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Original Article

Spectrum and Immunophenotypic Profile of Acute Leukemia: A Tertiary Center Flow Cytometry Experience

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Abstract. ***Background:*** **For diagnosis, sub-categorization and follow up of Acute Leukemia (AL), phenotypic analysis using flow cytometry is mandatory.**
Material and methods: **We retrospectively analyzed immunophenotypic data along with cytogenetics/molecular genetics data (wherever available) from 631 consecutive cases of AL diagnosed at our flow cytometry laboratory from January 2014 to August 2017.**
Results: **Of the total 631 cases, 52.9% (n=334) were acute lymphoblastic leukemia (ALL), 43.9% (n=277) acute myeloid leukemia (AML), 2.2% (n=14) mixed phenotypic acute leukemia (MPAL), 0.5% (n=3) acute undifferentiated leukemia (AUL) and 0.5% (n=3) chronic myeloid leukemia in blast crisis (CML-BC). ALL cases comprised of 81.7% (n=273/334) B-cell ALLs (95.2%, n=260/273 common B-ALLs and 4.8%, n=13/273 Pro B-ALLs). CD13 was the commonest cross lineage antigen, expressed in B-ALL (25.6%, n=70/273), followed by CD33 (17.9%, n=49) and combined CD13/CD33 (11.3%, n=31/273) expression. T-ALLs constituted 18.3% (n=61/334) of total ALLs and included 27.9% (n=17/61) cortical T- ALLs. CD13 was commonest (32.7%, n=20/61) aberrantly expressed antigen in T-ALLs, followed by CD117 (19.1%, n=9/47). AML cases included 32.1% (n=89/277) AML with recurrent genetic abnormalities, 9.0% (n=25/277) with FLT3/NPM1c mutation and 58.9% (n=163/277) AML NOS including 14.7% (n=24/163) AML M4/M5, 1.8% (n=3/163) AML M6 and 3.7% (n=6/163) AML M7. In AMLs, CD19 aberrancy was the most common (20.2%, n=56/277) followed by CD56 (15.8%, n=42/265).**
Conclusions: **In this study, we document the spectrum, correlate the immunophenotype with genetic data of all leukemias, especially concerning T-ALL where the data from India is scarce.**

Keywords: AML, MPAL, B-ALL, T-ALL, FLT3/NPM1c, AUL.

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Introduction. Acute leukemia (AL) is a clonal hematopoietic stem cell disorder characterized by an increase in immature cells (≥20%) in peripheral blood and/or bone marrow. Many classification systems have been proposed for AL. The first internationally accepted classification was the French–American–British (FAB) cooperative Group Classification (1976), entirely based on morphologic criteria. It was subsequently refined in 1981 and 1985.[1] These modifications of the classification system did not incorporate the characteristic immunophenotypic features seen in AL. In 2008, WHO gave a comprehensive classification system of AL (including morphology, cytochemistry, immunophenotyping, fluorescence in-situ hybridization (FISH), and reverse transcriptase polymerase chain reaction (RT-PCR).[2]

Flow cytometric immunophenotyping is the backbone of WHO classification and plays the most crucial role in the diagnosis, lineage characterization and sub-classification of AL. It also provides prognostic as well as predictive information aiding in modulating therapy appropriately. Leukemic blasts with specific genetic signatures show peculiar immunophenotype on flow cytometry. Knowledge and recognition of these associations can help save time and money, particularly in resource-constrained settings.[2,3]

In this study, we retrospectively analyzed 631 consecutive cases of AL diagnosed at our center, focusing on the spectrum of immunophenotypic features, aberrancy profiles and their correlation with cytogenetic/ molecular findings wherever available. Published data on immunophenotypic patterns associated with certain newer entities like ETP-ALL (Early T-cell precursor lymphoblastic leukemia) along with the importance of CD1a in T-ALL lack in the literature, especially from the Indian subcontinent. We have tried to incorporate the immunophenotypic signatures associated with these entities as well.

Material and methods. This study is a retrospective analysis of 631 consecutive cases of AL diagnosed by flow cytometry laboratory from January 2014 to August 2017. The diagnosis was given as per the WHO 2008 criteria.[2] Peripheral blood smears and bone marrow aspirates were air dried and stained with Wright-Giemsa stain. Myeloperoxidase was done routinely in all cases. Other cytochemical stains like periodic acid-Schiff (PAS); non-specific esterase and specific esterase were performed as and when required based on the morphological details of the leukemic blasts.[4] Immunophenotyping data was correlated with cytogenetics / molecular genetics data wherever available.

