The antigen receptor in the pathogenesis of B-cell non-Hodgkin's lymphomas
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Immunoglobulin diversification in a B-CLL: 
Alternative splicing of heavy chain variable region 
as a byproduct of somatic hypermutation

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Abstract

In this study we analyzed a case of B-cell chronic lymphatic leukemia (B-CLL) that was accompanied by severe auto-immune hemolytic anemia (AIHA). Molecular analyses revealed that the B-CLL cells expressed an IgMκ immunoglobulin consisting of mutated heavy (H) chain and light (L) chain variable (V) regions that showed closest homology to the V4-34 and DPK3/L11 germline V gene sequences, respectively. In the IgH locus, a triplification of the rearranged JH3 gene segment, including the putative N-region and some intronic sequences, was observed. Each JH3 gene segment had retained its normal 5' splice donor site. Consequently, different mRNA transcripts of the VH-CH region were found, depending on the number of JH3 copies present. In addition, we found a splice product of the VH region in which a sequence stretch located between the framework 1 (FR1) and complementarity determining region 2 (CDR2) was deleted. We prove that this was not due to a defect in the splicing machinery of the tumor cells but caused by specific somatic point mutations. One of these point mutations, located in the alternative 3' splice acceptor site in the CDR2 region, proved to be of critical importance. Thus, alternative splicing as a byproduct of somatic hypermutation may be an additional mechanism of IgV diversification.
Introduction

B Chronic Lymphocytic Leukemia (B-CLL) is a malignancy of mature B lymphocytes, characterized by expression of the CD5 antigen in combination with several B-cell markers and low levels of membrane Ig [1,2]. Initial studies indicated that B-CLL cells express Ig genes with little or no somatic mutations [3-10], although this finding was challenged by other investigators [11-14]. Recently, Fais et al [15] reported that, within a large panel of B-CLL, approximately 50% of the IgM+ B-CLL and 75% of the non-IgM+ B-CLL cells exhibit somatic mutations in their VH genes. These data indicate that a substantial percentage of the B-CLLLs derive from antigen-experienced post germinal center (GC) B cells and suggest that within the clinicopathological group of B-CLL, separate entities are included. Indeed, two recent studies indicate that patients with somatically mutated B-CLL IgV genes have a clearly better prognosis than those with unmutated B-CLL IgV genes [16,17].

During early B-cell development in the bone marrow, IgV regions of both heavy (H) and light (L) chain genes are formed by recombination of VH(D), and J gene segments. The potential recombinatorial repertoire, that includes the ‘random’ pairing of IgH and IgL chains, is significantly enlarged due to imprecise joining of the different gene segments. This junctional diversity depends largely on the enzyme terminal deoxynucleotidyl transferase (TdT), that is responsible for the addition of non-templated nucleotides to the V(D)J junctions [18,19]. In mature B cells, the IgV regions can be further diversified, particularly in the course of T-helper cell dependent antigen-specific responses in GCs. Here the Ig loci may be altered by somatic hypermutation and IgH chain class-switch recombination. It has recently been reported that the somatic hypermutation process, that is believed to occur in the GC environment exclusively [20-23], not only involves single nucleotide exchanges but also deletions and insertions of different size [24,25]. Finally, as the RAG genes may be (re-) expressed during the GC stage, it is presently not excluded that the Ig rearrangement processes can be reactivated, a process known as 'receptor revision' [26].

In this study, we analyzed the IgV genes in an extraordinary case of B-CLL. It was found that the rearranged IgVH locus contained a triplication of the JH3 gene segment including parts of the intronic sequences. The VH gene was mutated and displayed highest homology to the V4-34 germ line gene segment. From this IgVH locus, multiple transcripts were produced including an illegitimate splice variant of the mutated V4-34 gene segment. We demonstrate that the illegitimate splice variant was not due to a defect in the splicing machinery of the tumor cells but caused by specific somatic point mutations.
Results

Assessment of clonality, H chain isotype expression and \( V_H \) and \( V_L \) gene family usage.

