Immunoglobulin gene alterations in the progression of B cell lymphomas
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Antigen receptors and somatic hypermutation in B-cell Chronic Lymphocytic Leukemia with Richter’s transformation

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Antigen receptors and somatic hypermutation in B-cell Chronic Lymphocytic Leukemia with Richter’s transformation

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Abstract

Background and objective: Activation-induced cytidine deaminase is essential for somatic hypermutation and class switch recombination of the immunoglobulin genes in B cells. It has been proposed that aberrant targeting of the somatic hypermutation machinery is instrumental in initiation and progression of B-cell non Hodgkin’s lymphomas. In this study, we investigated the B-cell receptor and the role of the somatic hypermutation machinery in B-cell chronic lymphocytic leukemias (B-CLL) prior and after transformation to a lymphoma of a higher malignancy grade (Richter’s transformation).

Design and Methods: We investigated the activity of the somatic hypermutation machinery in 9 B-CLL and secondary diffuse large B-cell lymphomas by measuring the expression of Activation-induced cytidine deaminase, in combination with mutation analysis of immunoglobulin (Ig) and non-Ig genes. Furthermore, the structure of the antigen receptors of B-CLL known to have developed a Richter’s syndrome (RS B-CLL) was analyzed by comparing the most variable region of the Ig, the CDR3 region, to CDR3 sequences present on GenBank.

Results and Interpretation: Ig variable heavy chain (IgV_{H}) gene studies revealed that Richter’s transformation occurs almost exclusively in unmutated B-CLL. Furthermore, AID expression and somatic hypermutation activity of most RS B-CLL were found higher than those of control (non-transforming) B-CLL. Finally, comparison of the IgV_{H}-CDR3 regions showed a remarkable amino acid sequence homology between 2 RS B-CLL of our panel and 2 RS B-CLL described in literature.

Conclusion: The combined findings suggest a role for the Ig gene diversification apparatus during Richter’s transformation and show that distinct RS-B-CLL may recognize recurrent antigenic epitopes.
Introduction

Richter’s syndrome (RS) is the rare occurrence of a histologically and clinically aggressive secondary lymphoid malignancy in a patient with B-cell chronic lymphocytic leukemia (B-CLL) (1, 2). In approximately 3-5 % of B-CLL cases, a lymphoma of a higher malignancy grade develops, reducing the mean disease-free survival to 6 months (2). Usually the high-grade lymphoma is classified as a diffuse large B cell lymphoma (DLBCL) and less commonly as a Hodgkin’s lymphoma (3).

B-CLL is characterized by an accumulation of long-lived, monoclonal CD5+ CD23+ mature B cells that express low levels of membrane-bound immunoglobulin (1). About 50% of the B-CLL harbor somatic mutations in their immunoglobulin variable heavy chain (IgVH) genes (4). Although the difference in mutation status suggests a different cell of origin, gene expression profiling revealed that mutated and unmutated B-CLL are both most similar to normal memory B cells (5, 6). B-CLL express an IgVH repertoire clearly distinct from the IgVH repertoire of normal B cells of any lineage or maturational stage (4). It has been reported by others and us that ~ 19 % of B-CLL, mostly unmutated, express IgVH CDR3 amino acid sequences homologous with CDR3 regions of other B-CLL (so called inter-B-CLL CDR3 homology) (7-11). As yet, at least 8 B-CLL IgVH CDR3 homology groups have been defined (10). The occurrence of highly homologous B-cell receptors among B-CLL strongly suggests that they recognize a limited set of distinct antigenic determinants.

It is unknown to what extent the somatic hypermutation machinery is active in B-CLL. It is generally assumed that B-CLL have a low tendency to acquire additional mutations over time (12). Accordingly, the overall expression level of the enzyme that is essentially required for both somatic hypermutation and class switch recombination, i.e. Activation-induced cytidine deaminase (AID) (13, 14), is very low in blood-derived B-CLL samples as compared to those of purified germinal centre (GC) B cells (15, 16). It has been reported that only a small fraction (<1%) of circulating B-CLL cells, particularly of the IgVH-unmutated subgroup, expresses AID (17). However, in whole lymph node samples, where the CD40-expressing B-CLL cells are in close contact with CD40L-expressing CD4+ T cells, overall AID expression was found to be higher (18). In accordance, in vitro stimulation of B-CLL cells by CD4+ T cells and anti-B-cell receptor antibodies, induces somatic hypermutation in the IgVH genes (19).

