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Immunophenotypic profile and aberrancies in Acute Leukaemia: A study from tertiary oncology centre in south India

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Abstract
Introduction: *Flow cytometric immunophenotyping remains an indispensable tool for the diagnosis, classification and monitoring of acute leukaemias. Occurrence of aberrant phenotype has been reported in acute leukaemia with varying frequency. Aberrant expression has relevance to prognosis and can help in identification of minimal residual disease on follow up.*
Aims and Objectives: *To study the morphologic and immunophenotypic profile of acute leukaemia and to determine the incidence of various subtypes. This study also aims to find out the frequency of cross lineage antigen expression in acute leukaemias.*
Methods and Material: All consecutively diagnosed cases of acute leukaemias by flow cytometry during July 2017 to June 2018 were retrospectively reviewed and analysed.
Results: *Over a period of one year, 510 individuals were diagnosed with acute leukaemia. Among 510 cases, 53% (270) were acute myeloid leukaemia (AML), 45% (230) were acute lymphoblastic leukaemia (ALL) and 2% (10) were mixed phenotypic acute leukaemia (MPAL). Aberrant lymphoid expression was seen in 34% of AML and CD 7 was the frequently expressed marker. Out of 230 cases of ALL, 156(68%) were B-ALL and 74(32%) were T-ALL. CD 13 was the most common aberrant myeloid antigen expressed in ALL followed by CD 33.*
Conclusion: *AML accounted for 53% of all acute leukaemias, commonest subtype being AML-M4. Aberrant lymphoid expression was seen in 34% of AML and CD 7 was the frequently expressed marker. CD 13 was the most common aberrant myeloid antigen in ALL. Recently recognized entity, Early T- Precursor (ETP) lymphoblastic leukaemias accounted for 20% of all T-ALL.*
Keywords: *Acute leukaemia, Immunophenotyping, Acute lymphoblastic leukaemia, Acute myeloid leukaemia.*

Introduction

Acute leukaemias are a heterogeneous group of malignancies with varying clinical, morphologic, immunologic, and molecular characteristics.[1] Despite the increasing importance of molecular and genetic features in the sub-classification of acute leukaemias, morphologic and immunophenotypic analysis remains the main modality to diagnose acute leukaemia.[2] Flowcytometric immunophenotyping is an indispensable tool not only in determining the lineage of acute leukaemias but also in detection of mixed phenotype acute leukaemias and in evaluation of minimal residual disease and in assessment of prognosis.[3]

Myeloid lineage is suggested by expression of antigens including CD13, CD15, CD33, and MPO, whereas monocytic lineage is suggested by expression of antigens including CD4, CD14,CD 11b, bright CD33, and CD64.[1,4] Two unusual subtypes of AML include AML with erythroid or megakaryocytic differentiation. Early erythroid precursors (pronormoblasts) are recognized by expression of CD36, CD71, CD117, and CD235a.[4,5,6] Megakaryocytic blasts typically show expression of CD41 and/or CD61 on the surface and cytoplasm.[6]

Most cases of B-lymphoblastic leukaemia show strong expression of multiple B-lineage markers including CD19, CD22, and CD79a.[1,6,7] Expression of CD10, often somewhat brighter than for normal immature B lymphocytes (hematogones), is classic but neither specific nor required for diagnosis but is important prognostically.[1,7,8]

Moderate to bright expression of CD3, either on the cell surface or within the cytoplasm, is considered the most lineage-specific marker of T-celldifferentiation.[1,6,9] In addition, other T lineage–associated markers including CD1a, CD2, CD4, CD5, CD7, and CD8 are variably expressed.

Early T-cell Precursor (ETP) leukaemias have been recently recognized as a form of T-cell acute lymphoblastic leukaemia (T-ALL) with poor prognosis.[8,9] These are a group of immunophenotypically defined leukaemias characterized by the absence (<5% positive cells) of CD1a and CD8 expression, weak CD5 (<75% positive cells), and expression (>25% positive cells) of at least one or more of the following myeloid or stem-cell markers: CD117, CD34, (HLA)-DR, CD13, CD33, CD11b, or CD65.[1,8] Transcriptionally, ETP T-ALLs are characterized by very early arrest in T-cell differentiation and are most related to hematopoietic stem cells and myeloid progenitors.[8,10]

For Mixed Phenotype Acute Leukaemia, World Health Organization provides specific guidelines, that is, myeloperoxidase staining or expression of 2 or more monocytic antigens (CD11c, CD14, CD64, nonspecific esterase, lysosome) indicates myeloid lineage, strong cytoplasmic or surface CD3 expression indicates T lineage, and strong CD19 expression associated with weak expression of CD10, CD22, or CD79a or weak CD19 expression plus strong expression of 2 of the same markers indicates B-lineage.[1,11,12]