The patient was a 76 year old female who suffered from B-CLL with severe auto-immune hemolytic anemia, for which she was splenectomized. Microscopic examination of the resected spleen revealed that the PALS was colonised by small CD5\(^+\) B-CLL cells. Clonality was demonstrated immunohistochemically and confirmed by Southern blot, using a JH probe (data not shown) [31] as well as by a CDR3-specific PCR (Fig. 1a). This PCR makes use of clone-dependent length differences of the CDR3 regions in the \( V_H \) chains. A CDR3 PCR of a polyclonal B cell population yields products of variable size, visible as multiple bands or a smear pattern on agarose gel, whereas of a clonal B cell population a single band is obtained. The CDR3 region of the rearranged immunoglobulin \( V_H \) locus was amplified from both genomic DNA (gDNA) and copy DNA (cDNA), using a FR3 primer in combination with either a JH primer or CH-specific primers (\( C_{\mu}, C_{\delta}, C_{\gamma}, \) and \( C_{\alpha} \)). With FR3 - CH primer combinations a sharp band was observed only with the \( C_{\mu} \) and \( C_{\delta} \) primers, a result consistent with the immunohistochemically demonstrated co-expression of IgM and IgD. With both the \( C_{\gamma} \) and \( C_{\alpha} \) primer a smear pattern was obtained, most likely due to the presence of non-neoplastic B cells and/or plasmacells in the tumor tissue (Fig. 1b). However, with the FR3 - JH primer combination, from both cDNA and gDNA, unexpectedly three clonal products of 112 bp, 184 bp and 297 bp were amplified (Fig. 1b). Subsequently, the IgV genes were amplified with \( V_H \) or \( V_L \) family-specific leader primers in combination with the JH, \( C_{\mu} \) and \( C_{\delta} \) or Jk and Ck downstream primers, respectively. On gDNA, only JH or Jk downstream primers could be used, because of the presence of the JH - CH intronic sequences. The \( V_H \) family-specific PCRs revealed that the B-CLL expressed a VH4 family gene (data not shown). The PCR on gDNA with the VH4 leader primer in combination with the JH primer, yielded three clear products of 516, 588 and 701 bp, respectively, and a larger, albeit weaker product of 1071 bp (Fig. 1c). The same PCR on cDNA also resulted in at least three clear bands. The general length difference between the \( V_H \) family PCR products generated from either gDNA or cDNA is explained by the leader intron of 83 bp, which is only present in gDNA. With the \( C_{\mu} \) downstream primer, two bands were visible of which one was dominant. Finally, with the \( C_{\delta} \) primer only one product was detected (Fig. 1c). ByVk-family-specific PCR and subsequent sequencing it was determined that the B-CLL expressed a Vk1 family gene segment (data not shown).
Alternative splicing of heavy chain variable region in B-CLL

Figure 1 (a) Schematic representation of the IgH locus and the primers used for the V\textsubscript{H} family-specific PCR and the CDR3-specific PCR: L, leader sequence; V\textsubscript{H}, variable gene segment; D, diversity gene segment; J\textsubscript{H}, joining gene segment; C\textsubscript{H}, constant gene segment; N, non-templated nucleotide additions.

(b) Results of CDR3 PCR on cDNA and gDNA, using the FR3 upstream primer and JH, C\textsubscript{H}, Cy, Ca and C\ö primer as downstream primers.

(c) Results of VH4 family-specific PCR on cDNA and gDNA using the VH4 upstream primer and JH, C\textsubscript{H} and C\ö primer as downstream primers. The four different arrows and numbers indicate the four PCR products obtained on gDNA.