It has been proposed that promiscuous targeting of the somatic hypermutation machinery may be an initial event in the development of a number of DLBCL (20). It is not known whether this mechanism also applies to progression of low-grade B-non Hodgkin’s lymphomas (B-NHL). In the current study, we analyzed the B-cell receptor and the process of somatic hypermutation in a panel of B-CLL with documented transformation. Our data suggest restricted B-cell receptor specificities and an active somatic hypermutation machinery in B-CLL undergoing Richter’s transformation.
Material and Methods

Patient material.
All lymphomas were diagnosed according to the WHO classification system (1). Lymph node material of RS1, RS8 and the control B-CLL was freshly frozen in liquid nitrogen directly after surgical removal. Immunohistochemical analysis of RS1 and RS8 revealed that more than 80% of the tissue consisted of tumor cells. Of all other RS cases and of the peripheral blood samples of the control B-CLL, cell suspensions were frozen in 20% DMSO (Merck, Darmstadt, Germany) in FCS (Invitrogen, Breda, The Netherlands). This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

FACS analysis and cell sorting.
The following monoclonal antibodies (mAb) were used for FACS analysis: PE-conjugated anti-CD23 (clone EBVCS-5; Dako, Glostrup, Denmark), PE-conjugated or FITC-conjugated anti-CD5 (clone L17F12; Dako) and APC-conjugated or PerCP/Cy5.5-conjugated anti-CD19 (clone SJ25C1; Becton Dickinson Biosciences, Erembodegem-Aalst, Belgium). The following polyclonal antibodies were used: FITC-conjugated anti-Igκ, anti-IgM, anti-IgD and anti-IgA, PE-conjugated anti-Igλ, and anti-IgG (polyclonals from Southern Biotechnology Associates, Birmingham, AL). FACS analyses revealed that the cell suspensions of RS3, RS9, RS10, RS11c and RS12 consisted of >90% tumor cells, whereas the peripheral blood sample of RS11a consisted of 60% tumor cells. Of RS4 and RS6, the CD5+, CD19+ tumor cells were isolated using a FACS-Aria (BD Biosciences) cell sorter what resulted in more than 97% pure tumor samples. Germinal centre (GC) B cells were sorted as described previously (15, 21).

Immunohistochemistry.
AID was visualized in formalin-fixed, paraffin-embedded tissue sections using a rat monoclonal antibody (22). After deparaffination, blocking and antigen retrieval the slides were incubated overnight at 4°C with the primary antibody (1:1000), followed by application of an HRP-conjugated rabbit-anti-rat antibody (1:200, Dako). Subsequently, biotin-free tyramide signal amplification (Dako CSAII kit) enabled detection of AID, which was visualized with Nova Red (Vector). A hyperplastic tonsil functioned as a positive control, omission of the primary antibody as a negative control. Monoclonal antibodies specific for CD5 (Lab vision, Neomarkers, Fremont, CA), CD23, BCL6, and Ki67 (all from Dako) were used. Antibody detection was performed with the Powervision † system (ImmunoVision Technologies, Daly City, CA).
DNA isolation, RNA isolation and cDNA synthesis.
RNA and DNA were isolated using the Trizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer’s description. First-strand complementary DNA (cDNA) was synthesized as described previously albeit 5’-(dT)₁₄-d(A/G/C)-d(A/G/C/T)-3’ primers were used (15).

Amplification, cloning and sequencing.
IgVH transcripts were amplified using a mixture of forward primers located in the FR1 regions of the IgVH gene families VH₁ to VH₆ or alternatively in the FR3 region of VH₁ to VH₆ (23) in combination with one of the FAM-labeled reverse primers located in Cμ, Cδ, Cu or Cγ regions (24). The PCR reaction was performed as described previously using a 30 cycles program (24) and run on an ABI PRISM 3100 automated sequencer in the presence of either 1pM ROX 500 or 1pM ROX1000 marker (Applied biosystems, Warrington, UK). Results were analyzed using the program Genescan analysis (Applied Biosystems). When a monoclonal tumor population was present, IgVH amplicons were cloned into the pTOPO-TA vectors and transformed into TOP10 bacteria according to the manufacturer’s description (Invitrogen) and 8 to 16 clones were sequenced of each lymphoma. Sequencing on both strands was performed by an ABI PRISM 3100 automated sequencer (Applied Biosystems) using the big dye-terminator cycle-sequencing kit (Perkin Elmer Corporation). The consensus IgVH sequence is defined as the nucleotide sequence that is shared by more than 50% of the clones. Nucleotide alterations that are present in less than 50% of the clones are considered as intraclonal variation. Of note, according to our nomenclature, nucleotide alterations that are present in multiple clones (confirmed nucleotide differences) but are present in <50% of the clones are thus still regarded as intraclonal variation. The amount of intraclonal variation (ICV) was calculated as the mean number of nucleotide differences per clone compared to the consensus IgVH sequence and was considered significant when it was higher than the Taq error rate. To determine the Platinum Taq error rate of our experimental design, 48 clones of HPRT were sequenced using the primers 5’TTCCTCCTCCTGAGCAGTCAGC3’ and 5’GCGATGTCAATAGGACTCCAGATG3’. These clones were generated according to the same PCR and cloning procedures as used for the IgVH genes. The Taq error frequency thus established is 0.2 per 300 bp. BCL6 was amplified using the primers 5’CCGCTGCTCATGATCATT3’ and 5’CAGACTCGAGTCTTCCCATGGATCCACC3’. PIM1 was amplified and sequenced using the primers 5’AGCAGCAGGCAACCACACTAG3’ and 5’CTCTCCCAGTCCGAAATCC3’. The PCR mixtures contained 1x pfX amplification.
buffer, 1U platinum pfx DNA polymerase (Invitrogen), 1mM (BCL6) or 2.5mM (PIM1) MgSO₄, 0.2mM of each dNTP, 0.5mM of each primer and 1x enhancer solution. Both BCL6 and PIM1 PCR reactions started with 3 minutes at 94°C, followed by 39 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 68°C. The reaction was terminated for 4 minutes at 68°C. BCL6 and PIM1 amplicons were cloned as described above and 12-24 clones were sequenced. BCL6 was sequenced using the primers 5’CCGCGTCTCAGTCATTT3’ in combination with 5’GCAAGCGAGAAAAGAGGA3’ and 5’GTACGCGCTTGATCTCTCTT3’ in combination with 5’CAGACTCGAGTCTCAGTGATCCAC3’. BCL6 and PIM1 were amplified from DNA. Since DNA harbors two alleles of all genes, the consensus BCL6 and PIM1 sequence was defined as the nucleotide sequence that is shared by more than 25% of the clones.

