Trisomy 12 in Chronic Lymphocytic Leukemia Detected by Fluorescence In Situ Hybridization: Analysis by Stage, Immunophenotype, and Morphology

By T.H. Que, J. Garcia Marco, J. Ellis, E. Matutes, V. Brito Babapulle, S. Boyle, and D. Catovsky

Fluorescence in situ hybridization (FISH) with a chromosome 12 specific α-centromeric probe was performed on interphase cells from 183 patients with B-cell chronic lymphocytic leukemia (CLL). Twenty one cases with trisomy 12 (11.5%) were detected. The number of trisomic cells ranged from 5.5% to 76% (mean 38.5%). No correlation was found between the presence of trisomy 12 and white blood cell count, hemoglobin level, platelet count, a specific immunophenotype, clinical stage, sex, splenomegaly, or lymphadenopathy. Morphologic review of all cases with trisomy 12 showed seven (33%) with more than 10% prolymphocytes and three (14%) with CLL of mixed cell type. While trisomy 12 is the most common chromosomal abnormality in CLL, it is more frequent in morphologically atypical cases, some of which may be undergoing transformation. There was a statistically significant difference in the incidence of atypical cases between those with (47%) and without (7.6%) trisomy 12 (P < .001). It remains to be determined whether this abnormality is associated with a worse prognosis; this is currently being investigated in the context of a national therapeutic trial. The technique used is more sensitive than conventional cytogenetic analysis, which in this series failed to detect trisomy 12 in six cases. FISH allows the systematic study on a large number of patients without the need of metaphase preparations.

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B-CELL CHRONIC lymphocytic leukemia (CLL) results from the clonal expansion of mature looking lymphocytes. A degree of morphologic heterogeneity has been recognized for a number of years2;3; recently, this has been examined by the French-American-British (FAB) group4 in proposals for the classification of lymphoproliferative disorders in which CLL remains as a distinct entity. In addition, a degree of heterogeneity in membrane markers has been the basis for classifications proposed by other groups.5,6 The clinical course of CLL is also variable; although some patients have a survival of only months or a few years from diagnosis, others survive more than 10 years with stable disease. A number of parameters have been identified as important for prognosis,7,8 but the impact of cytogenetic abnormalities in the clinical course has not been fully established. This is possibly due to the difficulty in obtaining consistent chromosome preparations in CLL.9 Although the use of various mitogens has improved the cytogenetic results,10-17 clonal abnormalities can be detected only in half of the cases with this technique,17 while in the other cases either a normal karyotype or no divisions are found. Therefore, the precise incidence of chromosomal abnormalities, such as trisomy 12, the most commonly found in CLL, is still unknown.

Fluorescence in situ hybridization (FISH) using chromosome specific centromeric probes is a powerful tool for the detection of numerical chromosomal abnormalities, both in dividing and nondividing (interphase) cells. The application of FISH in CLL may establish more accurately the incidence of trisomy 12 by this technique.18-20 We present here a large series of cases studied by FISH and examine possible correlations of trisomy 12 with disease stage, morphology and immunophenotype.

MATERIALS AND METHODS

Patients. From June 1990 to June 1992 peripheral blood and/or bone marrow aspirates from 205 patients with CLL were sent to our laboratory for cytogenetic analysis, in situ hybridization, and immunophenotypic studies. Of these unselected cases, 201 were entered in the Medical Research Council CLL 3 treatment trial; the remaining four were retrieved from the Royal Marsden Hospital or sent to our laboratory in consultation. The diagnosis of CLL was based on clinical features, cell morphology, and immunologic markers.4 Patients with other B-cell diseases such as prolymphocytic leukemia (PLL),21 splenic lymphoma with villous lymphocytes (SLVL),22 and hairy cell leukemia23 or T-cell disorders were excluded. Staging was performed according to Binet et al.24

Immunophenotyping. Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque. Cells were analyzed by direct and indirect immunofluorescence with the following monoclonal antibodies: CD2 (RFTI) to detect T cells; CD37 (WR17), CD23 (MHM6), FMC7 (Sera-Lab), CD19 (RFB9), and/or CD22 (CLB/By1) to identify B cells; and CD5 (UCHT1), which reacts with B-CLL cells and T cells. The second layer antibody was a fluorescein (FITC) conjugated goat-antimouse F(ab')2 fragment (Cappel). FITC conjugated polyclonal antibodies against immunoglobulin heavy and light chain were used to detect the surface Ig (SmIg; Dako and Cappel, respectively). Reactivity with the various markers was analyzed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). When necessary, the alkaline phosphatase antialkaline phosphatase (APAAP) technique was applied on cytoplasmic preparations to demonstrate light chain restriction.

