A Strategy to Detect Chromosomal Abnormalities in Children With Acute Lymphoblastic Leukemia

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Abstract: Conventional cytogenetics (CC) can be used to identify chromosomal abnormalities that are predictors of treatment outcome in acute lymphoblastic leukemia (ALL). The detection of abnormalities in ALL is difficult because low mitotic index and poor-quality metaphases are obtained. Flow cytometry (FC) and fluorescence in situ hybridization (FISH) can be used to detect aneuploidy in any phase of the cell cycle, increasing the number of analyzable cells. The aim of this study was to develop a strategy combining these methods to improve the frequency of chromosome abnormality detection. One hundred children with newly diagnosed ALL were included. CC and DNA content analysis by FC were performed in all patients. The numerical abnormalities identified by both methods were compared and patients were classified as concordant or discordant. FISH was used to support aneuploidy results in discrepant cases using centromeric probes for the chromosomes most frequently involved in aneuploidy. CC and FC showed high concordance (86%). Fourteen cases were discrepant: nine showed hypodiploidy and low hyperdiploidy by cytogenetics and five showed high hyperdiploidy by FC. FISH confirmed aneuploidy in 12 cases in which it could be performed. High hyperdiploidy was the most common abnormality; the 31 cases showing this aneuploidy were identified by FC. The search for abnormalities must begin by measuring DNA content to detect this aneuploidy, which is useful to evaluate the patient's risk. However, it is important to screen for structural abnormalities by CC or molecular techniques. This strategy may detect chromosomal abnormalities, optimizing resources in laboratories where not all the screening methods are available.

Key Words: chromosomal abnormalities, acute lymphoblastic leukemia, DNA content, flow cytometry, fluorescence in situ hybridization

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Conventional cytogenetics (CC) has identified chromosomal abnormalities—numerical and structural rearrangements—that are independent predictors of treatment outcome in patients with acute lymphoblastic leukemia (ALL). In recent years, cytogenetic analysis has been shown to be a useful tool to stratify ALL children with increased or decreased risk of treatment failure.[1] High hyperdiploidy (the clonal gain of >50 chromosomes) and the t(12;21) rearrangement are abnormalities associated with a good prognosis; on the contrary, near-haploid karyotypes and the t(9;22) or 11q23 rearrangements are well established as poor prognosis factors.[2,3]

Unfortunately, conventional cytogenetic analysis is technically more demanding in ALL than in most other leukemias. Clonal abnormalities are difficult to identify because standard chromosome-processing techniques usually result in metaphases with fuzzy chromosomes and cultures lacking mitosis.[4,5] Moreover, clonal selection leads to a high proportion of normal metaphases, masking abnormal cells when a limited number of metaphases is analyzed. In fact, under optimal conditions karyotypically aberrant clones are detected in only 55% to 85% of ALL patients.[6–8] Therefore, the chromosomal classification of ALL is often incomplete, and it is not clear whether the abnormalities detected in a few analyzable metaphases are representative of the whole leukemic clone.[9]

Flow cytometry (FC) and fluorescence in situ hybridization (FISH) are important tools to identify chromosomal abnormalities in cells in any phase of the cell cycle. FC can detect chromosomal gains and losses in interphase cells based on nuclear DNA content, while FISH can analyze numerical or structural abnormalities on both metaphase spreads and interphase nuclei.[10–12]

FC is a fast and useful method to delineate gross quantitative deviations of DNA content; however, its ability to detect aneuploidy with gain or loss of only one or two chromosomes depends on the size of chromosomes involved.[13,14] Some reports recommend FISH analysis with numerous alpha-satellite probes to identify numerical abnormalities.[14–16] Although the analysis of a full set of chromosomes by FISH increases the detection of numerical abnormalities, the strategy is expensive to screen for aneuploidy.[9] Therefore, the aim of

this study was to develop a strategy combining three methods—CC, GC, and FISH based on the use of alpha-satellite probes of the chromosomes most frequently involved in ALL aneuploidy—to improve the frequency of chromosomal anomaly detection in these patients, and to optimize resources.

METHODS

Patients

One hundred ninety-five children with newly diagnosed ALL by the French-American-British (FAB) classification were seen at the National Pediatrics Institute in Mexico City between 1997 and 1999. In 34 patients, metaphases were not obtained for cytogenetic study, and they were excluded. Sixty-one of the 161 patients with successful cytogenetic study were also excluded because the samples were not suitable for FC analysis. Inconclusive cases (e.g., patients with normal karyotype in <20 cells and patients with only one cell with numerical abnormality [no clonal]) were included in the study trying to identify an aneuploid population by FC or FISH. These studies were performed under a research protocol approved by the Review Board and the Ethical Committee of the National Pediatrics Institute. Written informed consent was obtained from parents or legal guardians.

