



Evaluation of trisomy 12 by fluorescence *in situ* hybridization in peripheral blood, bone marrow and lymph nodes of patients with B-cell chronic lymphocytic leukemia

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ABSTRACT

Background and Objective. Trisomy 12 is the most common numerical chromosomal aberration in patients with B-cell chronic lymphocytic leukemia (B-CLL). Fluorescence *in situ* hybridization (FISH) has improved the detection of this cytogenetic abnormality and has made detection possible in all phases of the cell cycle. The presence of the trisomy 12 positive (+12) cell population has generally been investigated in leukemic cells obtained from the peripheral blood of CLL patients. To ascertain whether trisomy 12 is expressed homogeneously in cells of different hemopoietic tissues, we applied FISH to lymph node, peripheral blood and bone marrow samples obtained simultaneously from 23 untreated B-CLL patients.

Design and Methods. Twenty-three newly diagnosed patients with B-CLL, 15 in stage B and 8 in stage C, were included in the present study. Peripheral blood smears, bone marrow aspirate smears and lymph node touch imprints were collected from each patient at diagnosis. Cytologic preparations were examined by light microscopy in order to assess the lymphocyte morphology. Immunophenotyping was performed by cytofluorimetric analysis of the peripheral blood, bone marrow and lymph node mononuclear cell suspensions. The diagnosis was supported in all cases by histologic findings in bone marrow biopsy and lymph node biopsy specimens. Fluorescence *in situ* hybridization was performed on smears of blood and aspirated bone-marrow and lymph node touch imprints obtained by fresh tissue apposition.

Results. In 6 of the 23 cases (26%) trisomy 12 was clearly present in all tissues examined. A comparative analysis of the three different hemopoietic tissues was performed. A higher percentage of leukemic CD5⁺CD23⁺ cells was detected in lymph nodes than in peripheral blood and bone marrow. A significantly higher proportion of trisomic cells was observed in lymph nodes samples than in peripheral blood or bone marrow smears of trisomy 12 positive CLL patients.

Interpretation and Conclusions. Several previous reports show that only a proportion of malignant B-CLL cells carry trisomy 12 when analyzed by interphase FISH. The higher proportion of +12 cells in lymph nodes than in peripheral blood or bone marrow of CLL

patients with trisomy 12 could reflect different cell distributions in different tissues, or lymph node specific tropism, or proliferative advantage in selected tissue. At present, the role of trisomy 12 in the pathogenesis of lymphoproliferative disorders is unclear.
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Key words: B-CLL, trisomy 12, FISH

Trisomy 12 is the most common chromosome abnormality detectable in B-cell malignancies, occurring with a reported incidence of 10-30% in patients with B-cell chronic lymphocytic leukemia (B-CLL) investigated by conventional cytogenetic methods.^{1,2} Development of the fluorescence *in situ* hybridization (FISH) technique has improved the detection of this cytogenetic abnormality.^{3,4} Trisomy 12 is reported more frequently in *atypical* B-CLL than in the typical form.⁵⁻⁷ Some studies suggest that the presence of trisomy 12 may predict poor prognosis.^{1,8,9} Trisomy 12 is generally considered as a late event in the pathogenesis of B-CLL.¹⁰ Some studies have, however, reported that trisomy 12 is consistently found early in the disease¹¹ and that the trisomy 12 positive (+12) cell population does not increase in size during progression of the malignancy.¹²

The presence of the +12 cell population has generally been investigated in leukemic B-CLL cells obtained from the peripheral blood of CLL patients.

In order to ascertain whether trisomy 12 is present and expressed homogeneously in cells of different hemopoietic tissues, we evaluated the percentage of cells exhibiting trisomy 12 obtained simultaneously from the peripheral blood, bone marrow and lymph nodes of 23 untreated B-CLL patients investigated at diagnosis.

Design and Methods

Twenty-three newly diagnosed patients with B-CLL, 15 in stage B and 8 in stage C, were included in the present study (Table 1). These patients belong to a series of 97 consecutive B-CLL cases admitted to our Institution between 1995 and 1997.

