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Applicability of a Single 5 Color Cytoplasmic Markers Tube as Primary Panel in Routine Immunophenotyping of Acute Leukemia

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Abstract

Background: Immunophenotyping in acute leukemia is a routine practice for lineage assignment. Conventionally a primary panel with surface markers are applied first followed by cytoplasmic markers as secondary panel in the diagnosis of acute leukemia.We in this present study aim to assess the relevance of a single 5 color "CD45, MPO, CD79a, CD3, Tdt" cytoplasmic markers combination to be utilized as primary panel.

Methods: Sensitivity and specificity of different subset of positive negative combination of these markers were retrospectively analyzed in the 458 acute leukemia cases.

Results: MPO or cCD3 positivity alongwith cCD79a negativity was 100% specific diagnosis for AML and T-ALL respectively. Furthermore, cCD79a positivity along with MPO and cCD3 negativity was 97.2% specific for B-ALL diagnosis. MPO and cCD79a dual positivity was found 100% sensitive and 92.6% specific for MPAL (B-My) diagnosis. MPO and cCD3 dual positivity was 100% sensitive and specific for MPAL (T-My) diagnosis.

Conclusion: We found a good correlation of this single tube diagnosis when compared with standard morphology, cytochemistry, and flow cytometry based diagnosis. We hope our this cytoplasmic panel may help to design a precise extended immunophenotypic panel for acute leukemia diagnosis and may also be a cost effective approach in resource constrained developing countries.

Keywords: Acute leukemia; Flow cytometry; Immunophenotyping; Cytoplasmic antigens

Introduction

Immunophenotypic diagnosis of acute leukemia is performed based on a panel of lineage associated markers including cell surface as well as cytoplasmic antigens [1-3]. The standard approach is to apply first, primary panel of markers with cell-surface antigens then after secondary panel of markers with cytoplasmic antigens [3-5]. However, multiple reports have shown that cytoplasmic markers are the earliest identifiable marker of a particular cell development and they come on cell surface later [3,6,7]. Prior Information of cytoplasmic markers expression in the leukemic blasts may direct to apply a well systematic and extended secondary panel for accurate acute leukemia diagnosis. Such type of reports are very little available and nil in Indian Territory. We in this present study tried to investigate the diagnostic relevance of a single 5 color tube containing the markers MPO, cCD79a, cCD3, TdT (along with CD45-gating marker) as primary panel for acute leukemia immunophenotyping by comparing its sensitivity and specificity with standard morphology, cytochemistry and flow cytometry based diagnosis.

Materials and Methods

This study was conducted in the Department of Hematology, All india Institute of Medical Sciences, New Delhi, India during the period of January 2011 to December 2012. All the 458 cases of acute leukemia i.e ALL (B-ALL and T-ALL), AML and MPAL consecutively enrolled in this two year period were included in the study. All the cases were enrolled only after getting their written consent. Diagnosis of acute leukemia was based on morphological examination of bone marrow aspirate smears, including cytochemistry along with full panel of flow cytometric immunophenotyping. Almost of the samples received in our laboratory were bone marrow aspirate which were processed using a standard stain-lyse-wash method. All the samples were processed within 24 hour of collection. The monoclonal antibodies used in full panel were: CD45 (PerCP-Cy5.5), CD13 (PE), CD33 (APC), CD10 (PE-Cy7), CD19 (FITC), CD7 (FITC), CD117 (APC), CD34 (PE-Cy7), HLA-DR (PE), MPO (FITC), c CD79a (PE), c CD3 (PE-Cy7), Tdt (APC), CD64 (FITC) and CD11c (APC). The cytoplasmic tube contained CD45 as gating marker and MPO, CD3, CD79a, Tdt. All of these monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). Initial incubation of monoclonal antibodies for 15–30 min at room temperature in the dark followed by lysis and washing of debris/unlysed RBCs were performed and samples were acquired using a BD-FACS Canto (6 color, 2 LASER) system. For the cytoplasmic tube an additional step by incubating it with permeablizing solution (BD Biosciences) was carried out to render the easy passage of intracellular markers in cytoplasm of cells. An unstained control tube without adding any monoclonal antibody was prepared to set the quadrant for autofluorescence exclusion. All analyses and interpretation were carried out using the FACS-Diva software (BD Biosciences).

