Original Article

Clonality in B-Cell Lymphoid Malignancies

Comparative Evaluation of Immunohistochemistry and Flow Cytometry in Diagnosing Clonality in B-Cell Lymphoid Malignancies

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ABSTRACT

Objective: To assess various methods for clonality detection in patients with B-cell lymphoblastic leukemia.
Study Design: A diagnostic study
Place and Duration of Study: This study was conducted at the Department of Pathology and Forensic Medicine, Faculty of Medicine, University of Kufa from 1st January 2024 to 30th June 2024.
Methods: Diagnoses were based on the gold-standard flow cytometry test for B-cell acute lymphoblastic leukemia. Patient data, including age, gender, clinical features, complete blood count, blood smear, and bone marrow morphology reports, were collected. Immunohistochemistry panel (CD19, 20, 10, 34, MPO & TdT) was used on BM biopsies.
Results: The mean age was 30.74 years, with a male-to-female ratio of 1:2.04. Four diagnostic marker panels, incorporating IHC and cytochemistry, were evaluated. The first panel of five markers (CD10, 19, 20, 34, and TdT) showed a sensitivity of 46.27% and a specificity of 53.73%, second panel with three markers (CD10, 19, 20, and 34), demonstrated 52.24% sensitivity and 47.76% specificity and third panel (CD10, 19, 20, and TdT) yielded 53.73% sensitivity and 46.27% specificity.
Conclusion: The four-marker IHC panel (CD10, 19, 20, and TdT) is effective for diagnosing B-cell ALL, especially in settings where flow cytometry is inaccessible or unaffordable offers valuable diagnostic utility in low-resource laboratories lacking access to flow cytometry.
Key Words: Clonality detection, B-cell lymphoblastic leukemia, Flow cytometry, Immunohistochemistry, Cytochemical stain

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INTRODUCTION

Leukemia is a hematologic malignancy characterized by the uncontrolled proliferation of abnormal white blood cells, known as blasts, in the bone marrow and blood. Unlike normal white cells, leukemia cells are immature, rapidly proliferating, and dysfunctional, often replacing healthy blood cells and potentially infiltrating other organs such as the liver and spleen. The major types of leukemia include acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Acute Myeloid Leukemia (AML), and chronic myeloid leukemia (CML).[1]

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Leukemia is classified based on its rate of progressionacute or chronicand the blood cell lineage affected, either lymphoid or myeloid. Acute leukemia progresses quickly, while chronic forms develop more gradually. "Lymphoblastic" or "lymphocytic" leukemia arises from lymphoid stem cells and "myeloid" or "myelogenous" leukemia originates in myeloid progenitors.[2] ALL is a rapidly progressing leukemia derived from immature lymphoid cells. It primarily affects lymphocytes at an early developmental stage and accounts for about 85% of childhood leukemia cases.[3] Subtypes of ALL include B-cell and T-cell ALL, with B-cell ALL being the most common (85% of cases).[3,4] Advances in immunophenotyping have further classified B-cell ALL into Early pre-B (10%), Common ALL (50%), and Mature B-cell ALL (4%).[5]
The World Health Organization (WHO) no longer uses the French-American-British (FAB) classification for ALL due to its limited clinical utility and instead relies on immunophenotypic markers to define subtypes.[6]
B-ALL is characterized by the overproduction of immature B-cell lymphoblasts. It predominantly affects children aged 1–4 and accounts for 75% of ALL cases.

However, adults with B-ALL face lower survival rates and higher relapse risks.[7]

The incidence of ALL varies globally, with higher rates in North America and Europe compared to Asia and Africa. Genetic syndromes, such as Down syndrome, and environmental factors like radiation exposure and chemical toxins are associated with increased ALL risk.[8,9] Additionally, the "hygiene hypothesis" suggests that limited childhood infection exposure may influence leukemia risk.[10] Socioeconomic disparities also contribute to incidence and outcomes.[10]

