Flowcytometric and DNA Analysis Minimal Residual Disease (MRD) in Childhood B-Lineage lymphoblastic leukemia

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Abstract

Background: Induction Chemotherapy for Acute Lymphoblastic Leukemia achieves complete remission in over 90% of children. It is apparent therefore that many patients in clinical remission and without residual disease detectable by conventional light microscopy of peripheral blood or bone marrow films still harbor viable cells of the original disease MRD analysis does have a useful role to play in the risk directed treatment of childhood ALL and this is currently being investigated in large prospective studies.

Methods: We have investigated MRD in bone marrow samples by three color flowcytometry approach in 63 pediatric B-precursor ALL patients treated according to BFM ALL 95 protocol. Bone marrow samples were collected from children at three different times including: Day 28th, At the beginning of intensified therapy and at the end of therapy .Cells with leukemia associated Immunophenotype were investigated by DNA analysis for evaluation of DNA content.

Results: Among 63 children with diagnosis of B-lineage ALL and quantified for post induction residual disease study .We observed that the mean number of blast cells have significant differences among these groups. The mean number of Leukemic blasts counted on day 28 was 2.7± 0.4, at the beginning of intensified therapy 1.7±0.4, and at the end of treatment 0.5±.2. These patients were in complete remission in light microscopy examination. Relapse of ALL was demonstrated in six of 63 children (9.5%) whose MRD were more than one blast in 10^{-2} cells .Comparing this to light microscopic examination dill of these patients had 4-5% blasts vs. 1% for those who did not relapse.

Conclusion: MRD analysis does have a useful role in the risk directed treatment of child hood ALL and the investigation of levels and the dynamics of MRD by sensitive and quantitative flowcytometry and PCR methods reduce false negative results. However a good morphology is also valuable.

Keywords: Acute Lymphoblastic leukemia, Minimal Residual Disease, Flowcytometry, DNA analysis

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Introduction

Nearly a quarter of children with acute Lymphoblastic leukemia (ALL) who achieve complete remission by standard criteria eventually relapse and most die from disease.(1) To further increase the cure rate and to reduce unnecessary toxicity in children with ALL, efforts have been made to identify clinical and biological features of prognostic significance at diagnosis. Clinical features with recognized prognostic value in childhood ALL include sex, age at diagnosis, the initial WBC, Immunophenotype and early response to therapy(2). The imperfection of presenting features in predicting outcome is not unexpected because cytoreductive capacity of chemotherapy depends not only on the sensitivity of Leukemic cells to chemotherapy but also on pharmacokinetic and pharmacogenetic variables.(3) When ALL is diagnosed in a patient, the total number of leukemia cells is approximately 10^{12} to 10^{13} . A majority of patients reach complete remission (CR) after about 4 weeks of chemotherapy and leukemia cells is beyond the sensitivity of classical cytomorphologic methods (eg.1 T05%).(4) At this time, up to 10^{10} malignant cells can still remain in the

patient. They represent the minimal residual disease (MRD).[5] Techniques aimed at studying MRD are genetic markers which can be detected by PCR and Immunophenotype markers which can be detected by flowcytometry[6]. Flowcytometry of MRD is based on the identification of Immunophenotypes expressed on Leukemic cells but not on normal hematopoietic cells.[8] We established further that MRD determination simultaneously generated with FC and PCR based technology are highly concordant in qualitative as well as quantitative aspects.

Patients, Method and Materials

From June 1999 to November 2001 we conducted a population based study in Ali Asghar Children's Hospital.This single laboratory flowcytometry study was done in the Iranian Blood transfusion organization on MRD in consecutive series of 63 children with B– Lineage ALL. All children were treated with BFM 95 protocol. The study was approved by the ethical committee at Iran Medical University of Sciences with informed consent obtained from the parents. The cohort consisted of 36 boys and 28 girls between 2 and 17 years of age (Mean age: 8.25 ±0.7). Diagnosis of ALL was made on basis of morphologic evaluation of Wright stained bone marrow smears and flowcytometry. Patients were divided into three groups:

Group 1: On day 28 of induction, 28 patients.