**Sequence analyses of H- and L- chain loci.** The genomic V(D)J rearrangement, as obtained in the VH4 family PCR on gDNA, was cloned in bacteria. By CDR3-specific PCR, we identified cloned plasmids with CDR3 lengths of 112, 184 and 297 bp, thus identical to the products found in the CDR3-PCR on gDNA of the B-CLL (data not shown). One of the plasmid clones gave rise to an extra long CDR3 product of 667 bp. A VH4 family-specific PCR on this particular bacterial clone, yielded a product of a size comparable to the 1071 bp
product obtained in the VH4 family-specific PCR on total gDNA (Fig.1c). Clones, representative of the four different CDR3 lengths found, were sequenced. The identified V_H region expressed by the B-CLL had been formed by gene segments that displayed highest homology with the V4-34, D4 and JH3b germline gene segments, respectively (Fig.2) [28,32]. In the V_H gene segment, a total of 13 somatic mutations was identified. In the CDR1 and CDR2, 5 somatic mutations were found and the replacement versus silent (R/S) mutation ratio was 1.5. The FRs contained 8 somatic mutations with an R/S ratio of 0.3. The clone with the CDR3-PCR product of 112 bp harboured a normal V(D)J configuration, whereas in the clone with the 184 bp CDR3 length, a duplicated JH3b gene segment was found. The second JH3b (JH3b-II) gene segment was located 21 bp downstream of the JH3b-I, in the JH3-JH4 intron. The clone with the CDR3 length of 297 bp contained even three copies of the JH3b gene segment. This JH3b-III was located 55 bp downstream of the JH3b-II, again in the JH3-JH4 intron. It is noteworthy that the latter clone harboured a frameshift upstream the third JH3b gene segment. Thus, no functional immunoglobulin molecule can be produced from the putative messages containing all three JH3b gene segments. The clone with the extra long CDR3 sequence of 667 bp contained a PCR product that arose by annealing of the JH primer to the next JH gene in the JH locus, i.e. the JH4 gene segment. Interestingly, this clone contained the whole sequence of interest from the VH4 leader to the JH4 gene segment, including all three JH3b copies and the complete JH3 - JH4 intron (Fig. 2). The somatic mutations in the three successive JH3b gene segments with parts of the JH3 - JH4 intronic sequence, were all unique except for one mutation that was shared by JH3b-I and JH3b-III (Fig.2). Since all 5' splice donor sites after each of the three JH3b gene segments were preserved, putatively VH4 transcripts can be produced coupling one, two or three JH3b gene segments to CH gene segments.

The sequences of two Vk1 clones, derived from the Vk1 family-specific RT-PCR product, were identical to the consensus sequence which was obtained by direct sequencing of the whole PCR product. The expressed Vk1 gene harboured 10 somatic point mutations (4 in the CDRs and 6 in the FRs) compared to the most homologous germline Vk1 gene segment, DPK3/L11 (data not shown).
Figure 2. (a) Complete nucleotide sequence of the rearranged V<sub>H</sub> locus as determined on the large clone derived from a VH4-family PCR of gDNA. The upper line represents the germline V<sub>H</sub>, D and J<sub>H</sub> sequences. Below is the sequence as found in the B-CLL. Only somatic mutations are indicated. Indicated regions: FR, frame work region; CDR, complementarity determining region; N, non-templated nucleotide additions; 5’ splice site around codon 26; 3’ splice site around codon 64; (C). Somatic mutation which results in a closer homology to the 3’ splice consensus sequence; [GT]. The only shared mutation in JH3b1 and JH3bIII.

(b) Schematic overview of the rearranged IgV<sub>H</sub> locus of the B-CLL patient; L, leader sequence; V<sub>H</sub>, variable gene segment; D, diversity gene segment; J<sub>H</sub>, joining gene segment a, leader intron; i, intron.
**Alternative splice product of the V4-34 gene.** To analyze the expressed VH4 transcripts in more detail, bacterial clones were produced from the VH4 RT-PCR products obtained with the JH, Cμ and Cδ downstream primers, respectively. By CDR3-PCR on the cloned VH4 products, that had been generated with the JH downstream primer, we identified clones with one, two or three JH3b copies of 112, 184 and 297 bp, respectively. These three products were also detected in PCRs on cDNA that had been synthesized with oligo-dT primers, indicating that the product with three JH3b copies contain a normal polyA tail (data not shown). As the JH primer can anneal to each JH3b segment, it is however unclear whether the cloned products with two JH3b copies are representative of truly existing transcripts or just PCR products derived from the existing transcripts with three JH3b regions. The clones derived from VH4 PCR products generated with the Cμ and Cδ downstream primers, contained the short CDR3 length of 112 bp only. In these RT-PCRs, we used a CH1-Cμ primer. Also, when a CH3-Cμ downstream primer was used, only amplimers with one JH3b segment were found (data not shown), excluding the possibility that the two- and/or three-JH3b containing transcripts had been spliced to CH2-Cμ or CH3-Cμ. Thus, it seems that at least the majority of the μ- and δ transcripts contains functional IgVH regions with one JH3b segment in a normal V(D)J rearrangement.