Flow cytometry. Immunophenotyping was performed either on bone marrow (n=504) or peripheral blood (n=127) samples received in EDTA. The six-color analysis was performed on FACSCanto II (Becton Dickinson, San Jose, CA, USA) using the following fluorochromes; FITC (Fluorescein isothiocyanate), PE (Phycoerythrin), PerCP Cy5.5, APC (Allophycocyanin), PE-Cy7 and APC-H7. Two to four ml of EDTA sample was taken for the immunophenotypic analysis depending on the sample cell count. The standard technique of antibody staining (incubation at room temperature, 15-20 minutes) followed by lysis and washing was used. Cytoplasmic markers were permeabilized with BD FACS™ Permeabilizing Solution 2 (Becton Dickenson, San Jose, CA, USA). For data analysis and interpretation (BD FACS Diva version 6.1.3 software), a cut off of 20% expression for surface marker positivity and 10% for cytoplasmic markers was used as per the EGIL criteria.[2] For all AL cases, three tubes were used in common: 1) CD7 FITC, CD117 PE, CD34 PerCP Cy5.5, CD19 PE-Cy7, CD10 APC, CD45 APC-H7; 2) CD5 FITC, CD13 PE, CD20 PerCP Cy5.5, HLA-DR PE-Cy7, CD33 APC, CD45 APC-H7; 3) cMPO FITC, cCD79a PE, cCD3 PE-Cy7, TdT APC, CD45 APC-H7. Characterization of blasts was done based on CD45/ side scatter along with other markers like CD34, CD117, CD10, and CD19. Once delineated into either AML, B-ALL or T-ALL based on the above markers, in AML cases: 1) CD14 FITC, CD64PE, CD15 PE-Cy7, CD56 APC, CD45 APC-H7; 2) CD38 FITC, CD11c PE, CD123 PerCP Cy5.5, CD2 PE-Cy7, CD11b APC, CD45 APC-H7 were used for further characterizing Monocytic lineage AMLs and/or 3) CD235a FITC, cytoplasmic CD61 PE, cytoplasmic CD41a PerCP Cy5.5, CD71 APC and CD45 APC-H7 were used in suspected erythroleukemia (AML M6) and megakaryoblastic leukemia cases (AML M7). For B-ALLs, no further markers were used. For T-ALL cases: 1) CD8 FITC, CD4 PE, CD99 PerCP Cy5.5, CD2 PE-Cy7, CD11b APC, CD45 APC-H7; 2) CD57 FITC, CD16 PE, CD38 PerCP Cy5.5, sCD3 PE-Cy7, CD56 APC, CD45 APC-H7 were used for further characterization. All antibodies mentioned above were obtained from Becton Dickinson, San Jose, CA (USA).

Cytogenetics. Bone marrow samples were processed using standard cytogenetic protocols. For each sample, 15 – 20 GTG banded (G banding with trypsin using Leishman stain) metaphases were obtained from at least two un-stimulated overnight cultures of bone marrow (with and without colcemid). An automated karyotyping system (MetaSystems GmbH, Altlussheim, Germany) was used for analysis. Karyotypes were reported in accordance with the International System for Human Cytogenetic Nomenclature (ISCN) 2008.

FISH analysis (where applicable) was performed for confirmation of translocations using dual color dual fusion probes for *BCR-ABL1 (Zytovision*, Bremerhaven, Germany), t(8;21) (Metasystems, Germany) and t(15;17) (Abbot Vysis, Illinois, U.S.A.), locus-specific

dual color break apart probes for MLL and inv(16) (Abbot Vysis Illinois, U.S.A.) and locus-specific dual color extra signal ETV6/RUNX1 probe (Abbot Vysis Illinois, U.S.A.) according to manufacturer's instructions following standard techniques.[5]

Molecular testing. DNA was extracted from BM/ PB samples using the Qiagen DNA Mini kit (Qiagen, Hilden, Germany). The ITD (Internal tandem duplications) and tyrosine kinase domain (TKD) containing regions of the FLT3 and exon 12 of the NPM genes were amplified using fluorescent labeled primers. The size of the ITD/ NPM PCR product was determined by ABI 3500 DNA analyzer (Applied Biosystems, Foster City, California, USA). The TKD PCR product was digested with EcoRV, and the presence of the mutation was determined on agarose gel electrophoresis. RNA was extracted from WBCs using Trizol reagent, complementary DNA was then synthesized using reverse transcriptase, followed by nested RT-PCR (PML-RARA) using Roche First Start Taq polymerase and the products were separated by agarose gel electrophoresis.[6]

Results. Six hundred thirty one newly diagnosed cases of AL were retrospectively evaluated, of which 52.9% (n=334) were ALL, 43.9% (n=277) AML, 2.2% (n=14) MPAL, 0.5% (n=3) acute undifferentiated leukemia (AUL) and 0.5% (n=3) chronic myelogenous in blast crisis (CML-BC). Overall males out-numbered females (M: F ratio, 1.5:1). Adults (≥ 15 years) constituted 58.7 % (n=375) patients while 41.3% (n=256) belonged to pediatric (<15 years) age group. AML was the commonest (59.7%, n=224/375) AL in adults, while ALL was the commonest (59.0%, n=197/334) in pediatric age group (**Table 1**). Based on WHO 2008 defined categories, the immunophenotypic expression pattern in all AL cases except T-ALL is depicted in **Table 2** and that of T-ALL cases in **Table 3**.