Results

Out of the total 458 acute leukemia patients recruited, 222 were diagnosed as AML, 176 as B-ALL, 44 as T-ALL and 16 as MPAL by standard morphology, cytochemistry and immunphenotyping based criteria. We retrospectively analyzed the applicability of various intracellular combinations of immunophenotypic markers for independent diagnosis compared with that of final combinatorial diagnosis. The 13 different intracellular immune phenotypic subset were made based on positive and negative status of each marker (Table 1).

Since we did not see positive or negative status of Tdt playing any crucial role in the diagnosis, we further shortlisted this Table by ignoring the Tdt status (Table 2). These 6 different subsets of cytoplasmic markers positive negative status as found in Table 2 were comprehensively examined separately for their diagnostic utility in AML, B-ALL, T-ALL, MPAL (B-My), MPAL (T-My) diagnosis.

S.N	Intracellular Immunophenotypic expression subset	AML (n=222)	B-ALL (n=176)	T-ALL (n=44)	MPAL	
					B-My (n=14)	T-My (n=2)
1	MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^-$	126 (56.7)	0 (0)	0 (0)	0 (0)	0 (0)
2	MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^+$	52 (23.4)	0 (0)	0 (0)	0 (0)	0 (0)
3	MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^+$	9 (4.0)	0 (0)	0 (0)	9 (64.2)	0 (0)
4	MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^-$	9 (4.0)	0 (0)	0 (0)	5 (35.7)	0 (0)
5	MPO$^-$ cCD79$^+$ cCD3$^-$ Tdt$^-$	8 (3.6)	21 (11.9)	0 (0)	0 (0)	0 (0)
6	MPO$^-$ cCD79a$^+$ cCD3$^-$ Tdt$^+$	0 (0)	153 (86.9)	0 (0)	0 (0)	0 (0)
7	MPO$^-$ cCD79a$^-$ cCD3$^-$ Td^t	12 (5.4)	0 (0)	0 (0)	0 (0)	0 (0)
8	MPO$^-$ cCD79$^-$ cCD3$^-$ Tdt$^+$	6 (2.7)	02 (1.1)	0 (0)	0 (0)	0 (0)
9	MPO$^-$ cCD79a$^-$ cCD3$^+$ Tdt$^+$	0 (0)	0 (0)	11 (25)	0 (0)	0 (0)
10	MPO$^-$ cCD79$^+$ cCD3$^+$ Tdt$^+$	0 (0)	0 (0)	17 (38.6)	0 (0)	0 (0)
11	MPO$^-$ cCD79a$^+$ cCD3$^+$ Tdt$^-$	0 (0)	0 (0)	8 (18.1)	0 (0)	0 (0)
12	MPO$^-$ cCD79$^-$ cCD3$^+$ Tdt$^-$	0 (0)	0 (0)	8 (18.1)	0 (0)	0 (0)
13	MPO$^+$ cCD79a$^-$ cCD3$^+$ Tdt$^+$	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)

Table 1: Different subset of tubes with variable positive negative expression of cytoplasmic markers.

S.N.	Intracellular Immunophenotypic expression subset	AML (n=222)	B-ALL n=176	T-ALL (n=44)	MPAL	
					B-My (n=14)	T-My (n=2)
1	MPO$^-$ cCD3$^+$ cCD79a$^{+/-}$ Tdt$^{+/-}$	0 (0)	0 (0)	44 (100)	0 (0)	0 (0)
2	MPO$^-$ cCD79a$^+$ cCD3 Tdt$^{+/-}$	8 (3.6)	174 (98.8)	0 (0)	0 (0)	0 (0)
3	MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$	178 (80.1)	0 (0)	0 (0)	0 (0)	0 (0)
4	MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^{+/-}$	18 (8.1)	0 (0)	0 (0)	14(100)	0 (0)
5	MPO$^-$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$	18 (8.1)	2 (1.1)	0 (0)	0 (0)	0 (0)
6	MPO$^+$ cCD79a$^-$ cCD3$^+$ Tdt$^{+/-}$	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)

Table 2: Different subset of cytoplasmic tubes with variable positive negative expression after ignoring Tdt status. Subset-1 had 100% sensitivity and 100% specificity for T-ALL diagnosis; Subset-2 had 98.8% sensitivity and 97.2 % specificity for B-ALL diagnosis; Subset-3 had 80.1% sensitivity and 100 % specificity for AML diagnosis; Subset-5 had 8.1% sensitivity and 99.1 specificity for AML diagnosis; Subset-3&5 in combination had 88.3% sensitivity and 99.1% specificity for AML diagnosis; Subset-4 had 100% sensitivity and 92.6% specificity for MPAL (B-My) diagnosis; Subset-6 had 100% sensitivity and 100% specificity for MPAL (T-My) diagnosis.

