Splenic Lymphoma With Villous Lymphocytes: Analysis of BCL-1 Rearrangements and Expression of the Cyclin D1 Gene

By Dalal Jadayel, Estella Matutes, Martin J.S. Dyer, Vasantha Brito-Babapulle, M. Tariq Khokhar, David Oscier, and Daniel Catovsky

The translocation t(11;14)(q13;q32) occurs in about 15% of patients with splenic lymphoma with villous lymphocytes (SLVL) or the closely related disorder lymphoplasmacytic lymphoma (LPL). To characterize the nature and frequency of rearrangements of the BCL-1 locus in SLVL/LPL and to document the effect of these genetic alterations on the expression of the cyclin D1 gene, we analyzed 22 cases of SLVL/LPL with defined cytogenetic abnormalities by both conventional electrophoresis (CE) and pulse-field gel electrophoresis (PFGE) and by Northern blotting. Four SLVL/LPL cases showed rearrangement of the BCL-1 locus, in two cases with t(11;14)(q13;q32), different breakpoints were identified; one mapped adjacent to the major translocation cluster (MTC) and the other within a 28-kb region telomeric of it. In a third case of SLVL with no cytogenetic abnormality of 11q13, a novel breakpoint approximately 100 kb telomeric of MTC was detected by PFGE. The fourth case, which had a normal karyotype, demonstrated rearrangement with a BCL-1 probe immediately telomeric of MTC. This case may have had a small deletion of 0.5 kb from within the BCL-1 locus. No rearrangement of the BCL-1 locus or within the cyclin D1 gene was detected by CE or PFGE in any of the remaining 18 SLVL/LPL samples. Northern blot analysis showed expression of a normal-sized cyclin D1 transcript in the two SLVL/LPL cases with t(11;14)(q13;q32). In cases that lacked a cytogenetically demonstrable t(11;14) translocation, no cyclin D1 transcript was detected. Analysis of the BCL-1 locus was also performed in three other cases of B-cell disorders with t(11;14)(q13;q32) detected cytogenetically. Two cases were analyzed by Southern blot and showed rearrangement of the BCL-1 locus. Expression of high-level normal-sized and/or truncated cyclin D1 transcript was also detected in these cases. These data show the importance of PFGE in the detection of rearrangements in the BCL-1 locus and show further the complexity of rearrangements in this locus.

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THE TRANSLLOCATION t(11;14)(q13;q32) is found in a variety of B-lymphoid malignancies. This chromosome translocation joins the Ig heavy chain gene locus (IGH) on 14q32 to the BCL-1 locus on chromosome 11q13. Rearrangements of the BCL-1 locus covering a region of 63 kb have been reported in several subtypes of B-cell non-Hodgkin's lymphoma (B-NHL), but are frequently found in diffuse centrocytic or intermediate B-NHL. These two closely related histologic subtypes of B-NHL are grouped under the term of mantle-cell (MC) lymphoma. Recently, a gene located 110 kb telomeric of the BCL-1 major translocation breakpoint cluster region (MTC) has been cloned. The gene is a member of the cyclin gene family and is now formerly designated CCND1 or cyclin D1. The gene encodes a small 295 amino acid protein and is expressed in a wide range of human tissues, but is not normally expressed in any cell line of lymphoid or myeloid lineages. Cyclin D1 is also overexpressed in breast and squamous cell carcinoma cell lines that show amplification of the 11q13 region.

Splenitic lymphoma with villous lymphocytes (SLVL) is a distinct B-lymphoproliferative disorder characterized by splenomegaly and circulating lymphocytes with short and thin cytoplasmic villi. A monoclonal serum Ig band and lymphoplasmacytic features are often present and SLVL may be considered within the spectrum of lymphoplasmacytic lymphoma (LPL). The marker profile of SLVL includes moderate to strong expression of surface Ig, CD22, FMC7, and CD24, whereas it lacks expression of CD5 and the hairy cell leukemia markers, HC2 and B-ly-7. The disease usually has a benign clinical course but occasionally undergoes transformation to large-cell lymphoma. We have shown by cytogenetic analysis that approximately 15% of SLVL cases carry the t(11;14)(q13;q32) translocation. To determine both the frequency and nature of rearrangements of the BCL-1 locus and their possible effects on expression of the cyclin D1 gene in SLVL/LPL, we analyzed 22 cases of SLVL/LPL, including 3 cases with t(11;14)(q13;q32), using both conventional electrophoresis (CE) and pulse-field gel electrophoresis (PFGE) and probes from the BCL-1 and cyclin D1 loci. Northern blot analysis was used in these cases to determine the level of expression of the cyclin D1 gene and in particular to investigate any association of SLVL/LPL phenotype with cyclin D1 overexpression. Our analysis also included 5 cases of other B-cell disorders, 3 of which have chromosome 11q13 abnormalities.

