BCL-6 Mutations Are Associated with Immunoglobulin Variable Heavy Chain Mutations in B-Cell Chronic Lymphocytic Leukemia

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Abstract

The cell of origin of B-cell chronic lymphocytic leukemia (B-CLL) is still uncertain. Recent studies have indicated that a fraction of B-CLL displays somatically mutated immunoglobulin variable heavy chain (IgVH) genes, which suggests an origin from a post-germinal center (GC) B cell. It has been shown that the 5′ noncoding region of the BCL-6 proto-oncogene is affected by mutations in normal GC B-lymphocytes and in lymphoid malignancies displaying GC/post-GC phenotype. To further explore the cellular origin of B-CLL, we have analyzed 34 cases for mutations in the BCL-6 5′ noncoding region and in the IgVH genes. We found somatically mutated IgVH genes in 24 (73%) of 33 samples (average frequency, 6.5 × 10⁻³/2bp) and BCL-6 mutations in 8 (24%) of 34 cases (average frequency, 0.14 × 10⁻³/2bp in the mutated cases). The occurrence of BCL-6 mutations was restricted to those cases displaying IgVH mutations. Analysis of BCL-6 protein expression as a marker of GC phenotype showed that, regardless of the presence of IgVH or BCL-6 mutations, B-CLLs express BCL-6 at levels clearly below those found in normal or transformed GC B cells. These results indicate that a subset of B-CLL derives from a cell that has been exposed to the somatic hypermutation mechanism and support the hypothesis that BCL-6 mutations result from the same process that targets immunoglobulin genes.

Introduction

B-CLL is an indolent disorder that arises from the clonal expansion of small mature B-lymphocytes expressing the cell surface markers CD5, CD23, CD19, and low levels of surface IgM/IgD (1, 2). Because the neoplastic cells invariably express the CD5 antigen, it has been presumed that B-CLL originates from normal CD5+ B cells, which are usually characterized by unmethylated IgV genes (3). However, recent studies have documented the presence of IgV somatic mutations in a substantial fraction (up to 75%) of B-CLL cases (4–7). This finding has led to the hypothesis that the tumor clone may derive from a lymphocyte that transited the GC, the site where immunoglobulin somatic hypermutation occurs (8). In particular, it has been suggested that B-CLL carrying IgV mutations may represent the transformed counterpart of memory B cells (5).

Until recently, the process of somatic hypermutation was believed to be restricted to the immunoglobulin loci, including heavy (H) and light chain V region genes. However, it has been shown that another locus, BCL-6, can be targeted by somatic mutations in the GC (9–11). BCL-6 is a proto-oncogene encoding for a POZ/Zinc finger transcriptional repressor expressed at high levels in GC B-lymphocytes and required for GC development (12–22). Mutations of its 5′ regulatory region (~3.5 kb spanning the first noncoding exon and the first intron) are frequently found in normal GC and memory B cells, as well as in lymphoid malignancies displaying GC/post-GC phenotype and harboring mutated IgVH genes, but not in naive B cells or in other tumor types (9–11, 23, 24). BCL-6 mutations are multiple, often biallelic and heterogeneous, and display features of the immunoglobulin somatic hypermutation process, which suggests a common mechanism (9–11, 23). On the basis of these findings, BCL-6 protein expression has been considered a marker of GC phenotype in B cells and B-CLL gene mutations have been proposed as a molecular marker of transit through the GC.

As a further approach to determining the cellular origin of B-CLL, this study was aimed at investigating the presence and distribution of BCL-6 mutations in this disease. In addition, we assessed the relationship between the presence of BCL-6 and IgVH mutations, and the expression of the BCL-6 protein.

Materials and Methods

B-CLL Samples. PBMCs were collected during standard diagnostic procedures from 34 patients with clinical, morphological, and immunophenotypic features of B-CLL. In all cases, the neoplastic lymphocytes expressed the surface markers CD19, CD5, and CD23. The fraction of malignant cells corresponded to more than 80% in most cases and to ~40% in two cases. Genomic DNA was prepared from Ficoll-separated PBMCs by the “salting-out” procedure as described previously (25).

