Proposed minimal panel of antibodies for cost-effectiveness and accuracy in acute leukemias immunophenotyping: Prospective study at a tertiary care center

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Introduction: Flowcytometry has an essential role in the diagnosis and classification of acute leukemias. However, there exists a great degree of inter-laboratory variability on issues like panel selection, antibody combinations, gating strategies, fluorochromes, and clonal selection. Aims: The primary aim of this study was to derive a minimal panel of antibodies and evaluate its diagnostic usefulness in acute leukemias by flowcytometry by using the detailed immune-phenotype of different lineage-specific or non-specific markers.

Materials and methods: This prospective observational study involved 400 newly diagnosed cases of acute leukemias. Bone marrow aspirate samples were subjected to morphological evaluation, cytogenetics and flow cytometric immunophenotyping.

Results: A minimal panel of eight antibodies comprising of CD45/CD34/CD19/MPO/cytoCD3/CD64/CD117/CD79a was derived by applying different permutations and combinations with a diagnostic yield of 97.5%. The minimal panel was further validated by testing in an independent cohort of patients with similar demographic characteristics, where it showed a high diagnostic yield of 98% in comparison with the screening panels proposed by other recently published studies.

Conclusion: It may be concluded that the diagnostic performance of the eight antibody panel is better than most other panels used across the different laboratories in terms of yield, number of antibodies used and the scientific approach used to derive and validate the results and so henceforth may be applied in any setting with limited resources for better diagnostic accuracy.

Keywords: Immunophenotyping, Acute leukemia

Introduction

Immunophenotyping together with cyto-morphology is crucial for the detection and lineage assignment of blast cells in suspected samples, including the definition of acute leukemias of ambiguous lineage. Antibody panel selection is a critical step in specimen processing. However, there exists a great degree of inter-laboratory variability on issues like panel selection, antibody combinations, gating strategies, fluorochromes, and clonal selection. There is a need to formulate a minimal screening panel, which is cost-effective and provides an accurate diagnosis of acute leukemias, in most cases. Additional antibodies should be reserved for small percentage of patients who cannot be characterized with the limited panel.

Most consensus leukemia and lymphoma antibody panels consist of lists of markers based on expert opinions, but they have not been validated. The primary aim of this study was to obtain a minimal panel of antibodies and evaluate its diagnostic usefulness in acute leukemias by flowcytometry by using the detailed immune-phenotype i.e. the expression of different lineage-specific or non-specific markers in acute leukemias and further validating the results of the proposed panel in an independent cohort of acute leukemia patients.

Materials and methods

It was a prospective observational study in which 400 newly diagnosed cases of acute leukemias were enrolled in the Department of Hematology, All India Institute of Medical Sciences, New Delhi. Partially treated or relapsed patients of acute leukemia were
excluded from the study. The study was approved by the Institute’s Ethics Committee and written informed consent was obtained from the patients prior to the procedures.

Bone marrow (BM) aspirates from patients with clinical suspicion of acute leukemia because of clinical features, blood counts and peripheral blood picture were subjected to morphological evaluation, cytogenetics, and flow cytometric immunophenotyping. The morphological evaluation was performed and diagnosis of acute leukemia was made in accordance with WHO criteria, 2008. For each case, routine well-prepared Jenner-Giemsa-stained smears and cytochemical stains mainly myeloperoxidase (MPO), non-specific esterase (NSE) stains were evaluated. For flowcytometry, BM aspirate (0.5 ml) or peripheral blood (2 ml) samples were collected in EDTA and were processed using the standard stain-lyse-wash method. Sample to be taken in different tubes was based on the type of specimen received. In case of BM, 50 μl and in case of peripheral blood, 100 μl of sample was taken. (If the patient had high leukocyte count, amount was reduced.)

For all specimens, six-color flow cytometry was performed on BD FACScount™ (BD Biosciences) and analyzed using the inbuilt BD FACSDiva™ software. Comprehensive panel of antibodies was applied in all acute leukemia patients for making a definitive diagnosis. The antibodies primarily included CD19, CD7, CD117, HLA-DR, CD45, CD33, CD13, CD10, CD34, MPO, CD79a, cytoCD3 TDT, CD5, CD11c, and CD64. In selective cases where diagnostic dilemma persisted, few other antibodies were also applied such as CD41, CD61, CD2, cytoplasmic μ, CD22, CD4, CD8, CD1a, CD56, TCRαβ/γδ etc. [Table 1]. AML-M7 patients mostly yielded a dry tap, making way for use of immunohistochemistry (IHC) on BM biopsy for establishment of primary diagnosis so the existing immunophenotyping data may not be representative of its actual incidence.

