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MORPHOLOGICAL, CYTOCHEMICAL AND IMMUNOPHENOTYPING PATTERN OF ACUTE MYELOIDLEUKEMIA: A STUDY OF 95 CASES

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ABSTRACT

Aim: AML is a heterogenous disease characterized by arrest of normal hematopoetic maturation by dysregulated clonal expansion of karyotypically distinct neoplastic cells. The aim was to characterise clinical, morphological and cytochemical features of AML and corelate with the diagnosis provided by immunophenotyping method. **Methods:** A total of 95 patients of AML for a duration of one year(2014-2015) were included and immunophenotyping was done. **Results:** Majority cases (65.3%) were of pediatric age, with mean age of 27.2 years and male:female ratio of 1.3:1. Bleeding was raised in APML. Aleukemic cases were 1/4th(23.16%). AMLM6 was present in greater percentage as compared to literature. Mean Hb was 6.03g/dl) and Mean TLC was 37.87 X 10^9/L. CD33 was most common in AML(93.98%) followed by CD13, CD117, MPO, CD34 and HLADR. APML showed strong MPO, CD13 and CD33 positivity while CD34 and HLADR negativity. CD14, CD11b and CD64 positivity was seen in AMLM4. Auer rods were significant in identifying AMLM1, M2 and M3 subtypes with concordance of 54.74%. 100% concordance was seen between morphological and Immunophenotypic diagnoses while 76.19% between cytochemical and Immunophenotypic diagnoses. 33.68% showed aberrant markers, most common being CD19(31.43%) followed by CD7(18.18%) and CD2(9.09%) and were associated with worse hematological features. As per cytogenetic data, t(15;17) was most common in APML and t(8;21) in AMLM2. Conclusions: Aleukemic cases were significant(23%), hence, scrupulous PBS examination is recommended. We cannot trivialise the contribution of cytochemical studies where flowcytometry cannot be done. Immunophenotyping has become indispensable and is a boon to the diagnosis of AML.

KEYWORDS: Acute Myeloid Leukemia, Immunophenotyping, Myeloperoxidase, Aberrant.

Abbreviations: AML- acute myeloid leukemia, APML- acute promyelocytic leukemia, PB- peripheral blood, BM- Bone Marrow, MPO- myeloperoxidase, TLC- Total leucocyte count, CBC- Complete Blood Count, CD- Clusterof differentiation.

INTRODUCTION

AML is the most common type of leukemia in adults (about 25% of all leukemia) in the western world. Worldwide the incidence is approximately 3.7/1,00,000 population per year. The median age of diagnosis is 65 years. In pediatric age, AML comprises of 15-20% cases of leukemia with a peak incidence at 3-4 years of age.[1]

AML is a heterogenous disease clinically, morphologically, genetically and differs in course and prognosis. With recent advances, classifying it into homogenous subtypes allows us to diagnose it, distinguish the prognostic parameters and thereby refine the treatment strategies. The classification of AML has revolutionised over years. While FAB classification provided a subclassification of myeloid leukemia, the 4th edition of WHO classification(2008) incorporated new criteria for the recognition of some previously described neoplasms as well as added entities defined principally by genetic features. With the advent of flowcytometry, immunophenotyping analysis of antigens expressed by BM or PB cells has become a standard tool in the assessment of patients with leukemia.[2] In our study, we have used flowcytometry in conjunction with clinical, morphological features and cytochemical staining for the diagnosis of AML classified according to the FAB classification.

MATERIALS AND METHODS

A total of 95 newly diagnosed cases of AML were evaluated through a prospective and retrospective descriptive study at a tertiary care centre for a duration of one year(2014-2015). Evaluation of clinical features like fever, fatigue, weight loss, infection and bleeding,

lymphadenopathy, hepatosplenomegaly was done. CBC and peripheral smear evaluation was done. In addition, BM aspirates were collected in heparinized vials. PBS and BM aspirate smears were stained by Leishmann stain for morphological evaluation and cytochemical staining was done by Myeloperoxidase(MPO) using 3-3' Diaminobenzidene (Sigma D-8001)(DAB). Immunophenotyping was done by a four color flowcytometer BD FACS Caliber (Becton Dickinson-Fluorescence Assisted Cell Sorter) using appropriate pairs of monoclonal antibodies(CD45, CD2, CD7, cCd3, CD10, CD20, CD19, CD34, CD117, HLADR, CD13, CD33, MPO, CD14, CD11b, CD64, CD36 and CD16) conjugated to fluorescein isothiocyanate(FITC), phycoerythrin(PE), peridinin-chlorophyl-protein(PerCP) and Allophycocyanin(APC). Cytogenetic data obtained from patient record. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version15.0 statistical Analysis Software. The values were represented in Number(%) and Mean±SD.

