Comparison of Immunohistochemistry, Cytochemistry, and Flow Cytometry in AML for Myeloperoxidase Detection

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Abstract Acute Myeloid Leukemia (AML) as per World Health Organization (WHO 2008) classification is on the basis of the antigenic characterization, enzymes restriction in the neoplastic myeloid cells and the specific translocations/mutations. AML can be assessed and differentiated by flowcytometry (FCM)/immunohistochemistry (IHC)/cytochemistry techniques. Myeloperoxidase (MPO) is an unequivocal marker to differentiate AML from the acute lymphoblastic leukemia. Despite FCM popularity, it has its limitations, in form of ‘dry-tap’, cost, and inability of being performed by retrospective analysis. IHC, though an old technique has overcome

these disadvantages of FCM. Cytochemistry, on the other hand has its own advantages in being cost-effective; technically easy to do while its disadvantages are its inability to be carried out in the old samples, ‘dry-tap’ conditions in aleukemic leukemia. There has been non-uniformity in the literature among these techniques especially concerning their sensitivity for MPO. A prospective study was done at All India Institute of Medical Sciences New Delhi from 01 July 2014 to 30 Nov 2015 to include 120 diagnosed acute myeloid leukemia cases. Myeloperoxidase stain was done by cytochemistry, immunohistochemistry and flow cytometry and results were compared. There were 28 cases which showed discrepancies. Out of these 28 cases immunohistochemistry showed positivity in majority (22 cases) followed by flow cytometry (14 cases). Therefore it is important to employ more than one technique and IHC must be included for detection of MPO in all suspected cases of AML especially when negative with FCM.

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Introduction

Acute myeloid leukemia (AML) not otherwise specified (NOS) as per 2008 edition of the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues is diagnosed by the demonstration of myeloid specific antigen and the myeloperoxidase enzyme restriction in the blasts either by flowcytometry (FCM) or immunohistochemistry (IHC) or cytochemistry techniques.

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Though FCM is more popular, it has inherent limitations which include difficulty in interpretation of borderline positivity, absence of blasts in dry tap bone marrow aspirate (BMA), and stringent requirement of fresh sample. Therefore value of IHC to determine diagnosis of AML sometimes is the only available source for the diagnosis and classification of acute leukemia [1–3]. But there has been non-uniformity in the literature among the sensitivity of these techniques for the detection of MPO to diagnose AML [4, 5]. When the importance of cytochemistry and IHC are waning this has special significance. Our study has thus focused in analyzing the above techniques for the demonstration of MPO in the leukemia blasts. The present study will try to ascertain importance of techniques like IHC and cytochemistry. Another lacunae is regarding the cut-offs of MPO positivity by FCM, our study therefore has tried to compare and analyzing the various cut-offs by previous studies thus helping to choose the best cut-offs.

Materials and Methods

A prospective study was performed from 01 July 2014 to 30 Nov 2015, after the due approval by the All India Institute of Medical Sciences (AIIMS) New Delhi Ethics Committee. A total of 120 bone marrow aspirates and bone marrow biopsy (BMB) of newly diagnosed AML patients were received for evaluation of MPO by IHC, FCM and cytochemistry with the exclusion of treated/partially treated/other leukemia.

BMAs were stained with Jenner-Giemsa followed by cytochemical stains for MPO (Benzidine dihydrochloride as substrate, Giemsa as counterstain) and non-specific esterase (NSE). Morphological evaluation was performed and diagnosis of acute leukemia was made. The percentages of positive blast cells were based on a 500-cell differential count under oil immersion by two pathologists. BMAs for FCM analysis were processed immediately/within 12 h by six color-dual lasers, BD FACS Canto II instrument. All monoclonal antibodies for FCM were obtained from BD Biosciences (CA, USA). The antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanin 7 (PE-Cy7), PerCP-Cy5.5, APC and APC-H7. CD13, CD33, HLA-DR, CD 117, CD11c, CD 64, CD 34, CD19, CD 10, CD 79a, cCD 3, CD 7, TdT antibodies were used in flow cytometry. MPO was conjugated to FITC. In all cases, at least 100,000 events were acquired from each tube. IHC was performed using Antibody: Anti-human MPO (Thermo Scientific United Kingdom). Internal quality control was also performed by running the immunobeads every week. MPO positivity was defined as proportions of blast cells by cytochemistry and IHC ($\geq 3\%$) and FCM ($\geq 10\%$) respectively [6]. Besides

MPO by IHC CD 34 by IHC was also used. Interpretation of the results of cytochemistry, IHC and FCM were analyzed by two pathologists.

