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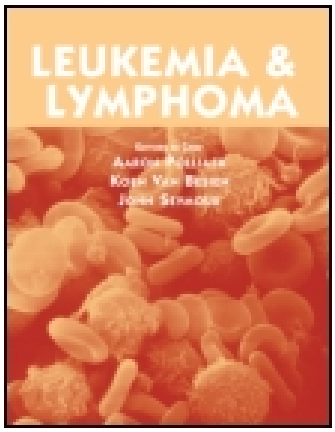

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Trisomy 12 in Chronic Lymphocytic Leukemia and Hairy Cell Leukemia: A Cytogenetic and Interphase Cytogenetic Study

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Trisomy 12 in Chronic Lymphocytic Leukemia and Hairy Cell Leukemia: a Cytogenetic and Interphase Cytogenetic Study

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Fluorescent in situ hybridization (FISH) with a chromosome 12-specific pericentromeric probe was performed in 42 patients with B-cell chronic lymphocytic leukemia (CLL) and in 10 patients with hairy cell leukemia (HCL). In all cases, a normal karyotype in more than 10 metaphase cells was obtained by conventional chromosome study.

FISH documented that 6/42 patients with CLL in fact had trisomy 12 in 15–49% interphase cells. Sequential FISH studies were performed in 2 cases, showing an increase of percentage of trisomic cells over a 2-month to 4-year period. Two out of 10 patients with HCL, one of whom had morphologic features consistent with a diagnosis of HCL variant, showed 5.5 and 10% interphase nuclei with three fluorescent signals, a finding suggestive of the presence of trisomy 12.

Combined immunophenotyping and FISH staining in these patients with HCL documented that trisomic cells were CD11c-positive, CD13-negative, and CD2-negative.

We conclude that FISH is a sensitive technique allowing for the detection of trisomy 12 in a fraction of cytogenetically normal patients affected with CLL and HCL.

KEY WORDS: Trisomy 12 FISH CLL HCL

INTRODUCTION

The role of conventional chromosome analysis in patients with some low-grade lymphoproliferative disorders such as B-cell chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL) is limited by difficulty in stimulating neoplastic cells into mitosis. Indeed, combined cytologic and cytogenetic studies have yielded increasing evidence that despite employment of mitogens, normal cells may proliferate in culture, raising the possibility that some abnormal clones may be overlooked under these circumstances due to inadequacy of culture conditions.[1,2]

Over the last 3 years, the introduction of fluorescent in situ hybridization (FISH) technique allowed for the detection of some structural and numerical chromosome aberrations in interphase cells,[3] documenting that some CLL patients with normal karyotype may in fact be carrier of acquired trisomy 12.[4–9]

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Thus, trisomy 12 represents a non-randomly occurring chromosome aberration in CLL[10] the detection of which may be difficult in some patients due to low mitotic index of neoplastic cells carrying this chromosome abnormality. In HCL, the matter is further complicated by the low number of cells available for cytogenetic analysis, impeding the study of adequate number of metaphases.[11] Consequently, cytogenetic data on few patients are available[11] and the cytogenetic profile of this B-cell neoplasia is poorly defined. Furthermore, because only few patients have been studied by FISH,[12] it is not known whether or not trisomy 12 may occur in HCL more frequently than expected on the basis of conventional chromosome studies.

We therefore elected to perform a cytogenetic and interphase cytogenetic study on 42 patients with B-CLL, and 10 patients with HCL in whom cytogenetic analysis yielded normal karyotype or no mitoses, in order to better define the role of combined FISH/cytogenetic study for the detection of trisomy 12 in these low-grade B-cell neoplasias.

PATIENTS AND METHODS

Chromosome studies were performed at our Institution during the last 5 years in 78 patients with B-CLL and in 10 cases of HCL. Included in the present report are 42 patients with CLL and 10 patients with HCL with a normal karyotype or inadequate number of analysable mitoses.

Diagnoses were made according to usual clinical and hematologic criteria.[13] Immunophenotyping was performed by cytofluorimetric analysis of the pertinent cell population as previously reported.[5]

The following antigens were tested in patients with a diagnosis of CLL using commercially available monoclonal antibodies: B-cell antigens: CD22, CD23, CD24; T-cell antigens: CD5, CD2. Double labelling with monoclonal antibodies recognizing the CD23/CD5 antigens, normally coexpressed by CLL lymphocytes, was performed on all patients. Surface immunoglobulins (Ig) were detected by a direct immunofluorescence assay under a Diaplan fluorescence microscope (Leitz).

