IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 19, Issue 5 Ser.1 (May. 2020), PP 01-08
www.iosrjournals.org

Corelation of Minimal Residual Disease Detection in Paediatric B Cell All Patient with Their Overal Survival and Prognosos-Experience in Tertiary Care Centre in Eastern India

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Abstract:

Objectives and Background*:To describe clinical outcomes and prognosis i.e overall survival and relapse in the patients with paediatric B cell ALL (pBALL)with respect to minimal residual disease detection on day 15, day 29 and post consolidations in a tertiary care centre in eastern India.* ***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 all over the world. This is the study where 8 colour flow cytometry (FCM) is used to detect MRD to assestreatment, diagnosis and prognosis of BcellALL.* ***Method****:Our Study had been running on 52 paediatric patients with the age group of 1 to 12 years with the MRD analysis on day 15, day 29 and post consolidations and a follow-up of 3 years where we have used 8 colour FCM to detect the MRD analysis. FCM 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 PC5,4.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 The patient has gone through a 3 years follow-up and number of patient relapse is determined..****Result:*** *The study includes 52 patients. In the 52 patients 59.6% patients are alive with a p value of 31. MRD was checked in every 15th and the 29th dayand post consolidation of the treatment where in day 15(p=23), 53.4% positive and 46.5% negative. In day 29(p=31), 22.5 positive and 77.5 negative, in post consolidation positive in 20% and Negative is 80%. MRD value below 0.01 is taken as negative and above is taken as positive. The overall survival(OS) is of 32.88+_ 8.59 with a 6 to 36 months duration. In relapse, no haemorrhagic relapse is found and 2 CNS relapse is found.* ***Conclusion****: It is a study of 52 patients of paediatric B cell ALL with a detection of MRD by FCM.MRD negative patient have a good prognosis and comparatively lower rate of relapse than the one with positive MRD. Effort should be made to adhere to recommendation of MRD testing in clinical guidelines.*

KEY WORD: *Paediatric ALL(pALL), MRD, Flowcytometry(FCM), Clinical Outcomes, Prognosis, overall survival*

Date of Submission: 19-04-2020 Date of Acceptance: 03-05-2020

I. Introductions

Acute Lymphoblastic Leukaemia (ALL) is the one of the most common cancer in the children. Chromosomal abnormalities are the hallmark in ALL and genetic alterations involved in differentiation and proliferation of lymphoid precursor cells.It includes 75% B cell lineage and remaining 25% are T cell lineage (1). Age, immunologic subtype, clinical, genetic and molecular features affect ALL(1).Treatment response assessment via MRD has been a corner stone for the risk stratification in pALL.(2) MRD has shown very prognostic and very useful when its appear in the early state of treatment.

Recent International Berlin-Franklin-munster trial (IBFM) for acute lymphoblastic leukaemia stratified paediatric patient into three main risk groups largely based on treatment response criteria, particularly minimal residual disease. In this IBFM intervention trial Italy (Conter et al, 2010, [3]), Australia-New Zealand haematology/oncology group (ANCCHOG) (Marshal et al 2013[4]), Dutch childhood oncology group (Pieters et al, 2016[5]) shows only 11% are in high risk are treated with multiple intensive chemotherapy and sometimes bone marrow transfer in needed. After doing MRD based treatment 5 years survival increased from 30% to 76-

80%(Marshal et al 2013[4] and petiers et al 2016[5]). 60% of the patient also shows relapse during the trial. (Karsa et al 2013[6])

During remission phase of leukaemiano disease symptoms are present in patientsundertreatment though small number of leukemic cells arestill present in bone marrow. That is referred to as **Minimal Residual Disease** (MRD) and those small number of leukemic cells remain in the body after treatment (7). These population of cells are too small that they don't show any significance symptoms in the body. Studies showed that in the treatment of paediatric B cell ALL treatment MRD is a strong prognostic factor (7-9). There are several conventional methods of measuring MRD like morphology; clonogenic Assay; immunophenotypeanalysis; **flowcytometry**,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(10).The advantage of using FCM is getting fast analysis data, easy storage of data information.However, the disadvantage is variable sensitivity, due to similaritiesof marker expression between normal and malignant cells (10).MRD detection is not only useful for the assessment of initial treatment response and subsequent definition of MRD-based risk groups. MRD helps us 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(11).

