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Detection of Minimal Residual Disease in Childhood B-Acute Lymphoblastic Leukemia by 4-Color Flow Cytometry

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Abstract

Monitoring of minimal residual disease (MRD) is today considered the most powerful predictor of outcome in acute leukemias, including acute lymphoblastic leukemia (ALL). The study aimed to determine whether panel of antibodies combination are more suitable than others for detection of MRD in Childhood B-lineage ALL. Eighty four (84) patients of ALL (B-lineage subtype) were enrolled in this study. Normal template for B. Cell precursors were been established in 15 control Patients by using 4 panels of monoclonal Abs (Mo Abs), {CD22, CD45, CD58 and CD97 in combination with CD10, CD19, CD34}. At diagnosis CD22 having the lowest incidence expression between the patients in 50% only, but CD45, CD58, and CD97 were expressed in 80.9%, 52.3% and 92.8% respectively. Analysis of MRD was performed for each Mo Abs combination at day 0 and day 14 post induction of chemotherapy by 4-color flow cytometer (FCM). The incidence of MRD were 61.9%, 70.6%, 60.0% and 55.5% for CD22,CD45,CD58 and CD97 respectively. Seventy-six from total 84 cases studied (90.0%) had at least one LAIP. Of these, 22 (29.0%) had only one LAIP and 54 cases (71.0%) had ≥ 2 LAIPS

Conclusion: In the B-ALL patients (CD10/CD19/CD34/CD45)+ and (CD10/CD19/CD34/CD97)+, represented the highest incidence markers expression of leukemic cells with a significant correlation with blasts count, so it's the more specific for MRD detection.

Keywords: B-lineage ALL; MRD; 4-color flow cytometry

Introduction

Acute lymphoblastic leukemia (ALL) is the most common leukemia in childhood with a peak incidence at 2-5 years of age. The overall cure rate in children is 85% [1]. MRD is a powerful predictor of the overall response to treatment in childhood ALL.

The most reliable and validated methods to assess MRD in ALL are FCM analysis of leukemia-associated immune-phenotypes (LAIP) and polymerase chain reaction (PCR) amplification of antigen-receptor gene rearrangements [2].

MRD studies by flow cytometry rely on panels of antibodies to define unique immunophenotypic signatures of leukemic cells which must distinguish leukemic blasts from their normal counterparts, the CD19 and CD10 lymphoid progenitors of the bone marrow "hematogones". Standard 4-color flow cytometry can detect 1 leukemic cell in up to 10 000 normal bone marrow or peripheral blood cells but this task typically requires considerable interpretative expertise [3].

Incorporating CD 97 as a marker into investigations of leukemia cells may allow discrimination of leukemia cells from normal haemopoietic stem cells. In B-ALL patients the CD34+/CD97+ subpopulation represented the bulk of leukaemic cells. Recent studies have highlighted potential markers that may improve the sensitivity of MRD detection by flow cytometry, CD 97 is one of these markers which show over expression in pediatric ALL [4].

Aim of the Work: To determine whether panel of antibodies combination are more suitable for detection of MRD in B-lineage all.

Patients and Methods

This study was carried out at Clinical Pathology and Pediatric Oncology Departments of Zagazig University hospitals. Ninety (90) patients of ALL (B-lineage subtype: Seventy (70) patients were of common ALL category, 14 patients of pro B ALL and 6 patients were pre B ALL), were participated in this study. Their ages ranged from 1 year to 17 years with mean age 7.4-4.5 years. They were 52 males and twenty 48 females with male to female ratio 1.08:1. Diagnosis of B-lineage ALL was based on morphological, cytochemical, immunophenotypic and cytogenetic characteristics of leukemic blasts, (WHO 2008 classification).

Written consent was taken by patient's parents to share in this study. For control, bone marrow was obtained from 15 patients with non-hematological malignancies undergoing bone marrow aspiration as a part of routine investigation e.g. idiopathic thrombocytopenic purpura (ITP) or lymphoma, for staging, without bone marrow infiltration (Proven by bone marrow biopsies).

