panel of antibodies was as follows: CD34, HLA-DR, CD13, CD14, CD33, CD19, CD10, CD2, CD3, CD4, CD7, CD8. Immunostaining of at least 20% of the cells was required for a surface marker to be considered positive. Coexpression of the surface markers was analysed when antibodies of more than one lineage were present. According to the results obtained at the initial screening, the second level of investigation was assessed. The second panel was used to identify stage of differentiation, prognosis features or aberrant phenotypes. The reactivity with fluorescent conjugated monoclonal antibodies directed against lymphoid and myeloid associated antigens was evaluated on the surface of leukemic cells. The intracytoplasmic Immunoglobin (Ig), CD3, CD79a and myeloperoxidase (MPO) antigens, as well as nuclear terminal deoxynucleotidyl transferase (TdT) staining, were evaluated by fluorescent conjugated monoclonal antibodies after fixation and permeabilization of leukemic cell. Blasts initially were gated for analysis by using CD45 versus side scatter, according to the gating strategy[16,17].

Leukemic samples were considered positive for a particular antigen if 20% or more of leukemic cells reacted with a particular antibody.

Statistics

All the relevant clinical data including were obtained from the medical records for each case and documented on Microsoft excel sheet. Statistical comparisons were performed on continuous variables with paired't' test within group and unpaired 't' test between groups, and chi-square test. All data analysis was performed with SPSS for Windows Software Package (Statistical package for social science), release 17. Pearson method for bivariate correlation was used to find out correlation coefficient (r) and thereby to determine the correlation between different parameters. Values of $p<0.05$ were considered to be statistically significant.

Results:

The study population included a total number of 75 cases of newly diagnosed cases of acute leukemia, selected on the basis of inclusion and exclusion criteria as mentioned in material and methods.

In the present study a twofold male predominance was seen, with 27 females and 48 males of total 75 cases. The age group in our study showed a wide range of distribution, ranging from a minimum of 2 to a maximum of 64 years (mean age 27.97 years).

Acute myeloid leukemia comprised 31 of 75 cases (41.3%), acute lymphoblastic leukemia comprised of 40 of 75 cases (53.3%) and biphenotypic leukemias comprised the rest of 4 cases (5.3%). The relative percentages were 41.3%, 53.3% and 5.3% respectively. Out of total 40 cases of acute lymphoblastic leukemais,72%(29) cases had B cell phenotype while 28% (11) cases had T cell phenotype. Relative frequency of various types of ALL is shown in figure 4. B cell ALL was

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predominantly seen in children constituting 75.8 % while T cell ALL was predominantly seen in adults (72.7 %). Immunophenotypic analysis of B cell ALL is shown in figure 3.

The age distribution of the different ALL subtypes showed that pre B-cell ALLs patients were younger with peak in incidence between 3 and 7 years (5.85 years). There was a trend for patients with T-cell ALL to have a more advanced age (mean age 26.8 years). Out of the total 5 cases of pro B-cell ALL, 4 expressed CD22 while 2 cases showed cCD79a positivity. Both were positive in 2 out of 5 cases(figure 5).

Eleven cases with T-cell ALL immunophenotype were studied. There were 9 males and 2 females (male/female ratio of 4.5: 1) with ages ranging from 7 to 64 years and median age of 25 years. Based on their reactivity with various anti-T-cell monoclonal antibodies , all tested T-cell ALL cells had surface CD7 and cytoplasmic CD3 (cCD3) antigens. CD5 was found in 90.9% cases and 9% expressed TdT. CD10 was found in 5 cases (45.4%) and absent in 6 (54.5%) of 11 cases of T-cell ALLs cases tested for CD10. CD10 positivity was found in 27 cases of B cell ALL (93.1%) and 5 cases of the T-ALL (45.4%). CD10 expression in pre B-ALL was frequent in patients with mean age of 11.64 years in age range of 1–10 years and having low leukocyte count. CD10 positivity was less common in T cell lineage and was associated with female sex and lower leukocyte count. The lack of CD10 in cases of T cell ALL was significantly associated with patients between 1 and 10 years. About 85% of case of ALL presented with fever, followed by pallor (72.5%) and malaise (62.5 %). Mediastinal mass was only seen in T cell ALL patients in 5 out of 11 cases (45.4 %). Lymphadenopathy was noted in 22.5 % seen most commonly in cervical group of lymph nodes. The clinical parameters studied showed a mean total lecocyte count of 100849/cu mm with a maximum count seen in T cell ALL. Blasts in bone marrow were higher (mean 81.7%) as compared to those seen in AML ($p>0.05$) (table 3).

