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Flow Cyometric Detection of Minimal Residual Disease (MRD) in Acute Lymphoblastic Leukemia: Correlation to Other Prognostic Parameters

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ABSTRACT

Background: Although clinical and biologic parameters can be used for treatment stratification of acute lymphoblastic leukemia (ALL), none of these prognostic factors is ideal. Measuring response to therapy as reflected by minimal residual disease (MRD) is now considered the most reliable prognostic parameter.

Objectives: To verify the value of MRD detection at different time points as a prognostic parameter in precursor-B ALL.

Patients and Methods: In this study flow cytometric detection of MRD was performed on 97 newly diagnosed precursor-B ALL cases (70 children and 27 adults) at day (D) 15, D28 and/or D42. The relationship between MRD and other clinical and biological prognostic factors was evaluated, as well as the clinical significance of MRD and its impact on the outcome of treatment regarding disease free survival (DFS) and overall survival (OS).

Results: In children, MRD positivity at D15 and D28, was significantly associated with cerebrospinal fluid (CSF) infiltration (p=0.03 and 0.01 respectively).

At D42 MRD positivity was significantly associated with CSF infiltration (p=0.01) and t(9;22) (p=0.045). Patients with MRD <0.01 at D28 and D42 had significantly better DFS (p=0.0002 and <0.0001 respectively) and OS (p=0.02 and 0.001 respectively).

In adults, a significant association was demonstrated between MRD D15 positivity and male gender (p=0.01) which was lost at D28 and D42. At D15, there was a trend for better OS in patients with MRD <0.1 (p=0.058) but no impact on DFS, however, it achieved significance for both at D28 (p=0.05). At D42, we demonstrated significant influence on OS (p=0.01) and DFS (p=0.02).

Conclusions: Using flow cytometry for MRD monitoring is a well-suited approach for the specific detection of minimal numbers of leukemic cells and, hence, could help obtain a more precise and early evaluation of response to therapy in patients with acute leukemia. A redefinition of complete remission according to MRD status is highly recommended.

Key Words: *ALL – MRD – Flow cytometry.*

INTRODUCTION

Despite recent advances in the treatment of acute leukemia, the disease remains a major cause of cancer-related mortality. In childhood acute lymphoblastic leukemia (ALL) there are still around 20% of patients who develop relapse, and those who survive suffer from major treatment related toxicities **[1,2]**, which reflects unadjusted treatment of the disease as a result of the lack of accurate prediction of response to therapy. Introducing methods for minimal residual disease (MRD) detection has revolutionized monitoring of treatment response in acute leukemia. The prognostic significance of MRD in childhood ALL was reported in many studies involving newly diagnosed patients, patients with first-relapse ALL, and those undergoing hematopoietic stem cell transplant **[3,4]**. It is considered the strongest prognostic predictor both in newly diagnosed or relapsed and in standard or intermediate risk ALL **[5,6]**. There is also strong evidence pointing to the clinical significance of MRD in adult ALL **[7-9]**. One of the distinctive markers of ALL cells is the clonal rearrangement of the genes encoding immunoglobulin and T-cell receptor proteins **[10]**. The test is accurate and sensitive (it allows the routine detection of one leukemic cell in 10,000 to 100,000 normal cells), however, the complexity of its set-up limits its routine appli-

cation. Leukemic lymphoblasts can also be recognized by the presence of chromosomal abnormalities and their resulting gene fusions and transcripts, such as *BCR/ABL*, *MLL/AF4*, *TCF3/PBX1*, and *ETV6/RUNX1* **[10]**. The most recurrent abnormalities are found in about one-third or less of patients and allow the detection of one leukemic cell in 1,000 to 100,000 normal bone marrow (BM) cells by reverse transcription-polymerase chain reaction (RT-PCR) **[10]**. Finally, ALL cells can be recognized by virtue of leukemia-associated cell markers combinations visualized with monoclonal antibodies and flow cytometry (FCM) at a sensitivity of detection of 1 leukemic cell in 10,000 normal cells **[11]**.