A total of 20 cDNA-derived PCR clones of the different lengths were sequenced. We did not detect intraclonal point mutation variation between the IgV-sequences of the bacterial clones. This lack of intraclonal variation is in accordance with a previous study [33]. In addition to the described differences in the number of included JH3b copies, we found in eight of these 20 clones a deletion within the V4-34 gene segment (Table 1). This deletion started at codon 26 in FR1 and ended in codon 64 in CDR2 (Figs. 2, 3). By analyzing the sequences flanking this deletion, it appeared that the 5’ site at codon 26 beared strong homology for the 5’ splice site consensus sequence 5’ A/C A G ↓ G U A/G A G U 3’ whereas the 3’ site at codon 64 showed homology for the 3’ splice site consensus sequence 5’ (Y), N Y A G ↓ G 3’ [34]. In these splice acceptor and donor sites only the underlined nucleotides are absolutely required for RNA splicing. One somatic mutation in codon 64 (A → C) resulted in a closer homology to the consensus 3’ splice acceptor site. In addition, the mammalian branch-point consensus sequence, which maps 18-38 nucleotides upstream of the 3’ splice site (5’ U N C U R A C 3’), was present in the mutated V4-34 and also here one somatic mutation contributed to a closer homology (Fig. 3) [34]. The clones with this deletion were all devoid of the VH4 leader intron, proving the RNA derivation of the cloned fragments. This, combined with the fact that we did not isolate gDNA-derived clones with the V4-34 deletion, indicates that this
deletion is the result of illegitimate splicing in the mutated V4-34 gene segment of this B-CLL. In the alternatively spliced V4-34, a frame-shift is introduced at the 5' site of the deletion. Thus, no functional immunoglobulin can be produced from these mRNA species. Figure 4 summarizes the transcripts identified in the B-CLL.

Table 1. Summary of plasmid clones generated from RT-PCR

<table>
<thead>
<tr>
<th>PCR</th>
<th>CDR3 length in CDR3 PCR</th>
<th>No. of clones sequenced</th>
<th>No. of clones with a deletion in V4-34</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH4 - JH</td>
<td>112 bp</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>VH4 - JH</td>
<td>184 bp</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>VH4 - JH</td>
<td>297 bp</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>VH4 - Cμ</td>
<td>112 bp</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>VH4 - Cδ</td>
<td>112 bp</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of part of the V₄ sequence of the B-CLL with the consensus 5' and 3' splice sites; FR1, frame work region 1; CDR2 complementarity determining region 2; * mutation that resulted in a closer homology to the splicing consensus sequence; + mutation which did not result in a closer homology to the splicing consensus sequence; | nucleotide matching the consensus sequence; x nucleotide not matching the consensus sequence. The somatic mutation in codon 64 which resulted in a closer homology to the 3' splice acceptor site is indicated with an arrow.
The alternative V4-34 splicing depends on a single somatic mutation. To distinguish whether the alternative V4-34 RNA processing was due to an aberrant splicing machinery in the B-CLL, or an intrinsic property of the particular V4-34 sequence, we transfected SP2/0 myeloma cells with the B-CLL-specific Ig. The B-CLL V4-34 genes with one JH3b (1J) and two JH3b (2J) gene segments were introduced into the plgH(γ3) expression vector. The introduced V_{H} sequence included the leader sequence and the normal JH 5' splice donor site. The insert is flanked at the 3' end by an intron, including the normal Ig enhancer sequence, and the Cγ3 sequence, respectively. Similarly, the patient’s Vκ gene was introduced into the plgL(κ) expression vector. The plgH(γ3)-1J and plgH(γ3)-2J were separately co-transfected with plgL(κ) in SP2/0 myeloma cells. The transfecteds were selected with genicin. On cDNA derived from these transfecteds, we performed VH4 family-specific PCRs with a Cγ downstream primer or, for a more accurate detection of the V4-34 splice variant, a B-CLL specific downstream FR3 primer (Fig. 5a). In the PCRs on the plgH(γ3)-2J transfecnt with the Cγ as downstream primer, three products were detectable, of which the largest product of 530 bp possessed two JH3b gene segments directly coupled to the Cγ3 sequence (Fig. 5b, lane 1). This is thus different from the B-CLL, in which transcripts with two JH3b gene segments
were not found in contiguity with Cμ or Cδ. The lower 458 bp product was derived from a normal V(D)J coupled to Cy3 whereas the smallest product of 342 bp represents the V4-34 splice variant with one JH3b gene segment coupled to Cy3. Theoretical transcripts, representing the splice variant of V4-34 with two JH3b gene segments coupled to Cy3 with a predicted length of 414 bp, were not detected. In the PCR on the plgH(γ3)-1J transfectant, we detected a dominant product of 458 bp and a very weak product of 342 bp, representing the unspliced and spliced V_H region coupled to Cy3, respectively (Fig. 5b, lane 2). Using the B-CLL specific FR3 downstream primer, the V4-34 splice variant is also detectable in the 2J and the 1J transfectants (Fig. 5c, lanes 1 and 2). Here, no difference is visible between products with one JH3b or with two JH3b gene segments since in this PCR the downstream primer anneals 5' of the JH region. It is remarkable that in the 2J transfectant, the ratio spliced : unspliced transcripts is significantly higher than in the 1J transfectant. This suggests that the abnormal size and structure of V_H,D-2J favours the use of the cryptic splice sites in V4-34.