Mean haemoglobin concentration in ALL was 8.2 g/dl . Myeloperxidase stain was negative in all cases of ALL with none showing positivity in more than 3 % of blasts(figure1). Mean platelet count was 45000/cumm. Serum urea and creatinine levels showed mean values of 30.12 mg/dl and 0.75mg/dl respectively.

Table 1: Morphology of ALL

Subtype	Bone Marrow Morphology
ALL	
ALL-L1	Small cells with minimal cytoplasm and no granules;rare nucleoli; TdT positive
ALL-L2	Small and large cells with moderate cytoplasm and prominent nucleoli; TdT positive
ALL-L3, B-cell or Burkitt's type leukemia	Large round cells with deeply basophilic cytoplasm and vacuoles; TdT negative

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Table 2: French- American-British Classification of ALL

Feature	Description	Scoring
N:C ratio	>20 % of cell area is cytoplasm in > 25% of cells < 20 % of cell area is cytoplasm in > 75% of cells	-1(Favours L2) +1 (Favours L1)
Nucleoli	One or more prominent in > 25% of cells Absent or inconspicuous in >75% of cells	-1 +1
Nuclear membrane	Irregular in > 25% of cells	-1
Cell size	>50% of large cells (>2 x diameter of lymphocytes)	-1

L1: Sum of 4 features is 0 to + 2, L2: Sum of 4 features is -1 to -4, L3: Scoring not relevant

Table 3: Clinical parameters in acute lymphoblastic leukemias

Laboratory parameter	ALL Mean
Hemoglobin(G/DL)	7.56
TLC(per cumm)	100849.80
Platelets(per cumm)	45200.00
Blasts in BM(%age)	81.70
Urea(mg/dl)	30.12
Creatinine(mg/dl)	0.75
Uric acid(mg/dl)	5.11
Bilirubin(mg/dl)	1.01

Discussion:

In this study 75 cases of acute leukemias were analysed and morphological,cytochemical, and flowcytometric analysis together with clinical parameters were studied. A majority of cases studied occurred in males (64%) with a twofold male predominance. This was also seen individually in different types; 70% in acute lymphoblastic leukemia(ALL), 51.6% in acute myeloid leukemia(AML) and 100% in acute leukemias of ambiguous lineage. This is comparable to the different studies which show a male predominance, especially in developing countries [18].

Acute lymphoblastic leukemia constituted 53.3 % of all leukemias(figure 4) in all age groups, 86.2 % in children and 32.6% in adults. B-cell ALL constituted 73 % and T- cell ALL 28%, an incidence similar to as reported by Advani et al[18]. Incidence of B-cell ALL and T- cell ALL from three major Indian cancer centres has been reported as 75, 60 and 45 % and 21,32 and 43 %,

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respectively[19]. The proportion of T-cell ALL was higher in Chennai as compared to our study. More than two third of our cases of B-cell ALL were in age group of 3-7 years, as reported elsewhere[20]. T-cell ALL comprised 14 % of childhood and 25 % of adult ALL and was found to be more common in adolescent males[20]. We confirmed the close association between T-cell phenotype with older age, male gender,and mediastinal mass[21]. As it has been described in children[21], the majority of cases were from pro B-ALL (55%) with the dominance of early pre-B phenotype (69% of pro B-ALL).

