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Flow Cytometric Immunophenotyping of Mixed Phenotype Acute Leukemia in a Tertiary Care Hospital of Eastern Odisha

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ABSTRACT

Background: Acute lymphoblastic leukemia affects both adults and children. Mixed phenotype acute leukemia (MPAL) is a rare subset featuring blasts with multiple lineage-specific antigens. Diagnosis is made via flow cytometric immunophenotyping with specific CD markers. This study aims to correlate MPAL's incidence, hematological findings, clinical profiles, and immunophenotypic features with treatment outcomes and prognostic significance.

Materials and Methods: A total of 750 cases of acute leukemia involving pediatric and adult patients were examined at SCB Medical College and Hospital in Cuttack, Odisha. Based on the WHO 2008 criteria, twenty-nine cases of MPAL were identified using morphological, cytochemical, and immunophenotypic features. The study covered all age groups admitted to the Department of Hematology from September 2011 to April 2021.

Results: Flow cytometric analysis of 750 cases showed B lymphoblastic leukemia as the most common subtype. Of the 29 MPAL cases (3.86%), 15 were B/myeloid, 13 T/myeloid, and one B/T/myeloid. Twenty-three cases received induction chemotherapy, with 12 achieving complete remission. The median survival was 11months, with a 15-month survival rate of 39%. Pediatric patients had a 60% survival rate at 15 months, compared to 30% for adults.

Conclusion: MPAL is a rare acute leukemia diagnosed through flow cytometry. Prognostic factors include age at onset, CD34 negativity, HLA-DR presence, BCR-ABL fusion, and MLL rearrangement, which indicate a poor prognosis. Children tend to have better outcomes and complete remission than adults, with therapies for ALL being more effective than those for acute myeloblastic leukemia.

Keywords: MPAL; HLA-DR; BCR-ABL fusion; Flow cytometer; Immunophenotyping

INTRODUCTION

Acute leukemias are classified as myeloid, B-cell acute lymphoblastic leukemia (B-ALL), or T-cell acute lymphoblastic leukemia (T-ALL), depending on the lineage of the leukemic cells[1]. In some rare cases, differentiation towards more than one blast lineage is observed. These cases are described using various terms, including hybrid acute leukemia, bilineal leukemia, biphenotypic acute leukemia (BAL), undifferentiated leukemia, and mixed lineage leukemia. The WHO classification (2008) of hematopoietic and lymphoid tumors has modified the diagnostic criteria and introduced a new designation, MPAL (Mixed-phenotype acute leukemia). MPAL leukemia presents separate populations of blasts from more than one lineage, termed bilineage, or blasts co-expressing antigens from multiple lineages, known as biphenotype[2].

Flow cytometry is a crucial diagnostic tool for classifying acute leukemias. Immunophenotypingand flow cytometry are routinely employed to classify different cell lineages, assess treatment responses, identify residual disease, and evaluate prognosis[3]. Mixed phenotype acute leukemia accounts for 3 to 5% of acute leukemias across all age and sex groups, but it is especially prevalent at 2.4 to 3.7% in children. Adults are more frequently affected, with a male predominance. The prognosis for MPAL is relatively poor compared to other acute leukemias, with an overall survival of 1 year and 6 months[4].

The current study aims to investigate the incidence, clinical signs, symptoms, hematological and immunophenotypic findings of MPAL, along with their treatment protocols and prognosis.

MATERIALS AND METHODS

In this longitudinal study, we considered 750 cases of acute leukemia involving both pediatric and adult patients of both sexes admitted to SCB Medical College and Hospital in Cuttack, Odisha. According to the WHO (2008) criteria, twenty-nine cases of MPAL were diagnosed based on morphological, cytochemical, and immunophenotypic features to establish neoplastic lineage and maturation.

Study period: From September 2011 to April 2021.

Inclusion Criteria: All cases of acute leukemia admitted to the hospital.

Exclusion criteria: Cases excluded from the study included secondary leukemia, CML in blast crisis, acute myeloblastic leukemia (AML) following myelodysplasia, therapy-related myeloid neoplasms, and relapse cases.