Results
A total of 200 consecutive patients of acute leukemias were selected for formulation of the minimal panel of antibodies (Group 1), and an independent cohort of 200 patients were selected for validation of the results of the minimal panel (Group 2).

Group 1 analysis
The patient age ranged from 12 months to 78 years with the median age at presentation being 28 years. Majority of the patients were males (124/76); male to female ratio was 1.63:1.

The distribution of different subtypes of acute leukemias in the Group 1 and the percentage expression of antigens with at least dim marker positivity is shown in Table 2.

The primary criterion for antibody selection of the minimal panel was based on lineage-associated specificity and sensitivity of the recognized antigens. Keeping this in mind, those markers which were lineage-specific and showed least cross-lineage reactivity were chosen in the first list of antibodies, with gradual addition of more markers for improving the yield of samples (Table 3).

A minimal panel of eight antibodies comprising of CD45/CD34/CD19/MPO/cytoCD3/CD64/CD117/CD79a was derived by applying different permutations and combinations with a diagnostic yield of 97.5%. Only 5/200 patients required addition of expanded list of antibodies for establishing the final diagnosis such as CD10, CD11c, CD41, CD61,

Table 1 List of antibodies used in comprehensive panel for flowcytometric diagnosis of acute leukemias

| Antibodies    | Fluorochromes | Source |
|---------------|---------------|--------|
| CD45          | PerCP-Cy5.5   | BD     |
| CD19          | FITC/APC-H7   | BD     |
| CD10          | PE-Cy7        | BD     |
| CD13          | PE            | BD     |
| CD33          | APC           | BD     |
| HLA-DR        | PE            | BD     |
| CD117         | APC           | BD     |
| CD34          | PE-Cy7        | BD     |
| CD7           | FITC          | BD     |
| CD11c         | APC           | BD     |
| CD64          | FITC          | BD     |
| CD5           | APC           | BD     |
| cytoCD79a     | PE            | BD     |
| CytoCD3       | PE-Cy7        | BD     |
| MPO           | FITC          | BD     |
| TDT           | APC           | BD     |
| CD8           | APC           | BD     |
| CD4           | PE            | BD     |
| CD2           | FITC          | BD     |
| CD41          | FITC          | BD     |
| CD61          | PE            | BD     |

Table 2 Showing percentage of acute leukemias with at least dim marker positivity in Group 1 (n = 200)

| Antibodies | AML-M0/M7 | AML (Others) | T-ALL | B-ALL | MPAL |
|------------|-----------|--------------|-------|-------|------|
| CD19       | 6.67      | 04           | NIL   | 100   | 100  |
| CD10       | 26.67     | 45           | 90    | 92.8  | 60   |
| CD13       | 89        | 96           | 50    | 61.4  | 80   |
| CD33       | 80        | 97           | 30    | 28.6  | 80   |
| CD7        | 13.3      | 11           | 100   | 2.85  | NIL  |
| CD117      | 73.3      | 79           | 20    | 5.7   | 40   |
| CD34       | 100       | 86           | 80    | 95.7  | 100  |
| HLA-DR     | 100       | 79           | 100   | 100   | 100  |
| cytoCD79a  | 26.67     | 07           | 70    | 97.1  | 100  |
| MPO        | NIL       | 92           | NIL   | NIL   | 100  |
| cytoCD3    | NIL       | NIL          | 100   | NIL   | 100  |
| TDT         | 40        | 18           | 100   | 94.3  | 100  |
| CD11c      | NIL       | 33           | NIL   | NIL   | 100  |
| CD64       | NIL       | 37           | NIL   | NIL   | 20   |
| CD41/CD61  | 6.67      | NIL          | NIL   | NIL   | NIL  |
| CD5        | 20        | 02           | 70    | 1.42  | NIL  |
Table 3  Yield of the different combinations of antibodies in 200 patients of acute leukemia