AML subgroup

Out of total 222 AML patients diagnosed, 196 (88.3%) were positive for MPO, rest 26 (11.71%) were negative for MPO. AML with MPO positivity exhibited 4 different expression subset of intracellular markers. MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^-$ expression subset was detected in 126 (56.7%) cases which was nil in all other cases. MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$ subset was detected in 52/ 222 (23.4%) which was again nil for all other cases. MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^+$ subset was detected in 9 (4%) cases which was nil in B-ALL, T-ALL, MPAL (T/My) but was detected in 9 (64.2%) cases of MPAL (B/My). MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt- subset was found in 9 (4%) AML cases which was nil in B-ALL, T-ALL, MPAL (T/My) but detected in 5 (35.7%) cases of MPAL (B/My) also.

MPO negative subgroup of AML exhibited 3 different expression subset of intracellular markers. MPO$^-$ cCD79$^+$ cCD3$^-$ Tdt$^-$ subset was detected in 8 (3.6%) cases of AML which was also expressed by 21 (11.9%) B-ALL but absent in T-ALL or MPAL. MPO$^-$ cCD79a$^-$ cCD3$^-$ Tdt subset was detected in 12 (5.4%) AML which was nil in all other cases. MPO$^-$ cCD79$^-$ cCD3$^-$ Tdt$^+$ was detected in 6 (2.7%) AML cases, which was also expressed by 2 (1.1%) B-ALL and nil in T-ALL and MPAL.

Total 67 (30.45%) of AML patients were found to have Tdt co-expression and 26 (11.81%) with cCD79a co-expression. 100% of the AML were negative for cCD3 co-expression. Tdt expression was found non-contributory for myeloid lineage determination. 18 (8.10%) AML cases had MPO positivity along with cCD79a co-expression which was also seen in all 14 (100%) cases of MPAL (B-My) thus making their discrimination difficult. cCD79a co-expression was detected in 8 (3.63%) AML patients having MPO negativity which overlapped with 21 (11.9%) cases of B-ALL.

By ignoring the positive/negative status of Tdt, 4 different subsets of intracellular expression could be achieved. MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$ expression subset was 80.1% sensitive and 100% specific for AML diagnosis. MPO$^-$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$ subset was 8.1% sensitive and 99.1% specific. MPO$^-$ cCD79a$^+$ cCD3 Tdt$^{+/-}$ subset was 3.6% sensitive and 26.3% specific. MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^{+/-}$ subset was 8.1% sensitive and 85.38% specific. Combination of these 4 subsets had 100% sensitivity but only 25.42% specificity for AML diagnosis. However, an intracellular combination of first two subsets only i.e MPO$^+$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$ and MPO$^-$ cCD79a$^-$ cCD3$^-$ Tdt$^{+/-}$ together had 88.3% sensitivity and 99.15% specificity for AML and cases diagnosed by this were found concordant with final combinatorial morphology, cytochemistry and immunophenotyping based diagnosis.

B-ALL subgroup

In the total 176 B-ALL patients, 3 different intracellular subset of immunophenotypic expression could be detected. MPO$^-$ cCD79$^+$ cCD3$^-$ Tdt$^-$ expression was detected in 21 (11.9%) of B-ALL, 8 (3.6%) of AML and nil in T-ALL and MPAL subgroup. MPO$^-$ cCD79a$^+$ cCD3$^-$ Tdt$^+$ expression was detected in 153 (86.9%) of B-ALL which was nil for all other cases. MPO$^-$ cCD79$^-$ cCD3$^-$ Tdt$^+$ expression was found in 2 (1.1%) B-ALL, 6 (2.7%) AML and nil in T-ALL and MPAL. These 3 intracellular expression subset in combination had 100% sensitivity and 89.36% specificity to detect B-ALL. cCD79a expression was found highly sensitive as 98.9% B-ALL cases expressed it. Tdt expression had 88.06% sensitivity in B-ALL. All of the B-ALL cases were negative for MPO as well as cCD3. By ignoring the positive/negative status of Tdt, a

single intracellular combination with MPO$^-$ cCD79a$^+$ cCD3 Tdt$^{+/-}$ was found to have 98.8% sensitivity and 97.2% specificity for the diagnosis of B-ALL.

