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Significance of minimal residual disease detection by flow cytometry in Paediatric B cell ALL treatment in tertiary care centre in Eastern India

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Abstract: *Background: The good prognosis of paediatric B cell acute lymphoblastic leukaemia (pALL) is considered as a great progress of medical science in the field of oncology and haematology. Minimal Residual disease (MRD) refers to the presence of disease in cases deemed to be in complete remission by conventional pathologic analysis. Prognostic importance of MRD in paediatric ALL is well accepted. This is the study where 8 colour flow cytometry (FCM) is used to detect MRD to asses treatment, diagnosis and prognosis of Bcell ALL. Method: Our Study has used 8 colour FCM which contains CD 19, CD 34, CD 10, CD58, CD 45, CD13, Anti TDT, CD33. 8 panels i.e1.CMPO-FITC/ cCD79a-PE/ cCd3ECD, 2. CD20-FITC/ cCD10-PE/ cCd19ECD, 3.CD34-FITC/ cCD117-PE/ cCd45 ECD/CD2 PC 5, 4. CD15 FITC/ CD33PE/ CD45ECD,5.CD14 FITC/ CD13 PE/CD45 ECD,6. HLADR FITC/CD7 PE/CD45 ECD,7. TdT FITC/CD45 ECD (IF CD 34 NEG), 8.CD58 FITC/CD 45 ECD (IF BOTH CD34 AND TdT NEG) are used to prepare the marker. Result: The study includes 52 patients. In the 52 patients 59.6% patients are alive with a p value of 0.031. MRD was checked at 15th, 29th day and post consolidation of the treatment where in day 15 (p=0.023), 53.4% positive and 46.5% negative. In day 29 (p=0.031), 22.5% positive and 77.5% negative, in post consolidation positive in 20% and Negative is 80%. MRD value below 0.01 is considered as negative. The overall survival is of 32.88±8.59 months with 6 to 36 months duration. Conclusion: It is a study of 52 patients of paediatric B cell ALL with a detection of MRD by FCM. According to this study day 29 and post consolidation results of MRD show a significant change with that of day 15. This significant early MRD negativity has any role in relapse and prognosis will be look into future study.*

Key Word: *Paediatric ALL (pALL), MRD, Flowcytometry (FCM)*

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I. Introductions

ALL is the most common cancer in children. The hallmark of ALL is chromosomal abnormalities and genetic alterations involved in differentiation and proliferation of lymphoid precursor cells including 75% B cell lineage and remaining 25% are T cell lineage (1). ALL is affected by different host related factors including age, immunologic subtype, clinical, genetic and molecular features (1).During remission phase of leukaemiano disease symptoms are present in patientsundertreatment though small number of leukemic cells are still present in bone marrow. That is referred to as Minimal Residual Disease (MRD) those small number of leukemic cells remain in the body after treatment (2). These population of cells are too small to show any physical symptoms. Studies showed that MRD is a strong prognostic factor in the treatment of Pediatric acute Lymphoblastic Leukaemia (2-4). There are several conventional methods of measuring MRD like morphology; clonogenic Assay; immunophenotype analysis; karyotype analysis; FISH and PCR. Flow-cytometric detection (FCM) of MRD is based on the identification of immunophenotypic combinations expressed on leukemic cells but not on normal hematopoietic cells. It is considered as less labour intensive and faster detection technique (5).The advantage of using FCM is getting fast analysis data, easy storage of data information However, the disadvantage is variable sensitivity, due to similarities of marker expression between normal and malignant cells

(5). MRD detection is not only useful for the assessment of initial treatment response and subsequent definition of MRD-based risk groups, but also to monitor disease burden in the setting of stem cell transplantation (SCT), for early recognition of impending relapse, and as potential end point in clinical trials (6).