RESULT

95 cases of AML diagnosed on immunophenotyping were evaluated for clinical, morphological and immunophenotypic characteristics.

Epidemiology: The mean age was 27.2 year(7 month-78 year). 65.3% were less than 16 years of age. The overall male:female ratio was 1.3:1(adult 1.06:1; pediatric 1.48:1). The minimum age was seen in AMLM7 subtype (7 months). As per FAB classification AMLM2 was the most common subtype(11/33) in adult patients. AMLM2 and AMLM1 both were equally common(20/62 each) in pediatric population. AMLMO(5/33) and M6(4/33) were more common in adult population as compared to pediatric age group(6/62 and 4/62, respectively). One case each of AMLM7 was seen in adult and pediatric population. APML and AML with monocytic differentiation showed similar distribution in adults and pediatric population(Table 1: Distribution of FAB subtypes in paediatric and adult age group).

Clinical features: Nonspecific marrow failure symptoms were seen. Most common was fever followed by fatigue, weight loss and infections. Lymphadenopathy was seen in 26% cases. Hepatomegaly and splenomegaly was seen in 18% and 15% cases respectively. Other abnormal mass comprising of orbital swelling was seen in 3/95 (3.16%) cases (6.45% of all cases of AMLM2, 2 cases and 25% of all cases of AMLM4, 1 case). Bleeding was seen most commonlyin M3 subtype(83%).

Morphology

Mean Hb was 6.03g/dl(pediatric 6.4; adult 5.3). Mean TLC was 37.87 X 10^9/L(pediatric 44.85 X 10^9/L; adult 24.75 X 10^9/L). A reduced TLC(< 11,000/cumm) was observed in approximately one-third i.e.32.63% cases(31/95), distributed chiefly among AMLM3(50% of M3 cases), M7(50% of M7cases) and M1 cases(42.31% of all M1 cases). Peripheral blasts was ≥20% in majority cases. Aleukemic presentation(<20% blasts) was seen in 1/4th cases (22/95);distributed amongst AMLM5> M4> M7>M2>M6>M1>M3>M0. Mean platelet count was 9.6 X 10^9/L (pediatric 39.1 X 10^9/L, adult 40.7 X 10^9/L). Eight cases had normal platelet count. Predefined morphological characteristics like monocytoid lobulated blasts with lacy chromatin were seen in AMLM4,M5; lobulated pleomorphic promyelocytes in AMLM3, and blasts with cytoplasmic vacuolation and blebs in AMLM7(Figure 1). Dysplastic blasts were observed in AMLM1 & M2, Auer rods were most frequently seen in AMLM3(83% cases), followed by AMLM2(71% cases), and AMLM1(46% cases). Positive cytochemical staining for MPO was useful in identifying 76% cases(64/84; excluding AMLM5, M6 and M7). Figure2.

Immunophenotype

The immunophenotyping results in AML subtypes are shown in Table 2. Most commonly expressed markers were CD33(94% cases) followed by CD13 (85% cases). MPO expression was seen in 48/61 cases(78.69%) distributed maximally amongst M3>M2>M1 CD34 expression was seen in 74% cases. All cases of AMLMO were positive for CD34.The expresssion was lower in AMLM1 and M2(81% cases) followed by AMLM4(75% cases) and M6 (66%). CD34 expression was seen in 20% cases of AMLM3. HLADR expression was seen in 72% cases. 20% cases of AMLM3 were positive for HLADR.