Cytogenetic analysis. Whole blood samples or bone marrow aspirates were cultured in RPMI with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal calf serum (FCS) in the presence of TPA (final concentration 0.05 μg/mL). After 3 and 5 days of culture, cells were harvested and treated with hypotonic solution

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(KCl 0.075 mol/L, 10 minutes, 37°C) after mitotic arrest (colcemid 0.1 μg/mL, 60 minutes) and fixed with methanol/acetic acid (3:1). G band metaphase chromosomes were performed according to standard methods. The remaining cells from these cell cultures were stored at -20°C until FISH was performed.

**FISH.** Cells from the methanol/acetic acid stored cell cultures were dropped onto methanol cleaned glass slides that were allowed to age for at least 7 days. The slides were incubated with RNase (RNase A; Sigma, St Louis, MO), 100 μg/mL in 2X (standard saline citrate (SSC): 20 × SSC + NaCl 175 g, Na₂ citrate 88 g dissolved in 1 l distilled water, pH 7.4) at 37°C for 1 hour and subsequently dehydrated (ethanol series of 70%, 90%, and 99%). Ten microliters of a hybridization mixture was layered on each slide, covered with a 10 × 10 mm coverslip, and sealed with rubber solution. The hybridization mixture contained a biotinylated chromosome 12 specific α satellite DNA probe 53PCR12α, 1 ng/μL (gift of Dr J. Fantes, Western General Hospital, Edinburgh, UK), formamide 50%, dextran sulphate 10% (wt/vol), Triton X 1% (BDH) and salmon sperm DNA, 0.5 ng/μL (Sigma) in 2X SSC. The slides were denatured at 70°C for 10 minutes. After overnight hybridization at 45°C the slides were washed in 50% formamide in 2X SSC (4 × 3 minutes) at 50°C and in 2X SSC (4 × 3 minutes, 50°C) and were incubated in 5% nonfat dry milk in 4X SSC for 5 minutes. The hybridized probe was detected with a FITC conjugated avidin (Vector) and the fluorescence intensity was reinforced with a sandwich amplification and avidin-FITC. The cells were counterstained with propidium iodide (2 μg/mL) (Sigma).

The slides were evaluated under a Zeiss Axioscop fluorescence microscope. At least 200 nuclei were analyzed in each case. Cells from three healthy donors with a normal karyotype were used as controls.

**Statistical methods.** Fisher exact and χ² tests were applied to investigate the correlations between several clinical and laboratory data and the presence of trisomy 12.

**RESULTS**

**Immunophenotyping.** Immunophenotyping was performed on samples from all patients. Cases were considered positive with a marker when ≥20% of the cells were reactive with CD5, CD22, CD23, CD37, and FMC7, and when ≥30% of cells expressed SmIg. As shown in Table 1, cells from 98% of the CLL cases were CD5 positive, 94% CD23 positive, 57% membrane CD22 positive, and 29% FMC7 positive. The latter two markers were, as a rule, weakly expressed. The two markers characteristic of B-cell CLL, CD5 and CD23, were simultaneously expressed in 161 of the 171 cases (94%) tested. In all but three cases the expression of SmIg was weak. The k/λ ratio was 1.7.