PCR Amplification of the BCL-6 Sequence. A 781-bp genomic fragment, located within the first intron of the BCL-6 gene and previously reported to represent the major cluster for mutations (23), was amplified using 100 ng high molecular weight DNA and the following primers: sense, 5′-GCTCTCTGC-CAAATGCTTTG; and antisense, 5′-CTCTCGTTAGGAAGATCACG. The reaction was carried out in a 50-μl volume containing Expand High Fidelity buffer (BMB), 1.75 mM MgCl₂, 200 μM each dNTP, 10 pmol of each primer, and 2.5 units Taq DNA polymerase (Life Technologies, Inc.). Amplification conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 45 s, and a final step at 72°C for 7 min.

PCR Amplification of IgVH Sequences. The protocol for amplification of the IgVH genes has been reported previously (26). Briefly, a set of six VH family-specific primers annealing to sequences in the framework region I was used in separate reactions, along with a J₅ primere mixer. PCR was performed for 34 cycles, and a 5-μl aliquot of the reaction mixture was analyzed on ethidium bromide-stained 2% agarose gel. In case of amplification failure, the sense primers were replaced with oligonucleotides complementary to the Leader sequences of the VH genes (27).

DNA Sequencing Analysis. PCR products were purified directly or by gel excision using the QiAquick PCR purification kit (QiAGEN) and were directly sequenced from both strands using the same primers as in the amplification reaction. The procedure was accomplished by the dyeoxy chain termination method on an ABI377 sequencer (Perkin-Elmer, Applied Biosystem Division, Norwalk, CT). Sequencing analysis and alignments were performed using the GCG software (Genetics Computer Group, Madison, WI) and the GenBank

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3 The abbreviations used are: B-CLL, B-cell chronic lymphocytic leukemia; GC, germinal center; IgV, immunoglobulin variable (region); IgVH, IgV heavy chain; PBMC, peripheral blood mononuclear cell; BL, Burkitt lymphoma; DLCL, diffuse large cell lymphoma.

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Unpublished observations.

Results

BCL-6 Mutations in B-CLL. To investigate the distribution of BCL-6 mutations in B-CLL, a panel of 34 cases was screened for sequence variants occurring in the BCL-6 5’ noncoding sequence. The genomic region analyzed corresponded to a 781-bp fragment of the BCL-6 cDNA (GenBank accession nos. AF304488–AF304517). The first nucleotide of the BCL-6 cDNA (GenBank accession no. U00115) was defined as position +1.

Western Blot Analysis. Cell lysates were obtained from exponentially growing cell lines and from the PBMCs of 10 B-CLL patients, as described previously (28). Total protein lysates (50 μg) were fractionated by 8% SDS-PAGE, transferred to nitrocellulose membrane (Schleicher & Schuell), and immunostained according to standard methods. Briefly, the membrane was blocked in Tris-buffered saline (TBS)-0.5% Tween with 5% milk and was incubated overnight at 4°C with a 1:1000 dilution of the anti-BCL-6 polyclonal antibody N3 (Santa Cruz) in TBS-0.2% Tween with 3% BSA. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated antirabbit IgG antibody (1:5000) in TBS-0.2% Tween with 5% milk. Reactive bands were detected using an ECL system (Amersham).

The results of the sequence analysis are shown in Fig. 1B and Table 1. Three previously described population polymorphisms were detected: a T insertion at position +876 (50% of the cases), a G to C substitution in position +753 (26% of the cases) and a G to A substitution in position +858 (2 cases; not shown; 9, 23). Case 1331 harbored an insertion T within a poly-T stretch in position 1104–1111, that could also be ascribed to a genetic polymorphism because it was previously seen in all IgD-CD27- naive B cells individually sorted from the peripheral blood of a healthy individual.3 These changes were found alone or in combination and were not considered in the calculation of the mutation frequency.