The pathogenesis of B-ALL involves genetic, epigenetic, and microenvironmental factors. Chromosomal translocations, including BCR-ABL1 and MLL rearrangements, are common in high-risk cases.[11] Epigenetic alterations, such as DNA methylation and histone modifications, and dysregulated non-coding RNAs also play critical roles in disease progression.[12] The bone marrow microenvironment supports leukemic cells through stromal cell interactions and cytokines like IL-7, contributing to therapy resistance.[13]

Diagnosis of B-ALL integrates clinical, cytological, and molecular assessments: (1) Clinical evaluation and CBC identifies anemia, thrombocytopenia, and leukocytosis. (2) Peripheral blood smear highlights characteristic lymphoblasts.[14] (3) Bone marrow biopsy confirms leukemic infiltration. (4) Flow cytometry, a gold-standard technique for immunophenotyping markers such as CD19, CD10, CD34, and TdT. It is also used for Minimal Residual Disease (MRD) monitoring.[15] (4) Cytogenetics and molecular testing detect chromosomal translocations and mutations (e.g. Philadelphia chromosome, hyperdiploidy) to guide prognosis and therapy.[16]

IHC aids in diagnosing and subclassifying B-ALL by identifying surface markers such as CD19: A universal B-cell marker, CD10: Indicates early B-cell precursor stages, TdT: Associated with immature lymphoid cells and PAX5 and CD20: Confirm B-cell lineage and guide therapy.[17]

Treatment of B-ALL involves chemotherapy, targeted therapies, and immunotherapies. Chemotherapy regimens include agents like vincristine and asparaginase, while tyrosine kinase inhibitors (TKIs) such as imatinib are effective for Philadelphia chromosome-positive B-ALL.[18] Novel therapies, including CAR T-cell therapy, offer hope for relapsed or refractory cases.[19] Prognosis depends on age, genetic factors, and MRD status. Children aged 1-9 years show high remission rates (>80%), while adults face poorer outcomes due to adverse genetic profiles.[20] Favorable genetic markers like hyperdiploidy improve outcomes, while CNS involvement worsens prognosis.[21]

METHODS

This study was conducted in the Department of Pathology and Forensic Medicine at the Faculty of Medicine, University of Kufa, from 1st January 2024 to 30th June 2024 vide letter No. 3234/QM/Approval/JKEIRU dated 2nd September 2023. The cases were collected from the Hematology Department at Baghdad Medical City and Baghdad Educational Laboratories. All cases diagnosed as B-cell Acute Lymphoblastic Leukemia (B-ALL) by a hematopathologist using clinical findings, complete blood count, blood film, bone marrow examination, and flow cytometry (FCM) and newly diagnosed patients, of both genders, with no age limitation were included. All patients undergoing treatment, uncertain diagnoses, other malignancies and cases without complete data were excluded. All cases were retrieved with permission from the department, and patient information was kept confidential.

Blood samples and bone marrow aspirates were collected for testing. The following diagnostic methods were used: complete blood count for evaluating general blood parameters, bone marrow aspiration for FCM analysis, bone marrow biopsy to understand the cellular makeup of the bone marrow and immunohistochemistry to detect cellular markers and signs of clonality in paraffin-embedded tissue samples. The IHC markers used were CD19, CD20, CD10, CD34, TdT, and MPO. Sampling including CBC, blood films, BMA, BMB, and FCM results, were collected. Fresh blood films were taken for further staining if needed, along with 2-3 slides of unstained BM aspirates. Paraffin blocks were sectioned into 6-7 slices for IHC analysis.

Immunohistochemistry is a technique used to detect antigens in tissues using specific antibodies. The steps involved in IHC are as follows:

1. Deparaffinization and Rehydration: Removal of paraffin wax and rehydration of tissue sections.
2. Antigen Retrieval: Treating tissue sections to unmask antigens.
3. Blocking: Preventing non-specific antibody binding.
4. Primary Antibody Incubation: Incubating with primary antibodies (e.g, CD10, CD20, CD19, CD34, MPO, TdT).
5. Washing: Removing unbound primary antibodies.
6. Secondary Antibody Incubation: Adding a secondary antibody with an enzyme or fluorophore for detection.
7. Washing: Removing excess secondary antibodies.
8. Detection and Visualization: Adding a substrate to reveal the target antigen.
9. Counterstaining: Optional step to provide contrast.
10. Mounting: Sealing the slide with a mounting medium.

