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ORIGINAL ARTICLE

Multiparametric Flow Cytometry in Mixed Phenotype Acute Leukemia

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Abstract Mixed phenotype acute leukaemia (MPAL) is a diverse group of leukemia of ambiguous lineage diagnosed when blasts in peripheral blood and/or bone marrow have antigens of more than one lineage or a mosaic of blasts belonging to more than one lineage. Retrospective analysis of 218 consecutive cases of acute leukaemia diagnosed by multiparametric flow cytometry (FCM) was done. MPAL cases were identified in accordance with European Group for the Immunological Classification of Leukaemias Criteria and World Health Organization 2008/2016 guidelines for lineage assignment. Nine out of 218 (4.1%) cases were classified as MPAL. Eight out of nine patients (88.8%) were male and 4/9 (44.4%) were < 20 years of age. There were three cases of B/T and T/myeloid MPAL each. Two cases were B/myeloid MPAL and one case was chronic myeloid leukaemia (CML) in B/myeloid blast crisis. B/myeloid MPAL and CML in B/myeloid blast crisis cases were Philadelphia chromosome positive. The latter case had a complex karyotype as well. Seven cases were treated with acute lymphoblastic leukaemia treatment regimen; two of them achieved complete remission (CR). The patient with CML in B/myeloid blast crisis was treated with imatinib based regimen, attained CR, underwent allogenic bone marrow stem cell transplantation, but developed graft versus host disease. Five patients died due to complications of febrile neutropenia early in the course of treatment (62.5%). The last patient (B/T MPAL) refused therapy and was lost to follow-up. Early accurate diagnosis of MPAL requires FCM. It may be misdiagnosed if a limited panel of antibodies is used.

Keywords Mixed phenotype acute leukemia · Multiparametric flow cytometry · Immunophenotype · Leukemia of ambiguous lineage

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Introduction

Acute leukemia are accurately diagnosed and classified into myeloid, T-lymphoblastic or B-lymphoblastic leukemia by meticulous examination of morphology, immunophenotyping, karyotyping and molecular analyses [1]. When the blasts show differentiation towards more than one lineage [2, 3], they are termed as mixed phenotype acute leukemia (MPAL) by the European Group for the Immunological Classification of Leukaemias (EGIL) Criteria [4] and World Health Organization (WHO) 2008/2016 guidelines [5, 6]. They constitute 2–5% of all types of acute leukemias [2]. MPAL have worse prognosis when compared to acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) [7]. Multiparametric flowcytometry (FCM) analysis is important in elucidating

aberrant markers in a single lineage leukemia as well as demonstrating the presence more than one lineage antigens in one blast population (biphenotypic) and presence of two blasts population of two different lineages (bilineal). In this study, we describe clinical, laboratory, morphological, immunophenotype and cytogenetic features of nine cases of MPAL diagnosed in our institute.

Materials and Methods

The study was reviewed and approved by institutional ethics committee. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. Retrospective analysis of consecutive acute leukemia cases diagnosed by FCM from January 2015 to June 2017 was conducted. Cases where definitive diagnosis of acute leukemia was not possible or where morphological diagnosis was unavailable were excluded from the study. The diagnosis of MPAL was given on the basis EGIL and WHO 2008/2016 criteria.

Clinical data included sex, age at presentation and mode of presentation of the disease, presence of hepatosplenomegaly, lymphadenopathy, therapeutic regimen given and clinical outcome.

Review of air dried peripheral blood and bone marrow aspirate smears stained with Giemsa stain was done for all the cases. The cellularity, the percentage of blasts along with morphology of blasts were tabulated. Myeloperoxidase (MPO), Sudan black B (SBB) and periodic acid Schiff (PAS) stains were done in all cases.