The present study was approved by ethical committee of Faculty of Medicine, Zagazig University. Unfortunately 1 patient was died before

day 14, 3 patients not achieved complete morphological remission (CR) and 2 patients were missed due to other reasons e.g. inadequate bone marrow samples or refusal to complete the study. For remission induction, all patients were treated with a chemotherapy protocol including Prednisone, Vincristine, Daunorubicin and L-asparaginase.

Inclusion criteria

Newly diagnosed patients of B-lineage ALL and then after complete hematological remission (CHR), (day 14). Age more than 1 years and less than 18 years, both sex were included.

Exclusion criteria

Patients suffering from other malignancies as T-ALL or bilineage leukemia, patients starting chemotherapy, patients more than 18 years.

All the members of the study were subjected to the following:

Complete blood count (CBC), bone marrow aspiration, Leishman stained peripheral blood (PB), bone marrow smears examination and conventional cytogenetic analysis.

Special investigations

Four (4) tubes well prepared for each patient, each tube contain a panel of 4 monoclonal antibodies, {tube 1 (CD10 FITC, CD19 PE, CD34 Per-CP and CD22 APC), tube 2 (CD10 FITC, CD19 PE, CD34 Per-CP and CD45 APC), tube-3 (CD10 FITC,CD19 PE,CD34 Per-CP and CD58 APC) and tube 4 (CD10 FITC, CD19 PE, CD34 Per-CP and CD97 APC)}, defined at diagnosis and day 14 post induction for tracing of MRD.

Sampling

Sample preparation and staining procedures

The whole blood lies staining method was performed.

Detection of surface markers by direct staining

Plastic Falcon tubes (12 × 75 mm) were labeled with lab no and staining antibody including controls. Ten µl labeled Mo Ab antibodies was dispensed into all appropriately labeled tubes. One hundred µl samples was added and incubated in the dark for 20 minutes. Three and half ml lysing reagent to each tube was added, inverted once, and kept for 3 minutes. Centrifuge at 1500 x g (3200 rpm) for 3 minutes. Three and half ml PBS was added and centrifuged at 1500 x g (3200 rpm) for 3 minutes.

Analysis of MRD

Sample analysis was performed by multicolor FCM (FACS Calibur flowcytometer Becton Dickinson, USA).

Gating strategy

The rational for MRD detection is to use sequential gating strategy. ALL cases first tight lympho-population gate applied on SSC *vs.* FSC and CD19 co expressing CD34 population, then CD19 co expressing CD10, then subsequently gating on (CD34 and CD22), (CD34 and CD45), (CD34 and CD58) and (CD34 and CD97) respectively. Leukemic events were defined at dot plot in a region with estimated number of events from statistics.

Methodology of minimal residual disease detection

Patients were evaluated at diagnosis, Mo Abs combination were used to define leukemia associated immunophenotype (LAIP), this step served to define a leukemia phenotypic fingerprint to be used in follow up samples. For establishing the diagnostic LAIP 50,000 events were acquired and 500,000 events were necessary for detection of MRD.

LAIP is identified to allow the discrimination of leukemic blasts from normal B lymphocyte progenitors (normal template for each Mo Abs combinations was drawn) and relies on qualitative or quantitative differences in antigen expression between leukemic cells and their normal counterparts.

The LAIP present in an individual case has been identified by using multi floresence colors (MFC) with a comprehensive panel of Mo Abs, So MRD during the course of treatment and follow-up can be assessed by the quantification of the frequencies of these cells by MFC.

Statistical Analysis

Data were entered checked and analyzed using Epi-Info version 6 and SPP for Windows version 8 [5]. Qualitative data were represented as frequencies and relative percentages.

The Wilcoxon signed-rank test is the nonparametric test equivalent to the dependent t-test used for comparison between the patients at day 0 and 14 of treatment. The significance Level for all above mentioned statistical tests done. P value of >0.05 indicates non-significant results. P value of <0.05 indicates significant results.