T-ALL subgroup

4 different intracellular immunophenotypic expression subsets were seen in the total 44 T-ALL patients. MPO$^-$ cCD79a$^-$ cCD3$^+$ Tdt$^+$ expression was detected in 11 (25%) T-ALL which was nil in all other cases. MPO$^-$ cCD79$^+$ cCD3$^+$ Tdt$^+$ was detected in 17 (38.6%) T-ALL and nil in all other cases. MPO$^-$ cCD79a$^+$ cCD3$^+$ Tdt$^-$ subset was detected in 8 (18.1%) T-ALL and nil in all other cases. MPO$^-$ cCD79$^-$ cCD3$^+$ Tdt$^-$ expression was found in 8 (18.1%) T-ALL which was again nil in all other cases. Combination of these 4 different subsets had 100% sensitivity and 100% specificity for diagnosis of T-ALL. cCD3 expression was detected in 100% cases of T-ALL and its co-expression could not be seen anywhere else. Tdt was found co-expressed in 63.6% and cCD79a in 56.8% cases of T-ALL. By ignoring positive/negative status of cCD79a and Tdt, a distinctive immunophenotypic subset of MPO$^-$ cCD3$^+$ cCD79a$^{+/-}$ Tdt$^{+/-}$ independently had 100% sensitivity as well as 100% specificity for T-ALL diagnosis.

MPAL subgroup

Out of total 458 acute leukemia cases recruited, 16 (3.5%) cases were diagnosed as Mixed phenotypic acute leukemia (MPAL) and 14 (87.5%) among them were MPAL (B-My) and 2 (12.5%) were MPAL (T-My). In the 14 MPAL (B-My) subgroup, two immunophenotypic subsets were found. MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^+$ expression subset was detected in 9 (64.2%) MPAL (B-My), and also in 9 (4%) AML, but nil in all other cases. MPO+ cCD79a+ cCD3- Tdt expression subset was detected in 5 (35.7%) MPAL (B-My) cases, and also in 9 (4%) B-ALL, but nil in all other cases. We found expression of Tdt in 9 (64.2%) and cCD79a in 100% MPAL (B-My) cases. By ignoring the positive/negative status of Tdt, a single intracellular subset with MPO$^+$ cCD79a$^+$ cCD3$^-$ Tdt$^{+/-}$ immunophenotype had 100% sensitivity and 92.6% specificity for MPAL (B-My) diagnosis. In the total 2 MPAL (T-My) patient subgroup, both cases had the identical intracellular expression subset i.e MPO$^+$ cCD79a$^-$ cCD3$^+$ Tdt$^+$ which had 100% sensitivity as well as 100% specificity for MPAL (T-My) diagnosis.

Discussion

Flow cytometry based immunophenotpying plays a crucial role in accurate classification and diagnosis of acute leukemia and has become a routine tool in hematologic laboratories worldwide [1-7]. An extensive panel of cell surface as well as intracellular CD markers are required for precise lineage assignment and subtyping of acute leukemia. Intracellular immunophenotypic markers are reported to be expressed at earliest phase of disease and holds a remarkable specificity for myeloid and lymphoid lineage determination in acute leukemia blasts [3,6,7]. Conventional practice is to use cell-surface markers as primary panel followed by cytoplasmic markers in second line to establish the diagnosis of acute leukemia [3-5]. Increasing number of the laboratories now a days are rapidly transforming from 3-4 to 5-6 color flow cytometry. We in this present study tried to attempt the applicability of a single 5 color cytoplasmic marker tube of the combination MPO, cCD79a, cCD3, TdT as primary panel and correlated the diagnosis made by this tube with that of final diagnosis made based on standard combinatorial morphology, cytochemistry and flow cytometry. By having multiple positive-negative combinations of all the cytoplasmic markers as demonstrated in Table

