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ROLE OF CYTOPLASMIC MARKERS AS PRIMARY PANEL FOR IMMUNOPHENOTYPING OF ACUTE LEUKEMIA IN PEDIATRIC POPULATION- A SINGLE CENTRE STUDY

Oncology

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ABSTRACT

INTRODUCTION: Flow cytometry is highly sensitive for detection and quantitative analysis of surface and intracellular antigens in malignant hemopoieticcells and is used for classification and lineage assignment of acute leukemia. AIM- To identify the role of a single 5 colour, CD45, myeloperoxidase (MPO), cCD79a, cCD3, and Tdt, cytoplasmic markers combination as a primary tube and comparison with final diagnosis done on the basis of morphology and primary and secondary panels in immunophenotyping.

MATERIALS AND METHODS: We have included 200 new cases of pediatric acute leukemia. We analyzed sensitivity and specificity of different subsets with combination of positive and negative markers.

RESULTS: MPO was positive in 76.9% of acute myeloid leukemia (AML) cases. All 39 (100%) cases of the AML were negative for cCD3 andcCD79a co-expression. cCD79a expression was highly sensitive as 97.7% B-acute lymphoblastic leukemia (B-ALL) expressed it. cCD3 expression was detected in 100% cases of T-ALL, and its co-expression was not seen in B-ALL and AML.

CONCLUSION: Our study indicates that there was very good correlation of 5-color cytoplasmic tube-based diagnosis versus final diagnosis based on morphology, cytochemistry, and flow cytometry. We can use this 5-color cytoplasmic tube method to make immunophenotyping cost-effective.

KEYWORDS

Acute leukemia, cytoplasmic antigens, flow cytometry

INTRODUCTION

Acute leukemias are a heterogeneous group of malignancies with varying clinical, morphological, immunological, and molecular characteristics.[1] Leukemias account for 0.15–0.6% of the total medical admissions in many general hospitals in India.[2] Acute myeloid leukemia (AML) accounts for approximately 20% of acute leukemia in children and 80% acute leukemia in adults.[3] Immunophenotypic panel of acute leukemia based on as panel of lineage-related markers including cell surface and cytoplasmic markers. Many studies have shown cytoplasmic markers are the earliest identifiable marker of a particular cell development and later come on surface of the cell.[4-8] Several advances in flow cytometry, including availability of new monoclonal antibodies, improved gating strategies, and multiparameter analytic techniques, have all improved the utility of flow cytometry in the diagnosis and classification of leukemia.[9] Morphological diagnosis of acute leukemia may be incorrect up to 9% in comparison to flow cytometry.[10] Myeloperoxidase (MPO) detection is probably the most specific technique for differentiating myeloid from lymphoid antigens.[11] The current study tried to identify the role of the flow cytometry study of acute leukemias by a single 5 colour cytoplasmic markers tube, containing the markers MPO, cCD79a, cCD3, and Tdt (along with CD45-gating marker) as primary panel of acute leukemiaimmunophenotyping. We also compared sensitivity and specificity of the markers with the final diagnosis based on morphology, cytochemistry, and flow cytometry

MATERIALS AND METHODS

This was a prospective study which was conducted in the Department of Pathology, Gujarat Cancer and Research Institute, Ahmedabad, India, from September 2016 to October 2018. 200 cases of paediatric acute leukemia, i.e., acute lymphoblastic leukemia (ALL: B-ALL and T-ALL), AML, and mixed phenotypic acute leukemia (MPAL) were enrolled in this 2-year period.

Sample collection and preparation

The bone marrow or peripheral blood was collected in ethylenediaminetetraacetic acid vacutainer for peripheral smear examination and immunophenotyping. A morphological evaluation was done from the Wright-stained peripheral smears and bone marrow aspirates using French–American–British classification of acute leukemias. Special relevant cytochemical stains were performed on the bone marrow aspirates in all cases. Final diagnosis of acute leukemia was based on morphological examination, cytochemistry along with full panel of flow cytometric immunophenotyping. All the samples were processed within 24 hours.

