Antigen receptors and somatic hypermutation in B-cell chronic lymphocytic leukemia with Richter's transformation

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Background and Objectives. 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 to 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 nine 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 in GenBank.

Results. Ig variable heavy chain (*IgV$_H$*) gene studies revealed that Richter's transformation occurs almost exclusively in unmutated B-CLL. Furthermore, activated-induced cytidine deaminase expression and somatic hypermutation activity of most RS B-CLL were found to be higher than those of control (non-transforming) B-CLL. Finally, comparison of the IgVH-CDR3 regions showed a remarkable amino acid sequence homology between two RS B-CLL of our panel and two RS B-CLL described in the literature.

Interpretations and Conclusions. 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.

Key words: activation-induced cytidine deaminase, B-cell chronic lymphocytic leukemia, Richter's transformation, antigen receptor, intraclonal variation.

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Richter's syndrome (RS) is the rare occurrence of a histologically and clinically aggressive secondary lymphoid malignancy in 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 (*IgV$_H$*) 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 *IgV$_H$* gene repertoire clearly distinct from the *IgV$_H$* 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 IgV$_H$-CDR3 amino acid sequences homologous with CDR3 regions of other B-CLL (so called inter-B-CLL CDR3 homology).[7-11] So far, at least eight B-CLL IgV$_H$-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 center B cells.[15,16] It has been reported that only a small fraction (<1%) of circulating B-CLL cells, particularly of the IgV$_H$-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-cell 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.

Design and Methods

Patient material

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

FACS analysis and cell sorting

The following monoclonal antibodies 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 PerCPCy5.5-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, USA). 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. The CD5+, CD19+ tumor cells from *RS4* and *RS6* were isolated using a FACS-Aria (BD Biosciences) cell sorter that resulted in more than 97% pure tumor samples. Germinal center 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 horseradish peroxidase-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, USA), CD23, BCL6, and Ki67 (all from Dako) were used. Antibody detection was performed with the Powervision+ system (ImmunoVision Technologies, Daly City, CA, USA) and succeeded by peroxidase visualization with 3,3'-diaminobenzidine (DAB) (Sigma), 0.03% H_2O_2 in Tris-HCl pH 7.6.

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, although 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 the Cμ, Cδ, Cα or Cγ regions.[24] The polymerase chain reaction (PCR) was performed as described previously using a 30-cycle 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 Genescan analysis program (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 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 was 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'AGCAGCAGCAGCAACCACTAG3' and 5'CTCTCCCCAGTCGGAAATCC3'. The PCR mixtures contained 1x *pfx* amplification buffer, 1U platinum *pfx* DNA polymerase

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(Invitrogen), 1mM (BCL6) or 2.5mM (PIM1) M_gSO_4, 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'CCGCTGCTCATGATCATT3' in combination with 5'GCAAGCGAGAAAAGAGGAA3' and 5'GTACGGCTTGTGATCTCTCT3' in combination with 5'CAGACTCGAGTCTTCCCATGGATCCACC3'. *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.

Quantitative reverse transcription (RT) PCR reactions

Quantitative RT-PCR analyses were performed using a LightCycler (Roche, Almere, The Netherlands). AID was amplified using the primers 5'AGAGGCGTGACAGTGCTACA 3' and 5'TGTAGCGGAGGAAGAGCAAT 3' 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 the whole exons 3 and 4.[25] β-actin was amplified using the primers 5'GGATGCAGAAGGAGATCACTG 3' and 5'CGATCCACACGGAGTACTTG 3'. The PCR reactions for both AID and β-actin were performed in a volume of 10 μL containing 2μL 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 mRNA 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-well plates (Costar, Corning NY, USA). Each well contained 2×10^5 B-CLL cells and 1×10^5 L cells as a control or 1×10 CD40L-transfected L cells and 400 U/mL interleukin-4 (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 nine cases of 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^{low} 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]

IgV_H 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* V_HDJ_H 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 IgV_H genes of all nine RS cases in our panel were unmutated (< 2% consensus mutations) (Table 4). The consensus *Ig* V_HDJ_H 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).

The IgV_H-CDR3 region is the most hypervariable region of Ig and is considered to contribute most to its antigenic specificity. Nevertheless, ~ 19% of B-CLL, mostly unmutated, express CDR3 sequences with homology to CDR3 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 in GenBank (Table 3). For this purpose, we used the NCBI Protein-Blast program with the option *search for short nearly exact matches* (BLASTP2.2.6[apr-09-2003]) as reported previously.[10] Briefly, CDR3 regions consisting of at least seven amino acids were analyzed. An IgV_H-CDR3 sequence was considered to be homologous to other CDR3 sequences if it shared at least 75% amino acid sequence homology. A length difference between the CDR3 sequences was allowed if not exceeding three amino acids (maximum gap of three amino acids).