CD22 was more sensitive than CD79a as a lineage specific marker for pro B ALL. CD10 was detected in blast cells from 27 of 29 (93.1%) patients with B-lineage ALL and 5 of 11 (45.4%) patients with T-cell ALL. This was in consonance with the earlier study done by Consolini et al[22]. Studies of the prognostic significance of CD10 expression in ALL have showed the CD10 expression in childhood B lineage ALL is associated with several favorable presenting features but is not an independent prognostic factor[22]. In T cell lineage, the expression of CD10 was independently associated with favorable clinical outcome[21]. However, for the larger subgroup of patients with T-lineage ALL, CD10 expression has no independent prognostic significance[21].

Limitations

There was a limitation of this study as we did only 12-16 antibodies per case for immunophenotyping, a number less than the recommended 20-24 antibodies[23]. This was due to limited resources available and the cost incurred on the patients.

Conclusion:

We confirm the excellent practicability of the morphologic, cytochemical and flowcytometric correlation for classification of acute leukemias, especially in countries with limited resources. We recommend atleast 10 antibodies mainly CD 13,CD 33, CD 117, CD 10, CD 19, HLA-DR, CD 7, CD 5 (or CD 2), CD 45 and CD34 as a primary minimal panel for all cases of acute leukemias. Additional antibodies may be used in the secondary panel as and when required. Cytogenetic and molecular studies should be done as per WHO guidelines as these can make a marked difference in the therapeutic decision making whenever possible. Finaly, the diagnostic accuracy and reproducibility of morphological diagnosis and cytochemistry varies between 70- 90% and this is further increased to greater than 95% by the incorporation of the flowcytometry in a resource poor country like ours where molecular and cytogenetic studies are available in limited centres.

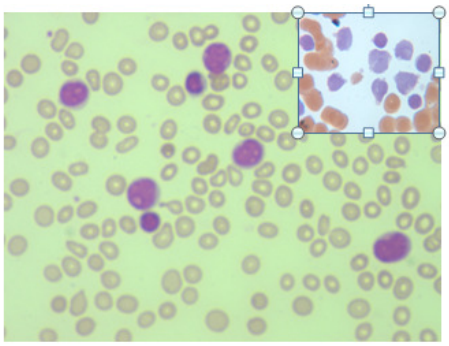

Figure1: lymphoblasts in peripheral blood. Inset shows MPO negativity.

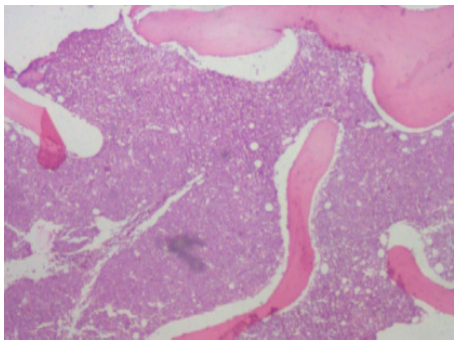

Figure 2: bone marrow biobsy showing infiltration by lymphoblasts.

