International Journal of Hematological Disorders, 2015, Vol. 2, No. 1, 10-17
Available online at http://pubs.sciepub.com/ijhd/2/1/3

DOI:10.12691/ijhd-2-1-3

Flowcytometric Immunophenotypic Characterization of Acute Myeloid Leukemia (AML) in Sudan

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Received December 29, 2014; Revised January 15, 2015; Accepted January 20, 2015

Abstract **Introduction**: Acute Myeloid Leukaemia (AML) accounts for approximately 20% of acute leukemia in children and 80% of acute leukemia in adults. Immunophenotyping has become extremely important not only in diagnosis and subclassification of AML but also in the detection of the minimal residual disease. Immunophynotypic pattern of AML in Sudanese patients have not been addressed before. This study was conducted to characterize immunophenotypic patterns of AML in Sudanese patients. Multiparameter flow cytometry and CD45/SSC gating were used to analyze the surface and cytoplasmic antigen expressions in 106 cases of AML during the period mid2010 to mid2011 at Radioisotope Centre Khartoum (RICK). The following antigens: CD45, HLA-DR, CD34, CD117, CD13, CD33, CD19, CD7, cytoplasmic markers (CD3, CD79a, MPO), CD11c, CD14, CD64, CD42a, CD41 and CD61 were used. **Results:** Almost all AML blasts were expressing CD45 with no significant differences between the subtypes. CD34 have different expressions in AML subtypes. CD13 and CD33 were also studied among the blast population having mean positivity of 51.5% and 49.8% respectively in all AML subtypes collectively. CD33 was found to have higher positivity among AML-M4 and AML-M5 with mean positivity of 75.9% and 76.6% respectively. CD13 and CD33 had no correlation for all AML subtypes except for AML-M5 with very strong negative correlation(r=-0.913). Apparent expression of CD7 and CD19 were expressed in 45.1% and 13.6% of all cases respectively. CD7 was mostly expressed in AML-M2 and AML-M3 (75%) and least in AML-M5, while CD19 was only expressed in cases of AML-M0 and AML-M7. **Conclusion:** Flowcytometric analysis of acute leukemia by combining the patterns and intensity of antigen expression improved the diagnosis of AML in our centre. Immunophenotyping results and FAB classification of our AML patients were comparable to international published studies.

Keywords: *acute myloidleukemia Immunophenotyping flowcytometry*

Cite This Article: Ihsan M. Osman, Amira A. K. Humeida, Osama Eltayeb, Inaam Abdelrhman, and Taghreed A. Elhadi, "Flowcytometric Immunophenotypic Characterization of Acute Myeloid Leukemia (AML) in Sudan." *International Journal of Hematological Disorders*, vol. 2, no. 1 (2015): 10-17. doi: 10.12691/ijhd-2-1-3.

1. Introduction

Acute myeloid leukemia (AML) is a complicated, heterogeneous disease involving the presence of a clonal expansion of neoplastic myeloid cells with variable degrees of differentiation and varying clinical, morphologic, immunologic, and molecular characteristics. It is a common hematological malignancy, accounts for approximately 80% of acute leukemia in adults and 20% of acute leukemia in children [1]. Immunophenotyping has become extremely important not only in diagnosis and subclassification of AML but also in the detection of the minimal residual disease. It is also suggested to have prognostic significance [2]. The prognostic value of immunophenotyping in AML is controversial [3]. Flowcytometric immunophenotyping of haematologic malignancies involving the blood and bone marrow is rapidly gaining prominence as an adjunct to traditional morphologic examination and cytochemistry. Multiparameter flowcytometry allows one to identify and characterize individual cells in suspension by using fluorescent-labelled antibodies to cell lineage and differentiation associated antigens expressed by these cells. It provides a rapid methodology for the assignment of cell lineage and stage of differentiation in acute leukaemia or blast crisis of chronic myeloid leukemia, this is particularly useful in those cases where the morphologic and cytochemical examinations do not clearly indicate lymphoid or myeloid lineage. The role of flowcytometry in subclassification of AML was improved by utilization of a variety of gating strategies including the use of CD45/side scatter gating. [4].