2, we found its higher degree of correlation with the final diagnosis. MPO or cCD3 positivity alongwith cCD79a negativity heralded 100% specific diagnosis of AML and T-ALL respectively. Furthermore, cCD79a positivity along with MPO and cCD3 negativity was 97.2% specific for B-ALL diagnosis. a single intracellular combination with $MPO^- cCD79a^+ cCD3^- Tdt^{+/-}$ was found to have 98.8% sensitivity and 97.2% specificity for the diagnosis of B-ALL. A single intracellular combination of $MPO^- cCD3^+ cCD79a^{+/-} Tdt^{+/-}$ independently had 100% sensitivity as well as 100% specificity for T-ALL diagnosis. A single tube of $MPO^+ cCD79a^- cCD3^- Tdt^{+/-}$ expression subset was 80.1% sensitive and 100% specific for AML diagnosis. MPO negative AML were little a bit tough to assign, although negativity of all the cytoplasmic markers was observed 99.1% specific for AML diagnosis. MPO and cCD79a dual positivity was found 100% sensitive and 92.6% specific for MPAL (B-My) diagnosis and a single tube of $MPO^+ cCD79a^+ cCD3^- Tdt^{+/-}$ immunophenotype had 100% sensitivity and 92.6% specificity for MPAL (B-My) diagnosis. Similarly, MPO and cCD3 dual positivity was 100% sensitive and specific for MPAL (T-My) diagnosis as a single tube with $MPO^+ cCD79a^- cCD3^+ Tdt^+$ combination had 100% sensitivity as well as 100% specificity for MPAL (T-My) diagnosis.

Since at the very immature stage of development the antigens are expressed in cytoplasm first then after they manifest to the cell surface a primary panel for lineage affiliation and then secondary panel to determine degree of cell differentiation is standard approach for acute leukemia immunophenotyping [3-7]. Studies have indicated intracellular expression of MPO, cCD3, cCD79a and cCD22 are the earliest expressing myeloid, B and T cell markers [8-13]. Some studies have also reported that CD19 is not the first differentiation antigen in human B-cell development [14,15]. B-cells were shown to be grown from a progenitor pool with $CD19^- CD34^+ CD10^+$ cells which support the concept of immaturity markers gaining at the earliest stage [10]. Immunophenotypic features in early ($CD34^+ TDT^+$) lymphoid cells have demonstrated that many of these precursors which lack CD19, they already express cCD79a in combination with CD10 [16]. These types of reports led us to comprehensively evaluate the diagnostic relevance of these cytoplasmic markers as primary panel in acute leukemia immunophenotyping. Our results are in support of these reports as our 98.9% B-ALL cases expressed CD79a and 100% T-ALL cases expressed cCD3 (Table 3). MPO expression in AML was seen in 100% of AML after excluding MO, M5 and M7 subtype which are known to characteristically lack it (Table 3). Similar finding were also reported by Drach et al. [17]. Tdt expression as immaturity marker was rather confounding and did not help much in lineage assignment. Our results strongly support that lineage specificity in acute leukemia at a greater extent can be determined by cytoplasmic antigens of the cells and their application as primary panel in routine immunophenotyping laboratories may be an attractive approach of implementation, although pertaining the necessity of full cell surface marker combination for the confirmation and proper classification. Our 5 color cytoplasmic markers based combination may also help a lot as cost-effective method of leukemia diagnosis in the resource-constrained developing countries.

Diagnosis	Total no. of cases	MPO	cCD79a	cCD3	Tdt
AML	222	196 (88.3%)	26 (11.71%)	0 (0%)	67 (30.45%)
B-ALL	176	0 (0%)	174 (98.9%)	0 (0%)	155 (88.1%)
T-ALL	44	0 (0%)	25 (56.81%)	44 (100%)	28 (63.64%)
MPAL (B-My)	14	14 (100%)	14 (100%)	0 (0%)	9 (64.2%)
MPAL (T-My)	2	2 (100%)	0 (0%)	2 (100%)	2 (100%)

Table 3: Frequency of different cytoplasmic markers in their particular lineages.

Conclusion

In nutshell, we in the present study evaluated the applicability of a single 5 color cytoplasmic tube based methodology to use it as primary panel in routine acute leukemia immunophenotyping. We found a good correlation of this single tube based diagnosis with final diagnosis made on the basis of standard morphology, cytochemistry and flow cytometry assessment. We assume this single tube based methodology will be very helpful in designing needful secondary extended panel protocol for precise diagnosis of acute leukemia and could also be particularly useful as cost-effective approach in the resource constrained countries.

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