CC and DNA content analysis by flow cytometry were performed in 100 patients. The numerical abnormalities identified by both methods were compared and the patients were classified according to the concordance or discrepancy of the results obtained. To support aneuploidy results in discrepant cases, FISH assays with alpha-satellite probes were performed.

CC

Cytogenetic studies were performed according to standard methods.[8] Cells from bone marrow aspirates were used for a direct technique and a 24-hour culture in RPMI-1640 medium. Cells were harvested using Colcemid (10 μg/mL), hypotonized with 0.075 mol/L KCl, and fixed in methanol and glacial acetic acid (3:1). Slides were prepared for cytogenetic analysis with Giemsa-trypsin banding, and the remaining pellet was stored at 4°C.

Karyotypes were analyzed according to the International System for Human Cytogenetic Nomenclature, 1995.[17] As mentioned above, patients with normal karyotype in fewer than 20 cells and patients with one cell with numerical abnormality were included in the study exploring the possibility of detecting an aneuploid clone, even though they did not meet conventional cytogenetic criteria.

DNA Quantification by FC

Fixed cell pellets previously washed in Dulbecco's phosphate saline buffer were used for DNA analysis[18] and the CycleTEST PLUS DNA Reagent Kit (Becton Dickinson) was used for DNA quantification. The analysis was performed in a FACScan (Becton Dickinson) cytometer, calibrated and adjusted with the DNA QC Particles Kit (Becton Dickinson). For each assay, normal male and female lymphocytes were used as controls and 20,000 events were analyzed per sample (from patients and controls). The coefficient variation of the DNA distribution curves was less than 5. The data were evaluated by Cellfit software (Becton Dickinson) and the DNA index was determined by dividing the mean channel number of the aneuploid peak by the mean channel of the diploid peak.[19]

Interphase Cytogenetics

FISH was performed on fixed bone marrow cells according to the manufacturer's instructions.[20] Alpha-satellite probes (ONCOR) were used to determine hyperdiploidy, and the selection of the probes was based on the strategy described by Moorman et al.[9] DNA probes for chromosomes X, 13/21, and 18 were used to screen for high hyperdiploidy, and probes for chromosomes X, 8, and 13/21 were used to screen for low hyperdiploidy. According to the literature,[2,5,21] hypodiploidy was screened with probes for chromosomes 20 and X. Cutoff values for aneuploidy were determined on five control bone marrow samples and were calculated as follows: cutoff = mean + 3(standard deviation).[22] One thousand cells were screened per assay in a doubled-blinded manner.

RESULTS

Patient Characteristics

Table 1 gives the gender, FAB classification, age, and white blood cell (WBC) counts of all patients included in the study. Sex ratio was 1.86 (boys/girls); most patients were diagnosed with ALL-L1, were aged 3 to 10 years, and had WBC counts less than 10×10^9 cells/L at diagnosis.

TABLE 1. Patient Characteristics

Gender		Cytomorphology				Age (years)			WBC Count (10^9/L)			
Female	Male	L1	L2	L1/L2	L3	0–2	3–10	11–16	≤10	11–25	26–50	>51
35	65	89	8	2	1	17	63	20	42	22	11	25

TABLE 2. Cytogenetic Analysis of the 100 Patients Included in the Study

	Normal Karyotypes	Abnormal Karyotypes	Total
With 20 analyzed cells or clonal abnormality	17	59	76
With <20 analyzed cells	16	0	16
Nonclonal abnormality	0	8	8*

*Patients with one hyperdiploid cell.

Cytogenetic Study

The 161 patients with successful cytogenetics presented normal karyotypes in 49 patients; 16 of the 49 were inconclusive because fewer than 20 metaphases were analyzed. In 112 patients abnormal karyotypes were found; 8 of the 112 presented nonclonal abnormalities. In patients with abnormal karyotype, the following alterations were observed: 13 patients with fewer than 46 chromosomes, 25 patients with 47 to 50 chromosomes (4 with Down syndrome by free 21 trisomy), 38 patients with more than 50 chromosomes, 1 patient with tetraploid cells, 2 patients with multiploidy, and 33 patients with structural abnormalities; in this group, 7 patients had t(9;22), 5 patients t(1;19), and 1 patient t(4;11).

In 100 of the 161 patients from the described group, it was possible to perform the DNA content analysis by FC to detect or confirm the presence of aneuploid clones. The characteristics of cytogenetic analysis from these patients are presented in Table 2.