Acute lymphoblastic leukemia. Of the total 334 ALL cases, 81.7% (n=273) were B-cell ALL and 18.3% (n=61) were T-cell ALL. B-cell ALLs included common-B ALL (95.2%, n=260) and CD10 negative Pro-B ALL (4.8%, n=13) cases (Table 2). Immaturity markers like HLA-DR, TdT, and CD34 were expressed in 97.4%, 97% and 81.3% of cases respectively. CD13 was the commonest aberrantly expressed marker in B-cell ALL, seen in 25.6% (n=70/273) cases followed by CD33 in 17.9% (n=49/273) cases. CD13/CD33 co-expression was seen in 11.3% (n=31/273) cases (**Table 4**). Cytogenetic data was available in 249 cases of which 15.7% (n=39) had a normal karyotype. Remaining cases showed numerical/structural abnormalities or chromosomal translocations. Overall, 24.9% (n=68) cases of B-ALL revealed hyperdiploidy of which 97% (n=66/68) belonged to the pediatric age group. CD13 and CD33 were frequent aberrancies observed in 22.1 % (n=15/68) and 8.8% (n=6/68) hyperdiploid cases respectively, while 4.4% (n=3/68) co-expressed CD13/CD33. Philadelphia positivity was found in 8.8% (n=22/249) cases, of which the majority (86.4%, n=19/22) were adults. CD13 and CD33 aberrancies were noted in 40.9% (n=9/22) and 36.9% (n=8/22) cases respectively while CD13/ CD33 co-expression was seen in 31.8% (n=7/22) cases. Total 5.5% (n=15/270) cases showed t(12;21) by FISH and CD13 was the most frequently (73.3%, n=11/15) aberrantly expressed marker in this subgroup of patients also.

T-ALL subgroup (n=61) included 9.8% (n=6) early thymic precursor T-ALL (ETP-ALL), 8.2% (n=5) pro T-ALL, 29.5% (n=18) pre T-ALL, 27.9% (n=17) cortical T-ALL and 24.6% (n=15) medullary T-ALL (**Table 3**). All the cases of T-cell ALL consistently expressed cCD3. CD10 was expressed in 37.7% (n=23/61) cases. CD13 was aberrantly expressed in 32.7% (n=20/61) cases and CD117 in 19.1% (n=9/47) cases (which were positive for CD13 also in 88.8%, n=8/9 cases). CD4/CD8 co-expression was seen in 39.3% (n=24/61) (**Table 4**). Cytogenetic analysis was carried out in 51 cases (with n=7 culture failures), of which 47.7% (n=21/44) had a normal karyotype. One case had a complex karyotype while remaining (n=22) cases revealed various structural and numerical abnormalities

Table 1. Subtype distribution of acute leukemia in adult (n=375) and pediatric (n=256) age groups.

Diagnosis	Adults (58.7%)(≥15yrs) n=375	Pediatric (41.3%)(<15yrs) n=256	Overall (n=631)
B-ALL	104 (27.8%)	169 (66%)	273 (43.2%)(M=164, F=109)
T-ALL	33 (8.8%)	28 (10.9%)	61 (9.6%)(M=52, F=9)
AML	224 (59.7%)	53 (20.7%)	277 (43.8%)(M=147, F=130)
MPAL	8 (2.1%)	6 (2.3%)	14 (2.25%) (M=9, F=5)
CML BC	3 (0.8%)	-	3 (0.5%)(M=2,F=1)
AUL	3 (0.8%)	-	3(0.5%)(M=2,F=1)
Total	375 (100%)	256 (100%)	631 (100%) (M=376, F=255)

ALL: Acute Lymphoblastic Leukemia, AML: Acute Myeloid Leukemia, M: Male, F: Female, AUL: Acute undifferentiated leukemia, MPAL: Mixed phenotypic acute leukemia, CML-BC: Chronic myeloid leukemia in blasts crisis.

Table 2. Immunophenotypic expression profiles of common antibodies in various leukemias (excluding T-ALL) classified based on WHO 2008.

S. No.	Subtypes of AL	Total cases	MPO	CD34	HLA DR	CD13	CD33	CD117	CD19	CD10	CD3	CD4	CD8	CD7	CD14	CD64	CD11b	CD11c	CD56
1	AML t(8;21)	33	33	31	33	33	33	32	24	0	0	2 / 15	NA	0	3	3	1 / 5	3 / 5	11
2	AML inv(16)	8	6	7	8	8	8	7	0	0	0	3 / 6	NA	0	3	5	2 / 3	3 / 3	1
3	AML t(15;17)	34	34	7	2	32	32	31	3	0	0	2 / 14	NA	0	0	0	0 / 5	0/ 5	1/22
4	AML (MLL)	11	7	6	10	7	11	6	2	0	0	5 / 7	NA	0	1	5	3 / 4	2 / 3	0
5	AML inv 3	3	3	3	3	3	3	3	1	0	0	NA	NA	0	0	0	NA	NA	0
6	AML NPM1c	8	6/8	0	3	8	8	6	0	0	0	3/6	NA	0	2	3	2/5	3/5	3
7	AML FLT3-ITD	8	8	8	8	8	8	8	0	0	0	4/4	NA	3	2	3	4 / 7	2/4	0
6	AML FLT3&NPM1c	9	8	3	4	8	9	9	0	0	0	2/2	NA	0	0	2	0	0	0
6	AML M4/M5	24	17	14	18	24	24	20	0	0	0	09 / 17	0	2	14	19	7 /14	13/17	6
7	AML M6	3	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
8	AML M7	6	0	2	2	3	3	3	1	0	0	0	0	0	0	0	0	0	2
9	AML NOS	130	113	72	105	123	125	127	25	4	0	7 / 73	0	25	5	12	1 /25	8 / 31	18
10	Pre B-ALL	155	0	120	148	31	22	2	155	155	0	0	0	0	NA	NA	NA	NA	NA
11	Pre B-ALL with t(9;22)	22	0	20	22	9	8	1	22	22	0	0	0	0	NA	NA	NA	NA	NA
12	Pre B-ALL with t(12;21)	15	0	13	15	11	7	0	15	15	0	0	0	0	NA	NA	NA	NA	NA
13	Pre B with hyperdiploidy	68	0	59	66	15	6	0	68	68	0	0	0	0	NA	NA	NA	NA	NA
14	Pro B-ALL	13	0	10	13	4	6	0	13	0	0	0	0	1	NA	NA	NA	NA	NA
16	AUL	3	0	2	3	2	3	3	1	0	0	NA	NA	2	0	0	NA	NA	1/1
17	CML-BC	3	2	3	3	3	3	2	1	2	0	0	NA	1	0	0	0	0	1
18	MPAL	14	11	12	14	11	9	4	10	7	5	1	0	4	0	0	NA	NA	3/6

