Molecular Cytogenetic Analysis in Splenic Lymphoma with Villous Lymphocytes: Frequent Allelic Imbalance of the RB1 Gene but not the D13S25 Locus on Chromosome 13q14

José A. García-Marco, Amara Nouel, Blanca Navarro, Estella Matutes, David Oscier, Cathy M. Price, and Daniel Catovsky

ABSTRACT

Structural abnormalities of chromosome 13q are one of the most frequent genetic aberrations in human tumors. 13q rearrangements are, however, infrequent in splenic lymphoma with villous lymphocytes (SLVL) by karyotype analysis. We have investigated the incidence of 13q14 deletions in a series of 74 SLVL cases by interphase fluorescence in situ hybridization using unique sequence probes for the RB1 and the D13S25 loci, which are frequently deleted in chronic lymphocytic leukemia. Chromosome 12 was also evaluated by fluorescence in situ hybridization using a pericentromeric DNA probe. 13q14 deletion was detected in 37 of 74 (50%) tumors. Thirty-five cases (47%) exhibited monoailelic loss of RB1, and 9 (12%) showed hemizygous D13S25 deletion. Seven cases displayed coexistence of RB1 and D13S25 deletion. Trisomy 12 was detected in 2 of 74 (3%) tumors. G-banding analysis in 40 tumors showed no interstitial deletion of 13q14 in any case. In contrast with the molecular findings observed in chronic lymphocytic leukemia, our results indicate that trisomy 12 is an uncommon chromosomal aberration in SLVLs, and microdeletion of 13q14 at the RB1 locus but not D13S25 is a frequent and specific genetic event in this disease, suggesting that allelic loss of the RB1 gene may play a role in the pathogenesis of SLVL.

INTRODUCTION

Chromosome banding and molecular studies have shown that structural rearrangements and interstitial deletions of the long arm of chromosome 13, and particularly those involving 13q14, are recurrent chromosomal aberrations in solid tumors and hematological malignancies (1—6). The high incidence of allelic loss suggests the presence of one or several tumor suppressor genes involved in the growth control of many human tumors. The RB1 susceptibility gene (RB1), located on chromosome band 13q14.2, is a tumor suppressor gene directly involved in the control of cell proliferation (7—9). Deletion and/or inactivation of the RB1 gene and its protein product have been postulated to be important in the development or progression of tumors (10). Recently, several studies have reported a variable incidence of RB1 deletion in acute and chronic leukemias, but the significance of this genetic abnormality in leukemogenesis remains to be elucidated, because inactivation of the RB1 gene is not a common feature in these tumors (11—16).

SLVL is a low-grade B-cell lymphoma with distinct clinicopathological characteristics (17—19). Molecular cytogenetic studies in SLVL have allowed the identification of certain chromosomal aberrations associated with this disease, such as deletions or translocations involving 7q, 2p11, iso17q, and t(1;14)(q13q32), but structural abnormalities of 13q have been reported rarely (20—22). Despite the frequency of genetic changes, the nature and prognostic significance of the chromosomal abnormalities in SLVL have not been established. Therefore, the detection of consistent genetic rearrangements would be of importance to understand the pathogenesis of this disease. Recent molecular cytogenetic studies in CLL, another chronic B-cell neoplasm, have demonstrated that trisomy 12 and interstitial rearrangements of chromosome 13q are the most frequent numerical and structural abnormalities in this disease, which have an impact on survival. Analysis of allelic imbalance at chromosome band 13q14 has shown frequent monoailelic RB1 loss and in a higher number of cases, hemi- and homozygous deletion of the D13S25 locus, which lies ~1.6 cm telomeric to RB1, suggesting that this is a critical region for a novel putative tumor suppressor gene involved in CLL and related chronic lymphoid disorders (23—29). We have investigated the frequency of RB1 and D13S25 (13q14.2—q14.3) deletions as assessed by interphase FISH in a series of 74 SLVL cases and its correlation with cytogenetic findings in 40 tumors. In addition, we have also analyzed the prevalence of trisomy 12 in these tumors to characterize the cytogenetic correspondence with other B-cell disorders.

