Original Research

EVALUATION OF ACUTE LEUKAEMIAS BY FLOW CYTOMETRY AND ITS ASSOCIATION WITH DIAGNOSIS USING MORPHOLOGICAL AND SPECIAL STAINING TECHNIQUES

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ABSTRACT

Background: Acute leukemia (AL) is a group of hematological malignancies with the common feature of the uncontrolled proliferation of immature cells. It is crucial to diagnose and lineate a specimen accurately to be able to classify and manage it properly. The conventional methods such as morphology and cytochemical staining have remained useful and flow cytometry has played a more prominent role in the immunophenotypic characterization of leukemic blasts.

Objective: To assess the value of cytochemical staining and flow cytometry in the diagnosis of acute leukemia and to correlate morphological and cytochemical with flow cytometric diagnosis.

Materials and Methods: This is a cross-sectional study of 60 suspected cases of acute leukemia. In all cases, peripheral smear examination, bone marrow morphology and periodic acid Schiff (PAS) and myeloperoxidase (MPO) cytochemical staining were carried out. Definitive lineage was then determined by flow cytometric immunophenotyping. The flow cytometric findings were correlated with the morphological and cytochemical diagnosis**.**

Results: We observed that on flow cytometry analysis 31 cases (51.7%) were diagnosed as AML and the remaining 29 cases (48.3%) were diagnosed as ALL. Morphological diagnosis was in good agreement with flow cytometry. Of the 28 cases morphologically diagnosed as AML, 100% of these cases were confirmed as AML by flow cytometry. Contrary to this, 32 cases diagnosed as ALL, 29 cases (90.6%) matched with flow cytometric diagnosis while three were re-classified as AML. When the discordant ALL cases were observed, the expression of myeloid markers CD13, CD33 and CD117 was evident in the absence of good cytochemical staining.

Conclusion: Morphology and cytochemical staining are still valuable preliminary diagnostic procedures in acute leukemia. Flow cytometry, however, offers greater diagnostic accuracy and is an important tool when the diagnosis is difficult and the morphology is poorly differentiated. The integrated use of morphology, cytochemistry and immunophenotyping is still essential for the correct classification of acute leukemia.

INTRODUCTION

Leukaemia is a group of haematological malignancies in which abnormal haematopoietic cells multiply in an uncontrolled manner resulting in progressive infiltration of bone marrow, and in some cases, the lymphoid tissues.[1] This is a fundamentally clonal disease characterized by accumulation of immature blast cells that are unable to differentiate correctly, the unchecked growth of which seriously disrupts the normal process of hematopoiesis, and, if the disease progresses without prompt treatment, it can become rapidly progressive with fatal outcome.[2]

www.ajmrhs.com
eISSN: 2583-7761

Date of Received: 12-10-2025
Date of Acceptance: 24-12-2025
Date of Publication: 17-02-2026

Leukaemias can be classified broadly into lymphoid and myeloid. The clinical presentation, disease course, response to treatment and prognosis differ for each type depending on the precursor cell involved. Moreover, these cancers are often divided into acute and chronic types, depending on the progression rate and the nature of the disease. The diagnosis of acute leukemia has been largely based on the examination of peripheral smear and bone marrow morphology and cytochemical staining for decades.[3] Economical, technically uncomplicated stains even now routinely used to determine blast lineage and maturation characteristics like myeloperoxidase (MPO), Sudan Black B (SBB), periodic acid Schiff (PAS) and non specific esterase (NSE) work very well and remain useful in many laboratories, especially in areas where advanced diagnostic facilities are lacking.[4] But cytochemistry can not always be reliable in distinguishing poorly differentiated leukemias, mixed phenotype leukemias, or minimally differentiated AML. Over