Diagnoses of HCL were made on the basis of typical morphologic and histologic features supported by positivity for the tartrate resistant acid phosphatase stain (TRAP)[14] and by positivity for monoclonal antibodies detecting the CD11c and CD25 antigens.

Cytogenetic studies

Protocols for cytogenetic study of CLL and HCL adopted at our Institution were published previously.[15] Peripheral blood (PB) and/or BM cells were cultured for 72 hours at a concentration of 2×10^6 cells/ml in RPMI 1640 with 10% fetal calf serum. Mitogens employed were: lipopolysaccharide (LPS) (0.1 mg/ml), pokeweed mitogen (PWM) (0.1 mg/ml), phytohemagglutinin (PHA) (0.1 mg/ml). Phorbol miristate acetate was employed since 1991 at a final concentration of 50 ng/ml.

At least 10 metaphases, and usually more than 20, were studied before a karyotype was defined as normal.

Detection of trisomy 12 by FISH

FISH was performed on 42 patients with CLL and 10 patients with HCL with a normal karyotype or inadequate number of analysable mitoses. For the detection of trisomy 12 in interphase cells, the chromosome 12-specific alpha satellite probe pBR 12 was used (courtesy of Dr. M. Rocchi, Istituto Gaslini, Genova). The probe was biotinylated using the BRL kit (Gaithersburg, Maryland) following the manufacturer's instructions. This probe detected less than 1% cells with three signals and less than 10% cells with 1 signal on control slides from normal volunteer donors. In accord with other authors[16] the cut-off point for classifying a patient as trisomic was set at 3% of all nuclei showing well-delineated signals.

FISH techniques employed in our laboratory were described previously.[5] Briefly, slides for conventional cytogenetic analysis, aged at room temperature for 2–30 days were pretreated by RNAse, dehydrated in methanol and denatured in 70% formamide in 2 × SSC at 70°C for 2 minutes. The biotynated probe (100 ng/slide) was dissolved in 45 ul hybridization mixture (total volume) consisting of 60% formamide in 2 × SSC, 10% wt/vol dextran sulfate and 30 ug salmon sperm DNA.

Following denaturation at 75°C for 5 minutes the hybridization mixture was layered onto each slide. Slides were covered with a coverslip and sealed with nail polish. Hybridization was allowed to procede overnight at 37°C in a moist chamber.

Unspecifically bound probe was removed through repeat wash in 45% formamide in 2 × SSC with intermittent agitation. Specific signal was visualized by incubation with FITC-avidin (Vector, Burlinghame) followed by amplification with biotynated anti-avidin

monoclonal antibody (Vector, Burlinghame) and a second layer of FITC-avidin. Propidium iodide was used as counterstain. Slides were examined under a fluorescence microscope (Diaplan, Leitz). At least 200 cells with well-delineated fluorescent spots were counted in each patient for signal screening.

Combined FISH and immunophenotyping

In order to directly identify which cell lineage carried trisomy 12 in HCL, PB mononuclear cells were submitted to membrane staining according to a standard indirect immunofluorescence technique. The LeuM5 monoclonal antibody (Becton-Dickinson, Mountain View, California) detecting the CD11c surface antigen was used to label hairy cells, whereas T-lymphocytes and granulomonocytic cells were labelled by the OKT11 (CD2) (Ortho Diagnostic System, Raritan NJ) and by the My7 (CD13) (Dakopatts, Copenhagen, Denmark) monoclonal antibodies, respectively. These reagents were handled and used following the manufacturer's instructions. In order to minimize non-specific Fc binding, cell suspensions were pre-incubated with 5% normal rabbit serum (Dakopatts). Specific binding of the primary antibody was visualized with rabbit anti-mouse immunoglobulins conjugated with tetramethyl rhodamine isothiocyanate (TRITC) (Dakopatts).

Cytospin preparations were made from each immunolabelled cell suspension and fixed by immersion for 30 minutes in methanol/acetic acid at a concentration 19:1 and by a second immersion for 15 minutes in methanol/acetic acid at a concentration 3:1. The same hybridization protocol as described above was employed for the detection of trisomy 12 in immunolabelled cells. No counterstaining was used.

RESULTS

Chronic lymphocytic leukemia

Conventional cytogenetic studies yielded a normal karyotype in more than 10 metaphases in 32 cases and no analysable mitosis in 10 cases. No evidence of trisomy 12 was detected by FISH in 36/42 cases showing two fluorescent signals in more than 89% interphase cells and three signals in less than 1% interphase nuclei.