As we suspected that the A→C mutation in codon 64 was pivotal by causing a closer homology to the consensus 3' splice acceptor site (Fig. 3), we also transfected cells with 1J and 2J constructs in which this somatic mutation had been reversed. By RT-PCR we indeed found that in neither the 1J nor the 2J containing revertants, alternatively spliced V4-34 were detectable (Fig. 5b and 5c, lanes 3 and 4). We conclude that the alternative processing of V4-34 RNA is not invoked by a disturbed splicing machinery of the B-CLL but is V4-34 encoded and critically depends on one single point mutation.
Figure 5 (a) Schematic representation of the PCRs performed on cDNA derived from the transfectants.

(b) Results of VH4 - Cy PCRs on cDNA derived from SP2/0 transfected with plgH(y3) containing V_HD-2J and V_HD-1J respectively (lanes 1 and 2). In the lanes indicated with "Rev." the somatic mutation in codon 64 was reversed into the V4-34 germline nucleotide.

(c) Results of VH4 - FR3 PCRs on the same samples as in Fig.5b.

Figure 6 Comparison of the different 'N' regions proximal to each JH3b gene segment; | , nucleotide identical to the N-region; x, nucleotide not identical to the N-region.
Discussion

We analyzed IgH and IgL chain of an IgM/IgD-expressing B-CLL. The rearranged $V_H$ and $V_K$ genes contained 13 and 10 somatic mutations compared to the most homologous germline genes, V4-34 and DPK3/L11 respectively. Moreover, in the IgV$H$ locus a triplication of the JH3b gene segment that included parts of the JH3-JH4 intron was found. Directly upstream of the JH3b-II and the JH3b-III segments stretches of respectively 5 bp and 13 bp were present, of which each contained 4 bp homologous to putative N nucleotides directly upstream of the first JH3b segment (Fig. 6). This suggests that the JH3b triplication took place in an already rearranged IgV$H$ locus. Moreover, this N-region may have served as the target region in the triplication process that most likely took place in replicating cells, possibly via a repeated mechanism of DNA double strand break followed by homologous recombination. These alterations may have occurred in dividing precursors in the bone marrow after the initial IgH rearrangement had been completed. As mentioned, we found a stretch of 13 bp directly upstream of the JH3b-III segment which, in addition to the 4 bp sequence with homology to the JH3b-I N region, included 9 bp with no homology to sequences of the JH3-JH4 intron nor to any other region. Potentially, this 9 bp stretch may have been added due to expression of TdT and could be considered as a 'secondary' N region. Alternatively, the finding that this B-CLL exhibited somatically mutated IgV genes, indicates that the neoplastic cells were derived from a germinal center-experienced B cell, as has been described for approximately 50% of IgM-expressing B-CLLs [15]. Alternatively, as it has been demonstrated that the somatic hypermutation process not only involves nucleotide substitutions but also deletions and insertions/duplications of one or more nucleotides [24,25], the triplication could as well have occurred during the hypermutation process in a germinal center. An important argument that duplications occur during the GC reaction is the finding of both unique as well as shared point mutations in duplicated regions. In this B-CLL we found one mutation, shared only by the JH3b-I and JH3b-III segments. Assuming that the JH3b-I segment served as template, it is difficult to envisage why this mutation was not shared by the JH3b-II segment.

