

IMMUNOPHENOTYPIC PROFILE OF ACUTE LEUKEMIA: A TERTIARY CARE HOSPITAL EXPERIENCE

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Article Received on 12/03/2017

Article Revised on 02/04/2017

Article Accepted on 22/04/2017

ABSTRACT

Introduction: Acute leukemia comprises a heterogeneous group of conditions which differ in aetiology, pathogenesis, molecular mechanisms and prognosis. Assignment of lineage is critical in the diagnostic evaluation of acute leukemia, as treatment for acute myeloid leukemia and acute lymphoblastic leukemia differs. The present study was conducted with the aim to evaluate the immunophenotypic profile of acute leukemia patient by flowcytometric methods. **Material and Methods:** A total of 58 new cases of acute leukemia patients constituted the study material. A detailed clinical history, examinations were done for all the patients. Cytomorphological analysis was done by examination of peripheral blood smears and bone marrow aspirate smears. Immunophenotyping was performed on 8 Color Flowcytometer BD FACS Canto II. **Result:** In our study FCA analysis in 58 cases of acute leukemia was done and compared with their morphological diagnosis. Cytomorphologically, 48.2% ALL and 51.8% AML cases were diagnosed which were classified according to FAB classification. On FCA, out of 28 cases of ALL 78.6% and 21.4% cases were diagnosed as B-ALL and T-ALL respectively while 30 (51.8%) cases were diagnosed as AML. **Conclusion:** Present study proved to be helpful in assigning correct lineage to leukemia cells and support the use of particular panel of CD markers as a better diagnostic tool after preliminary investigations. Although considered superior, flowcytometric analysis must always be performed in conjunction with cytomorphology.

KEYWORDS: Acute leukemia, Flow cytometry, Immunophenotyping, Bone marrow, Acute myeloid leukemia, Acute lymphoid leukemia.

INTRODUCTION

Acute leukemia comprises a heterogenous group of condition which differ in etiology, pathogenesis, molecular mechanism and prognosis.^[1] Assignment of lineage is critical in diagnostic evaluation of acute leukemia, as treatment for Acute Myeloid Leukemia (AML) and Acute lymphoblastic leukemia (ALL) differs. Myeloid and lymphoid lineage may be distinguished based on cellular morphology, cytochemical staining and expression of lineage specific antigens.^[2]

Immunophenotyping of leukemia cells played a crucial role in identifying leukemia cell line, maturation stage and any possible aberrant antigens which in turn contributes to individual treatment, monitoring and detection of residual disease. Earlier, the diagnosis and classification of AML and ALL was almost exclusively based on well-defined morphologic features and

cytochemistry. Though most cases can be diagnosed by these two methods, there is only a modest correlation between morphologic types and treatment responsiveness and prognosis. Due to expansion of therapeutic options, improved remission induction and disease-free survival for both ALL and AML, an emphasis is given on defining good and poor treatment response groups. This is most effectively achieved by a multifaceted approach to diagnosis and classification with the help of immunophenotyping, cytogenetics, and molecular analysis in conjunction to the traditional methods.^[3-5] Now a days, IPT for AL cases has become more important in the determination of the lineage of leukemia and trends to become universal when facilities are readily available. The present study was done to evaluate the immunophenotypic profile of the acute leukemia patients in a tertiary care hospital.

MATERIAL AND METHODS

The present study was conducted in the Department of Pathology, in a Tertiary Institute of North India. A total of 58 new cases of acute leukemia patients constituted the study material. A detailed clinical history, examinations were done for all the patients. Cytomorphological analysis was done by examination of Leishman stained peripheral blood smears and bone marrow aspirate smears. Cytochemical analysis includes special stains; Myeloperoxidase, Sudan Black and Periodic acid Schiff etc.