Eight (24%) of 34 samples displayed sequence variants in the BCL-6 5’ noncoding region. These variants were represented by 18 distinct nucleotide substitutions, with no deletions or insertions. Ten of the changes were transitions, whereas 8 were transversions, in agreement with previous observations showing a bias for transition mutations (9–11, 24). The overall frequency of mutations in the mutated cases was 0.14 × 10−2/bp (range, 0.064–0.38 × 10−2/bp, corresponding to 1–6 mutations per case), representing a >10-fold increase over normal DNA controls (P < 0.01; Ref. 9). The mutation frequency observed in B-CLL is comparable with that reported in normal GC centroblasts and centrocytes (0.16 × 10−2/bp in the mutated cells; Ref. 9) as well as in memory B cells (−0.12 × 10−2/bp; Ref. 10), which suggests that there was no apparent selection for BCL-6 mutations during tumorigenesis. Sequencing analysis of cloned fragments from 2 of the 4 cases carrying more than one nucleotide change indicated that mutations were biallelically distributed (data not shown), as previously observed in normal B cells and B-cell-derived tumors (9, 23).

Analysis of IgVH Genes. To assess the relationship between the occurrence of BCL-6 mutations and the immunoglobulin somatic hypermutation process, we characterized the rearranged IgVH genes of the B-CLL cases by PCR amplification and sequencing of genomic DNA. Clonal rearrangements were obtained from 33 samples; one case (case 1330, Table 1) did not yield a positive amplicon with either framework region I or leader-specific primers and, therefore, was not included in the comparative analysis. In seven cases (21.2%), we detected more than one rearranged IgVH gene (Table 1). Sequencing

4 Internet address: www.genetik.uni-koeln.de.

5 Unpublished observations.
analysis showed that in five of these seven cases, the two isolated V_H
sequences corresponded to the productively and nonproductively re-
arranged allele; in the remaining two cases (6%), both of the VDJ
rearrangements amplified—three in sample 1287—were productive
and displayed distinct VH genes, which was consistent with a lack of
allelic exclusion (29), or amplification of V genes from bystander
normal B cells, or oligoclonality of the leukemic population.

As shown in Table 1, 9 (27%) of 33 cases displayed unmutated V_H
genes, whereas significant levels of somatic mutations were found in
the remaining 24 cases (73%). Among the mutated sequences, 19
(63%) differed by 5% or more from the closest germ line immuno-
globulin gene. The average frequency of mutations in the IgVH genes,
including both of the alleles in cases with more than one rearrange-
ment, was 6.5 \times 10^{-2}/bp. The V_H3, V_H4, and V_H1 families were the
most commonly used in our B-CLL panel, being observed in 56, 27,
and 12%, respectively, of the rearranged sequences.

**BCL-6 Mutations Are Associated with IgV_H Mutations.** On the
basis of the occurrence of BCL-6 and IgV_H mutations, the B-CLL
panel can be divided into three groups (Table 2). The first, represented
by 8 (24%) of 33 samples, displays mutations in both BCL-6 and IgV_H
sequences; the second, accounting for \sim49% of the cases, is charac-
terized by mutated IgV_H genes and germ line BCL-6 sequences; in the
third group, which includes 9 cases (27%), both of the genes were in
germ line configuration. None of the cases investigated displayed
BCL-6 mutations in the absence of IgV_H Mutations (Table 2). In the
mutated cases, the average mutation frequency corresponded to 0.14
and 6.5% for the BCL-6 and the IgV_H genes, respectively, consistent
with previous reports of a 10- to 100-fold difference between the
frequency of nucleotide changes in the two loci (9–11). The total
number of mutational events in the BCL-6 gene (18) was too limited
to define the features of mutations. Taken together, these observations
indicate that, analogous to normal GC cells and to GC/post-GC-
derived B-cell malignancies, BCL-6 mutations are restricted to B-
CLLs displaying IgVH mutations.

**BCL-6 Expression in B-CLL.** In the B-cell lineage, BCL-6 pro-
tein expression is restricted to GC B cells and, thus, represents a