Staging procedures were based on the system recommended by the International Workshop on CLL.¹³

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Table 1. Clinical stage and laboratory data of 23 patients with B-CLL.

Case	+12	S/A	Stage Binet/RAI	% PB lymph. (%CD5/CD23)	smlg I.c.	Morphology B cell (PB)	% BM lymph. (%CD5/CD23)	Histology BM LN	% LN cell suspensions lymph. (% CD5/CD23)
1 B.D.	No	M/65	B/II	93 (90)	κ	t.	48 (89)	n.d. d.	96(89)
2 B.N.	No	M/52	B/II	86 (72)	κ	t.	45 (96)	n.d. d.	94(86)
3 C.F.	Yes	M/74	C/IV	81 (95)	κ	t.	80 (90)	d. d.	94(83)
4 D.F.C.	No	F/68	C/III	81 (73)	κ	a.	81 (70)	n.d. d.	97(85)
5 D.N.F.	Yes	M/80	B/II	75 (81)	λ	a.	82 (86)	n.d. d.	95(83)
6 F.G.	No	M/69	C/IV	79 (96)	κ	t.	81 (85)	d. d.	94(82)
7 G.G.	No	M/51	B/II	75 (84)	κ	t.	83 (84)	d. d.	96(90)
8 G.G.	No	M/70	B/III	90 (90)	κ	a.	84 (93)	d. d.	95(87)
9 M.M.	No	M/51	C/IV	93 (96)	λ	t.	85 (80)	n.d. d.	98(85)
10 M.C.	Yes	M/72	B/II	76 (79)	λ	a.	98 (93)	d. d.	90(81)
11 P.P.	No	M/76	C/III	92 (97)	κ	t.	90 (95)	d. d.	92(86)
12 P.L.	Yes	M/78	B/II	61 (60)	κ	a.	88 (64)	n.d. d.	91(80)
13 R.V.	No	M/52	B/II	90 (84)	λ	a.	80 (84)	n.d. d.	98(88)
14 R.A.	No	F/58	B/II	90 (96)	κ	t.	88 (95)	n.d. d.	96(89)
15 S.A.	No	F/75	B/II	93 (94)	κ	t.	76 (88)	n.d. d.	94(88)
16 B.F.	No	F/67	C/III	88 (86)	κ	t.	68 (70)	n.d. d.	93(88)
17 C.R.	No	M/65	B/II	92 (91)	κ	t.	75 (79)	d. d.	91(87)
18 C.A.	Yes	M/53	C/IV	83 (81)	κ	t.	83 (80)	d. d.	90(82)
19 L.D.	No	M/73	B/III	77 (74)	λ	t.	79 (86)	n.d. d.	94(88)
20 N.C.	No	M/60	C/IV	78 (76)	κ	t.	85 (83)	d. d.	90(81)
21 Q.G.	Yes	F/59	B/II	79 (78)	λ	a.	82 (88)	n.d. d.	91(83)
22 M.P.	No	F/74	B/II	92 (89)	κ	t.	84 (90)	n.d. d.	92(90)
23 P.V.	No	M/53	B/II	88 (85)	κ	a.	86 (91)	n.d. d.	90(86)

S/A: sex/age; PB: peripheral blood; BM: bone marrow; LN: lymph node; smlg: surface membrane immunoglobulin staining; I.c.: light chain; t.: typical; a.: atypical; n.d.: non-diffuse; d.: diffuse pattern; lymph.: lymphocytes.

Peripheral blood and bone marrow aspirate smears were assessed morphologically according to FAB criteria. The lymph node specimens were examined as touch imprints and whole cell suspensions. The lymph node touch imprints were carefully performed in order to evaluate the whole lymph node cut surfaces. Cytologic preparations of lymph node cell suspensions and touch imprints were examined by light microscopy in order to assess the lymphocyte morphology.

Immunophenotyping was performed by cytofluorimetric analysis of the peripheral blood, bone marrow and lymph node mononuclear cell suspensions. A panel of monoclonal antibodies (Becton-Dickinson) detecting T cell (CD2, CD3, CD5) and B cell antigens (CD19, CD23) was employed. Double-labeling with monoclonal antibodies recognizing the CD5 and CD23 antigens was performed on all patients using a fluorescence-activated cell sorter (FACS). Expression of surface immunoglobulin was tested with the FACS analyzer.