The immunophenotypic expression of various aberrant markers in FAB subtypes is shown in table 3. The most common aberrant antigens expressed were CD19(31% cases), followed by CD7(18% cases) and CD2(9% cases). The aberrant antigen expression was more common in pediatric cases(23/62) as compared to adult cases(2/33) aberrant antigen expression was more common in M0, M1, M2 subtypes. CD2 was the most aberrant antigen in AMLM3. Lymphoid antigen expression in AML was associated with adverse hematological features(WBC count>50,000/cumm, peripheral blasts>50%).

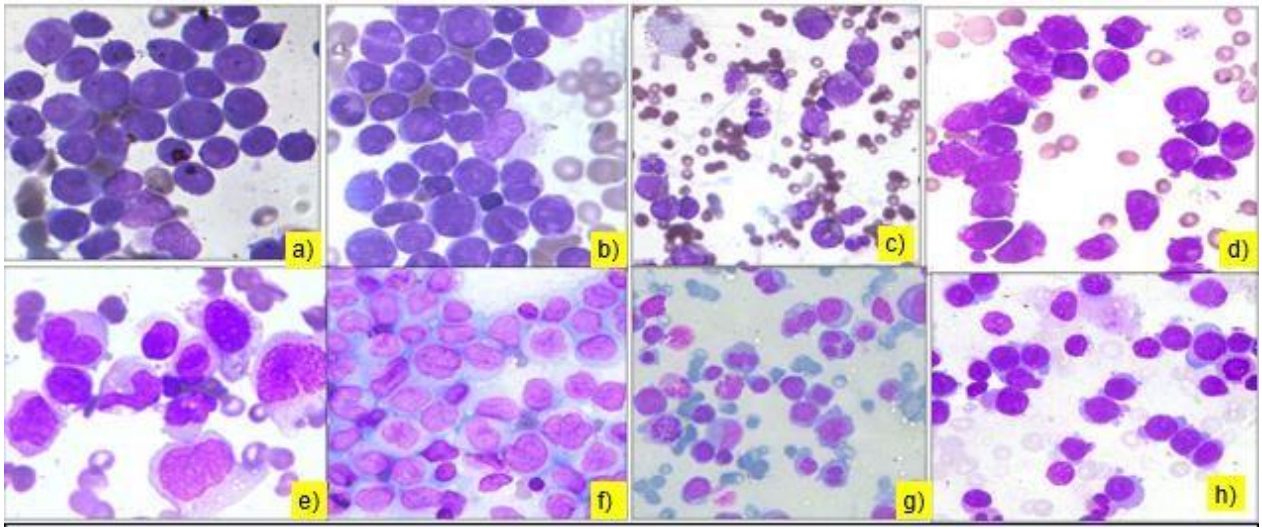

Figure 1: a).M0 without evidence of myeloid maturation b).M1>90%blasts <10% granulocytic maturation c).M2 >10%granulocytic maturation d)M3.lobulated Promyelocytes &f).M4&M5 Monocytoid blasts with lacy chromatin g)M6 Erythroid blasts with sieve like chromatin h)M7 Blasts with vacuolation& blebbing.

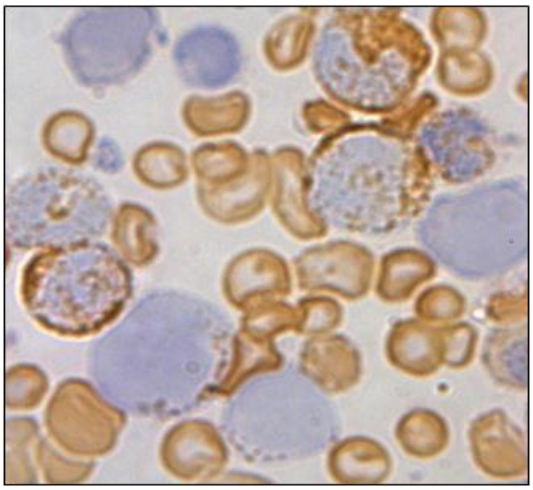

Figure 2: Myeloperoxidase positive myeloid blasts.

Table 1: Distribution of FAB subtypes in paediatric and adult age group.