MATERIALS AND METHODS

Patients. Peripheral blood and/or spleen from 27 patients with a B-lymphoproliferative disorder were investigated. These cases included 16 cases with SLVL, 6 with LPL, 4 with Mc-NHL, and 1 case of chronic lymphocytic leukemia with more than 10% prolymphocytes (CLL/PL). All cases analyzed had peripheral blood involvement on the basis of light microscopy morphology and immunologic markers that demonstrated a clonal B-cell population with light chain restriction. In all the cases, diagnosis was based on peripheral blood

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morphology, bone marrow, and, in many cases, spleen histology. Diagnosis was made according to the French-American-British (FAB) proposals.25 Details of the histologic material reviewed, the immunophenotypic markers, and cytogenetics of some of the cases analyzed are shown in Table 1.

**Samples.** Peripheral blood mononuclear cells were isolated by Lymphoprep gradient centrifugation and used for immunophenotyping, cytogenetics, and molecular analysis.

**PFGE.** High molecular weight DNA was prepared from mononuclear cells resuspended in 1% low melting temperature agarose at 3 × 10⁷ cells/mL. The agarose blocks were then incubated at 50°C for 48 hours in a solution of 0.5 mol/L EDTA, pH 9.5, containing 1% sodium lauryl sarcosine and 1 mg/mL proteinase K. The solution was changed every 24 hours. Blocks were stored at 4°C in a fresh solution of 0.5 mol/L EDTA containing 1% sodium lauryl sarcosine without proteinase K. Before digestion, blocks were washed overnight at 4°C in 10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA (TE) containing 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Blocks were washed for an additional 3 hours in TE without PMSF and one-third of a block containing 10⁶ cells from each sample was used for digestion with 30 to 40 U of enzyme for 6 hours or overnight. The blocks were then placed in 1% agarose gel and subjected to electrophoresis in a clamped homogenous electric field (CHEF) at 18°C for 24 hours at 200 V with two different pulse time (60 seconds for 15 hours and 90 seconds for 9 hours). Concatemers of λ (New England Biolabs, Beverly, MA) and *Saccharomyces cerevisiae* were used as size standards.

Table 1. Immunophenotype and Cytogenetic Results of SLVL/LPL and Other B-Cell Disorders

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Spleen</th>
<th>BM</th>
<th>CD5</th>
<th>CD10</th>
<th>FMC7</th>
<th>No. of Cells Analyzed</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SLVL</td>
<td>NA</td>
<td>NA</td>
<td>30</td>
<td>ND</td>
<td>80</td>
<td>IgMK</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>SLVL</td>
<td>+</td>
<td>+</td>
<td>15</td>
<td>83</td>
<td>80</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>SLVL</td>
<td>NA</td>
<td>+</td>
<td>13</td>
<td>0</td>
<td>61</td>
<td>IgMK</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>SLVL</td>
<td>NA</td>
<td>+</td>
<td>15</td>
<td>3</td>
<td>71</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>LPL</td>
<td>+</td>
<td>+</td>
<td>89</td>
<td>10</td>
<td>25</td>
<td>IgK</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>Mc-NHL</td>
<td>+</td>
<td>+</td>
<td>10</td>
<td>31</td>
<td>66</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>Mc-NHL</td>
<td>+</td>
<td>+</td>
<td>76</td>
<td>ND</td>
<td>76</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>27</td>
<td>CLU-L</td>
<td>NA</td>
<td>+</td>
<td>0</td>
<td>ND</td>
<td>71</td>
<td>-</td>
<td>8</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; NA, not available; ND, not determined; +, tissue used for histology; -, negative results.

* Immunophenotype results refer to the percentage of positive cells with a given monoclonal antibody. Diagnosis on each case was made using peripheral blood only.

† The Ig λ light chain locus was analyzed in the sample by CE and PFGE and was found in germline configuration.