Results

Ninety (90) patients included in this study, 6 patients were missed, 84 patients were traced for presence of MRD. At diagnosis, in combination with (CD10, CD19, CD34), CD22 was expressed in 42 cases (50%) tube 1, CD45 was expressed in 68 patients (80.9%) tube 2, CD58 was expressed in 50 patients (59.5%) tube 3, while CD97 was expressed in 78 patients (92.8%) tube 4.

The frequencies of aberrant phenotypes by MFC showed, 24 patients (28.6%) had one aberrant marker and 60 patients (71.4%) had 2 or more aberrant markers.

MRD level <0.01% was considered negative, while positive MRD was classified according to its level into 2 groups: ≥ 0.01- <0.1 and ≥ 0.1, (Table 2) For assessment of MRD, in tube-1 MRD was absent in 38.1% but recorded in 61.9% of cases {≥ 0.01-<0.1,(28.6%) - ≥ 0.1, (33.3%)}, tube-2 MRD was negative in 29.4% but positive in 70.6% of cases {≥ 0.01-<0.1, (23.5%) - ≥ 0.1, (47.1%)}, tube-3 MRD was negative in 40.0% but positive in 60.0% of cases {≥ 0.01-<0.1,(24.0%) - ≥ 0.1, (36.0%)}, tube-4 MRD was negative in 45.5% of cases but recorded in 55.5% of cases {≥ 0.01-<0.1, (20.0%) - ≥ 0.1, (25.5%)},(Table 3).

Seventy six (76) from total 84 cases studied (90.0%) had at least one LAIP. Of these, 22 (29.0%) had only one LAIP and 54 cases (71.0%) had ≥ 2 LAIPS.

There was a highly significant decline in BM blasts and blast events at day 14 of chemotherapy induction compared to baseline level (before treatment), (Table 1), (Figures 1-4).

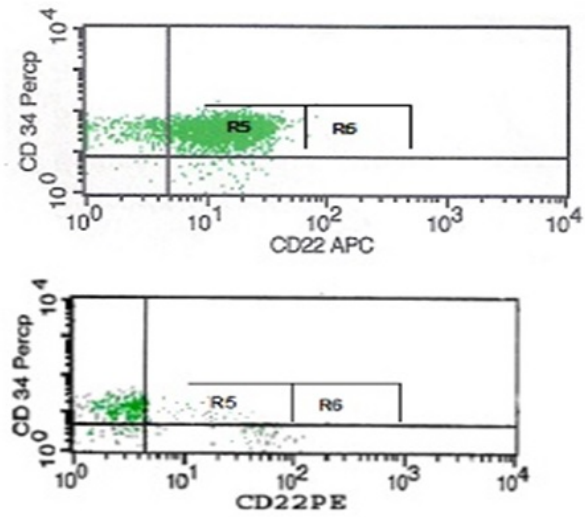

Figure 1: Comparison between CD34 andCD22 blast events (tube 1) at (day 0) and (day 14); (A) Before therapy (day 0): CD34 and CD22 positive blast events was 3143 (Blast: 69%); (B) After therapy (day 14): CD34 and CD22 positive blast events was 25=0.25 positive MRD.

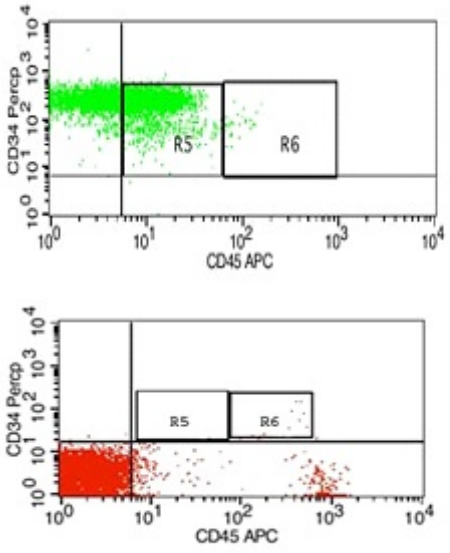

Figure 2: Comparison between CD34 and CD45 blast events (tube 2) at day 0 and day 14. (A)Before therapy (day 0): CD34 and CD45 positive blast events was 4567(Blast:77%) R5 represent CD 34 positive blasts with dim expression of CD45. (b)After therapy (day 14): CD34 and CD45 positive blast events was 8=0.08 positive MRD. R6 represent hematogones (CD45 positive with high expression of CD45).

