13q14 Deletions Are Not Primary Events in B-Cell Chronic Lymphocytic Leukemia: A Study of 100 Patients Using Fluorescence in Situ Hybridization

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ABSTRACT

Fluorescence in situ hybridization with a chromosome 12-specific a-centromeric probe and a 13q14 yeast artificial chromosome probe was performed on interphase cells from 100 patients with B-cell chronic lymphocytic leukemia. Thirty-one patients exhibited a 13q14 deletion. No correlation was found between 13q14 deletions and clinical stage, sex, or morphology. Sixteen patients had trisomy 12, including 6 (of 12) with an atypical morphology. Trisomy 12 and 13q14 abnormalities were detected concomitantly in three patients only. The analysis of patients with deletions clearly showed that in five cases a significant number of cells retained two signals with the yeast artificial chromosome probe, indicating a genetic heterogeneity among the leukemic population. Our data confirm that the 13q14 deletion is a frequent event, indicate that the concomitant occurrence of 13q14 deletion and trisomy 12 is rare but possible, and show that both abnormalities are secondary events in B-cell chronic lymphocytic leukemia.

INTRODUCTION

B-CLL³ is the most frequent leukemia in adults from Western countries. Chromosomal abnormalities are found in approximately 50% of the cases (1). The most common chromosomal abnormalities are trisomy 12 (2–4) and structural aberrations affecting 13q14 (4–7). In the other 50%, only normal metaphases are found, corresponding to the division of nonleukemic T cells (8, 9).

Cytogenetic studies revealed trisomy 12 in 18–30% of the cases with evaluable metaphases (4, 10), whereas FISH studies demonstrated it in 11–35% of the cases (11, 12). These differences may reflect different inclusion criteria or patient selection. Since trisomy 12 is a secondary event (13), the selection of patients with a more advanced disease stage would be expected to increase the frequency of this abnormality. Furthermore, this abnormality seems to be more frequent in patients with an atypical morphology (11) and could be associated with a poorer prognosis (11, 12).

Large cytogenetic studies have revealed structural abnormalities of chromosome 13 to be at least as frequent as trisomy 12, with a frequency of 17–29% (5–7). Two-thirds are deletions and one-third translocations, both involving band q14 (1). Since the Rb gene is localized in this region, it was an obvious candidate gene. However, several studies have shown that, if involved, one allele is usually retained (14–16). Moreover, Rb protein is detected in the large majority of B-CLL analyzed to date (17–19). Thus, these 13q14 rearrangements probably affect a more telomeric gene. Several authors showed that a locus (D13S25), telomeric to Rb, was deleted in at least 40% of the patients (20–22). Using different anonymous markers, we and others have defined more precisely the deleted region containing a new putative tumor suppressor gene (23). To date, no published study has concomitantly analyzed the presence of trisomy 12 and deletion of the D13S25 region using interphase FISH at the single-cell level. The sole publication using this approach compared trisomy 12 and deletion of the Rb gene (25). Another study analyzing trisomy 12 and deletion of the Rb gene has been reported as an abstract (26). We have isolated a YAC probe containing the D13S25 and D13S19 loci and used it as a fluorescent probe on interphase nuclei. We show that 31% of the cases have a monoallelic loss of the 13q14 region, 16% display trisomy 12, and that these two events may occur concomitantly, although rarely. Moreover, we show that 13q14 deletions are not primary clonal events in B-CLL patients.

