Pathology Section

Morphological Characteristics, Immunophenotyping and Cytogenetics in Acute Myeloid Leukaemia Patients at a Tertiary Care Centre, Gujarat, India

RAHUL RANKA[1], BEENA BRAHMBHATT[2]

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ABSTRACT

Introduction: Acute Myeloid Leukaemia (AML) is a group of disorders characterised by a spectrum of clinical, morphological, immunophenotypic and associated chromosomal abnormalities. The identification of cytogenetic abnormalities at diagnosis is important for the evaluation of the response to therapy and the identification of an early re-emergence of disease.

Aim: To examine the morphological characteristics of AML and emphasise the role of immunophenotyping and cytogenetics in subtyping AML.

Materials and Methods: This cross-sectional study was a prospective study, that was conducted from June 2018 to November 2021 at a tertiary care cancer centre, Department of Oncopathology in Gujarat, India. Following the inclusion and exclusion criteria, a total of 232 patients were diagnosed, with 21 being eliminated owing to the unavailability of samples for cytogenetic testing. Diagnosis of AML was based on morphology of Bone Marrow (BM) aspirates and flow cytometric immunophenotyping. Chromosomal analysis was performed on BM and peripheral blood by using standard cytogenetic technique. Descriptive statistics were used to analyse the data and calculated in number and percentages.

Results: There were 115 (54.50%) males and 96 (45.49%) females with age group between 1-72 years. The most common subtype was diagnosed as AML M1. Flow cytometry was done on 178 (84.36%) bone marrow and 33 (15.63%) peripheral blood samples. The CD33, CD13, MPO and CD117 were expressed in the vast majority of AML patient, aberrant expression of CD7and CD19 was seen. Total case 121 (57.34%) had normal karyotypes (the majority of cases), 86 (40.75%) cases had anomalous karyotypes consistent with t (8;21), t (15;17), and Inv16, and 4 (1.89%) cases were non informative.

Conclusion: The study concluded that, flow cytometry and cytogenetics should be performed routinely in all cases of AML. A multimodal diagnostic approach combining cytomorphology, multiparametric flow cytometry, accompanied by cytogenetic is needed to arrive at definitive diagnosis of AML.

INTRODUCTION

In terms of clinical characteristics, morphology, immunophenotype, and molecular genetics, AML is a heterogeneous disease [1]. Based on morphological characteristics, blast proportion, blast maturation, and cytochemistry, the FAB (French-American-British) classification method divides AML into eight subtypes [1]. Several more subgroups defined by recurring chromosomal abnormalities have been identified as a result of the development of immunophenotyping, cytogenetics, and molecular genetics [2-3]. As a result, AML is divided into six major groups by the World Health Organisation (WHO) [4].

The diagnosis and classification of acute leukaemia begins with morphology, which may be complemented by cytochemistry. Multiparameter flow cytometry is the preferred method for immunophenotypic analysis in AML because it can analyse a large number of cells in a short amount of time while simultaneously recording information about several antigens for each individual cell. At the time of diagnosis, cytogenetic and molecular genetic tests are required not only to identify specific genetically defined entities, but also to provide a baseline against which follow-up studies can be read to assess disease progression and prognosis [5-7]. Research efforts in the last decade have expanded the pathophysiologic molecular subsets of AML, through identification of prognostic, predictive and targetable molecular abnormalities [8,9].

The present research examines the morphological characteristics of AML and emphasises the role of immunophenotyping and cytogenetics in subtyping AML and provide prognostic importance for illness prediction.

MATERIALS AND METHODS

The current study was a cross-sectional study, that was conducted from June 2018 to November 2021 at a tertiary care cancer centre, Department of Oncopathology in Gujarat, India.

Inclusion and Exclusion criteria: All adult and paediatric patients having a complete blood count, peripheral smear, bone marrow aspirate showing any blast cell population, as well as samples for immunophenotyping and cytogenetics, met the inclusion criteria. Patients without sufficient samples or data were excluded. A total of 232 patients were diagnosed, with 21 being eliminated owing to the unavailability of samples for cytogenetic testing.