DNA probes. The MTC or BCL-1 probe "b" is a 2.1-kb Ssp I fragment. Probes q13-1 and q13-7 are 1.0-kb EcoRI/Sst I and 2-kb BamHI/Sst I fragments, respectively. p94PS is a 450-bp Pvu II-Sma I fragment that lies approximately 23 kb telomeric of MTC.27 The location of these probes within the BCL-1 locus is shown in Fig 4. pPL-8 is a 1.4-kb EcoRI cDNA probe from the cyclin D1 gene and is a subclone of the Lpl-4.26 Each probe was analyzed with the restriction enzymes BamHI, EcoRI, HindIII, and Sst I. The probe for IgH joining region 3'tH/C76R51A is a 2.5-kb EcoRI-Bgl II. Cmu is a 1.2-kb EcoRI fragment from the Ig heavy chain constant region. GAPDH is a cDNA probe of the rat glyceraldehyde-3-phosphate dehydrogenase gene.

RNA extraction and analysis. Total RNA was isolated by the guanidinium isothiocyanate method or by the guanidinium isothiocyanate-cesium chloride method.30 RNA was electrophoresed through denaturing formaldehyde agarose gels and transferred to nylon filters. RNA was immobilized by irradiation with UV light and hybridized to [32P]-labeled DNA probes in 0.5 mol/L sodium phosphate/7% SDS buffer. Hybridization with the GAPDH probe was used to document the quality and equal loading of RNA in each lane.

RESULTS

All 27 cases studied exhibited clonal rearrangement of *JH* and *Cμ*. All samples were analyzed for rearrangement of the BCL-2 gene within the major and minor breakpoint cluster region and no rearrangements were identified (data not shown).

Rearrangement of BCL-1 and cyclin D1 in SLVL/LPL without t(11;14)(q13;q32) translocation. A total of 19 cases of SLVL/LPL without t(11;14) were analyzed for rearrangement of the BCL-1 locus and the cyclin D1 gene using 4 BCL-1 probes and a cDNA clone from the cyclin D1 gene.

Using CE, BCL-1 rearrangement was detected in only 1 case (case no. 4, Table 2) with the q13-1 probe in Sst I and EcoRI restriction digests (Fig 2). Eight of the 19 SLVL/LPL cases were analyzed further by PFGE, using the MTC probe
Fig 1. (A) Short-range restriction map of the BCL-1 locus. The MTC, q13-1, q13-7, and p94PS probes are indicated above the map. Arrows refer to the precise location of the breakpoints in some of the samples analyzed, determined from the hybridization results of the probes to EcoRI, HindIII, BamHI, and Sst I digests. Numbers at the end of arrows refer to cases numbers. *The Sst I site that might be deleted in case no. 4. (B) Long-range restriction map of the BCL-1 locus. The sizes of restriction fragments are from Lammie et al. and Rosenberg et al. The vertical arrow on the far left refers to the breakpoints of case no. 3. The other vertical arrow refers to the breakpoints of case no. 17.

Table 2. Summary of Results on 27 Cases Analyzed for Rearrangement of the BCL-1 Locus and Expression of the Cyclin D1 Gene

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Disease</th>
<th>MTC</th>
<th>q13-1</th>
<th>q13-7</th>
<th>p94PS</th>
<th>pPL-8</th>
<th>PF Analysis</th>
<th>Transcript Size</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>SLVL</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>R</td>
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<tr>
<td>2</td>
<td>SLVL</td>
<td>G</td>
<td>G</td>
<td>ND</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>SLVL</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>SLVL</td>
<td>G</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5-16</td>
<td>SLVL</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>LPL</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>G</td>
<td>–</td>
<td>4.4, 1.7</td>
</tr>
<tr>
<td>18-22</td>
<td>LPL</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>++</td>
</tr>
<tr>
<td>23</td>
<td>Mc-NHL</td>
<td>G</td>
<td>G</td>
<td>R</td>
<td>G</td>
<td>R</td>
<td>++</td>
<td>4.4</td>
</tr>
<tr>
<td>24</td>
<td>Mc-NHL</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>G</td>
<td>R</td>
<td>+++</td>
<td>4.4, 1.7</td>
</tr>
<tr>
<td>25</td>
<td>Mc-NHL</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>26</td>
<td>Mc-NHL</td>
<td>G</td>
<td>G</td>
<td>ND</td>
<td>G</td>
<td>G</td>
<td>++</td>
<td>1.7</td>
</tr>
<tr>
<td>27</td>
<td>CLL-PL</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+++</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available; ND, not determined; –, expression of the cyclin D1 gene is not detected; +, low expression; ++, moderate expression; ++++, strong expression.