CC and FC Analysis in 100 Patients

The CC and FC results of 100 patients in whom both studies were performed are described below. Table 3 summarizes the chromosomal abnormalities identified by CC and FC according the number of chromosomes and DNA index.

Patients With Concordant Results

Results were concordant according to both methods in 86 patients (see Table 3). CC identified in three patients only hyperdiploid clones and no normal metaphases, while FC revealed a normal diploid population in addition to the hyperdiploid clone; these cases were considered concordant because both methods detected hyperdiploidy. Only one hyperdiploid cell was observed by CC in eight patients. FC supported aneuploidy in six, and the two remaining patients (patients 11 and 28 in Table 4) were considered as discrepant.

Discrepant Patients

Results were discordant in 14 patients (see Table 3). Aneuploidy was found only by CC in nine patients (hyperdiploidy in seven, hypodiploidy in two). The five remaining patients showed aneuploidy by FC only, high hyperdiploidy being suspected based on the DNA content (DNA index > 1.16).[1] Table 4 describes the karyotypes and DNA content of discrepant cases; a Down syndrome patient with free trisomy 21 was included in this group.

FISH in Discrepant Patients

Hyperdiploid Patients Determined by CC Only

FISH analysis revealed aneuploidy in all patients in whom hyperdiploidy was determined only by CC (Table 5). The DNA probes for chromosomes X, 8, 10, and 13/21 identified only hyperdiploid clones in two patients (patients 28 and 93) and both trisomy and monosomy in four patients (patients 8, 12, 57, and 58). This set of selected probes failed to identify hyperdiploidy but determined a chromosome 8 monosomy in only one patient (patient 11). Because cytogenetic analysis had identified trisomy 16 in this patient, a probe for this specific chromosome was used to confirm this aneuploidy. Chromosomes 13/21 were analyzed in an independent assay in patient 8.

Hypodiploid Patients Determined by CC Only

Table 6 shows FISH results with probes for chromosomes X and 20 in the two patients in whom hypodiploidy was determined only by cytogenetics. Monosomy for chromosome X was identified in patient 73, but both patients failed to show monosomy for chromosome 20. To identify the chromosome loss in patient 78, we started the screening with probes for

TABLE 3. Patient Classification Based on Chromosome Number and DNA Index

Numerical Abnormalities Detected by	Number of Chromosomes					
	2N	<46	47–50	>50	Multiploidy	Total
Both studies (concordant)	42*	7	9	26	2	86
Conventional cytogenetics (discordant)	—	2	7	—	—	9
Flow cytometry (discordant)	—	—	—	5**	—	5

13* = patients with structural abnormality
5** = one patient with structural abnormality

TABLE 4. Chromosomal Abnormalities and DNA Index in Discrepant Patients

Patient No.	Karyotype	Numerical Abnormalities Determined by:		
		DNA Index	Flow Cytometry	Cytogenetics
8	46~47,XX,+add(1),del(3), del(6), add(11),−21[cp20]	1.00	−	+
9	46,XX[20]	1.00/1.18	+	−
11	47,XY,−6,−12,+16,+mar1, +mar2[1]/46,XY[7]	1.00	−	+
12	47,XY,−?B,+6, +21c[3]/46,XY[5]	1.00	−	+
22	46,XX[11]	1.00/1.20	+	−
28	48,XY, +mar1, +mar2[1]/46,XY[7]	1.00	−	+
57	47,XY, +mar[2]/46,XY[19]	1.00	−	+
58	46~48,XX,t(1;19), +mar1, +mar2[4]	1.00	−	+
68	46,XX,del(6q)[2]/46,XX[3]	1.00/1.20	+	−
73	45,X,−?C[10]/46,XX[17]	1.00	−	+
78	45,XY,−?20,−G,+mar[7]/46,XY[3]	1.00	−	+
80	46,XX[5]	1.00/1.30	+	−
93	47~48,XY,+mar1,+mar2[4]/46,XY[19]	1.00	−	+
94	46,XX[10]	1.00/1.18	+	−

chromosomes 8 and 10 because the cutoff values for these chromosomes had already been determined. Chromosome 10 monosomy was found in this patient.

Hyperdiploid Patients Determined by FC Only

FISH analysis could be performed in only three of the five patients in whom hyperdiploidy was determined only by FC (Table 7). The three patients analyzed showed hyperdiploid clones with trisomies, double trisomies, and/or tetrasomies. Percentages of aneuploidy (see Tables 5, 6 and 7) were higher than the cutoff values obtained with each probe.