QRT-PCR reactions.
Quantitative RT-PCR analyses were performed using a LightCycler (Roche, Almere, The Netherlands). AID was amplified using the primers 5’AGAGGCGTGACATCGACTACA 3’ and 5’TATAGCGGAGGAGGACAT3’ matching sequences in the 3’ end of exon 2 and 5’ end of exon 3, respectively. All reported AID splice variants are detected by this PCR (but not discriminated) except for the splice variant lacking the 3’ end of exon 2 and whole exon 3 (25). β-Actin was amplified using the primers 5’GGATGCAGAAGGAGATCACTG 3’ and 5’CGATCCACAGGAGTACTTT3’. The PCR reactions for both AID and β-actin were performed in a volume of 10 μl containing 2 μl cDNA, 1 μl FastStart DNA Master PLUS SYBR Green I mix (Roche) and 0.5 pM forward and reverse primers. The PCR protocols to amplify AID and β-actin started with 95°C for 6 minutes, after which 40 cycles of amplification were performed, i.e. successively 10 seconds at 95°C, 5 seconds at 60°C (AID) or 61°C (β-actin) and 5 seconds at 72°C (AID) or 8 seconds at 72°C (β-actin). Melting curve analysis was performed to check for PCR specificity. Starting concentrations of mRNAs and PCR efficiencies for each sample were calculated using the LinRegPCR computer program as described before (26). Results are expressed as ratios of the calculated values of AID and β-actin.

In vitro stimulation of B-CLL cells.
B-CLL cells were cultured for four days in 24-wells plates (Costar, Corning NY, USA). Each well contained 2 x 10⁵ B-CLL cells and 1 x 10⁵ L cells as a control or 1 x 10⁵ CD40L-transfected L cells and 400 U/ml IL4 (Strattmann, Hannover, Germany) with and without anti-IgM (clone MH15/1)(Sanquin, Amsterdam, The Netherlands) coupled CNBR-activated sepharose beads (Amersham biosciences, Uppsala, Sweden). As a positive control peripheral blood B cells of healthy volunteers were stimulated with each experiment.
Results

B-CLL with Richter’s transformation.
Tumor samples of 9 B-CLL that underwent clinical and histological progression to a DLBCL were analyzed. RS1, RS3, RS4 RS6 and RS10 presented as a monoclonal population of small CD5+, CD19/CD20+, sIg<sup>+</sup> B-CLL cells which over time transformed into a DLBCL (Table 1). RS8 already showed signs of transformation at presentation with, next to small tumor cells, a subpopulation of centroblast-like cells with abundant basophilic cytoplasm and irregular nucleoli. In a lymph node sample of RS8 one year later, the percentage of centroblast-like cells had clearly increased. RS9, RS11 and RS12 have been described previously as case 9, case 3 and case 8 respectively (27).

Table 1. 9 B-CLL with clinical and histological progression to DLBCL.