In 6 patients with normal karyotype from PHA- and PWM-stimulated cultures, FISH documented the presence of 15–49% nuclei with 3 fluorescent signals, a finding suggesting that these patients in fact had trisomy 12 in interphase cells. Detailed results are summarized in Table 1. Clinical, cytogenetic and cytologic features in 4 of these patients were described previously.[5]

FISH studies were performed on 3 different occasions during the course of the disease in patient 1 and 2 (see Table 1) in order to monitor the relative size of the trisomic clone. Percentage of interphase cells with 3 fluorescent signals varied over the time, ranging from 21–49% in the former patient and from 25–45% in the latter patient. As shown in Table 1, an increase of trisomic cells was detected following administration of chemotherapy in both cases, whereas the size of the trisomic clone relative to the total lymphocyte population did not change when FISH was performed after chemotherapy had been stopped for more than 3 months.

Hairy cell leukemia

Two fluorescent signals were detected by FISH in more than 90% interphase nuclei in 8 cases, in which no cell with three signals was seen.

Evidence of trisomy 12 was found in 2/10 patients showing 10% and 6% interphase cells with three signals. Peripheral blood hairy cells at the time of FISH study were 55% and 15% of total cellularity, respectively.

Trisomy 12 was found in 20% and 16% CD11c positive cells by combined membrane immunophenotyping and FISH staining in these two patients, whereas no cell that was labelled by the anti-CD2 and CD13 monoclonal antibody showed three fluorescent signal. Details are reported in Table 2.

One patient with trisomy 12 showed abnormal lymphocytes with morphology that was intermediate between hairy cells and prolymphocytes. In these cells short cytoplasmic projections and a visible central nucleolus were present (Figure 1). TRAP reaction was positive (Figure 1) and immunologic studies documented a B-cell phenotype with CD11c positivity and CD25 negativity.

These findings were consistent with a diagnosis of HCL variant.[17]

Cytologic diagnoses and clinical features as well as results of fluorescent signal screening in these 2 patients with trisomy 12 are summarized in Table 2.

DISCUSSION

This study extends previous observations on the role of FISH in the detection and monitoring of trisomy

Table 1 Cytogenetic and interphase cytogenetic findings in 6 patients with B-CLL and trisomy 12 in interphase cells

No.	*Sex/age*	*Diagnosis (FAB) date, Rai stage*	*% clonal B-cells in PB samples**	*No. of normal karyotypes (mitogens)*	*No. of fluorescent spots in interphase cells*		
					1	*2*	*3*
1	F/72	CLL/PLL					
		Dec. 1988, III	85%	26(PWM), 8(PHA)	12	67	21
		Dec. 1990,† III	92%	NA	5	55	40
		Aug. 1993,‡ III	95%	NA	9	42	49
2	M/70	CLL/PLL					
		Aug. 1990, II	81%	21(PWM), 10(PHA)	8	67	25
		Oct. 1990†, II	80%	8(PHA)	9	46	45
		Nov. 1993‡, III	85%	NA	9	50	41
3	F/50	CLL/PLL					
		Dec. 1988, III	83%	16(PWM), 12(PHA)	11	74	15
4	M/68	Dec. 1988, III	87%	10(PWM), 8(PHA)	7	68	25
5	M/80	Oct. 1992, III	67%	3(PWM), 10(PHA)	7	53	20
6	M/63	Oct. 1992, I	40%	15(PHA)	10	61	29

*As assessed immunologically based on the % of cells with coexpression of CD23/CD5 antigens.
†Treated by alkylating agents before the FISH study.
‡Untreated for the last 3 months preceding the FISH study.
NA: not available.

Table 2 Clinical, cytologic and interphase cytogenetic findings in two patients with HCL and trisomy 12 in interphase nuclei

Patient, sex, age, diagnosis	*Immunophenotype**	*WBC × 10^{-9} l/ splenomegaly†/ % PB hairy cells*	*No. normal karyotypes (mitogen)*	*No. of fluorescent spots in interphase cells‡*			*No. of CD11c+ cells/ no. of cells with 3 spots*
				1	*2*	*3*	
1, m, 78 HCL variant	CD19+41%; CD11c+55% FMC7+33%; IgM/k+34% CD5−; CD25−	2.3/+++/55%	no mitosis (LPS, PHA)	6	84	10	50/10
2, m, 85 HCL	CD22+33%; CD11c+29%; IgM/l+10%	4.0/+/15%	12(PHA)	5	89	6%	50/8

*% positive cells in the lymphocyte gate, −: <5% positive cells.
†−: not palpable; +: 1–5 cm; ++: 6–10 cm; +++: >10 cm below costal margin.
‡400 nuclei observed.