Sensitivity, specificity, positive predictive and negative predictive values were calculated using EPR-Val Test Pack 2 version. McNemar test was used to compare the proportions of the cases by Med Calc software. *p* value of <0.05 was considered to be significant.

Results

Total 120 AML cases including 77 males and 43 females, age ranging from 1 to 75 years (median 36 years) were analyzed according to FAB sub-classification. Morphologically AML-M2 cases predominated (32 = 26.6%) followed by AML-M4 (28 = 23.3%), AML-M5 (22 = 18.3%), AML-M1 (22 = 18.3%), AML-M0 (8 = 6.6%), AML-M3 (3 = 2.5%), AML-M7 (3 = 2.5%), AML-M6 (2 = 1.6%) respectively. Morphologically AML was diagnosed in 60 cases due to presence of Auer rods.

Among these 120 cases, 77 (64.2%) were positive for MPO while 15 (12.3%) were negative by all three techniques. In the remaining 28 cases (23.3%) discrepancies occurred i.e. when any one or more technique(s) showed negativity, while another showed positivity for MPO (Table 1). Among these IHC and FCM were alone positive for MPO in 7 and 5 cases respectively. None of the cases were positive by cytochemistry alone when the other two modalities were negative. IHC and FCM together were positive in 08 while IHC and cytochemistry together were positive in 07 cases when the third technique was negative (Table 1). No discrepancies were seen in AML-M3. No isolated positivity by cytochemistry were seen in 28 discrepant cases noted (Table 1).

Because of non-availability of uniform acceptable cut-offs value for IHC and FCM, present study has considered the cutoff of $\geq 3\%$ for the IHC and $\geq 10\%$ MPO positive blast cells for FCM as was suggested by Saravanan et al. [7] and Bene et al. [2] respectively. With the use of cutoff of $\geq 10\%$ for the IHC as suggested by British task force, 1994 [8], the discrepant cases has reduced to 26 from the 28. Our result has shown that if we take the cut-offs of 10% rather than 3% then IHC would be missing 05 case which were MPO positive (Table 2).

We in our study have compared the cut-offs of 10% (as suggested by Bence et al.) [2] with 5.4% as suggested by Prabhu et al. [9] and have found that 15 cases which were negative for MPO by all the three techniques with the cut-offs of 10% were reduced to 10 cases with the cut-offs of 5.4% (Table 3).

Number of cases with different cut-offs for MPO positivity in our study were ($\geq 3\%$ = 66.7% and

Table 1 Number of discrepant cases showing positivity for MPO with one or more techniques but negativity for MPO by at least one

AML Subtype	Isolated Cytochemistry MPO positivity	Isolated FCM MPO positivity	Isolated IHC MPO positivity	Cytochemistry and IHC positivity	Cytochemistry and FCM positivity	IHC and FCM MPO positivity	Total (n = 28)
M-0	0	0	5	0	0	0	5
M-1	0	3	0	3	0	2	8
M-2	0	0	0	0	1	0	1
M-3	0	0	0	0	0	0	0
M-4	0	0	0	1	0	3	4
M-5	0	1	2	1	0	3	7
M-6	0	0	0	1	0	0	1
M-7	0	1	0	1	0	0	2
Total cases	0	5	7	7	1	8	28

Table 2 Comparison of two different cutoffs of IHC for detection of MPO

	Cutoff of 3%	Cutoff of 10%
Total discrepant cases	28	26
IHC alone positivity	7	5
FCM alone positivity	5	6
Cytochemistry alone positivity	0	2
IHC + Cytochemistry positivity	7	5
IHC + FCM	8	7
FCM + Cytochemistry	1	1
Total IHC Positivity	22	17

>10% = 51.5% by IHC), ($\geq 3\%$ = 24.2 by cytochemistry) and ($\geq 10\%$ = 42.4%, 5.4% = 76.19% by FCM) (Figs. 1 and 2). While the overall sensitivity of cytochemistry remains the lowest, the sensitivity of FCM became the highest when the cutoff of 5.4% was taken.

