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Original Research Article

Flow Cytometric Analysis of Surface and Cytoplasmic Markers in Acute Monoblastic Leukemia – GCRI experience

Biren Parikh[1], Nisha Khanna[2], Dhaval Jetly[3], Hemangini Vora[4], Abhishek Kumar[2], Priya Patel[2]
[1]Assistant Professor, [2]Resident, [3]Professor and Head Department of Pathology, Gujarat Cancer Research Institute, Ahmedabad
[4]Associate Professor and Head Immunohistochemistry and Flow Cytometry Division, Gujarat Cancer Research Institute, Ahmedabad

***Corresponding author**
Dr. Biren Parikh
Email: birenparikh2002@rediffmail.com

Abstract: Flow cytometry is highly sensitive for detection and quantitative analysis of surface and intracellular antigens in malignant hematopoietic cells. Immunophenotyping of leukemia cells play crucial role in identification of leukemia cell line, which in turn serves for individual treatment monitoring and detection of residual disease. The main purpose of this study is to identify the expression and frequency of various cytoplasmic and surface markers in acute monoblastic leukemia. We have included 30 cases of acute monoblastic leukemia with applied primary and secondary panels of markers for immunophenotyping. We analysed frequency and expression of different subsets with combination of positive and negative markers. CD15 and CD11c were found to be positive in all cases while expression of CD14 was found to be variable. CD19 was aberrantly expressed in 13.3% cases**.** Our study indicates that all 30 cases were negative for B cell and T cell markers. Amongst the secondary panel for monocytic markers CD15 and CD11c were found to be highly sensitive and specific while CD14 was variable in its expression.
Keywords: Acute monoblastic leukemia, flow cytometry, immunomarkers

INTRODUCTION:

Acute myeloid leukemia (AML) is a complicated heterogenous disease involving the presence of a clonal expansion of neoplastic myeloid cells with variable degrees of differentiation and varying clinical, morphological, immunologic and molecular characteristics [1]. The characterization of acute leukemias is based on a multiparametric analysis which includes clinical features, cell morphology, genetics and immunological markers [2]. Immunophenotyping has become extremely important not only in diagnosis but also sub classification of acute myeloid leukemia. It is particularly useful in those cases where the morphologic and cytochemical examination do not clearly indicate lymphoid or myeloid lineage [1]. The subtype of acute myeloid leukemia classified by French-American-British classification as M5 or acute monocytic leukemia is a distinct subtype with characteristic clinical features [3]. AML-M5 is further sub classified depending on whether the monocytic cells are predominantly monoblasts [>80%] acute monoblastic leukemia i.e. M5a or a mixture of monoblasts and promonocytes [< 80% blasts] M5b. AML-M5a comprises 5-8% of cases of AML. The main purpose of this study is flowcytometric analysis of surface and cytoplasmic markers in acute monoblastic leukemia.

MATERIALS AND METHODS:

This retrospective study was conducted in the department of Pathology, Gujarat Cancer and Research Institute, Ahmedabad, India from March 2015 to October 2016. A total of 30 cases of AML-M5a were studied in this period.

Sample collection and preparation

The bone marrow or peripheral blood was collected in EDTA 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 leukemia.

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 flowcytometric 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 immuno phenotyping

For surface markers, respective antibody (20 µl) mentioned above was added in six-color combination to the bone marrow or peripheral blood (100 µl, 1×10^6) 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 suspended in 500µl of PBS. For cytoplasmic markers, 2 ml lysing solution was added to 100 µl 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 (20µl) 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 suspended 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 FACS Canto II flow cytometer (6-color, 2-Laser, BD Biosciences, USA) and analyzed using FACS Diva software (BD Biosciences, 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 marker.