Morphologicaldiagnosis	Pediatric group	Adults	Total
AML M0	6	5	11
	9.7%	15.2%	11.6%
AML M1	20	6	26
	32.3%	18.2%	27.4%
AML M2	20	11	31
	32.3%	33.3%	32.6%
AML M3	8	4	12
	12.9%	12.1%	12.6%
AML M4	3	1	4
	4.8%	3.0%	4.2%
AML M5	0	1	1
	.0%	3.0%	1.1%
AML M6	4	4	8
	6.4%	12.1%	8.4%
AML M7	1	1	2
	1.6%	3.0%	2.1%
Total	62	33	95
	100.0%	100.0%	100.0%

Table 2: The immunophenotyping results in various AML subtypes.

	CD34	HLAD R	CD13	CD33	CD117	MPO	CD7	CD19	CD2	CD11	CD14	C64
AML M0	100	80	81.82	90.91	70	62.50	27.27	40	10	--	0	0
AML M1	81.82	90.91	79.17	91.67	86.96	88.89	31.82	36.36	12.5	--	0	0
AML M2	83.33	82.35	85.19	92.59	92.59	94.12	8	34.78	4.76	0	0	0
AML M3	20	20	90	100	44.44	100	11.11	12.50	25	--	0	--
ML M4	75	75	100	100	50	66.67	0	50	0	100	50	100
AML M5									--	--		--
AML M6	66.67	80	100	100	100	0	16.67	0	0	0	0	100
AML M7	0	100	100	100	100	78.69	0	0	--	---	0	0
Mean	74.36	71.70	85.54	93.98	81.25	78.69	18.18	31.43		60	15.38	

Table 3: Comparison of the percentage of aberrant markers in different studies.

Year	Author	No. of AMLcases	CD19 +	CD7+	CD2+
2010	Mukda et al.	30	64.2%	35.71%	----
2011	Ihsan et al	106	13.6	45.1	
2007	Abdelhaleem et al	59	22%	25.8%	15.5%
2015	Our study	95	31.43%	18.18%	9.09%

DISCUSSION

Evaluation of demographic pattern of AML shows an increasing proportion of paediatric cases(65%) as compared to previous reports.[3] Our patient group has a lower mean age group than previously reported[4,5] AML in adults aged> 45 years has worst prognosis. In our population, 14% patients fell under this category. The overall male: female ratio had slight inclination towards male gender as reported previously.[3,6]

AMLM2 was the most common morphological subtype accounting for approximately 1/3rd of our cases, in coherence with previous reports.[3] In paediatric population, AMLM1 contributed to an equal proportion. Ihsan et al reported a higher proportion(21% of AML M1).[7] The AMLsubtypes with monocytic differentiation (AMLM4, M5) constituted 4% and 1% respectively which is lower than compared to the previous reports of 16-25%.[8] AMLM4 constituted 4% of our cases coherent to reports.[3] AMLM5, however, contributed to a minimal of 1% as compared to 20% in previous reports.[3,9] AMLM6 was seen in higher proportion(8.4%) as compared to previous reports i.e. 1.6%.[3,9] APML contributed for 12.6% which is higher than the reported frequency in literature.

Non specific marrow failure symptoms were most common clinical presentation as reported previously. Bleeding was overall seen in less than half of the cases(40%), which is higher than the previous reports[3] Bleeding was most commonly seen in AMLM3, coherent to previous reports. Lymphadenopathy(26%) and organomegaly (15%) was seen in a lower proportion of cases as compared to previous reports of 36% and 26%, respectively.[3] AMLM1 and M2 were more commonly associated with lymphadenopathy and organomegaly as compared to other subtypes. t(1;22) is associated with marked organomegaly[10](WHO). Inv16 (p13.1; q22) or t(16;16)(p13.1; q22) is associated with predisposition to cervical lymphadenopathy.[11] All our cases with t(16,16) had cervical lymphadenopathy. In inv3(q21; q 26.2) hepatosplenomegaly is more frequent than other subtypes of AML.[12] However, no such finding was observed in our study. Extramedullary involvement is usually seen in monocytic leukemia and so was in our patient group. Orbital granulocytic sarcoma was seen in only 3 cases. (2AMLM2 and 1AMLM5), much lower than previous case reports.[13]