Bone marrow samples collected after a temporary stop in chemotherapy, after the end of treatment, or after hematopoietic stem cell transplantation may contain a high proportion of recovering immature lymphoid cells whose morphology resembles that of ALL lymphoblasts "hematogones" **[12-15]**. Therefore, morphologic assessment of these samples is difficult and may result in erroneous conclusions; the application of MRD assays can clarify the identity of the morphologically ambiguous cells. Among MRD methods, flow cytometry is the one that is most affected by the state of bone marrow recovery **[16]**. In this regard, it is critical that flow cytometric analysis of MRD relies on markers that truly distinguish ALL cells from normal cells, including lymphoid progenitors; otherwise, the risk of false-positive MRD results is high **[17,18]**.

In this study we aimed to determine the relationship between MRD and other clinical risk factors of precursor-B pediatric and adult ALL. We also aimed to find out the impact of the MRD status at different time points on the outcome of treatment regarding overall survival (OS) and disease free survival (DFS) in precursor-B ALL.

PATIENTS AND METHODS

The study was approved by the Institutional Review Board (IRB) of the National Cancer Institute (NCI), Cairo University and was conducted according to Helsinki declaration for studies involving human subjects. Informed consent was obtained from all patients, patients' parents/guardians.

Patients:

From March, 2009 to February, 2011, a total of 97 newly diagnosed precursor-B ALL patients were recruited from NCI. Among the 97 ALL cases, 70 were pediatric patients including 43 males and 27 females with an age range of 2 months – <18 years with a mean of 6±5.3 and a median of 7 years (Group I). The other 27 ALL cases were adults including 17 males and 10 females with an age range of 19-56, a mean of 30.6±10.78 and a median of 30 years (Group II).

Methods:

The diagnosis of ALL was based on standard morphologic, cytochemical, immunophenotypic and genetic studies. Flow cytometric immunophenotyping of BM aspirates at diagnosis was performed using a standard panel of antibodies and analyzed on Coulter EPICS XL-MCL flow cytometer. The monoclonal antibodies panel included CD45, CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, cyto-μ, k, λ, CD13, CD14, CD33, MPO, TdT, anti class II MHC, CD56, CD38 and CD58 with relevant isotype controls. The antibodies were FITCI, PE, PerCP or Cy5 labelled, obtained from Coulter Hialeah, FL; Immunotech, Marseille, France; DACO, An Agilant Technologies company and/or/Becton Dickinson, Mountain View, California.

The presence of fusion genes in ALL including t(1;19) *TCF3 (E2A)/PBX1*, t(12;21) *ETV6 (TEL)/RUNX1 (AML1)*, t(9;22) *BCR/ABL* and t(4;11) *MLL/AF4* was examined following the standardized RT-PCR analysis of fusion gene transcripts for chromosomal aberrations in acute leukemia **[19]**.

Patients were treated according to standard NCI Cairo University treatment protocols (www.nci.cu.edu.eg).

Disease free survival (DFS) and overall survival (OS) were estimated at 27 months in Group I and at 18 months in Group II.

Flow cytometric MRD analysis:

After initial immunophenotyping at diagnosis, monoclonal antibodies combinations were used to define leukemia- associated phenotypes (expressed on >50% of the blast cells). This step served to define a leukemia phenotypic fingerprint to be used in follow up samples. At

least 2 antibodies combinations were used to minimize pitfalls due to phenotypic switches. The panels included:

- CD34/CD19/CD10/CD58.
- CD34/CD19/CD10/CD38.
- CD34/CD19/CD10/CD45.
- Any aberrant myeloid (CD13 or CD33) or T markers (CD7, CD2).
- CD34/CD22/CD10/CD19: This one is helpful to recognize hematogones by the differentiation pattern.

Data acquisition was performed using the Coulter EPICS XL-MCL flow cytometer. At least 10,000 events were acquired and analyzed for identification of aberrant leukemic phenotypes at diagnosis, and at least 100,000 events were needed for MRD measurements. A detection limit of 0.01% (10/100,000 cells) could be achieved in most cases. MRD level was evaluated at day (D) 15, D28 and D42 and classified as negative (<0.01%) and positive (≥0.01-<0.1 and ≥0.1%). Examples of MRD detection by Flow Cytometry are presented in Fig. (1).