the years, flow cytometry has become a valuable tool as it has revolutionized the diagnostic approach to acute leukaemia (AL) with rapid immunophenotyping of blast cells and has greatly aided in establishing blast lineage more accurately with monoclonal antibodies directed against surface and cytoplasmic antigens.[5] It demonstrates abnormal antigenic expression, can aid in the identification of mixed lineage leukemias, and can aid in the subclassification of acute leukemias based on the current WHO and ICC classification. Furthermore, flow cytometry is an important tool for the prognostication and monitoring of minimal residual disease (MRD).[5,6] Although the main role of immunophenotyping has grown, cytochemical staining remains useful in limited situations, with several studies showing there is significant overlap in the two methods, and with flow cytometry showing excellent sensitivity and specificity in challenging or indeterminant cases.[3,6,7] Both methods are frequently used in conjunction in routine hematopathology. The other important practical point is resource availability because in many developing regions, advanced immunophenotyping panels may be cost- or infrastructure-limited and cytyochemical staining is an important preliminary diagnostic step. Meanwhile, the increasing availability of flow cytometry has greatly enhanced diagnostic accuracy and minimized misclassification. Thus, the integration of morphology, cytochemistry and immunophenotyping continues to be crucial for a correct diagnosis of acute leukemias. Therefore the present study was conducted to be an evaluation of the role of cytochemical staining and flow cytometry in the diagnosis of acute leukemias and the usefulness of these techniques in lineage assignment and diagnostic classification of leukemic blasts.

MATERIAL AND METHODS

A cross sectional study was done in Department of Pathology, Government Medical College, Jammu for one year in this hospital (March 2022-Feb 2023) . A total of 60 newly diagnosed suspected cases of acute leukemia were included in the study were referred for peripheral blood smear, bone marrow aspiration and immunophenotypic evaluation in the Hematology section. Patients of all age groups and both genders were included in the study after obtaining informed consent. The study was approved by the Institutional Ethics Committee of GMC Jammu. Those cases with over 20% blasts in the peripheral blood and/or bone marrow aspirate on morphological evaluation were selected for the study. Those who had leucoerythroblastic reaction and the others who refused to participate were excluded. Patient records were used to collect clinical data and demographic data. Peripheral blood smears and bone marrow aspirate smears were made and stained with Leishman stain and examined for morphological evaluation. MPO (myeloperoxidase) and PAS (periodic acid Schiff) stains were used to evaluate blast cells for lineage differentiation. MPO was positive and PAS was positive, which was interpreted as suggestive of myeloid and lymphoid lineage respectively. Flow cytometric immunophenotyping was carried out on EDTA anticoagulated peripheral blood and/or bone marrow samples using a multiparameter flow cytometer. Antibodies to lineage specific markers (CD3, CD19, CD10, CD13, CD33, CD117 and others) were applied as panels depending on the suspected lineage. Blasts were detected with CD45 and side scatter gating strategy and an antigen expression of more than 20% of the blast cells was defined as positive. The morphological and cytochemical diagnoses were correlated with flow cytometric diagnosis and the degree of concordance was assessed with the aim of determining the place of flow cytometry in the accurate lineage assessment and classification of acute leukemia. Data collected were presented and analysed descriptively and results presented as frequencies and percentages.

RESULTS

Table 1: Age Distribution of AML and ALL Patients [N=60]

Age (Years)	AML		ALL		Total	
	No.	%age	No.	%age	No.	%age
0-10 Years	1	3.2	18	62.1	19	31.7
11-20 Years	3	9.7	8	27.6	11	18.3
21-30 Years	6	19.4	2	6.9	8	13.3
31-40 Years	5	16.1	0	0.0	5	8.3
41-50 Years	8	25.8	0	0.0	8	13.3
51-60 Years	5	16.1	0	0.0	5	8.3
61-70 Years	2	6.5	1	3.4	3	5.0
> 70 Years	1	3.2	0	0.0	1	1.7
Total	31	100	29	100	60	100

We assesed the age distribution of patients with AML and ALL wherein we found that acute lymphoblastic leukemia (ALL) was evident predominantly in children, with 62.1% of cases occurring in the 0–10 year age group, this was followed by 27.6% occurring in 11–20 years. Acute myeloid leukemia (AML), however, was more common in adults, the age group 41-50 years constituted (25.8 %), 19.4 % in the age group 21-30 years, 16.1 % in the age group 31-40 years and 16.1 % in the age group 51-60 years as observed in table 1. Overall, a majority of the respondents were in the age group 0–10 (31.7%).