Each marker's expression was evaluated based on staining intensity and distribution in the tissue.

Scoring System: Two scoring systems were used to assess marker expression:

- **Positivity Score:** Measures the percentage of cells showing positivity for a specific marker:
  - Score 0: Less than 5% of tumor cells
  - Score 1: 5%-25% of tumor cells
  - Score 2: 26%-50% of tumor cells
  - Score 3: 51%-75% of tumor cells
  - Score 4: More than 75% of tumor cells
- **Intensity Score:** Measures the strength of staining:
  - 0: Negative
  - 1: Weak
  - 2: Intermediate
  - 3: Strong

A final score is calculated by combining the positivity and intensity scores, with values between 0-12. Scores between 0-8 indicate reduced immunoexpression, and scores between 9-12 indicate strong immunoexpression.

Statistical analysis was done using SPSS-26. T-tests were used for comparisons between two groups, and ANOVA was used for comparisons across multiple groups. Regression analysis (e.g, linear regression) was performed to explore the relationship between marker expression levels and patient outcomes and $p<0.05$ was considered significant.

RESULTS

The mean age of the patients is 30.74 years, with a standard deviation of 15.79 years, indicating a wide age range among the patients. The youngest patient is 13 years old, while the oldest is 75 years old. The study found that there are 67.16% females (45 patients) and 32.83% males (22 patients), indicating a higher representation of females in this patient group, with ratio (1:2) [Table 1].

Table 2 shows the hematological features of the patients including their hemoglobin (HB) levels, platelet counts, total white blood cell (WBC) counts, absolute neutrophil (NE), absolute lymphocyte (LY), and blast cell percentage.

Table 3 presents the distribution of positive and negative markers among the patients, as identified through Flow Cytometry (FCM) and Immunohistochemistry (IHC). All patients (67) were positive for CD10 using both FCM and IHC, indicating a 100% positivity rate with no negatives. For CD20, FCM also showed 100% positivity, while IHC revealed that 94.02% (63 patients) were positive and 5.33% (4 patients) were negative. Similarly, CD19 showed 100% positivity by FCM, but IHC indicated that 67.16% (45 patients) were positive and 29.34% (22 patients) were negative. CD34 was positive in 85.07% (57 patients) by FCM and 83.58% (56 patients) by IHC, with corresponding negative rates of 13.34% (10 patients) and 14.67% (11 patients). MPO exhibited 100% negativity with both FCM and IHC. For TDT, FCM showed 85.07% positivity (57 patients) and 13.34% negativity (10 patients), while IHC showed 91.04% positivity (61 patients) and 8% negativity (6 patients).

Both flow cytometry and immunohistochemistry demonstrated a 100% positive detection rate for CD10. CD20 showed a 100% positive detection rate with Flow Cytometry, but a slightly lower positive rate of 94.02% with Immunohistochemistry. The positive detection rate for CD19 was 100% with Flow Cytometry; whereas it was 67.16% with Immunohistochemistry. Flow Cytometry detected CD34 in 85.07% of cases, while Immunohistochemistry showed a slightly lower detection rate at 83.58%. Both Flow Cytometry and Immunohistochemistry did not detect MPO. TDT was detected in 85.07% of cases using Flow Cytometry and in 91.04% of cases using Immunohistochemistry (Fig. 1).