| Antibody combinations | Accuracy/yield (in %) |
|-----------------------|-----------------------|
| CD45/CD19/CD34/cytoCD3/ MPO | 84.5%  
[70/70 (100%) B-ALL+ 10/ 10(100%) T-ALL+ 89/ 100(89%) AML-Others] |
| CD45/CD19/CD34/cytoCD3/ MPO/ CD64 | 88%  
[70/70 (100%) B-ALL+ 10/ 10(100%) T-ALL+ 96/ 100(96%) AML-Others] |
| CD45/CD19/CD34/cytoCD3/ MPO/ CD64/CD117 | 93.5%  
[70/70 (100%) B-ALL+ 10/ 10(100%) T-ALL+ 96/ 100(96%) AML-Others + 11/15(73.3%) AML-M0] |
| CD45/CD19/CD34/cytoCD3/ MPO/CD64/CD117/cytoCD79a | 97.5%  
[70/70 (100%) B-ALL+ 10/ 10(100%) T-ALL+ 11/ 15(73.3%) AML-M0+ 5/ 5(100%) MPAL+ 99/ 100(99%) AML-Others] |

*Seven patients of AMLs with monocytic differentiation which were MPO negative were picked up. They were confirmed by using other Monocytic markers such as CD11c, NSE etc.  
*11/15 (73.3%) AML-M0 were CD34+/CD117+/CD19−/ cytoCD3−/ MPO−/CD64−.  
*15/5 MPAL patients showed CD19+/cytoCD79a+/MPO−.  
3/100 AML (Others) with aberrant CD79a were confirmed in the absence of CD79a. Diagnosis in these patients was further confirmed by application of additional markers such as CD10 and cytoCD22.

CytoCD22, CD13, CD33 etc. These five patients included the three patients with AML-M0 immunophenotype (CD117−/CD13+ or C117−/CD33+), one patient with acute megakaryoblastic leukemia (CD41+/CD61+) and one CD19+ patient with MPO positivity found on cytochemistry. Various other permutations and combinations were explored to evaluate for the accuracy of results and co-linearity of antibodies. However, the yield did not improve further with the addition of more markers.

It should be noted that six patients (6/100) of Acute Promyelocytic Leukemia (APML) incorporated in this study showed strong MPO positivity both by cytochemistry and immunophenotyping, classical tear-drop pattern on CD45/SSC scatter plot and all of them were HLA-DR negative. However, the diagnosis of APML was confirmed only by molecular analysis for PML-RARA fusion product or cytogenetic studies exhibiting APML translocations such as t(15;17), t(11;17) or t(5;17) and flowcytometry served only as an adjunct in establishing the final diagnosis.

The minimal panel of eight antibodies was further validated by testing in an independent cohort of 200 patients with similar demographic characteristics. The patients’ age ranged from 6 months to 67 years with the median age at presentation being 29 years. Male to female ratio was 1.1:1. The validation group (Group 2) comprised of 25% B-ALL, 12% T-ALL, 04% AML-M0, 50% AML-Others, 08% MPAL and 01% Undifferentiated leukemia cases. When applied to the validation group (Group 2), the diagnostic yield of the minimal panel of eight antibodies was found to be 98%. The remaining four cases where additional markers were required for establishing the diagnosis included one patient with Mature B-ALL (CD20+/CD3−/Surface Immunoglobulin+, Burkitt morphology), another patient with surface CD3+/TCRαβ+/CD4+/cytoCD3+ mature T-cell neoplasm (T T-Prolymphocytic Leukemia) and two patients with Mixed-Phenotype Acute Leukemia (a) MPO−/CD19+/CD79a+ but MPO positive by cytochemistry and (b) CD19+/CD79a+/MPO−/CD11c+/NSE+ by cytochemistry).

When the final combination of eight antibodies in the minimal panel was compared with the screening panels recommended by recently published studies by Haycocks et al.3 and Dongen et al.4 as shown in Table 4, it was found to be significantly efficacious in establishing the final diagnosis in the validation group (98% vs. 96% and 91.5%, respectively). Most other recommendations discourage the use of cytoplasmic markers and rely on 10 or more surface antibodies for preliminary grouping of acute leukemias.