DISCUSSION

The results obtained by CC and FC showed high concordance (86%). Results were discrepant in 14 patients: aneuploidy was identified only by CC in 9 patients and only by FC in 5 (see Table 4). Two of nine patients (patients 11 and 28) presented only one cell with numerical abnormalities by CC,

TABLE 5. FISH Analysis in Patients with Hyperdiploidy Determined Only by Cytogenetics

Patient no.	8	11	12	28	57	58	93
Number of chromosomes	46/46~47	46/47	46/47	46/48	46/47	46/48	46/47~48
% of aneuploid cells	16.7	36.7	19.3	12.5	12.0	53.6	49.2
% of normal cells	83.3	63.3	80.7	87.5	88.0	46.4	50.8
−X	0	0	0	0	0	9.6	0
+X	0	0	0.8	12.3	0	36.0	0
−8	0	24.4	1.1	0	8.0	0	0
+8	0	0	0	0.2	2.0	0	0
−10	0	0	0	0	2.0	0	0
+10	0.8	0	0	0	0	8.0	45.6
+10, +10	0	0	0	0	0	0	0.6
+8, +10	4.4	0	0	0	0	0	3.0
+13/21	0	0	17.4	0	0	0	0
−13/21	11.5	0	0	0	0	0	0
+16		12.3					

Cutoff value: −X = 0.17 +X = 0; −8 = 0.41; +8 = 0; −10 = 0.84; +10 = 0.20; −13/21 = 2.96; +13/21 = 0.52, +16 = 0.
Notes: Chromosomes 13/21 were analyzed in an independent assay in patient 8. Chromosome 16 was analyzed only in patient 11 (■).

TABLE 6. FISH Analysis in Patients with Hypodiploidy Determined Only by Cytogenetics

Patient no.	73	78*
Number of chromosomes	45/46	45/46
% of aneuploid cells	56.0	1.6
% of normal cells	44.0	98.4
−X	56.0	0
−10	—	1.6
−8	—	0
−20	0	0

Cutoff values: −X = 0.17; +X = 0; −8 = 0.41; +8 = 0; −10 = 0.84; +10 = 0.20; −20 = 0.9; +20 = 0.

Chromosome 8 was analyzed only in patient 78 ( ).

*Patient 78 showed a probable −20 by conventional cytogenetics, and was suspicious to present the dic(9;20); this abnormality was discarded by FISH analysis with centromeric probes for chromosomes 9 and 20 (data not shown).

and in three patients who were hyperdiploid by FC and normal by CC (patients 22, 80, and 94) fewer than 20 cells were analyzed. These patients are considered inconclusive because not all the cytogenetic findings met the standard definition of a clone, or the number of analyzed cells was limited. However, they were included in the study to support by other methods the findings that were observed by cytogenetics and trying to detect aneuploid populations in these samples with low mitotic index.

Aneuploid patients detected only by CC had low hyperdiploidy, with modal numbers of 47 or 48 chromosomes, or hypodiploidy, with 45 chromosomes. Previous reports indicate that FC consistently detects the gain or loss of more than two chromosomes[13,14]; however in the present study FC was able to identify low hypodiploidy in three patients and low hyperdiploidy in two. The ability of FC to detect aneuploidy depends on the size of chromosomes gained or lost, the presence of unbalanced structural abnormalities, and the combination of monosomies and trisomies.[14] Five of the nine aneuploid patients detected only by CC showed alterations that may have eliminated the differences in DNA content and avoided the identification of the aneuploid population by FC (see Table 4).

FISH revealed only chromosome gains in patients with high hyperdiploidy. In contrast, five of seven patients with low hyperdiploidy (patients 8, 11, 12, 57, and 58) showed both monosomies and trisomies. The presence of both types of aneuploidy could limit the detection of alterations in DNA content.[14]

Probe selection to confirm hyperdiploidy by FISH was based on the most frequently gained chromosomes in ALL using the strategy designed by Moorman et al,[9] which considers that certain chromosomes are often gained together in one ploidy subgroup. We thus selected alpha-satellite probes for chromosomes X, 18, and 13/21 to identify high hyperdiploidy and probes for chromosomes X, 8, 10, and 13/21 to identify low hyperdiploidy. This strategy was successful in identifying the expected aneuploidy in 9 of the 10 hyperdiploid cases: 4 cases showed monosomy and trisomy and 5 only trisomy. One patient (patient 11) showed an unexpected monosomy 8 by FISH, but no trisomies were observed with the analyzed probes; a trisomy 16, detected by conventional cytogenetics, indicated the next probe to be tested in this patient, and the trisomy was confirmed by FISH (see Table 5). This case demonstrates the usefulness of conventional cytogenetics to select the most suitable probes to solve inconclusive cases.[12]

In only two patients (patients 58 and 93) included in the low hyperdiploidy group, about 50% of the cells showed aneuploidy detected by FISH. Trisomies for chromosomes X and 10 were found, respectively; this abnormality could be related to the supernumerary markers that were found by conventional cytogenetics, but the other markers remain to be identified. In the remaining cases of this group, FISH analysis supported the CC results, although in a lower percentage of cells. Patient 8 had −13/21, patient 11 +16, and patient 12 +13/21; all these findings agree with the CC study. Patients 28 and 57 showed trisomies for chromosomes X and 8, respectively, which could correlate with the markers observed by CC.