ALL: Acute Lymphoblastic Leukemia, AML: Acute Myeloid Leukemia, AUL: Acute undifferentiated leukemia, MPAL: Mixed phenotypic acute leukemia, CML-BC: Chronic myeloid leukemia in blasts crisis, NA: Not available.

Table 3. Immunophenotypic expression profiles of common antibodies in T-ALL cases (n=61).

Diagnosis	Total Cases	CD45	CD34	CD1a	CD2	cCD3	sCD3	CD4	CD5	CD7	CD8	CD10	CD13	CD33	HLA-DR	CD117	CD16	CD56	CD57	CD99	TdT
ETP-ALL	6	6	6	0	2	6	0	0	0	5	0	1	5	3	4	6	0	1	0	3	3
Pro T-ALL	5	5	2	0	0	5	2	5	5	4	1	2	3	1	2	1/ 4	0	0	0	2	4
Pre T-ALL	18	17	10	0	15	18	3 /16	9	17	18	7	6	5	1	5	1 / 17	0	0	0	7/ 10	15
Cortical	17	17	2	17	14	17	7	13	17	17	11	9	4	1	1	0	0	0	0	9 /12	15
Medullary	15	15	7	0	13	15	14	12	15	15	8	5	3	3	4	1/ 13	1	1	1	5	14

ALL: Acute Lymphoblastic Leukemia, ETP: Early thymic precursor.

Table 4. Overall expression of common antibodies in different subtypes of acute leukemia.

Antibody	Non-APL AML (N=243)	APL (n=34)	B-ALL (n=273)	T-ALL (n=61)
cMPO	82.3%	100%	0.00%	0.0%
CD34	60.8%	20.6%	81.3%	44.2%
HLA DR	83.2%	5.8%	97.4%	26.2%
CD13	92.1%	94.1%	25.6%	32.7%
CD33	95.8%	94.1%	17.9%	14.7%
CD117	90.9%	91.1%	1.0%	19.1%
CD19	21.8%	8.8%	100%	3.2%
CD10	2.0%	0.0%	95.2%	37.7%
cCD3	0.0%	0.0%	0.0%	100%
CD4	21.1%	13.3%	0.0%	60.6%
CD8	NA	NA	NA	44.2%
CD7	12.3%	0.0%	0.03%	96.7%
CD14	10.1%	0.0%	NA	0.0%
CD64	25.9%	0.0%	NA	0.0%
CD11b	42.3%	0.0%	NA	0.0%
CD11c	51.8%	0.0%	NA	0.0%
CD56	16.8 %	4.5%	NA	3.2%

ALL: Acute Lymphoblastic Leukemia, APL: Acute Promyelocytic Leukemia, AML Acute Myeloid Leukemia, NA: Not available.

and balanced and unbalanced translocations with unknown prognostic significance.

Acute myeloid leukemia. A total of 277 cases were diagnosed as AML by flow cytometry and further sub-classified based on the cytogenetic profiles. AML with recurrent cytogenetic abnormalities constituted 32.1% (n=89/277) cases including 11.9% (n=33/277) AML with t(8;21); 12.3% (n=34/277) AML with t(15;17) i.e. APLs; 2.9% (n=8/277) AML with inversion 16/ t(16;16); 3.9% (n=11/277) *MLL* gene rearranged AMLs; and 1.1% (n=3/277) cases of AML with 3q abnormalities. Normal Karyotype AMLs with mutated *NPM1/FLT3* constituted 9.0% (n=25/277) cases. Rest of the cases were categorized into AML NOS (58.8%, n=163/277) which included 8.6% (n=24/277) cases of AML with monocytic differentiation, 1.1% (n=3/277) acute erythroid leukemias and 2.2% (n=6/277) acute megakaryoblastic leukemias (**Table 2**).

Flow cytometric evaluation of all non-APL cases as a group (243/277) showed consistent expression of CD33, CD13, CD117 and MPO in 95.8%, 92.1%, 90.9% and 82.3% of cases respectively. CD34 positivity was noted in 60.8 % of cases while HLA-DR was expressed in 83.2 % of non-APL AMLs. Other markers like CD11b, CD11c, and CD64 were expressed in 42.3%, 51.8% and 25.9% of cases respectively. CD19 was the commonest aberrantly expressed marker seen in 21.8% (n=53/243) of cases followed by CD56 (16.8%, n=41/243) and CD7 (12.3%, n=30/243). Amongst 33 cases with t(8;21), aberrant CD19 expression on flow cytometry was seen in 72.7% (n=24/33) of cases.