Immunophynotypic pattern of acute myeloidleukaemia in Sudanese patients have not been addressed before. This study was conducted to characterize Immunophenotypic pattern of AML presented to the flowcytometry unit at Radio Isotope Centre Khartoum (RICK). RICK is the main referral centre for cancer and the only centre running the service of flowcytometry Immunophenotyping of haematological malignancy in Sudan.

2. Materials and Methods

A total of 106 acute myeloid leukemia (AML) cases immunophenotyped at flowcytometrylaboratory, Radioisotope centre Khartoum (RICK), using 4 coloursflowcytometer during the period mid2010 and mid2011 were reviewed retrospectively. The analyzed samples were either of peripheral blood (PB) or bone marrow aspirate (BMA) according to availability and presence of blast cells. Relapsed or recurrent cases of AML were excluded. Morphological examinations to all films was first done followed by cytochemical stains (Sudan Black B).

2.1. Sample Collection and Preparation

Venous blood sample: 2.5 ml of venous blood sample was collected in EDTA vacotainer (5ml). Bone marrow aspiration: 2 ml of bone marrow aspiration was collected in Lithium Heparin vacotainer (5ml).

2.2. Lysing Procedure for Whole Blood and B.M Aspiration Monoclonal Antibody Combination

The tubes were labeled for analysis. 20 µL of monoclonal antibody was added into each tube. 100 µL of sample was added containing no more than 1 x 104 leukocytes / ml. (Counted by hematology analyzer - SYSMEX). Each tube was vortexed for 5 seconds. Each tube was incubated at room temperature (18-25 °C) for 15 minutes. Add 1.0 ml of the "fix-and-lyse" mixture was added to the tube and vortexed immediately for three seconds. Each tube was incubated at room temperature for at least 10 minutes and was protected from light. Centrifugation of the tubes was done at 150 x g for 5 minutes and the supernatant was discarded. 3.0 mL of PBS was added. All tubes were centrifuged in 150 x g for 5 minutes and the supernatant were discarded by aspiration. The pellets were re-suspended by addition of 0.5 to 1.0 mL of 0.1% formaldehyde. All tubes were vortexed for 5 seconds. All tubes were analyzed by the flowcytometer.

2.3. Flowcytometric immunophenotyping

Samples were stained by monoclonal antibodies for the following antigens: CD45, HLA-DR, CD34, CD117, CD13, CD33, CD19, CD7, cytoplasmic markers (CD3, CD79a, MPO), CD11c, CD14, CD64, CD42a, CD41 and CD61 (Immunostep-Spain).

2.4. Quality Control

Depending up on pilot study in the quality control results (that saved in the Q.C system II software file) of EPICS XL flow cytometer, which adjusted the cut off points between negative and positive scale for every marker, Positivity was considered when ≥20% of the population expressed the marker. The parentheses was also recorded for most of the markers.

2.5. Data Analysis

Statistical analysis was done using SPSS 17, correlation tests were done using Pearson correlation coefficient.

3. Results

- **Frequency of AML:** Out of the 420 cases that were analyzed by flowcytometry at RICK centre during the period of mid-2010 till mid-2011, 105 cases were diagnosed as AML, having the frequency of 25.0% of all cases and 46.8% of all acute leukemia cases. AML-M0 was the most frequent among AML cases (27.6%), followed by AML-M1(21%), the least frequent was AML-M3 hypogranular (2.95%).
- **Age group:** The mean age was 35 years. The most frequent age group was 17-40 Table 3.
- **CBC findings: Hb:** AML-M7 showed the lowest mean ofHb(6.9g/dl),

TWBC: mean TWBC was 73.5x10^9/ml among AML-M7 and the lowest mean was 16.6x10^9/mlamong AML-M3v cases.