The diagnosis was supported in all cases by histologic findings in bone marrow biopsy and lymph node biopsy specimens. In cryostat sections of lymph nodes, as well as the touch preparations, the expression of T cell and B cell antigens was evaluated by immunocytochemistry with monoclonal antibodies (CD2, CD3, CD5, CD19, CD23).

FISH and α -satellite probes

Aliquots of blood and aspirated bone-marrow were smeared on the glass slides, while lymph node touch preparations were obtained by fresh tissue apposition. For each patient three specimens were collected from blood, bone marrow and lymph nodes.

Peripheral blood smears from 5 healthy subjects (aged 48 to 72 years) were used as negative controls.

Fluorescence *in situ* hybridization was performed using published methods.⁴ The probe used was α -satellite DNA probe pBR12, highly specific for the centromeric region of chromosome 12. The probe was labeled by nick-translation using biotin-11-dUTP (Ortho) or biotin-21-dUTP (Clonotech) according to the suppliers' instructions. The hybridized probe was detected with fluorescein-conjugated avidin (Vector).

Evaluation and counting of the hybridization spots were carried out according to the criteria described previously.^{3,14} The number of unhybridized cells was lower than 3% and only those spots with a similar size, intensity and shape were counted; nuclei with unclear morphology or an ambiguous signal were excluded. At least 300 interphase nuclei for each patient tissue were scored under the fluorescence microscope. The percentage of cells was then calculated from the total of scored nuclei per preparation (Table 2).

Table 2. *In situ* hybridization signal frequencies (%) among 300 cells analyzed from peripheral blood, bone marrow and lymph nodes from 6 B-CLL patients.

Case	Sources*	% With no. of signals			
		≤1°	2	3	≤4#
1 B.D.	PB		100		
	BM	5	85	3	7
	LN		97		3
2 B.N.	PB	9	90	1	
	BM		100		
	LN		99	1	
3 C.F.	PB		56	44	
	BM		68	32	
	LN		30	70	
4 D.F.C.	PB	6	86	6	2
	BM	3	87	7	3
	LN	2	94	3	1
5 D.N.F.	PB		46	54	
	BM		63	37	
	LN		29	71	
6 F.G.	PB	2	97	1	
	BM	1	98	1	
	LN	1	98	1	
7 G.G.	PB	1	98	1	
	BM	19	81		
	LN	5	93	2	
8 G.G.	PB		98	2	
	BM		94	6	
	LN	8	90	2	
9 M.M.	PB	3	96	1	
	BM	4	95	1	
	LN	3	96	1	
10 M.C.	PB		68	32	
	BM		66	34	
	LN		55	45	
11 P.P.	PB	2	97	1	
	BM		91	5	4
	LN		92	1	7
12 P.L.	PB		53	47	
	BM		56	44	
	LN		30	70	

(continued to next column)

Paired samples Student t-test was applied to evaluate the differences between the mean percentages of trisomic cells obtained from the peripheral blood, bone marrow and lymph node of *trisomy 12 positive* patients. The statistical significance limit was set at ≤ 0.05.

Table 2. (continued).

Case	Sources*	% With no. of signals			
		≤1°	2	3	≤4#
13 R.V.	PB	15	83	2	
	BM	4	92	4	
	LN	5	93	2	
14 R.A.	PB		90	4	6
	BM		92	4	4
	LN		94	2	4
15 S.A.	PB	2	93	3	2
	BM	5	57	7	31
	LN	2	95	2	1
16 B.F.	PB	2	96	2	
	BM	5	94	1	
	LN	3	96	1	
17 C.R.	PB	3	96	1	
	BM	4	89	5	2
	LN	2	96	1	1
18 C.A.	PB		50	50	
	BM		62	38	
	LN		32	68	
19 L.D.	PB	6	92	2	
	BM	8	92		
	LN	5	93	2	
20 N.C.	PB	1	98	1	
	BM	1	97	2	
	LN	2	97	1	
21 Q.G.	PB		52	48	
	BM		54	46	
	LN		28	72	
22 M.P.	PB	3	88	4	5
	BM	2	90	4	4
	LN	3	91	3	3
23 P.V.	PB		93	5	2
	BM		95	4	1
	LN		93	3	4

*PB = Peripheral blood; BM = bone marrow; LN = lymph node; °includes cells with no signal; #includes cells with several signals or ambiguous hybridization spots.

