

Flow Cytometric Analysis of Nuclear DNA Ploidy and Proliferative Activity in Acute Lymphoblastic Leukemia

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ABSTRACT

Purpose: We have planned this work to determine DNA ploidy and S-phase fraction (SPF) in acute lymphoblastic leukemia using flow cytometry and also to correlate these parameters with FAB classification, immunophenotyping, other prognostic factors and response to induction chemotherapy.

Materials and Methods: Twenty de novo ALL patients were selected and classified into 3 groups: L₁, L₂ and L₃ according to FAB classification and into 4 groups: common ALL, pre B-ALL, B-ALL and T-ALL according to immunophenotyping. All patients were subjected to the following: full history and clinical examination, laboratory investigations including: complete blood picture, bone marrow examination, cytochemistry, immunophenotyping of blast cells and the study of FCM DNA ploidy, DNA index and S-phase fraction (SPF) done on ALL blast cells. All patients were treated with ALL induction protocol.

Results: The results were statistically analyzed and came to the following findings: aneuploidy was found in 5 cases of L₁ group (45.5%) in one case of L₃ (50%), in 5 cases of common ALL group (45.5%) and in one case of B-ALL (50%). A significant statistical difference was detected between the percentage of S-phase and both FAB and immunophenotyping subgroups. There was a significant association between response to induction chemotherapy and FAB subgroups, immunophenotyping subgroups and percentage of S-phase fraction, whereas, there was no association between response to therapy and ploidy status or DNA index.

Conclusion: We can conclude that quantitative DNA analysis reflects the total chromosomal content of malignant cells and estimates the proliferative index of the blast cells which can now be determined rapidly and reliably using flow cytometry. It can be used as an additive tool besides conventional methods helping in the diagnosis and in predicting patients outcome in acute lymphoblastic leukemia.

Key Words: Flow cytometry - DNA Ploidy - Acute lymphoblastic leukemia.

INTRODUCTION

The clinical features of neoplasia are due to the capacity of tumor cells to proliferate. Neoplastic cells often have an abnormal DNA content (DNA aneuploidy) in comparison to normal (diploid) cells [2]. Aneuploidy is believed to reflect the numerical or structural chromosomal aberrations present in neoplastic cells [4].

DNA analysis is one of the important applications of flow cytometry with a prognostic implication. It includes DNA content measurement, which determines ploidy status and also cell cycle analysis which provides information about the proliferative activity of the cell population of interest [9].

Cytogenetic abnormalities of leukemic cells can be characterized as changes involving chromosome number (ploidy) or chromosome structure. Ploidy is determined either by quantitation of the modal number of chromosomes in metaphase preparation or by flow cytometry. Chromosome classification adds significant prognostic information to WBCs count at diagnosis, age, FAB classification and immunophenotyping [23].

In ALL, children with higher ploidy (> 50 chromosomes) carry the best prognosis while children with hyperploidy (47-50 chromosomes) have an intermediate prognosis and those with hypoploidy have a relatively poor prognosis [22].

The aim of this work is to determine DNA ploidy and S-phase fraction in ALL patients using flow cytometry and to correlate these parameters with FAB classification, immunophenotyping

notyping, other important prognostic factors and response to chemotherapy.

PATIENTS AND METHODS

This study was conducted on twenty de novo ALL patients attending the Hematology and Medical Oncology Unit of the Internal Medicine Department, Zagazig University (Table 1).

All patients were subjected to the following:

- 1- Thorough history taking and full clinical examination.
- 2- Complete blood picture (Sysmex SF 3000), bone marrow examination and cytochemistry (Peroxidase stain) for diagnosis of ALL and classification according to FAB system [3].
- 3- Immunophenotyping of leukemic bone marrow cells using dual color monoclonal antibodies (Becton Dickinson); CD10, 19, 20, 5, 3, 22, 7, 34, HLA DR, TDT, on FACScan flow cytometer (Becton Dickinson) [10].
- 4- Flow cytometric DNA analysis, peripheral blood mononuclear cells stained with DNA Con3 staining kit (19700) CONSUL TS, on FACScan flow cytometer (Becton Dickinson) [24].