Table 2: Gender Distribution of AML and ALL Patients [N=60]

Gender	AML		ALL		Total	
	No.	%age	No.	%age	No.	%age
Male	18	58.1	19	65.5	37	61.7
Female	13	41.9	10	34.5	23	38.3
Total	31	100	29	100	60	100

In our study, males outnumbered females in both AML and ALL with males making up 58.1 % of cases in AML and 65.5 % of cases in ALL, as females made up 41.9 % of AML cases and 34.5 % of ALL cases. In total, there were 61.7% male patients.

Table 3: Correlation of Morphological Diagnosis with Flow Cytometry Diagnosis

Morphological Diagnosis	Flow Cytometry Diagnosis					
	AML		ALL		Total	
	No.	%age	No.	%age	No.	%age
AML	28	100	0	0.0	28	46.7
ALL	3	9.4	29	90.6	32	53.3

Out of 28 cases, which were morphologically diagnosed as AML, all the cases were confirmed as AML on flow cytometry. Of the 32 cases initially diagnosed as ALL, 29 (90.6%) were concordant with flow cytometric diagnosis and 3 cases were diagnosed as AML, demonstrating a high degree of agreement between the morphology and flow cytometric diagnosis.

Table 4: Non-Concordant Cases between Morphology and Flow Cytometry

Morphological Diagnosis	No. of Cases	Cytochemistry	Flow Cytometry Findings	Final Diagnosis
ALL	3	MPO negative, PAS weak positivity	CD13, CD33, CD117 positive	AML

We assessed the cases in which the initial morphological diagnosis was not in agreement with the flow cytometry findings. Three (03) cases initially labelled as ALL demonstrated MPO negativity with weak PAS positivity, while flow cytometry showed myeloid markers including CD13, CD33 and CD117, leading to a final diagnosis of AML. These discordant cases underline the importance of flow cytometry in accurately establishing blast lineage, particularly in diagnostically challenging cases.

DISCUSSION

In the present study, acute lymphoblastic leukemia (ALL) was observed predominantly in the pediatric age group, with 62.1% of ALL cases occurring between 0–10 years and an additional 27.6% between 11–20 years. In contrast, acute myeloid leukemia (AML) was more frequently encountered in adults, particularly in the 41–50 year age group where 25.8% of AML cases were recorded, 19.4 % in the age group 21-30 years and 16.1 % in the age group of 31-40 years and 51-60 each. These findings are in accordance with the well-established epidemiological pattern of acute leukemias, where ALL predominates in childhood while AML is more common among adults. A large pediatric study by Jamal et al., reported that nearly 75.7% of B-ALL cases occurred in children between 1–10 years of age, which closely parallels the predominance of childhood ALL observed in our study.[8] Similarly, a review of childhood acute leukemia in India by Arora et al., noted that the median age of presentation for childhood ALL ranged between 5–10 years in most Indian series, further supporting our observations.[9] Ahmad et al., reported that ALL has a greater predilection for lower age groups, with the maximum number of cases in the age group 0-10 years (n = 17, 60.7%), whereas nine cases (32%) were found to be in the age group of 11-20 years, which is consistent with our study.[3] With regard to AML, our findings of increased frequency in

middle-aged and older adults are comparable to studies showing that AML incidence rises progressively with advancing age. Nagel et al., demonstrated that AML occurs predominantly in adult and elderly populations, with age-related increase in disease frequency.[10] Likewise, a tertiary care flow cytometry study by Gupta N et al., observed that AML constituted 59.7% of acute leukemia cases in adults.[11] Similarly, Ahmad N et al documented that AML, cases were clustered in the age group 21-60 years (n = 23, 79.3%), which is in consonance with our study.[3] The overwhelming predominance of ALL in younger age groups could have implications on the biological susceptibility of immature lymphoid precursor cells in childhood years, while AML is more likely to be associated with cumulative genetic changes, other environmental factors and age-related changes in the marrow in adults. The age-wise distribution of the present study is thus consistent with the age wise distribution reported in Indian literature and in international literature.