All of the specimens were acquired using conventional procedures. The Complete Blood Count (CBC) was performed using the BC 6800 Plus Mindray cell counter (seven parts) and the LH 750 Beckman coulter (five parts). Peripheral Smears (PS) and bone marrow aspirate smears were stained with the Wright stain.

The revised 2017 edition of the World Health Organisation's haematopoietic and lymphoid tissues was used to report AML diagnosis and subtyping [4].

Study Procedure

Total 178 bone marrow and 33 peripheral blood samples were collected in a Potassium Ethylenediamine Tetraacetic Acid (K$_2$EDTA)

vial for immunophenotyping. Total 100 µL of whole blood and/or bone marrow samples were obtained for the immunophenotyping assay and treated with the appropriate panel of AML antibodies for 10 minutes at room temperature (CD45, CD117, CD33, CD13, CD15, HLADR, CD14, CD34, and others). Following incubation, the cells were treated with an erythrocyte lysing solution (1:10) and incubated at room temperature for 10 minutes. The White Blood Cells (WBCs) were pelleted and washed twice with 2 mL of sheath fluid after centrifugation at 1000 rpm for five minutes.

Before being acquired, the final pellet was resuspended in a 500 µL Phosphate Buffered Saline (PBS) solution. On a Fluorescence Activated single Cell Sorting (FACS) Canto II eight-color flow cytometer with BD Diva software, data was collected and analysed [6,7]. Blasts were subtyped based on marker expression and side-scattered versus CD45 plots.

Traditional cytogenetic and Fluorescent In-Situ Hybridisation (FISH) techniques were used to analyse chromosomal data from pretreatment BM. For traditional cytogenetics, atleast 20 metaphases were examined, and 200 cells in interphase were examined using FISH. The worldwide system of cytogenetic or cytogenetic nomenclature was used to report the diagnoses {International System for Human Cytogenomic Nomenclature (ISCN)} [7].

STATISTICAL ANALYSIS

Descriptive statistics were used to analyse the data and the data was represented as number and percentages.

RESULTS

Adults accounted for 169 (80.09%) cases and children accounted for 42 (19.90%) cases, with the age range ranging from 1 to 72 years (median 37 years). There were 115 (54.50%) males and 96 (45.49%) females among the 211 cases. The M:F ratio of 1.2:1 showed a male preponderance.

Haematological findings: The average Haemoglobin (Hb) level was 7.5 gm/dL, accounting for 86% of cases and normal Hb levels accounting for the remaining instances. A total leucocyte count of more than 11,000/cumm was found in 60% of the patients, whereas leucopoenia (4000/cumm) was found in 31%. A total of 70% of the

people had moderate thrombocytopenia (platelet counts greater than 20,000×10³), while the rest had normal platelet counts.

Most common type was AML M1 accounting for 61 (28.90%) of the cases and AML M2 contributing to 56 (26.54%).

Flow cytometry: It was done on 178 (84.36%) bone marrow and 33 (15.63%) peripheral blood samples. The following antigens were the most commonly expressed

- Myeloid markers CD33, CD13, MPO, and CD117 were expressed in the vast majority of AML patients, with frequencies of 80.5% (170), 90.04% (190), 78.19% (165), and 81.99% (173), cases respectively. A significant proportion of AML M5 cases, 39.39% (13) were reported as MPO negative.

- Human Leukocyte Antigen (HLA)-DR and CD34 were expressed at a rate of 151 (71.56%) and 181 (85.78%), respectively, in stem/progenitor cells. Five of the 34 AML M3 cases expressed CD34, while one expressed HLA-DR.

- Aberrant expression of CD7 was the most commonly expressed lymphoid marker 48 (22.74%) in AML patients, followed by CD19 15 (7.1%) [Table/Fig-1].