All cases were analysed by FCM using six color antibody panel, either on peripheral blood or bone marrow aspirate. The analysis was performed on BD FACS Canto II (Becton, Dickinson and company, San Jose, CA, USA). All reagents were obtained from the same manufacturer. Fluorochromes used were fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCPCy5.5, PE-Cy7, allophycocyanin (APC) and APC-H7. Antibodies used were cluster differentiation (CD) 45 for gating blasts, MPO, CD117, CD13, CD33, CD15 for myeloid lineage; CD64, CD14, CD36 and CD11c for monocytic lineage; CD3, CD7, CD2, CD1a, CD5, CD4 and CD8 for T-cell lineage; CD79a, CD19, CD10 and CD20 for B-cell linage; and CD34 and human leukocyte antigen-DR (HLA-DR) for the stem/progenitor cell clone. Stain–lyse–wash protocol was used for sample processing of surface antibodies. When simultaneous staining for surface (CD45) and cytoplasmic markers (MPO, CD79a and CD3) was to be done, surface staining was done followed by cytoplasmic staining where these markers were permeabilized with Perm 2. Both cytoplasmic and surface CD34 testing was done in one of the cases as it was part of the antibody panel which was found to be injudicious and abandoned later. The data was stored in list mode and analysed with BD FACS Diva version 8. After doublet discrimination, low side scatter and dim/negative CD45 events were gated as blasts. They were further noted for CD34 positivity. If negative for CD34, expression of CD117 or CD10–CD19 co-expression were considered as immaturity markers. A cut off of 20% positivity for surface markers and 10% for cytoplasmic markers was used as per EGIL criteria.

Conventional cytogenetic analysis was carried out on direct preparations or 24 h unstimulated culture of bone marrow cells according to standard techniques. A complex karyotype was defined when three or more clonal structural chromosomal abnormalities were present.

Results

This study included nine cases (4.1%) diagnosed as MPAL out of 218 acute leukemia cases as per the EGIL/WHO 2008-2016 criteria.

Clinical and Laboratory Details

Eight patients were male. Four patients were < 20 years of age. Total leucocyte count ranged between 4.6 and 313.3 $\times$ 10^3/µl. Mean peripheral blood blast % was 59.9. Two patients had peripheral lymphadenopathy and hepatosplenomegaly. All patients had varying degrees of anemia and thrombocytopenia. The clinical and laboratory findings are summarised in Table 1.

Bone Marrow Morphology

Bone marrow aspirates and biopsies of all cases showed hypercellular marrow with markedly elevated myelopoiesis and eight out of nine cases showed more than 50% blasts. Based on the morphology and cytochemistry, three cases were classified as AML, one case as chronic myelogenous leukaemia (CML) in blast crisis, 3 as ALL and 2 as acute leukemia (AL) (Table 2).

Immunophenotyping by FCM

There were two cases with B-lymphoid and myeloid (B/M) phenotype. Both patients showed MPO positive blasts. One patient had blasts with CD10–CD19 co-expression and CD13 positivity but CD33 was negative. The other case was CD10 negative but positive for cytoplasmic CD79a and CD19 along with monocytic markers. The third case

Table 1 Clinical and laboratory details

Case number	Sex	Age	Liver	LN	Spleen	TLC × 10^3/μl	PLT × 10^3/μl	Hb (gm/dl)	PB[!] blasts (%)
1.	M	38	+	−	−	8.1	101	9.3	30
2.	M	30	−	−	+	19.9	63	7.1	79
3.	F	8	+	+	+	4.6	31	4.9	20
4.	M	49	+	+	+	23.7	52	11.1	70
5.	M	35	−	−	−	71.3	83	4.9	80
6.	M	14	−	−	−	313.3	27	11.1	87
7.	M	16	−	−	+	95.4	68	9.1	24
8.	M	38	−	+	−	167	61	7.5	96
9.	M	14	−	+	+	6.6	5	3.1	53

LN lymphadenopathy, *TLC* total leucocyte count, *PLT* platelets, *Hb* hemoglobin, *PB* peripheral blood