MATERIALS AND METHODS

Patients. From March 1989 to December 1992, we have selected frozen peripheral blood specimens from 100 consecutively diagnosed and previously untreated patients with B-CLL from the Department of Clinical Hematology. The diagnosis of B-CLL was based on clinical features, cell morphology, and immunological markers (concurrent expression of CD5 and CD23). Staging was performed according to Binet et al. (27). Seventy-eight patients had Binet stage A, 16 had Binet stage B, and 6 had Binet stage C. According to the French-American-British classification (28), 79 patients had a common B-CLL and 21 patients had a mixed cell type CLL [including 9 patients with >10% prolymphocytes (PL)]. All of these patients
were analyzed for expression of CD5, CD19, CD22, CD23, and surface immunoglobulins as described previously (29). For none of them was FMC7 expression analysis performed. As proposed by Matutes et al. (30), patients were classified as immunologically typical with a total score of 3 or 4 (1 point for the expression of CD5, 1 for the expression of CD23, 1 for a negative or weak expression of surface immunoglobulin, and 1 for a weak expression of CD22). Patients with a score <3 were classified as immunologically atypical. Ninety-two patients were in the typical group and 8 in the atypical one. Among these eight immunologically atypical patients, four were morphologically atypical, two were classified as CLL/PL, and two had a typical morphology. Conventional cytogenetics was not performed in these patients.

**FISH.** Peripheral blood mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque and frozen in liquid nitrogen. After thawing, cells were fixed with methanol-acetic acid (3:1) and dropped onto ethanol-cleaned glass slides. Slides used before 2 weeks were incubated in 2X SSC for 30 min at 37°C before denaturation. Older slides were directly denatured. The YAC probe 933E9 was obtained from the Centre d'Etude du Polymorphisme Humain and ALU-PCR-amplified before labeling. This probe is a mega-YAC (approximately 1.7 Mb) containing both D13S25 and D13S319 loci. Another YAC from the Centre d'Etude du Polymorphisme Humain (854E2), mapping to 16p13, was used as a control. The 933E9 YAC clone was labeled with biotin and 854E2 with digoxigenin using nick translation. Four μl of each amplified product were ethanol precipitated with 10 μg of unlabeled human Cot-1 DNA, denatured at 72°C, preannealed at 37°C for 30 to 120 min, and applied onto denatured dehydrated slides. After overnight hybridization at 37°C, slides were washed three times in 50% formamide, 2X SSC at 45°C for 10 min, then in 0.1X SSC at 55°C for 10 min and PBD buffer (Appligene-Oncor) at room temperature for 5 min. The 12-centromere digoxigenin-labeled probe (Appligene-Oncor) was denatured and applied directly onto denatured slides. After overnight hybridization at 37°C, these slides were washed for 5 min in 2X SSC at 72°C and for 5 min in PBD buffer at room temperature. The hybridized probes were detected with Cy3-streptavidin (Sigma) and a FITC-conjugated antidigoxigenin antibody (Boehringer Mannheim). To prevent false-positive results arising from inadequate hybridization or chromatin loss, analysis was performed on slides with a high hybridization efficiency, indicated by two 854E2 signals in >90% of the nuclei. In the event of inadequate hybridization, cells were pretreated with pepsin (0.01 mg/ml).

**RESULTS**

The 933E9 YAC was first tested on metaphase spreads from normal lymphocyte preparations and showed bright hybridization signals on each chromosome 13 chromatide on the 13q14 band. No signal was detected on other chromosomes, confirming that this YAC was not chimeric. On six control samples (patients without any B-cell malignancy), we detected two bright signals on >95% ± 2.6 nuclei. We classified a sample as deleted if only one signal was seen in >13% of the cells (mean percentage plus 3 SDs). We found a monoallelic 13q14 deletion in 31 of the 100 patients. The percentage of cells per patient exhibiting one 933E9 signal ranged from 31 to 90%, with a mean of 71%, suggesting the presence of two clones, one deleted and one nondeleted. To prove that this was not due to an inadequate hybridization of the probe, cells were cohybridized with a differentially labeled 16p YAC probe that gives similar fluorescent signals on controls. Among the cells exhibiting one 933E9 signal only, we found two 854E2 signals in >90% of them, confirming the true monoallelic deletion of this region. We then analyzed these results according to other patient characteristics (clinical stage at diagnosis and morphology). No correlation could be drawn between 13q14 deletions and stage or morphology (Table 1).