Aberrant phenotypes in acute leukaemia are defined as patterns of antigen expression on neoplastic cells different from the process of normal hematopoietic maturation.[3,4] In ALL, these aberrancies include: Cross- lineage antigen expression (expression of myeloid antigens in ALL, B- lineage antigens in T- ALL or T-lineage antigens in B- ALL); asynchronous antigen expression where early antigens are co-expressed with mature ones.[4,6] Immunophenotyping plays a major role in identifying these aberrant phenotypes. The importance of identifying these aberrant phenotypes is to generate sufficient data about their incidence as well as to know about their clinical and prognostic significance.[13] It also helps to detect minimal residual disease on follow up.[14] Characteristic immunophenotypes have been associated with the presence of recurrent genetic abnormalities.[22,25] In AML, characteristic antigens have been related to particular morphological FAB subtypes and associated with the presence of recurrent genetic abnormalities[23,24]. AML with t(8;21) shows aberrant expression of lymphoid markers CD19 and cCD79a as well as CD56, which is associated with poor prognosis[22] . In AML with inv(16) or t(16;16), Co-expression of CD2 is frequently observed. AML-M5 with t(9;11) is reported to have high expression CD56. Regarding ALL, t (9; 22) was reported to be more frequently observed in myeloid antigen positive B ALL than in myeloid antigen negative B ALL. Immunophenotypic aberrancies have been explored to predict treatment outcome in AML. The results are not unequivocal: some studies reported an adverse prognostic association with a multitude of markers while others failed to show any association between immunophenotype and

treatment outcome[22,25]. CD56 expression in AML has been associated with poor prognosis[25]. CD7 is often aberrantly expressed in AML and, in most publications, associated with adverse outcome. In ALL, poor prognosis has been reported in myeloid antigen positive ALL than in myeloid antigen negative ALL.

Aims and Objectives

To study the morphologic and immunophenotypic profile of acute leukaemia and to determine the incidence of various subtypes. This study also aims to find out the frequency of cross lineage antigen expression in acute leukaemias.

Materials and Methods

A retrospective study was conducted in the Department of Pathology. All consecutively diagnosed cases of acute leukaemias during July 2017 to June 2018 were included in the study. Diagnosis of acute leukaemia was made on the basis of peripheral blood counts, peripheral smear examination, bone marrow aspiration, cytochemistry and immunophenotyping by flowcytometry. Peripheral blood smears and bone marrow aspirates were air dried and stained with Giemsa stain. Myeloperoxidase was done routinely in all peripheral smears and bone marrow aspirate smears. Other cytochemical stains - periodic acid-Schiff and nonspecific esterase were done wherever necessary. Nonspecific esterase stains were done in 3 cases in which cytochemistry for myeloperoxidase was negative and flow cytometry was inconclusive. PAS stain was done in 5 cases where we had difficulty in distinguishing B ALL from Burkitt lymphoma. EDTA was the anticoagulant used for flowcytometry samples. Six color flow cytometry analysis was performed using BD FACS Verse flow cytometer (Becton Dickinson, San Jose, CA, USA). Standard lyse-wash procedure was used. The cells were stained with various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein (PerCP), Allophycocyanin (APC) , phycoerythrin – Cy 5.5(PE-Cy 5.5), and Allophycocyanin- H7 (APC-H7) labeled monoclonal antibodies. All the monoclonal antibodies were obtained from Becton Dickinson (San Jose, California, United states). The panel of antibodies used were as follows: CA-CD13 PE-Cy 5.5 (L138), CD14 PE-Cy7 (MoP9), CD33 PE (BD P67.6), CD64 FITC (10.1), CD117 APC (104D2), CD11c APC (S-HCL-3), CD10 PE (HI10a),CD19 PE-Cy7 (SJ25C1),CD20 FITC (L27), CD2 FITC (S5.2), CD3 PerCP-Cy 5.5 (SK7), CD5 PE-Cy7 (L17F12), CD7 APC (4H9), CD56 PE (MY31), CD34 APC (My10) , CD4 PE-Cy7 (SK3), CD8 FITC (SK1),and HLADR PerCP-Cy 5.5 (L243). Cytoplasmic markers-anti MPO FITC (5B8), cytoplasmic CD79a PE (2ST8.5H7), cytoplasmic CD3 Per CP-Cy 5.5 (SK7) and cytoplasmic CD22 PE (5-HCL-1) - were included in the primary flow cytometry panel. Glycophorin A PE (GA-R2), CD61 FITC (RUU-PL7F12), and terminal deoxynucleotidyl transferase (Tdt) FITC (E17-1519) were done in secondary panel when indicated. CD45 gating was used to identify the blast population. BD FACSuite software was used in Flowcytometer for data analysis. The distribution of acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL) and Mixed Phenotype Acute Leukaemia (MPAL) was studied. Incidence of recently recognized entity, Early T- Precursor (ETP) lymphoblastic leukaemias was noted. Frequency of various FAB subtypes of AML was also noted. The aberrant expression of antigens in AML and ALL were studied.