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(12). A subset of patient with a more guarded prognosis using this approachwho may benefit from haematopoitic allogeneic stem cell transplantation(allo-HSCT).Normally 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. The relapse can be Hematologic or can be CNS type. According to various studies MRD levels are strongly associated with treatment outcome and clinical remission(13-14).

II. Materials and Methods

Subject selection: Total52paediatric patientsof 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 determinantsexposed 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 otherfluorescences.Thus the positively stained cells are distinguished from unstained cells.The resultsfinally 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. Survival analysis (SPSS software) were done at different time points of MRD analysis and compared with MRD positive and negative subjects. P value <0.05 and < 0.001 were considered as statistically significant.

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 werecommon acute lymphoblastic leukemia-associated antigen (CALLA) positive, 2 patientsare CALLA negative (Table 1). In risk stratification 88% patientsare risk stratified(SR) and 11% patientsare 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	<0.001S
>10	1	1.9	
Gender			
Male	32	61.5*	<0.001S
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	<0.001S
CALLA positive	37	71.2	
CALLA negative	2	3.8	
Others	3	5.8	
Risk Stratification			
Risk stratified	46	88.5*	<0.001S
High risk	6	11.5	
Treatment Protocol			
MCP	47	90.4*	<0.001S
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, though32% 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	
Dead	4	7.7	0.031
Incomplete treatment	17	32.7	
Response to treatments			
Complete Remission by morphology	32	61.5*	<0.01
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 atPost-consolidation (n= 25)			
Positive	5	20	0.020
Negative	20	80	

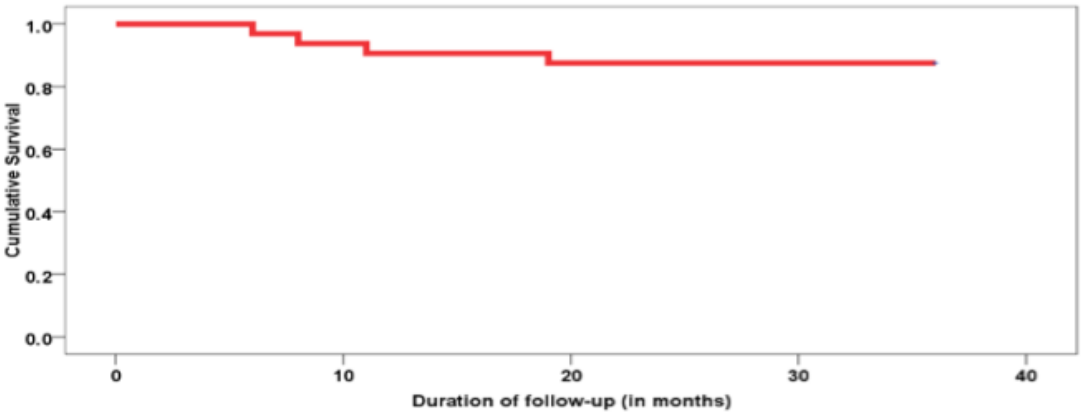
Table 2: This table shows treatment outcome and MRD status among patients at different time points.
The mean (±s. d) duration of overall survival of the patient was 32.88±8.59 months with range 6-36 months and median was 36 months

Adverse effect or Relapse (n=52)
Out of 52 patients, 2 patients have shown relapse. The 2 patients (100%) have shown CNS relapse and no patients have shown hematologic relapse. So pvalue has come as 2.

Adverse Effect/ Relapse	Number	Percentage	pValue
Hematologic	0	0	0.02
CNS	2	100	

KM survival curve for Overall survival (OS) of the patient in the completion of the treatment