Because the triplicated region included parts of the JH3-JH4 intronic sequence, the normal 5′ splice donor site downstream of each JH3b copy was preserved. Thus, of this locus potentially three VH4 transcripts can be produced with either one, two or three JH3b gene segments coupled to the CH region genes. The transcripts with one JH3b gene segment,
encode a functional $V_H$ fragment. Also the transcripts containing two JH3b can potentially encode a $V_H$ fragment with an extended junctional region. By contrast, no functional $V_H$ fragment are to be expected from transcripts harbouring three JH3b gene segments, due to a frameshift just upstream of the third JH3b copy. The CDR3 PCRs performed on bacterial clones that had been derived from VH4 - JH RT-PCR products, yielded 112, 184 and 297 bp products, corresponding to $V_H$ regions with one, two and three JH3b copies, respectively. However, as the JH primer can anneal to each JH3b segment it is uncertain whether the cloned products with two JH3b segments are representative of truly existing transcripts or just PCR products derived from the existing transcripts with three JH3b regions. By using cDNA synthesized with oligo-dT primers, we confirmed that the transcripts with three JH3b copies contain a normal polyA tail. Furthermore, it was found that the three-JH3b containing transcripts were not spliced to the second or third $C\mu$ domain (CH2, CH3). Possibly, these $V_{\mu}$D-3J products represent remnants of primary transcripts of the rearranged locus. The CDR3-specific PCRs on the bacterial clones derived from the VH4-$C\mu$ and -$C\delta$ RT-PCR products, yielded only 112 bp products i.e. CDR3 lengths corresponding to $V(D)J$ joints with one JH13b copy only. This suggests that the majority of the synthesized $\mu$ and $\delta H$ chains, and thus the IgM and IgD proteins, are normal.

In eight of twenty RT-PCR derived clones, a deletion in the V4-34 gene segment was found (Table 1). It appeared that the sites flanking the deletion showed significant homology to the consensus splice acceptor, splice donor and branchpoint sequences. Two somatic mutations were of special interest as they brought about a closer homology to the consensus 3' splice acceptor and branch point sequences, respectively [34]. This, combined with the fact that we did not isolate clones with the V4-34 deletion out of gDNA PCR products strongly suggested that the deletion was the result of illegitimate splicing. In transfection studies of the V4-34 sequence in SP2/0 we demonstrated that the observed splicing of V4-34 was an intrinsic property of the particular V4-34 sequence itself and therefore could not be ascribed to an aberrant splicing machinery of the B-CLL. Moreover, as reversion of a single point mutation abolished the alternative splicing, we conclude that this particular somatic nucleotide substitution in V4-34 was critically important for the generation of a functional 3' splice acceptor site.

Alternative splicing of Ig genes has been previously described in human heavy chain disease (HCD), Burkitt's lymphoma cell lines and in multiple myeloma [35-39]. In all these malignancies, extensively truncated H chains are produced, often without associated L chain. The truncation of the abnormal H chain in $\gamma$- and $\alpha$-HCD usually involves part or all of the $V_H$ region and the whole CH1 domain. In most of these cases, the $V_H$ leader and a small part
Alternative splicing of heavy chain variable region in B-CLL

of the $V_H$ region is directly spliced to the hinge region. In most cases of $\alpha$-HCD also inserts of unknown origin have been found [40,41]. Furthermore, two intracellular $\mu$-HCD producing Burkitt's lymphoma cell lines have been described expressing truncated VH4 family genes in which the same cryptic 5' splice donor site at codon 26 was used as in our B-CLL [39,42]. However, also in these cell lines the 3' acceptor site was located in the $C_\mu$-CH1 exon. Thus, to our knowledge, this B-CLL provides the first example of alternative splicing within a $V_H$ gene. This mode of IgH splicing was due to the creation of a 3'splice acceptor site by a critical somatic point mutation.