Multicolour monoclonal antibody combination

The monoclonal antibodies used in the primary panel were CD45 (PerCP), CD22 (FITC), CD34 (PE), CD5 (PE Cy7), CD10 (APC), CD19 (APC-H7), CD7 (FITC), CD13 (PE), CD33 (PE Cy7), CD117 (APC), HLA-DR (APC- H7), MPO (FITC), cCD79a (PE), cCD3 (PE Cy7), and Tdt (APC) and in the secondary panel were CD11b (PE Cy7), CD11c (PE), CD14 (APC-H7), CD15 (FITC), CD2 (FITC), CD4 (PE Cy7), CD8 (APC-H7), CD1a (PE), CD41a (PE), CD41b (FITC), and CD61 (FITC). The CD45 was used for blast gating for both surface and cytoplasmic markers. The antibodies were procured from BD Biosciences, USA.

Flow cytometric immunophenotyping

For surface markers, respective antibody (20 µl) mentioned above was added in six-colour combination to the bone marrow or peripheral blood (100 µl, 1 × 106) and incubated for 15 min. After incubation, 2 ml of erythrocyte lysing solution (1:10 dilution with double distilled water; BD Biosciences, USA) was added and incubated for 15 min at room temperature. Then, cells were centrifuged at 400 *g* for 5 min and supernatant was discarded. Remaining pellet was washed twice with phosphate -buffered solutions (PBS) and then resuspended in 500 µl of PBS. For cytoplasmic markers, 2 ml lysing solution was added to 100 µ l of bone marrow or peripheral blood to lyse red blood cells and incubated for 15 min. After centrifugation, to the pellet, 1 ml perm/wash buffer was added to permeabilize the cells for intracellular staining and incubated for 20 min. After centrifugation, to the pellet, respective antibody (20 µl) was added to the pellet and incubated for 15 min. Then, 2 ml PBS was added and the samples were centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 500 µl of PBS. For surface and cytoplasmic markers, negative control tubes were run simultaneously with the addition of

sample and CD45 antibody.

Acquisition and data analysis

The cytometer setup and tracking beads were (BD Biosciences, USA) used for daily calibration of the instrument. The samples were then acquired in FACSCanto II flow cytometer (6-colour, 2-Laser, BD Biosciences, San Jose, CA 95131, USA) and analysed using FACSDiva software (BD Biosciences, San Jose, CA 95131, USA). At least 30,000 total cells were acquired, and the side scatter versus CD45 PerCP dot plot was used for blasts gating. The percentage of positive cells more than 20% was considered positive for that surface or intracellular markers.

RESULTS

In this study, 200 cases of paediatric acute leukemia patients were enrolled, of which 132 (66%) cases were diagnosedas B-ALL, 25 (12.5%) as T-ALL, 39(19.5%) cases as AML and 4 (2%) as MPAL by morphology, cytochemistry, and immunophenotyping [Table 1]. These patients were subgrouped according to 11 different subsets listed in Table 2 based on marker expression.

Acute myeloid leukemia subgroup

Out of 39 AML patients diagnosed, 30 (76.9%) were positive for MPO and remaining 9 (23.1%) were negative for MPO. Further, MPO+cCD79a−cCD3−Tdt−phenotype was detected in 29 (74.3%) and MPO−cCD79a−cCD3−Tdt−in 7 (17.9%) of AML patients [Table 1]. Tdt positivity was noted in 3 (7.7%) patients with a phenotype of MPO− cCD79a−cCD3−Tdt+in two patients and MPO+cCD79a−cCD3−Tdt+in one patient. Hence, MPO+cCD79a−cCD3+/−Tdt+/− phenotype was detected in 30 (76.9%) cases. These AML patients did not show co- expression of cCD3 and cCD79a. It was found that MPO had76.9% sensitivity, 100% specificity, 100% positive predictive value, 94.6% negative predictive value along with 95.4% accuracy for diagnosis of AML [Table 3].

B-acute lymphoblastic leukemia sub group

Out of 132 B-ALL patients diagnosed, 129 (97.7%) were positive for cCD79a and remaining 3 (2.3%) were negative for cCD79a.MPO−cCD79a+cCD3−Tdt+phenotype was detected in 94 (71.2%) and MPO−cCD79a+cCD3−Tdt−phenotype in 35 (26.5%) of B-ALL patients. MPO− cCD79a−cCD3−Tdt+was detected in none of the cases and MPO− cCD79a−cCD3−Tdt−in 3(2.3%) of B-ALL patients. Hence, MPO−cCD79a+cCD3−Tdt+/− was noted in 129 (97.7%) and MPO−cCD79a−cCD3−Tdt+/− in 3 (2.3%) of B-ALL patients. These B-ALL patients did not show co-expression of MPO and cCD3 [Table 3]. It was found that CD79a has 97.7% sensitivity, 100% specificity, 100% positive predictive value, 95.5% negative predictive value along with 98.5% accuracy for diagnosis of B-ALL [Table 3].