Of the 18 RS B-CLL that were thus studied, eight (44 %) fulfilled our criteria for CDR3 homology with CDR3 amino acid sequences present in GenBank (Table 3). The CDR3 regions of these eight RS cases were homologous to the CDR3 regions of 12 normal B-cell clones and nine B-CLL without reported transformation (Table 3). These latter B-CLL all expressed unmutated IgV_H genes, except B-CLL $YarV_H$[28] whose IgV_H genes harbored five mutations. Interestingly, we also observed IgVH-CDR3 amino acid

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

RS	Time*	Diagnosis	Source	CD5	CD23	Ig class
RS1a		B-CLL	nasopharynx	+	+	IgM, IgD
RS1b	20	DLBCL	nasopharynx	+	+	IgM, IgD
RS3a		B-CLL	LN	nd	nd	IgM, IgD
RS3b	5	DLBCL	LN	nd	–	IgM, IgD
RS4a		B-CLL	BM	+	+	IgG
RS4b	14	B-CLL/DLBCL	PB	+	nd	IgG
RS6a		B-CLL	LN	+	+	IgM, IgD
RS6b	86	DLBCL	LN	+	+	IgM, IgD
RS8a		B-CLL/DLBCL	LN	+	+	IgG
RS8b	4	B-CLL/DLBCL	LN	+	–	IgG
RS9a‡		B-CLL	PB	+	+	IgM, IgD
RS9b‡	2	DLBCL	colon	+	+	IgM, IgD
RS10a		B-CLL	PB	+	+	IgM, IgD
RS10b	10	DLBCL	PB	+	+	IgM, IgD
RS11a‡		B-CLL	PB	+	+	IgM, IgD
RS11c‡	13	DLBCL	BM	+	+	IgM, IgD
RS12a‡		B-CLL	PB	nd	nd	IgD
RS12b‡	50	DLBCL	PB	+	+	IgD

*BM: bone marrow; LN: lymph node; nd: not done; PB: peripheral blood; *Time interval in months between samples; ‡RS9, RS11 and RS12 have been previously described as case 9, case 3 and case 8.*[27]

Table 2. Immunoglobulin variable heavy chain genes and CDR3 amino acid sequences of nine B-CLL with clinical and histological progression.

RS	V_H	D	J_H	CDR3 (n. of amino acids)
RS1	V_H1-69 (V_H1.2)	3-10 (fr.1)	J_H6b	GGRQELLWFGEFDYYYGMDV[21]
RS3	V_H4-4b (V_H4.22)	5-12 (fr.1)	J_H4b	GLNIVATGDY[10]
RS4a	V_H4-39 (DP79)	6-13 (fr.1)	J_H5b	NSGYSSSWFRGYSWFDP[17]
RS4b	V_H4-39 (DP79)	6-13 (fr.1)	J_H5b	NSGYTSSWFRGYNWFDP[17]
RS6	V_H5-51 (DP73)	n.a.	J_H2	RPLQWPLERYWYFDL[15]
RS8	V_H3-30/30.5(DP49)	3-22 (fr.2)	J_H6c	GGDYYDSSGYGLYYYYYYMDV[22]
RS9	V_H1-69 (DP10)	2-21 (fr.2)	J_H4b	VAGVAYCGGDCYWREYYFDY[20]
RS10	V_H3-74 (DA8)	3-16 (fr.2)	J_H3b	DAWRPARPAYYDYV[14]
RS11	V_H3-11 (DP35)	3-09 (fr.2)	J_H5b	DSVWYYDILTGYSPQLVSYNWFDP[24]
RS12	V_H1-8 (DP15)	n.a.	J_H2	ASSYDSGDYYYSLCLL[16]

CDR indicates complementarity determining region; n.a., the D gene was not assigned.

Table 3. Homology between IgV$_H$-CDR3 amino acid sequences of the eight RS B-CLL and IgV$_H$-CDR3 amino acid sequences present in GenBank.