In APLs (n=34), MPO expression was seen in all cases (100%) and CD13 and/or CD33 in 94.1% (n=32/34) of cases. CD34 and HLA-DR were expressed in 20.6% (n=7/34) and 5.8% (n=2/34) of cases respectively. CD11b and CD11c were analyzed in 5 cases of APL and found to be consistently negative in all 5 cases (100%). We also found aberrant CD4 expression in 13.3% (n=2/15) cases of APL and unusual aberrant CD19 expression in 8.8% (n=3/34) of cases. In our cohort of total n=34 APLs, RT-PCR PML/RARA analysis was available in n=24 cases, of which 54.2% (n=13/24) had BCR1 transcript (all cases were $CD34^{neg}/CD56^{neg}$), 4.2% (n=1/24) had BCR2 ($CD34^{neg}/CD56^{neg}$) and 41.2% (n=10) had BCR3 transcript (only n=1/10 case revealed CD34 positivity, while only one case had CD56 positivity). Conventional karyotyping was available in n=33/34 cases, of which n=29/33 cases showed t(15;17) on conventional karyotyping and were also FISH PML/RARA positive. In the remaining five APL cases, FISH PML/RARA was negative and karyotype (which was available in 4/5 cases) did not reveal any structural abnormality. All these 5 cases were found to be RT-PCR PML/RARA (BCR1 transcript) positive. We did not have any patients with variant APML translocations in this cohort.

Amongst the cases of AML with normal karyotype (20.6%, n=57/277), *FLT3/NPM* mutation analysis was carried out in 44 cases and found positive in 56.8% (n=25/44) cases. Eight of these cases (32%) were only NPM1c mutated (all n=8/8 were CD34 negative, and n=6/8 were HLA-DR positive), 32% (n=8) were only FLT3 ITD mutated (all 8 were CD34 and HLA-DR positive) and 36% (n=9) were both FLT3 ITD/ NPM1c

mutated (showing CD34 expression in n=3/9 cases and HLA-DR in n=3/9 cases). Amongst the cases tested, FLT3/ NPM1mutations were not found in any of AML case with recurrent cytogenetic abnormalities.

Acute Leukemia of ambiguous lineage. Of the total 631 cases studied, 2.7% (n=17/630) cases were classified as Acute Leukemia of Ambiguous lineage. Of these 17 cases, 82.4% (n=14) cases were MPALs. Majority of these were males (57.1%, n=8) and adults (78.5%, n=11). These cases were further classified as: B /myeloid (64.3%, n=9/14), T / myeloid (28.6%, n=4/14) and B / T lymphoid (7.1%, n=1/14). Cytogenetic data was available in 12 of 14 cases, of which 25% (n=3) had normal karyotype and 33.3% (n=4) had t(9;22)(q34; q11).We also came across a single case of B/T MPAL which had MLL (mixed lineage leukemia) gene rearrangement, t(v;11q23). Certain cases (17.6%, n=3/17), where none of the lineage-defining markers was expressed, were classified into acute undifferentiated leukemia (AUL). All these cases with treatment profiles have been already published earlier.[7]

CML in blasts crisis. CML in blast crisis constituted 3 cases. Two of these had myeloid lineage blast crisis and one lymphoid lineage (B-lymphoid).

Discussion. Flow cytometric immunophenotyping is a highly sensitive and specific technique and has become an integral part of diagnostic workup and sub-classification of AL.[2] The immunophenotypic profile also provides strong prognostic and predictive information. To the best of our knowledge, published literature from the Indian sub-continent on detailed AL immunophenotyping (including T-ALL) and their corresponding surrogate marker profiles for genetic lesions is sparse.

Total 631 consecutive cases of AL diagnosed at our center were retrospectively evaluated. The overall patient demographic profile and the frequency distribution of various AL subtypes were concordant with published Indian and western literature.[8-17]

B-ALL immunophenotypic features and genetic findings. B-ALL is defined by the presence of either strong CD19 along with one other strongly expressed B- cell markers (i.e., CD79a, cCD22, CD10); or weak CD19 and two other strongly expressed B-cell markers.[2] In our cohort, the overall B-ALL immunophenotypic profile was similar to that previously reported in the literature.[18-23]