T-acute lymphoblastic leukemia subgroup

Out of 25 T-ALL patients, MPO−cCD79a− cCD3+Tdt+phenotype was detected in 16 (64%) and MPO−cCD79a−cCD3+Tdt−phenotype in 9 (36%) of T-ALL patients. cCD3 expression was detected in 100% cases of T-ALL. It was found that cCD3 has 100% sensitivity,100% specificity, 100% positive predictive value, 100% negative predictive value along with 100% accuracy for diagnosis of T-ALL [Table 3].

Mixed phenotypic acute leukemia subgroup

Out of 200 cases of acute leukemia, 4 cases were diagnosed as MPAL. Two cases were T-myeloid and two were of B-myeloid leukemia. MPO+cCD79a−cCD3+Tdt− and MPO− cCD79a −cCD3+ Tdt +phenotype was detected in one case of T-myeloid acute leukemia each. MPO+cCD79a+cCD3-Tdt+ and MPO-cCD79a+cCD3-Tdt+phenotype was detected in one case of B-myeloid each.

Table 1: Lineage Specific Markers

MARKERS	AML (n=39)	B-ALL (n=132)	T-ALL (n=25)	MPAL (n=4)
CD 79a	0	129 (97.7%)	0	1(25%)
CD 3	0	0	25 (100%)	1(25%)
MPO	30(76.9%)	0	0	2(50%)

Table 2: Different Subset Of Tubes With Variable Positive And Negative Expression Of Cytoplasmic Markers

MARKERS	AML (n=39)	B-ALL (n=132)	T-ALL (n=25)	MPAL (n=4)
MPO+ cCD79a− cCD3−Tdt−	29(74.3%)	0	0	0
MPO+ cCD79a− cCD3−Tdt+	1(2.6%)	0	0	1(25%)
MPO− cCD79a+ cCD3−Tdt−	0	35 (26.5%)	0	0
MPO− cCD79a+ cCD3−Tdt+	0	94 (71.2%)	0	1(25%)
MPO− cCD79a− cCD3−Tdt−	7(17.9%)	3 (2.3%)	0	0
MPO− cCD79a− cCD3−Tdt+	2 (5.2%)	0	0	0
MPO− cCD79a− cCD3+Tdt+	0	0	16(64%)	1(25%)
MPO+ cCD79a− cCD3+Tdt+	0	0	0	0
MPO+ cCD79a+ cCD3+Tdt+	0	0	0	0
MPO− cCD79a− cCD3+Tdt−	0	0	9(36%)	0
MPO+ cCD79a− cCD3+Tdt-	0	0	0	1(25%)

Table 3: Sensitivity And Specificity Of Cytoplasmic Markers

VARIABLE	MPO	CD79a	CD3
SENSITIVITY	76.9%	97.7%	100%
SPECIFICITY	100%	100%	100%
PPV	100%	100%	100%
NPV	94.6%	95.5%	100%
ACCURACY	95.4%	98.5%	100%

DISCUSSION

Flow cytometry-based immunophenotyping plays an important role in accurate classification and diagnosis of acute leukemia. Various cell surface and intracellular CD markers are required for primary and secondary panels. Intracellular markers expressed at the earliest phase of disease and had good specificity for myeloid and lymphoid lineage determination in acute leukemia blasts.[4-6] In this current study, we tried to identify the applicability of a single 5 colour cytoplasmic markers tube of the combination of MPO, cCD79a, cCD3, and Tdt as primary panel. The diagnosis based on the cytoplasmic tube was correlated with the final diagnosis. Our final diagnosis was based on morphology, cytochemistry, and surface and cytoplasmic markers on immunophenotyping. We found that cCD3 and MPO positivity with cCD79a negativity provides 100% specific diagnosis of T-ALL and AML, respectively. Single intracellular combination MPO−cCD3+cCD79a+/−Tdt+/− independent had 100% sensitivity and 100% specificity for T-ALL. A single tube of MPO+cCD79a−cCD3+/−Tdt+/− has 76.9% sensitivity and 100% specificity for AML.