MATERIALS AND METHODS

Peripheral blood and/or spleen samples from 74 previously untreated patients submitted to our institution were investigated. Thirty-five patients were male and 39 were female, with a median age of 68 years. All of the cases had peripheral blood involvement as assessed by morphology and immunological profile. The diagnosis of SLVL was based on peripheral blood and bone marrow examination, immunological markers, and spleen histology (30). Peripheral blood samples from 20 healthy donors were used as normal controls.

FISH. Mononuclear cells for interphase analysis were directly treated with hypotonic solution for 20—40 min at 37°C, resuspended in fixative (methanol: glacial acetic acid, 3:1), and stored at —20°C until use.

Probes. Dual-color FISH was performed using directly labeled fluorescent probes in a pairwise manner. We used a probe mixture including an satellite DNA probe specific for chromosome 12 (CEP12 labeled with spectrwm green; Vysis, Downers Grove, IL) and a single-copy probe for the RB1 and/or D13S25 loci at 13q14 band (LSI-RB1 labeled with spectrum orange, LSI-D13S25 spectrum orange; Vysis). Furthermore, to evaluate the clonal distribution of trisomy 12 and 13q14 deletion among the neoplastic population, triple-color FISH was carried out in those cases bearing both abnormalities by using a pericentromeric chromosome 12 PCR33 probe, directly labeled RB1 probe, and a cosmId probe specific for the D13S25 locus. PCR33 and D13S25 probes were labeled with biotin 11-dUTP or digoxigenin 11-dUTP, respectively, using nick translation according to the manufacturer’s protocol (Boehringer Mannheim, Bell Lane, UK). D13S25-labeled probe (250 ng/hybridization spot) was mixed with COT! human DNA (100-fold excess DNA/probe) and 1 mg of herring sperm DNA. The mixture was precipitated in ethanol and resuspended in 5 μL of hybridization mixture (50% deionized formamide, 2× SSC, and 10% dextran sulfate, pH 7). In cases with both RB1 and D13S25...
deletion, dual-color FISH using biotinylated cosmid D13S25 and LSI-RBI probes was also performed.

**FISH Method.** Hybridization combining directly labeled chromosome 12 and 13q14 probes was done in pairs (chromosome 12/RBI or chromosome 12/D13S25) in all 74 cases as recommended by probe manufacturers following an overnight hybridization procedure (Vysis).

Cohybridization using biotinylated PCR53, LSI-RBI, and digoxigenin-labeled D13S25 probes was performed by denaturing the probes for 10 min at 70°C, and the latter was allowed to preanneal at 37°C for 30 min. After overnight hybridization, PCR53 was detected by incubating slides in AMCA-conjugated avidin (1:40, 4× SSC/low fat milk, pH 8.5; Vector, Peterborough, United Kingdom), and where necessary, the signal was amplified with additional layers of biotinylated anti-avidin (1:50, Vector) and avidin-AMCA. D13S25 digoxigenin-labeled probe was detected by FITC-conjugated sheep antidigoxigenin (1:10; Vector), and where necessary, a second layer of FITC-conjugated rabbit anti-sheep antibodies (1:75; Vector) was used. Nuclei were mounted in fluorescence antifade or counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. Chromosome 12, LSI-RBI, and D13S25 signals were scored using a Zeiss Axioscop microscope (Oberkochen, Germany) equipped with epi-fluorescence and a triple band pass filter. Pictures were captured by analysis of digital images obtained with a high resolution CCD camera (Photometrics, Tucson, AZ) and imaging software (Digital Scientific, Cambridge, UK). Two hundred nuclei were scored from each sample. For a reliable estimation of RBI and D13S25 probe copy number, we only scored those cells that showed hybridization signals for chromosome 12 and/or a control cosmid probe for chromosome 21.

**Chromosome Analysis.** Mononuclear cells from peripheral blood and/or spleen were cultured at 37°C in the presence of tetradecanoylphorbol-13-acetate (0.05 μL/ml) for 3—5 days, and standard cytogenetic preparations were made, G-banded, and karyotyped according to the International System for Cytogenetic Nomenclature (31). If available, 10—20 metaphases were evaluated for each case.