**FISH.** In each control case 500 cells were analyzed. Zero, 1, 2, 3, or 4 hybridization signals were seen in an average of 0.1%, 3.8%, 95.7%, 0.4%, and 0% of the cells, respectively. The standard deviation for cells with three hybridization signals was 0.2. Theoretically, we could set our threshold value for trisomy 12 at ≥2 (mean ±3 × SD). There is evidence, however, that nonleukemic (control) samples differ from leukemic (CLL) samples in hybridization properties.26

Of the 205 cases with CLL sent to our laboratory, FISH was successful in 183; due to cell loss and degeneration of DNA, 22 stored specimens were inadequate for analysis. Analysis of the distribution of cells with three spots in our CLL cases showed 28 with 0.5% to 1%, three cases with 1.5% to 2%, and one case with 3% of cells with three hybridization signals. There were no cases with a percentage of three spots between 3.5% and 5%. Therefore, we have arbitrarily set the cut off point at >3 to avoid overestimating the incidence of trisomy 12. Chromosome analysis showed trisomy 12 in a case in which FISH detected 6.5% of trisomic cells (Table 2, no. 21), and did not show any trisomy 12 in the cases with a percentage lower than the threshold value. These karyotypic results justify our choice of the threshold value of >3, which also agrees with the literature.27,28

Trisomy 12 was demonstrated by FISH in 21 cases (11.5%). The median number of trisomic cells was in these cases 38.5% (range 5.5% to 76%) (Table 2).

**Cytogenetic studies.** Cytogenetic analysis was available in 128 cases; these included 18 cases in which trisomy 12 was detected by FISH. Cytogenetic analysis in these 18 cases showed trisomy 12 in 12, although in two of these only 1 metaphase out of 10 to 14 cells examined showed an extra chromosome 12; one case had a clonal abnormality other than trisomy 12, one had a normal karyotype, and four showed no divisions (Table 2). Thus, cytogenetic analysis could not detect trisomy 12 in 6 of 18 cases (33%) with this abnormality. Trisomy 12 was not detected by conventional cytogenetics in any case that was not found by FISH.

**Morphology.** Morphologic examination of May Giemsa Grünwald stained peripheral blood films in 180 cases showed that cells from 158 had a typical CLL morphology with small lymphocytes with clumped nuclear chromatin, regular outline, and no visible nucleolus. Twenty two cases were classified as atypical and included: 14 cases of CLL with more than 10% prolymphocytes (CLL/PL) and 8 cases

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* + indicates the incidence at which a marker is positive in ≥20% of cells (>30% of Ig lsotypes).
† Total number of patients in which marker studies were performed.
‡ Weak expression in all but three cases.
§ No expression of heavy chains.

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characterized by an admixture of lymphocytes of different size\(^4\) (Table 3). In some of these mixed cell CLL cases a small percentage of the cells showed lymphoplasmacytoid features.

**Correlations.** Clinical data were available in all but two cases (Table 4). The relationship between the various clinical and laboratory features and the presence of trisomy 12 is examined in Tables 1 through 4. Cases with trisomy 12 tended to have more frequently a positive phenotype, but this difference was not statistically significant. The percentage of trisomic cells found by karyotype analysis was not correlated with the number of cells with three spots detected by the FISH technique (Table 2). There was no correlation between the presence of trisomy 12 and clinical parameters such as age, sex, stage, lymphadenopathy, or splenomegaly nor with the peripheral blood counts (Table 4).

In contrast, morphologic differences were found between the cases with and without trisomy 12. An atypical morphology, in particular CLL/PL features, was more frequent in cases with trisomy 12. This difference was statistically significant (\(P < .001\)) (Table 3). However, the percentage of atypical cells was not correlated with the number of cells with three spots (Table 2).

**DISCUSSION**

This is the largest CLL series in which the frequency of trisomy 12 was estimated by FISH and which correlates the

| Table 2. Laboratory Data from 21 Patients With Trisomy 12 Detected by FISH |
|------------------|------------------|------------------|------------------|
| Patient No. | Karyotype | Number of Metaphases | FISH | Ig | Morphology | Atypical Cells (%) |
| No. reviewed (180) | 21 | 159 |
| Typical CLL (158) | 11 (52) | 147 (92) |
| Atypical CLL (22)* | 10 (48)\* | 12 (7.6)\* |

* Fourteen cases with CLL/PL (7 with trisomy 12) and 8 with CLL of mixed cell type (3 with trisomy 12).