Cytogenetics: In 211 cases of AML, karyotype tests revealed that 121 cases (57.34%) had a normal karyotype, 86 cases (40.75%) had an aberrant karyotype, and 4 cases (1.89%) were non informative. The AML M2, AML M3, and AML M4 were reported to have karyotype aberrations consistent with t (8;21), t (15;17), and Inv16, respectively. The relationship between morphology (FAB subtype) and immunophenotyping and cytogenetics is summarised in [Table/Fig-2].

All the findings of three methods i.e. blood smear, flow cytometry and FISH technique is depicted in [Table/Fig-3].

DISCUSSION

The 211 cases of AML were diagnosed by cytomorphology, flow cytometry, and cytogenetics in the present analysis included 19% of childhood cases and 81% of adult cases, which differed somewhat from Basharat M et al., [10] findings (27.3, 72%, respectively). In contrast, paediatric cases were 24% and 76%, in research by Ghosh S et al., [11]. AML M1 (28.90%) was the most common subtype in the present study, which is similar to Faleh AA et al., [12]

Classification (FAB subtype) (n=number of cases) N=211	MPO	CD79a	CD34	HLADR	CD64	CD14	CD15	CD13	CD117	CD33	CD19	CD7	CD41a	CD61
M0 (n=4) 1.89%	2/4	0	1/4	2/4	0	0	2/4	4/4	3/4	4/4	0	1/4	0	0
M1 (n=61) 28.90%	41/61	0	41/61	54/61	1/61	11/61	33/61	50/61	59/61	54/61	4/61	20/61	0	0
M2 (n=56) 26.54%	49/56	0	41/56	50/56	5/56	14/56	35/56	52/56	53/56	48/56	9/56	17/56	0	0
M3 (n=34) 16.11%	34/34	0	5/34	1/34	0	2/34	30/34	34/34	29/34	34/34	2/34	1/34	0	0
M4 (n=19) 9%	17/19	0	12/19	18/19	8/19	10/19	12/19	17/19	12/19	16/19	0	3/19	0	0
M5a (n=23) 10.90%	12/23	0	9/23	21/23	7/23	8/23	11/23	20/23	7/23	2/23	0	1/23	0	0
M5b (n=10) 4.73%	8/10	0	4/10	9/10	3/10	4/10	7/10	10/10	6/10	9/10	0	4/10	0	0
M6 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M7 (n=4) 1.89%	2/4	0	3/4	2/4	0	0	1/4	3/4	4/4	3/4	0	1/4	4/4	4/4

[Table/Fig-1]: Distribution of cases by marker positivity in AML.

Classification (FAB subtype) n (%)	M0 4 (1.89%)	M1 61 (28.90%)	M2 56 (26.54%)	M3 34 (16.11%)	M4 19 (9%)	M5 33 (15.63%)	M7 4 (1.89%)
Normal karyotype (sum=121)	2	44	34	0	11	29	1
Cytogenetic abnormalities	Inv16=1+8=1	t (8;21=-2 Inv16=5 +11=2 t (9;22)=3 Monosomy 7=1 Hyperdiploidy=1	t (8;21)=11 Inv16=6 Trisomy 11=1 t (2;19)-=1 Monosomy 16=1 -5q=1 Hyperdiploidy=1	t (15;17)=28 vt (15;17)=4 +8, t (15;17)=1 +21, t (15;17)=1	t (8;21)=1 Inv16=3 Mono7=1 Mono16=1 -5q=1 -7q=1	t(8;21)=1 Inv16=1 +8=1 Complex=1	+21=1 Isochr17=1 Non informative=1