Platelets: the highest mean was 94X10^9/ml in AML-M0 and least mean was 38.5X10^9/ml in AML-M3 Table 1.

Table 1. The mean of hemoglobin, TWBC and platelets count among all AML cases and AML subtypes

Flow Diagnosis	HB g/dl	TWBC x10^3/µl	Plts x10^3/µl
AML-M0	8.4	39.7	94.0
AML-M1	9.0	52.5	83.2
AML-M2	7.8	35.0	39.8
AML-M3	7.2	34.1	38.5
AML-M3V	7.5	16.6	69.0
AML-M4	8.1	30.1	74.9
AML-M5	9.1	62.2	63.8
AML-M7	6.9	73.5	50.8
Total	8.3	42.6	70.8

- **Immunophenotyping markers**:
- **CD45:** All AML blasts were expressing CD45 with the mean positivity of 90.8% with no significant differences between the subtypes.

HLA: HLA was expressed in 70.2% of all cases, 84.5% of AML-M2, 66.7% of AML-M3v and 20% of AML-M3 with the strongest positivity in AML-M5 (mean positivity 64.7%) and weakest in AML-M3 (mean positivity 13.1%).

CD34: CD34 was positive in 78.7% of all cases (Figure 2), and in all AML-M0, only 33.3% of AML-M3v cases were positive for CD3, the strongest mean positivity was in AML-M0 (68.8%), followed by AML-M1, weakly positive in AML-M5 and AML-M7 (23.4% and 32.4% respectively)and lowest in AML-M3 and AML-M3v (17.3% for both).

CD117: CD117 was positive in 83.8% of cases tested. All AML-M2 and AML-M3v were positive for CD117, only 25% of cases of AML-M5 and AML-M7 were positive. The highest mean positivity (61.5%) for CD117 was found in AML-M0 and the least in AML-M5 (13.1%).

CD34, CD117 correlation: CD34 was studied in correlation to CD117 using Pearson correlation and was found to be of very strong positive correlation with AML-M3v, AML-M4 and AML-M7(r=1.0, 0.928 and 0.962 respectively), and strong positive correlation in AML-M2 (r=0.763) and strong negative correlation in AML-M3 (r=-0.689).

CD13, CD33 expression and correlation: CD13 and CD33 were also studied among the blast population having mean positivity of 51.7% and 50.3% respectively

in all AML cases. CD 13 was positive in 90% of AML-M4 cases, with the strongest positivity in AML-M3 (mean positivity of 59%) andweakest in AML-M7 (mean positivity 33.7%). CD33 was 100% positive in AML-M3v and AML-M5 and found to have higher positivity among AML-M4 and AML-M5 with mean positivity of 65.9% and 76.6% respectively and least weak in AML-M0 (38.4%). CD13 and CD33 were also studied for correlation using Pearson correlation and was found ofno correlation with all AML subtypes except for AML-M5 where there was negative very strong correlation(r=-0.913).