12 in B-CLL,[5] and shows that some patients with HCL with normal karyotype may in fact carry trisomy 12 in interphase nuclei. Since FISH was widely adopted in most cytogenetic laboratories, increasing evidence was provided that a significant fraction of CLL patients with normal karyotype in fact had trisomy 12 in interphase cells.[18] Besides our 6/42 CLL patients with normal karyotype in whom trisomy 12 in interphase cells was documented by FISH, similar discrepancy between FISH and chromosome analysis was documented in 2/13 CLL cases by Perez-Losada *et al.*,[4] in 5/30 cases by Anastasi *et al.*,[6] in 3/45 cases by Dohener *et al.*,[9] in 6/128 by Que *et al.*[8] and in 65.4% of 117 cases studied by Escudier *et al.*[7]

Interestingly, conventional chromosome studies failed to detect trisomy 12 in this series in patients with no mitoses from LPS-stimulated cultures, whereas no evidence of trisomy 12 was detected by FISH in 30/42 chromosomally normal patients showing analysable mitoses from cultures additioned with this mitogen. Unfortunately, only 10 patients in which phorbol esters were used could be included in this study precluding a reliable analysis of the efficacy of this mitogen for the detection of trisomy 12.

Dohener *et al.*[9] and Que *et al.*[8] found trisomy 12 by FISH in 6.5% and 4.8% and patients with normal metaphases originating from cultures stimulated by phorbol esters, whereas frequent failure of conventional cytogenetic to detect trisomy 12 was found in the study by Escudier *et al.*[7] in which PHA and LPS were used. These authors, however, do not report the number of analysable mitoses obtained from LPS-

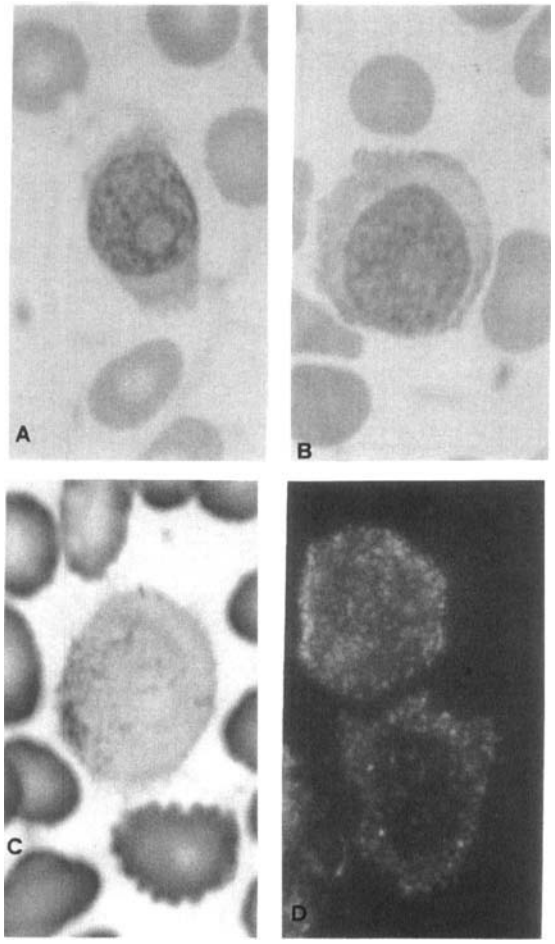


Figure 1 Morphologic, cytochemical and combined immunologic/FISH studies in 1 patient with HCL variant. Two cells with short cytoplasmic villi and morphology that is intermediate between that of typical hairy cells and prolymphcytes are shown (a) and (b). Note the prominent central nucleolus. TRAP positivity is shown in (c). Positivity for the CD11c antigen (red membrane stain) in two cells with three fluorescent spots (green nuclear spots) is shown in (d). (See Colour Plate IX at the back of this publication.)

stimulated culture in their patients with normal karyotype.

Our data and those reported in the literature confirm our previous observation that trisomy 12 may be present in a CLL subclone, percentages as a low as 5–15% of trisomic interphase cells having been found in several patients.[5,7,9] Thus, trisomy 12 may represent a cytogenetic event possibly conferring growth advantage to already transformed monoclonal B-lymphocytes.