Hybridization on control slides with the 12-centromere probe showed three signals in 1.2% ± 0.75 cells. We classified a patient as trisomic for chromosome 12 if three spots were detected in >5% cells (mean plus 3 SDs). Sixteen patients (16%) displayed a trisomy 12 in 23–76% of the cells (mean, 56%). Nine of these trisomic patients had a morphologically typical B-CLL, six were classified as morphologically atypical (P < 0.02), and one had a CLL/PL. Three patients simultaneously presented a trisomy 12 and a 13q14 rearrangement (two deletions and one probable 13q14 translocation). Two of these patients had a typical morphology and one an atypical one. Cohybridization with 933E9 and a 12-centromere probe revealed the presence of different clones in these three patients (Fig. 1). We found cells that were disomic for each probe, other cells were monosomic for 933E9 and disomic for the 12-centromere, other cells were monosomic for 933E9 and trisomic for the 12-centromere, and cells that were disomic for 933E9 and trisomic for the 12-centromere. These results were confirmed in at least one other independent experiment for each of these three patients.

**DISCUSSION**

We have performed a combined FISH study on the most frequent cytogenetic abnormalities found in 100 B-CLL patients, i.e., 13q14 rearrangements and trisomy 12, with the purpose to analyze the respective frequency of each one and the occurrence or not of these abnormalities in the same clone.

Our data indicate that a chromosomal deletion of the 13q14 region occurs in 31 of these 100 patients. A very recent study using FISH revealed a deletion of the D13S25 region in 50% of the patients (16). This higher percentage may be explained by the size of the probe used (two overlapping cosmids covering approximately 50 kb). Another interesting point is the absence of detected biallelic deletion. This is in contrast with other

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**Table 1** Correlations between 13q14 status, morphology, and clinical presentation

<table>
<thead>
<tr>
<th></th>
<th>Patients with a 13q14 deletion (n = 31)</th>
<th>Patients lacking any 13q14 deletion (n = 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical morphology</td>
<td>27 (87%)</td>
<td>52 (75%)</td>
</tr>
<tr>
<td>Atypical morphology</td>
<td>3 (10%)</td>
<td>9 (13%)</td>
</tr>
<tr>
<td>CLL/PL</td>
<td>1 (3%)</td>
<td>8 (12%)</td>
</tr>
<tr>
<td>Binet stage A</td>
<td>26 (84%)</td>
<td>52 (75%)</td>
</tr>
<tr>
<td>Binet stage B</td>
<td>4 (13%)</td>
<td>12 (17%)</td>
</tr>
<tr>
<td>Binet stage C</td>
<td>1 (3%)</td>
<td>5 (7%)</td>
</tr>
</tbody>
</table>

*No statistical difference.
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Fig. 1 Analysis of chromosome 12-centromere and 13q14 region in patient 43 using FISH. Red signals represent 12 α-satellite probes and green signals represent the 13q14 probe. A, a cell with two signals for each probe. B, a cell with three red signals (trisomy 12) and one green signal (hemizygous 13q14 deletion). C, a cell with two red signals and one green signal (hemizygous 13q14 deletion). D, a cell with three red signals (trisomy 12) and two green signals.

studies which described homozygous 13q14 deletions in 10–50% of the patients. We may have missed some biallelic deletions because of the large size of the YAC probe. Analysis of typical tumor suppressor genes (i.e., Rb) usually shows large deletions affecting one allele and point mutations or smaller deletions on the second allele (31, 32). Deletions of a few kb would not be detected with our probe. This absence of homozygous deletion could also reflect differences in patient selection or differences in genetic expression among our population. In a previous molecular study (23), we reported only one patient (of 25) with a D13S25 homozygous deletion. Therefore, we cannot exclude this possibility.