Discussion

Myeloperoxidase, an enzyme which is present in the primary granules of myeloid cells is an unequivocal marker of myeloid lineage. Its importance is being emphasized in mixed phenotypic acute leukemia. Techniques like cytochemistry, immunophenotyping by FCM and immunohistochemistry have been compared in the past for the

detection of MPO. Our study revealed that in an overall 28 discrepant cases, IHC alone could diagnose 7 (25%) cases, while FCM alone diagnosed 5 (18%) cases. Comparison of three techniques in our study, showed IHC to be better than FCM (with the cutoff of 10%) and cytochemistry numerically in the discrepant cases, but statistically there was only significant difference between the IHC and cytochemistry (Table 4). This depicts important role of IHC if not superior advantage over FCM. It gives us an opportunity in future to apply IHC in ‘dry tap’ sample and in the set up where the facility of FCM is not available. IHC under the strict protocol can be done in any peripheral centre and being less expensive than FCM, gives an edge over FCM. Though cytochemistry couldn’t diagnose even a single case on a solo basis in our study, however in conjunction with other techniques it could diagnose 8 out of 28 (28.6%) discrepant cases (Table 1). The absence of MPO positivity by cytochemistry is a known fact in AML M-0 (<3%) and acute monocytic/monoblastic leukemia (AML-M5) [10, 11]. The low sensitivity of the cytochemistry in present and other studies [7, 10, 11] in contrast to Peffault de Latour et al. [4], Kheiri et al. [5] and Nguyen et al. [12] can be due to technical flaw or alteration of the MPO enzymatic activity in leukemic blasts while maintaining immunological activity [13, 14]. The false negativity can be further reduced by repetition and careful analysis of the internal control. Interestingly 14 cases failed to demonstrate MPO positivity by FCM while it was detected using the IHC and/

Table 3 Comparison of two different cutoffs for FCM for detection of MPO

	Cutoff of 5.4%	Cutoff of 10%
Total discrepant cases	32	28
Negative MPO by all three techniques (With FCM showing positivity for CD13, CD33, CD117, CD34, HLA-DR and negativity for CD19, cCD79a, cCD3)	10	15
IHC + Cytochemistry positivity	4	7

Fig. 1 MPO positivity by cytochemistry stain

or cytochemistry. The negativity of the MPO by FCM in the discrepant cases can be explained by technical error including diluted or degenerated sample, inadequate antibody, inadequate incubation time of antibody, exposure of incubated sample to light and poor quality antibody. This can be minimized by increasing the number of the total events to 2,00,000 in FCM to create an appropriate blast window in cases of the diluted bone marrow with blasts up to 20%, processing the samples within 12 h and taking care of all pre-analytical variables by strictly following of the laboratory standard operation procedure for instance performing in the dark, air-conditioned room. FITC as fluorochrome for MPO was used and has an advantage of its stability and brighter expression. The internal quality control can also be performed by running the immunobeads every week to reduce the technical flaws.

Secondly, the negativity of MPO by FCM can be explained by the lack of consensus in the cut off of MPO positivity by FCM. In a series of 219 patients by Prabhu et al. the cutoff of 5.4% for MPO positivity by FCM has emerged out to be the most sensitive and most specific [9].

In the present study the cutoff of 5.4% was able to detect 3 additional cases from the discrepant and 5 additional cases which were otherwise initially negative by the three

techniques (Table 3). Interestingly all these cases which were added from the triple negative MPO were of AML-M5. After this cutoff, the overall sensitivity of the flow-cytometry became superior. Kheiri et al. observed the lesser sensitivity of FCM as compared to cytochemistry with the current recommendation of threshold of 10% cutoff. With 3% cutoff of MPO by FCM, sensitivity of FCM was increased in comparison to cytochemistry, however IHC was not included in their study [5]. Similarly Peffault de Latour et al. reviewed 136 cases and found FCM to be less sensitive than the cytochemistry using a cutoff of 10% for MPO [4]. In fact various studies have been conducted with different cut-offs for FCM, highlighting the lack of consensus among them [2, 15, 16]. Therefore more and larger studies would be required to define the threshold for MPO by FCM.

Among FAB sub-classification of AML, AML minimally differentiated (AML-M0), acute monocytic/monoblastic (AML-5), and acute megakaryocytic leukemia (AML-M7), carried the most discrepancies while AML with maturation (AML-M2), acute promyelocytic leukemia (AML M3) carried the least discrepancies (Table 1). Among two out of three AML-M7 discrepant cases, one showed positivity for MPO by the

Fig. 2 Flow cytometry of AML Cases with varied MPO positivity

Table 4 Comparison and difference between techniques in the discrepancies

Techniques*	Difference (%)	95% confidence interval (%)	'p' value
IHC versus cytochemistry	50	22.59–56.96	0.0005
FCM versus cytochemistry	21.43	–13.17 to –49.44	0.2632
IHC versus FCM	28.57	–6.11 to –54.44	0.115

* With cut-off of for MPO positivity in our study ($\geq 3\%$ for IHC and cytochemistry and $\geq 10\%$ for FCM)

immunophenotyping alone, while other case showed MPO positivity by immunohistochemistry. The third AML-M7 case showed negativity by all the three techniques. The diagnosis of AML-M0 cases becomes difficult in inadequate sample for the flowcytometry, since the cytochemistry will always reveal MPO negative. The importance of IHC takes the leading role in the diagnosis of AML-M0 when FCM can't be performed. In our study we have found that IHC was able to detect 5 out of 8 AML-M0 cases. Thus with the IHC, we can sometimes overcome the common dilemma, which any laboratory can face in the absence of flowcytometry. While in the case of AML-M5, IHC and FCM were able to detect 6 out of 7 and 4 out of 7 discrepant cases. Our finding concurs with Jaffe et al. showing variable positivity of AML-M5 cases [17].