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**RESULTS**

**Controls.** The probes showed practically 100% hybridization efficiency to both homologues of chromosomes 12 and 13 in metaphase spreads. Interphase FISH on 20 control specimens showed a mean percentage of cells with one hybridization signal for RBI in 1.87 ± 0.25 and D13S25 in 2.21 ± 0.68 and three signals in 1.02 ± 0.53 (RBI) and 1.17 ± 0.36 (D13S25). No hybridization signal was found in 0.8 ± 0.4 and 1.04 ± 0.26 for the RBI and D13S25 probes, respectively. Controls for chromosome 12 exhibited one signal in 1.55 ± 0.68 and three signals in 0.36 ± 0.26 of the cells. Monoallelic deletion of RBI and D13S25 was defined when >4% (mean ± 3 SD) of the cells had one hybridization signal, and trisomy when >3% of the cells had three hybridization signals. Based on our previous analysis (32), the cutoff level for definition of homozygous deletion was arbitrarily set up when >5% of the cells showed no hybridization signal for the target probe and two signals for the control probe. Monosomy 12 and trisomy 12 in patient samples were defined when >4% and >2% (mean ± 3 SD) had one and three hybridization signals, respectively.

**Detection of 13q14 Deletion.** Interphase FISH revealed 13q14 allelic loss in 37 (50%) of 74 SLVL tumors. Monoallelic RBI loss was detected in 35 of 74 (47%) cases (one case had a subclone with homozygous RBI deletion), whereas only 9 (12%) of 74 cases exhibited monoallelic loss at the D13S25 locus (P = 0.04 by χ²; Fig. 1). The mean percentage of cells with one hybridization signal was 42.7 ± 14.2 (range, 20—93%) for RBI and 37.27 ± 19.87 (range, 19—85%) for D13S25. Seven (19%) of 37 cases with 13q14 allelic loss showed coexistence of monoallelic RBI and D13S25 deletion.
and four of them displayed a similar percentage of deleted cells for both probes evaluated on different hybridization experiments (Fig. 1, SLVL 3, 21, 56, 62). Cohybridization using both 13q14 probes in the remaining three cases (SLVL 5, 12, and 74) showed a clone with RBl and D13S25 deletion, a subclone with only monoallelic RBl loss, and another subclone with diploid RBl and D13S25 (Table 1). In 2 (5%) of 37 cases with 13q14 loss, the deletion was distal to the RBl locus. Monosomy 12 was detected in 2 of 74 (3%) cases and trisomy 12 in the other 2 (3%) cases. One case with trisomy 12 also exhibited monoallelic RBl and D13S25 deletions, and three different clones were observed as assessed by triple-color FISH (Fig. 2): one clone with trisomy 12 and diploid RBl/D13S25 (39% of the cells); another subclone with trisomy 12 and monoallelic loss of RBl/D13S25 (34%); and a minor subclone with two copies for chromosome 12 and RBl/D13S25 (27%). Because these features are more distinctive of CLL, the diagnosis of SLVL was confirmed by immunopathological review in these two cases.

Correlation between FISH and Cytogenetic Findings. Chromosomal abnormalities demonstrated by G-banding analysis and allelic loss at 13q14 as assessed by FISH were compared in 40 of 74 cases. Chromosome 13 abnormalities were detected in 3 (10%) cases, but interstitial deletion of 13q14 band was not observed in any case (Table 2). One case with a t(9;13(q32;q12) translocation had monoallelic RBl loss (45% of the cells) and diploid D13S25 on interphase cells, suggesting the presence of a 13q12 rearrangement and allelic loss of the RBl gene at the 13q14 locus or a larger segment of cytogenetic deletion including 13q14 band and ensuing genetic loss as a result of the translocation (SLVL28; Table 2). One case with monosomy 13 and a balanced translocation t(2;13)(q11—q12p11) exhibited hemizygous loss of RBl and D13S25 by FISH (SLVL12; Table 2). Cohybridization using both 13q14 probes showed two abnormal clones: one clone with one signal for both probes; and another clone with one signal for the RBl and two for the D13S25 (Table 1). This finding suggests that monoallelic RBl loss was the larger clone, and D13S25 deletion might be present as part of the clone showing monosomy 13. One case with trisomy 13 exhibited one signal for the RBl probe in 42% of the cells and three signals for the D13S25 probe in 24% of the interphase cells (SLVL 31; Table 2). Cohybridization with both probes showed one clone with monoallelic RBl loss and diploid D13S25, another subclone with three copies for D13S25 and diploid RBl, and a subclone with two copies for both probes (Table 1). This finding suggests the occurrence of two different genetic events on chromosome 13 in this case.