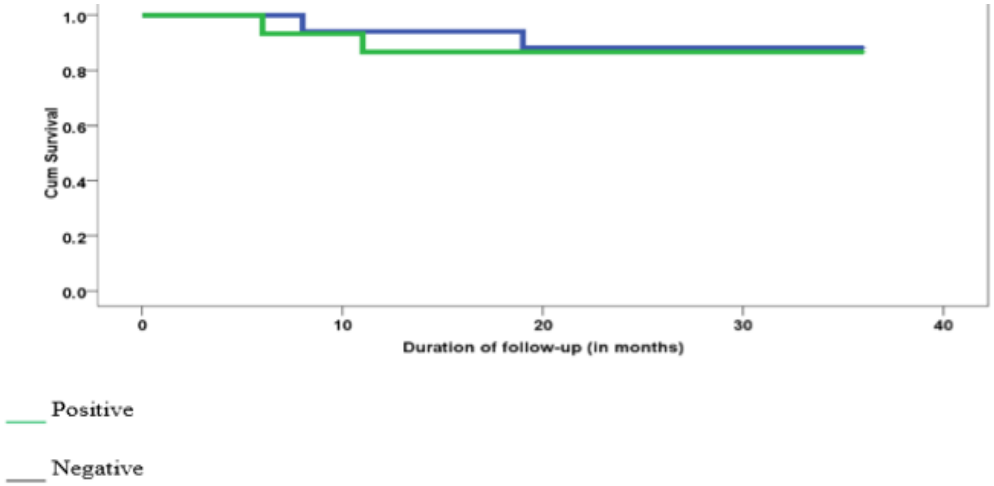
The Kaplan- Mier curve also known as the product limit estimator is a non-parametric statistic used to estimate the survival function from lifetime data. In our study n is 52. In this curve X axis represents duration of follow-up in months and Y axis represents cumulative survival.



KM survival curve for Overall survival (OS) of the patient in the completion of the treatment (n=52)

Comparison of OS of patients who completed the treatment of according to the status of MRD day 15

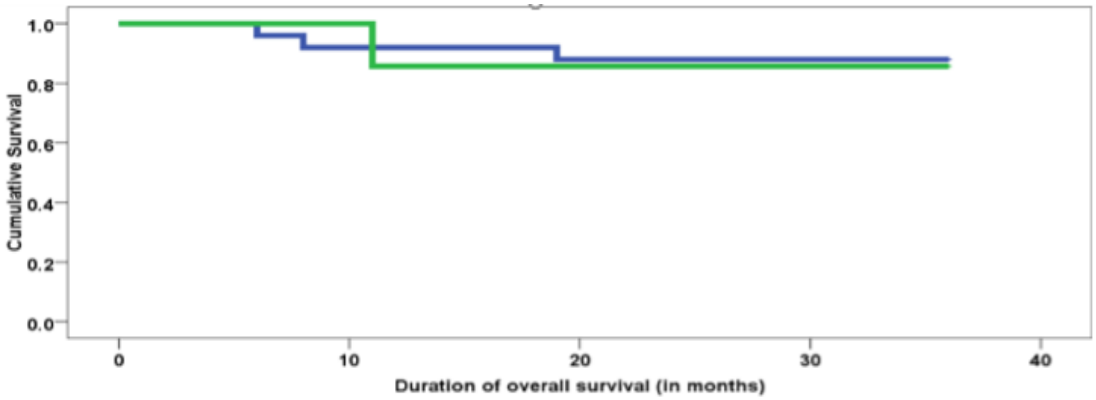
X axis represents duration of follow up in months and Y axis represents cumulative survival.



Comparison of OS of patients who completed the treatment of according to the status of MRD day 15 (n=52), As per Log Rank Test there was no significant difference in the pattern of OS according to status of MRD day15. (p=0.86)

Comparison of OS of patients who completed the treatment of according to the status of MRD day 29

X axis represents duration of follow up in months and Y axis represents cumulative survival.



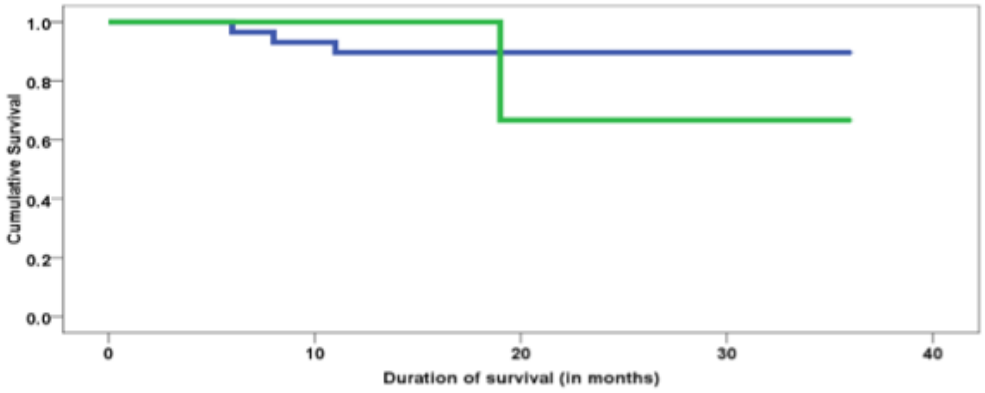
Positive

Negative

Comparison of OS of patients who completed the treatment of according to the status of MRD day 29 (n=52), As per Log Rank Test there was no significant difference in the pattern of OS according to status of MRD day29. (p=0.89)