Aberrant myeloid antigen (MyAg) expression has been reported to range from 4.3% to 64% in B-ALL.[24] There is no reported difference between $MyAg^{+}$ and $MyAg^{-}$ groups in complete remission (CR) achievement or Overall Survival (OS).[20,24,25] In our series, CD13 was the commonest aberrantly expressed marker in B-cell ALL, seen in 25.6% (n=70/273) cases followed by CD33 in 17.9% (n=49/273) cases while CD13/CD33 co-expression was seen in 11.3% (n=31/273) cases. The cryptic (12;21)(p13;q22) translocation (TEL/AML1 fusion) found in > 20 % pediatric ALL and < 1–3 % of adult ALLs carries a favorable prognosis, particularly in pediatric population.[3,22] Borkhardt et al. in their study on 334 pediatric ALLs showed that the overall incidence of the t(12;21) was 18.9% and 24.6% cases co-expressed at least two myeloid antigens (CD13, CD33, or CD65) in more than 20% of the gated blast cells.[26] The most predictive immune profile described in the literature for this favorable risk subtype applicable for both pediatric and adult population is $CD10^{Pos}$, $CD20^{Neg}$, $CD34^{Neg}$, $cIgM^{Neg}$, frequently CD33 and $CD13^{Pos}$ and $CD11b^{Neg}$.[3] In our study, t(12;21) was seen in 5.5% (n=15/270) cases (all pediatric). CD13 was the most frequent (73.3%, n=11/15) aberrantly expressed marker in this subgroup of patients followed by CD33 (46.6%, n=7/15) and CD13/CD33 co-expression (33.3%, n=5/15). Our numbers for t(12;21) positive cases were small in this cohort, and we did not have cIgM and CD11b in our B-ALL panel so these markers could not be evaluated.

BCR/ABL fusion gene is a dominant negative prognostic factor in ALL.[3,20] The best surrogate marker profile described in literature for BCR/ABL^{Pos} lymphoblasts includes $CD25^{Pos}$ / $CD34^{high}$ / $CD10^{high}$ / $CD66c^{Pos}$ / $CD38^{weak}$ blasts with dual expression of myeloid antigens CD33 and CD13.[3] CD25 (alpha-chain of the interleukin-2 receptor) expression has been described as an independent prognostic factor having negative impact on OS, Event free survival (EFS) and low rates of CR in.[3,27-29] In our cohort, Philadelphia positivity was found in 8.8% (n=22/249) cases, of which majority i.e. 86.4% (n=19) were adults. CD13 and CD33 aberrancies were noted in 40.9% (n=9/22) and 36.9 % (n=8/22) cases respectively while CD13/ CD33 co-expression was seen in 31.8% (n=7/22) cases. CD34 was positive in 90.9% (n=20/22) cases while 100% (n=22/22) cases were $CD10^{pos}$. We however, did not have CD25 and CD66c, in our diagnostic B-ALL panel.

CD10 (also known as the common acute lymphoblastic leukemia antigen, CALLA) negativity in B-ALL (i.e., Pro B-ALL cases) is a strong negative prognostic factor, frequently associated with MLL rearrangements and are known to have a significantly low OS, EFS and low rates of CR.[3,22,24,27,28] In our series, 4.8% (n=13/273) of all B-ALLs were $CD10^{neg}$.

CD20 expression, other than serving as a potential target for immunotherapy, has known prognostic implications and is associated with shorter remission duration and OS particularly in adults.[3] In our cohort, CD20 was found to be positive in 53.5% (n=146/273) cases similar to that reported in Indian literature[8-10] but higher than that reported in western literature (20-40%). These differences could be due to the choice of fluorochromes used in different studies.[30]

T-ALL immunophenotypic features and genetic findings. T-lineage ALL is diagnosed based on the presence of cytoplasmic CD3 in leukemic blasts.[2] Traditionally recognized T-ALL subsets based on different stages of intra-thymic differentiation include pro-T ALL EGIL T-I (cCD3^{pos}, CD7^{pos}); pre-T ALL EGIL T- II (cCD3^{pos}, CD7^{pos} and CD5/CD2^{pos}); cortical T ALL EGIL T-III (cCD3^{pos}, CD1a^{pos}, sCD3$^{pos/neg}$); and mature-T ALL EGIL T-IV (cCD3^{pos}, sCD3^{pos}, CD1a^{neg}).[31-33] In our cohort of T-ALLs (n=61/631), there were 8.2% (n=5) Pro T-cell ALL, 29.5% (n=18) Pre T-ALL, 27.9% (n=17) Cortical T-ALL and 24.6% (n=15) Medullary T-ALLs.

ETP-ALL (Early T-cell precursor ALL) is a recently identified subtype of T-ALL characterized by distinctive immunophenotype, gene expression profile (lower frequencies of prototypical T-ALL lesions such as CDKN2A/B deletions, activating mutations in NOTCH-signalling pathway, higher prevalence of mutations typically associated with the pathogenesis of AMLs like NRAS/ KRAS/ FLT3), poor response to chemotherapy and very high risk of relapse. ETP-ALLs show a uniquely high prevalence of 5q, 13q, and 11q chromosomal deletions and in contrast, lack deletions involving the short arm of chromosome 9 which delete the *CDKN2A/B* tumor suppressor gene in over 70% of all T-ALLs. ETP-ALL immunophenotype resembles the earliest thymic precursors with both T and myeloid lineage potential.[31-34] Corroborating with this truly bi-phenotypic potential, around 5-10% blasts in these cases express cMPO and occasionally can show Auer rods, the majority of these cases being FLT3 mutated.[3] In our series, 9.8% (n=6/61) cases were classified into ETP-ALL based on the typical immunophenotype characterized by expression of cCD3 (100% cases), CD7 (83.3% cases), TdT (50% cases), CD99 (50% cases) along with one or more stem cell/ myeloid markers like CD34 (100% cases), CD117 (100% cases), HLA-DR (66.6% cases), CD13 (83.3% cases), CD33 (50% cases) associated with lack of CD1a (100% cases), CD8 (100% cases) and weak CD5 (100% cases). ETP-ALLs also show significantly lower expression of CD2, sCD3, CD4 and CD10,[3] as seen in 33.3%, 0%, 0% and 16.6% of our cases respectively. One of our ETP-ALL patients also had cMPO positivity in 5% blasts and showed occasional blasts with Auer rods, possibly hinting towards FLT3 mutated ETP-ALL, which was not tested in any of the patients with T-ALL. This immuno-morphological correlation in FLT3 mutated cases has implications concerning innovative treatment strategies like tyrosine kinase inhibitors (TKI) for this otherwise high-risk subgroup of adult ETP-ALLs.[3]