Table 1: Frequency of Various Surface and Cytoplasmic Markers Expression in Acute Monoblastic Leukemia

Immuno markers	Positive, N (%)	Negative, N (%)	
CD 13	23 (75.67%)	07(23.33%)	
CD 33	30(100%)	-	
CD 117	12(40.0%)	18(60.0%)	
MPO	17 (56.67%)	13(43.33%)	
CD 11b	28(93.33%)	02(06.67%)	
CD 11c	30(100%)	-	
CD 14	19(63.33%)	11	(36.67%)
CD 15	30(100%)	-	
CD 34	14 (46.67%)	16(53.33%)	
HLA-DR	29 (96.67%)	01(03.33%)	
TdT	-	30	(100%)
CD 19	04(13.33%)	26	(86.67%)
CD 79a	-	30(100%)	
CD 3	-	30	(100%)

Table 2: Frequency of Various Monocytic Markers in Acute Monoblastic Leukemia

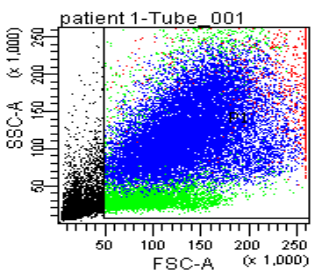
Combined monocytic markers	Number of cases	Percentage (%)
$CD11b^+/CD11c^+$	28	93.3%
$CD11b^+/CD11c^-$	-	-
$CD11b^-/CD11c^+$	02	6.7%
$CD11b^-/CD11c^-$	-	-
$CD14^+/CD11b^+$	19	63.3%
$CD14^+/CD11b^-$	-	-
$CD14^-/CD\ 11b^+$	09	30%
$CD14^-/CD11b^-$	02	6.7%
$CD11b^+/CD15^+$	28	93.3%
$CD11b^+/CD15^-$	-	-
$CD11b^-/CD\ 15^+$	02	6.7%
$CD11b^-/CD\ 15^-$	-	-
$CD11c^+/CD\ 15^+$	30	100%
$CD14^+/CD15^+$	19	63.3%
$CD14^+/CD\ 15^-$	-	-
$CD14^-/CD15^+$	11	36.7%
$CD14^-/CD\ 15^-$	-	-
$CD11c^-/CD\ 15^-$	-	-

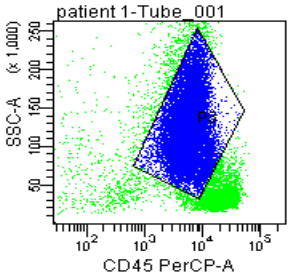
Table 3: Different Subset of Tubes with Variable Positive and Negative Expression of Immune-Markers

Immunomarkers expression subset	Number of cases	Percentage (%)
$MPO^+/CD117^+/CD11c^+/CD15^+$	06	20%
$MPO^+/CD117^-/CD11c^+/CD15^+$	11	36.7%
$MPO^-/CD117^+/CD11c^+/CD15^+$	06	20%
$MPO^-/CD117^-/CD\ 11c^+/CD15^+$	07	23.3%

RESULTS

Thirty cases of acute monoblastic leukemia were diagnosed at GCRI Ahmedabad between March 2015 and October 2016 by immunophenotyping. Figure 1 shows immunophenotypic analysis of a patient of AML-M5a. The blasts mainly expressed markers CD13, CD33, CD11b, CD11c, CD15 along with HLA-DR. The frequency of various surface and cytoplasmic markers with their expression in all the 30 cases is depicted in Table1. Out of these cases 43.3% cases were positive for cytoplasmic marker MPO and remaining 56.7% were negative for MPO. CD33 and CD117 were found to be positive in 100% and 40 % cases respectively. Markers of monocytic lineage such as CD 11c and CD15 were found to be positive in 100% cases. Other monocytic markers such as CD11b and CD14 were found to be positive in 93.3% and 63.3% cases respectively. CD34 showed positivity in 46.7% cases, remaining 53.3% cases were negative for CD34. HLA-DR was to be positive in 96.7% cases. Other cytoplasmic markers such as CD3, CD79a and TdT were negative in 100% cases. Surface B cell markers such as CD19 was aberrantly expressed in 13.3% cases while remaining 86.7% cases were negative for CD19. Frequency of various monocytic markers with their expression is depicted in Table 2. Combination of CD11c and CD15 was found to be positive in 100% cases followed by combination of CD11b and CD11c which was expressed in 93.3% cases. Combinations of $CD11c^-$ and $CD11b^+$, $CD15^-$ and $CD14^+$, $CD14^-$and $CD15^-$, $CD11c^-$ and $CD15^-$ and $CD11b^-$ and $CD15^+$ were not expressed in any case thereby indicating high sensitivity and specificity of CD11c and CD15. Table 3 shows different subsets of immunomarkers with their expression. $MPO^+CD117^-CD11c^+CD15^+$ was found to be positive in maximum number of cases i.e. 36.7% followed by $MPO^-CD117^-CD11c^+CD15^+$ was expressed in 23.3% cases while $MPO^+CD117^+CD11c^+CD15^+$ and $MPO^-CD117^+CD11c^+CD15^+$ were found to be expressed in equal number of cases i.e. 20% each.