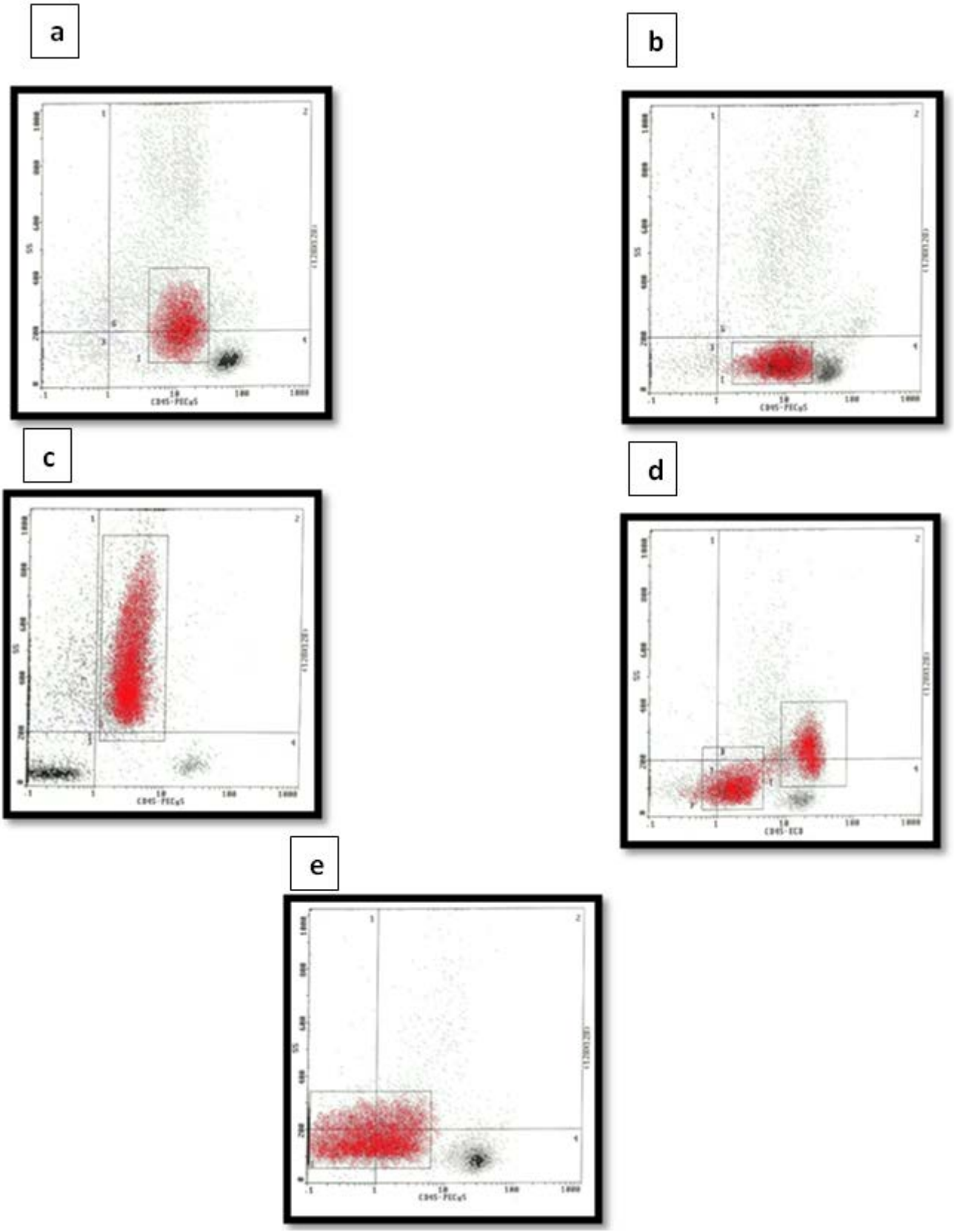

Figure 1. shows the CD45 expression with side scatter dot plot in different subtypes of AML with gating of the abnormal population labelled in red. (a): AML-M1 (b): AML-M2 (c):in AML-M3 (d): AML-M4 (e): AML-M7