Patients with acute leukemia underwent detailed evaluations of their past and present medical and family histories. They were clinically examined for symptoms such as low-grade fever, pallor, bone pain, lymphadenopathy, hepatosplenomegaly, and bleeding manifestations. Hematological parameters such as hemoglobin percentage, WBC/platelet counts, lactate dehydrogenase, and alkaline phosphatase levels were assessed using a fully automated 5-part cell counter (SYSMEX XT 2000-1). Bone marrow aspiration was performed in a fully aseptic environment for morphological studies, immunophenotyping, and flow cytometry (BD FACSCalibur) to determine the type of leukemia, ranging from ALL (L1 or L2) and AML (M1 to M5), and to assess myeloperoxidase positivity. All patients with acute leukemia were classified by correlating hematological data with the morphology of the cells observed in peripheral and bone marrow smears, cytochemical studies, and immunophenotyping markers for lineage assessment. Peripheral blood smears and bone marrow aspirate slides were morphologically examined after air drying and staining with Giemsa stain. Myeloperoxidase assessment was routinely conducted for all bone marrow aspirates and peripheral blood smears.

Flow cytometric analysis was conducted using a BD FACSCalibur flow cytometer (Becton Dickinson, USA) and CellQuest Pro software for data acquisition and analysis. Bone marrow aspirates were prepared using a standard Lyse Wash procedure. The cells were stained with various combinations of monoclonal antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin chlorophyll protein (PerCP), and Allophycocyanin (APC) against specific antigens. Fluorescence and scatter signals from 10,000 to 20,000 nucleated cells per sample were acquired as ungated list mode events. Forward and side light scatter were used as threshold parameters to best discriminate between nucleated cells. Subsequently, the acquired data were gated on CD45 dim versus side scatter to isolate the blast population. Surface antigen expression was considered positive when at least 20% of blasts showed positive labeling; for cytoplasmic antigen expression, the threshold was set at 10%. The core monoclonal antibodies investigated in our study include:

Non lineage - CD45, CD34, HLA DR
Myeloid - CD13, CD33, CD117, ANTI MPO
Lymphoid - B Cell- CD19, CD10, cy CD79a
Lymphoid -T Cell- CD5, CD7 cy CD3

Treatment protocol

In this study, both AML and ALL regimens were employed. The AML regimen consisted of cytarabine and daunorubicin (3+7 protocol).

Cytarabine was administered as an intravenous bolus at 100 mg/m² twice daily from day 1 to day 7, and daunorubicin was given at 50 mg/m² on days 1, 3, and 5. This regimen was repeated over four cycles in four weeks.

Induction 1:

- Intravenous (IV) Vincristine 1.4 mg/m² administered on days 1, 8, 15, 22, and 29 IV.
- Daunorubicin 30 mg/m² on days 8, 15, and 29.
- Subcutaneous (SC) L-Asparaginase 60,000 IU/m² administered on alternate days from day 2 to day 20.
- Oral (PO) Prednisolone 40 mg/m² daily from day 1 to day 28.
- Intrathecal (IT) Methotrexate 12 mg/m² on days 8, 15, and 22.

Induction 2:

- Oral (PO) 6-Mercaptopurine 75 mg/m² daily from days 1-7 and 15-21.
- IV Cyclophosphamide 750 mg/m² on days 1 and 15.
- IT Methotrexate 12 mg/m² on days 1, 8, 15, and 22.
- Cranial Irradiation at 2000 cGy over 10 days.

Repeat Induction -1 (RI1) consists of two parts: Consolidation and Maintenance, starting on the 15th day post-chemotherapy.

Consolidation:

- IV Cyclophosphamide 750 mg/m² on days 1 and 15.
- IV Vincristine 1.4 mg/m² on days 1 and 15.
- SC Cytarabine 70 mg/m² every 12 hours for 6 doses on days 1-3 and 15-17.
- Oral (PO) 6-Mercaptopurine 75 mg/m² daily from days 1-7 and 15-21.
- Oral (PO) Prednisone 40 mg/m² daily from days 1-7.