Statistical analysis:

SPSS version 17.0 was used for data management and data analysis. Mean±Standard deviation with median and range when appropriate described quantitative data. Parametric and non-parametric *t*-tests and ANOVA were used for comparing independent groups. Numbers with percentages described qualitative data. Chi-square test and Fisher exact tested proportion independence. Kaplan and Meier method estimated overall and disease free survival and log rank test compared survival curves. *p*-value, or calculated significance level, was considered significant at 0.05.

RESULTS

The study was performed on 97 newly diagnosed precursor-B ALL patients. Among the 97 ALL cases, 70 were pediatric patients including 43 males and 27 females with an age range of 2 months – <18 years with a mean of 6±5.3 and a median of 7 years (Group I). The other 27 ALL cases were adults including 17 males and 10 females with an age range of 19-56, a mean of 30.6±10.78 and a median of 30 years (Group II).

Group I: 70 Pediatric precursors B ALL:

According to risk stratification, patients were subdivided as 36 of favorable age group (≥1-<10 years) and 34 of unfavorable age group (<1-≥10 years).

Immunophenotyping revealed 39 common ALL (cALL), 27 Pre B and 4 Pro B cases. Aberrant CD33 expression was found in 7 cases while, CD2 and CD56 were found each in 2 cases.

DNA index of 1.06-1.16 was found in 23 patients and 47 had DNA index <1.06 or >1.16.

Cerebrospinal (CSF) fluid was free in 67 cases and 3 cases had infiltration.

Molecular studies by RT-PCR revealed t(12;21) in 8 and t(9;22) in 3 patients.

MRD level at different time points is presented in Table (1)

At D15 MRD was positive in 11/58 (19%) patients with no CSF infiltration and in all the 3 patients with CSF infiltration, (p=0.03).

There was no significant association between MRD and molecular studies. Neither was there an impact of MRD status on DFS or OS (Table 2).

At D28, MRD was positive in 10/50 (20.0%) patients with no CSF infiltration and in both patients with CSF infiltration, (p=0.01).

There was no significant association between MRD and molecular studies.

Patients with negative MRD had significantly better DFS (p=0.0002) and OS (p=0.02) than those with positive MRD (Table 2).

At D42, MRD was positive in 11/52 (21.2%) patients with no CSF infiltration and in both patients with CSF infiltration, (p=0.01).

MRD was positive in both cases with t(9;22) and in none of the 6 cases with t(12;21) (p=0.045).

Patients with negative MRD had significantly better DFS (p<0.0001) and OS (p=0.001) than those with positive MRD (Table 2).

There was no statistically significant association between MRD at D15, D28 or D42 and age, gender, total leucocytic count (TLC), lymphadenopathy, hepatosplenomegaly, DNA index or immunophenotyping.

Group II: 27 Adult precursors B ALL:

Immunophenotyping revealed 14 cALL and 13 Pre-B. Aberrant CD33 was found in two cases and CD2 in one case. Two patients had t(9;22).

At day 15, MRD was positive in 11/14 (78.6%) males and 2/9 (22.3%) females (p=0.01). The significance was lost at D28 and D42.

At D15, there was a trend for better OS in patients with MRD <0.1% (p=0.058) but no impact on DFS (Table 2).

At D28, at a cutoff of 0.1%; patients with lower MRD level had significantly better DFS and OS (p=0.05); the corresponding values at D42 were p=0.02 and p=0.01 (Table 2).

Lymphadenopathy, hepatosplenomegaly, TLC, immunophenotyping and molecular genetics showed no significant association with MRD.

Table (1): Minimal residual disease (MRD) level at different time points in precursor-B ALL.

Time point	Children			Adults		
	D15	D28	D42	D15	D28	D42
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
MRD level % \ No.	61	62	54	24	16	16
<0.01	31 (50.8)	49 (79.0)	44 (81.5)	9 (37.5)	8 (50.0)	7 (43.75)
>0.01<0.1	20 (32.8)	7 (11.3)	6 (11.1)	3 (12.5)	3 (18.75)	2 (12.5)
>0.1	10 (16.4)	6 (9.7)	4 (7.4)	12 (50)	5 (31.25)	7 (43.75)

Table (2): Disease free survival and overall survival in ALL patients in relation to minimal residual disease.