All patients were treated with induction protocol which consisted of vincristine, prednisone, daunorubicin and L-asparaginase. High dose methotrexate was used only for B-ALL.

Flow cytometric DNA analysis:

Leucocytes, isolated with simple gravimetric method, were stained with DNA Con3 kit. This kit uses the intercalating properties of propidium iodide (PI), a DNA binding fluorochrome, to determine DNA content in cells or nuclei.

Samples were analyzed on Becton Dickinson FACScan flow cytometry. At least 10,000 cells were measured with the use of consort 32 software for DNA ploidy and cell cycle analysis.

DNA flow cytometry measures the relative DNA content compared to a standard value. In our study the normal diploid cells from samples are used as an internal standard to calculate the DNA index.

Data analysis:

The DNA content was categorized as either normal (diploid) or abnormal (aneuploid). Aneuploidy was defined by the presence of one or

more distinct separate peaks compared with G_0/G_1 and G_2/M peaks of the diploid cell population [12]. The fraction of cells in each phase of the cell cycle was measured using the rectangle mathematical model [5].

The DNA index (DI) is used to derive a quantitative estimate of the relative DNA content between the tumor specimens and the diploid controls. It is defined as the ratio of the mean channel position of the G_0/G_1 peak of the tumor sample divided by the mean channel position of the G_0/G_1 peak of the diploid reference population [5].

Statistical analysis:

Data were entered, checked and analyzed using Epi-info version (6.02) computer package [6]. Data were expressed as mean \pm standard deviation for quantitative variables, number and percentage for qualitative ones. Chi-square, ANOVA, *t* test and correlation coefficient were used for analysis of results.

RESULTS

In L_1 group there were 11 common ALL cases (100%), in L_2 there were 3 cases Pre B-ALL (42.9%) and 4 T-ALL cases (57.1%) while in L_3 the 2 cases were B-ALL (100%), a highly statistically significant difference was found between immunophenotyping and FAB ($p = 0.001$).

The number of hyperdiploid cases was 6 out of 20 (30%) and 14 out of 20 were diploid cases (70%). No hypodiploid cases was detected in this study. Hyperdiploid cases were divided according to its prognostic relevance into hyperdiploid cases with $DI \geq 1.16$ and ≤ 1.6 (3 cases) and hyperdiploid cases with $DI > 1.6$ (3 cases).

As regards the percentage of S-phase fraction (SPF), the mean \pm SD was 4.06 ± 1.6 in L_1 , 5.07 ± 2.4 in L_2 and 9.15 ± 4.6 in L_3 with statistically significant difference between FAB and S-phase ($p = 0.03$). The SPF was 4.06 ± 1.6 in common ALL, 3.8 ± 2.2 in pre B-ALL, 9.15 ± 4.6 in B-ALL and 6.03 ± 2.4 in T-ALL with a statistically significant difference between immunophenotyping and S-phase ($p = 0.03$).

Mean \pm SD of S phase fraction was 4.55 ± 2.2 in diploid cases and 4.23 ± 1.46 in hyperdiploid cases with $DI \leq 1.6$ and 7.37 ± 4.4 in hyperdiploid cases with $DI > 1.6$. There was no statistically

significant difference between the three groups as regards S-phase fraction ($p = 0.21$).

The median value of SPF (4.5) was chosen to delineate low S phase from high S phase fraction [17]. At this value, 11 out of 20 cases had high SPF (55%) and 9 cases had low SPF (45%).

S-phase fraction was positively correlated with WBCs count ($r = 0.64$), Hb concentration ($r = 0.66$) and negatively correlated with platelet count ($r = -0.58$), $p < 0.001$, whereas, no significant correlation was observed with age ($r = 0.001$), or DI ($r = 0.1$), $p > 0.05$.

There was no significant correlation between DI and age, WBCs count, Hb concentration or platelet count ($r = -0.27, -0.19, 0.6, -0.15$ respectively, $p > 0.05$).