Maintenance (beginning on day 15):

- IV Vincristine 1.4 mg/m² on day 1.
- IV Daunorubicin 30 mg/m² on day 1.
- SC L-Asparaginase 60,000 IU/m² on days 1, 3, 5, and 7.
- Oral (PO) Methotrexate 15 mg/m² once a week, skipping every fourth week for a total of 12 weeks.
- Oral (PO) 6-Mercaptopurine 75 mg/m² daily for 3 weeks out of every 4, for a total of 12 weeks.

Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows, Advanced Statistics Version 27 (IBM). Frequency distribution and percentage calculations were utilized to analyze clinical and laboratory variables.

Ethical clearance

Ethical clearance was obtained from the Institutional Ethics Committee of SCB Medical College, Cuttack, vide IEC/IRB No. 94/24/02/2011.

RESULT

In the present study, 750 patients with acute leukemia were analyzed. B-ALL was the most common subtype, diagnosed in 316 (41.13%) cases, followed by AML in 230 (30.67%) cases, and T-ALL in 175 (23.3%) cases. Only 29 (3.86%) cases of MPAL were observed (Table 1).

Table 1: Age/Sex distribution of acute leukemia in the study population

Type of leukemia	Total No.	Total %	Male %	Female %
B ALL	316	41.13%	201(26.8)	115(15.33)
T ALL	175	23.33%	99(13.2%)	76(10.13%)
AML	230	30.67%	133(17.73%)	97(12.93%)
MPAL	29	03.86%	16(2.13%)	13(1.73%)
Total	750	100%	449(59.86%)	301(40.13)

According to the WHO 2008 criteria, MPAL was diagnosed in 29 (3.86%) of the 750 acute leukemia cases, ranging in age from 5 to 55 years. There were 15 (51.7%) cases of B/Myeloid, 13 (44.8%) cases of T/Myeloid, and one case of B/T Myeloid identified in this study. Among the 29 cases, 11 (37.93%) were children (age <14 years), and 18 (62.07%) were adults. Male patients accounted for 16 (55.17%) of the MPAL cases, while 13 (44.82%) were female (Table 2).

Table 2: Age/Sex distribution 29 cases of MPAL (B/Myeloid n=15, T/Myeloid n=13 B/T Myeloid n=1)

Total cases	N= 29	B/M	T/M	B/T/M	Male	Female
Age <14 child	11(37.93%)	6	5	0	9 (31.03)	7(24.13)
≥14 Adult	18(62.07%)	9	8	1	7(24.13)	6 (20.69)
Total	29 (100%)	15(51.7%)	13(44.8%)	1(3.4%)	16(55.17%)	13(44.82%)

The most common presenting symptoms were fever in 23 (79.31%) patients, generalized weakness in 20 (68.97%), bleeding tendencies in 16 (55.17%), and bone pain in 11 (37.95%). Anemia, frequently noted as pallor, was present in 27 (93.10%) patients, followed by lymphadenopathy in 15 (51.72%), sternal tenderness in 13 (44.82%), splenomegaly in 12 (41.37%), gum hypertrophy in 11 (37.93%), hepatomegaly in 10 (34.48%), and subcutaneous bleeding in 10 (34.48%) of the study population (Table 3).

Table 3: Common signs and symptoms among the study population

Common Symptoms	Cases	Percentage
Pyrexia	23	79.31%
Generalized weakness	20	68.97%
Bleeding manifestations	16	55.17%
Bone pain	11	37.95%
Common Signs		
Anemia	27	93.10%
Enlarged lymph nodes	15	51.72
Tenderness over sternum	13	44.82%
Splenomegaly	12	41.37%
Hypertrophy of Gum	11	37.93%
Hepatomegaly	10	34.48%
Subcutaneous bleeding	10	34.48%

Routine blood examinations revealed leukocytosis (>12,000) as the most common finding, along with decreased platelet counts (<100,000/cmm) and hemoglobin levels (<10 g/dl). Elevated levels of serum lactate dehydrogenase and alkaline phosphatase were also noted. Bone marrow smears indicated Acute Lymphoblastic Leukemia features (L1 or L2) in 13 out of 29 (44.82%) cases and acute myeloid leukemia features (M1 or M5) in 16 (55.17%) cases. Cytochemical examinations confirmed Myeloperoxidase positivity in 11 (37.93%) cases (Table 4).