Table 4 critically evaluates various biomarkers using different diagnostic methods, specifically Flow Cytometry and Immunohistochemistry. For CD10, CD20, CD19, and TDT assessed by FCM, the results reveal a perfect sensitivity of 100% but a specificity of 0%. This indicates these markers are excellent at identifying true positives, with a high PPV of 90%, but fail to correctly identify true negatives, resulting in an overall accuracy of 90%. In contrast, the IHC method for CD20 demonstrates a more balanced performance with 93.65% sensitivity, 85.71% specificity, 98.36% PPV, 54.55% NPV, and an accuracy of 92.86%. The IHC method for CD19, with a high sensitivity of 98.41% and moderate specificity of 28.57%, achieves a PPV of 92.86% and an accuracy of 90.48%. The CD34 marker shows a similar pattern, with FCM yielding high sensitivity (98.41%) but low specificity (28.57%), resulting in 90.48% accuracy. In contrast, IHC for CD34 achieves lower sensitivity (80.95%) but higher specificity (85.71%), leading to an accuracy of 82.14%. MPO, evaluated by both methods, fails to detect malignancies effectively, with 0% sensitivity, specificity, PPV, NPV, and accuracy.

Table No. 1: General features of the patients

Variable	No.	%
Gender: Male	22	32.83
Female	45	67.16
Age (years)	30.74±15.79	

Table No. 2: Hematological characteristics of the patients

Parameter	Mean±SD
Hemoglobin	8.16±1.32
Platelets x 109 /L	40.74±21.32
WBC x 109 /L	12.74±10.37
NE %	12.50±6.43
LY %	41.79±14.47
Blast cell %	31.65±10.55

IHC identified 31 cases as positive (46.27%) and 36 cases as negative (53.73%). In terms of sensitivity, the IHC panel correctly identified 46.27% of the cases that were positive according to IHC, meaning it missed 53.73% of the positive cases, indicating low sensitivity. The specificity of the IHC panel was 53.73%, which implies it correctly identified negative cases 53.73% of the time, suggesting a moderate ability to avoid false positives (Table 5).

Table No. 3: Number of positives and negatives of IHC markers

IHC markers		No. Positives	%	No. Negatives	%
CD10	FCM	67	100	-	-
	IHC	67	100	-	-
CD20	FCM	67	100	-	-
	IHC	63	94.02	4	5.33
CD19	FCM	67	100	-	-
	IHC	45	67.16	22	29.34
CD34	FCM	57	85.07	10	13.34
	IHC	56	83.58	11	14.67
MPO	FCM	0	100	67	-
	IHC	0	100	67	-
TDT	FCM	57	85.07	10	13.34
	IHC	61	91.04	6	8

Table No. 4: Sensitivity, Specificity, PPV and NPV of the markers

Marker	Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CD10	FCM	100	0	90	N/A
CD10	IHC	100	0	90	N/A
CD20	FCM	100	0	90	N/A
CD20	IHC	93.65	85.71	98.36	54.55
CD19	FCM	100	0	90	N/A
CD19	IHC	98.41	28.57	92.86	66.67
CD34	FCM	98.41	28.57	92.86	66.67
CD34	IHC	80.95	85.71	98.15	30
MPO	FCM	0	0	0	0
MPO	IHC	0	0	0	0
TDT	FCM	100	0	90	N/A
TDT	IHC	90.48	28.57	92.31	25

Table No. 5: Panel Test for CD10, CD20, CD19, CD34, TDT

Result	+ve	-ve	Sensitivity	Specificity	NPV	PPV	Total
IHC Panel	31	36	46.27%	53.73%	53.73%	46.27%	67

DISCUSSION

This study explored the efficacy of various diagnostic methods for clonality detection in B-cell lymphoid malignancies. We specifically compared flow cytometry, Immunohistochemistry, to assess their diagnostic reliability. While FCM was confirmed as the gold standard, the integration of IHC helped further diagnosis, particularly in ambiguous cases. These findings highlight the advantage of employing multiple diagnostic tools in combination, rather than relying on a single method.[22,23]

In the present study, mean age was 30.74±15.79 years and 45 (67.16%) were females and 22 (32.83%) males. This sex distribution aligns with typical B-ALL trends, where females are more frequently diagnosed in younger age groups, while males show higher incidence rates in older age groups. Age is a significant factor in prognosis, as pediatric patients typically benefit from specialized treatment protocols that yield better outcomes, while adult patients face more challenges, particularly those aged over 45.[22] Our results reflect these age-related patterns in B-ALL.