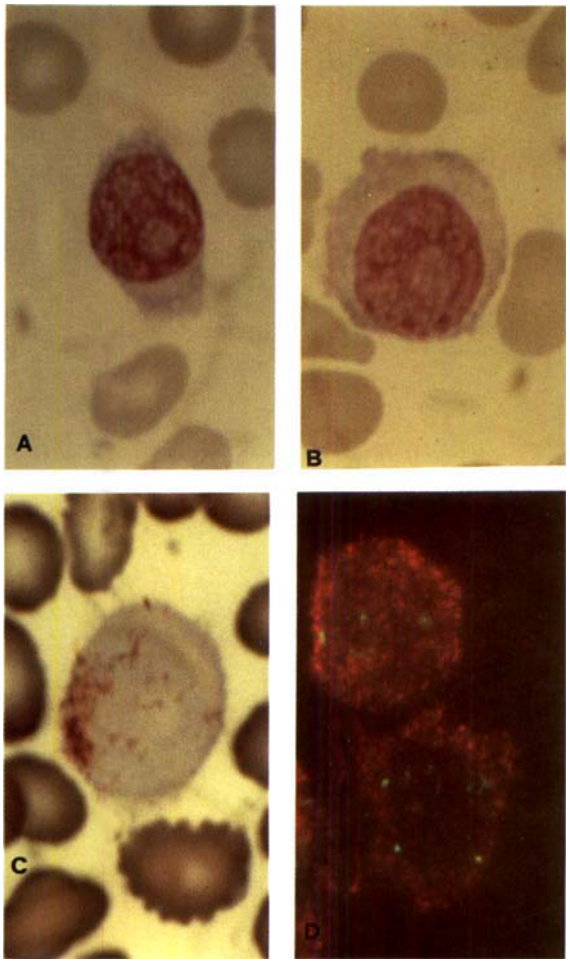
Given the prognostic role of this acquired chromosome change[19] it was of interest to assess whether or not the size of the trisomic clone increased over the time in patients showing mosaicism of a trisomic and disomic leukemic clone.

We previously detected a 1.8- to 3-fold increase of the size of trisomic clone over a 2-month to 2-year period, following administration of alkylating agents, suggesting that lymphocytes with trisomy 12 may be more resistant to some cytotoxic drugs than clonal lymphocytes without this chromosome abnormality. In this study we were able to extend follow-up on two patients (nos. 1 and 2) performing FISH after therapy had been stopped for the last 3 months. Under these circumstances no change of the size of the trisomic clone was detected. This finding is in keeping with the report by Raghoebier *et al.*,[20] showing the persistence of mosaicism of trisomy 12 in untreated CLL patients. Sequential studies of more cases are warranted to clarify whether or not the relative proportion of trisomic cells may increase over the time in untreated patients.

Chromosome studies in HCL have been few not only due to the rarity of the disease, but also because the cells are difficult to stimulate into mitosis. In addition, few cells are usually obtained from BM aspirate and/or PB samples. Sandberg reviewed available cytogenetic data in 1990,[11] showing that a normal karyotype, sometimes with nonclonal aberrations, is seen in most cases. However, a number of non-recurring chromosome changes have also been described, including structural aberrations of chromosome 14 with breakpoint usually located at band q32 and trisomy 12.[21,22] To our knowledge, the presence of a minority of cells with 3 signals in 2/10 cases with normal karyotype is the first demonstration of trisomy 12 in interphase cells in HCL. The percentage of trisomic cells in these patients was only 10% and 6% of all nuclei. This finding, however, is not surprising if one considers that morphologically abnormal cells were only 55% and 15% respectively in samples submitted to FISH study.

Combined FISH and immunophenotyping directly demonstrates that the clone with trisomy 12 bears the CD11c, a surface antigen normally expressed by hairy cells and monocytes. Because CD13 positive cells only showed two signals, it is likely that trisomy 12 was confined to hairy cells in both patients. Recently, Lewis *et al.* did not detect trisomy 12 by FISH in 16 cases of HCL.[12] Unlike these patients, cytologic features in one of our 2 cases with trisomy 12 were consistent with a diagnosis of HCL variant, a subset of HCL usually associated with resistance to IFN therapy.[23,24]

We conclude that FISH is a sensitive technique allowing for the detection of trisomy 12 in a fraction



Colour Plate IX (see page 171 Figure 1) Morphologic, cytochemical and combined immunologic/FISH studies in 1 patient with HCL variant. Two cells with short cytoplasmic villi and morphology that is intermediate between that of typical hairy cells and prolymphcytes are shown (a) and (b). Note the prominent central nucleolus. TRAP positivity is shown in (c). Positivity for the CD11c antigen (red membrane stain) in two cells with three fluorescent spots (green nuclear spots) is shown in (d).

of chromosomally normal patients affected with CLL and HCL. Its role as a tool complementary to conventional chromosome analysis in the definition of the karyotype of other subtypes of low-grade B-cell neoplasias is worth exploring.

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