* Comigrated with JH.
breakpoint in this case is 100 kb centromeric to MTC (Fig 1B).

All the SLVL/LPL cases were analyzed for rearrangement within the cyclin D1 gene, but no rearrangement was detected in any of the samples analyzed by CE or PFGE.

**Rearrangement of BCL-1 and cyclin D1 in SLVL/LPL with t(11;14)(q13;q32) translocation.** Two SLVL and 1 LPL had t(11;14)(q13;q32) identified cytogenetically (cases no. 1, 2, and 17; Table 1). Material for PFGE analysis was available from cases no. 1 and 17. In both cases, a germline band and an additional rearranged band were detected with the MTC probe in Not I digests (Fig 3A). These 2 cases were also analyzed by PFGE with the probe q13-7, which detected rearranged fragments of approximately 100 kb in both of them (Fig 3B), suggesting that the breakpoint is located within a 28-kb region that separates the MTC and the q13-7 probes. Moreover, the rearranged fragments of different sizes detected by the MTC and the q13-7 probes may indicate that each of these probes detected a different derivative chromosome. The breakpoints in case no. 1 were also detected by CE with the MTC probe in BamHI, HindIII, and EcoRI digests, and these results mapped the breakpoints immediately telomeric of MTC. However, no rearrangements in case no. 17 were detected by CE. The reasons for this are unclear; lack of suitable material has precluded further analysis. No rearrangement was detected by CE with the MTC and p94PS probes in case no. 2. All samples were further analyzed with the cyclin D1 cDNA probe and no rearrangement was observed in any of the samples analyzed by CE or PFGE.

**Rearrangement of the BCL-1 locus and the cyclin D1 gene in other B-lymphoproliferative disorders.** Material for PFGE analysis was available from 4 cases of Mc-NHL (cases no. 23 through 26). t(11;14)(q13;q32) was detected cytogenetically in cases no. 23 and 24. BCL-1 rearrangements were detected in these 2 cases in Not I and Eag I digests and in the MTC probe (Fig 3A and C). The precise localization of breakpoints in these cases was also determined by CE and is shown in Fig 1A. No rearrangements were detected in cases no. 25 and 26 even by PFGE analysis.

**Involvement of the IgH locus.** In the cases with t(11;14)(q13;q32) that have been cloned and sequenced, the breakpoints in the IgH locus are located in the joining regions, ie, J4, J9, J5, or outside the JH within or near the switch region. Therefore, the cases with BCL-1 rearrangement were analyzed for IgH involvement by re-probing the Not I and Eag I PFGE filters with the JH probe. In each sample, one or more hybridizing bands were detected. None of the rearranged Not I fragments observed with the MTC probe comigrated with any of the fragments hybridizing to the JH probe. The same hybridizing pattern as for JH was obtained with the Cμ probe (data not shown), suggesting no breakpoint between the JH and the Cμ probes in the samples analyzed. However, in case no. 24, a clear comigration of the Eag 1 rearranged band obtained with MTC and JH probes was detected (Fig 3C). Comigration between rearranged bands obtained with MTC and JH probe was also detected by CE in this patient and in case no. 1.
Expression of the cyclin D1 gene in SLVL/LPL and other B-NHL. The results of the expression of cyclin D1 gene in some of the cases analyzed are summarized in Table 2 and Fig 4. Expression of a normal-sized cyclin D1 transcript of 4.4 and 1.7 kb \( ^{34} \) was detected in 1 SLVL and 1 LPL (cases no. 2 and 17) with t(11;14)(q13;q32). However, the cyclin D1 gene was not expressed in any of the SLVL/LPL cases without the t(11;14)(q12;q32) and/or BCL-1 rearrangement. The major 4.4-kb mRNA transcript was absent in case no. 27; instead, an abundant but truncated form of the cyclin D1 transcript of approximately 2.6 kb was detected (Fig 4). Cyclin D1 expression was seen in 3 of 4 cases of Mc-NHL, including case no. 26, which showed no BCL-1 rearrangement by CE or PFGE analyses.

