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Haematological profile of patients with mixed-phenotype acute leukaemia from a tertiary care centre of north India

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***Background & objectives*: Mixed-phenotype acute leukaemia (MPAL) is a rare neoplasm with no definite treatment protocols and a distinctly poor outcome. Advancement in polychromatic flow cytometry has made its identification easier. This prospective study was designed to identify cases of MPAL and study their clinical presentation and haematological profile in a tertiary care hospital in north India.**

***Methods*: Ethylenediaminetetraacetic acid (EDTA)-anticoagulated bone marrow aspirate samples of patients diagnosed as acute leukaemia (AL) on the basis of morphology were utilized for immunophenotyping. A comprehensive panel of fluorochrome-labelled monoclonal antibodies targeting myeloid, B-cell, T-cell and immaturity markers was utilized. The patients diagnosed to have MPAL, on the basis of the World Health Organization 2008 classification, were selected for further analyses.**

***Results*: There were 15 (2.99%) patients with MPAL of the total 501 cases of AL. Seven were children, all males and mean age of 5.08±3.88 yr. Eight were adults, male:female=6:2 and mean age of 21.43±5.74 yr. Eight were diagnosed as B/myeloid and seven were T/myeloid. No association was observed between age and immunophenotype of MPAL. On morphology, 11 were diagnosed as AML and four as ALL, and no specific morphology of blasts was predictive of a MPAL.**

***Interpretation & conclusions*: MPAL appeared to be a rare neoplasm (2.99% of AL cases). A comprehensive primary panel of monoclonal antibodies should be used to identify this neoplasm known to have a poor outcome.**

Key words Acute leukaemia - flow cytometry - haematological profile - immunophenotyping - mixed-phenotype acute leukaemia

Most cases of acute leukaemia (AL) can be classified as acute myeloid leukaemia (AML) or acute lymphoid leukaemia (ALL) based on morphology, cytochemistry and a comprehensive panel of immunological markers. There are, however, some cases that remain difficult to classify with these techniques[1-3]. This is due to the co-expression of several myeloid and lymphoid antigens in the same cells or in two populations of cells. With the availability of increasing number of monoclonal antibodies, many such cases started being described in the literature[4]. These cases were designated previously with different terminologies - hybrid leukaemia or

mixed lineage[5-8], myeloid antigen-positive ALL[9,10], lymphoid antigen-positive AML[9,11,12], biphenotypic AL and mixed-phenotype acute leukaemia (MPAL). Various studies on MPAL showed an incidence of 2-5 per cent of all ALs[2,3,13-16]. Most of these studies have, however, utilized old scoring systems to recognize MPALs which have a tendency to overestimate its incidence. The World Health Organization (WHO) 2008[17] provided stringent criteria for diagnosis of MPAL, and very few studies have documented cases on the basis of WHO 2008 criteria[18,19]. Patients with MPAL have a distinctly poor prognosis, and there is the absence of a specifically designed, globally accepted treatment protocol. It seems that MPAL is a complex entity with heterogeneous clinical, immunophenotypic, cytogenetic and molecular genetic features[19]. This study was designed to identify patients of MPAL at a tertiary care hospital based on the WHO criteria and analyze their clinical presentation, haematological and immunophenotypic profile.

Material & Methods

This was a prospective study conducted in the department of Hematology, Postgraduate Institute of Medical Education & Research, Chandigarh, India. All consecutive cases of AL from July 2010 to June 2012, diagnosed on the basis of bone marrow and/or peripheral blood examination were included in the study. The clinical details and results of haematological investigations including complete blood counts and findings of bone marrow aspirate and trephine biopsy done from posterior superior iliac spine were noted. The aspirate smears were stained with May-Grunwald-Giemsa stain and the trephine biopsy were stained with Haematoxylin and Eosin stain for light microscopic evaluation. All MPAL patients were analyzed morphologically according to the French-American-British (FAB) criteria[20]. Part of bone marrow aspirate sample (approximately 1 ml) was utilized for extensive immunophenotyping by flow cytometry. In minority of paediatric cases with a very high leucocyte count and high percentage of blast, 3 ml of ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood sample was used for flow cytometry.