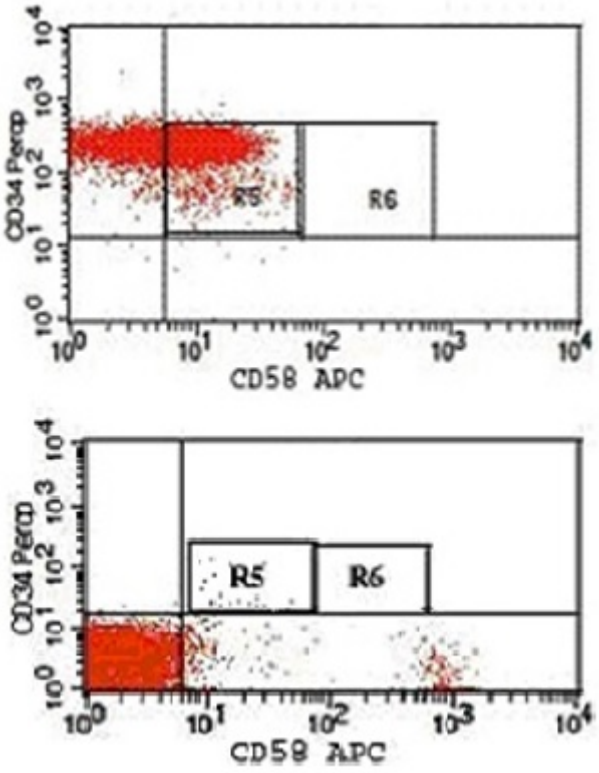

Figure 3: Comparison between CD34 and CD58 blast events (tube 3) at (day 0) and (day 14) (A) Before therapy (day 0): CD34 and CD97 positive blast events was 6999(Blast: 90%); (B) After therapy (day 14): CD34 and CD97 positive blast events was 19=0.19 positive MRD.

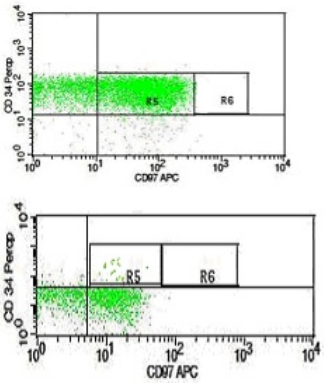

Figure 4: Comparison between CD34 and CD97 blast events (tube 4) at day 0 and day 14. N B: R5 represent CD 34 positive blasts- R6 represent hematogones.

Blasts (%)	Studied ALL patients		Test•	p-value (Sig.)
	At day (0)	At day (14)		
BM (%)				
Mean ± SD	73.29 ± 15.90	1.00 ± 1.46	-4.544	<0.001 (HS)
Median (Range)	71 (39 - 100)	1 (0 - 5)		
FCM (%)				
Mean ± SD*	72.11 ± 11.90	5.68 ± 4.90	-4.541	<0.001 (HS)
Median (Range)	75 (43 - 90)	2.0 (0.03 - 12)		

Table 1: Comparison between blast count at day (0) and at day (14) by morphology and flow cytometry (FCM, Wilcoxon signed ranks test. p<0.05 is significant; Sig: Significance.

Type of panel	Tube-1 (10/19/34/22)	Tube-2 (10/19/34/45)	Tube-3 (10/19/34/58)	Tube-4 (10/19/34/97)
Patients (%) with aberrant phenotype (N=84)	(42)-50%	(68)-80.9%	(50)-59.5%	(78)-92.8%

Table 2: Incidence of aberrant phenotype within panel of antibodies used for MRD detection.