<table>
<thead>
<tr>
<th>RS</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;</th>
<th>D</th>
<th>J&lt;sub&gt;H&lt;/sub&gt;</th>
<th>CDR3 (no. amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;1-69 (V&lt;sub&gt;H&lt;/sub&gt;1.2)</td>
<td>3-10 (fr.1)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;6b</td>
<td>GGRQELLFGEFDYNYGMDV (21)</td>
</tr>
<tr>
<td>RS3</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;4-4b (V&lt;sub&gt;H&lt;/sub&gt;4.22)</td>
<td>5-12 (fr.1)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;6b</td>
<td>GLNIYATGDY (10)</td>
</tr>
<tr>
<td>RS4a</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;4-39 (DP79)</td>
<td>6-13 (fr.1)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;5b</td>
<td>NSGYSWFRGYSFWDPL (17)</td>
</tr>
<tr>
<td>RS4b</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;4-39 (DP79)</td>
<td>6-13 (fr.1)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;5b</td>
<td>NSGYSWFRGYNFDPL (17)</td>
</tr>
<tr>
<td>RS6</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;5-51 (DP73)</td>
<td>n.a.</td>
<td>JH2</td>
<td>RPLQWPLRYWFDL (15)</td>
</tr>
<tr>
<td>RS8</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-30/30.5 (DP49)</td>
<td>3-22 (fr.2)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;6c</td>
<td>GGDYDSSYGGLYNYMMDV (22)</td>
</tr>
<tr>
<td>RS9</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;1-69 (DP10)</td>
<td>2-21 (fr.2)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;6b</td>
<td>VAGVAYCGDCYVRSEFYFDY (20)</td>
</tr>
<tr>
<td>RS10</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-74 (DA8)</td>
<td>3-16 (fr.2)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;3b</td>
<td>DAWRPAPAYDYFV (14)</td>
</tr>
<tr>
<td>RS11</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;3-11 (DP35)</td>
<td>3-09 (fr.2)</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;5b</td>
<td>DSVWYKDILTGYSPQLVSNNFDPL (24)</td>
</tr>
<tr>
<td>RS12</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;1-8 (DP15)</td>
<td>n.a.</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;2</td>
<td>ASSYDSGDDYLYSLCLL (16)</td>
</tr>
</tbody>
</table>

BM indicates bone marrow; LN, lymph node; nd not done; PB, peripheral blood. * Time interval in months between samples. † RS9, RS11 and RS12 are previously described as Case 9, Case 3 and Case 8 (27).

Ig<sub>H</sub> genes and CDR3 regions of B-CLL with Richter’s transformation.
To establish the clonal relationship between the tumor populations at presentation and after relapse, the rearranged Ig <sub>H</sub> DJ<sub>H</sub> genes were amplified by RT-PCR and sequenced (Table 2). In all RS cases, the B-CLL and DLBCL cells proved clonally related. Interestingly, sequence analysis revealed that the Ig<sub>H</sub> genes of all 9 RS cases in our panel were unmutated (<2% consensus mutations) (Table 4). The consensus Ig <sub>H</sub> DJ<sub>H</sub> sequences of most of the RS cases remained unaltered over time. However, RS4 and RS6 had each acquired an additional consensus mutation (i.e. a mutation found in more that 50% of the molecular clones) in
their respective IgV_{H} genes after transformation (Table 4). RS4 also acquired an additional replacement mutation in the third complementary determining region (CDR3) after transformation in more than 50% of the clones (Table 2).

Table 2. Immunoglobulin variable heavy chain genes and CDR3 amino acid sequences of 9 B-CLL with clinical and histological progression. CDR indicates complementarity determining region; n.a., The D gene was not assigned.

<table>
<thead>
<tr>
<th>RS</th>
<th>time*</th>
<th>diagnose</th>
<th>Source</th>
<th>CD5</th>
<th>CD23</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1a</td>
<td>20</td>
<td>B-CLL</td>
<td>nasopharynx</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS1b</td>
<td>5</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>nd</td>
<td>nd</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS2a</td>
<td>5</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>nd</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS3a</td>
<td>5</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS3b</td>
<td>5</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS4a</td>
<td>14</td>
<td>B-CLL/DLBCL</td>
<td>BM</td>
<td>+</td>
<td>+</td>
<td>IgG</td>
</tr>
<tr>
<td>RS4b</td>
<td>14</td>
<td>B-CLL/DLBCL</td>
<td>PB</td>
<td>+</td>
<td>nd</td>
<td>IgG</td>
</tr>
<tr>
<td>RS5a</td>
<td>86</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS5b</td>
<td>86</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS6a</td>
<td>4</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS6b</td>
<td>4</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>IgG</td>
</tr>
</tbody>
</table>

The IgV_{H}-CDR3 region is the most hypervariable region of the Ig and is considered to contribute most to its antigenic specificity. Nevertheless, ~19% of B-CLL, mostly unmutated, express CDR3 sequences with homology to CDR3s of other B-CLL (inter-B-CLL CDR3 homology), which suggests that a limited set of distinct antigenic determinants is recognized by these B-CLL. This prompted us to investigate the IgV_{H}-CDR3 region of B-CLL known to have developed a Richter’s syndrome (henceforth called RS B-CLL). The IgV_{H}-CDR3 amino acid sequences of the RS B-CLL described in this study and all RS B-CLL described in literature were compared to all CDR3 amino acid sequences available on GenBank (Table 3). For this purpose, we used the NCBI Protein-Blast program with the option “search for short nearly exact matches” (BLASTP 2.2.6 [apr-09-2003]) as reported previously (10).
Briefly, CDR3 regions consisting of at least 7 amino acids were analysed. An IgVH-CDR3 sequence was considered to be homologous to other CDR3 sequences (i) if sharing at least 75% amino acid sequence homology. (ii) A length difference between the CDR3 sequences was allowed if not exceeding 3 amino acids (maximum gap of 3 amino acids).