Some aneuploidies detected by FISH were not observed by CC and were presented in low percentages of cells. Those abnormalities could represent secondary changes originated by clonal evolution, more than part of a main leukemic cell line. Furthermore, it has been reported that cytogenetic results were occasionally inconsistent with those obtained by FISH; this cytogenetic limitation is mainly due to the poor quality of chromosomal structure, which makes it difficult to distinguish

TABLE 7. FISH Analysis in Patients with Hyperdiploidy Determined Only by Flow Cytometry

Patient no.	22	80	94
DNA index	1.00/1.20	1.00/1.30	1.00/1.18
% of aneuploid cells	38.0	84.2	48.3
% of normal cells	62.0	15.8	51.7
+X	0	6.1	0
+18	38.0	0	35.1
+13/21	0	0.1	0
+X, +18	0	31.2	0.2
+18, +13/21	0	0	7.4
+18, +13/21, +13/21	0	0	5.5
+X, +18, +18	0	0	0.1
+X, +X	0	41.7	0
+X, +X, +13/21	0	5.1	0

Cutoff values: −X = 0.17; +X = 0; −13/21 = 2.96; +13/21 = 0.52; −18 = 0.66; +18 = 0.

between chromosomes of similar size, consequently producing a wrong assignment of the chromosome.[15]

The ploidy-level classification of the patients was based on the results obtained by the CC study, but in some cases the FISH analysis revealed numerical abnormalities that were not detected by this method. Patients 8, 11, 12, and 57 showed a low percentage of cells with the combination of monosomy and trisomy by FISH. CC revealed only one or few abnormal cells with chromosome gains, or metaphases with the combination of chromosomal gains or losses. It is difficult to define the ploidy level in this group and to establish whether some of the abnormalities detected by both methods are secondary or not; however, the FISH and CC results could explain the diploid DNA index value obtained by FC. The monosomy 10 found in a low percentage of cells in patient 78 also suggests that could be a secondary change.[23]

The confirmation of aneuploidy by FISH was more difficult in hypodiploid patients. A well-represented monosomic X clone was identified only in patient 73. Unfortunately, there is no characteristic pattern of chromosomal loss in ALL.[21] Although a large series of patients analyzed by CC suggest that chromosomes X, Y, and 20 are the most frequently involved, this study and comparative genomic hybridization (CGH) show that the chromosomes involved in hypodiploidy are not specific.[24,25]

High hyperdiploidy with 51 to 65 chromosomes detected by flow cytometry was confirmed by CC or FISH. FC is a quick and appropriate method for identification of numerical aberrations with prognostic value and is useful for patient management.[14,15,26]

Based on our results, we propose the following approach to identify chromosome anomalies in ALL patients. Numerical aberrations should be analyzed first, measuring cellular DNA content by FC. This method detects high hyperdiploidy in 31% of patients, low hyperdiploidy in 16%, and hypodiploidy in 9%. Because structural aberrations of prognostic value could be present in high hyperdiploidy karyotypes,[2,27] DNA content analysis in combination with RT-PCR may give to the oncologist enough information to assess the patient's risk to make therapeutic decisions. Low hyperdiploid, hypodiploid, and diploid patients must be analyzed by CC supported by FISH to detect chromosomal rearrangements in this disease. With the combination of these techniques, we would expect to detect chromosome aberrations in 71% of patients. However, because of the technical limitations of cytogenetics and the high cost of FISH,[12] common chromosome rearrangements can be screened by RT-PCR in institutions where molecular biology techniques are available. Only patients without translocations or those with rearrangements involving only one of the translocation genes would be analyzed by cytogenetic methods.

The assignment of patients to specific risk groups based on genetic abnormalities is a difficult and expensive process that requires resources, special equipment, and the expertise of a number of professionals.[9,12,28] The strategy described here may allow the detection of numerical and structural abnormalities in only a few days. It may optimize resources in laboratories that do not have all the screening methods for chromosomal abnormalities with diagnostic and prognostic significance in ALL.

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