Processing for immunophenotyping by flow cytometry: Samples for flow cytometry were collected in EDTA-anticoagulated vials. These samples were processed by standardized lyse-stain-wash technique using in-house ammonium chloride-based lysis buffer (8.26 g ammonium chloride, 1 g potassium bicarbonate, 0.037g EDTA in 1 l distilled water, *p*H 7.2). A panel of monoclonal antibodies conjugated with four fluorochromes *i.e*., flourescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein (PerCP) and allophycocyanin (APC) was used. Four colour cocktails of pre-titrated antibodies used for lineage assignment comprised CD19 (Clone HIB19), CD10 (Clone HI10a), CD20 (Clone 2H7), CD22 (Clone S-HCL-1), cytoplasmic CD79a (Clone HM47), kappa (Clone TB28-2) and lambda (Clone 1-155-2) light chains, IgM (Clone G20-127), IgD (Clone IA6-2), CD1a (Clone HI-149), CD2 (Clone RPA-2.10), CD3 (Clone SK7 & UCHT1), CD4 (Clone RPA-T4), CD5 (Clone UCHT2), CD7 (Clone M-T701), CD8 (Clone HIT8a), T-cell receptor αβ (TCRαβ) (Clone T10B9.1A-31), TCRϒδ (Clone B1), cytoplasmic CD3 (Clone SK7 & UCTH1), CD13 (Clone WM15), CD33 (Clone WM53), CD117 (Clone YB5.B8), CD15 (Clone HI98), CD14 (Clone M5E2), CD64 (Clone 10.1), CD41 (Clone HIP8), CD61 (Clone VI-PL2), CD235a (Clone GA-R2), anti-myeloperoxidase (MPO) (Clone 5B8), human leucocyte antigen-D related (HLADR) (Clone L243), CD34 (Clone 581), CD38 (Clone HIT2), CD123 (Clone 7G3), terminal deoxynucleotidyl transferase (TdT) (Clone E17-1519) and CD45 (Clone 2D1) (BD Biosciences, San Jose, CA, USA). CD45 PerCP was the anchor marker in each tube to assist gating of blasts in majority of cases. The primary panel included both surface and cytoplasmic markers. The tubes with cytoplasmic markers *i.e*., anti-MPO, TdT, cytoplasmic CD3 and cytoplasmic CD79a were processed by treating lysed sample with BD FACS Permeabilizing Solution 2 (BD Biosciences, USA) buffer for 10 min followed by staining with monoclonal antibodies.

Since demonstration of MPO expression in cytoplasm of leukaemic cells remains critical for the diagnosis of MPAL, an isotype control was run in all cases, along with the tube containing 7 µl (pre-titrated) of FITC-conjugated anti-MPO antibody. A cut-off percentage (10%) was used to report a positive expression of MPO in leukaemic cells. Isotype control was used to exclude non-specific binding. In addition, normal lymphocytes in each sample were gated as a negative control for anti-MPO staining and neutrophils were gated to demonstrate a positive staining. At least 10,000 events were acquired in all cases of AL and the number of events was increased in any sample diluted with peripheral blood. The samples were

acquired on dual-laser BD FACSCanto II and analyzed by BD FACSDiva software. Both cytochemical and immunophenotyping results were corroborated for lineage determination of the blasts and diagnosis of a mixed lineage AL was made based on the WHO 2008 criteria of lineage assignment[21].

The study was carried out after approval of the protocol from the Ethics Committee of the institute and included patients only after obtaining written informed consent.

Statistical analysis: Statistical analysis was performed using SPSS for Windows version 17 (SPSS Inc., Chicago, IL, USA). All continuous variables were assessed for normal distribution and equality of variances and then subjected to independent sample *t* test or ANOVA. All categorical variables were subjected to Pearson Chi-square test or Fischer's exact test to assess significant variability.

Results

Five hundred and one patients with AL were included in the study. Of these, 221 (44.12%) were paediatric and 280 (55.89%) were adult patients, with an overall male:female ratio of 1.9:1. There were 303 (60.4%) cases of ALL, 183 (36.5%) of AML and 15 (2.99%) cases of MPAL. Amongst ALL cases, 59 per cent (179/303) were paediatric and 40.9 per cent (124/303) were adult patients with an overall male:female ratio of 2.5:1. Amongst AML cases, 80.9 per cent (148/183) were adults, whereas 19.1 per cent (35/183) cases were paediatric with an overall male:female ratio of 1.2:1. Of the 15 patients with MPAL, seven (46.7%) were paediatric (all male patients) with a mean age of 5.08±3.88 yr, whereas eight (53.3%) were adult patients (male:female=3:1) with a mean age of 21.43±5.74 yr. Overall, the male:female ratio of MPAL cases was 6.5:1. The mean age of presentation for paediatric AL cases was similar and averaged around 5.6 yr in ALL, AML and MPAL subtypes. Among the adults, the AML cases presented at a significantly higher age (mean=39.1±17.18 yr) (P<0.01) as compared to ALL cases (mean=26.7±13.80 yr) and MPAL cases (mean=21.4±5.74 yr).