[Table/Fig-2]: Cytogenetics profile of AML cases.
v-Variant, inv-inversion, t-translocation, +trisomy, -deletion
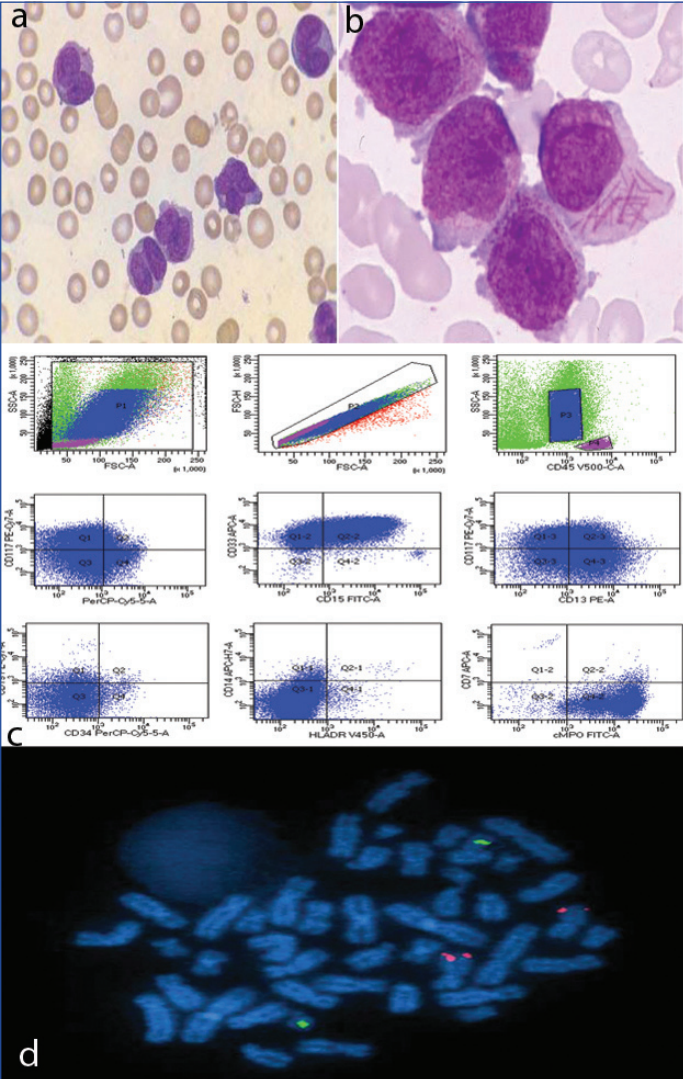
[Table/Fig-3]: Morphology, flow cytometry and cytogenetic in a case of AML M3. (a) PS shows bilobedpromyelocytes with intense azurophilic granules. (b) promyelocytes with bundles of numerous auerrods. Wright Stain 100X (oil immersion) (c)-flowcytometry -In BM blasts were gated using CD45 vs SSC A blasts expresses Myeloid markers MPO, CD13, CD33, CD117, CD15 and lacks expression of CD34 and HLADR (d) FISH technique, PML-RARA translocation using locus specific probe identifies t(15;17).
Red=PML locus (15q22);, Green=RARA locus (17q12)

Each FAB subtype has distinct immunophenotypes, including AML M3, which can be distinguished from other AML FAB subtypes. Five out of 34 cases of AML M3 in the present investigation exhibited CD34, which is similar to the findings of Faleh AA et al., [12] and Zheng J et al., [13] (1/4, 3/31, respectively). The AML-M4 and other the AML subtypes have varying levels of CD14 and CD15 expression. Strong CD64 expression distinguished AML M5 from M0 to M4 subtypes. However, dim or moderate CD64 expression did not distinguish M0 to M4 subtypes from M5 [5]. However, in the study by Dunphy CH [14], any CD64 expression associated with strong CD15 expression distinguished AML M4 or M5 from other AML subtypes, which was a similar finding in the present study.

The presence of CD41, a marker for megakaryocytic lineage, in AML-M7 [5] was consistent with Faleh AA et al., findings [12]. In the present study, CD7, a T-cell antigen known to have aberrant expression, was the most commonly expressed 48 (22.74%), followed by CD19 15 (7.10%). The same pattern was observed in studies conducted by Faleh AA et al., [12] (10%, 8%), Zheng J et al., [13] (14%, 10%), and Basharat M et al., [10] (26.4%, 1.2%).

Although morphological evaluation of BM aspiration and biopsy is still important for AML diagnosis, the presence or absence of specific cytogenetic abnormalities and acquired genetic mutations is clearly a cornerstone in predicting prognosis (favourable, intermediate, and unfavourable risk groups) and treatment [15,16].