According to flow cytometric DI results, the cases were divided into diploid with $DI = 0.85 - 1.15$ (Figs. 1,2) and hyperdiploid cases with $DI > 1.15$ (Figs. 3,4).

As regards chemotherapy, 11 ALL cases (55%) responded to chemotherapy (complete remission), 2 cases (10%) showed no response and 7 cases (35%) died early during induction therapy. As the number of cases is low for statistical analysis we combined the two subgroups that didn't respond to chemotherapy into one

group (9 cases). A statistically significant association was observed between both FAB classification ($p = 0.02$) and immunophenotyping ($p = 0.013$) and response to chemotherapy.

A significant association was observed between S-phase fraction and response to chemotherapy ($p = 0.02$), while no significant association was found between ploidy status and response to chemotherapy ($p = 0.2$) (Table 4).

Table (1): Patient characteristics.

Parameters	Numer of patients (%)
<i>Age (years):</i>	
Range (1-18)	
Median (4.0)	
<i>Sex:</i>	
Male	14 (70)
Female	6 (30)
<i>FAB classification:</i>	
L ₁	11 (55)
L ₂	7 (35)
L ₃	2 (10)
<i>Immunophenotyping:</i>	
Common ALL	11 (55)
Pre B-ALL	3 (15)
B-ALL	2 (10)
T-ALL	4 (20)

Table (2): DNA ploidy and S-phase fraction according to different FAB subtypes and immunophenotypes.

Parameters	Ploidy status						S phase fraction				X ²	p
	Diploid		Hyperdiploid (1.16-1.6)		Hyperdiploid > 1.6		Low SPF		High SPF			
	No.	%	No.	%	No.	%	No.	%	No.	%		
L ₁ (N = 11)	6	54.5	3	27.3	2	18.2	6	54.5	5	45.5		
L ₂ (N = 7)	7	100	0	0	0	0	3	42.9	4	57.1	2.05	0.36 NS
L ₃ (N = 2)	1	50	0	0	1	50	0	0	2	100		
Common ALL (N = 11)	6	54.5	3	27.3	2	18.2	6	54.5	5	45.5		
Pre B-ALL (N = 3)	3	100	0	0	0	0	2	66.7	1	33.3	3.26	0.35 NS
B-ALL (N = 2)	1	50	0	0	1	50	0	0	2	100		
T-ALL (N = 4)	4	100	0	0	0	0	1	25	3	75		

NS: Not significant.

Table (3): Hematological parameter according to ploidy status and S phase fraction.

Parameter	Ploidy status			S phase fraction				<i>t</i>	<i>p</i>
	Diploid X±SD (Range)	Hyperdiploid (1.16-1.6) X±SD (Range)	Hyperdiploid > 1.6 X±SD (Range)	F	<i>p</i>	Low SPF X±SD (Range)	High SPF X±SD (Range)		
WBCs x 10 ⁹ /L	74±70 (13.6-208.8)	57.4±53.8 (19.8-119)	72.9±68.9	0.07	0.93 NS	28.4±14.6 (13.6-63)	106.5±69.2 (19.6-208.8)	3.31	0.004 HS
Hb g/dl	6.15±1.37 (4-8.4)	5.53±1.89 (3.4-7.0)	7.1±0.52 (6.8-7.7)	1.001	0.38 NS	5.03±1.03 (3.4-6.8)	7.15±0.73 (5.9-8.4)	5.39	0.001 HS
Platelets x 10 ⁹ /L	78.3±60.8 (11-199)	45.0±15.4 (28-58)	43.3±21.7 (20-63)	0.84	0.54 NS	102±63.3 (46-199)	40.3±18.8 (11-63)	3.09	0.006 HS

S: Significant. HS: Highly significant. NS: Not significant.

Table (4): Association between different parameters and response to chemotherapy.