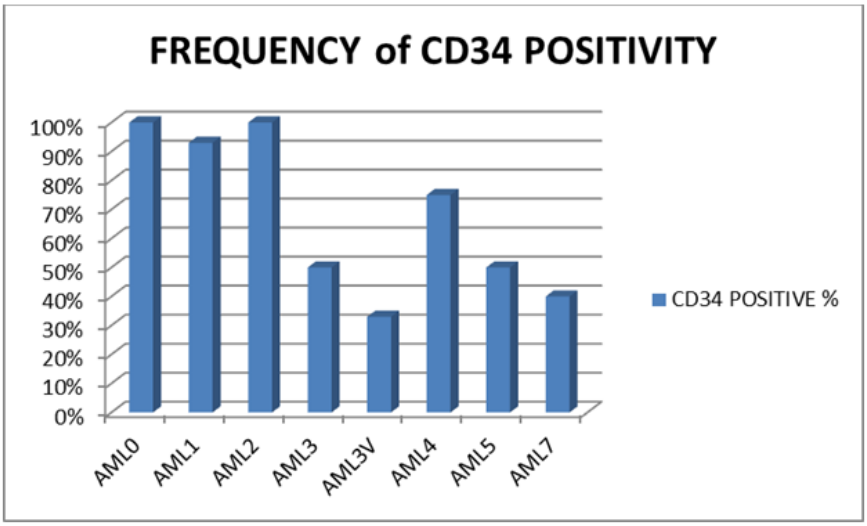

Figure 2. CD34 was positive in 100% of AML-0 and AML-2 while least positive in AML-M variant among population studied

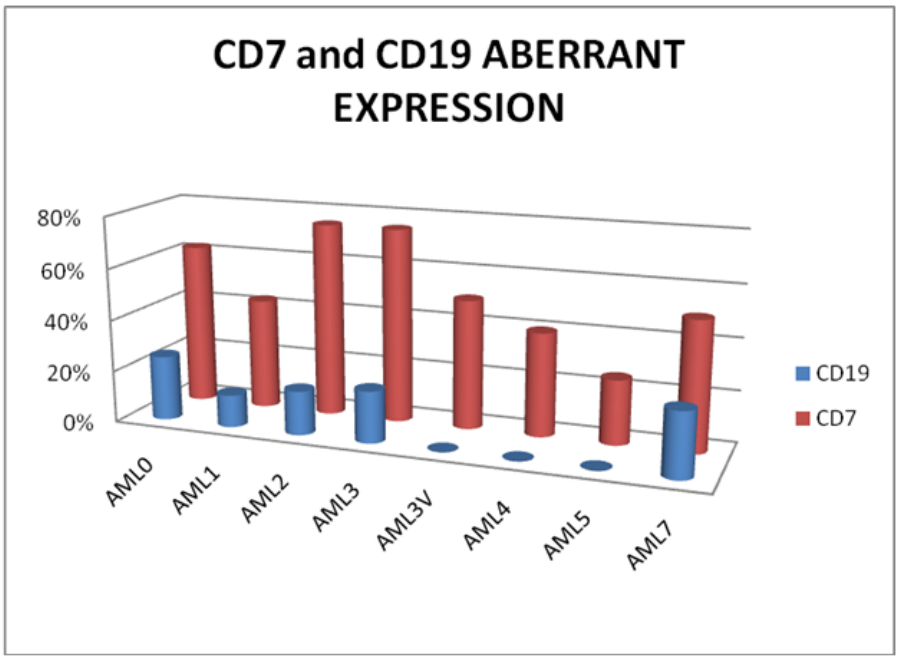

Figure 3. CD19 was totally negative in AML-M3V,M4 and M5 while CD7 was expressed in 75% of AML-M2 and M3 cases

CD7: CD7 expression was tested in 51 cases, being positive in 45.1% of them, and in 75% of AML-M3 followed by AML-M7 (60%) and in only 25% of AML-M5 patients. The expression was weak in most of the cases with mean positivity of only 27.4% among all cases and 38.4% inAML-M7 as the highest mean positivity (Figure 3).

CD19: only 13.6% of the 59 cases had positive expression. AML M2 showed the highest frequency followed by M3& M7. Twenty five percent of AML-M2 showed CD19positivity while none of the cases of AML-M3v, AML-M4 and AML-M5 were positive for this marker (Figure 3).

4. Discussion

4.1. Epidemiology

AML can occur at any age, however the overall incidence and the proportion of myeloid acuteleukemias increase with age [5]. In S. Ghosh study [6], adult AML accounted for 76% of all acute leukemias and childhood AML stood at 24%. In Mansoura experience study [7], in all acute leukemia cases, 68.9% were classified as AML.