Most children with acute lymphoblastic leukaemia (ALL) in first complete remission (CR1) have an excellent prognosis with multi-agent chemotherapy in induction, consolidation, re-induction and maintenance therapy (7). There is a subset of patient with a more guarded prognosis using this approach who may benefit from haematopoitic allogeneic stem cell transplantation (allo-HSCT).Usually remission of ALL is termed when the blast cell population is less than 5% in the bone marrow cells. After that MRD is tested in 15^{th}, 29^{th} day and post consolidations. MRD value bellow 0.01 (1 leukemic cell out of 10,000 normal cell) is considered as negative and less chance of relapse thus disease prognosis is better. But MRD value more than 0.01 is considered as positive and chance of relapse of disease is much higher in this case. According to various studies MRD levels are strongly associated with treatment outcome and clinical remission (8-9).

II. Materials and Methods

Subject selection: Total 52 paediatric patients of either sex with age group between 1-12 yrs with newly diagnosed B cell ALL are enrolled in this study within the time period of Jan 2016-Dec 2018.The subjects were diagonosedon the basis of the peripheral blood smear report of anemia, thrombocytopenia, leucocytosis and lycocytopenia with more than 5% blast cell in bone marrow aspiration sample. Bone marrow aspirates are also taken for MRDon day 15, day 29 and post consolidation. Informed consent was taken from parents or legal guardiansof the patients took part in the study according to following criteria.

Inclusion Criteria:

- The subjects who qualified for the study should meet the following inclusion criteria.
- Subjects of either sex aged between > 1 to 12 years.
- Subjects of B-cell Acute Lymphoblastic Leukemia proved from peripheral blood smear & bone marrow aspiration sample for morphology, cytochemistry&immunophenotyping
- Subjects screening and baseline laboratory test as defined by Haemoglobin ≥ 9 gm%.
- Serum SGPT &SGOT ≤ 1.5XUNL.
- Serum total bilirubin ≤ 1.5 x UNL
- Serum Creatinine ≤ UNL

UNL: Upper Normal Limit

- Subjects are willing and able to adhere to the study visit schedule and other protocol requirements as evidence by signed written informed consent.

Exclusion Criteria:

- Subjects with Ph+ chromosome positive B-cell ALL.
- The subjects which who are taking olsalazine, mesalazine or sulphasalazine, warfarin, thioguanine and drugs whose primary or secondary toxicity is myelosuppression.
- Anemia (Hemoglobin) < 9 gm%.
- Subjects with inherited deficiency of the TPMT enzyme.
- Subjects who cannot adhere to the protocol due to drug allergy & sensitivity.
- Subjects who have current signs and symptoms of severe, progressive, or uncontrolled renal, hepatic ,hematologic, endocrine, pulmonary, cardiac, neurologic or cerebral disease.
- Subjects who are participating in any other clinical study or who have received treatment with any investigational drug or device within one month prior to screening.
- Any other condition that in investigational judgement might increase the risk to the subject or decrease the chance of obtaining satisfactory information needed to achieve the objectives of the study.
- Subjects who have a known infection with human immunodeficiency virus (HIV) and or Hepatitis B or Hepatitis C.

Sample Collection: Bone marrow aspirations were taken by physician and sent to designated laboratory on the same day. The diagnosis is done based on standard morphologic, cytochemistry and immunophenotyping.Bone marrow aspirate was examined before starting of the treatment, on day 15 time point1 (TP-l)and day 29 (TP-2)and post consolidation(TP-3).. Patients who failed to achieve MRD -veIN POST CONSOLIDATION SAMPLEwere assigned to high - risk group;and treatment to be shifted to higher regime.

A**aining:**The air-dried blood films are stained with Leishman Giemsa stain. Double volume of giemsa Phosphate buffer is used and washed in the tap water and dried.

Flow Cytometry (FCM): Here in this study 8 colour flow cytometry [CD10, CD13, CD19, CD33, CD34, CD 45, CD 58 and anti TdT) is used to detect the immature B cells.