Table 3. Homology between IgVH-CDR3 amino acid sequences of the 8 RS B-CLL and IgVH-CDR3 amino acid sequences present on GenBank.

<table>
<thead>
<tr>
<th>RS</th>
<th>Reference</th>
<th>CDR3 homology</th>
<th>Patient/Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1</td>
<td>this study</td>
<td>1 normal B cell clone</td>
<td>ya0208</td>
</tr>
<tr>
<td>RS4a</td>
<td>this study</td>
<td>1 RS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 B-CLL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 normal B cell clone</td>
<td></td>
</tr>
<tr>
<td>RS4b</td>
<td>this study</td>
<td>1 RS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 B-CLL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 normal B cell clone</td>
<td></td>
</tr>
<tr>
<td>RS8</td>
<td>this study</td>
<td>1 RS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 B-CLL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-polysacc. of N. Meningitis Ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC15</td>
<td></td>
</tr>
<tr>
<td>case3</td>
<td>Matolesy et al.(29)</td>
<td>RS8 (this study)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 EBV B cell in AITL</td>
<td>case2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-polysacc. of N. Meningitis Ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-natural Sm Ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-Rota virus Ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 normal B cell clones</td>
<td></td>
</tr>
<tr>
<td>B-CLL57</td>
<td>Ghiotto et al.(11)</td>
<td>RS4a/b (this study)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 B-CLL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 normal B cell clones</td>
<td></td>
</tr>
<tr>
<td>RS 3557</td>
<td>Matolesy et al.(30)</td>
<td>normal B cell clones</td>
<td>2CB4N2, A29A29</td>
</tr>
<tr>
<td>B-CLL4</td>
<td>Aoki et al.(39)</td>
<td>1 B-CLL</td>
<td>YarVH*</td>
</tr>
<tr>
<td>case2</td>
<td>Ohno et al.(38)</td>
<td>1 normal B cell clone</td>
<td>PBT-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-Staphylococcal protein A Ab</td>
<td>4D5</td>
</tr>
</tbody>
</table>

Ab indicates antibody; AITL, angioimmunoblastic T cell lymphoma.

* B-CLL YarVH was 73% homologous to B-CLL4 instead of at least 75% like the other cases in this table.

* GenBank accession numbers: ya0208, AB067329; CLL57, X84339; CLL8, AY486198; CLL9, AY486207; CLL202, AY268373; HI81, Y09249; CLL114, AY268372; CLL209, AY300037; CLL32, AY486216; SC15, AF115134; BUD94, Z46379; RVI-22, AY86908; 102-17, AF208108; MBT-159, U32960; CLL39, X84336; CLL7, AY486206; SC77U-44, AF174118; 2CB4N2, AY671324; A29A29, AF460484; YarVH, AF999199; PBT-16, U3220; 4D5, PH1650.
Of the 18 RS B-CLL that were thus studied, 8 (44%) fulfilled our criteria for CDR3 homology with CDR3 amino acid sequences present on GenBank (Table 3). The CDR3 regions of these 8 RS cases were homologous to the CDR3 regions of 12 normal B-cell clones and 9 B-CLL without reported transformation (Table 3). These latter B-CLL all expressed unmutated \( \text{IgV}_{\mu} \) genes, except B-CLL YarVH (28) whose \( \text{IgV}_{\mu} \) genes harbored 5 mutations. Interestingly, we also observed \( \text{IgV}_{\mu} \)-CDR3 amino acid homology among different RS B-CLL. RS8 expressed a \( \text{V}_{\mu3-30}/\text{D3-22}/\text{JH6} \) rearrangement and the \( \text{IgV}_{\mu} \)-CDR3 amino acid sequence showed ≥75% homology to the CDR3 sequence of RS case 3 of which the \( \text{V}_{\mu}\text{DHJ} \) rearrangement was unfortunately not described (29). In addition, the \( \text{IgV}_{\mu} \)-CDR3 amino acid sequence of RS8 showed 68% homology to the \( \text{IgV}_{\mu} \)-CDR3 amino acid sequence of RS 3557 (30) although it must be noted that the latter expressed a \( \text{V}_{\mu3-74}/\text{D3-09}/\text{JH6} \) rearrangement (Figure 1).

RS4 showed \( \text{IgV}_{\mu} \)-CDR3 amino acid homology (≥75%) with RS B-CLL57 (31). RS4 and RS B-CLL57 not only expressed the same \( \text{V}_{\mu}\text{DHJ} \) rearrangement (i.e. \( \text{V}_{\mu4-39}/\text{D6-13}/\text{JH5} \)) but also the same \( \text{V}_{\mu} \)-CDR3 homology (data not shown). Based on the \( \text{IgV}_{\mu} \)-
CDR3 homology that is observed between B-CLL, 8 homology groups have been defined (10). B-CLL57 is an IgG+ B-CLL that shows homology with 4 other unmutated V_h4-39 expressing IgG+ B-CLL (11). The IgV_h-CDR3 region of the first time point of RS4 (RS4a) was homologous to 2 B-CLL of this homology group (B-CLL57 and B-CLL202).