Table 2 Bone marrow morphology

Case number	Cellularity	Blasts (%)	Size of blasts	N:C ratio	Number of nucleoli	PAS	SBB	MPO	Morphological diagnosis
1.	Hypercellular	68	L + M	I	2–3	+	+	−	AL
2.	Hypercellular	84	L + M	H + I	2–3	+	+	+	AML M2
3.	Hypercellular	96	L + M	H	1	−	−	−	ALL
4.	Hypercellular	63	L + M	I + H	2–3	−	+	+	AL
5.	Hypercellular	89	L	I	2–3	−	+	+	AML M2
6.	Hypercellular	90	L + M	H	1	+	−	−	ALL
7.	Hypercellular	47	M	H	1	−	+	+	CML blast crisis
8.	Hypercellular	96	L	I	2–3	+	+	+	AML M1
9.	Hypercellular	96	L + M	H	1	+	−	−	ALL

L large, *M* medium, *H* high, *I* intermediate, *AML* acute myeloid leukemia, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukaemia, *MPO* myeloperoxidase, *SBB* Sudan black B, *PAS* periodic acid Schiff

was of CML in B/M mixed blast crisis where blasts expressed CD19, CD10 and were moderately positive for MPO which was confirmed by IHC. Three cases with T-lymphoid and myeloid (T/M) phenotype were identified. In 2 cases, MPO was positive by FCM. The third case was a bilineal leukaemia where in addition to T-lymphoblasts, there were blasts of monocytic lineage. In this case, MPO was negative by MFC, but positive on IHC. For cases where MPO positivity was confirmed by IHC, a cut-off of 3% positive blasts was considered as positive MPO. One of the T/M MPAL showed lineage switch to AML at the end of 4 weeks of therapy. All three cases were negative for surface CD34 although one case showed cytoplasmic CD34 positivity. Three cases had a phenotype of B-lymphoid and T-lymphoid (B/T). One of these patients was an 8 year old female child with Down's syndrome who had a mixture of blasts of B and T lineages. Another patient with B/T MPAL had relapsed after 18 months of disease free interval when follow-up FCM showed blasts of only T lineage. The details are mentioned in Table 3.

Cytogenetics

Cytogenetics information was available for six out of nine cases. Two cases of B/myeloid MPAL were positive for t(9; 22) (q34; q11.2). One case additionally showed deletion in the long arm of the same chromosome 1 at the region q25q44. 3 and translocation between the long arms of one of the chromosome 1 and 4 at the region q21 and q12. 2. Rest of the 4 cases showed normal karyotype (Table 3).

Treatment and Outcome

Five patients were treated with acute lymphoblastic leukaemia (ALL) treatment regimen. Two of these patients achieved complete remission as assessed by minimal residual disease evaluation by RT-PCR. One patient of B/T MPAL relapsed after 18 months of event free period and succumbed to death. The remaining three patients died due to complications arising from febrile neutropenia early in the course of treatment. The patient with CML in

Table 3 Lineage specific immunophenotype markers and cytogenetic features in each case