Comparison of OS of patients who completed the treatment of according to the status of MRD in post consolidation

X axis represents duration of follow up in months and Y axis represents cumulative survival.



Comparison of OS of patients who completed the treatment of according to the status of MRD in post consolidation (n=52), As per Log Rank Test there was no significant difference in the pattern of OS according to status of MRD in post consolidation. (p=0.31)

IV. Discussion

Minimal residual disease(MRD) analysis for patient of acute leukaemia has evolved as a significant prognostic factor. Based on the results of MRD, patient receive the most therapeutic effect during chemotherapy and in the post consolidation period. (15). 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 acute lymphoblastic leukaemia (ALL).(16)In USA, 1 in 285 will be diagnosed with cancer before reaching 20 years of age (17). Multiparameter flow cytometry (MFC) has the potential for a rapid and sensitive identification of high risk patients. (18) 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.(19)

95% of MRD is detected via FCM which has a sensitivity of 10-4. FCM method has the advantange of rapidness and high accuracy level. Disadvantage is operator dependent and needs further standerization.(20)

28% of all the childhood cancer is laukaemia. The most common type is acule lymphoblastic leukaemia(ALL). According to WEBMD, acute lymphoblastic leukaemia is the most common type of cancer in the children. It affects the immune system i.e B cell. 75% are B cell lineage and 25% are T cell lineage. A study from FDA highlighted a need for interventions in MRD+ patients and suggested that MRD response could be used as an early end point to assess novel therapies, a concept that the US Food and Drug Administration had previously resisted.(21). A fully-standardized EuroFlow 8–color antibody panel and laboratory procedure was stepwise designed to measure minimal residual disease (MRD) in B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) patients with a sensitivity of ≤10^-5 (22).

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.(23,24) In Karawajew et al(19) and Shaver et al. study 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.

At day 15, a level of minimal residual disease in blood lower than 10^{-4} was associated with an excellent 5-year relapse-free survival in 78 investigated patients (100% *versus* 69±7%; *P*=0.0003). Subgroups defined by the level of minimal residual disease in blood at day 15(25) In general, measurements during remission induction therapy (typically 2 weeks after diagnosis) provide an early identification of good responders and of 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.(26). 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) (27)

The OS is this study is approximately 2.5 years. The Kaplan Meier curve indicated a good OS in the day 15, 29 and post consolidations of the treatment. Patients who are MRD positive are high risk of relapse and death. In a KM curve we got a good plateau which signifies the success of the procedure. In case of post-consolidation the plateau is lost with a p value of 0.31 which signifies that MRD negative patients are positive again. This signifies in the 5 years of OS, it's a good challenge to maintain the plateau although MRD is the best prognostic method to understand the treatment prognosis.

In India the testing of MRD is expensive and its costs around 30000 INR, so in the resource restricted countries it is very tough to avail MRD successfully in all patients.

V. 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 15 day 29 and post consolidation results of MRD show a change with that of day 15 significantly. This study shows 2 CNS relapse cases and no hematologic relapse is seen. So MRD negative patients have a good prognosis and MRD positive patients have poor prognosis with high chance of relapse. This study should continue in a large number of subjects and with a 5 years of duration.

Acknowledgement

This research and paper is acknowledged by the West Bengal University of Health Sciences and special thanks to Director of Chittaranjan National Cancer Institute for allowing this research work in his institute and provide us funding to continue this research work.

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Kalyan K Mukherjee,etal. "Corelation of Minimal Residual Disease Detection in Paediatric B Cell All Patient with Their Overal Survival and Prognosos- Experience in Tertiary Care Centre in Eastern India." IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), 19(5), 2020, pp. 01-08.