Table 4: Hematological and bone marrow profile of the study population

Hematological profile	Cases	Percentage	Value
Increased white cell count	25	86.20%	>12 thousands
Decreased platelet counts	24	82.75%	< 1 lakh
Hemoglobin percentage	27	93.10%	< 10g/dl
Serum lactate dehydrogenase	19	65.51%	Elevated
serum alkaline phosphatase	12	41.73%	Elevated
Morphology of lymphocytes(BM)	-		
ALL (L1 or L2)	13	44.82%	
AML (M1 or M5)	16	55.17%	
Myeloperoxidase positive	11	37.93%	

BM: bone marrow

In the present study, out of 29 cases of MPAL, 15 (51.72%) displayed a B lymphoid + myeloid immunophenotype (B/myeloid), 13 (44.83%) had a T lymphoid + myeloid immunophenotype (T/myeloid), and one case (3.45%) exhibited a trilineage immunophenotype (B/T/myeloid), in accordance with WHO guidelines for lineage specificity. All cases showed co-expression of markers from at least two different lineages. T Lymphoid markers were estimated by positive cytoplasmic CD3 (cyt CD3) expression in 12 out of 13 patients. One case of B/T/myeloid was observed in this study. In the T/myeloid series, CD5 was positive in 7 cases, and CD7 was positive in 9 out of the 13 patients. B lymphoid lineage was associated with strong expression of at least one other B cell marker, such as cytoplasmic CD79a in 8 out of 15 cases and CD10 in 12 out of 15 patients, including one case of the B/T/myeloid subtype. Immaturity markers HLA DR was expressed in 16 (10 B/myeloid, 6 T/myeloid and 1 B/T/myeloid) cases, CD 34 was expressed in 15 (9 B/myeloid, 6 T/myeloid) cases and CD 45 was expressed in 17 (9 B/myeloid, 7 T/myeloid and 1 B/T/myeloid) cases respectively.

Myeloid markers demonstrated myeloperoxidase positivity in 11 cases. CD13 was positive in 21 cases, CD33 in 21 cases, and CD117 in 17 cases across the B/myeloid (n=15) and T/myeloid (n=13) series. All markers were also positive in the one case of the B/T/myeloid series (n=1) (Table 5).

Table 5: Flow Cytometric Immunophenotyping of the study population

Markers	B/myeloid (n=15)	T/myeloid (n=13)	B/T/myeloid (n=1)
T-Lymphoid marker			
CD3		12/13	1/1
CD 5		7/13	
CD7		9/13	
B-Lymphoid marker			
CD79a cytoplasmic	8/15		
CD 10	12/15		1/1
Immaturity marker			
HLA DR	10/15	6/13	
CD 34	9/15	6/13	
CD 45	9/15	7/13	1/1
Myeloid marker			
CD 13	11/15	10/13	1/1
CD 33	11/15	10/13	1/1
CD 117	9/15	8/13	1/1
Total	15(51.72%)	13(44.83%)	1 (3.45%)

In the present study, cases of MPAL were treated using standard AML and ALL regimens—specifically, daunorubicin and cytarabine (3+7 protocol) for AML, and the MCP 841 protocol for ALL. Of the 29 MPAL patients, 23 received induction chemotherapy while 6 were given palliative care. Of those who received induction chemotherapy, the ALL protocol (MCP 841) was administered to 13 patients (7 B/myeloid, 5 T/myeloid, and 1 B/T/myeloid) and the AML regimen (3+7 protocol) to 10 patients (6 B/myeloid and 4 T/myeloid). Palliative treatment was provided for 6 cases (3 B/myeloid, 2 T/myeloid, 1 B/T/myeloid).

Among the 13 patients treated with the ALL regimen, 9 (5 B/myeloid and 4 T/myeloid) achieved complete remission (CR), defined as less than 5% blast cells in the bone marrow and a complete absence of blast cells from peripheral blood after induction chemotherapy. Following the MCP 841 protocol, consolidation chemotherapy was administered based on the patients' performance status, tolerance, recovery of blood counts, and maintenance of the CR phase. Seven patients who achieved CR during the induction phase received consolidation treatment. During the last follow-up, two patients had passed away during the consolidation phase (one due to disease relapse and one due to sustained neutropenia and severe infection), and one case was lost to follow-up. Five pediatric cases (3 T/myeloid and 2 B/myeloid) who achieved CR continue their treatment and have not relapsed.