patient 1-Tube_001
SSC-A (x 1,000)
FSC-A (x 1,000)
P1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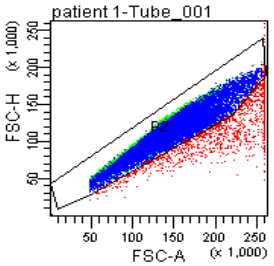
SSC-A (x 1,000)
CD45 PerCP-A
P3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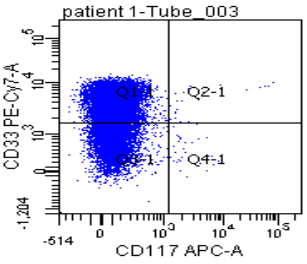
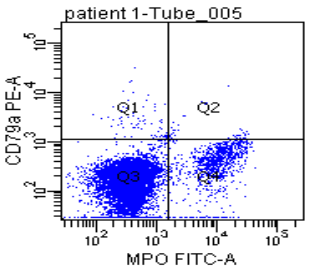
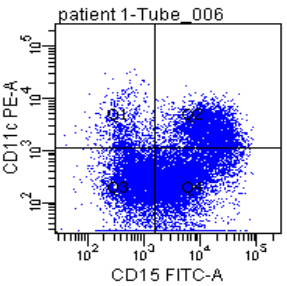
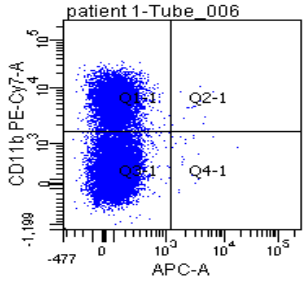
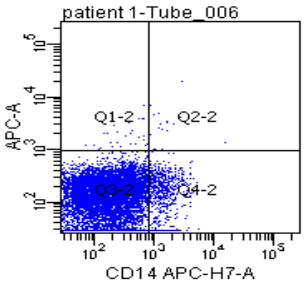
FSC-H (x 1,000)
FSC-A (x 1,000)
P2