Majority of patients with AL presented with fever, hepatomegaly, splenomegaly and/or lymphadenopathy. Significant difference between presentations of ALL, AML and MPAL was noted only for incidence of fever and lymphadenopathy. Fever was significantly more common in ALL as compared to MPAL patients (P<0.05) and lymphadenopathy was more often seen in MPAL than AML patients (P<0.001). Hepatomegaly and splenomegaly did not differ in MPAL patients when compared to ALL or AML cases.

Baseline investigations in MPAL cases revealed mean haemoglobin (Hb) of 8.22±0.57 g/dl, mean platelet count of 148.40±32.67 × 10^3/µl and mean white blood cell (WBC) count of 21.54±7.29 × 10^3/µl. Leucocytosis (WBC count >11 × 10^3/µl) was seen in six patients. Mean Hb and mean leucocyte counts did not significantly differ between MPAL and ALL/AML patients; however, mean platelet counts in MPAL patients were significantly high as compared to other two groups (MPAL vs. ALL, P<0.001 and MPAL vs. AML, P<0.001).

May-Grunwald-Giemsa-stained bone marrow aspirate smears were studied along with cytochemical stains *i.e.*, MPO and periodic acid-Schiff to arrive at a morphological diagnosis. In 14 patients blasts were seen in the peripheral blood smear ranging from 15 to 94 per cent in differential counts. All 15 MPAL patients were analyzed morphologically according to the French-American-British (FAB) criteria[20]. Table I summarizes the morphological characteristics of the blasts, and Figs. 1 and 2 show representative microphotographs of two cases of MPAL. Eleven of the 15 (73.3%) cases were considered as myeloid on morphology, of whom nine had ≥3 per cent blasts showing cytochemical MPO positivity, and the remaining two MPO-negative cases had morphological evidence of monocytic/monoblastic differentiation. One of the 11 MPO-positive patients also had clear evidence of monoblastic differentiation on morphology

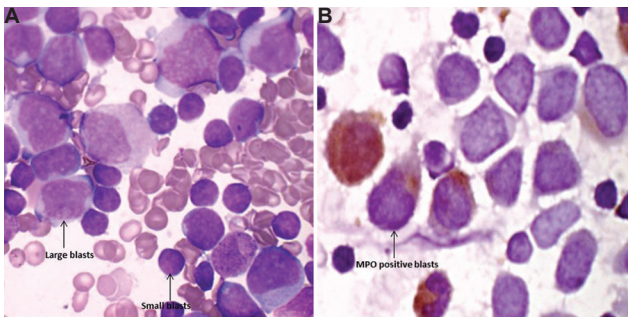

Fig. 1. (A) A case of mixed-phenotype acute leukaemia (B/myeloid) showing heterogeneous blast populations (small and large). The small blasts (arrow) with scanty cytoplasm, round nuclei, homogenous chromatin and inconspicuous nucleoli appear lymphoid in origin, whereas the large blasts (arrow) with moderate amount of granular cytoplasm, irregular nuclear contours, opened up chromatin and presence of nucleoli appear myeloid in origin; **(B)** myeloperoxidase (MPO) stain shows granular positivity in some of the large blasts (arrow).

Table I. Morphological, cytochemical and immunophenotypic profile of 15 patients with mixed phenotypic acute leukaemia (MPAL)

Patient no.	Blast in periphery (%)	Type of blast populations (morphology)	FAB subtype	MPO stain	Immunophenotyping
1	35	Heterogeneous	AML-M5b	Negative	B/monocytic
2	60	Heterogeneous	AML-M1	Positive	T/myeloid
3	84	Heterogeneous	AML-M2	Positive	B/myeloid
4	Nil	Homogeneous	ALL-L1	Negative	T/myeloid
5	47	Homogeneous	ALL-L1	Negative	T/myeloid
6	14	Heterogeneous	AML-M2	Positive	T/myeloid
7	55	Homogeneous	ALL-L3	Negative	B/myeloid
8	30	Heterogeneous	AML-M2	Positive	T/myeloid
9	85	Heterogeneous	AML-M5a	Negative	B/monoblastic
10	21	Homogeneous	ALL-L1	Negative	B/myeloid
11	15	Heterogeneous	AML-M2	Positive	B/monoblastic
12	50	Homogeneous	AML-M1	Positive	T/myeloid
13	30	Homogeneous	AML-M2	Positive	B/myeloid
14	88	Heterogeneous	AML-M1	Positive	B/myeloid
15	7	Homogeneous	AML-M5a	Positive	T/myeloid