Parameter	Complete remission N = 11		No response N = 9		X ²	<i>p</i>
	No.	%	No.	%		
Male (N = 14)	7	50	7	50	0.47	0.42 NS
Female (N = 6)	4	66.7	2	33.3		
L ₁ (N = 11)	9	81.8	2	18.2	7.62	0.02 S
L ₂ (N = 7)	2	28.6	5	71.4		
L ₃ (N = 2)	0	0.0	2	100		
Common ALL (N = 11)	9	81.8	2	18.2	10.69	0.013 S
Pre B-ALL (N = 3)	2	66.7	1	33.3		
B-ALL (N = 2)	0	0.0	2	100		
T-ALL (N = 4)	0	0.0	4	100	5.31	0.02y S
Diploid (N = 14)	7	50	7	50		
Hyperdiploid (1.16-1.6) (N = 3)	3	100	0	0.0		
Hyperdiploid (> 1.6) (N = 3)	1	33.3	2	66.6	3.16	0.2 NS
Low SPF (N = 9)	8	88.9	1	11.1		
High SPF (N = 11)	3	27.3	8	72.7		
		X±SD		X±SD	<i>t</i>	<i>p</i>
Age		6.45±5.3		5.6±5.01	0.42	0.68 NS
WBCs x 10 ⁹ /L		52.9±54.3		93.8±72.4	1.45	0.17 NS
Hb g/dL		5.7±1.5		6.7±1.17	1.51	0.14 NS
Platelets x 10 ⁹ /L		77.3±62.1		56.6±41.4	0.84	0.58 NS
DNA index		1.17±0.81		1.13±0.33	0.28	0.77 NS
S phase fraction		3.5±1.6		6.61±2.7	3.17	0.005 HS

S : Significant.
NS: Not significant.

HS: Highly significant.
y: yates correction

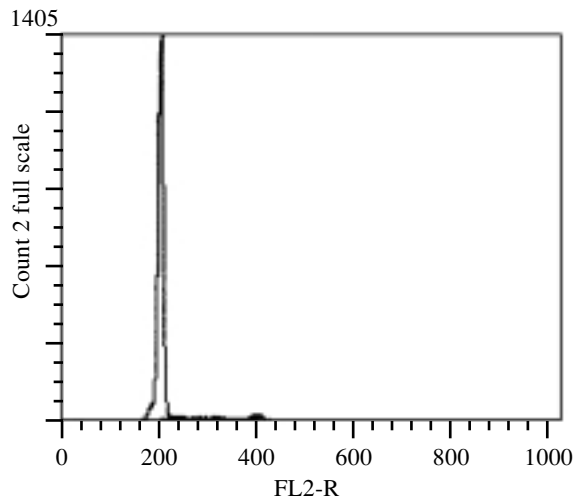


Fig. (1): Diploid DNA histogram with high SPF (7.8%).

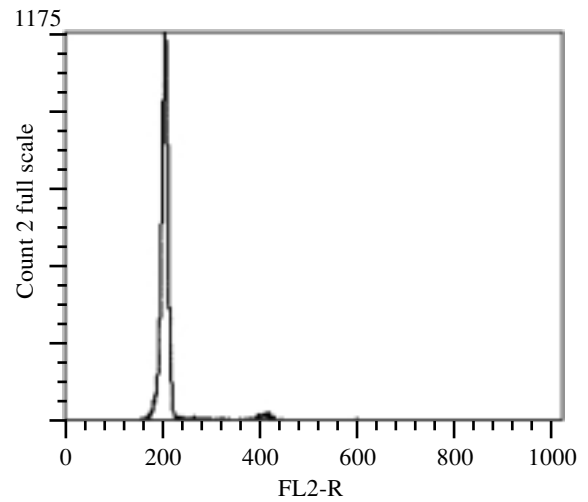


Fig. (2): Diploid DNA histogram with low SPF (2.1%).