Survival Months	MRD level: Mean±SE			p
	<0.01	0.01-<0.1	>0.1	
Pediatrics:				
Day 15				
DFS	NC	22.8±1.3	20.3±2.97	0.299
OS	NC	25.4±1.04	22.8±2.3	0.22
Day 28				
DFS	25.7±0.8	–*	18.3±5.4	0.0002
OS	26.5±0.5	–*	20.8±1	0.02
Day 42				
DFS	25.5±0.7[a]	20.0±4.3[a]	10.2±3.4[b]	<0.0001
OS	NC	25.4±0.87	NC	0.001
Adults:				
Day 15				
DFS	NC	13.4±2.1	7.1±1.2	0.17
OS	NC[a]	13.3±1.7[a]	6.47±1.7[b]	0.058
Day 28				
DFS	14.5±2.2[a]	13.8±2.1[a]	6.1±1.1[b]	0.05
OS	14.7±2.1[a]	13.5±1.8[a]	6.3±1.2[b]	0.05
Day 42				
DFS	14.1±2.3[a]	14.4±1.5[a]	5.0±1.1[b]	0.02
OS	15.1±1.8[a]	14.8±1.97[a]	7.1±1.2[b]	0.01

NC: Survival estimates cannot be computed as all observations are censored.
*Only one case.

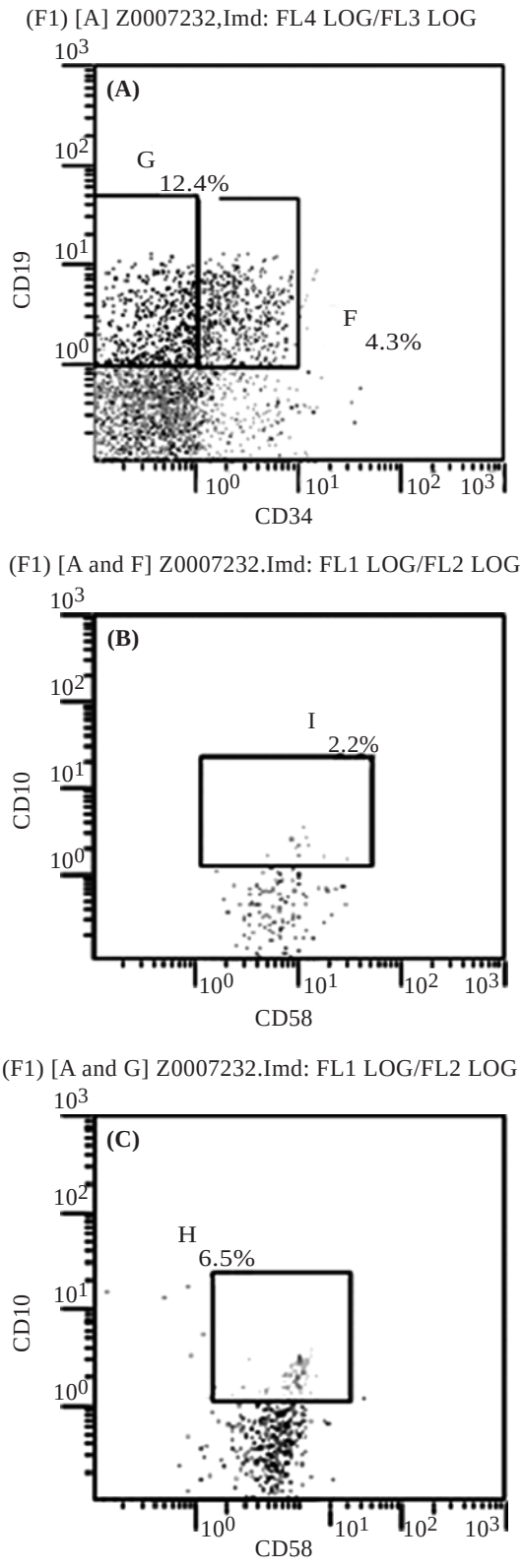

Fig. (1): MRD Detection in precursor B ALL case (CALL) at D15. A: Cells are gated: CD19+/CD34– (Gate G) and CD19+/CD34+ (Gate F). B: CD19+/CD34– in Gate F are analyzed for CD10 and CD58: Region I shows 2.2% coexpression of CD10 & CD58. C: CD19+/CD34+ cells in gate G are analyzed for CD10 & CD58 expression, region H shows 6.5. Both populations belong to the malignant clone as indicated by CD58 expression.