FAB, French-American-British; MPO, myeloperoxidase; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia

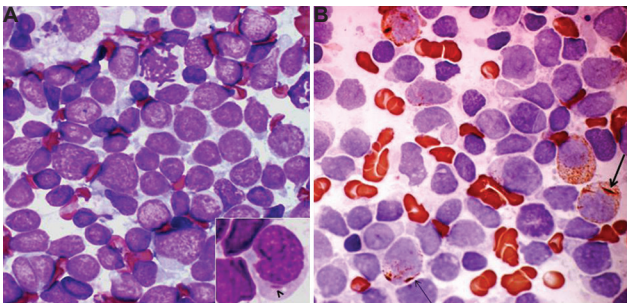

Fig. 2. (A) A case of mixed-phenotype acute leukaemia (T/myeloid) showing relatively homogeneous blast population comprising small to intermediate-sized blasts with scanty agranular cytoplasm, mostly rounded nuclei and absence of nucleoli. Inset shows a blast with the presence of Auer rod (arrowhead); **(B)** myeloperoxidase (MPO) stain shows sparse granular positivity in blasts (thin arrow) and highlights the presence of Auer rods (thick arrow).

with 3 per cent blasts showing faint MPO stain positivity seen as a few scattered cytoplasmic granules. Four of the 15 (26.7%) patients were considered morphologically as lymphoid. Morphological examination showed 8 of 15 cases with a heterogeneous blast populations comprising large and small blasts, whereas seven showed a homogeneous population of blasts (Table I).

On immunophenotyping, seven of the 15 MPAL patients (46.7%) were T/myeloid and eight (53.3%) were B/myeloid. None of the cases were diagnosed as B/T lymphoid or triphenotypic (B/T/myeloid). B/myeloid was seen more commonly in paediatric cases (5/8, 62.5%) as compared to paediatric T/myeloid cases (2/7, 28.6%); however, the difference was not significant though this could be attributed to the small sample size of MPAL cases. All eight patients with B/myeloid were males and five of seven patients with T/myeloid were males. There was no significant difference between B/myeloid and T/myeloid with respect to sex of patients, mean age, mean total leucocyte counts, mean platelet counts and mean percentage of blasts in the peripheral blood. Mean Hb of 94 g/l in T/myeloid patients was slightly higher than the mean Hb of 72 g/l in B/myeloid patients ($P<0.05$). No significant association was seen between morphological diagnosis (AML or ALL) and subtype of MPAL (B/myeloid or T/myeloid). However, two of the three cases of MPAL diagnosed as AML-M5 based on morphology turned out to be B/monocytic on immunophenotyping.

The immunophenotypic expression profile of B/myeloid and T/myeloid cases was compared (Table II). The immaturity markers CD34 and HLA-DR were expressed in all cases of B/myeloid leukaemia, however, were expressed in much lower frequency in T/myeloid cases. The expression of myeloid lineage-associated markers CD13 and CD33 was more frequent in B/myeloid when compared to T/myeloid cases. Aberrant expression of B lineage-associated markers such as CD19, CD10 and cytoplasmic-CD79a was seen

Table II. Comparison of immunophenotypic expression profile of B/myeloid and T/myeloid cases

Markers	B/myeloid (n=8)	T/myeloid (n=7)
Immaturity markers (%)		
CD34	8/8 (100)	4/7 (51.7)
HLA-DR	8/8 (100)	1/7 (14.3)
TdT	5/8 (62.5)	6/6 (100)
Myeloid markers (%)		
Anti-MPO	7/8 (87.5)	7/7 (100)
CD33	8/8 (100)	3/7 (42.8)
CD13	5/6 (83.3)	4/6 (66.6)
CD117	3/6 (50)	3/7 (42.8)
B-lymphoid markers (%)		
cCD79a	5/7 (71.4)	2/5 (40)
CD22	7/8 (87.5)	0/5 (0)
CD19	8/8 (100)	1/7 (14.3)
CD10	3/8 (37.5)	4/7 (57.1)
T-lymphoid markers (%)		
cCD3	0/8 (0)	7/7 (100)
CD2	-	6/7 (85.7)
CD7	0/7 (0)	7/7 (100)

MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase. Values in parentheses denote percentages

with variable frequencies in T/myeloid cases, whereas the T-lineage markers CD7 and cytoplasmic CD3 were not found in B/myeloid cases (Table II).