MRD	Tube-1	Tube-2	Tube - 3	Tube-4
	(10/19/34/22)	(10/19/34/45)	(10/19/34/58)	(10/19/34/97)
Negative(<0.01)	38.10%	29.40%	40.00%	45.50%
Positive	-61.90%	-70.60%	-60.00%	-55.50%
≥0.01-<0.1	28.60%	23.50%	24.00%	20.00%
≥0.1	33.3.%	47.10%	36.00%	25.50%

Table 3: Positivity of MRD among the four tubes used.

Discussion

Acute lymphoblastic leukemia is the most common malignancy in children, accounting for almost one third of newly diagnosed pediatric cancer cases. The annual incidence is approximately four cases per 100,000 children per year in national cancer institute (NCI), Cairo University, Egypt. All constitutes 30% of all pediatric malignancies and 70% of pediatric leukemia [6]. Minimal residual disease (MRD) studies in childhood acute lymphoblastic leukemia (ALL) give highly significant prognostic information superior to other standard criteria as age, gender and total leucocytic count (TLC) in distinguishing patients at high and low risk of relapse [7]. The approach used in this study was to determine whether panel of antibodies combination are more suitable for detection of MRD in childhood B lineage ALL. In our study the mean marrow blasts at diagnosis was 73.29 ± 15.9 while at day 14 post induction therapy it was 1.0 ± 1.46. By flow cytometer the mean no of BM blasts was 72.11 ± 11.9, while at day 14 post induction therapy it was 5.68 ± 4.9. There was a highly significant decline in BM blasts at day 14 of induction chemotherapy compared to baseline level (before giving treatment) (p<0.001). The difference between blast count at day 14 by morphology and Immunophenotyping may be due to miscounting of some blasts as hematogones by morphology that proved to be blasts by immunophenotyping. In addition to the difference in number of cells counted by morphology maximum 500 while by immunophenotyping 10,000 making it more sensitive and accurate method. This allows reducing the limit of blasts to 0-1% as CHR, as proved later on only those with less than 1% blasts are actually in CR by immunopheno typing. From 1-5% are grey zone that should be assessed for MRD. Regarding Mean ± SD of BM blasts were in agreement with Mikhail et al. [8] who reported that the relative proportion of abnormal blasts in bone marrow in his study declined during treatment from day 0 (median >90%) to day 29 (median 1.1%). Also Oudot et al. [9] reported that the vast majority of children with ALL achieve complete morphologic remission by the end of the first month of treatment. In many ALL protocols, days 8 and 15 of induction therapy are considered as the first checkpoints to test the *in vivo* sensitivity of the therapy in the individual patient. The elimination or fast reduction of the leukemia accounts for prediction of relapse-free survival [10]. Choice of day 14 post induction to assess MRD is in agreement with Neale et al. [11] who found that in general measurements of MRD 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. Our results showed that at day 14 not all patients undergo complete morphological remission, so it is better to use MRD on day 15 and day 42 for treatment assignment as reported by St Jude Children's Research Hospital. At diagnosis in combination with (CD10, CD19 and CD34), CD22 having the lowest incidence expression between the patients in 50% only and positivity of MRD were 61.9%. Patkar et al. [12] reported that CD22 is weakly expressed by early CD34 positive B cell precursors and aberrant strong expression was seen in 26.5% of our patients. As a part of multiparametric analysis, however, this tube (containing combination 22/34/45/19) was found to be applicable to 57.1% of our cases. CD22 when seen solely as a marker of aberrant expression seems to be of limited utility as shown by Campana D (13). (20–30%), Irving et al., [13,14] (5%). Loretta et al. [15] however reported a much higher applicability of 46.2%. According to Coustan-Smith et al. [16] the 0.01% threshold is commonly used to define MRD positivity, simply because this represents the typical limit of detection for routine flow cytometric and molecular assays. Nevertheless, it is possible to achieve a routine sensitivity of 0.001% by PCR in clinical samples. With improvements in technology, it is likely that such threshold could also soon be achieved by flow cytometry. The current 0.01% threshold has proven to be clinically informative. For example, they found that patients who had MRD of 0.01% or higher in bone marrow at any time point during treatment had a significantly higher risk of relapse. The highest incidence of markers expression in our patients were reported in tubes 2 and 4 (CD45 and CD97 in combination with CD10/CD19/CD34) were positive in 80.9% and 92.8% respectively and a significant correlation with blasts by FCM at day 0 and day 14 post induction chemotherapy was observed. Analysis of MRD was performed, 70.6% and 79.5% of patients had positive MRD for tube-2 and tube-4 respectively. Choice a combination of (CD10/CD19/CD34/CD45) for assessment of MRD was in agreement with Jmili et al.[1] who reported that two combinations are suitable for