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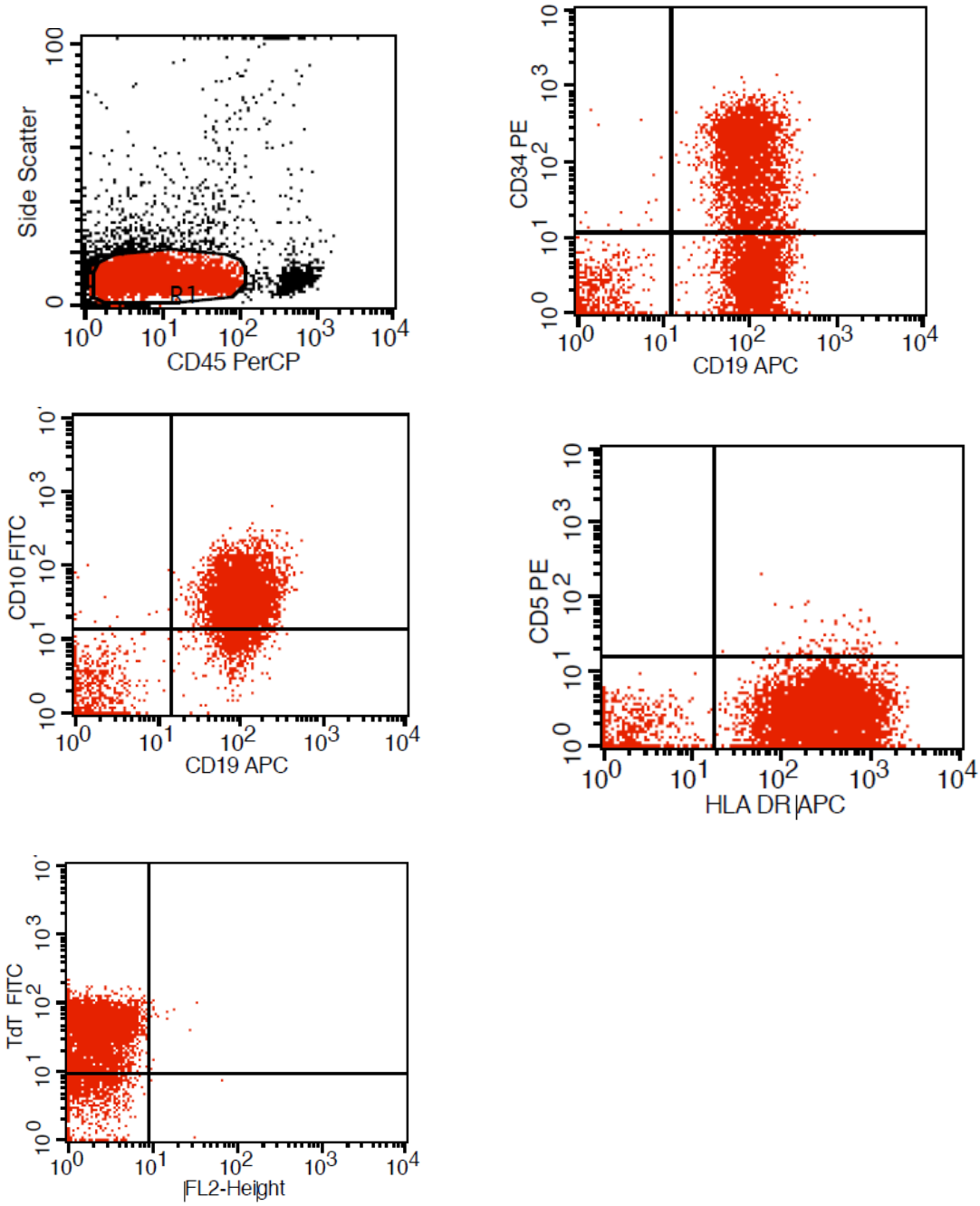

Figure 3: immunophenotype of Pro B-cell ALL

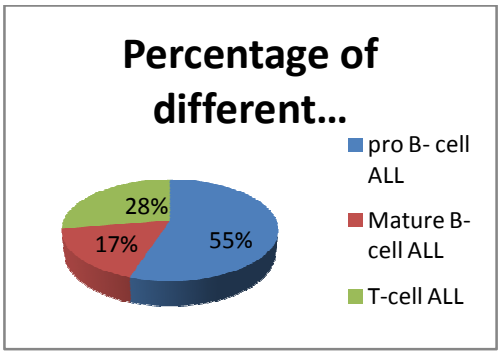

Figure 4: Percentage of different phenotypes of ALL

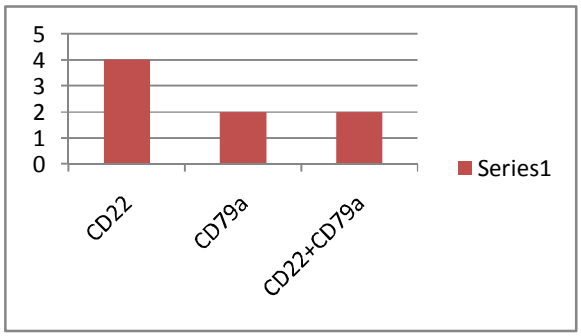

Figure 5: Expression of CD22 and CD79a in pro B-cell ALL

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