Chromosomal abnormalities were found in 39.6%of AML cases in the present study. Balanced translocation was found in proportions comparable to other large series and was found to be correlated with FAB subtypes. The t (15;17) was observed in 13.27% of the cases in the present study, whereas it was observed in 7% of the cases in study by Faleh AA et al., [12], 14.3% of Y Chang [17], and 11% of Enjeti AK et al., [18] studies.The benefit of cytogenetic analysis is that it has the fundamental potential to detect structural or numerical anomalies, as well as, unique and uncharacterised abnormalities. Total 10% of AML patients have chromosomal abnormalities. The relevance of cytogenetic abnormalities and multilineage dysplasia in leukaemia subtyping was also highlighted in a recent WHO classification [19-21].

In AML M3, the present study found variant abnormalities such as {t (15;17) and t (15;11)} (0.5%), as well as, additional chromosomal abnormalities such as [t (15;17), trisomy 8] (1%) and [t (15;17), trisomy 21] (0.5%). Faleh AA et al., [12] and Y Chang [17] found an incidence of t (8.21%) in their AML patients (8.0% and 8.3%, respectively), which was confirmed in the present study (7%). In comparison to Ahmed's study, the authors found a higher incidence of AML with Inv16 (8%) in the present study (1%). The comparision of karyotypic pattern in AML patients in the present study with various previous study has been shown in [Table/Fig-4] [12,17,18,22-24].

study (AML M1=86%). In contrast, the M2 subtype predominated in a study by Basharat M et al., [10] and Ghosh S et al., [11]. (47.2% and 34.3%, respectively).

The use of flow cytometry in the diagnosis and subclassification of AML is essential. Flow cytometric immunophenotyping offers the advantage of great sensitivity and efficiency, when using a multiparametric method.

Karyotype pattern	Enjeti AK et al., [18] 2004 (N=454)	Faleh AA et al., [12] 2020 (N=180)	Y Chang, [17] 2017 (N=1432)	Rowley JD [23] 1984 (N=883)	Bacher U et al., [22] 2005 (N=2460)	Byrd JC et al., [24] 2002 (N=1311)	Present study (N=211)
Normal karyotype	59	29.4	42.3	46.5	58.2	44	39
t (15;17)	12	7	14.3	6.5	-	7	11
t (8;21)	7	8	8.3	6.7	4.3	6	7.5

t (9:22)	1.2	-	1.8	-	-	1	-
Inv 16	8	1	-	-	-	-	-
Del 5q	0.5	1	0.8	8.8	1.7	7	11
Del 7q	0.5	1.5	1.4	4.2	2.6	7	5
Trisomy 8	1	2.5	3.8	5.5	6.7	9	6
Trisomy 21	1	-	1.6	1.8	0.8	2	-

[Table/Fig-4]: Comparison of the karyotypic pattern in AML patients [12,17,18,22-24].
t- translocation, Inv- inversion

Limitation(s)

Limitation of study was the fact that molecular testing could not be performed due to non availability at the Institute.

CONCLUSION(S)

Flow cytometry and cytogenetics should be performed routinely in all cases of AML. Morphology combined with cytochemistry are used to reliably diagnose acute myeloid leukaemia. Flow cytochemistry is a very effective method for diagnosing and subclassifying AML efficiently and precisely. Cytogenetic analyses aid in the biological classification of AML and should be performed routinely, in all cases of AML, as required for risk stratification (prognostic index).

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PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Oncopathology, Gujarat Cancer and Research Institute, Ahmedabad, Gujarat, India.
2. Assistant Professor, Department of Oncopathology, Gujarat Cancer and Research Institute, Ahmedabad, Gujarat, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Beena Brahmbhatt,
100, Shivganesh Bunglows, Zydus Hospital Road, SG Highway,
Thaltej, Ahmedabad-380059, Gujarat, India.
E-mail: beena.brahmbhatt@gcriindia.org

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