Out of 10 patients who received the AML regimen (3+7 protocol) as induction therapy, CR was

achieved in only 4 cases (2 B/myeloid and 2 T/myeloid). Two patients died during the induction phase. All four cases that achieved complete remission underwent consolidation therapy with high-dose cytarabine according to the standard AML regimen. During the last follow-up, one patient died during the consolidation phase due to disease relapse. Two patients who achieved CR are continuing treatment and have not relapsed (Table 6).

Table 6: Response to therapy and outcome of the study population

MPAL phenotypes	ALL regimen	AML regimen	Palliative treatment	Complete remission		Incomplete remission	
Induction chemotherapy	N=13	N=10	N=6	ALL	AML	ALL	AML
B/myeloid	7	6	3	5	2	2	4
T/myeloid	5	4	2	4	2	1	2
B/T/myeloid	0	0	1	0	0	0	0
Total	12	10	6	9	4	3	6
Death	2	3					

The overall median survival of the study population was 11 months, with a 15-month survival rate of 39%. Pediatric patients had a median survival of 7 months, with a 15-month survival rate of 60%, compared to 30% for adults. Patients treated with ALL-directed therapy had a median survival of 11 months, with a 15-month survival rate of 56%. In contrast, patients treated with AML-directed therapy had a median survival of 10 months, with a 15-month survival rate of only 32% (Table 7).

Table 7: Relationship between treatment and survival of patients with MPAL

Phenotype	Median survival	>15 months survival
ALL (R) Regimen	11 months	56%
AML (R) Regimen	10 months	32%
≥14 year	7 months	30%
<14years	Not yet reached	60%
Overall	11 months	39%

DISCUSSION

MPAL occurs in 3-5% of acute leukemia cases across all age and sex groups. Adults are more susceptible to this disease, with a higher prevalence in males. The prognosis for MPAL is relatively poor compared to other forms of acute leukemia, with an overall survival rate of 18 months despite the availability of advanced medications[4].

In this study of 750 acute leukemia patients, MPAL, also known as aberrant phenotypes, was identified in 29 cases (3.86%). There were variable expressions of lymphoid antigens in acute myeloblastic leukemia. B-ALL was the most common subtype, found in 316 cases (41.13%), followed by AML in 230 cases (30.67%), and T-ALL in 175 cases (23.3%)[5] (Table 1).

MPAL was observed in 29 cases (3.86%) with ages ranging from 5 to 55 years. This included 15 cases (51.7%) of B/Myeloid, 13 cases (44.8%) of T/Myeloid, and one case of B/T Myeloid. Among these, 11 cases (37.93%) were children (age <14 years) and 18 cases (62.07%) were adults. Males comprised 16 cases (55.17%), showing a male predominance compared to 13 cases (44.82%) in females, corroborating findings from previous authors[5,6] (Table 2).

The most common presenting symptom among the patients was fever, followed by generalized weakness, bleeding tendencies, weight loss, and bone pain. Anemia, often referred to as pallor, was the most frequent sign, followed by lymphadenopathy, sternal tenderness, splenomegaly, gum hypertrophy, hepatomegaly, and subcutaneous bleeding[7](Table 3).

Repeated attacks of the common cold and generalized weakness led to increased white blood

cell counts (leukocytosis, >12,000) followed by anemia (<10 g/dL) and decreased platelet counts (<100,000/cmm). Elevated levels of serum lactate dehydrogenase, serum alkaline phosphatase, and other liver enzymes were observed, due to iron toxicity and the deleterious effects of chemotherapeutic drugs used for treatment, as suggested by Maruffi M[8]. Morphological and cytochemical examinations of bone marrow smears showed features of Acute Lymphoblastic Leukemia (ALL) L1 or L2 in 13 (44.82%) of the 29 cases. AML markers M1 or M5 in 16 (55.17%) cases (Table 4).Cytochemical examinations revealed myeloperoxidase positivity in 11 (37.93%) cases, similar to findings by OberleyMet al[9].