Immunophenotyping was performed on 8 Color Flowcytometer BD FACS Canto II (Becton Dickinson, San Jose, CA). Specimens used were 2 ml EDTA peripheral blood (PB) and bone marrow (BM) aspirate. Sample processed within 24 hours of collection by using Lyse and Wash technique for preparing the cells. 100 µl of PB or BM (about 1 million cells) were mixed with titrated volume of fluorescently labelled antibodies in each falcon tube and incubated in dark for 15 minutes. Lysing solution added and kept for 10 minutes and then centrifuged and supernatant was removed. Cells are washed once/twice with sheath fluid. resuspension of cells was done in 0.5ml of sheath fluid. For intracellular staining Perm 2 solution was used.

The fluorescent probes used were Allophycocyanin (APC-H7) for CD45, Fluoroisothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC), Peridinin-chlorophyll protein (PerCP) and PerCPCy5.5. Flow Cytometric Analysis (FCA) was performed using primary and/or a secondary panel of monoclonal

antibodies. The CD panel used was CD45, CD34, sCD3, cyCD3, CD5, CD2, CD7, CD4, CD8, CD10, CD20, CD19, CD79a, Tdt, CD13, CD33, CD117, HLA-DR, MPO, CD64.

The analysis was performed on FACS Diva software using 6 colour protocol. Gating strategy was side scatter (SSC)/ Forward scatter (FSC) and SSC/CD45 and results are expressed as the % of positive cells in the gate.

All the quality control measures were undertaken before starting the procedure such as checking the system pressure and vacuum gauges, checking the optical alignment, fluorescence standardization and colour compensation of instrument and testing the antibody integrity by verifying quality controls.

The collected data was compiled and entered in MS-excel. Statistical analysis was done by using Chi-Square. The software used was SPSS-20.

RESULTS

A total of 58 cases of acute leukemia were included in the study. Age ranged from 1.5 to 94 years. There were 30 males and 28 females with M:F 1.5:1. It was observed that fever in 41(70.6%) cases was the most common chief complaint followed by generalised weakness in 28(48.2%) cases and bleeding tendencies in 19(32.7%) cases of acute leukemia. Other less common presenting features were pain abdomen in 9(15.5%) cases, weight loss in 3(10.7%) cases of ALL and loss of appetite in 5(17.2%) cases of AML.(Table 1).

Table 1: Chief complaints in Acute Leukemia cases (n=58).

Chief complaints	ALL(n=28)	AML(n=29)	MPAL(n=1)	Total(n=58)
	No. (%)	No. (%)	No. (%)	No. (%)
Fever	20(71.4%)	20(68.9%)	1(100%)	41(70.6%)
Generalised weakness	12(42.8%)	16(55.1%)	0(0.0%)	28(48.2%)
Weight loss	03(10.7%)	0(0.0%)	0(0.0%)	3(5.1%)
H/O Bleeding /echymotic patches	09(32.1%)	10(34.4%)	0(0.0%)	19(32.7%)
Loss of appetite	0(0.0%)	5(17.2%)	0(0.0%)	5(8.6%)
Pain abdomen	2(7.1%)	6(20.6%)	1(100%)	9(15.5%)
others	6(21.4%)	12(41.3%)	0(0.0%)	18(31%)

Pallor 52 (89.6%), was the most frequently observed clinical sign followed by organomegaly in 29 (50%) cases, lymphadenopathy in 17 (29.3%) cases and bony

tenderness in 10 (17.2%) cases. Icterus was also observed in 4(6.8%) cases. Only pallor and organomegaly was observed in case of MPAL (Table 2).

Table 2: Clinical examination in Acute Leukemia patients(n=58).

	ALL(n=28)	AML(n=29)	MPAL(n=1)	Total(n=58)
Pallor	25(89.2%)	26(89.6%)	1(100%)	52(89.6%)
Icterus	02(7.1%)	02(6.8)	0(0.0%)	4(6.8%)
Organomegaly	15(53.5%)	13(44.8%)	1(100%)	29(50%)
Lymphadenopathy	07(25%)	10(34.4%)	0(0.0%)	17(29.3%)
Bony tenderness	03(10.7%)	07(24.1%)	0(0.0%)	10(17.2%)

On laboratory examination, mean Hb was 7.3 gm% (3.5-14.5 gm%), mean TLC $48 \times 10^9/L$ ($2 \times 10^9/L$ - $280 \times 10^9/L$), mean platelet count $47 \times 10^9/L$ ($2 \times 10^9/L$ - $130 \times 10^9/L$) and mean blast count in BM 66% (30%-90%).