![Table showing amino acid sequences and homology](image)

Figure 1. IgV_H-CDR3 amino acid sequence homology of RS4 and RS8 with IgV_H-CDR3 of 3 previously described RS B-CLL. The IgV_H-CDR3 amino acid sequence of RS4 is homologous to the IgV_H-CDR3 amino acid sequence of B-CLL57. Both RS B-CLL expressed the same V_HDJH gene rearrangement. The IgV_H-CDR3 amino acid sequence of RS8 is homologous to the IgV_H-CDR3 amino acid sequences of case 3 and 3557. Whereas RS8 expressed the same V_HDJH gene rearrangement as case 3, it differed from the V_HDJH gene rearrangement of 3557. Amino acids are depicted by the single letter code. FR3 and FR4 indicate framework region 3 and 4; N, amino acid encoded by the non-templated nucleotides; D, gene segment; JH, gene segment; | identical amino acid; +, similar amino acid; Hom, percentage of homologous amino acid; Id, percentage of identical amino acid; Gap, length difference in amino acid of the compared IgV_H-CDR3 sequences.

Remarkably, due to an extra mutation in the IgV_H-CDR3 region, the DLBCL of RS4 (RS4b) shared homology with a total of 4 B-CLL of this CDR3-homology group (B-CLL57, CLl202 and additionally CLl14 and CLl209) (11) (Figure 1). This B-CLL subgroup, previously denoted by us as homology group 6, is thus extended by our IgG+ RS4 and now includes a total of 6 B-CLL, 2 of which underwent Richter’s transformation over time. In conclusion, these data demonstrate that Richter’s transformation occurs preferentially in unmutated

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B-CLL. Furthermore, we found that the most hypervariable region of the Ig gene, the CDR3 region, was highly homologous among distinct RS B-CLL cases.

Figure 2. Relative AID expression levels of RS B-CLL and control B-CLL before and after stimulation. Quantitative RT-PCR analysis of AID and β-actin was performed on peripheral blood samples of a panel of 15 B-CLL without reported transformation and 5 B-CLL that transformed to a DLBCL. To induce AID expression, the B-CLL and RS samples were cultured for three days in the presence of IL4 and CD40L. RSa indicates the tumor sample before Richter’s transformation and RSb indicates the tumor sample after transformation to a DLBCL. Each dot represents the average value of at least three AID/β-actin ratio measurements.

Endogenous and induced expression of Activation-induced cytidine deaminase.

Next, we analyzed the role of the somatic hypermutation machinery during Richter’s transformation. To this end, we quantitatively measured the expression of AID in 5 RS B-CLL before and after transformation and compared these to the expression levels of peripheral blood samples of 15 control B-CLL and of sorted tonsillar germinal center B-cell fractions (Figure 2). AID expression was not quantifiable in any of the 9 (3 IgM+, 6 IgG+) mutated B-CLL, nor in 6 unmutated B-CLL (IgM+). Interestingly, 4 of 5 RS B-CLL did express measurable levels of AID, although the AID/β-actin ratios were clearly below the ratios observed in germinal center B cells (Figure 2).

It has been described that CD40 engagement induces AID expression in B cells(25, 32). To investigate if the malignant cells were still responsive to environmental stimuli with respect to their AID expression, 3 RS B-CLL (RS3, RS4, RS6) and 14 control B-CLL were cultured for three days on either untransfected or CD40L-transfected L cells in the presence of IL4 and anti-IgM coupled sepharose beads. As positive controls, peripheral blood B cells and an EBV B cell line were used. Under these conditions, AID expression was increased in healthy donor peripheral blood B cells, the EBV B-cell line (data not shown) and in the mutated (3 IgM+ and 6 IgG+) and unmutated (6 IgM+) B-CLL. However, the stimulated RS B-CLL expressed significantly higher levels of AID, both before and after transformation, as compared to both control B-CLL groups (Figure 2).
Figure 3. AID protein expression in RS8 and control B-CLL. Haematoxylin&Eosin, Ki67, AID and CD5 stainings on lymph node material of an unmutated B-CLL (upper panel), RS8a (middle panel) and RS8b (lower panel). Small B-CLL cells are in all cases negative for AID, whereas proportions of blastoid cells of RS8a and RS8b show clear cytoplasmic AID staining. Magnification 25x.