Most studies have found a higher incidence of AML in males although the male predominance is not as distinct as in ALL. In our series, no male preponderance was present, male to female ratio 1.3:1 Table 2, while in Ghosh series[6], the male to female ratio was 2.5:1 compared to Karachi hospital study [8] which was 1.5:1.

In our series, AML-M0 was the most frequent AML subtype (27.6%) specially in adults, while the commonest AML subtype in Ghosh series was AML-M2 (34%) which was slightly higher than the frequency reported in the literature(27-29%), while the commonest AML subtype in Mansoura series was AML-M4/5 at 34.5%. The low frequency of M2 in our series (13.3%) might be due to fact that majority of cases could be diagnosed by morphology and cytochemistry stains such as Sudan Black B.

Table 2. shows the gender distribution among the cases and AML subtypes

			Gender		Total
			Male	Female	
Flow Diagnosis	AML-M0	Count	13	16	29
		% of Total	12.5%	15.4%	27.9%
	AML-M1	Count	13	9	22
		% of Total	12.5%	8.7%	21.2%
	AML-M2	Count	9	5	14
		% of Total	8.7%	4.8%	13.5%
	AML-M3	Count	5	6	11
		% of Total	4.8%	5.8%	10.6%
	AML-M3V	Count	2	1	3
		% of Total	1.9%	1.0%	2.9%
	AML-M4	Count	8	5	13
		% of Total	7.7%	4.8%	12.5%
	AML-M5	Count	3	3	6
		% of Total	2.9%	2.9%	5.8%
	AML-M7	Count	5	1	6
		% of Total	4.8%	1.0%	5.8%
Gender		% of Total	55.8%	44.2%	100.0%

Table 3. The age distribution among the cases and subtypes

			agegroup						Total
			<1 yr	(1-5)year	(6-16)year	(17-40)year	(41-60)year	>60year	
Flow Diagnosis	AML-M0	Count	0	3	2	7	10	5	27
		% of Total	.0%	3.0%	2.0%	6.9%	9.9%	5.0%	26.7%
	AML-M1	Count	0	1	7	4	3	7	22
		% of Total	.0%	1.0%	6.9%	4.0%	3.0%	6.9%	21.8%
	AML-M2	Count	0	3	2	3	3	2	13
		% of Total	.0%	3.0%	2.0%	3.0%	3.0%	2.0%	12.9%
	AML-M3	Count	0	0	3	5	2	1	11
		% of Total	.0%	.0%	3.0%	5.0%	2.0%	1.0%	10.9%
	AML-M3V	Count	0	0	1	0	0	2	3
		% of Total	.0%	.0%	1.0%	.0%	.0%	2.0%	3.0%
	AML-M4	Count	0	0	6	4	1	2	13
		% of Total	.0%	.0%	5.9%	4.0%	1.0%	2.0%	12.9%
	AML-M5	Count	0	0	1	3	2	0	6
		% of Total	.0%	.0%	1.0%	3.0%	2.0%	.0%	5.9%
	AML-M7	Count	3	1	1	0	0	1	6
		% of Total	3.0%	1.0%	1.0%	.0%	.0%	1.0%	5.9%
Total		Count	3	8	23	26	21	20	101
		% of Total	3.0%	7.9%	22.8%	25.7%	20.8%	19.8%	100.0%

Table 4. shows the mean of parentheses of the different markers among the total AML cases and AML subtypes

	CD34	HLA	CD45	CD13	CD33	CD117	CD19	CD7
AML-M0	68.63%	47.14%	89.62%	56.81%	38.42%	61.50%	12.87%	31.89%
AML-M1	57.85%	48.80%	90.06%	51.12%	45.22%	55.81%	11.58%	19.94%
AML-M2	62.79%	51.99%	88.32%	49.85%	53.84%	56.68%	9.48%	33.37%
AML-M3	17.32%	13.13%	91.08%	58.94%	54.29%	22.49%	14.08%	22.26%
AML-M3V	17.32%	24.47%	96.87%	47.70%	67.50%	53.90%	1.52%	27.15%
AML-M4	41.64%	41.95%	93.86%	54.86%	65.98%	35.00%	2.88%	23.36%
AML-M5	23.38%	64.71%	94.88%	38.34%	76.64%	13.10%	0.84%	16.41%
AML-M7	32.35%	25.34%	91.92%	33.72%	56.12%	21.04%	7.72%	38.40%
Total	50.36%	42.87%	90.81%	51.73%	50.35%	49.44%	9.58%	27.37%