In the present study, males predominated in both AML and ALL. Male patients accounted for 58.1% of AML cases and 65.5% of ALL cases, while females constituted 41.9% and 34.5% respectively. Overall, 61.7% of the study population were males with the male-to-female ratio of 1.38:1 in AML, 1.9:1 in ALL, and 1.6:1 overall, indicating a clear male predominance among acute leukemia patients. These findings are comparable with several previously published studies. A study by Jamal S et al., evaluating 1379 pediatric acute leukemia cases reported an overall male prevalence of 63.7%, with male-to-female ratios of 1.5:1 in AML and 1.5:1 in B-ALL, which is comparable with our study. [8]Similarly, a clinicopathological study by Patel G et al., observed a male predominance with an overall male-to-female ratio of 1.7:1, while AML and ALL showed male-to-female ratios of 1.5:1 and 2.1:1 respectively, which is in consonance with our study. [12] Our findings regarding ALL are also in agreement with the large retrospective analysis conducted in Southern China by Li S et al., where 457 out of 705 ALL patients were males, yielding a male-to-female ratio of 1.84:1, which is akin to our study.[13] Likewise, Srivastava V et al., reported in a cytogenetic analysis of 1791 adult AML patients, that 60.6% of cases were males, which is consistent with our study.[14] The reason for this male predominance is not completely understood, although hormonal influences, genetic susceptibility, environmental exposure patterns and sex-related biological differences in hematopoiesis have all been proposed as contributing factors. Recent literature has also suggested that sex-specific molecular and epigenetic mechanisms may influence leukemia susceptibility and disease biology.

In the present study, morphology showed a high level of agreement with flow cytometric diagnosis. Among 28 cases initially diagnosed as AML on morphology, all these cases were confirmed as AML by flow cytometry. Similarly, of the 32 morphologically diagnosed ALL cases, 29 (90.6%) were concordant with flow cytometric findings and three cases were ultimately categorized as AML. These findings indicate strong diagnostic concordance between conventional morphology and immunophenotyping, while also highlighting the important role of flow cytometry in resolving diagnostically ambiguous cases. Comparable observations have been reported in earlier studies. Belurkar et al., evaluated 50 cases of acute leukemia and observed an overall concordance rate of 86% between morphologic/cytochemical diagnosis and flow cytometric diagnosis, with complete concordance in 58% and partial concordance in 22% of cases and only 4% of their cases were completely discordant, which is in consance with our study.[7] Similarly, Basharat et al. reported nearly 90% concordance between morphology and flow cytometry in AML cases, emphasizing that immunophenotyping substantially improves diagnostic accuracy when interpreted alongside morphology, which is consistent with our study. [15]Kheiri et al. also demonstrated a high degree of lineage agreement between cytochemistry and flow cytometry, reporting concordance in 95.8% of leukemias classified as AML or ALL by both methods, although overall agreement decreased when biphenotypic and non-diagnostic cases were included.[16] Browman G et al., in another analysis on assessing the contribution of immunophenotyping to leukemia classification, concordance between observers improved dramatically from 89% with morphology plus cytochemistry to 99% after addition of immunophenotypic data.[17] Nishat Ahmad et al., evaluating acute leukemias using morphology, cytochemistry and flow cytometry observed that all 25 morphologically diagnosed AML cases were confirmed by flow cytometry, giving 100% concordance for AML, while 28 out of 32 ALL cases (87.5%) correlated with flow cytometric diagnosis and four cases initially interpreted as ALL were later identified as AML on immunophenotyping which is compatible with our study.[3] The discordant cases observed in the present study further highlight the limitations of relying solely on morphology and cytochemistry. Three cases initially labelled as ALL demonstrated weak PAS positivity with absent MPO staining, but immunophenotyping established myeloid lineage through expression of CD13, CD33 and CD117. Similar findings have been reported by Nishat A et al., in minimally differentiated AML, where blasts may lack convincing MPO positivity and mimic lymphoblastic leukemia morphologically.[3] They