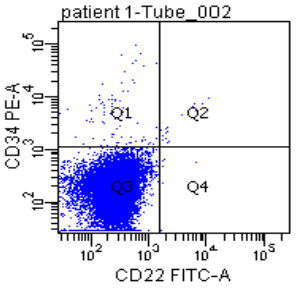

patient 1-Tube_003
CD33 PE-Cy7-A
CD117 APC-A
Q1-1
Q2-1
Q3-1
Q4-1

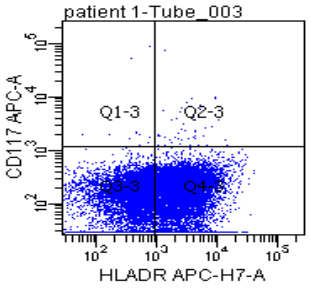

patient 1-Tube_005
CD79a PE-A
MPO FITC-A
Q1
Q2
Q3
Q4

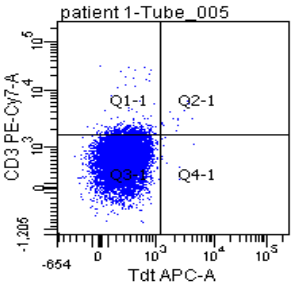

patient 1-Tube_006
CD11c PE-A
CD15 FITC-A
Q1
Q2
Q3
Q4

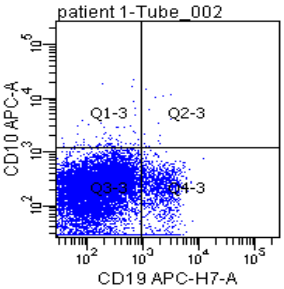

patient 1-Tube_006
CD11b PE-Cy7-A
APC-A
Q1-1
Q2-1
Q3-1
Q4-1


patient 1-Tube_006
APC-A
CD14 APC-H7-A
Q1-2
Q2-2
Q3-2
Q4-2


patient 1-Tube_002
CD34 PE-A
CD22 FITC-A
Q1
Q2
Q3
Q4


patient 1-Tube_003
CD117 APC-A
HLADR APC-H7-A
Q1-3
Q2-3
Q3-3
Q4


patient 1-Tube_005
CD3 PE-Cy7-A
Tdt APC-A
Q1-1
Q2-1
Q3-1
Q4-1


patient 1-Tube_002
CD10 APC-A
CD19 APC-H7-A
Q1-3
Q2-3
Q3-3
Q4-3

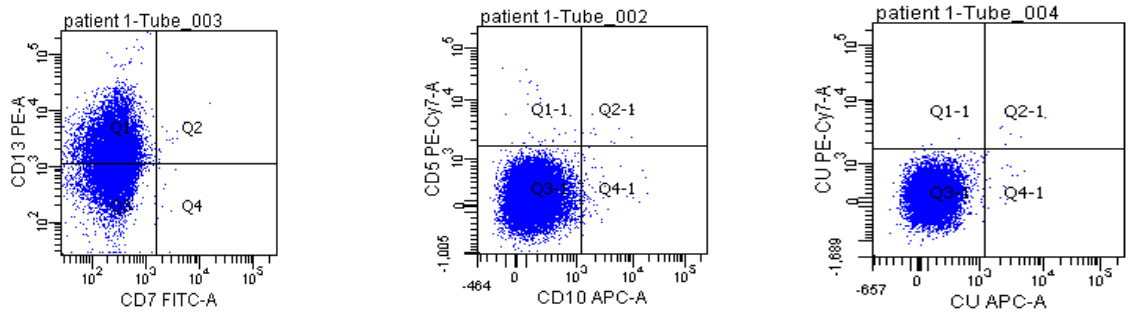


Fig 1: Flow cytometric immuno phenotyping in a patient of AML M5a. The blasts mainly expressed myeloid markers CD13, CD33, CD11b, CD11c, CD15 along with HLA-DR. MPO, TdT and CD79a were found to be negative.

DISCUSSION

Flowcytometry has become an indispensible tool for diagnosis and classification of acute leukemia. Lineage assignment is critical for optimal therapy of acute leukemia [4]. This study characterizes various cytoplasmic and surface markers expressed in acute monoblastic leukemia. Although CD14 is highly specific for monocytic differentiation there is controversy regarding its sensitivity [5]. As Eshoa *et al.;* concluded that although mature monocytes strongly express CD14, most immature leukemic monocytes lack its expression [6, 7], our study correlates with it as only 36.7% cases of AML-M5a were negative for CD14. As per Scott *et al.;* the combined immunophenotypic analysis of membrane CD11b and CD11c showed that their expression by leukemic myeloid cells is clearly associated and virtually all blast cells with moderate-strong CD11c expression are also CD11b positive [8]. Our study correlates very well with as 94.4% cases were found to be positive for both CD11c and CD11b. Combined immunophenotypic analysis of CD14 and CD11b with CD14 negative and CD11b positive expression was found in 30% cases which is comparable to Tallman et al in which this combination was seen in 32% cases [9]. In conclusion this study correlates with high sensitivity and specificity of CD11c and CD15 and variable expression of CD14 in AML-M5a.

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Conflicts of interest No author has any competing interest.

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