### Table 1 Mutational analysis of BCL-6 and IgV_H sequences in B-CLL

<table>
<thead>
<tr>
<th>Case no.</th>
<th>No. of mutations</th>
<th>%</th>
<th>V_H Gene</th>
<th>No. of mutations</th>
<th>%</th>
<th>Gene rearrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1297</td>
<td>3</td>
<td>0.20</td>
<td>4–59</td>
<td>9</td>
<td>3.7</td>
<td>In frame</td>
</tr>
<tr>
<td>1301</td>
<td>1</td>
<td>0.07</td>
<td>3–21</td>
<td>6</td>
<td>2.4</td>
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</tr>
<tr>
<td>1316</td>
<td>1</td>
<td>0.07</td>
<td>4–34</td>
<td>17</td>
<td>7.1</td>
<td>In frame</td>
</tr>
<tr>
<td>1283</td>
<td>2</td>
<td>0.26</td>
<td>3–33</td>
<td>23</td>
<td>9</td>
<td>In frame</td>
</tr>
<tr>
<td>1314</td>
<td>1</td>
<td>0.07</td>
<td>3–11</td>
<td>19</td>
<td>7.6</td>
<td>In frame</td>
</tr>
<tr>
<td>1291</td>
<td>6</td>
<td>0.38</td>
<td>4–59</td>
<td>26</td>
<td>10.6</td>
<td>In frame</td>
</tr>
<tr>
<td>1333</td>
<td>3</td>
<td>0.20</td>
<td>4–39</td>
<td>31</td>
<td>12.3</td>
<td>In frame</td>
</tr>
<tr>
<td>1331</td>
<td>1</td>
<td>0.07</td>
<td>3–43</td>
<td>17</td>
<td>6.9</td>
<td>In frame</td>
</tr>
<tr>
<td>1286</td>
<td>0</td>
<td>0.00</td>
<td>3–30</td>
<td>25</td>
<td>8.3</td>
<td>In frame</td>
</tr>
<tr>
<td>1287</td>
<td>0</td>
<td>0.00</td>
<td>3–30</td>
<td>1</td>
<td>0.14</td>
<td>Stop codon in V_H gene.</td>
</tr>
<tr>
<td>1288</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>CDR, complementarity determining region.</td>
</tr>
<tr>
<td>1292</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>Two deletions in V_H gene.</td>
</tr>
<tr>
<td>1293</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>NA, not amplified.</td>
</tr>
<tr>
<td>1299</td>
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<td>0.00</td>
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<td>1</td>
<td>0.26</td>
<td>Stop codon in CDR3</td>
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<tr>
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<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1320</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1321</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1323</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1324</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1325</td>
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<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
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<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
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<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1322</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1348</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1268</td>
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<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1300</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1302</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1326</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1284</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
<tr>
<td>1330</td>
<td>0</td>
<td>0.00</td>
<td>3–34</td>
<td>1</td>
<td>0.26</td>
<td>In frame. Stop codon in CDR3</td>
</tr>
</tbody>
</table>

* Stop codon in the V_H gene.

**Table 2 Distribution of BCL-6 and IgV_H mutations in B-CLL**

<table>
<thead>
<tr>
<th>Mutation pattern</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL-6+/IgV_H+</td>
<td>8/33 (24)</td>
</tr>
<tr>
<td>BCL-6+/IgV_H−</td>
<td>0/33 (0)</td>
</tr>
<tr>
<td>BCL-6−/IgV_H+</td>
<td>16/33 (49)</td>
</tr>
<tr>
<td>BCL-6−/IgV_H−</td>
<td>0/33 (0)</td>
</tr>
</tbody>
</table>

* Mutated; –, germ line.

The number of positive/tested cases is indicated.
BCL-6 mutations in B-CLL

The present study reports that the 5’ noncoding region of the BCL-6 gene is affected by somatic mutations in a fraction of B-CLLs, that mutations occur exclusively in cases exhibiting mutated IgV<sub>H</sub> genes, and that B-CLL cells express low levels of BCL-6 protein regardless of the status of mutations in the BCL-6 and IgV<sub>H</sub> loci. These data have implications for the understanding of the cellular origin of B-CLL and of the mechanism of somatic hypermutation in B cells.

BCL-6 mutations in B-CLL. The percentage of B-CLL cases carrying BCL-6 mutations reported here (24%) is not significantly different from that reported in a previous study (30%) as part of a survey involving major subtypes of B-cell malignancies (24). These percentages are also not significantly different from those observed in other categories of B-cell malignancies including BL (37%), mucosa-associated lymphoid tissue lymphoma (40%), and multiple myeloma (33%), whereas higher frequencies may be present in follicular lymphoma and DLCL (50–73%; Refs. 9, 11, 24). These values are also comparable with the percentage (30–40%) of normal GC and memory B cells carrying BCL-6 mutations (9–11). Thus, the number of B-CLL cases displaying BCL-6 mutations may reflect the fraction of normal B cells undergoing BCL-6 hypermutation. In terms of average frequency of mutations per mutated case, B-CLLs appear to have a lower load of mutations (0.14%) than do DLCLs (~0.5%; Ref. 23), which suggests a higher exposure of DLCL cells to the hypermutation process.