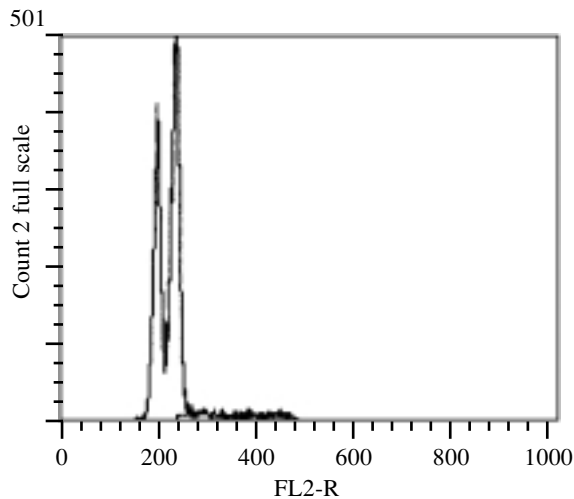


Fig. (3): DNA histogram with hyperdiploid population (DI = 1.20) and high SPF (12.4%).

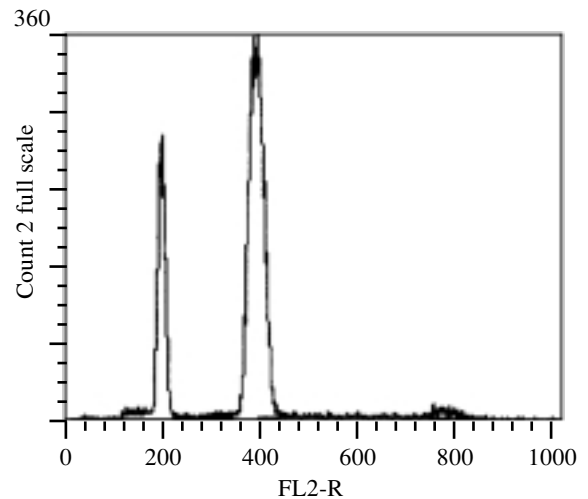


Fig. (4): DNA histogram with tetraploid population (DI = 1.98) and high SPF (5.6%).

DISCUSSION

In the present study, aneuploidy was found in 6 out of 20 ALL cases (30%), DNA index range was 1-1.98 with a median of 1.12. The aneuploid peaks had hyperdiploid DNA indices. Look et al. [17] reported aneuploidy in 36.21% of ALL cases: 34.1% hyperdiploid and 2% hypodiploid. Also, Khalifa et al. [15] found 40% aneuploid DNA index in ALL: 28.6% hyperdiploid and 11.4% hypodiploid with DI range 0.69-1.89 and a median of 1.18.

As regards FAB classification of ALL cases in our study, hyperdiploidy was found in 5 out of 11 L₁ cases, one of 2 L₃ cases and non of L₂ cases. This agreed with Williams et al. [26]. On

the other, hand Hammond et al. [11], reported an increased incidence of aneuploidy in L₂ FAB subtype compared to L₁ (71% VS 34%). This discrepancy could be explained by the fact that 4 out of our 7 L₂ cases were T-ALL phenotype which tended to be pseudodiploid or have small numerical changes that were undetectable by DNA FCM analysis [26].

A close association was observed between hyperdiploidy and CD10 expression. Five out of 11 C-ALL cases (45.5%) were hyperdiploid, with complete absence of hyperdiploidy in T-ALL. These results were parallel to that of Kaspers et al. [14] and Khalifa et al. [15]. The increased frequency of hyperdiploidy in C-ALL could mean that lymphoid progenitor cells with

this phenotype are more susceptible to transformation that produce hyperdiploidy or that hyperdiploid leukemia clones arise in pluripotent stem cells cannot differentiate beyond the C-ALL phenotypic stage due to the hyperdiploid chromosomal abnormality [16].

There was no significant difference in initial total leucocytic count, Hb concentration and platelet count between diploid and hyperdiploid groups and also no significant correlation between the degree of ploidy (DI) and the same parameters. These results agreed with Hammond et al. [11] and Khalifa et al. [15]. In contrast Hiddemann et al. [12] reported an association between hyperdiploidy and lower leucocytic count.

One of the most commonly used parameters of proliferative activity is the percentage of cells with S-phase DNA content measured by flow cytometry [18]. The number of S-phase cells represents the proliferation potential of the analyzed sample and is important in prognosis and treatment [5].