AML-M0 was the least frequent AML among children less than 16 years (12.6%) while AML-M7 was the most frequent in infants which was in concordance with Horibe study in Japan [9] with frequency of 33% among children

aged less than 4 years but not in concordance with Ghosh study where no case of AML-M7 was reported in children in their series.AML-M2 was the most common in pediatric group followed by AML-M4 and M5 Horibe study stated a relatively higher incidence of M5 and lower incidences of M1 and M2 were found among patients aged 1 to 4 years than among the other age groups.AML-M3 (hyper and hypogranular) constituted 13.4% of all AML cases studied while in Ghosh study, and other studies reported lower AML-M3 percentages ranging from 5 to 14% of all AML cases [9,10,11,12,13]. Other investigators stated higher percentage to our results (24%) [7,14,15]. This variation between studies could be explained by the geographical variation of the different countries.

4.2. Immunophenotypic Markers

CD45: All AML subtypes were positive for CD45 with high mean positivity ranging from 88.3%-98.9% and found to be the commonest and the strongest expressed marker and showed different expression patterns in different AML subtypes. CD45 was expressed in 97.2% of AML cases in Khalidi [16] series. CD45 expression in our AML case was similar to other studies.

AMLs are defined immunologically by the expression of 2 or more of the following myeloid markers: myeloperoxidase (MPO), CD13, CD33, CDw65, and CD117 [17].

CD13: CD13 is normally expressed on hematopoietic stem cells, on the mature and immature elements of the myeloid and monocytic lineages, on eosinophils and basophils. During myeloid maturation, CD13 appears before CD33 on CD34 myeloid precursors.[18] Although frequently expressed, CD13 cannot be demonstrated in all cases of AML, its absence is related to a good prognosis[19].CD13 was positive in 82% of all AML cases in this series while it was found higher in Brian [20] series (91%) and was even higher in Ollivier [17] study (95%) and lower than those in Mansoura study[7] Bradstock [21] series (77.9% and71% respectively). CD13 was mostly expressed in AML-M4 (90%) followed by AML-M1 then AML-M0 and least in AML-M7 (50%) which also showed the lowest mean positivity as seen in parentheses (33.7%) denoting the weak positivity and 51.7% in all cases collectively. The sensitivity for CD13 was 0.82 and specificity 0.63 emphasizing the importance of CD13 expression in diagnosis of AML especially if combined with CD13, CD117 and MPO expression.

CD33: CD33is a myeloid antigen and appears during myeloid differentiation after CD13 at the hemopoieticprecursor level [18], The intensity of expression of CD33 is high on monocytes, and progressively decreasing on basophils, neutrophils and eosinophils [22]. CD33 was positive in 76.1% of AML cases of this study compared to 91% [17], 87% [20] and 79% [21] obtained by other series. CD33 was the myeloid marker that most commonly present in all AML subtypes in Mansoura study with a percent of 89.4% unlike this study that found CD117 being the most common myeloid marker expressed in the majority of cases of AML (83.8%). Expression was 100% in AML-M5 and AML-M3v followed by AML-M2 (90%). The expression pattern of CD33 contributes to some extent in the distinction between APL and AML-M1/2 in Mansoura [7] because none of their 26 APL cases lacked this antigen in contrast to approximately 11.1% of AML-M1/2 cases. In our study, only 60% of hypergranular expressed CD33 but was positive in all cases of hypogranular M3 this could be explained by the low number of AML cases in this study (3 cases for AML-M3 and 2 cases for AML-M3v).