the monitoring of MRD by FC in B-ALL: CD10/CD38/CD19/CD45 and CD10/CDCD34/CD19/CD45 because they allow the screening of LAIP which are frequent and distinguish B-lymphoblasts from normal hematogones as hematogones are precursors which were defined by CD19 positivity and CD45 bright, so overexpression of CD45 is expected in regenerating bone marrow. Our results showed under expression of CD45 (on CD34 +ve blasts) was found in 60.1% of patients. Also Irving et al. [14] found the under expression of CD45 to be useful in 75% of B-ALL. Patkar et al. [12] (2012) found that CD20, CD10, CD19 and CD45 combination was applicable to 71.4% of B-ALL cases. Coustan-Smith et al. [16] found combination including (CD19, CD34, CD10, CD97) to be informative in 27.2% only of cases compared to our results 92.8%. This difference may be attributed to our small sample size (n=42) compared to Coustan-Smith study, no. of cases (81). According to (Compana et al. [16] a significantly lower and/or an undetectable level of expression of CD97 marker in the second specimen relative to that of the first specimen obtained prior to the initiation of the therapy can be a positive indication of the efficacy of the therapy and this in agreement with our study where we found that there's a highly decrease in expression of CD97 after therapy which may be a sign of good response to treatment. CD58 was expressed in 60% of our patients. Veltroni et al. [17] showed that, CD58 expression was significantly higher in all blasts than in normal B lymphocytes, while no significant differences between regenerating and normal B lymphocytes were observed. CD58 was expressed in 99.4% of the precursor-B ALL cases and 93.5% of this showed over-expression compared to normal. No significant modulation of CD58 expression during remission induction therapy was noted. Finally, 66 (95.6%) of 69 BM samples simultaneously analyzed using both FC and RQ-PCR at day +78 showed concordant results regarding MRD. Zhongguo et al. [18] indicating that CD58 could be an effective marker in MRD detection. The CD58/CD10/CD34/CD19 was the second most effective combination next to TdT/CD10/CD34/CD19 in B-ALL MRD detection with flow cytometry. Meanwhile, the positive rate of MRD detection by flow cytometry was significantly lower in CD58 over expression group ($P<0.05$). It is concluded that CD58 may be used as an indicator for detection of MRD in B-ALL patients, which would enrich the combination of MRD detection. The CD58 over expression may be considered as a marker of a favorable prognosis in childhood B-ALL. Our results not in agreement with the previous two studies, as showed that tube [3] contains CD58 were informative in 59.5% only and overexpression of CD58 (on CD34+ve blasts) was recorded in 37.3% of studied patients. Recent study showed that the most applicable antibody combination was TdT/CD10/CD34/CD19 (87/139, 62.6%), followed by CD38/CD10/CD34/CD19 (85/139, 61.2%) and CD45/CD10/CD34/CD19 (58/139, 41.7%). they have identified a relatively effective MRD panel, combined with TdT, CD38, and CD45 as key markers, that is applicable to the majority of newly diagnosed B-lineage all [19].

Conclusion

In the B-ALL patients (CD10/CD19/ CD34/CD45)+ and (CD10/CD19/CD34/CD97)+, represented the highest incidence markers expression of leukemic cells with a significant correlation with blasts count, so it's the more specific for MRD detection.

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