Hematological parameters

Majority of patients had severe anemia(mean Hb less than 7 gm%) and significant thrombocytopenia (mean platelet count <50X 10^9/L). AML with inv3(q21q26.2) or t(3;3)(q21;q26.2) is associated with normal platelet count. In our patient group, eight cases had a normal platelet count, however, no correlation with cytogenetic abnormality could be done.

t(15;17) and t(6;9) are associated with low TLC.[14] In our study population, approximately 1/3rd cases fell under this category of low TLC. Half of these were promyelocytic leukemia, but for the others, no cytogenetic confirmation could be done.

t(6;9) is usually associated with AMLM2 or AMLM4 morphology while our cases showed AMLM7 and AMLM1 morphology.[15]

AML with t(8;21), inv 16, inv 3, t(3,3) can be diagnosed even if the percentage if blasts is< 20%. 1/4th of our cases had less<20% blasts in the PB. These findings highlight a careful examination in patients with lower TLC. Also, identification of a small proportion of blast cells can avoid unnecessary time delay in diagnosis of these critical cases.

In our study, 54.7% cases showed Auer rods while Neelam et al reported 40 % cases with Auerrods. 76.19% cases sowed concordance with Myeloperoxidase staining in our study while the other studies such as Mukda et

al[16], Gujral et al[6] and Belurkar et al[17] showed 89.6%, 75% and 91.6% MPO concordance, respectively.

Immunophenotype

Immunophenotyping was done for all cases. In our study population immunophenotyping showed 100% concordance with morphology. The previous reports have shown concordance ranging from 89 - 100%.(Belurkar et al[17], Kresno et al[18], Qadir et al[19], Kheiri et al[2], showed 100%, 89.1%, 96.52% and 99.43% respectively) CD33 was the most commonly i.e.93.98%(78/83 cases) expressed myeloid marker for all the AML subtypes Maximum positivity of CD33 was seen in M3,M4,M6 and M7 (100% cases) of AML followed by AMLM2, M1 and least in M0. In a study by Ihsan et al[7], CD33 was found to have higher positivity among AML-M4 and AML-M5 with mean positivity of 65.98% and 76.6% respectively, followed by M3, M7, M2, M1 and M0. CD13 was the next most common marker i.e.85.54%(71/83 cases) present in all the AML subtypes. Similar expression pattern has been previously reported.[3] Expression of MPO was seen in 78.69% cases (48/61). Maximum positivity was seen in M3(100%cases), followed by AMLM2 and M1. 62.5% cases of AMLM0 were positiv'0e for MPO on flowcytometry while on cytochemical staining by MPO, only 36.36% cases of AMLM0 were positive. Hence, flowcytometry is a more sensitive method for MPO identification. CD14 and CD36 positivity was more commonly associated with the monocytic leukemias.CD14 expression ranged between 11% to 87% and the highest was seen in the AMLM4 subtype. In study by Ghosh et al. CD14 expression ranged between 3.7% to 44.4% and the highest was seen in the AML M4. CD36 expression was highest in the AMLM5 and less in AMLM4 categories.[3]

Expression of immaturity marker CD34 was seen in 74.36% of cases(58/78). Maximum positivity was seen in M0 (100%) of AML followed by AMLM2 and M1.Least positivity was seen in AMLM3 and AMLM7. Expression of HLADR was seen in 71.70% of cases(38/53). Maximum positivity was seen in M7, M1, M2 types of AML. Least positivity was seen in AMLM3. Ghosh et al[3] reported immunophenotyping results in 260 cases of AML, which showed the positivity of CD34 in the range of 18.5% to 66.7% among the various subtypes. The highest positivity was seen in AMLM0 and AML M6. HLA-DR positivity was highest in case of AMLM2 and was less in AMLM0 and M4 subtypes. CD117 had maximum positivity seen in M6 and M7(100% cases) of AML followed by AMLM2, M1, M0, M4 and M3. In our study, HLADR, CD34, CD117 and CD13 were negative in 80%, 80%, 55.6% and 10% cases. Gujral[6] has reported similar negative expression in 95%, 95%, 51% and 2% cases, respectively.