CD34: CD34is expressed on many different cell types, more specifically on myeloblasts and very weakly on promyelocytes [23]. CD34 cells can be detected in cord blood, bone marrow and in the peripheral blood of normal subjects, where they constitute respectively about 1.5% and 0.1–0.01% of the elements [24]. The expression of CD34 has poor prognostic value, its absence was associated with a higher percentage of complete remissions, and it turned out to be an independent prognostic factor of best survival [19]. CD34 was positive in 78.7% of all cases studies and being expressed in all cases of AML-2 followed by AML-M0 (92.3%) and AML-M1 (90%). Variability in expression of CD34 was found in different studies: 71% [25], 68% [17], 52% [16]45% [26], and as low as 25% [6]. CD34 was reported by Mansoura study to have the highest positivity in AML-M0 followed by AML-M1/M2 subtypes. In AML-M3 in this series, CD34 was positive in 50% while was expressed in 33.3% of AML-M3v with weak expression in both types with mean positivity of 17.3%. this finding is opposite to what was reported by Gorczyca [27] who found CD34 positive in 4% of AML-M3 and67% in AML-M3v. Double negativity for CD34 and HLA-DR in AML-M3 was seen in 50% and in 33.3% of AML-M3v cases, while in Mansoura study it was higher (80.8%) this variation could be explained by the decreased number of AML-M3 and AML-M3v cases (11 and 3 cases respectively) included in the recent study.

CD117: CD117 is normally expressed by bone marrow hemopoieticprecursors, and can be detectable throughout the myeloid lineage until the promyelocyte maturation step and in the erythroid lineage until the pro-erythroblast stage [23]. According to the FAB classification, CD117 is preferentially associated with the AML-M0, AML-M1 and AML-M2 subtypes [28]. In AML-M3 subtype it is often positive, especially in the M3-variant form [29]. CD117 was positive in most cases tested (83.8%), in Mansoura study the percentage of positive expression was 74.3% and 80% in Brian series. In this series it was positive in all cases of AML-M2 and AML-M3v, this is followed by AML-M0 (96.2%) and least expression in AML-M5 and AML-M7(25%)

Lymphoid markers (CD19, CD7): CD7, It is the first T-associated antigen to appear during the maturation of T lymphocytes [30] and is a T cell antigen known to show aberrant expression in a subset of AML cases. Its expression in AML correlates with a lower incidence of complete remission [31]. In our series, CD7 (45.1%) was more commonly expressed among AML cases than CD19 (13.6%).CD 19 appears very early during the maturation of B lymphocyte precursors and is constantly expressed on mature B lymphocytes [32]. Expression of CD19 has been reported in 2–22% of observed cases, CD19 expression is relatively frequent in AML-M2 with translocation t(8;21) [18]. AML-M3 was the commonest AML expressing CD7 in 75% and was least expressed in AML-M5 (25%). CD7 was positive in 44% of AML cases in Launder series and CD19 in 13% within the same

population [33]. CD7 was positive in 28% [20], 23% [7], only 16% [16]and 11% [6]of cases while CD19 was found positive in 9.8% of cases included in Khalidi study [16], 8% in Brian study[20] and as low as 1.8% in Mansoura study [7].

5. Conclusion

Flowcytometry is a powerful tool for diagnosis and classification of AML, as proved by the current study which enabled us to subclassify AML in our centre specially AML-M0 and AML-3. Our results were comparable with that of other series. WHO 2008 classification is dependent on cytogenetics and molecular studies and in a laboratory where these facilities are not available, flowcytometry will be an important tool to diagnose AML. Further studies correlating together the flowcytometric and genetic findings are recommended.

Acknowledgement

The authors are thankful to Professor Hussein M. Ahmed (ex manager of Radio-Isotope Centre Khartoum) and to the sector donated the flowcytometry for RICK laboratory, who without their efforts and donation it would be impossible to provide flowcytometric service to our patients.

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