Cytomorphological profile of acute leukemia cases were categorized according to FAB classification. In ALL, it

was observed that ALL L1 23 (82.2%) cases were more common than ALL L2 5 (17.8%). In AML, M2 was more common i.e. 23 (76.7%) cases followed by M4/M5 4 (13.4%) and M0, M1, M3 each constitute 1 (3.3%) cases. No case of ALL L3, AML M5, M6 and M7 was detected. All the cases of AL were subjected to FCA. (Table 3).

Table 3: Summary of Acute Leukemia cases on Cytomorphology and FCA.

	Diagnosis	No. of cases	Percentage (%)
ALL(n=28)	B-ALL	22	37.9%
	T-ALL	6	10.3%
AML(n=29)	AML M0	1	1.7%
	AML M1	1	1.7%
	AML M2	22	37.9%
	AML M3 with t(15;17)	1	1.7%
	AML M4/M5	4	6.8%
MPAL(n=1)	MPAL	1	1.7%
Total		58	100%

We observed the frequency of CD markers expression in ALL cases as CD45 Antigen, showed dim expression in 24(84.7%) cases and heterogenous expression in 3(10.7%) cases. The most common CD markers expressed were CD19 in 22(78.5%) cases followed by CD10 in 19(67.8%) cases, CD34 in 17(60.7%) cases,

CD79a in 15(53.5%) cases, Tdt in 15(53.5%) cases, CD20, cyCD3, CD5 each in 6(21.4%). CD 19, cyCD3, sCD3, CD5 and CD7 were significant statistically.($p < 0.05$). CD19 was expressed in all 22 (100%) B-ALL cases and in T-ALL, all 6 (100%) cases expressed cyCD3 and CD5. (Table 4)(Figure 1, 2).

Table 4: Frequency of antigen expression in ALL cases on FCA.

Antigen		B-ALL(n=22)	T-ALL(n=6)	ALL(n=28)	p value
		No. of cases (%)	No. of cases (%)	Total No. of cases (%)	
CD 45 ⁺	Dim	19(86.7%)	5(83.4%)	24(85.7%)	0.982
	Heterogenous	2(9.1%)	1(16.7%)	3(10.7%)	0.868
CD 10 ⁺		19(86.4%)	0(0.00%)	19(67.8%)	0.128
CD 19 ⁺		22(100%)	0(0.00%)	22(78.5%)	0.021
CD 20 ⁺		6(27.3%)	0(0.00%)	6(21.4%)	0.631
CD 34 ⁺		15(68.2%)	2(33.4%)	17(60.7%)	0.301
sCD 3 ⁺		0(0.00%)	4(66.7%)	4(14.2%)	0.006
cyCD3 ⁺		0(0.00%)	6(100%)	6(21.4%)	0.006
CD 5 ⁺		0(0.00%)	6(100%)	6(21.4%)	0.000
CD 7 ⁺		1(4.5%)	4(66.7%)	5(17.8%)	0.002
CD 4 ⁺		0(0.00%)	0(0.00%)	0(0.0%)	0.000
CD 8 ⁺		0(0.00%)	0(0.00%)	0(0.0%)	0.000
CD79a ⁺		14(63.6%)	1(16.7%)	15(53.5%)	0.124
Tdt ⁺		13(59.1%)	2(33.4%)	15(53.5%)	0.533

Fig 1: B - Acute lymphoblastic leukemia (B-ALL)