Case no.	Myeloid markers	B lymphoid markers	T lymphoid markers	Monocytic markers	Immaturity markers	EGIL Score	WHO 2008/2016 diagnosis	Cytogenetics
1	MPO, CD13	CD19, CD10	–	–	CD34, HLA-DR	B = 2; myeloid = 3	B/myeloid	46, XY, t(9; 22) (q34; q11.2) [10]
2	CD117, CD13, CD33, MPO		Cytoplasmic CD3, CD4	CD11c	Cytoplasmic CD34, HLA-DR	Myeloid = 5; T = 2	T/myeloid	46, XY [19]
3	–	CD19, CD20, cytoplasmic CD79a	CD3, CD7, cytoplasmic CD3, CD4, CD5	–	CD34, HLA-DR	B = 2; T = 5.5	B/T	Not available
4	CD13, CD33, MPO	–	Cytoplasmic CD3, CD5, CD7	CD64, CD11c, CD14	–	Myeloid = 4.5; T = 3.5	T/monocytic	46, XY [19]
5	MPO, CD13, CD33	CD19, cytoplasmic CD79a	–	CD11c, CD64, CD36	CD34, HLA-DR	Myeloid = 4.5; B = 3	B/ myelomonocytic	46, XY [15], t(9; 22)(q34; q11.2), 46XY [4]
6[a]	–	CD19, CD10, CD20	CD3, cytoplasmic CD3, CD4, CD7	–	CD34	B = 3; T = 4.5	B/T	Not available
7	MPO	CD19, CD10	–	–	CD34, HLA-DR	Myeloid = 2; B = 2	CML in MPAL (B/ myeloid)crisis	46, XY, der(1)t(1; 4)(q21; q12), del(1)(q25q44), t(9; 22)(q34; q11.2) [19]
8[b]	MPO, CD117, CD13	–	Cytoplasmic CD3, CD7	CD64	HLA-DR	Myeloid = 4; T = 2.5	T/myeloid	46, XY [15]
9	CD117, CD13	CD19, cytoplasmic CD79a, cytoplasmic CD22	Cytoplasmic CD3, CD7, CD5	–	CD34, HLADR	B = 5; T = 3.5	B/T	Not available

MPAL mixed phenotype acute leukemias, *EGIL* European Group for the Immunological Classification of Leukaemias, *WHO* World Health Organization, *CD* cluster differentiation, *HLA-DR* human leukocyte antigen-DR

[a]Relapse after 18 months of event free period with T cell acute lymphoblastic leukemia

[b]Lineage switch to acute myeloid leukemia at the end of 4 weeks of therapy. FCM on cases 1, 3 and 7 was done on peripheral blood; for rest of the cases bone marrow aspirate was used for FCM

B/myeloid blast crisis was treated with imatinib based regimen, attained complete remission (CR), underwent allogenic bone marrow stem cell transplantation, had developed graft versus host disease and is alive after 12 months of follow-up. One patient with T/myeloid was started on acute myeloid leukaemia treatment protocol, but died within a week after the beginning of therapy. The remaining two patients (T/myeloid and B/T) refused therapy and were lost to follow-up (Table 4).

Discussion

Most of the acute leukemia can be classified as myeloid, B or T lymphoid leukemia based on immunophenotyping. The problem arises when there are more than one lineage specific markers detected in a single population of blasts or there a mixture of blasts belonging to two different lineages. These cases have been historically labelled by a variety of names, such as mixed lineage, bilineal and biphenotypic leukemia. WHO 2008 classification of myeloid neoplasms and acute leukemia suggest the use of designation 'MPAL' [4]. The information about MPAL is limited as it accounts for only about 2–5% of all the

Table 4 Treatment and outcome

Case number	Diagnosis	Treatment	CR	Current status
1	B/myeloid	Vincristine +	No	Upper gastrointestinal bleed, pneumonia, death
		Daunorubicin +		
		Intrathecal		
		Methotrexate +		
		L-asparaginase +		
		Imatinib		
2	T/myeloid	Refused treatment	LFU	
3	B/T	BFM95 protocol	Withheld treatment due to pneumonia	Pneumonia, sepsis, massive pulmonary haemorrhage, death
4	T/myelomonocytic	Vincristine +	Withheld treatment due to pneumonia	Fungal pneumonia, thrombus in right subclavian vein, death
		Daunorubicin +		
		L-asparaginase		
5	B/myelomonocytic	BFM95 protocol + radiotherapy	Yes	Alive
6	B/T	BFM95 protocol + radiotherapy	Yes, relapsed after 18 months of event free period	Death
7	CML in MPAL (B/Myeloid)crisis	Imatinib +	Yes	Alive
		Mycophenolate moefetil +		
		Tacrolimus		
		Allogenic bone marrow transplant		
8	T/Myeloid	Vincristine +	Withheld treatment due to pneumonia	Bilateral Klebsiella pneumonia, death
		Daunorubicin +		
		L-asparaginase +		
		Cytarabine C		
9	B/T	Refused treatment	LFU	

CR complete remission, *LFU* lost for follow-up, *MPAL* mixed phenotypic acute leukaemia, *BFM* Berlin–Frankfurt–Munster, *CML* chronic myelogenous leukaemia

leukemias. There are two large studies which describe the characteristics of MPAL in People's Republic of China (n = 117) [8] and Europe (n = 100) [9]. There are no larger series studied in India [10, 11].