Eight tumors with a normal karyotype exhibited monoallelic loss at 13q14 (four had RBl loss and four cases RBl/D13S25 loss), and another tumor with a normal karyotype displayed a similar proportion of cells with three hybridization signals for RBl and D13S25 (Table 2). Cohybridization using both 13q14 probes demonstrated that cells with three copies for RBl and D13S25 were part of the same clone, suggesting the presence of a subclone with trisomy 13 that was not detected by G-banding. A case with a complex karyotype without evidence of 13q abnormalities underwent transformation into a diffuse large cell lymphoma, and interphase FISH performed on directly fixed mononuclear cells showed homozygous (40%) and hemizygous (53%) RBl deletion with two copies for D13S25 in 97% of the cells (SLVL35; Table 2). Thus, the allelic loss in this tumor was centromeric to the D13S25 locus and involved a small and karyotypically invisible interstitial deletion within a structurally normal chromosome 13.

DISCUSSION

SLVL is a B-cell disorder characterized by splenomegaly, circulating lymphocytes with villous cytoplasmic projections, and a benign clinical course, although in a minority of patients, transformation to a higher grade lymphoma may be seen (33–35). The differential diagnosis of this disease with hairy cell leukemia and its variant, atypical CLL, prolymphocytic leukemia, and mantle cell lymphoma is in some instances difficult to establish, and a combination of clinical features and immunopathological characteristics should be sought to distinguish these entities. Therefore, the recognition of genetic markers is

<table>
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<tr>
<th>Case</th>
<th>RBl/D13S25</th>
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<th>RBl/D13S25</th>
<th>RBl/D13S25</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 signal/1 signal</td>
<td>1 signal/2 signals</td>
<td>2 signals/2 signals</td>
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important and helpful in the differential diagnosis of these chronic B-cell neoplasms.

Chromosomal studies in SLVL exhibit a high frequency of single and complex clonal aberrations, but there is not a consistent recurrent abnormality in this disease. Evaluation of chromosomal loss is one of the most important approaches to identify tumor suppressor genes involved in the initiation or progression of human tumors. Previous studies have shown that translocations or deletions involving the long arm of chromosome 13 are not a frequent cytogenetic finding in SLVL (20, 36), and our review of the results in this series confirms that finding. We have observed chromosome 13 abnormalities in 10% of the analyzable cases. Of them, two cases showed chromosome 13 translocations, and there was no case displaying 13q interstitial deletion. In contrast, FISH on interphase cells detected submicroscopic interstitial 13q14 deletions in 50% of the SLVL cases. Thirty five (47%) cases had monoallelic RB1 loss, and none of them had corresponding karyotypic losses. In addition, 7 cases with RB1 and D13S25 deletion, which represents a region of specific allelic loss, also appeared normal on G-banding after thorough examination of these cases. Thus, it seems likely that the genetic changes leading to these losses are below the detection limit of conventional cytogenetic analysis. This discrepancy between conventional cytogenetics and FISH to evaluate genetic alterations has been described in other series (37–39). One explanation for the finding is that FISH is not reliant on dividing cells to assess clonal populations and, therefore, gives a better estimation of known chromosomal abnormalities or that the mitotic population studied by G-banding might be different from the overall population of nondividing cells evaluated by FISH. In the case of structural abnormalities such as balanced translocations, allelic loss would only be expected if the structural breakpoint involved the region encompassing the RB1/D13S25 loci. However, in this study, we outlined a case with a balanced 13q12 translocation and 13q14 loss by FISH. This anomaly might have occurred as a result of chromosomal instability, or more likely, it might represent a different and cumulative genetic event in the evolution of the disease (40).

The differential diagnosis of SLVL with other chronic lymphoid disorders, and particularly CLL, is sometimes difficult to establish on the basis of clinical, morphological, and immunopathological grounds. Trisomy 12 is a characteristic chromosomal abnormality in CLL and less frequently in follicular and diffuse non-Hodgkin’s lymphoma, as shown by interphase FISH (41). In contrast, previous chromosomal analysis in SLVL have reported trisomy 12 as an infrequent cytogenetic abnormality. To evaluate whether trisomy 12 might help as a cytogenetic marker in the differential diagnosis between SLVL and CLL, we studied the incidence of trisomy 12 in all 74 cases. Interphase FISH detected trisomy 12 in only 3% of the tumors, and peripheral blood morphology and immunopathological studies of the spleen demonstrated that the diagnosis was consistent with SLVL. These results confirm previous cytogenetic studies (20) and demonstrate that conversely to CLL, trisomy 12 is a rare genetic abnormality in SLVL.