Results

Morpho-immunophenotypic and clinical data

Twenty-three consecutive patients, 15 with *typical* B-CLL (Table 1, cases #1, 2, 3, 6, 7, 9, 11, 14, 15, 16, 17, 18, 19, 20, 22) and 8 (Table 1, cases #4, 5, 8, 10, 12, 13, 21, 23) with *atypical* B-CLL (CLL of mixed cell type), according to the FAB criteria, were included in the present study.

All patients showed peripheral blood lymphocytosis higher than $5 \times 10^9/L$ (mean 70.7; range 10.0-

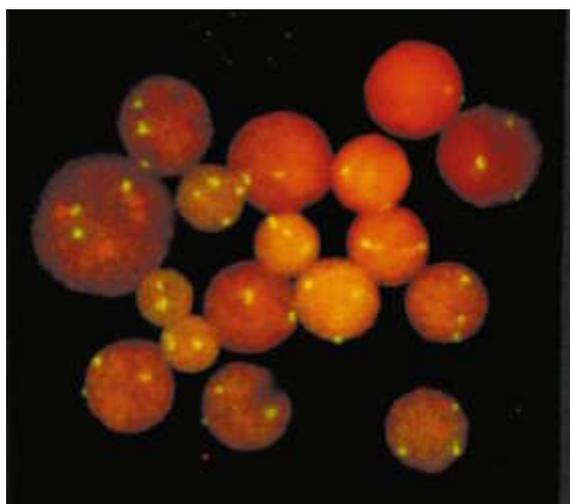


Figure 1. CLL patient (CF, #3); lymph node touch preparation. FISH analysis using α -satellite DNA probe pBR12 specific for the centromeric region of chromosome 12. Almost all the cells display three signals (+12 positivity).

260.0). The mean peripheral blood lymphocyte percentage was 84.0% (range 61-93) of which more than 60% (mean 85.8, range 60-97) were B-CLL cells as judged by immunologic marker analysis with coexpression of CD5 and CD23. The intensity of light chain (l.c.) surface immunoglobulins (smlg) expression was weak to very weak in all cases.

The clinical stage and laboratory data are reported in Table 1. Seventeen patients were male and 6 female. Their mean age was 65 years (± 9.70) (range 51-80). Clinical staging according to the Binet classification was stage B in 15 patients (65.2%) and stage C in 8 (34.8%). According to the Rai classification, 13 patients were in stage II, 5 in stage III and 5 in stage IV. The mean hemoglobin level was 120 g/L (± 17.5) (range 78-151), mean platelet level $155.0 \times 10^9/L$ (range 62.0-275.0).

The mean bone marrow lymphocyte percentage was 79.6% (range 45-98). The mean percentage of CD5⁺CD23⁺ bone marrow lymphocytes was 85.1% (range 64-96).

At histology, the bone marrow infiltration pattern was diffuse in 9 patients (39.1%) and non-diffuse in 14 (60.9%).

The mean percentage of lymphocytes observed in lymph node touch preparations was 95.0% (range 93-98). The mean percentage of lymphocytes observed in lymph node histologic sections was 94% (range 92-96). In lymph node cell suspensions, the mean percentage of lymphocytes was 93.0% (range 90-98). Immunophenotyping performed on lymph node cell suspensions showed 80-90 percent CD5⁺CD23⁺ cells (mean 85.5%).

The lymph node histologic pattern was diffuse in all cases, consistent with B-CLL histology. In histologic

sections the cells expressed CD19 (mean 86.3%), CD23 (mean 85.7%) and CD5 (mean 97.3%) antigen. The percentage of T lymphocytes (CD2, CD3) was low (range 2-10%). In our CLL cases there were no differences in the lymph nodes histologic pattern and in the immunophenotype of typical and atypical cases with and without trisomy 12.