Detailed flow panel is as follows: CMPO-FITC/cCD79a-PE/cCd3ECD, CD20-FITC/cCD10-PE/cCd19ECD, CD34-FITC/cCD117-PE/cCd45 ECD/CD2 PC5, CD15 FITC/CD33PE/CD45ECD, CD14 FITC/CD13 PE/CD45ECD, HLADR FITC/CD7 PE/CD45 ECD, TdT FITC/CD45 ECD (IF CD 34 NEG),CD58 FITC/CD 45 ECD (IFBOTH CD34 AND TdT NEG)
Following criteria are considered to analyse the sample for the study:

Acceptance Criteria for FCM
- Specimenshould be labelled properly.
- Specimen collected in EDTA vacutainers as per guidelines and well preserved until tested.
- Specimen submitted alongwith complete request form.
- Adequate specimen volume by protocol.
- Specimen to be sent to the lab at correct time.
- Adequately prepared patient with respect to test requirements.

Rejection criteria for FCM:
- Insufficient quantity.
- Specimen collected in wrong container or not properly labelled.
- Contamination suspected.
- Inappropriate transport.
- Sample not reached in proper time.

The test is based on the ability of specific monoclonal antibody to bind to the antigenic determinants exposed by leukocytes. Then specific staining of leukocytes is performed by incubating the samples. The red cell are removed, lysed and analyzed by FCM followed by CD45 staining and other scatterplots combining two of the different parameters available on the cytometer are also used in gatingstage. The cell population thus gated is subdivided into subpopulations using two other fluorescences. Thus the positively stained cells are distinguished from unstained cells. The results finally exposed as a percentage of fluorescent cells in relation to all the events acquired by gating. The basic set of antibodies used for MRD detection in B-lineage ALL (B-ALL) usually include CD45, CD34, CD19, CD58 and CD10 and T-lymphoid or myeloid cell markers such as CD13, CD33, and Anti TdT.

Statistical analysis: Results are expressed as mean ± standard deviation. P value <0.05 and < 0.001 were considered as statistically significant. Log rank statistical analysis (SPSS software) was done to analyze the overall survival.

III. Results

Demography of the patients:

We have enrolled 52 patients according the criteria discussed before. Demography of the subjects is listed in Table 1. Most of the patients are below 5 yrs age group (69%) and male (61%). ~71% patients were common acute lymphoblastic leukemia-associated antigen (CALLA) positive, 2 patients are CALLA negative (Table 1). In risk stratification 88% patients are risk stratified (SR) and 11% patients are high risk (HR) (Table 1). In treatment protocol 90% patients are treated according to MCP protocol and only 9% patients are treated in BFM(Berlin-Frankfurt-Munster treatment) treatment protocol (Table1).

Table-1

Parameters	Number	Percentage	P value
Age (in years)			
1 – 5	36	69.2*	
5 – 10	15	28.8	<0.001
>10	1	1.9	
Gender			
Male	32	61.5*	<0.001
Female	20	38.5	
Subtype of B cell ALL by FCM			
Aberrant ex CD 15	4	7.7	
Aberrant ex CD 33	1	1.9	
Aberrant ex CD19A	1	1.9	
Aberrant ex CD15, CD33	2	3.8	
Aberrant ex CD13,CD33	2	3.8	<0.001
CALLA positive	37	71.2	
CALLA negative	2	3.8	

Others	3	5.8	
Risk Stratification			
Risk stratified	46	88.5*	<0.001
High risk	6	11.5	
Treatment Protocol			
MCP	47	90.4*	<0.001
BFM	5	9.6	

Table 1: Distribution of different parameters among patients (n=52). *-Statistically Significant. The mean (± s.d) age of the patients was 4.99±2.67 years with range 1 – 11 years and the median age was 4.0 years.