To date, no data have been published on the evaluation of the molecular abnormalities affecting 13q14 in SLVL. However, this chromosomal band has extensively been characterized in solid tumors and hematological malignancies. In CLL, monoallelic loss of the RB1 gene has been reported in 30–35% of the cases (23, 26, 41), but the inactivation of the RB1 has not been fully addressed. Recent studies have shown frequent homozygous deletion of a region telomeric to the RB1 and close to the D13S319 locus encompassing the D13S319 and D13S25 loci, suggesting this is a critical area for the localization of a new putative tumor suppressor gene (DBM) involved in the pathogenesis of chronic lymphoid disorders (14, 24). In contrast to the pattern of deletion observed on 13q14 in CLL where hemi/homozygous D13S25 loss is more frequent than RB1 deletion, here we report a higher incidence of RB1 loss (47%) compared with D13S25 deletion (12%). None of the nine tumors with D13S25 loss showed a clonal population with homozygous deletion. In addition, allelic loss of 13q14 in these 37 cases appears to be a secondary event, because either monoallelic RB1 or D13S25 deletion was observed in a variable proportion of clonal B cells (Fig. 1). In seven cases, the allelic loss was seen at both loci, although only four cases showed similar proportion of deleted cells, and the remaining three cases had a larger cell population with RB1 loss. This finding might suggest that these genetic losses comprise an area of a larger deletion in these cases, or that loss of DNA sequences at the D13S25 locus represents a bystander event or deletion of a neighboring gene still to be cloned.

Homozygous deletions of the RB1 gene leading to inactivation of its protein product have been reported in acute leukemias (11, 15, 42). In this study, we report molecular cytogenetic data on a case that underwent transformation into a diffuse large cell lymphoma with bone marrow and central nervous system involvement. FISH at the time of transformation showed allelic RB1 loss in >90% of the interphase cells with homozygous deletion in 40% of them, and cytogenetic studies demonstrated a complex karyotype without evidence of 13q abnormalities. Although it is tempting to hypothesize that RB1 might be responsible for this transformation, we cannot make that assumption, because there were no previous studies and there was insufficient material for further analysis. However, the presence of allelic loss in the majority of the clonal malignant cells suggests that the RB1 gene could have been involved in the molecular mechanism responsible for the transformation.

In summary, our data reveal that allelic loss at 13q14 in sporadic SLVL is a recurrent genetic abnormality that is below the detection limit of conventional cytogenetic analysis because there was no corresponding chromosomal structural abnormalities in any case. Nevertheless, allelic imbalance at 13q14 shows that there is a preferential loss of RB1 with respect to the D13S25 locus. Further studies are needed to evaluate whether RB1 is the true target of these deletions in SLVL. If that is the case, then based on Knudson’s model (43), one would expect inactivation of the second RB1 allele by intragenic deletion, mutation, or methylation.

### Table 2 Chromosome 13 abnormalities detected by karyotype analysis and/or interphase FISH in five SLVL cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Karyotype</th>
<th>% 1 signal (RB1)</th>
<th>% 3 signals (RB1)</th>
<th>% 1 signal (D13S25)</th>
<th>% 3 signals (D13S25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLVL12</td>
<td>45,XX,−13q(2;13q11−12;p11), del(21)(q11) [5], 46,XX [5]</td>
<td>73</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>SLVL28</td>
<td>46,XY,t(9;13q32q12), i(11;14q)(13q32) [6], 46,XY [4]</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SLVL31</td>
<td>46,XY,t(11;14q)(13q32) [12], 46,XY,−11,+1p, +13,t(11;14q)(13q32) [3]</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>SLVL34</td>
<td>46,XX, +10p</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>SLVL35a</td>
<td>45,XX,−3(2;2)(q27;q27), del(7q)(q21) [3], 46,XY [7]</td>
<td>53</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

* FISH for 13q 14 probes was performed in separate hybridization experiments.
* a This case showed homozygous RB1 deletion in 40% of the interphase cells.

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