**Discussion**

Immunophenotyping requires careful selection of unique combinations of individual markers based on their degree of specificity for the identification of a given cell lineage, maturation stage and aberrant phenotype, as well as the selection of appropriate antibody clones and fluorochrome conjugates to be used in multicolor combinations.4 Multiple consensus panels have been proposed in the last two decades,3-9 but they typically included largely overlapping lists of cluster of differentiation...
(CD) markers per disease category. Virtually all consensus proposals lack information about reference antibody clones for the proposed CD markers and they only provide limited information on the most appropriate combinations of relevant markers in multicolor antibody panels for immunophenotypic diagnosis and classification of hematological malignancies. Moreover, neither of these recommendations have validated their results nor have they incorporated the lineage-specific cytoplasmic markers in their panels.

This present study resulted in validated and flexible eight antibody minimal panel for multidimensional identification and characterization of normal and aberrant cells, optimally suited for immunophenotypic screening and classification of hematological malignancies. The proposed panels were initially designed based on the experience and knowledge accumulated in the literature as well as in the institutional laboratory.

The criteria for antibody selection in order to optimally orientate the acute leukemia sample were based on lineage-associated specificity and sensitivity of the recognized antigens. An ideal orientation marker is constantly expressed by all cells of a single lineage and shows no cross-lineage reactivity.

Based on previous reports and on our knowledge and experience, all potentially valuable markers were considered. These included, among others, CD7, CD5, CD10, CD13, CD19, CD33, CD117, nuclear TdT, HLA-DR, CyCD3, CyCD79a, CyMPO etc. As lineage commitment has to be assessed by flow cytometry on well-identified immature cells, markers that could contribute to the definition of immaturity and the identification of blast cells were considered independently (for example, CD34, CD45, CD117, and TdT).

The proposed minimal eight antibody panel that showed 97.5% diagnostic yield in the Group I of 200 acute leukemia patients was then prospectively applied to an independent cohort of 200 freshly collected acute leukemia cell samples for validation of results. Analysis of the 100 well-characterized B-ALL, T-ALL and AML cases, including AUL/MPAL samples, highlighted excellent discrimination of B-ALL from both AML and T-ALL (97% cases were diagnosed correctly). Consequently, the few cases that could not be characterized by the minimal panel require further evaluation with comprehensive antibody panels for complete characterization.

Only one study by Dongen et al. 2012 has till date validated their eight antibody panel similar to the present study where they showed an unprecedented orientation efficiency of 98.3% for non-ambiguous lineage cases in their series of 483 newly diagnosed acute leukemia cases, tested prospectively at different centers. However, it was observed that the eight antibody panel proposed in our study performed better in our patients (Group 2) than the panel proposed by Dongen et al. (98% vs. 91.5%). Moreover, the diagnostic accuracy of the 10-antibody panel proposed by Haycocks et al. was comparable to ours (96%) in terms of diagnostic efficacy but they used more number of antibodies. There is only one study published from India by Gujral et al. touching upon the issue of cost-effective screening panels in which they have proposed a 10-antibody minimal screening panel comprising of CD10, CD19, CD7, CD5, CD13, CD33, CD117, CD34, HLA-DR, CD45. However, they have not used lineage-specific cytoplasmic markers in their panel nor have they validated their results. Moreover, when we applied their panel in our patients, the diagnostic yield did not improve either.

This study also re-instates the fact that immune-phenotyping should never be performed in isolation. Morphology and cytochemistry are useful adjuncts to correct diagnosis of acute leukemias due to the fact that there may be discrepancy in the expression of MPO by cytochemistry, flowcytometry or IHC and also variation in the antigenic profile of monocytic leukemias requiring at least two out of these five markers such as CD14, CD64, NSE, Lysozyme or CD11c for confirming the lineage. Moreover, in less frequent acute leukemias such as AML-M6/M7, Early-T-Precursor ALL and undifferentiated leukemias etc., application of the proposed minimal panel or other existing panels worldwide yielded less convincing results and so use of additional antibodies is recommended in such cases.

Conclusions
From these observations it can be stated clearly that the diagnostic performance of the eight antibody panel is better than most other panels used across the different laboratories in terms of yield, number of antibodies used and the scientific approach used to derive and validate the results and so henceforth may be applied in any setting with limited resources for better diagnostic accuracy. It needs to be mentioned herein that the classification efficiency using the proposed antibody combination remains to be assessed with a larger independent validation cohort and will also require collection of a vast number of acute leukemia cases in order to achieve relevant characterization. Altogether, the minimal panel with an integrated analysis of only eight markers appeared to be an unprecedentedly strong tool for acute leukemia diagnosis.
Disclaimer statements
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