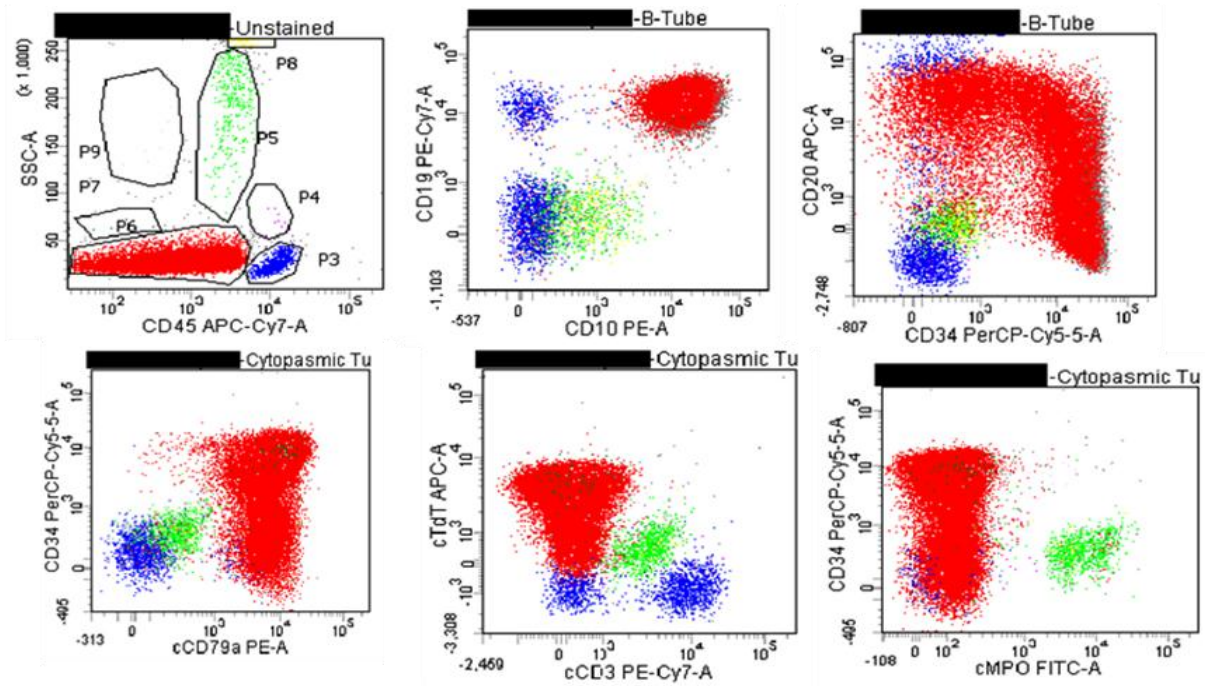


Figure 1: Dot plot SSC/CD45 gating showing heterogeneous expression of CD45, coexpression of CD10 and CD19, positive for CD34, CD79a, Tdt and negative for MPO and cCD3.

Fig 2: T- Acute lymphoblastic leukemia (T-ALL)