CD1a antigen is expressed on 60-90% thymocytes, some T-cell leukemias/ lymphomas, Langerhans cells of the skin and some dendritic cells. Based on CD1a along with CD13, from a prognostic point of view, particularly in the adult population, two major subclasses of T-ALL which exhibit minimal immunophenotypic overlap are relevant: CD1a^{Pos} Cortical/ Thymic T-ALL subtype (lacking CD13) and CD1a^{neg} (CD13^{Pos} CD34^{Pos} positive) T-ALL. Positivity of blasts for CD1a (lacking CD13 expression) has been associated with significantly higher OS, CR rate and low risk of relapses compared with the CD1a^{Neg} (CD13 expressing) T-ALL. Overall, negativity for CD1a, positivity for CD34, and the presence of myeloid antigens combined confer inferior prognosis in T-ALL of all ages.[31,35]

Of our total 61 T-ALL cases, CD13 was the most common aberrantly expressed myeloid marker seen in 32.7% cases followed by CD33 (14.8% cases). In the study by Marks DI et al, aberrant myeloid antigen expression i.e. CD13 was seen in 51% and CD33 in 30% of T-ALL patients.[31] Expression of CD10 and CD34 was observed in 34.4% and 32.7% of our cases respectively. Published literature mentions expression of CD10 in approximately 15-22% cases and CD34 in 10 to 42% T-ALLs.[18,19,21,32,33] In our series, CD4 / CD8 co-expression was seen in 39.3% (n=24/61) cases, CD4 / CD8 double negativity in 32.8% (n=20/61), CD4^{Pos}/CD8^{Neg} in 21.3% (n=13/61) and CD8^{Pos}/CD4^{Neg} in 6.6% cases (n=4) similar to that previously reported.[18,32,33]

Cytogenetic analysis was carried out in 51 cases (n=7 cases with culture failure). Of the remaining 44 cases, 47.7% (n=21/44) cases had a normal karyotype. One case had a complex karyotype while remaining (n=22/44) cases revealed various abnormalities including both euploidy and aneuploidy. From a prognostic point of view, only patients with a complex karyotype have been reported to have a significantly lower OS compared with patients with normal karyotypes, irrespective of age and WBC counts.[3,35]

AML subtypes with immunophenotypic features. AML is diagnosed based on the presence of either myeloperoxidase (by flow cytometry, immunohistochemistry or cytochemistry) or at least two markers of monocytic differentiation (NSE, CD11c, CD14, CD64 or lysozyme) in leukemic blasts.[2] Of our total 277 AML cases (with n=243 Non APLs and n=34 APLs) expression of CD13, CD33 and CD117 was seen in 93.9%, 94.8% and 90.9% respectively, similar to that in published literature.[8,36-38]

In our study, CD7 was aberrantly expressed in 10.8% of all AML cases (n=30/277). CD7 is first to appear during the maturation of T lymphocytes and is reported to be aberrantly expressed in an 11-28% of AML cases.[9,13,36,39] CD34, the stem cell marker, is known to be an independent negative prognostic indicator for overall survival and remission achievement in AMLs.[3,32] CD34 positivity in non-APL cases varies between 55.8 and 69.1%.[8,14,15] In our cohort, 60.8% of non-APL cases were CD34^{pos}.

APL was the first leukemia whose characteristic features were described immunophenotypically. As per the ECOG guidelines, the most accepted surrogate marker profile for PML/RARA includes lack of HLA-DR and CD133 (two antigens expressed at differentiation levels more immature than that of promyelocytes during normal myelopoiesis); the absence of adhesion molecules, such as CD11a (α_L subunit of the leukocyte integrin LFA-1), CD18 (β_2 subunit of LFA-1), and CD11b ($\alpha_M$subunit of Mac-1 integrin); the expression of carbohydrate molecule, CD15, only in the sialylated form (CD15s); dim expression of CD45 and CD38 and strong CD117 expression. These features of PML/RARA APL cells are valid for all currently known variant APL translocations that involve rearrangement of the RARA gene like the ATRA un-responsive PLZF/RARA derived from t(11;17)(q23;q21), the ATRA responsive NPM/RARA (nucleophosmin) APL, derived from t(5;17)(q35;q21) and also the FISH negative, RT-PCR positive cases.[3,40] In our cohort of APL cases, HLA-DR, CD34 and CD117 expression was seen in 5.8%, 20.6%, and 91.1% cases respectively. Double negativity of CD34 and HLA-DR was seen in 77.1% of APL cases. Other markers like CD11c and CD11b, tested in 5 out of our 34 cases, showed negativity in all 5 cases (100% negativity). Our findings are in concordance with published literature.[8,13,16,17]