Discussion

MPAL/ALAL are defined as a biologically different group of leukaemia arising from precursor stem cell, co-expressing more than one lineage-specific markers and associated with poor prognosis, early relapse and extramedullary infiltration[1,17,22]. The first published reports on mixed leukaemias appeared in 1981 when monoclonal antibodies were first used to characterize leukaemic cells. This study noted co-expression of MPO and TdT in AML[23]. One of the earliest large series of BAL was published by Mirro *et al*[6] in 1985 who analyzed the frequency and significance of AL displaying both lymphoid and myeloid characteristics in 123 patients. Before WHO 2008 classification system, the diagnosis of MPAL was mostly based on scoring systems which gave different weightage for different markers according to generally accepted lineage specificity of markers. The first such system was described by Catovsky *et al*[24] in 1991 and was followed by a similar scoring system called European Group for the Immunological Characterization of Leukemia (EGIL) in 1995[4]. St. Jude's criteria for the diagnosis of MPALs for the first time utilized a combination of lineage-specific markers without scores to diagnose MPALs[25].

The frequency of MPAL was 2.99 per cent and was similar to that seen by Lee *et al*[13] (2.2%), Al-Seraihy *et al*[26] (1.7%) and Yan *et al*[19] (2.4%). The mean age of occurrence of MPAL was 12.5 (range 0.58-21) yr and indicated that MPAL was a disease seen in children and young adults. There was marked male predominance (male:female=6.5:1). Although a higher incidence in males has been reported by other studies[18,26], but the highly skewed gender ratio in the present study could be due to social factors leading to a biased presentation of male patients in the hospital.

Morphological diagnosis on bone marrow aspirates from MPAL patients was made in accordance to FAB classification. Eleven cases of AML were subtyped either as AML-M1 or M2 or M4/5. None of the cases represented AML-M3, M6 or M7. One of the MPAL patients in the present study, subtyped as T/myeloid, revealed the presence of Auer rods in blasts, highlighted especially on cytochemistry for MPO stain. Matutes *et al*[18] reviewed morphology in 90 MPAL cases and found ALL morphology in 39, whereas 38 cases were identified as AML, mainly M1 and M5 and rarely M2 or M4. No case of M3, M6 or M7 was identified. Thirteen cases had a dual population which was undifferentiated and difficult to classify on morphology. Overall, no particular morphology of blasts was indicative of MPAL although heterogeneous blast population comprising primarily large and small blasts would evoke the possibility of MPAL.

The analysis of immunophenotypic profile of the MPAL cases revealed eight B/myeloid and seven T/myeloid cases. Of the 100 cases of MPAL reported by Matutes *et al*[18], 59 were B/myeloid, 35 were T/myeloid, four were B/T lymphoid and two cases were identified as trilineage MPAL. Yan *et al*[19] showed that of the 117 MPAL cases, 64 (55%) were B/myeloid, 38 (33%) were T/myeloid, 14 (12%) were B/T lymphoid and one (0.9%) case was trilineage MPAL. Al-Seraihy *et al*[26] performed a retrospective reanalysis of 32 cases of MPAL and found that only 11 (1.7%) could be categorized as having MPAL according to the WHO 2008 criteria. Five of the 11 cases were classified as B/myeloid, another five were T/myeloid and one case was diagnosed as B/T MPAL. Overall,

B/myeloid MPAL appears to be the more common subtype; however, analysis of more number of patients would be required to confirm it. Gujral *et al*[27] showed that the polymorphous mixed blast morphology was more commonly associated with T/myeloid MPAL, whereas monomorphic morphology was associated with B/myeloid MPAL. However, no such association was seen in our study as well other studies[18,19].

In this study, a cut-off of 10 per cent was used in defining MPO positivity on flow cytometry for the purpose of myeloid lineage identification in categorizing a case as MPAL. However, no single cut-off percentage has been uniformly accepted. Literature reports the use of a wide variety of cut-off percentages, ranging from 3 to 20 per cent in different studies[28]. Since defining MPO positivity on flow cytometry is often critical for identification of MPAL, each flow laboratory needs to carefully standardize sample processing.

The understanding of MPAL has vastly improved over the years, which is attributed to the advancement in diagnostic modalities. This study was an attempt to understand the characteristic features of this unique subgroup of AL, however, was based only on flow cytometric evaluation. A definite subclassification of MPAL, in accordance to WHO 2008 criteria[17], requires cytogenetic/molecular workup, which was not undertaken in the present study. Another limitation of this study was a lack of long-term follow up of patients. Both of the above shortcomings need to be addressed in the future studies for better characterization of this neoplasm and better evaluation of response to treatment.

***Conflicts of Interest*:** None.

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