Blood parameters, such as hemoglobin (Hb), neutrophil-to-lymphocyte ratio (NLR), and platelet counts, are increasingly recognized as important prognostic indicators in B-ALL. Low Hb levels, for instance, are associated with more aggressive disease, while an elevated NLR often correlates with poorer outcomes. Platelet abnormalities are common in B-ALL, serving as a marker of bone marrow dysfunction. Although these blood tests can signal potential leukemia, they are not sufficient for a definitive diagnosis and should be followed by more specific methods like FCM and IHC.[24]

The IHC panel used in this study comprising CD10, CD20, CD19, CD34, and TdT demonstrated moderate sensitivity and specificity for B-ALL diagnosis. Of the 67 cases, 31 tested positive (46.27%), and 36 were negative (53.73%). The panel's sensitivity of 46.27% means that it correctly identified less than half of the true positives, while its specificity of 53.73% indicates that it identified just over half of the negative cases correctly. While these results suggest that IHC may not be the most sensitive method, it remains valuable for excluding negative cases. It also provides a cost-effective alternative when more expensive tests like FCM are not accessible.

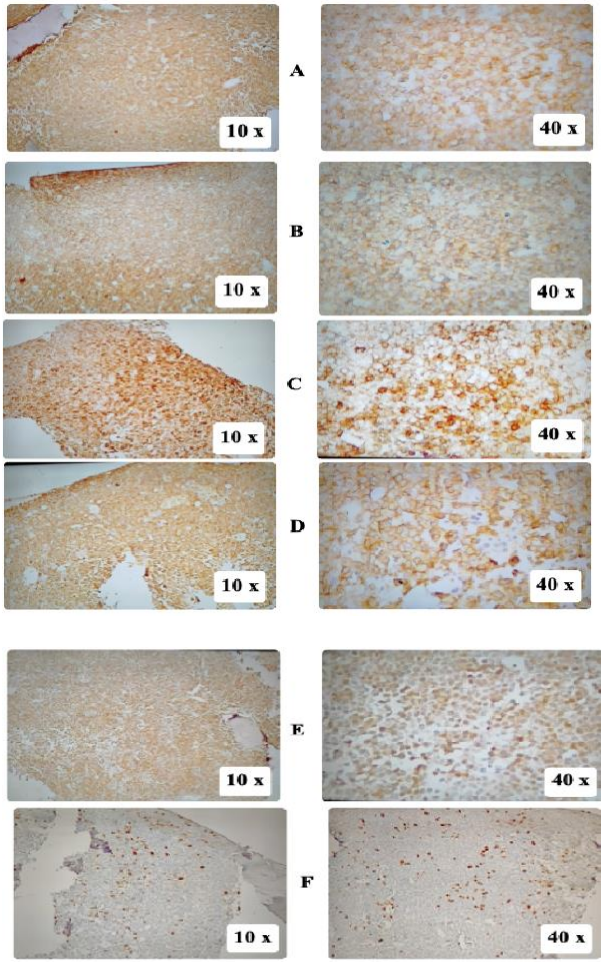

Figure No. 1: (A) Slides of Positive CD10, (B) Slides of Positive CD19, (C) Slides of Positive CD20, (D) Slides of Positive CD34, (E) Slides of Positive TDT, (F) Slides of negative MPO

IHC is supported by several studies, such as the work by Allen et al, which advocate for a streamlined IHC panel to reduce both costs and turnaround time, without compromising diagnostic accuracy.[25] Other research also supports the combination of various markers to improve sensitivity, particularly in cases where other tests are inconclusive.[26] The use of IHC in conjunction with other diagnostic techniques like FCM can offer more robust results and reduce misdiagnosis.

CONCLUSION

The use of 4-panel immunohistochemistry (CD10, 19, 20, TdT) is more suitable in the diagnosis of B-cell acute lymphoblastic leukemia when the flow cytometry method is unavailable or cost-related limitations occur.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Bahaulddin Hassan Abbood, Rahem Mahdy Rahem
Drafting or Revising Critically:	Kaswr Musa Jaafar Al tariahi
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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