DISCUSSION

We have investigated the molecular genetics in 27 cases of B-lymphoid malignancy with particular emphasis on SLVL to see whether (1) DNA analysis may disclose cases with 11q13 involvement not demonstrable by cytogenetics, (2) the breakpoints within the BCL-1 are distinct in SLVL/LPL from those in Mc-NHL, and (3) these alterations affect the expression of the cyclin D1 gene. We have used PFGE to expand the DNA region of the BCL-1 locus analyzed, thus increasing the possibility of detecting rearrangements. In this respect, the breakpoints in cases no. 3 and 17 could only be detected by PFGE. The long-range restriction map of the BCL-1 locus and the presence of an HTF island \( ^{36} \) on one side of the gene coding region show that a DNA region of 350 kb centromeric of the cyclin D1 gene can be easily analyzed using one or more of the rare cutting restriction enzymes mapped in this region \( ^{36} \) (Jadayel et al, unpublished results).

The rearranged bands detected by PFGE analysis are not caused by technical artifact because DNA from 4 different controls were studied without similar findings. Moreover, the rearranged bands cannot be caused by polymorphism because there is variation in the relative intensity of the normal and rearranged bands that reflects the proportion in the DNA region of the BCL-1 locus analyzed.
the blood sample of leukemic cells with the rearrangement. Furthermore, different-sized rearranged fragments were obtained with different BCL-1 probes, indicating that the altered restriction fragments are caused by true rearrangements.

The results presented here indicate that the breakpoints in SLVL/LPL are scattered, as in the case of Mc-NHL; they involve the MTC; or are located within a 28-kb region telomeric of it. We have also demonstrated a new breakpoint 100 kb centromeric of MTC that was not detectable by CE (Fig 5). Of the 4 cases of SLVL/LPL and Mc-NHL with t(11;14)(q13;q32) translocation and a breakpoint in the BCL-1 locus, 2 showed comigration with the JH probe. The lack of comigration would suggest that the activation of the cyclin D1 gene does not always result from its juxtaposition to Ig sequences but may result from the elimination, by the translocation, of a distant negative control element located near or beyond the MTC.14,20

This study has widened the spectrum of B-cell malignancies in which the BCL-1 locus is rearranged to include SLVL and LPL. RNA analysis of the expression of cyclin D1 gene in these subtypes of B-NHL showed that, in samples with t(11;14)(q13;q32), as for Mc-NHL lymphoma,14,15 deregulation leading to overexpression of the cyclin D1 gene mRNA seems to be a functional consequence of such rearrangements. However, the effect of the translocation on the expression of the cyclin D1 appears to differ in different cell types. High levels of normal-sized cyclin D1 transcripts were observed in Mc-NHL, whereas high levels of truncated transcripts were found in a case of CLL/PL (this study), in 2 prolymphocytic variant cell lines with t(11;14)(q13;q32),16 and in a lymphoma cell line.16 In contrast, the 2 SLVL/LPL cases analyzed had low to moderate levels of cyclin D1; in these cases, the transcripts were apparently of normal size. Expression of the cyclin D1 gene in SLVL/LPL may indicate an aggressive clinical course, because case no. 2 developed generalized lymphadenopathy but with no histologic evidence of transformation, whereas case no. 17 transformed to large-cell lymphoma with a very aggressive disease course. However, case no. 1 is clinically stable and has not required therapy. As preliminary data, these observations may suggest that detectable levels of cyclin D1 in SLVL/LPL may be associated with a more aggressive clinical course.

Our analysis of cases of SLVL/LPL and Mc-NHL showed two further points. Firstly, the absence of cyclin D1 expression in case no. 3 suggests that breakpoints centromeric of MTC do not deregulate the cyclin D1 gene. Secondly, the detection of a shortened form of cyclin D1 transcript in case no. 26, with no BCL-1 rearrangement even using PFGE, may suggest that activation of the cyclin D1 gene is not always associated with a breakpoint in the BCL-1 locus but may result from the loss, because of the use of different polyadenylation signals, of the 3' regulatory sequences implicated in mRNA stability.14,20 Alternatively, deregulation of cyclin D1 expression could be caused by mutations of regulatory elements of the gene.

Finally, the finding of BCL-1 rearrangements and of detectable levels of cyclin D1, albeit in a small proportion of SLVL/LPL, and its possible indication of an aggressive clini-
cal course is a further proof of the involvement of cyclin D1 in B-cell lymphomagenesis.

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