RS	Reference	CDR3 homology	Patient/Clone†
RS1	this study	1 normal B-cell clone	ya0208
RS4a	this study	1 RS	CLL57
		3 B-CLL	CLL8, CLL9 and CLL202
		1 normal B-cell clone	1HI81
RS4b	this study	1 RS	CLL57
		4 B-CLL	CLL8, CLL114, CLL202, CLL209
		1 normal B-cell clone	1HI81
RS8	this study	1 RS	case 3
		1 B-CLL	CLL32
		1 anti-polysacc. of *N. meningitis* Ab	SC15
case 3	Matolcsy *et al.*[29]	1 RS	RS8 (this study)
		1 EBV B cell in AITL	case2
		1 anti-polysacc. of *N. meningitis* Ab	SC15
		1 anti-natural Sm Ab	BUD94
		1 anti-Rota virus Ab	RVI-22
		2 normal B-cell clones	102-17 and MBT-159
B-CLL57	Ghiotto *et al.*[11]	1 RS	RS4a/b (this study)
		6 B-CLL	CLL39, CLL114, CLL209 CLL7, CLL8 and CLL9
		2 normal B-cell clones	1HI81 and SC77U-44
RS 3557	Matolcsy *et al.*[30]	2 normal B-cell clones	2CB4N2, A29A29
B-CLL4	Aoki *et al.*[39]	1 B-CLL	YarVH*
case 2	Ohno *et al.*[38]	1 normal B-cell clone	PBT-16
		1 anti-Staphylococcal protein A Ab	4D5

*Ab: 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; 1HI81, Y09249; CLL114, AY268372; CLL209, AY300037; AY486216; SC15, AF115134; BUD94, Z46379; RVI-22, AY686908; 102-17, AF028108; MBT-159, U32960; CLL39, X84336; CLL7, AY486206; SC77U-44, AF174118; 2CB4N2, AY671324; A29A29, AF460484; YarVH, AF099199; PBT-16, U3220; 4D5, PH1650.*

homology among different RS B-CLL. *RS8* expressed a V$_H$3-30/D3-22/JH6 rearrangement. The IgV$_H$-CDR3 amino acid sequence showed ≥75% homology to the CDR3 sequence of RS case 3 of which the V$_H$DJ$_H$ rearrangement was unfortunately not described.[29] In addition, the IgV$_H$-CDR3 amino acid sequence of *RS8* showed 68% homology to the IgV$_H$-CDR3 amino acid sequence of RS 3557[30] although it must be noted that the latter expressed a V$_H$3-74/ D3-09/J$_H$6 rearrangement (Figure 1). *RS4* showed IgVH-CDR3 amino acid homology (≥75%) with RS B-CLL57.[31] *RS4* and RS B-CLL57 not only expressed the same V$_H$DJ$_H$ rearrangement (i.e. VH4-39/D6-13/J$_H$5) but also the same Vκ012/02 -Jκ1 gene rearrangement (*data not shown*). Based on the IgV$_H$-CDR3 homology that is observed between B-CLL, eight homology groups have been defined.[10] *B-CLL57* is an IgG$^+$ B-CLL that shows homology with four other unmutated VH4-39 expressing IgG$^+$ B-CLL.[11] The IgV$_H$-CDR3 region of the sample of *RS4* (*RS4a*) taken at the first time point was homologous to two B-CLL of this homology group (*B-CLL57* and *B-CLL202*). Remarkably, due to an extra mutation in the IgV$_H$-CDR3 region, the DLBCL of *RS4* (*RS4b*) shared homology with a total of four B-CLL of this CDR3-homology group (*B-CLL57*, *CLL202* and additionally *CL114* 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 six B-CLL, two of which underwent Richter's transformation over time. In conclusion, these data demonstrate that Richter's transformation occurs preferentially in unmutated B-CLL. Furthermore, we found that the most hyper-

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Table 4. Mutations and intraclonal variation of IgV_H, BCL6 and PIM1 in five RS B-CLL and 15 control B-CLL.

RS		IgV_H		BCL6		PIM1	
	AID/β-actin×10^{04}	mut*	ICV†	mut‡	ICV†	mut‡	ICV†
RS1a	36.1	0	≤0.2 (12)	1	0.4 (12)	0#	≤0.2 (16)
RS1b	nd	0	≤0.2 (11)	2	0.4 (7)	0#	0 (6)
RS3a	0.9	2	≤0.2 (9)	0	0.5 (23)	nd	nd
RS3b	0.9	2	≤0.2 (16)	0	0.5 (12)	nd	nd
RS4a	10.1	0	0.3 (12)	0¶	0.3 (17)	nd	nd
RS4b	140.0	1	≤0.2 (23)	0¶	≤0.2 (16)	nd	nd
RS6a	nd	0	≤0.2 (12)	nd	nd	nd	nd
RS6b	50.0	1	0.3 (12)	nd	nd	nd	nd
RS8a	8.3	0	≤0.2 (23)	0¶	≤0.2	0#	≤0.2 (7)
RS8b	5.0	0	≤0.2 (23)	0¶	≤0.2	0#	≤0.2 (9)
CLL M§	0.7	17	na	na	na	na	na
CLL UM"	0.2	0	na	na	na	na	na