Group 2: On day 0 of late intensification, 17 patients.

Group 3: the end of therapy (after 3 Years) 18 patients.

Treatment Protocol:

All patients were treated according to the Modified BFM 95 protocol.

Flow cytometry:

In these cases markers were measured by three color coulter Epics flowcytometry. That includes:

Tdt/CD10/CD19, Tdt/CD10/CD34,
Tdt/CD19/CD34 and CD10/CD34/CD19

Sampling preparation for flowcytometry:

Two milliliter bone marrow aspirate were collected in EDTA anticoagulant tubes during day 28, onset of intensification and at the end of therapy and maintained at 2-7 ^{c} until processed at maximum 24 hours. For each sample two tube was prepared (Test and negative control). In each tube 200 µl of bone marrow plus 1 ml paraformaldhyde were mixed and incubated in 4 °C and the dark for 10-15 minutes.

The samples were washed with PBS and were centrifuged for 10 minutes at 2000 rates. Upper layer of tubes were removed and cells were mixed with 0.5 ml triton then incubated for another 1 minute then washed two times with PBS. The cells were stained Nuclear Tdt. All cases were isotype matched immunoglobulins with no reactivity against bone marrow cells and triple color cells with negative control were counted in flowcytometry.

These CD markers were surveyed: Tdt/CD10/CD19, Tdt/CD10/CD3, Tdt/CD19/CD34, CD10/Cd34/Cd19. The expression of leukemia associated Immunophenotypes among the selected population was then analyzed, sampling preparation for DNA content: For each patient one tube was set with a healthy control. In each tube 50 µl bone marrow was added to 500 µl RNASE, 50 landa triton 10% and 500 µl propidium iodide. This mixture was incubated for 30 min in 37°C after this time, tubes were prepared for survey.

Data acquisition and statistical analysis

Data acquisition was performed by using 3-color flowcytometer. Distributions of presenting features according to the degree of residual disease during clinical remission were compared in three groups by Fisher exact test. The probability of surviving without ALL relapse was estimated

Results

We identified cells with leukemia associated Immunophenotypes in 28.6% in group A (one blast cell in 10^{-4} normal cell) and 71.4% in group B (One blast cell in 10^{-2} normal cell) of bone marrow samples taken on the 28th day of therapy. Leukemic cells were identified in 35.3% group B and 64.7% group A at onset of intensification period while level of detection was 94.4% group A and 5.6% group B at the end of treatment.

All patients achieved morphological complete remission by light microscope and blasts cells were under 5 blast cells. Rates of residual disease detection did not differ significantly in comparison based on sex, age, and leukocyte count.

Table 2:Median and Standard deviation of CD Markers

Groups M±SD	CD34	HLA DR	CD20	CD19	CD10	CD7	CD3	CD2
1	3.5±0.5	10.2±1.3	13.3±3.9	8.9±1.4	1.6±0.3	14±2.1	2.03±1.9	7.5±1.6
2	4±0.8	12.5±1.1	4.9±1.2	4.1±0.8	2.9±0.5	7.2±1.2	10.7±0.9	3.7±0.7
3	3.7±0.9	13±1.8	4.9±0.9	5.6±1.2	2.9±0.5	6.6±1.8	9.1±1.1	3.9±0.9
Total	3.7±0.4 P=0.841	11.6±0.8 P=0.32	8.6±1.8 P=0.067	6.6±1.8 P=0.027	2.3±0.2 P=0.025	10.1±1.1 P=0.004	14.9±1.2 P=0.000	5.4±0.8 P=0.065

Table 3: Incidence of Blast in group

Group	Blast <2	Blast 2-200	Total
1	8	20	28
2	11	6	17
3	17	1	18

In our study based on immunophenotyping, there were 63.3% in early pre B ALL, 24.5% in pre B ALL and 12.2% in pro B ALL.