Results

Data was analyzed using IBM SPSS advanced statistics version 20. Numerical data were expressed as mean and standard deviation for variables with normal distribution or median and range as for variables deviating from normal distribution appropriate. Qualitative data were expressed as frequency and percentage. Over a period of one year, 510 individuals were diagnosed with acute leukaemia. Out of 510, 327 were adults and 183 were children. Male to female

ratio was 1.1:1 in adults and 1.6:1in children (Table 1). Flowcytometry was done in peripheral blood in 173 Cases and bone marrow in 337 cases. Morphology coupled with cytochemistry for Myeloperoxidase (MPO), we grouped acute leukaemias into MPO positive and MPO negative cases. Among 510 cases, 228 (45%) were MPO positive and 282(55%) were MPO negative. Among the MPO negative cases, 230(81%) turned out to be ALL, 18 cases (6%) AML-M5, 12 cases (4%) AML-M0, 6 cases (2%) AML-M4, 5 cases(1.77%) AML-M7 and one case(0.35%) AML-M6 by flowcytometry. In 510 cases of acute leukaemia, 53% (270) were classified as acute myeloid leukaemia (AML) and 45% (230) were acute lymphoblastic leukaemia (ALL), while remaining 2% (10) of cases were mixed phenotypic acute leukaemia (MPAL) (Table 1 and 2).

Acute myeloid Leukaemia

AML accounted for 53% of acute leukaemia. The commonest FAB subtype in AML was Acute Myelomonocytic Leukaemia (AML-M4 FAB type) (32%) followed by M5, M2, M1, M3, M0, M7, M6 respectively (Table 3). AML with myelodysplasia related changes was present in two cases. Among 270 cases of AML, aberrant expression was present in 93(34%) of cases (Table 4). CD7 was the frequently expressed (19%) antigen. CD5 antigen was aberrantly present in 5(2%) cases. CD 19 was aberrantly expressed in 37(14%) cases. In 14 cases with AML-M3, CD64 was present in 13(93%), CD 11C in 3(21%), CD 14 in 9(64%). In AML-M3, CD 34 positivity was seen in 2(14%) and HLA-DR in 2(14%)

Acute Lymphoblastic leukaemia

In ALL, 156(68%) were B-ALL. CALLA positivity was present in 142 (91%) of B ALL. In B-ALL, aberrant expression of CD 13 was present in 57 (37%) cases, CD 33 in 26(17%), CD 117 in 4(3%), CD 4 in 14(9%), CD5 in 2 (1%), CD7 in 2 (1 %), CD14 in 25(16%) and CD 11c in 5 (3%) cases.

Out of 230 cases of ALL, 74(32%) were T-ALL. 25 (42%) cases of T-ALL were CALLA positive. Aberrant antigens expressed in T-ALL included CD13, CD33, CD117, CD14, CD11C and CD19. CD 13 expression was present in 13 (22%) cases, CD 33 in 12 (20%),CD 117 in 7(12%),CD14 in 6 (10%), CD 11c in 3 (5 %) and CD 19 in 2 (3%) cases.

ETP-ALL

Of the 74 cases of T-ALL, 15(20%) cases were ETP-ALL. Patients with ETP-ALLs were identified on the basis of the following immunophenotypes: $CD1a^{-}$, $CD8^{-}$, $CD5^{-}$ (dim), and positivity for 1 or more stem cell or myeloid antigens. All 15cases of ETP-ALL showed positivity for CD34. Out of 15 cases 14 showed CD 13 and CD 33 positivity, 10 cases expressed CD 117 and 7 cases expressed HLA-DR. CD 5 was negative or dim positive in most cases (67%). Bright CD5 expression was present in 5 (33%) cases which were labelled as near ETP- ALL.

Mixed phenotype acute leukaemia

A diagnosis of MPAL was made based on WHO guidelines. There were 10 cases of MPAL, which accounted for 2% of all acute leukaemias. Age ranged from 1 year to 60 years. A diagnosis of MPAL-T/myeloid was made in 5 cases and B/myeloid in 3 cases. A diagnosis of B/T was given in 2 cases.