Overall Treatment Outcome:

During the study time (36 months follow up) most of the patients (59%) are alive, though 32% patients couldn't complete their treatment (Table 2). Complete remission was found in 32 patients (61%). The MRD levels at different time points are listed in Table 2. It is found that at day 15 and post consolidation most of the patients showed MRD positive 53% and 80% respectively. At day 29 most patients showed MRD negative (77%).

Table-2

Outcome	Number	Percentage	p value
Status at last contact			
Survival Status			
Alive	31	59.6	0.031
Dead	4	7.7	
Incomplete treatment	17	32.7	
Response to treatments			
Complete Remission by morphology	32	61.5*	<0.001
MRD Status at Day 15 (n=43)			
Positive	23	53.4	0.023
Negative	20	46.5	
MRD Status at Day 29 (n= 40)			
Positive	9	22.5	0.031
Negative	31	77.5	
MRD Status at Post-consolidation (n= 25)			
Positive	5	20	0.020
Negative	20	80	

Table 2: This table shows treatment outcome and MRD status among patients at different time points. The mean (±s. d) duration of overall survival of the patient was 32.88±8.59 months with range 6-36 months and median was 36 months. Positive MRD levels were significantly different from each other at different days (paired Student t test: P = .30). Correlation analysis showed r of 0.964 (95% confidence interval: 0.857-0.991; P < .0001).

IV. Discussion

Minimal residual disease (MRD) testing by higher performance techniques such as flow cytometry and polymerase chain reaction (PCR) can be used to detect the proportion of remaining leukemic cells in bone marrow or peripheral blood during and after the first phases of chemotherapy in children with ALL (10). In USA, 1 in 285 will be diagnosed with cancer before reaching 20 years of age (11). In the report of American Cancer Society, it has shown that leukaemia is the most common childhood cancer. In India also leukaemia is the most common childhood cancer (12).

In FCM-MRD the marker expression of CD10, CD19, and CD34 or TdT is normally used to identify premature B cells (10). CD45 used to differentiate between quantitative expressions among normal leukemic origin of premature B cells (10). Finally we have used 8 colour flow cytometry to detect MRD in a tertiary health care centre in eastern India. CD19, CD34, CD58, CD45, CD10, CD13, anti TDT, CD33 are used to detect MRD. Before starting of 8 colours FCM, mostly 4 colours and 6 colours FCM are widely used (13). In Karawajew et al (14) and Shaver et al. study (15), CD38 is present in all proposed panels and was proven to be relevant. 8 panels i.eCMPO-FITC/cCD79a-PE/cCd3ECD, CD20-FITC/cCD10-PE/cCd19ECD, CD34-FITC/cCD117-PE/cCd45 ECD/CD2 PC5, CD15 FITC/CD33PE/CD45ECD, CD14 FITC/CD13 PE/CD45 ECD, HLADR FITC/CD7 PE/CD45 ECD, TdT FITC/CD45 ECD (IF CD 34 NEG),CD58 FITC/CD 45 ECD (IF BOTH CD34 AND TdT NEG) are used to prepare the marker.

Previously it was shown that at day 15, MRD lower than 10^{-4} was associated with an excellent 5-year relapse-free survival in 78 investigated patients (16). In general, measurements during remission induction therapy (typically 2 weeks after diagnosis) provide an early identification of good responders and very poor responders, which can be further refined by assessing MRD at the end of induction therapy and during the early

phases of continuation therapy (17). It was also observed that the presence of MRD in day-8 blood and day-29 marrow MRD was associated with shorter event-free survival (EFS) in all risk groups; even patients with 0.01% to 0.1% day-29 MRD had poor outcome compared with patients negative for MRD patients (59% ± 5% vs 88% ± 1% 5-year EFS) (18,19).

V. Conclusion

It is a study of 52 patients of paediatric B cell ALL with a detection of MRD by FCM. This study revealed that MRD detection at day 29 and post consolidation showed a significant change with that of day 15. This is a novel preliminary study to find an early MRD negativity. The effect this early detection on disease relapse and prognosis will be studied further.

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