To further investigate the role of AID in Richter’s transformation, the expression of this protein was visualized immunohistochemically in paraffin-embedded tissue sections of two RS patients (RS1 and RS8), of 11 lymph node samples of control B-CLL and of a tonsil (Figure 3). In the latter, AID was found within the germinal center blasts and in scattered extrafollicular centroblast-like cells (not shown), as was previously reported by Greiner et al (22). In accordance with the mRNA expression data of blood-derived B-CLL samples, in 9 out of 11 B-CLL lymph node specimens no AID-expressing cells were present. In one unmutated control B-CLL, sporadic AID-expressing paraimmunoblasts were found in some proliferation centers (Figure 3, upper panels). In another control B-CLL, more AID-expressing centroblast-like cells were observed. However, since in this biopsy scattered residual germinal centers were present we could not exclude that these centroblast-like cells were GC related (data not shown). No QRT-PCR data were available on these patients to confirm these findings. In contrast to the control B-CLL and in accordance with the QRT-PCR data, AID-expressing cells were present in both RS cases of which lymph node material was available, i.e. RS1 and RS8 (Figure 3, middle and lower panel). It is noted that in both tumors, cytoplasmic AID expression was never observed in the small B-CLL cells but was confined to the centroblast-like cells. Immunohistochemical staining for BCL6, CD21 and BCL2 excluded the presence of residual germinal centers in the tissues (data not shown).
Somatic hypermutation in IgV_{H} and non-Ig genes in B-CLL undergoing Richter’s transformation.

Since both quantitative RT-PCR and immunohistochemistry demonstrated that AID is expressed in RS B-CLL, we searched for evidence that the somatic hypermutation machinery indeed has been active in the tumor cells. Individual molecular clones of the amplified IgV_{H} genes of RS1, RS3, RS4, RS6, and RS8 were sequenced. The degree of intraclonal sequence variation in IgV_{H} was compared with that of 6 unmutated and 9 mutated B-CLL without reported transformation. We found significant intraclonal variation (i.e. a mutation frequency higher than the Taq error rate determined in our laboratory) in several IgV_{H} mutated- and unmutated B-CLL and in 2 of the 9 RS B-CLL (RS4\textsuperscript{a} and RS6\textsuperscript{b}). The degree of intraclonal variation was low with a mean of 0.3 nucleotide differences per clone (Table 4). The observed nucleotide differences were present in only a minority of the clones. Finally, the nucleotide differences that accounted for the ICV in the RS B-CLL were non-confirmed and present in single clones only. The IgV_{H} genes are not the only genes that can be targeted by the somatic hypermutation machinery. Since BCL6 and PIM1 are described to be mutated in DLBCL as well (20), we amplified, cloned and sequenced these genes in selected RS B-CLL (Table 4). Of RS1, RS3, RS4 and RS8, 790 basepairs (bp) downstream of the transcription initiation site of BCL6 were analyzed. This region includes part of the first intron (position 358 to 1148 according to GenBank AY189709). In RS4 and RS8 one polymorphism (G \rightarrow C) at position 754 was found in all clones. The consensus sequence of RS1 already harbored one mutation (C \rightarrow T) at position 897 before Richter’s transformation. Interestingly, after transformation an additional (T \rightarrow C) mutation was found in the consensus sequence at position 1075. Although RS1 and RS3 showed a low degree of intraclonal variation in BCL6 (0.4 and 0.5 per 300 bp per clone respectively, all nucleotide alterations were non-confirmed and found in single clones only), this was significant and higher than the intraclonal variation observed in their IgV_{H} genes (≤0.2 per IgV_{H} gene). No intraclonal variation was observed in BCL6 of RS4 and RS8. For PIM1, 600 bp downstream of the transcription initiation site was analyzed in RS1 and RS8 (position 859 to 1623 according to GenBank AF386792). In both RS B-CLL the consensus sequence harbored a polymorphism (C \rightarrow G) at position 1039. Neither mutations nor intraclonal variation were found in this region in either of these lymphomas. Taken together, quantitative RT-PCR and immunohistochemistry both demonstrated that AID is expressed in RS B-CLL. Furthermore we observed a low but distinct degree of ongoing hypermutation in either the IgV_{H} genes or BCL6, indicating that the hypermutation machinery indeed has been active during Richter’s transformation.
Discussion

To our knowledge, a total of 97 transformed B-CLL have so far been described (27, 29, 30, 33-52). In 74 B-CLL patients (76%), the secondary lymphoma was classified as a DLBCL whereas in 23 B-CLL patients (24%) a Hodgkin’s lymphoma developed. Overall, in 67 of the 97 RS cases (69%), the high-grade lymphomas were of the same clonal origin as their low-grade precursors. Clonality was in most studies assessed by southern blot analyses. IgVH-CDR3 sequences of only 9 of these 67 RS B-CLL were available. Here we present 9 additional RS B-CLL in which the high-grade lymphomas were all of the same clonal origin as the preexistent B-CLL. Interestingly, of these altogether 18 RS B-CLL, 16 belonged to the unmutated subgroup, indicating that Richter’s transformation occurs almost exclusively in this subset of B-CLL. It is noted that this finding does not necessarily account for the well documented poor prognosis of the unmutated B-CLL subset, since Richter’s transformation is a rare phenomenon (53, 54).