WHO classification 2008 specified the cutoff of $<3\%$ MPO positivity in the blasts by the cytochemistry for the diagnosis of AML-M0, but the cutoff for MPO positivity by FCM or by IHC was not mentioned [6]. Saravanan et al. and Guy et al. suggested cutoff of MPO positivity by IHC of $\geq 3\%$ and 10% respectively. In the present study with cutoff percentage of MPO positivity by IHC of $>10\%$, 11 AML cases would have been missed while with the cutoff of $\geq 3\%$ only 6 AML cases would have been missed. FCM with cutoff of $\geq 10\%$ would have missed 14 AML cases. Thus the sensitivity of IHC is far ahead than that of FCM and cytochemistry even when the cut off was 10% instead of 3% therefore supporting the literature [18]. But as mentioned, the overall sensitivity of FCM became superior if the cutoff of MPO positivity is taken to be 5.4% . By increasing total sample size, more cases of AML including AML M0 and AML M5 would be diagnosed with a single technique. The newer cutoffs may also help us to diagnose the missed MPAL cases.

Above discussion has reflected an importance of using two or more techniques for the detection of MPO. Each technique has its advantages and disadvantages. Starting with IHC the common drawback of IHC is nondiscrimination between MPO positivity in blasts and mature myeloid cells, particularly in low blast counts. Another drawback is interpretation and analysis of $5\text{--}10\%$ positivity by IHC. In our study we faced this problem especially in AML M0 with isolated IHC positivity wherein the IHC positivity percentage was between 5 and 10% only. The above drawbacks were overcome by meticulous correlation

with H&E stained sections and using additional IHC markers like CD34. Additionally we can use CD 117. We had 8 isolated cases of MPO positivity by IHC out of which 7 cases were CD 34 positive by FCM, thus by doing CD 34 simultaneously we confidently analysed MPO positivity by IHC. One case was CD 34 negative by FCM had 56% blasts with more than 50% MPO positivity, thus made our analysis easy. All the isolated positive MPO cases by IHC also showed CD 117 positivity by FCM. So in doubtful cases CD 117 along with CD 34 can be utilized thus making interpretation of MPO by IHC easier. Similarly in AML M5 cases additional markers like CD14, CD64 positivity was utilised for the analysis. In the two cases of AML-M5 with an isolated positivity of MPO for IHC, Non specific esterase (NSE) was positive by cytochemistry along with CD 13, CD33, CD16 and CD 64 positivity and negativity for CD 19 and cCD3 markers. Another disadvantage of IHC is its longer turnaround time as compared to cytochemistry and FCM where the results are available within a few hours. However this much delay is not clinically significant in the majority of case of AML.

Our study is one of the largest to date (120 cases) from India which included all FAB subtypes of acute myeloid leukemia. It is also the only study from India to include IHC as a marker of MPO positivity and has demonstrated it to be the most sensitive method employed.

The above discussion reflects the importance of using two or more techniques and highlights the role of IHC particularly in MPO negative cases by cytochemistry and FCM. Though FCM as method of choice for routine diagnosis of AML is recommended, however the role of cytochemistry and IHC cannot be ignored. While cytochemistry is a rapid and an inexpensive method of determining myeloid lineage, IHC has appeared to be sensitive method in our study. Our study has emphasized the stress on lowering of cutoff especially for FCM so as to increase its sensitivity. It was depicted in cases where FCM was negative and either IHC or cytochemistry was positive. In developing countries where FCM facility is yet not available, IHC for MPO, CD 34 and Cd117 carries an important role in diagnosing AML cases. Our study has stressed upon an important issue regarding acute undifferentiated leukemia that all the three modalities should be utilized before labeling as acute undifferentiated leukemia as isolated positivity are not uncommon scenarios.

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Author Contribution AA and ST were involved in conceptualization, designing, writing and critical review of the manuscript and were responsible for overall supervision. TS, HPT, GPSG, PT and VS were involved in literature search, writing and manuscript editing. ST is the overall guarantor of the article.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Ethical Approval The present study is in compliance with Ethical Standards.

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