found that four cases morphologically diagnosed as ALL were subsequently identified as AML after flow cytometric analysis.[3] Their study demonstrated that morphology and cytochemistry correctly classified 92.9% of acute leukemia cases, while the remaining discordant cases mainly represented minimally differentiated AML (M0/M1), which lacked characteristic morphologic and cytochemical features. The pattern observed in their study closely resembles our findings, where cases initially interpreted as ALL were ultimately confirmed as AML on immunophenotyping because of myeloid marker expression. Larger flow studies also confirm our discordant AML immunophenotypes. In a tertiary care analysis of 631 cases of acute leukemia, Gupta et al. showed that CD13, CD33 and CD117 were highly consistent, with a positivity rate of 93.9%, 94.8% and 90.9%, respectively, in AML.[11] On the other hand CD19 and CD10 are well known B-lineage ALL markers and are routinely used in lineage assignment. The discordant cases in the present study may be attributed to the overlapping nature of immature leukemic blasts in terms of their morphology and cytochemical reactions. Occasional AML cases may express aberrant lymphoid antigens, and in some instances, AML might be morphologically identical to ALL and yet show weak or no expression of MPO. Hence, while morphology and cytochemistry are still valuable first-line diagnostic aids, flow cytometry is more specific and is an important part of the process for the correct diagnosis of the more difficult acute leukemias.

CONCLUSION

In the present study, a very high concordance was obtained between the morphological assessment and the flow cytometric diagnosis, suggesting that traditional morphological diagnosis is still a valuable first-line tool for the diagnosis of acute leukemia. A few cases, however, were found in which the morphology and the cytochemistry were not enough to establish a definite lineage. In these diagnostically difficult cases, flow cytometry proved to be a breakthrough tool and helped to confirm the final diagnosis by detecting the expression of lineage specific antigens. Based on the results of the present study, it may be concluded that the use of morphology, cytochemistry and flow cytometry is complementary in the diagnosis of acute leukemia. An additional advantage of flow cytometric immunophenotyping is that it adds considerably to the accuracy of diagnosis, especially when morphology is not completely suggestive or cytochemical markers are equivocal, and that it helps to classify cases with greater accuracy; although conventional methods still yield valuable preliminary information.

Limitations:

The present study was limited by its relatively small sample size and single-center design, which may restrict the generalizability of the findings. The cross-sectional nature of the study did not permit assessment of treatment outcomes, survival, or minimal residual disease monitoring. Only MPO and PAS cytochemical stains were employed, while additional stains such as Sudan Black B and non-specific esterase were not evaluated. Furthermore, cytogenetic and molecular investigations, which form an integral part of contemporary leukemia classification and prognostication, were not included. Future multicentric studies with larger sample sizes and integrated molecular analysis are recommended to validate and extend these findings.

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How to cite this article: Dr. Bindoo Goria, Dr. Pratibha Shalini, Dr. Komal Yadav, EVALUATION OF ACUTE LEUKAEMIAS BY FLOW CYTOMETRY AND ITS ASSOCIATION WITH DIAGNOSIS USING MORPHOLOGICAL AND SPECIAL STAINING TECHNIQUES, Asian J. Med. Res. Health Sci., 2026; 4 (2):622-627.
Source of Support: Nil, Conflicts of Interest: None declared.