DISCUSSION

In this study flow cytometric detection of MRD was performed on 97 precursor-B ALL cases (including 70 pediatric patients and 27 Adults). The purpose of this study was to verify the value of MRD detection at different time points as a prognostic parameter in precursor-B ALL and to demonstrate the relationship between MRD status and other prognostic parameters. In children with ALL, measurements of MRD provide unique information on treatment response and have become a crucial component of contemporary treatment protocols. Flow cytometry-based assays are rapid and provide an accurate quantification of MRD while gaining information on the status of normal hematopoietic cells at the same time. Abnormal phenotype that can be used for MRD detection could be applied in 98% of cases of ALL with sensitivity up to 10^{-4}. The sensitivity of this approach depends on two main factors: The degree of dissimilarity between the immunophenotypes of leukemic cells and those of normal cells, and the number of cells available for study **[20]**. In the pediatric cohort, associations between MRD at D15, D28, and D42 post induction and other clinical and biological risk factors including age, gender, TLC, lymphadenopathy, hepatospleomegaly, molecular studies and DNA index were of no statistical significance. This is in agreement with other studies **[21-26]**, except for one that reported statistical association between gender and MRD **[27]**. This might be attributed to the different treatment protocols or to ethnic differences. In our study, no association was encountered between D15 MRD and OS or DFS at a cut off value of 0.01; however it was evident at D28 and D42. This suggests that D28 might be a good early indicator of early responders. Although this is consistent with another study **[22]**, it is not in line with others **[26,28-30]** The difference may be attributed to the short follow-up period as well as the sample size. Furthermore, these studies included both T and B ALL in their analysis and patients received different treatment protocols. In the current study, we detected statistically significant association between MRD positivity at D42 post induction and molecular findings (p=0.045). This is in agreement with some reports **[23,24]**. However, these issues were not addressed in other studies **[22,26]**. In the current study, we reported statistically significant

association between MRD positivity and CSF infiltration. These results are in concordance with previous reports **[23,24]**. In the current study, presence of MRD level of ≤0.01% at D42 post-induction had a significant impact on DFS and OS; this is consistent with one report **[22]**. but not in line with others **[26,28-30]**. The prognostic value of MRD detection in childhood ALL was most convincingly demonstrated by 3 large prospective studies **[28-30]**. They concluded that, flow cytometric MRD >0.1% on day 15 bone marrow was the most powerful early predictor of relapse. Multivariate analysis demonstrated that the MRD level was an independent prognostic factor with borderline significance **[26]**.

Campana and his team recommended that patients with MRD at 0.1% level have to be reclassified as high-risk group who need therapy intensification and those with ≥1% are eligible for allogeneic BMT in the first remission **[31]**.

In our cohort of adult ALL, a significant association was demonstrated between MRD on D15 post induction and gender (p=0.01). This was not reported in other studies **[21,25]**. The disagreement may be due to small sample size, as well as studying different ethnic groups.

At a cutoff of <0.1% there was a trend (p=0.058) for better OS at D15 but not DFS, border line for both at D28 (p=0.05) and significantly better at D42 (p=0.01 and 0.02 respectively). This is in line with other studies **[32,33]**.

Associations between MRD at D42 post induction and other clinical and biological risk factors including gender, TLC, lymphadenopathy, hepatosplenomegly, and molecular findings were of no statistical significance. These results are in agreement with other studies **[21,25]**.

Although the clinical significance of MRD has been studied less extensively in adult patients with ALL, there is considerable evidence supporting its potential usefulness **[25,34]**. In further work, it was claimed to be an independent prognostic parameter in both standard- and high-risk Philadelphia-negative ALL at a cutoff of 0.1% **[33]**.

In conclusion, our study has validated the efficiency and practicability of Flow cytometry in evaluation of MRD status. It has further emphasized the prognostic value of MRD detection. The differences encountered between our study and others might be attributed to different treatment protocols or different response pattern in patients' cohorts from different countries. Accordingly, the time of testing and the cutoff of MRD that could best serve as a prognostic indicator has to be worked out in context of the specific patient cohort and the treatment protocols adopted. A longer follow-up period is needed to accurately determine the prognostic significance of MRD measurements at different time points and using different cutoff levels.

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