In the previously reported cases, alternative splicing most likely rescued B cells harbouring non-functional $V_H$ genes with stop codons and/or frameshifts due to deletions or insertions. The mode of splicing observed in this B-CLL may theoretically have significance as an additional mechanism of somatic Ig diversification. It can be envisaged that in a V gene with an intrinsic insertion/duplication, alternative splicing may be a way to compensate for non-functional frameshifts or length aberrations. This is also suggested by the increased ratio spliced : unspliced $V_H$ in the 2J transfectant as compared to the 1J transfectant (Fig. 5c). On the other hand, alternative splicing implies that a B cell can potentially co-express different IgV molecules encoded by a single rearranged allele. Although it is doubtful whether this has any relevance during normal B cell selection processes, it may have pathophysiological significance. If e.g. such an alternatively spliced V gene product forms aberrant membrane complexes resulting in constitutive signaling, it could be instrumental in cellular transformation.

Materials and methods

Patient material. The patient was a 76 year old female who suffered from B-CLL with severe auto-immune hemolytic anemia, for which she was splenectomized. Spleen tissue was obtained from the department of pathology, the Westeinde Hospital, the Hague.

DNA -, RNA isolation and cDNA synthesis. DNA and RNA was isolated from frozen tissue sections or from transfected cells using the DNAZOL or the TRIZOL reagent, respectively (Life Technologies, Breda, the Netherlands) according to the manufacturer's instructions. cDNA was synthesised with Pd(N)6 random primers (Pharmacia Biotech, Roosendaal the Netherlands) or for some experiments with an oligo-dT primer (Promega, Leiden the Netherlands) as described [27].
**PCR reactions.** The complementary determining region 3 (CDR3) was amplified using a forward primer specific for the framework region 3 (FR3) in combination with one of the different downstream primers specific for JH, Cµ, Cγ, Cα or Cδ. For the V_H family-specific PCR, the reactions were performed with V_H family specific leader primers as described [27] combined with the appropriate reverse primer either JH, Cµ, Cδ or the B-CLL specific FR3 reverse primer. The different PCRs were performed with 1 µl of cDNA or 500 ng genomic DNA in a volume of 25 µl. The PCR mixture contained 1X Taq buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 0.2mM of each dNTP, 1.5 mM MgCl2 and 1 unit of recombinant Taq polymerase (Life technologies). In the CDR3-specific PCR and in the V_H family-specific PCR the primers were used in a concentration of 0.5 µM and 0.25 µM respectively. The PCR reactions were performed with a thermal cycler (PTC-100, MJ research Inc., Watertown, MA, USA). For the CDR3-specific PCR, 10 cycles were performed, 30 s at 95 °C, 20 s at 57 °C and 20 s at 72 °C. Next, 40 cycles of amplification were executed, 30 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C. The reaction was completed for 6 min at 72 °C. PCR products were analysed on a 3% Metaphor agarose gel (FMC Bioproducts, Rockland, ME, USA). For the V_H family-specific PCR, 30 cycles were performed, 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. The reaction was terminated for 6 min at 72 °C. The PCR products were analysed on a 1 % standard agarose gel (Sigma, St.Louis, MO, USA). Primer sequences:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR3</td>
<td>5'-GACACGGCC(T/C)(T/G)ATATTACTG-3'</td>
</tr>
<tr>
<td>JH</td>
<td>5'-GGACTAGTCTTCTTACCTGAGGAGCGTACC-3'</td>
</tr>
<tr>
<td>Cµ</td>
<td>5'-GGGTCCACCGAGGAGCGGAGGGGAAAAG-3'</td>
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<td>Cγ2</td>
<td>5'-AGACCGATGGGGCCTTTGGTG-3'</td>
</tr>
<tr>
<td>Cα</td>
<td>5'-GGGAAAGCGCTTGGGGCTG-3'</td>
</tr>
<tr>
<td>Cδ</td>
<td>5'-TGTCTGACCTTGATATGATG-3'</td>
</tr>
<tr>
<td>VH4</td>
<td>5'-AAATCGATACCACCACATGAACACCTGTTGGTCTT-3'</td>
</tr>
<tr>
<td>B-CLL FR3</td>
<td>5'-CGGCGGTTCAGAGCCTCAA-3'</td>
</tr>
<tr>
<td>Vk1</td>
<td>5'-AAATCGATACCACCACATGAGGAGGGTCCCC-3'</td>
</tr>
<tr>
<td>Jk</td>
<td>5'-GCGGCCGCCACTAACGGTCTGATCTCCACCTTG-3' [27]</td>
</tr>
</tbody>
</table>

