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Detection of trisomy 12 by fluorescent *in situ* hybridization (FISH) in chronic lymphocytic leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) presents a varying incidence of karyotypic abnormalities whose detection is complicated by difficulties in obtaining mitosis for analysis in this type of mature lymphocyte disorder. Since the introduction of molecular cytogenetics (FISH = fluorescent *in situ* hybridization), applying centromeric probes for chromosome 12 has made it possible to detect a higher percentage of trisomy 12 cases. The objective of the present study was to detect trisomy 12 by FISH (alpha satellite probe) in 13 patients with CLL whose karyotypes by G-banding were either normal or inadequate. Using this method trisomy 12 was detected in three patients in a percentage of positive cells varying from 55.5% to 79%, showing that FISH is a sensitive and highly specific method for trisomy detection and should be routinely performed when the karyotype is normal.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder characterized in 98% of the cases by clonal expansion of B lymphocytes CD 5, CD 19 and CD 23 positive.

Cytogenetic study is important in prognosis, but may depend on disease stage (Zwiebel and Cheson, 1998). The most frequent abnormalities found, totaling 60%, are trisomy 12, t(14q), t/del(13q), del(6q) and 11q22-23 (Hallek *et al.*, 1997; Juliusson and Merup, 1998). Patients with chromosomal abnormalities, particularly when complex, have shorter survival (Escudier *et al.*, 1993; Xue *et al.*, 1993) while those with normal karyotypes respond significantly better to chemotherapy (Han *et al.*, 1988). Karyotype evolution has been observed in 20% of the cases and generally in Richter's transformation (Robert *et al.*, 1982; Tsujimoto *et al.*, 1985; Giles *et al.*, 1998).

Chromosomal abnormalities are found by conventional G-banding in only 50% of the patients (Hallek *et al.*, 1997). Some difficulties exist in obtaining mitosis in CLL due to low mitotic index or because metaphases are not always part of the malignant clone. Since the introduction of molecular cytogenetics (FISH), applying centromeric probes for chromosome 12 (alpha satellite) has made possible the detection of a higher percentage of trisomy 12 cases (Hamblin, 1997).

The objective of the present study was to detect trisomy 12 by FISH in 13 patients whose chromosomal analysis by conventional G-banding was either normal or failed.

MATERIAL AND METHODS

Thirteen CLL patients with normal karyotypes or without results due to lack of metaphases were studied. Table I shows some diagnostic data. The control group was constituted of 3 bone marrow donors with normal karyotypes.

The CLL diagnosis resulted from the presence of absolute lymphocyte count greater than 5,000/dl, bone marrow aspiration showing infiltration by lymphoproliferative cells, phenotyping with CD 5, CD 23 and SIg positive cells, and bone marrow biopsy showing massive lymphocyte infiltration (Brittinger *et al.*, 1997).

Study of karyotype was done using peripheral blood whose buffy coat was divided and placed in 2 culture flasks with 8 ml RPMI 1640 medium, pH 7.2, 20% fetal calf serum, 1% antibiotics and 100 µl L-glutamin. To the first flask it was added Pokeweed mitogen (0.1 mg/ml) and, to the second, phorbol 12-myristate 13 acetate (TPA, 50 ng/ml) and PHA (0.1 mg/ml) for 72 h. Colcemid was added during the last 60 min followed by hypotonic treatment with 0.075 mol/l KCl for 20 min and fixation with methanol and acetic acid (3:1) solution repeated 4 times.

The slides were banded for trypsin G-banding (GTG); 10 metaphases from each culture were analyzed. Chromosomal abnormalities were described following ISCN (1995).

Part of fixed cell (0.5 ml) suspension was separated for FISH. This material was centrifuged again and resuspended; the slides were made by cytocentrifuging (Incibras). After air drying, these slides were washed in 2X SSC for 30 min at 37°C and dehydrated in 70, 80 and 95% ethanol for 2 min in each. The probe for chromosome 12 alpha satellite D1273 (Oncor) (1.5 µl) was mixed with 30 µl hybrisol VI (Oncor), both previously heated to 37°C, for 5 min. The probe mixture was then denatured at 72°C for 5 min, dropped into the cytospin smear, coverslipped, sealed with rubber cement and incubated for 16 h in a humidified cham-

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Table I - Patient diagnostic data.

	Sex	Age	Date of diagnosis	Stage	Chemotherapy	Δts/qt	L x 10^9/dl
1	F	46	9/93	B	S	1	9.000
2	F	60	8/95	A	N	-	17.000
3	F	86	11/93	B	S	3	18.900
4	M	60	7/94	A	N	-	17.000
5	M	77	5/94	A	N	-	42.000
6	M	70	5/97	A	N	-	21.000
7	F	72	5/98	B	S	3	27.000
8	F	70	1/92	A	S	4	56.000
9	M	73	3/98	C	S	1	39.000
10	F	83	9/99	A	N	-	12.000
11	M	60	2/99	C	N	-	85.000
12	F	83	5/69	C	S	2	98.000
13	M	68	1/92	C	S	2	58.000

F = Female; M = male; Δts/qt = period of time without chemotherapy in years; L x 10^9/dl = lymphocytes total count /dl.