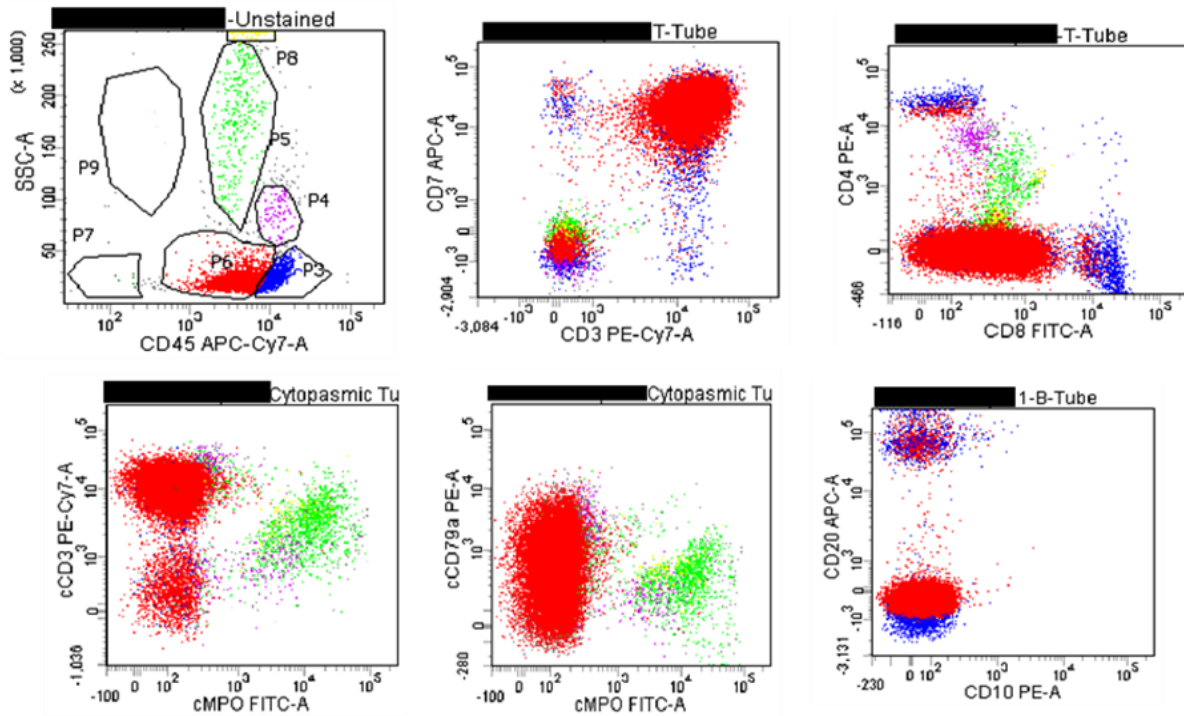


Figure 2: Dot plot SSC/ CD45 gating showing dim CD45, positive for CD3, CD7, CD8 and CD79a, negative for CD4, CD10, CD20 and MPO.

Similarly the frequency of antigen expression in AML cases, a dim CD45 expression was observed in all the AML cases. CD13 was the most common antigen expressed i.e. in 27(93.1%) cases followed by CD117 in 24(82.7%) cases, CD34 in 23(79.3%) cases, HLA-DR in

22(75.8%) cases, MPO in 22(75.8%) cases and CD33 in 21(72.4%) cases. CD 45, CD34, CD13, CD33, HLA-DR and MPO were highly significant statistically. (Table 5)(Figure 3).

Table 5: Frequency of antigen expression in AML cases on FCA.

Antigen	AML(n=29)						P value
	M0 (n=1)	M1 (n=1)	M2 (n=22)	M3 with t(15;17) (n=1)	M4 (n=4)	Total (n=29) No. (%)	
CD 45 ⁺	1(100%)	1(100%)	22(100%)	1(100%)	4(100%)	29(100%)	0.000
CD 34 ⁺	0(0.00%)	1(100%)	20(91%)	0(0.00%)	2(50%)	23(79.3%)	0.000
CD 13 ⁺	1(100%)	1(100%)	20(91%)	1(100%)	4(100%)	27(93.1%)	0.000
CD 33 ⁺	1(100%)	1(100%)	14(63.6%)	1(100%)	4(100%)	21(72.4%)	0.000
HLA-DR ⁺	1(100%)	0(0.00%)	18(81.8%)	0(0.00%)	3(75%)	22(75.8%)	0.000
MPO ⁺	0(0.00%)	0(0.00%)	17(77.2%)	1(100%)	4(100%)	22(75.8%)	0.000
CD 117 ⁺	1(100%)	0(0.00%)	18(81.8%)	1(100%)	4(100%)	24(82.7%)	0.000
CD 64 ⁺	0(0.00%)	0(0.00%)	0(0.00%)	1(100%)	4(100%)	5(17.2%)	0.058