Based on the differential breakpoints in the PML gene, the L- (Long, bcr1), S- (Short, bcr3), or the V- (Variable, bcr2) transcript isoform of PML/RARA are formed. The classical surrogate marker profile described applies to all these three molecular isoforms. However, only the leukemic promyelocytes that contain S-form transcripts variably express CD34, CD2, and CD56 and are associated with a higher incidence of extra-medullary relapse, greater thrombotic complications and poorer prognosis.[3,40] These cases are also associated with FLT3 mutations. CD2 and CD56 at diagnosis are never seen in BCR1 and BCR2 PML/ RARA transcripts, however, can rarely have CD34 expression.[3] In our cohort of patients, of the total n=34 APLs, RT-PCR PML/RARA analysis was available in n=24 cases, of which 54.2% (n=13) had BCR1 transcript (all cases were $CD34^{neg}/CD56^{neg}$), 4.2% (n=1) had BCR2 ($CD34^{neg}/CD56^{neg}$) and 41.2% (n=10) had BCR3 transcript (n=1 case revealed CD34 positivity another case had CD56 positivity). We did not have CD2 in our diagnostic panel so could not be evaluated.

The characteristic feature of the t(8;21) AML immunophenotype is striking myeloid immaturity, with weak expression of CD33 and weak MPO consistent with the suggestion that the AML1 / ETO fusion event occurs at an early stem/ progenitor cell stage AML. The unique B-lymphoid aberrant antigenic signature of t(8;21) AML, with an expression of PAX5, CD19, and CD79a is however negated by the co-expression with CD7, and the CD19/CD7 double positivity has been associated with a predominantly normal karyotype and FLT3-ITD and NPM1 mutations.[3] Aberrant expression of CD19 was noted in 20.2% (n=56/277) AML cases in our cohort. It has been reported in 2-22% AMLs in literature, being relatively more frequent in AML with translocation t(8;21).[41] In our study, of the 33 cases of AML with t(8;21), CD19 aberrancy was seen in 72.7% (n= 24/33) cases while none of these cases (100%, n=24) had CD7 aberrant expression along with CD19. In the study by Gujral et al., CD19 aberrancy predicted t(8;21)(q22;q22) in 45% of cases.[8] CD56 (neural cell adhesion molecule) is associated with increased incidence of granulocytic sarcomas in t(8;21) AMLs.[3,37,39,41] In our cohort, 33.3% (n=11/33) cases revealed aberrant CD56 expression of which eight presented with the extra-medullary disease.

AML patients with normal cytogenetics are characterized by CD34 and HLA-DR negativity. These cases may carry poor prognostic genetic lesions (FLT3 mutations) or aberrations associated with better outcome (isolated NPM1 or CEBPA mutations). The distinctive "cuplike" nuclei (nuclear invaginations) are typically associated with AMLs having Normal Karyotype and mutated NPM1 and/or FLT3. NPM1/ FLT3 mutations can be seen irrespective of FAB AML subtypes. NPM1 mutations involve all but lymphoid hematopoietic cell lineages. However, FLT3 mutations can also be seen in T-ALL (more commonly in pediatric patients) and very rarely also seen in B-ALL.[3,42] In AML, CD34 expression is related to genes predictive of poor outcome (FLT3), while a lack of CD34 expression predicts favorable prognosis. In our cohort of AMLs with normal karyotype (20.6%, n=57/277), *FLT3/NPM* mutation analysis was carried out in 44 cases and found positive in 56.8% (n=25/44) cases. Eight of these cases (32%) were only NPM1c mutated (all eight were CD34 negative, and only 6/8 were HLA-DR positive), 32% (n=8) were only FLT3 ITD mutated (all 8 were CD34 and HLA-DR positive) and 36% (n=9) were both FLT3 ITD/ NPM1c mutated (showing CD34 expression in 3/9 cases and HLA-DR in 3/9 cases).

Mixed phenotypic leukemia. MPALs are thought to arise from multipotent progenitor stem cells capable of differentiating into both myeloid and lymphoid lineages.[7,43-45] The frequency of MPAL in our study was found to be 2.2% (n= 14/631) which is in concordance with published data, with documented frequency ranging from 2.2 to 2.6%.[43,44] CD34 expression was seen in 85.7% (n=12) cases. MPAL were classified into B/myeloid in 64.2% (n=9/14), T/Myeloid in 28.5% (n= 4/14) along with a single case of B+T lymphoid MPAL based on WHO 2008 criteria. A similar study by Matutes E, et al. consisting of a larger cohort of 100 MPAL, diagnosed using 2008 WHO criteria, revealed 59% of cases were B/myeloid, 35% T/myeloid and B+T

lymphoid included 4% cases.[43] Myeloid markers frequently co-expressed with MPO in our study included CD13/CD33 (69%) and CD117 (38%). Matutes et al. in their study found that majority of lymphoid/ myeloid MPALs frequently expressed myeloid markers like CD13 (74%), CD33 (66%), and CD117 (52%) along with MPO.[43] A description of MPAL cases in our cohort has been published recently.[7]

Conclusions. In this study, we document the spectrum, correlate the immunophenotype with genetic data of all leukemias, especially with respect to T-ALL where the data from Indian sub-continent is scarce.

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