No correlation could be drawn between 13q14 deletion and morphology or clinical presentation (Table 1). Analysis of patients with deletions revealed the presence of two clonal cell populations: one deleted and one disomic. Several hypotheses can be raised. The first one is the presence of contaminating nonclonal cells (i.e., T cells or monocytes). This hypothesis is probably the good one for the majority of patients since we detected two signals in only 10–20% of the analyzed cells, in agreement with the percentage of normal contaminating cells. But in a few instances (five patients) with a monoallelic deletion in only 31–64% cells, this is not likely since immunological analysis of these cases revealed >85% CD5-positive cells and <20% CD2-positive cells (Table 2). The second hypothesis is a heterogeneity in the genetic abnormalities of the leukemic cell population. In this view, the 13q14 deletion appears to be a secondary event. The latter hypothesis is corroborated by another finding. In three patients, we simultaneously detected trisomy 12 and a 13q14 rearrangement (a deletion in two cases and a probable translocation involving 13q14 in the third one). In each case, we found cells without any 13q14 deletion and disomic for the 12-centromere probe, other cells with trisomy 12 and 13q14 deletion, other cells with disomy 12 and 13q14 deletion, and cells with three chromosome 12 signals and two 933E9 signals. The latter cells clearly show that the 13q14 rearrangements are not the primary cytogenetic abnormality in B-CLL. The same conclusion has been recently reported using a Rb-specific probe (26).

Trisomy 12 was detected in 16% of the patients. This

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Table 2 Immunological characteristics of the five patients who clearly displayed a 13q14 deletion in a subclone only

<table>
<thead>
<tr>
<th>Patients</th>
<th>% of CD19-positive cells</th>
<th>% of CD5-positive cells</th>
<th>% of CD2-positive cells</th>
<th>% of cells with a 13q14 deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88</td>
<td>86</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>85</td>
<td>90</td>
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<td>4</td>
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<td>79</td>
<td>23</td>
<td>38</td>
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<tr>
<td>5</td>
<td>78</td>
<td>80</td>
<td>19</td>
<td>64</td>
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</table>
percentage is in agreement with most published series (11, 12). Furthermore, we confirm the correlation between trisomy 12 and an atypical morphology (11). Six of the 12 atypical cases (50%) were trisomic for the chromosome 12-centromere probe, whereas only 9 of 76 common cases (12%) had a trisomy 12 ($P < 0.02$). Trisomy 12 and 13q14 abnormalities seem to occur very rarely together but may exist. Only three patients had both abnormalities. These three patients do not differ from the other ones. We cannot tell which abnormality occurs first and could be the potential oncogenic event. In each case, we found cells monosomic for 933E9 and disomic for the 12-centromere probe and other cells trisomic for the 12-centromere probe and di- somic for 933E9. We may have missed other patients with both abnormalities because of the large size of the YAC probe, but these findings are in disagreement with a previous study claiming that 13q14 deletions were a clonal event, possibly primitive (33). In that report, the authors used a Southern blot-based approach, and even if they studied a highly purified cell population, they possibly missed cases with a double population (one with a D13S25 deletion and one without any deletion) because of technical limitations. The FISH technique allows a cell-by-cell analysis and can clearly demonstrate the presence of cells with trisomy 12 and two signals for 933E9. The only possible univocal explanation would be to suppose the presence in the same patient of cells with a large 13q14 deletion (detected with our YAC probe) and of another cell population with a smaller deletion not detectable with our probe. We cannot eliminate this hypothesis.

We analyzed the possible impact of each genetic abnormality on clinical presentation (i.e., Binet stage). Our study confirms previous analyses regarding the absence of any correlation between a 13q14 rearrangement and a Binet stage. The same conclusion may be drawn for patients with a trisomy 12. This study is in disagreement with a previous one which found a strong correlation between 13q14 abnormalities and a typical morphology (22). Here, we show that 13q14 deletions are frequently found in typical cases, but may also be present in morphologically atypical cases and in CLL/PL.

This FISH study shows that trisomy 12 and 13q14 abnormalities are rarely concomitant genetic abnormalities (but not exclusive) and that both are secondary events in the course of B-CLL. We confirm the high frequency of 13q14 deletions (31%) in B-CLL patients. This abnormality is not correlated with any morphological, immunological, or clinical feature. We also confirm the association between trisomy 12 and an atypical morphology. The next step will be to more precisely define the minimal deletion region (or to clone the involved gene) to better understand the significance of this 13q14 abnormality.

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