Fig 3: Acute Myeloid Leukemia

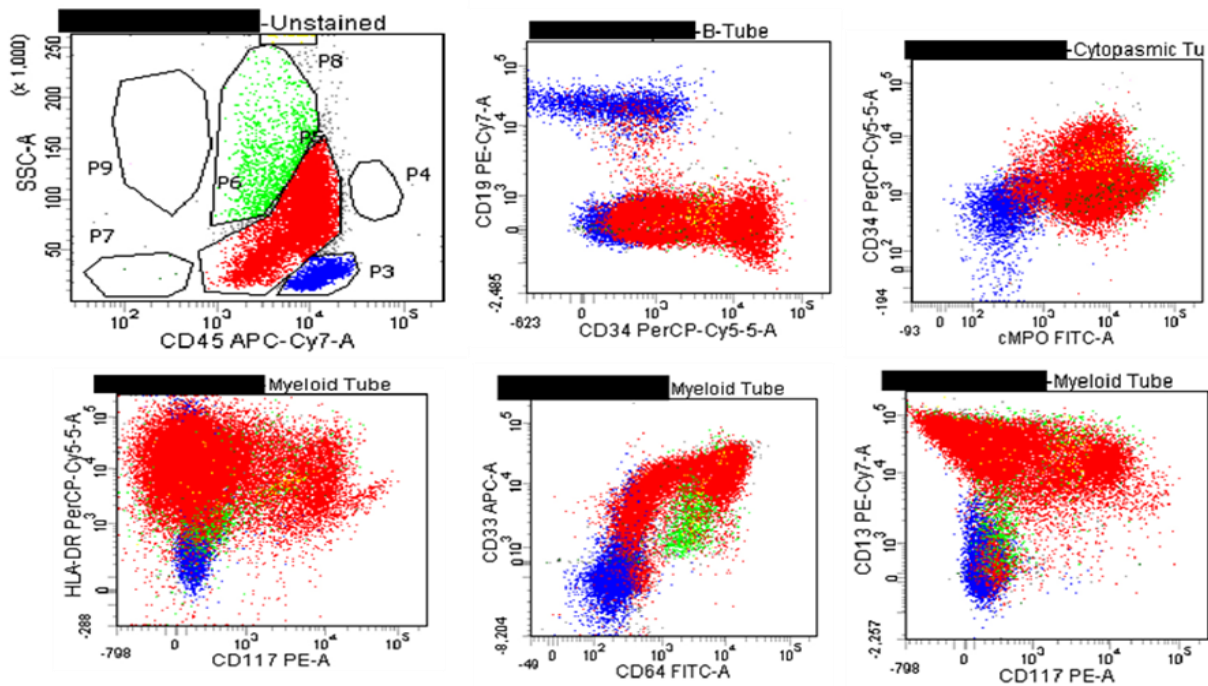


Figure 3: Dot plot SSC/ CD45 gating showing dim CD45, heterogenous positive for CD34, Positive for CD117, CD13, CD33, CD 64, HLA-DR, MPO, negative for CD19.

Two cases which were diagnosed as ALL L2 and AML M2 on morphology expressed different lineage on FCA. Hence a lineage correction was done. The ALL L2 on morphology, was diagnosed Mixed Phenotypic Acute Leukemia on immunophenotyping expressing both lymphoid and myeloid antigens. Another case of AML M2 on morphology was assigned lymphoid lineage of T cell type i.e. CD5, CD7, Tdt, cyCD3 on immunophenotyping. It was reviewed again morphologically and reported as T-ALL.

DISCUSSION

Immunophenotyping has become an essential diagnostic tool for classification, prognosis, patient management and for disease monitoring of acute leukemias besides clinical, morphological, cytochemical and cytogenetic analysis.^[4] The expansion of therapeutic options and improvement in remission induction and disease-free survival for both AML and ALL have stimulated

emphasis on defining good and poor treatment response groups.^[5]

In our study FCA analysis in 58 cases of acute leukemia was done and compared with their morphological diagnosis. Cytomorphologically, 48.2% ALL and 51.8% AML cases were diagnosed which were classified according to FAB classification. Among these ALL L1 (82.2%) and AML M2 (76.7%) were common FAB subtypes in our study. ALL L3, AML M6 and AML M7 subtypes were not diagnosed which could be attributed to small sample size. Koshaish *et al* reported a high frequency of ALL L1(67.7%) followed by ALL L2(31.4%) and AML M2(27.2%) followed by AML M4/M5(17.6%) which was comparable to our study.^[6] Selicean *et al* and Belurkar *et al* also showed similar results.^[7,8]