\* \(P < .001\).

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**Table 3. Correlation of Trisomy 12 and Morphology**

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<th>Morphology</th>
<th>Trisomy 12 (%)</th>
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<td>No. reviewed (180)</td>
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\* \(P < .001\).
presence of this abnormality with clinical, morphologic, and immunophenotypic features as well as conventional cytogenticics. The lack of correlation between the percentage of trisomic cells discovered by karyotyping and that found by FISH may reflect the heterogeneity of the trisomic clone in respect of responsiveness to the mitogen used.

Our results, like those of others,18-20 have shown that FISH is a valuable supplement of conventional cytogenticics for the detection of numerical chromosomal abnormalities such as trisomy 12. All of the cases with trisomy 12 demonstrated by chromosome analysis were also detected by FISH. Moreover, FISH allowed to demonstrate the presence of trisomy 12 in a relatively high proportion of cases with normal or non-analyzable metaphases.

The 11.5% incidence of trisomy 12 in CLL found here is lower than in most other published data in which the incidence of trisomy 12 varied between 20% and 32.5%16,20 by FISH, or in early reports by conventional cytogenticics. Recently, however, Raghoeber et al26 found in their series of 67 cases an incidence identical to ours. These differences might reflect different methods of patient selection, inclusion criteria, or, less likely, geographic differences. In our series, only bona fide CLL cases as shown by membrane markers and morphology were included. Of seven other cases, which were originally submitted with a diagnosis of CLL, six had trisomy 12 by FISH but were excluded after review of the morphology and immunologic markers (Table 5). Most of these patients showed a phenotype that was different from that of CLL with FMC7 expression in the majority and negative CD5 and/or CD23 and strong SmIg. All these cases were reclassified as non-Hodkkin's lymphoma (NHL) in leukemic phase after morphologic analysis (Table 5). If we had included them in our analysis, the incidence of trisomy 12 would have been 14.2%.

Because most of the cytogentic studies in the IWCCLL compilation17 were started in the early '80s, a precise classification of the B lymphoid diseases was not made, and possibly cases with a diagnosis other than CLL were included.12,14,30 Juliusson et al13 studied 17 patients with trisomy 12 by conventional techniques: 6 were classified morphologically as typical CLL; 10 patients had immunocytoma, which is the designation of lymphoplasmacytic lymphoma in the Kiel classification;21 and one was unclassifiable. Likewise, Han et al12 found a high incidence of trisomy 12 in patients with Waldenström's and CLL-associated macroglobulinemia, which are also classified as immunocytoma.31 Of the studies using FISH, which reported a higher incidence of trisomy 12,18-20 some have included cases with PLL,19,20 while one19 included a high proportion of morphologically atypical CLL cases (morphology reported in Bird et al4). Furthermore, one of the four cases with trisomy 12, reported by Cuneo et al,20 had PLL and two others showed atypical morphology (CLL/PL). However, these data are in concordance with our finding of an association between trisomy 12 and CLL with atypical morphologic features as well as with cases of low grade NHL in leukemic phase that we excluded as non-CLL on immunologic and morphologic grounds. It would appear, therefore, that patients with trisomy 12 tend to present with an increased proportion of prolymphocytes and others are associated with NHL with lymphoplasmacytoid differentiation. It appears likely that trisomy 12 is associated within CLL with morphologic changes associated with progressive disease. Studies with the monoclonal antibody Ki-67 have demonstrated a high percentage of cells in the cell cycle in the CLL/PL group.33 Further detailed morphologic analysis of trisomy 12 positive CLL cases should be performed to confirm our data.

Despite the above findings, we have not found a correlation between trisomy 12 with stage and other clinical and laboratory parameters. We are now examining the correlation of trisomy 12 with the response to therapy and/or survival within the context of the current Medical Research Council CLL 3 trial.

ACKNOWLEDGMENT

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TRISOMY 12 IN CLL


Trisomy 12 in chronic lymphocytic leukemia detected by fluorescence in situ hybridization: analysis by stage, immunophenotype, and morphology

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