Acute myeloid leukemia subgroup

In our study of AML subset, MPO+cCD79a−cCD3−Tdt− was 74.3% comparable with Sharma *et al* study (56.7%).[12] MPO+cCD79a−cCD3−Tdt+was very low 2.6% in our study against their study (23.4%). In AML, all four markers were negative, MPO−cCD79a− cCD3−Tdt−was 17.9% in our study against 5.4% in their study [Table 4]. When we ignore Tdt positivity/negativity in AML, MPO+cCD79a− cCD3+/−Tdt+/− was 76.9% in our study while 80.1% in their study [Table 2]. However, there was significant difference in AML subset; MPO−cCD79a−cCD3−Tdt+/− was 23.1% in our study against 8.1% in their study.[12] Table 4 depicts that in AML, we detected 76.9% positivity of MPO which is comparable with Salem *et al*. (67.2%) and Renate *et al* (70.5%).[1,13] The current study shows good correlation between expression of cCD79a, cCD3, and Tdt in AML cases with various authors [Table 4].

B-acute lymphoblastic leukemia

In our study of B-ALL, MPO− cCD79a+cCD3−Tdt+was 71.2% comparable with Sharma *et al*. study (86.9%).[12] MPO−cCD79a+cCD3−Tdt−was found in 26.5% in the current study against 11.9% of their study. Hence, both subsets correlated very well. When we ignore Tdt positivity/ negativity sensitivity, MPO−cCD79a+cCD3−Tdt+/− was 97.7% in our study while 98.8% in their study.[12] MPO− cCD79a−cCD3−Tdt+/− was 2.3% in our study against 1.1% in their study. It shows good correlation.[12] In total B-ALL cases, individual expression of MPO, cCD79a, cCD3, and Tdt in the present study which shows very good correlation with various authors [Table 4].

T-acute lymphoblastic leukemia

In T-ALL subset, MPO−cCD79a−cCD3+Tdt+was detected in 64% in the present study against 25% as per Sharma *et al*.[12]Our study shows MPO−cCD79a−cCD3+Tdt−in36% cases against 18.1% in their study. In T-ALL, if we ignore cCD79a and Tdt positivity/negativity, sensitivity in subset MPO−cCD3+cCD79a+/−Tdt+/− was 100% the same in their study 100%. We found 64% Tdt expression in T-ALL while it was 63.64% in their study.[12] In our study, cCD3 positivity was 100% sensitive and specific for diagnosis of T-ALL similar to Sharma *et al*. In total T-ALL cases, individual expression of MPO, cCD79a, cCD3, and Tdt in the present study showed as very good correlation with various authors [Table 4].[1,12,14] Various studies show that intracellular expression of MPO, cCD3, cCD79a, and CD22 is the earliest expression myeloid, B- and T-cell markers.[14-19]

Table 4: Comparison Of Sensitivity Of Cytoplasmic Markers In Various Studies

Cytoplasmic markers	Leukemia subtype	Sharma et al12 (Pediatric+ adult)	Renate et al13 (Adult)	Dalia et al1 (Pediatric +adult)	Present study (Pediatric)
MPO	AML	88.3	70.5	67.2	76.9
	B-ALL	0	0	0	0
	T-ALL	0	0	0	0
CD79a	AML	11.7	2.3	0	0
	B-ALL	98.9	100	100	97.7
	T-ALL	56.8	0	0	0
CD3	AML	0	1.2	0	0
	B-ALL	0	0	0	0
	T-ALL	100	100	100	100

CONCLUSION

In this study, we highlight the role and applicability of cytoplasmic markers tube-based method to use as primary panel in routine acute leukemia immunophenotyping. There was a good correlation of 5 colour cytoplasmic markers tube-based diagnosis versus final diagnosis based on morphology, cytochemistry, and flow cytometry. This single 5 colour cytoplasmic markers tube method to make immunophenotyping cost-effective; however, further studies are required from India.

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Statistical analysis

Statistical analysis was done using SPSS (Statistical package for social sciences) software.

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Conflict of interest

There are no conflicts of interest.

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