AMLM0, AMLM1 and AMLM2 cases were positive for CD34, CD13, CD33, CD117 and HLADR. Cases of APML showed strong MPO positivity. CD34 and HLA-DR expression was absent in all cases of APML than previous reports.Ghosh et al[3] Elisabeth Paietta[20] and Bakke et al.[21]

In our study, 33.68%(32/95) showed presence of aberrant markers, most common being CD19(31.43%) followed by CD7(18.18%) and CD2(9.09%). Despite the presence of aberrant markers, myeloid origin of blasts is not doubtful because of additional confirmation using MPO(cytochemical and immunophenotyping MPO). A study by[16] Mukda et al, showed presence of 46.67% cases with aberrant markers with most common being CD19(64.2%) followed by CD7(35.71%). Also, Mehdi Jaredi et al[22], showed presence of 57.1% cases with aberrant markers.

However, Ihsan et al[7] found the most common aberrant marker as CD7(45.1%) followed by CD19(13.6%). Similarly, Abdelheem[23] et al showed CD7 as most common(25.8%) followed by CD19(22%) in their study. In study by Ghosh et al, expression of lymphoid antigens was seen in 15% cases of AML. CD7 was the most commonly expressed lymphoid antigen (11%). Sharma et al[24] also found CD7 as most common aberrant lymphoid antigen.

In our study, CD7 was most commonly expressed in AMLM1 while CD2 in AMLM3. In a study by Mehdi Jaredi et al[23], both CD7 and CD2 were maximally expressed in AMLM1. Also, in a study by Ihsan et al[7], CD7 was mostly expressed in AMLM2 and AMLM3(75%) and least in AMLM5, while CD19 was only expressed in AMLM0 and AMLM7.

In our study, we found AMLM7 is usually not associated with aberrant lymphoid antigens while Sharma et al[24] found the same for AMLM3. It was found that aberrant lymphoid antigen expression in adult AML is associated with adverse hematological features(WBC count >50,000/mm^3, peripheral blasts >50%).(20/23) CD19 positive cases and CD2 and CD7 cases had peripheral blast count >50% while(43/95) cases had >50% blast count. (9/23) CD19 positive cases, (3/8) CD2 and (4/14) CD7 positive cases had TLC >50,000/cumm while only 22/total 95 cases had high TLC. Similar findings were stated by Sharma et al.[24]

Thus, sequence of expression was CD33> CD13>CD117> MPO>CD34>HLADR>CD19>CD7 in our study while Ihsan et al stated the sequence as CD13>CD34>CD33>CD117>HLADR>CD7>CD19.

Cytogenetics was done in 34 cases. Of these 7 were APML with t(15;17). The translocation t(8;21) was found to be most common in remaining cases subjected to cytogenetic evaluation. It was detected in 11.76%(4/34) of cases. Of these 44.4% (4/ 9) cases were AMLM2 in correlation with previous reports.

CONCLUSIONS

A major proportion of paediatric AML cases point

towards changing epidemiological pattern of acute leukemias. The clinical features are similar in subtypes of AML except bleeding being more common in AMLM3, however they are not of much importance in differentiation of the subtypes. AML cases presenting with as aleukemic leukemia warrant a careful morphologicalexamination of the PBS.

The evaluation of morphological features of myeloid blasts was helpful in categorising subtypes of AML. There was 100% concordance between morphological and Immunophenotypic diagnosis and 76.19% between cytochemical and ImmunophenotypicDiagnosis. In cases where morphological features overlap or are inadequate for establishing adefinitive lineage, cytochemistry comes into use. MPO is easily done and sensitive for identification of myeloid series of cells. MPO expression in immunophenotyping was helpful in identifying AMLM0 cases negative on cytochemistry. Knowledge of aberrant expression of lymphoid markers in AML is essential to avoid misclassification as leukemias of ambiguous lineage.

Thus concluding, our study emphasized the significance of morphological diagnosis of AML by scrupulous examination of PB and BM smears even in the era of immunophenotypicdiagnosis. Also, we cannot understate the significance of cytochemistry, as they provide a cheaper alternative where flowcytometry cannot be done because of financial restrictions. But, again immunophenotyping has become indispensable in the present scenario and is a boon to the diagnosis of AML.

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