The bone marrow study showed ALL immunophenotypic T/lymphoid markers: CD3 (cyt CD3) in 12/13 cases, CD5 in 7/13 cases, and CD7 in 9/13 cases. B-lymphoid markers included CD79a cytoplasmic in 8/15 cases and CD10 in 12/15 cases. One case of B/T/myeloid was positive for both CD3 and CD10 markers. Immaturity markers and AML immunophenotypic markers were present in all subtypes of MPAL. Immaturity markers such as HLA DR were observed in 10/15 B/myeloid and 6/15 T/myeloid cases, with CD34 and CD45 seen in 9/15 and 6/15 B/myeloid cases, respectively, and CD34 in 6/15, CD45 in 7/15 T/myeloid cases, and in one B/T/myeloid case. AML immunophenotypic markers such as CD13 were seen in 11/15 B/myeloid, 10/13 T/myeloid, and 1/1 B/T/myeloid cases; CD33 in 11/15 B/myeloid, 10/13 T/myeloid, and 1/1 case; and CD117 in 9/15 B/myeloid, 8/13 T/myeloid, and 1/1 case, respectively. The present study revealed more cases of the AML subtype than the ALL subtype of acute leukemia, which was inconsistent with previous studies[5].

MPAL is a multipotential hematopoietic stem cell capable of differentiating into any type of lineage. In this study, MPAL was shown to express CD34 and HLA DR, indicative of an early precursor stem cell origin. CD34 positivity was observed in 15 out of 28 cases, and HLA DR was positive in 16 out of 28 cases of MPAL. The reactivation of the lymphoid series and myeloid differentiation explains the development of MPAL (Table 5). Similar findings have been reported by Matthew P M and Gupta M et al[10, 11].

The response to therapy and outcome for MPAL cases remain uncertain, with decisions to treat using either ALL or AML regimens followed by hematopoietic stem cell transplantation. Of 29 patients, 23 received induction chemotherapy, and six received palliative care. The ALL protocol (MCP 841) was administered to 13 cases (7 B/myeloid and 6 T/myeloid), while the AML regimen (3+7 protocol) was used for 10 cases (6 B/myeloid and 4 T/myeloid). Palliative treatment was considered for 6 cases (3 B/myeloid, 2 T/myeloid, 1 B/T/myeloid)[12, 17, 18] (Table 6). CR was achieved in nine cases (5 B/myeloid and 4 T/myeloid) with ALL treatment, and four cases (2 B/myeloid and 2 T/myeloid) with the AML regimen. Two of the six patients on palliative care died early[13, 17, 18].

The median survival of the study population was 11 months, with a 15-month survival rate of 39%. Among pediatric patients, the survival rate at 15 months was 60%, compared to 30% for adults. Patients treated with ALL-directed therapy had a 15-month survival rate of 56%. In contrast, those receiving AML-directed therapy had a median survival of 6 months and a 15-month survival rate of 32%[14, 15]. The median survival observed in this study was 18 months (Table 7). The 5-year survival rate was 37%, with children experiencing better survival rates than adults[16,19, 20].

CONCLUSION

Accurate diagnostic criteria are essential in diagnosing MPAL, a rare subtype of acute leukemia with a poor prognosis. It is crucial to distinguish between acute myeloid and lymphoid leukemias with cross-lineage antigen expression through detailed immunophenotyping analysis. Cytoplasmic and lineage-specific markers should be meticulously analyzed using flow cytometry to accurately evaluate MPAL. Treatment should include induction chemotherapy followed by consolidation chemotherapy, maintenance therapy, and, if possible, hematopoietic stem cell transplantation during the first remission. It is concluded that CR rates in ALL-directed therapy are higher than those in AML regimens, particularly in pediatric patients compared to adults.

LIMITATIONS: Given the small size of the study group, it is difficult to draw definitive conclusions. To accurately diagnose MPAL, a more comprehensive panel of antibodies, including cytoplasmic markers, is required.

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