Association between BCL-6 Mutations and IgV<sub>H</sub> Mutations. When examining the relationship between IgV<sub>H</sub> and BCL-6 mutations, the analysis of B-CLL is particularly informative because, differently from other B cell tumor types, which display IgV<sub>H</sub> mutations in almost 100% of cases, B-CLL are heterogeneous, with a fraction of cases carrying unmutated IgV<sub>H</sub> sequences. Thus, assessing the distribution of the two types of mutations can support or disprove the hypothesis of their derivation from the same mechanism. Our results suggest that BCL-6 mutations are restricted to B-CLL cases displaying IgV<sub>H</sub> mutations, whereas in no instance, could they be detected in the subset of B-CLL that lacks IgV<sub>H</sub> mutations. This observation strongly supports the notion that BCL-6 mutations are introduced by the same mechanism that generates IgV hypermutation.

The finding that not all cases carrying mutated IgV<sub>H</sub> genes have BCL-6 mutations is entirely consistent with the above hypothesis, based on the observation that only 1 of 3 normal B cells displaying IgV<sub>H</sub> mutations also harbor BCL-6 mutations. Thus, the three groups shown in Table 2 may in fact represent two types of B-CLL cells: those that have been exposed to the somatic hypermutation mechanism and acquired IgV<sub>H</sub> mutations and, in a fraction of cases, BCL-6 mutations; and those that have not been exposed to the process and, therefore, lack both types of mutations.

Two studies addressing the same issues were reported while this article was in preparation/review. In complete agreement with our findings, Capello et al. (30) showed a concordant distribution of BCL-6 and IgV<sub>H</sub> mutations in B-CLL. Conversely, Sahota et al. (31) reported the presence of subclonally distributed BCL-6 mutations in 4 of 10 B-CLL patients with unmutated IgV<sub>H</sub> sequences and suggested that a diverse mechanism targets the two genes. Because, in the latter study, sequences were obtained after the cloning of PCR products (as opposed to direct sequencing; Ref. 30 and our analysis) the two sets of results are difficult to compare. However, the detection of a few subclonal BCL-6 mutations in the absence of IgV<sub>H</sub> mutations does not necessarily imply a distinct molecular mechanism, because a low activity of the same somatic hypermutation process may stochastically target one of the two loci, leading to a discordant pattern of mutations.

Role of BCL-6 Mutations in B-CLL. The role of BCL-6 mutations in normal GC B cells as well as in their various transformed counterparts is presently unknown. Initial results indicate that some specific BCL-6 mutations may be responsible for BCL-6 overexpression in DLCL. However, no differences were detectable between wild type and mutated alleles in five B-CLL cases tested; consistently, our analysis on BCL-6 protein expression showed no difference in the levels of BCL-6, irrespective of the BCL-6 mutation status. Thus, it is possible that BCL-6 mutations are not functionally significant in B-CLL or, alternatively, that they confer subtle regulatory disturbances to gene expression.

Implications for the Cellular Origin of B-CLL. Previous studies have proposed that B-CLL cases displaying IgV<sub>H</sub> mutations originate from antigen-selected memory B cells, whereas those carrying germ line IgV<sub>H</sub> sequences may derive from naive B cells or from memory B cells that were selected by antigens unable to induce IgV<sub>H</sub> hypermutation (5). The concordant distribution of BCL-6 and IgV<sub>H</sub> mutations shown here confirms the existence of two distinct subgroups of B-CLL. However, both groups express the BCL-6 protein, a marker of GC phenotype, at levels significantly lower than those detectable in normal GC B cells or in their transformed counterparts (follicular lymphoma, BL, DLCL; Ref. 17). A cell population with such BCL-6 expression levels and variable presence of IgV<sub>H</sub> and BCL-6 mutations has not been identified in normal B cells. This may be attributable to either the incomplete knowledge of the functional program of CD5+ B cells or to phenotypic differences associated with the transformed status of B-CLL cells.
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References

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