CLL M indicates CLL cases with >2% IgVH mutations; CLL UM, <2% IgVH mutations; ICV, intraclonal variation; mut., mutation; nd, not determined.
**IgVH mutations are defined as nucleotide differences present in more than 50% of the clones, as compared to the germline sequence. †The intraclonal variation is indicated as the mean number of nucleotide differences observed per ~300 nucleotides per clone. The number of clones that were sequenced is given in brackets.*
‡BCL6 and PIM1mutations are defined as nucleotide differences that were present in ≥25 % of the sequenced clones, as compared to the BCL6 and PIM1 sequences published in Genbank (AY189709 and AF386792 respectively). §Values are the average of nine mutated B-CLL. "Values are the average of six unmutated B-CLL. ¶A polymorphism was found in BCL6 at position 754. #A polymorphism was found in PIM1 at position 1039.

variable region of the *Ig* gene, the CDR3 region, was highly homologous among distinct RS B-CLL cases.

Endogenous and induced expression of AID

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 five 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 nine (three IgM^+, six IgG^+) mutated B-CLL, nor in six unmutated B-CLL (IgM^+). Interestingly, four of five 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 whether the malignant cells were still responsive to environmental stimuli with respect to their AID expression, three RS B-CLL (*RS3*, *RS4*, *RS6*) and 14 control B-CLL were cultured for 3 days on either untransfected or CD40L-transfected L cells in the presence of interleukin-4 and anti-IgM coupled sepharose beads. As positive controls, peripheral blood B cells and an Epstein-Barr virus B-cell line were used. Under these conditions, AID expression was increased in healthy donor peripheral blood B cells, the Epstein-Barr virus B-cell line (*data not shown*) and in the mutated (three IgM^+ and six IgG^+) and unmutated (six 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).

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 tonsil tissue section, 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

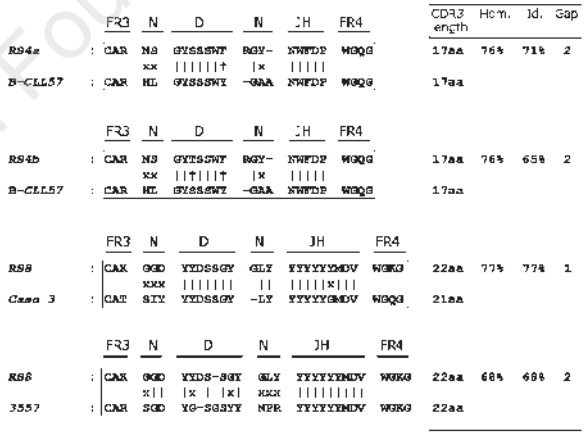

Figure 1. IgV_H-CDR3 amino acid sequence homology of *RS4* and *RS8* with IgV_H-CDR3 of three previously described RS B-CLL. The IgV_H-CDR3 amino acid sequence of *RS4* is homologous to the IgV_H-CDR3 amino acid sequence of *B-CLL57*. Both RS B-CLL expressed the same V_HDJ_H gene rearrangement. The IgV_H-CDR3 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_HDJ_H gene rearrangement as *case 3*, its V_HDJ_H gene rearrangement differed from that 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 acids of the compared IgV_H-CDR3 sequences.

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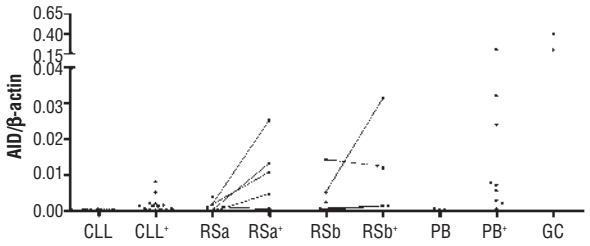

Figure 2. Relative levels of AID expression in RS B-CLL and control B-CLL before and after stimulation. Quantitative RT-PCR analysis of AID and β-actin was performed on peripheral blood samples of a panel of 15 B-CLL without reported transformation and five B-CLL that transformed to a DLBCL. To induce AID expression, the B-CLL and RS samples were cultured for 3 days in the presence of interleukin 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.