Figure4: Immunophenotype

Early Pre B ALL	63.3%
Pre B ALL	24.5%
Pro B ALL	12.2%

There were striking differences in CD markers including CD19 (P= 0.027, F= 3.83), CD10 (P= 0.025, F= 3.91), CD7 (P= 0.004, F= 6.171) and CD3 (P= 0.000, F=19.15).

Result of post HOC analysis demonstrated striking differences in CD19 in group 1 and 2 (P=0.011) and CD10 in group 1 and 3. CD3 and CD7 between different group had a similar difference to CD10 (P=0.001).The Blast group is demonstrated in table 3.

DNA analysis showe ddifferences between three groups on percent of G_2 M + S (P= 1.015) and G_0 G_1 (P=4.692).

These differences were between groups of 2 and 3 (P=0.05). DNA analysis was performed and 78.7%±1.3 of cells were in G_0 G_1 phase, while 21.3%±1.3 were in G_2 M+S phase. Median Blast was counted: group 1 2.7± 0.4, group 2 1.7±0.4 and group 3 0.5±0.2.

Relapse of ALL was demonstrated in six of 63 children. In five patients, the recurrence was demonstrated in the bone marrow. Four patients relapsed at the end of treatment. One patient relapsed at the end of intensification. One patient demonstrated extramedulary relapse in testis. Immunophenotype of all relapsed patients was Pro B ALL and higher MRD levels (one blast cells in 10^{-2} cells). The mean DFS for children who suffered relapse of ALL was 43 months. In our investigation, Leukemic cells in group 1 was 0.027%, group 2 was 0.017 % and group 3 was 0.005%.

Table 4: DNA analysis at initial diagnosis

Groups	G_0G_1	G_{2M} +S
1 R	0.389	0.389
P Value	0.189	0.189
2 R	0.172	0.172
P Value	0.172	0.172
3 R	0.648	0.648
P Value	0.237	0.237

Discussion

Detection of minimal residual disease during continuation chemotherapy was strongly predictive of Leukemic relapse. The follow-up samples were obtained from all patients with childhood ALL. Patients with MRD levels greater than one blast in 10^{-2} cells after the end of therapy or every time after induction therapy had significantly increased rate of relapse compared to patients with MRD value lower than one blast in 10^{-4} cells. Our main conclusion is that the presence or absence of detectable residual leukemia during clinical remission strongly influences treatment outcome suggesting that flowcytometric assays could be used to augment current methods of risk assessment.

The sensitivity of both molecular and immunologic MRD detection methods depends on the numbers of cells analyzed and on the probes applied.[9,10] Also, sample preparation and live gate acquisition should be performed with in 24 hours.[11] Detection of MRD in B lineage ALL requires a large panel of antibodies. We usually identify immature B cells by simultaneous expression of CD19, CD10 and CD34 or TdT.[12,13]

In our study, demonstrated that MRD positive patients in all MRD phases of treatment have been at risk of relapse, but in another study measurements days 14, 19, 28 and week 14 seem to be the best predictors for relapse.[14, 15, 16]

The flowcytometric techniques we used are well suited for clinical investigation of residual disease because of their availability and rapidity

Table 5: Blast counts and DNA analysis

Groups	G_0G_1	G_{2M} + S
1 R	0.061	0.061
P Value	0.549	0.810
2 R	0.021	0.155
P Value	0.938	0.567
3 R	0.101	0.101
P Value	0.797	0.797

of result but in conjunction with PCR ,the study has increased sensitivity and false negatives are decreased.[17,18,19,20] Finally sensitivity of the test with the markers used was limited when regenerating bone marrow samples were studied.[21]

In summary, immunological and DNA analysis investigation of MRD provides the best clinical information about the outcome and treatment protocol in child ALL. Individual approaches to treatment could reduce the risk of both relapse and therapy related late effects. We suggest that all patients in the course of therapy should be closely monitored by serial flowcytometric follow up which may easily create treatment response curves for clinical use and treatment adjustments.

Contributors

Dr.M.Aghaiepoor and M.Nikoogophtar were responsible for the flowcytometric analysis at the Iranian Blood Transfusion organization and basically conducted the study.

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