Previously, several groups including ours have reported that the IgVH-CDR3 amino acid sequences of a significant fraction of B-CLL are highly homologous, particularly within the group of unmutated B-CLL (7-11). This type of homology is unique for the group of B-CLL, and was not found within extensive cohorts of follicular lymphomas, DLBCLs, Burkitt’s lymphomas and multiple myelomas (10). This suggests that a proportion of B-CLL recognizes recurrent antigenic epitopes. Of the 18 RS B-CLL analyzed in this study, the CDR3 region of as many as 8 (44%) displayed homology to IgVH-CDR3 amino acid sequences present on GenBank. This frequency is not higher than that observed within the group of unmutated B-CLL (44%) (10). More remarkable is the finding that the IgVH-CDR3 amino acid sequence of 5 of these 8 RS B-CLL (i.e. RS4, B-CLL57 (11) and RS8, case3 (29), RS 3557 (30)) exhibited inter-RS group homology. Both RS4 and RS8 express an unmutated IgG. In fact, all RS B-CLL with CDR3 regions homologous to that of RS4 expressed unmutated IgG and all have a reported aggressive clinical course. Furthermore, whereas RS4 shared CDR3 homology with 2 of such IgG+ B-CLL before transformation, an additional mutation in the CDR3 region of RS4 after transformation resulted in CDR3 homology with a total of 4 of these IgG+ B-CLL. Altogether, these findings point towards selective forces that favor outgrowth and possibly also progression of tumor (sub)clones with B-cell receptors of restricted specificities. Identification of the antigens involved may further clarify the biological mechanism underlying tumor progression and provide tools for therapeutic intervention. Alternatively, IgVH gene analyses may thus be of value to identify B-CLL with a poor biological behavior.

Our quantitative RT-PCR demonstrated that in peripheral blood samples of the control B-CLL AID levels did not exceed background levels. Our findings seem to contradict several papers reporting on AID expression in B-CLL (25, 55, 56). In most of these studies however,
AID mRNA expression was measured by non-quantitative RT-PCRs and by consequence the actual expression levels are difficult to judge. Limiting dilution assays revealed that less than 1% of the B-CLL cells express AID and accordingly quantitative AID mRNA measurements demonstrated that the expression levels found in the B-CLL were in all cases less than 5% of those found in GC cells (17, 55). In addition, western blot analyses showed that AID protein could not be detected in B-CLL regardless of their IgV_{H} mutation status (16). In contrast to our findings in B-CLL, AID expression was quantifiable in most of the RS B-CLL at presentation. After transformation, the AID levels varied considerably among the different RS B-CLL. Moreover, in the transformed stage AID expression seemed less influenced by *in vitro* CD40 stimulation, suggesting that the tumor cells are more autonomous. In general, the AID mRNA expression data were highly compatible with the observed AID protein expression in tissues as assessed by immunohistochemistry. In lymph node material of 9 of the 11 control B-CLL (mutated and unmutated), no AID expressing cells were observed, not even in the proliferation centers. In both RS cases that were histologically analyzed, scattered AID-expressing tumor cells were present. These AID-positive cells all had a blastoid appearance, whereas the small tumor cells were devoid of AID expression. It is not clear whether the AID-expressing cells are B-CLL cells activated by CD40L and IL4 or represent already transformed cells. In conclusion, our analyses indicate that in B-CLL the presence of significant numbers of AID-expressing cells is exceptional, whereas in RS B-CLL AID is more abundantly expressed and in fact may predict an aggressive clinical course.

Sequence analyses of IgV_{H} and BCL6 demonstrated low but significant degrees of intraclonal variation in the RS B-CLL. In both genes, the nucleotide alterations were not equally spread over the different molecular clones, but clustered in a fraction of the clones. This suggests that the somatic hypermutation machinery is active in a minority of the B-CLL cells only, which fits the AID staining results. It is noteworthy that in two of the four RS B-CLL, the degree of intraclonal variation in BCL6 was twice as high as the intraclonal variation observed in the IgV_{H} genes. Moreover, in the IgV_{H}-unmutated RS1 an additional consensus mutation was obtained in BCL6 during transformation, whereas the IgV_{H} gene remained unaltered. This finding is remarkable since in normal GC B cells the BCL6 mutation rate is 10-100 times lower than the mutation rate in IgV_{H} (57-59). Mutations in BCL6 have been reported in IgV_{H}-mutated (58-62) and IgV_{H}-unmutated B-CLL (63). It thus seems that at least in these RS B-CLL, the somatic hypermutation machinery, and most likely AID, can target BCL6 (and potentially other non-Ig genes) while leaving IgV_{H} unaffected. This, together with the observation that AID expression, either spontaneous or induced, is higher in RS B-CLL and increases during transformation suggests a role for this genetic diversification mechanism during the ongoing transformation of the RS B-CLL.
References
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