In situ hybridization data

The results of the FISH analyses are summarized in Table 2.

Normal control cells (5 cases) had one hybridization signal in 5.5%, two signals in 91.2% and three signals in 3.3% of the cells.

Patients' samples were considered suitable for evaluation if hybridization spots were present in 90% of cells or if less than 20% of cells had zero or one signal. Lymph node touch preparations gave good results by FISH analysis, with a very low percentage of non-assessable cells, strong hybridization signals and good spatial resolution which allowed rapid enumeration of signals (Figure 1).

Trisomy 12 was clearly present in only 6 of the 23 patients (26.6 %) (Table 1, cases #3, 5, 10, 12, 18, 21). The percentages of cells with three hybridization signals in these patients ranged between 32-54% (mean 45.83) in the peripheral blood, 32-46% (mean 38.50) in bone marrow aspirate and 45-72% (mean 66.00) in the lymph node touch preparations (Table 2). In the peripheral blood and/or bone marrow aspirate from 9 patients (Table 2, cases #4, 8, 11, 13, 14, 15, 17, 22, 23), the number of trisomic cells ranged from 4% to 7%; however, lymph node FISH analysis demonstrated very low numbers of trisomies (< 3%) in these cases, comparable to those detected in normal controls. The percentages of trisomic cells in the other patients ranged from 1% to 3% in all tissues examined.

No major differences were observed with respect to patients without trisomy 12 regarding either the clinical stage, splenomegaly, lymphadenopathy or laboratory data including hemoglobin level, platelet count, pattern of bone marrow or lymph node involvement, number of CD5⁺CD23⁺ B cells and surface immunophenotyping results.

The percentages of cells with three hybridization signals were then compared between the different tissues analyzed. The percentage of +12 cells observed in the lymph nodes of the 6 cases with *trisomy 12 positivity* (Table 3), was considerably higher [mean 66.00 (± 10.37)] than in the peripheral blood [mean 45.83 (± 7.55)] or bone marrow aspirate [mean 35.50 (± 5.50)]. The differences between the percentages of +12 cells in the lymph nodes versus (vs) peripheral blood ($p=0.0001$) and between the percentages of +12 cells in the lymph nodes vs bone marrow ($p=0.0007$) were statistically significant (Table 3). No statistically significant difference between the percentages of +12 cells was observed in peripheral blood

Table 3. Comparison between +12 level (%) in different tissues from "trisomy 12 positive" cases (n=6).

	mean \pm SD	p value*
Peripheral blood vs Lymph node	45.83 \pm 7.55 66.00 \pm 10.37	0.0001
Bone marrow vs Lymph node	38.50 \pm 5.50 66.00 \pm 10.37	0.0007
Peripheral blood vs Bone marrow	45.83 \pm 7.55 38.50 \pm 5.50	0.06

*paired samples t-test; SD: standard deviation.

Table 4. Calculated percentages (%) of CLL cells and corrected percentages (%) of +12 cells per CLL cells in peripheral blood, bone marrow and lymph nodes from 6 B-CLL patients.

	CF	DNF	MC	PL	CA	QG	p*
Calculated %CLL cells [^]							
PB	77	61	60	37	67	62	
BM	72	70	91	56	66	72	
LN	78	79	73	73	73	75	
Corrected % +12 cells per CLL cells [#]							
PB	57	88	53	100	75	77	0.002
BM	44	53	37	78	57	64	0.0004
LN	90	90	62	96	93	96	

[^]% lymphocytes x % CD5⁺CD23⁺ cells; [#]% +12 cells/calculated % CLL cells
PB: peripheral blood; BM: bone marrow; LN: lymph node; *paired samples t-test.

vs bone marrow (p=0.06).