In our study, the mean \pm SD of S-phase DNA was $4.9 \pm 2.6\%$. Nearly similar results were detected by Orfao et al. [20], while Andreef [1] found that the mean value of S-phase was $5.50.3\%$. As regards FAB subtypes, L_3 (corresponding to B-ALL) showed significantly higher mean percentage of S-phase compared to both L_1 and L_2 . Similarly, Khalifa et al. [15] found that 2 out of 3 cases of L_3 had shown very high S-phase (35% and 17.9%).

As regards immunophenotyping, B-ALL had a significantly higher mean percentage of SPF and that of T-ALL was the next highest. Similar results were found by Look et al. [16]. In contrast, Smets et al. [25] and Khalifa et al. [15] found no significant difference between the percentage of SPF and immunophenotyping groups.

In the present study, the mean percentage of S-phase in diploid ALL was $4.55 \pm 2.2\%$. It was $4.23 \pm 1.46\%$ in hyperdiploid cases with $DI \leq 1.6$ and $7.37 \pm 4.4\%$ in hyperdiploid cases with $DI > 1.6$. Although the later group had a higher percentage of SPF, no statistical significant difference between the three groups was revealed. Similar results was obtained by Smets et al. [25].

The percentage of S-phase fraction was found to be positively correlated with initial total

leucocytic count and Hb concentration and negatively correlated with platelet count. Similar results were obtained by Dow et al. [7].

As regards response to chemotherapy, there was a statistically significant association between prognosis and FAB classification. The cases which showed complete remission were 81.8% L_1 , 18.2% L_2 and none of L_3 . FAB L_1 morphologic features are associated with more favorable prognosis than L_2 in childhood ALL, whereas L_3 has historically been predictive of remission induction failure and short survival in both adults and children [16].

Also, there was a highly significant association between immunophenotypic groups and response to chemotherapy. 100% of B-ALL and T-ALL cases died early during induction therapy. Pui et al. [21] found that 100% of children with mature B-ALL had chromosomal translocation (8;14) and had an extremely poor prognosis. On the other hand, Khalifa et al. [15] found no significant difference between immunophenotyping groups and event-free survival, overall survival or early death.

In our study, there was no significant association between ploidy status and response to chemotherapy and also no significant difference was found between DI and patient outcome. Our results go hand in hand with those found by Khalifa et al. [15]. In contrast, Smets et al. [25] found that children with hyperdiploid ALL stem lines had very long event-free survivals. It is unknown why patients with DNA index ≥ 1.16 and ≤ 1.6 (51-65 chromosomes) respond well to chemotherapy but it may be related either to a favorable intracellular metabolism of methotrexate or the marked propensity for hyperdiploid blasts to undergo apoptosis [13].

A statistically significant difference was observed between low and high S-phase and response to chemotherapy. At a cut-off value of SPF 4.5% , in low SPF cases 88.9% had complete remission, while in high SPF cases only 27.3% showed complete remission. Eight out of 9 cases who didn't respond to therapy in our study had high SPF. Khalifa et al. [15] put 5% cut off value of SPF and they found that patients with pre-treatment high S-phase had an adverse prognostic impact while those with low S-phase had a high probability of event-free survival. On the other hand, Andreef [1] and Duque et al. [8] failed to

demonstrate a significant effect of SPF on the outcome.

The lack of agreement on the prognostic value of SPF is best explained by difference in therapy and patients selection in different studies. Further clinical follow-up and analysis of a large sample number of ALL cases may clarify the combined influence of ploidy and S phase cell percentage on response to chemotherapy.

Finally, we can conclude that DNA analysis by FCM is an ideal way to detect aneuploidy in ALL. It is rapid, automated and an interpretable reading can be obtained in most patients. The percentage of SPF appears to be a parameter of potential prognostic value. Its routine recording in patients with ALL is highly recommended.

Also, we recommend that each newly diagnosed ALL patient should be analyzed by karyotyping in addition to FCM to detect pseudodiploid cells, with balanced chromosomal translocations which do not affect the net DNA content of the cells.

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