It is common for acute leukemias to aberrantly express protein markers more typically associated with other lineages, for example, expression of the myeloid markers CD13 and CD33 in B-ALL or T-ALL and expression of the T/NK-cell markers CD7 and CD56 in AML. The aberrant and complex patterns of marker expression in acute leukemia created a need for consensus criteria for lineage assignment. Major breakthrough in the diagnosis of MPAL came when the EGIL criteria was proposed in 1995 which has undergone several modifications although the weightage given to certain markers has been questioned by several investigators [3]. The WHO 2008/2016 criteria for lineage assignment has also been debated [4]. However, there was no discordance in the diagnosis with the use of either criteria in this series (Table 3).

In this study, eight patients were male and five were < 20 years of age. Such distribution was not observed in other studies [3, 8, 9]. There was no correlation between immunophenotypic subtypes and sex or age. Most consistent clinical feature was splenomegaly. Varying degrees of anemia were noted irrespective of the subtype of MPAL. Total leukocyte count was markedly increased in cases associated with T-lymphoid blasts. Morphologically, the patients were classified as AL or ALL or AML. Although five patients had blasts of two different morphology, the final diagnosis relied upon cytochemistry, specifically on

MPO. Apart from this, no other clinical or laboratory feature hinted the presence of MPAL. Hence the diagnosis of MPAL depended solely on immunophenotyping, provided AML with recurrent genetic abnormalities and myelodysplasia related changes were ruled out after molecular testing. Thus WHO 2008/2016 defined lineage specific markers have to be included in the routine acute leukemia immunophenotyping panel so that the cases of MPAL are not underdiagnosed [9].

There were 33.3% of B/myeloid, T/myeloid and B/T MPAL each in this series. This is in contrast to other major studies [8, 9] where B/myeloid MPAL accounted for $> 50\%$ cases.

Eight cases showed positivity for CD34 and HLA-DR indicating their origin from an early precursor. All cases with myeloid component had MPO positive blasts except for case with T/monocytic differentiation. The WHO criteria for lineage assignment mandates MPO testing by FCM, immunohistochemistry (IHC) or enzyme cytochemistry (EC) [5, 6]. The sensitivity of these methods vary greatly, hence these three techniques are complementary to each other [12]. The most sensitive method for detection of MPO is reverse transcription-polymerase chain reaction which can demonstrate MPO mRNA even in ALL lymphoblasts [13], although this method has not been recommended by WHO for MPO testing. Testing by FCM may also give varying results depending on what negative control was used when interpreting partial positivity for MPO by FCM. The reason for this is that most blasts have greater auto-fluorescence than mature lymphocytes. Hence, a negative blast population will have a higher median fluorescence intensity than a negative lymphocyte population [12]. Because of this uncertainty, current practice in our institution is to test for MPO positivity by EC in all cases of acute leukemia, followed by MPO testing by FCM. If the EC shows more than MPO positivity in more than 3% blasts or there is partial or weak expression of MPO by FCM, the positivity was confirmed by IHC. Since the sensitivity of each technique varies between laboratories, it is important for each laboratory to define the positive interpretation of MPO and the negative control.

There is no specific chromosomal abnormality that is seen in association with MPAL. However, t(9; 22) (q34; q11.2) is the most commonly observed abnormality followed by rearrangement in 11q23 MLL gene [9]. In this study, we had two cases which were positive for t(9; 22) (q34; q11.2). One of these case was CML in MPAL crisis who had additional complex karyotypic abnormality. No association was observed between clinical outcome and chromosomal abnormality although in larger studies poor outcome was noted in the presence of t(9; 22) (q34; q11.2) [14]. The common genetic mutations seen in AML and ALL are not found in MPAL [8].