During Flow cytometric analysis of acute leukemia cases, CD45 was used for gating of blasts. A dim CD45 expression was seen in majority of the cases i.e.91.3% (Other cases expression was heterogenous) which was also mentioned by other studies and thus explaining that during gating strategy using CD45 expression is the most specific marker for the identification of leukemic cell populations in peripheral blood or marrow specimen.^[9-11] Hematopoietic progenitor or immature cells express CD34 normally. These CD34 positive cells are less than 3% in a normal marrow and becomes a specific marker for monitoring of blast population.^[12] Around 60% of ALL and 79% of AML cases were CD34 positive. A total 17 cases of acute leukemia were CD34 negative. According to Repp *et al* CD34 negative cases were associated with higher complete remission rate.^[13]

In ALL cases, B-ALL and T-ALL must be separated, as it is clinically important for both therapeutic and prognostic implications.^[14] In our study, on FCA, out of 28 cases of ALL 78.6% and 21.4% cases were diagnosed as B-ALL and T-ALL respectively. All B-ALL cases expressed CD19 followed by CD10, and Tdt which was statistically significant and helped in B-lymphoid lineage assignment. Other studies also reported CD19 as the most sensitive marker in diagnosing B-ALL.^[8,14,15,16,17] Whereas Augilera *et al* reported CD79a the most often expressed antigen.^[18] On the other hand Mukda *et al* mentioned both CD19 and CD79a as the most often expressed B-ALL antigens.^[19] Nineteen (67.8%) of B-ALL cases expressed CD10 which is an antigen of longstanding and well known prognostic value.^[20] In our study 3(13.6%) cases of B-ALL were negative for CD10 which is associated with a poor prognosis according to literature.^[21]

In T-ALL cases, cyCD3 and CD5 were expressed in all cases i.e. 6(100%) each, followed by sCD3, CD7, Tdt, and CD79a revealing a significant association statistically. In similar studies, cyCD3 was reported as the most frequent expressed antigen along with CD3 and CD^[7,8,15,17,22] Augilera *et al* concluded that cyCD3 was

the best marker for T-ALL cases.^[18] Whereas Tiensiwankul *et al* observed CD5 expression by all the cases of T-ALL along with CD7.^[17] CD7 positive ALL must not be assigned T cell lineage because a large number of B-ALL and myeloid lineage has been associated with the expression of CD7 on the cell surface.^[23] Thus a diagnosis of T-ALL should be made when other T-cell markers(CD2, cyCD3 and CD5) are also present.

In our study, CD13, CD33 and CD117 were the most common myeloid markers expressed in AML cases, followed by HLA-DR and MPO. The results were statistically significant and consistent with the previous studies.^[24-27] On immunophenotyping our study revealed a predominance of AML M2 i.e. 22 (75.8%) cases followed by AML M4/M5 in 4(6.8%) cases. The results were consistent with Chen *et al* and Nakase *et al*.^[28,29] AML-M2 was reported as the commonest AML subtype by Ansari *et al*.^[30] Advani *et al* in two separate studies observed a higher frequency of AML-M2 in both pediatric and adult population from the same institution.^[31,32] Most published data indicates predominance of M1-M2 subtypes.^[33] However Salem *et al*, Harakati *et al*, Harani *et al* varied with our results in that their studies reported AML M4/M5 predominance.^[33,34,35] CD13, CD33, CD117, CD64 and MPO were expressed in all the 4 cases of AML M4/M5 and was comparable with Venketsvaran *et al*.^[14] Only one case (1.7%) of AML M3 was diagnosed. Since our institution had no facility, the patient was advised cytogenetic analysis from other laboratory and was found positive for t(15;17). Ansari *et al* reported a frequency of 5.4% AML M3.^[30] In a study by Laishram *et al* AML M3 was the most common type of AML.^[36] Salem *et al* reported 23% of AML M3 which was much higher as compared to our study which may be due to their large sample size.^[33] FCA showed positivity for CD13, CD33, CD117, CD64, MPO and was concomitant with other studies by Selicean *et al* and Kaleem *et al*.^[7,26] CD34 and HLA-DR were found negative which was consistent with Salem *et al*. They suggested that negativity of both CD 34 and HLA-DR may not favour a diagnosis of APL always as 6.9% of non-APL AML cases in their study were double negative for these two markers.^[33]