We observed that, in our cases, there were fewer lymphocytes and, specifically, fewer CD5⁺CD23⁺ cells in the peripheral blood and bone marrow than in lymph node samples. The percentage of trisomic cells in the peripheral blood and bone marrow could be lower because there was a lower number of CLL cells in these locations. Data for FISH were corrected for the denominator of calculated CLL cells (% lymphocytes x % CD5⁺CD23⁺ cells) at each site. When the data were corrected in this way, the pattern of distribution of trisomy 12 was the same as described by the uncorrected data in all but one case (Table 4; case PL). The differences between the corrected percentages of +12 cells (%+12 cells/calculated % CLL cells) in peripheral blood vs bone marrow (p=0.002) and bone marrow vs lymph nodes (p=0.0004) were statistically significant (Table 4). No statistically significant difference between the corrected percentages of +12 cells was observed in peripheral blood vs lymph nodes (p=0.06).

Discussion

Trisomy 12 is the most frequent numerical chromosomal abnormality in B-CLL cells. The different trisomy 12 incidences reported could be explained in part by differences in analysis methods (conventional cytogenetic versus FISH analysis) and in patient selection. FISH eliminates the potential bias introduced by a dependence on stimulation of B-cell division by appropriate mitogens. It enables rapid evaluation of a greater number of cells than is possible using conventional cytogenetic analysis and, consequently, permits faster assessment of the proportion of abnormal cells present. In large series of B-CLL patients in whom other chronic lymphoproliferative diseases have been excluded, a low frequency of trisomy 12 has been reported, together with a low proportion of *atypical* B-CLL cases.^{2, 15} Many studies^{5, 6, 7, 15, 16} have noted a higher occurrence of trisomy 12 in *atypical* than in typical B-CLL cases. Our data are in agreement with these observations.

The aim of the present study was to investigate whether significant differences were present with regard to number of +12 cells in different body tissues in trisomy 12 positive cases. The intriguing finding of our study is the presence of significantly more +12 B-cells in the lymph node samples of 6 trisomy 12 positive patients relative to the numbers in peripheral blood and bone marrow. The presence of trisomy 12 in 32-54% of peripheral blood interphase nuclei, despite the leukemic cells representing over 60% of mononuclear cells in all trisomic patients, clearly shows that only a proportion of the malignant cells are trisomic, as reported previously.^{2, 14} Similarly, in bone marrow we observed that the percentage of +12 cells of the 6 trisomy 12 positive patients ranged between 32-46%, despite the proportion of the CD5⁺CD23⁺ cells being over 80% of mononuclear cells present. Bone marrow lymphocytes and bone marrow CD5⁺CD23⁺ cells were both detectable in high proportion in trisomy 12 positive cases with more trisomic signals in the lymph node than bone marrow of each patient.

The higher proportion of +12 cells in lymph nodes than in peripheral blood or bone marrow of CLL patients with trisomy 12 could reflect different cell distributions in different tissues, or lymph node specific tropism, or proliferative advantage in selected tissue.

Some reported increased levels of +12 cells observed in the peripheral blood of CLL patients during the disease or in treated patients,^{15, 17} explained as proliferative advantage of trisomic cells, could also be attributed to a different distribution of +12 cells in the body tissues. At present, the biological significance of this different distribution of +12 cells in CLL is unknown, as is the role of this trisomy (if any) in the pathogenesis of lymphoproliferative disorders. A study of adhesion molecules in B-CLL cells, simultaneously evaluated in peripheral blood, bone marrow and lymph nodes, has shown that CD44 and CD54

on CD5⁺ CD23⁺ lymphocytes were more dominant in more numbers of cells in lymph nodes than in peripheral blood and bone marrow.^{18,19} In some patients with chronic lymphoproliferative diseases other than CLL, polysomy 12 detected in lymph node cells was clearly associated with progressive disease.²⁰ Accurate prolonged follow-up of selected patients may perhaps contribute to explaining some clinical and biological aspects of trisomy 12 in CLL.

Contributions and Acknowledgments

VL was responsible for the conception of the study. SC and AL contributed to FISH analysis. VL wrote the paper with SC's collaboration. SC, VP, AG and GS followed the patients clinically. All the authors contributed to the critical revision and approval of final version of paper.

Funding

This work was supported by Italy-USA project on "Therapy of Tumors", by AIL "Trenta ore per la vita" project and by MURST.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received September 4, 1998; accepted November 12, 1998.

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