Table (1)

Acute Leukemia according to Age and sex

	Adults			Children		
Types	**F**	**M**	**Total**	**F**	**M**	**Total**
ALL	27	60	87	52	91	143
AML	122	111	233	16	21	37
MPAL	1	6	7	1	2	3
Grand Total	**150**	**177**	**327**	**69**	**114**	**183**

Table (2)

Types of Acute Leukaemia	
AML	270
B-ALL	156
T-ALL	74
MPAL	10
TOTAL	**510**

Table (3)

Type of AML	M	F	Grand Total	%
AML-MDS	1	1	2	1
M0	8	4	12	4
M1	17	19	36	13
M2	16	24	40	15
M3	7	7	14	5
M4	43	43	86	32
M5	35	39	74	27
M6	1		1	0
M7	4	1	5	2
Grand Total	**132**	**138**	**270**	

Table (4)

Frequency of Aberrant Cross lineage antigen expression in acute leukaemias				
	Our Study (South India)		**Study by Surendra Koju et al[19] (North India)**	
Leukaemia	No, of cases n=500	Aberrant Case n=	No, of cases n=408	Aberrant Case n=159
AML	270	93 (34%)	153	40 (26.1%)
B-ALL	156	92 (59%)	214	85 (39.8%)
T-ALL	74	32(43%)	41	34 (83%)

Discussion

Flow cytometric immunophenotyping is important for the distinction between ALL and AML, identification of B-cell or T-cell lineage, and assessing response to treatment, including the identification of early responders and the detection of MRD. In addition, flow cytometric studies are also of value in the identification of megakaryocytic differentiation with expression of CD41, CD61, and pure erythroid leukaemia with expression of CD235a (glycophorin A).

Acute Myeloid leukaemia is more common in adult whereas Acute lymphoid leukaemia is common in children. In our study also we observed the same frequency of acute leukaemia based on age group. In this present study, incidence of ALL was 45%, AML was 53% and remaining 2% was MPAL.

In AML, 5 % cases were APML while remaining 95% were non APML. In most of the other studies also APML ranges from 5-14 %.[16,18] The commonest AML subtype in our series was AML M4 that account for 32% which is higher than the reported frequency of 5-10%. AML M5 constituted 27% where as other studies showed incidence of 2-9%.[19] Incidence of AML M2 was 14.8% as compared to 27-29% reported in literature. AML M1 was seen in 13% similar to other studies[16,19]. The proportion of AML M0 in our series was 4.4% similar to incidence (<5%) reported in literature.[16] AML M7 was reported in 1.85%, less than 5% as quoted in literature. Incidence of AML M6 was 0.37% similar to other studies. AML with myelodysplasia related changes were reported in 0.74%. In our study 34% of our AML cases showed lymphoid antigen expression which is comparable to Study from North India by Surendra Koju et al(Table 4). CD7 was the most commonly expressed (19%) lymphoid marker. Similarly, in case of ALL, B-ALL (68%) is predominant to T-ALL (32%). Similar study was observed in the West, the

predominant immunophenotype observed in ALL was B-ALL, accounting for 60-80% of total cases, whereas T-ALL comprised only of 15-20%.[17] However incidence of T-ALL in our centre was higher than other studies. CD 13 was the most common aberrant myeloid antigen followed by CD 33 as seen in other studies. Multiple immunophenotypic aberrancies were identified in ALL cases in current study. In B-ALL, 16 cases (10%) showed co expression of CD13 & CD33 and one case showed CD7 and CD5 co expression. Nine cases (12%) of T-ALL had CD13 and CD33 co expression. In AML, one case had co expression of CD4, CD5, CD7 and 3 cases (1%) showed co expression CD4 and CD5. Biphenotypic acute leukaemia or MPAL is a rare type of leukaemia which probably arises from hemopoietic pluripotent stem cell with the capability of differentiating along both myeloid and lymphoid (T or B) lineages of antigen expression.[11,12] In our study, MPAL accounted for 2%.

Conclusion

Flowcytometric immunophenotyping is a rapid reliable method for correct diagnosis and subclassification of acute leukaemia especially in cytochemical myeloperoxidase (MPO) negative cases and in the diagnosis of MPALs. In our study AML accounted for 53% of all acute leukaemias diagnosed at our institution. Commonest subtype in AML was AML-M4. Aberrant lymphoid expression was seen in 34% of cases of AML and CD 7 was the lymphoid marker most commonly expressed. Predominant immunophenotype observed in ALL was B-ALL in our study. Incidence of T-ALL in our centre was higher than other studies. CD 13 was the most common aberrant myeloid antigen in ALL. Recently recognized entity, Early T- Precursor (ETP) lymphoblastic leukaemias accounted for 20% of all T-ALL. MPAL constituted 2% of all acute leukaemias in our study.

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