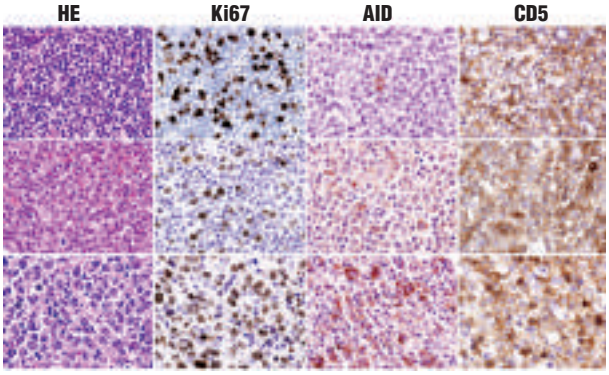

Figure 3. AID protein expression in *RS8* and control B-CLL. Hematoxylin and eosin, Ki67, AID and CD5 stainings on lymph node material of an unmutated B-CLL (upper panel), *RS8a* (middle panel) and *RS8b* (lower panel). Small B-CLL cells are in all cases negative for AID, whereas proportions of blastoid cells of *RS8a* and *RS8b* show clear cytoplasmic AID staining. Magnification 25×.

data of blood-derived B-CLL samples, no AID-expressing cells were present in nine of the 11 B-CLL lymph node specimens. 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 scattered residual germinal centers were present in this biopsy we could not exclude that these centroblast-like cells were germinal center-related (*data not shown*). No quantitative RT-PCR data were available for these patients to confirm these findings. In contrast to the control B-CLL and in accordance with the quantitative RT-PCR data, AID-expressing cells were present in both RS cases for which lymph node material was available, i.e. *RS1* and *RS8* (Figure 3, *middle and lower panel*). Cytoplasmic AID expression was never observed in the small B-CLL cells of either tumor 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 had indeed been active in the tumor cells. Individual molecular clones of the amplified IgV_H genes of *RS1*, *RS3*, *RS4*, *RS6*, and *RS8* were sequenced. TL.A. Smit et al.he degree of intraclonal sequence variation in IgV_H was compared with that of six unmutated and nine 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 two of the nine RS B-CLL (*RS4a* and *RS6b*). 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 intraclonal variation 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). We analyzed 790 basepairs (bp) downstream of the transcription initiation site of *BCL6* in samples *RS1*, *RS3*, *RS4* and *RS8*. This region includes part of the first intron (position 358 to 1148 according to GenBank AY189709). In *RS4* and RS8 one polymorphism (G→C) at position 754 was found in all clones. The consensus sequence of *RS1* already harbored one mutation (C→T) at position 897 before Richter's transformation. Interestingly, after transformation an additional (T→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→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 had, indeed, 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

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(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 assessed in most studies by Southern blot analyses. IgV$_H$-CDR3 sequences of only nine of these 67 RS B-CLL were available. Here we present nine additional RS B-CLL in which the high-grade lymphomas were all of the same clonal origin as the pre-existent 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. This finding does not, however, necessarily account for the well documented poor prognosis of the unmutated B-CLL subset, since Richter's transformation is a rare phenomenon.[53,54]

Several groups, including ours, have previously reported that the IgV$_H$-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 to the group of B-CLL, and was not found within extensive series of follicular lymphomas, DLBCL, Burkitt's lymphomas or 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 eight (44%) displayed homology to IgV$_H$-CDR3 amino acid sequences present in GenBank. This frequency is not higher than that observed within the group of unmutated B-CLL (44%).[10] The finding that the IgV$_H$-CDR3 amino acid sequence of five of these eight RS B-CLL (i.e. *RS4*, *B-CLL57*[11] and *RS8*, *case 3*,[29] RS *3557*)[30] exhibited inter-RS group homology is more remarkable. 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 two 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 four 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, *IgV$_H$* gene analyses may be of value for identifying B-CLL with a poor biological behavior.

Our quantitative RT-PCR demonstrated that AID levels in peripheral blood samples of the control B-CLL 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-PCR 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 levels of expression found in the B-CLL were in all cases less than 5% of those found in germinal center cells.[17,55] In addition, western blot analyses showed that AID protein could not be detected in B-CLL regardless of the *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. No AID-expressing cells were observed, not even in the proliferation centers, in lymph node material of nine of 11 control B-CLL (mutated and unmutated). 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 interleukin-4 or represent already transformed cells. In conclusion, our analyses indicate that the presence of significant numbers of AID-expressing cells is exceptional in B-CLL, whereas AID is more abundantly expressed and in fact may predict an aggressive clinical course in RS B-CLL.

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.

LAS and FvM performed the experiments and analyzed the data; MdW and SB contributed to the research; AWL, CEvdS and EC provided patient material used in the study; LAS, FvM, RJB and CJMvN wrote the paper; LAS, SB and CJMvN designed the study.

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