One case of each AML M0 (1.7%) and AML M1(1.7%) subtype were also diagnosed in the present study. Consistent with Mukda *et al* FCA in present study helped into the confirmatory diagnosis of AML M0 which was positive for SBB in<3% blasts and negative for MPO on cytochemistry as well as on immunophenotyping.^[19]

In our study, Six (21.4%) ALL cases expressed aberrant myeloid antigens of which 5(22.7%) B-ALL cases expressed CD13 and 1 (1.7%) T-ALL case expressed CD33. Various other studies too reported similar aberrant myeloid antigen expression.^[15,33]

Similarly, 11(37.9%) AML cases expressed aberrant lymphoid antigens. CD7 and CD4 were most frequently expressed antigen followed by CD10 and CD19. Aberrant expression on immunophenotyping have been analysed to predict treatment outcome in AML. CD7 is frequently expressed in AML as an aberrant marker and, in most publications, associated with adverse outcome.^[37] Three (75%) cases of AML M4/M5 showed aberrant expression for CD4. A study by Miwa et al reported 65.0% AML M4 and 78.3% AML M5 cases with aberrant expression of CD4 which was related to worse prognosis.^[38]

One case of mixed phenotypic acute leukemia (MPAL) on FCA expressed both lymphoid and myeloid antigens i.e. CD7, cyCD3, Tdt, and CD13, CD117, MPO, and HLA-DR which was diagnosed as ALL L2 on morphology. Augilera et al showed positivity of CyCD3, MPO, CD7, CD34, CD13, CD33 and CD79a in MPAL cases.^[18] Belurkar et al reported positivity of CD13, CD33 and CD19 in MPAL cases and also stated that survival rates of MPAL are not different from AML.^[8] Cytomorphological correlation with immunophenotyping a concordance rate of 96.5% was observed both on cytomorphology and immunophenotyping revealing that maximum number of cases had same diagnosis on morphology and FCA. Our study showed a high concordance rate as compared to studies by Belurkar et al, Kheri et al and Mhaweche et al. (Table 6)^[8,39,40]

Table 6: Cytomorphological correlation with immunophenotyping.

Studies	Concordance rates (%)
Kheiri et al ³⁹	77.4
Mhaweche et al ⁴⁰	80.32
Belurkar et al ⁸	58
Present study	96.5

Lineage correction was done for two cases which were diagnosed as ALL L2 and AML M2 on morphology expressed different lineage on FCA. The ALL L2 on morphology, was diagnosed Mixed Phenotypic Acute Leukemia on immunophenotyping expressing both lymphoid and myeloid antigens. Another case of AML M2 on morphology was assigned lymphoid lineage of T cell type on immunophenotyping. These two cases would have been misdiagnosed or remained unclassified without the aid of flowcytometry. Belurkar et al reported a case which was diagnosed as M0/ALL L2 on morphology and MPAL on FCA.^[8] Qadir et al also reported lineage correction in 2% cases.^[5]

Therefore, immunophenotyping with a limited panel must be routinely performed for the correct diagnosis of acute leukemia.

CONCLUSION

To conclude, our study proved to be helpful in assigning correct lineage to leukemia cells and support the use of

particular panel of CD markers as a better diagnostic tool after preliminary investigations. In our study, most useful markers for B-ALL is CD19, for T-ALL cyCD3 and for AML cMPO and CD13 were found. The expansion of therapeutic options and improvement in remission induction and disease free survival for both ALL and AML have stimulated emphasis on defining good and poor treatment response groups. This is most effectively accomplished by multifaceted approach to diagnosis and classification using immunophenotyping. As suggested by various studies, an antigen expression may have a prognostic significance and predict therapeutic outcome in acute leukemia, we need to elaborate the panel for CD markers to explore the role of each antigen. Although considered superior, flowcytometric analysis must always be performed in conjunction with cytomorphology.

ACKNOWLEDGEMENT

No financial support was received. The authors declare that there is no conflict of interest.

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