In this series only two patients out of nine were in remission. The MPAL blasts probably arise from a multipotent progenitor cell with an ability to differentiate along both lymphoid and myeloid lineages [1]. This may have rendered them chemoresistant due to slow replication. The other possible reason for poor response to therapy could be lineage plasticity as in one of the T/M MPAL cases which showed lineage switch to AML at the end of 4 weeks of therapy. Mortality is high in MPAL. High incidence of CD34 positivity, lack of definite recommendations for induction and high incidence of relapse has led to a lower overall and disease-free survival in patients of MPAL [15].

Special mention needs to be made of a particular case of the female child of 8 years who had Down's syndrome and developed B/T MPAL. Down's syndrome is associated with transient abnormal myelopoiesis and AML (with megakaryoblasts). There are only anecdotal reports of mixed phenotype blasts in Down's syndrome [16]. This is the first reported case of B/T MPAL in a child with Down's syndrome. However, further molecular testing was not possible in this case.

Another rare entity in this series was the case of B/myeloid blasts crisis in CML. The patient in question was a 16 year old boy with fever, night sweats and chills, along with pallor, massive splenomegaly (11 cm left subcostal margin) and multiple ecchymotic patches over flexor aspect of the knees. Total leukocyte count was 95,400/cmm, with 24% blasts, 19% myelocytes and 01% basophils with anemia and thrombocytopenia. Bone marrow examination showed granulocytic hyperplasia with 47% blasts with immunophenotype of MPAL (Table 3, case 7). RT PCR showed BCR/ABL1 e13a2 corresponding to p210 kd protein. FISH analysis showed a complex karyotype and t(9; 22) in both neutrophils and blasts. RT-PCR on follow-up also showed BCR/ABL1 p210 kd protein. Hence, the final diagnosis was chronic myelogenous leukemia in mixed phenotype blast phase with t(9; 22); BCR–ABL1. In contrast, in case of de novo MPAL with t(9; 22)(q34.1; q11.2), the p190 fusion transcript is more common than the p210 transcript. If the p210 transcript is present, CML in a mixed blast crisis should be considered in the differential diagnosis, especially if there are two distinct lymphoid and myeloid populations [5]. There are six such cases reported so far [17–19]. This is the seventh case of B/myeloid blast crisis reported till date.

There are several studies which debate the therapeutic strategies in MPAL. In spite of many differing opinions, consensus is that molecular testing for t(9; 22) (q34; q11.2) and FLT3/ITD must be done as they are the potential targets for therapy [20–22]. The poor response to therapy is also attributed to high proportion of patients with adverse cytogenetic abnormalities [15].

Due to low incidence of MPAL, large studies on treatment and prognosis of MPAL are lacking. It is important to follow a consistent system of lineage assignment as using different criteria for different cases may result in suboptimal treatment [23]. Majority of MPAL with T cell lineage as a component are pathobiologically similar to AML. However, they respond better with ALL directed regimen, even those who did not have good outcome with initial AML directed therapy [24]. ALL directed regimen should be followed by allogenic stem cell transplantation [25, 26]. Patients with t(9; 22) (q34; q11.2) may benefit with addition of tyrosine kinase inhibitors like dasatinib [27]. Majority of therapeutic approaches are based on uncontrolled, retrospective studies [28]. Immunophenotype and genetics based targeted therapy guidelines are required to be elucidated from prospective multicentric controlled clinical trials.

Conclusion

MPAL is a rare type of leukemia where diagnosis and treatment decisions may be difficult due to overlapping features between different types of ALL and AML along with heterogenous immunophenotypic profile. FCM immunophenotyping with a comprehensive panel of antibodies is crucial for the diagnosis. Criteria for positive MPO testing by FCM must be defined in individual laboratories to avoid misinterpretation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The study was reviewed and approved by institutional committee at Kasturba Medical College, Manipal. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

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