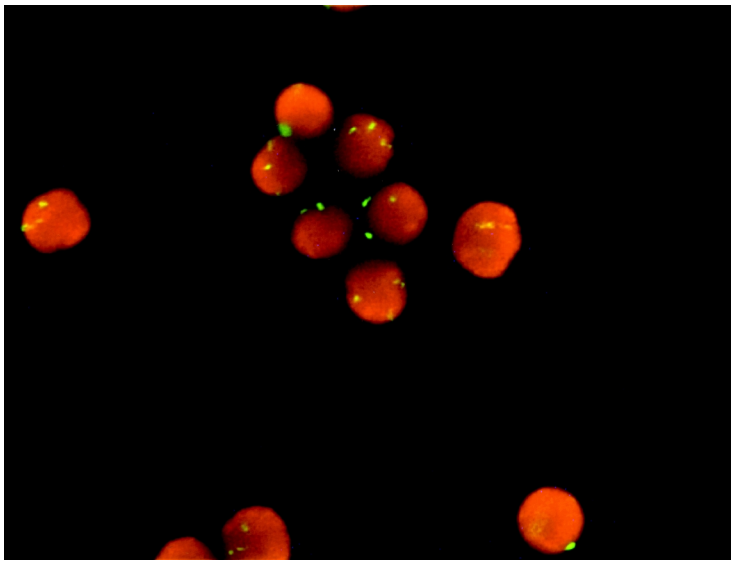

Figure 1 - FISH of case 11. The interphase cells present 3 fluorescent signals.

ber at 37ºC. Detection and signal amplification were made by removing coverslip, rinsing the slides in 0.25X SSC for 5 min at 72ºC and phosphate-buffered detergent (PBD) for 2 min at 37ºC. Incubation continued with FITC-labeled antidigoxigenin for 5 min at 37ºC covered with plastic coverslip. They were then rinsed three times in PBD for 2 min and incubated with 20 µl rabbit antisheep antibody for 15 min at 37ºC. The slides were washed three times in PBD, then incubated with 20 µl FITC antirabbit antibody, for 15 min at 37ºC, coverslipped. After three PBD washes, 10 µl propidium iodide with antifade (0.3 µg/ml) was added and the slides were again coverslipped. They were then analyzed with a fluorescent microscope (Olympus BMX60) with FITC and rhodamine filters (Chroma Technologies). The images were captured through a CCD camera (Cohu) assembled to a computer having karyotyping and FISH software (CytoVision-Applied Imaging). Slides showing more than 50% cells with fluorescent dots were selected for analysis. From each slide at least 200 cells were counted and all of them isolated to avoid overlapping signals (Figure 1).

RESULTS

Table II shows FISH analysis results of the 13 patients and controls. Each fluorescent dot in a cell represents 12 chromosome centromere (Figure 1).

Patients 4, 11 and 13 presented trisomy 12 with 55.5, 79 and 62% of positive cells, respectively.

In the control group the mean of 1.16 cells with three fluorescent dots was observed, and normal range was defined as from 0 to 3.24 cells (two standard deviations).

DISCUSSION

The main problem in karyotype study in CLL is the dif-

Table II - Results of FISH analysis.

Case	3 signals	2 signals
1	1%	98%
2	2%	91%
3	3%	90%
4	**55.5%**	42%
5	0	94.3%
6	2.5	94%
7	0	93.3%
8	2.5	94%
9	3	91%
10	0	98%
11	**79%**	18.6%
12	2.3	92%
13	**62%**	36%
Control 1	4 (2%)	188 (94%)
Control 2	0	189 (94.5%)
Control 3	3 (1.5%)	194 (97%)
X	1.16	95.16
SD	0-3.24	91.96-98.36

3 signals = Visualization of 3 fluorescent signals in cell; 2 signals = visualization of 2 fluorescent signals in cell; X = mean; SD = standard deviation.

ficulty in obtaining B lymphocyte in mitotic division, even using B cell mitogens like PWM or TPA (Hamblin, 1997).

Chromosomal abnormalities are described varying from 20 to 70% of CLL cases, occurring more frequently in patients with advanced stages of the disease (Zwiebel and Cheson, 1998).

The FISH method is sensitive and highly specific for trisomy 12 detection; subsequent to its implementation the number of cases reported with this abnormality increased around 10%, varying from 12 to 42.6% (Nair *et al.*, 1998). In the present report we detected trisomy 12 in 3 (around 23%) out of 13 patients whose karyotypes were normal or failed.

A variation in the percentage of cells with trisomy 12 detected by FISH has been reported and some authors have divided it into two groups: one with small percentage (<10%) and the other with high percentage (>10%). The second group correlates with a more advanced disease stage and, consequently, trisomy 12 would relate to an ongoing event (Reed, 1998; Nair *et al.*, 1998).

A trisomy 12 and atypical morphology correlation was suggested (nucleous cleavage, lymphoplasmatic characteristics and increased prolymphocyte number) (Matutes *et al.*, 1996) but no correlation with disease stage has been observed.

Case 4 showed increased CD 20 intensity and surface IgM in phenotyping. Great immunological marker variability has been described in CLL with trisomy 12, though no specific pattern exists (Döhner *et al*., 1997).

In conclusion, detecting trisomy 12 by FISH was possible in 3 patients (around 23%) with normal or unsuccessful karyotype showing that FISH should be used as a routine test in addition to karyotype. More extensive studies are needed to better estimate frequency of this abnormality in patients.

RESUMO

A leucemia linfocítica crônica (CLL) apresenta incidência variável de anomalias de cariótipo devido às dificuldades em se obter mitose para análise. Desde a introdução da citogenética molecular (FISH = hibridação *in situ* por fluorescência) usando sonda centromérica para o cromossomo 12 foi possível detectar uma maior porcentagem de casos com trissomia 12. O objetivo deste trabalho foi de detectar trissomia 12 por FISH (sonda alfa satélite) em 13 pacientes com CLL cujos cariótipos por banda G haviam sido normais ou sem resultado. Três pacientes apresentaram trissomia 12 por este método com uma porcentagem de células trissômicas variando de 55,5 a 79%, demonstrando que a FISH é um método sensível e altamente específico para detecção de trissomia 12.

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