**Cloning and sequencing** PCR products were cloned into pTOPO vectors and transformed into TOP10 bacteria, according to the manufacturer’s instructions (Invitrogen, Groningen, the Netherlands). The colonies were sequenced on both strands. Sequencing was performed with an ABI sequencer (Perkin Elmer Corporation, Norwalk, CT, USA) using the big dye-terminator cycle-sequencing kit. The sequences found were compared to published sequences.
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germline sequences, using the Vbase database [28] and DNAplot [29] on the internet (http://www.mrc-cpe.cam.ac.uk/imt-doc) to identify mutations.

**Expression vectors** The IgH(γ3) expression vector was produced by modifying the pRTM1 vector [30]. The IgH expression vector consists of a rearranged V(D)J gene flanked by ClaI and SpeI restriction sites, which are used to introduce the IgV\(_H\) gene of interest. Downstream, the C\(_{γ3}\) constant gene is located. The expression of the recombinant IgH is regulated by normal human IgH promoter and enhancer sequences. For selection in bacteria the β-lactamase gene is present that results in ampicillin resistance and for selection in mammalian cells the neomycin phosphotransferase gene is present which allows selection of stably transfected cells with geneticin. The IgL(κ) expression vector was produced starting from the pSVG-Vk3 vector [30]. The IgL(κ) expression vector consists of a rearranged VJ gene flanked by ClaI and NotI restriction sites. These restriction sites are used to introduce the IgV\(_\kappa\) gene of interest. Downstream, the κ constant gene is located. The expression of the recombinant Igκ is regulated by normal human Igκ promoter and enhancer sequences. Detailed description of the generation of these immunoglobulin expression vectors is available on request. The pRTM1 and the pSVG-Vk3 vectors were kindly provided by Dr. Kipps (University of California, San Diego, CA, USA) and the IgH(γ3) and IgL(κ) expression vectors were kindly provided by Drs. J. van Es and T. Logtenberg (University Hospital, Utrecht, the Netherlands).

**Generation of transfectants in SP2/0 cells** For transfection, 10 μg IgH(γ3) and 10 μg IgL(κ) were linearised with PvuI and mixed with 10x10^6 SP2/0 myeloma cells in Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS (HyClone), 100 IU/ml penicillin and 100 μg/ml streptomycin. The suspension was electroporated in a 0.4 cm gap cuvette using a Biorad gene pulsar with capacity extender at 960 μF and 250 V. The cells were selected and cultured in IMDM complete medium containing 400 μg/ml geneticin (Life technologies).

**Nucleotide reversion of the somatic mutation in codon 64** Primers were designed that matched around codon 64. One primer (B-CLL-R) included a mismatch that reversed the mutation in codon 64 to the germline nucleotide. The V4-34 of the B-CLL was amplified in two separate parts. The 5' part was amplified with the VH4 family specific leader primer and the downstream primer (B-CLL-R) with the mismatch that reversed the somatic mutation. The 3' part was amplified with a forward primer (B-CLL-F) located adjacent to the mismatch primer and combined with the JH downstream primer. These PCRs were performed according to the protocol used for the V\(_H\) family-specific PCR except that Pfu polymerase was used (Stratagene, La Jolla, CA, USA). The two parts were purified from gel, combined and used as
template in an amplification reaction with the VH4 family specific leader primer and the JH primer downstream primer using Taq polymerase. Bacterial clones were generated and from these clones the V4-34 segment was sequenced to confirm the reversion of the somatic mutation in codon 64. Next this B-CLL V4-34 fragment was cloned into the IgH(γ3) expression vector as described above. Primer sequences: B-CLL-F 5' - GAGTCACCATATCAGTGGAC-3', B-CLL-R 5' - GACTCTIGAGGGACGGGTTG-3'. The underlined nucleotide is a mismatch which reverts the somatic mutation in codon 64.

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References


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