Selective influences in the expressed immunoglobulin heavy and light chain gene repertoire in hairy cell leukemia

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Funding: the work was supported by an EHA clinical research grant 2004, and grants from the Hairy Cell Leukemia Foundation (Illinois, USA), Piano di Ateneo per la Ricerca (University of Siena), PRIN-MUR 2006 (Progetto di Ricerca di Interesse Nazionale - Ministry of University and Research), Ricerca Sanitaria Finalizzata, Regione Piemonte, Novara-AIL Onlus, Associazione Italiana per la Ricerca sul Cancro (AIRC, Italy), Siena-AIL Onlus. SSS is supported by the Leukaemia Research Fund UK.

Manuscript received September 24, 2007. Revised version arrived on November 22, 2007. Manuscript accepted December 27, 2007.

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background
We previously reported ongoing mutational and isotype switch events in the immunoglobulin (Ig) heavy chain (H) locus in hairy cell leukemia. Those analyses raised questions on the incidence and type of selective influences occurring on the tumor B-cell receptor of hairy cell leukemia.

Design and Methods
To further investigate this issue, we examined the full *IGH* and κ and λ light chains (*IG*κ and *IG*λ) variable and constant region transcripts expressed in a large cohort of patients with hairy cell leukemia (n=88).

Results
Multiple *IgH* isotypes were expressed in 46/56 (82%) cases of hairy cell leukemia. Comparison of tumor with normal B-cell repertoires revealed preferential usage of *IGHV3-21*, *IGHV3-30* and *IGHV3-33* in hairy cell leukemia (p=0.001, p=0.003 and p=0.001, respectively). Light chain analysis demonstrated preferential *Ig*λ use with an inverted *IG*κ:*IG*λ ratio (0.7:1) and universal usage of *IGLJ3*. Analysis of LCDR3 junctions revealed highly homologous motifs in 40% of *IGL*. Parallel analysis of *IGH* and *IGL* showed selective pairing of *IGHV3-21/30/33* segments to specific *LCDR3-J3* subsets (p=0.008). Of 40 cases of hairy cell leukemia, 38 had mutated *IGHV* and/or *IGK/LV*, with variations in 13/13 cloned cases, while two had 100% unmutated *IGHV* and *IGK/LV*.

Conclusions
Overall, biased *IGV* usage, preference for *Ig*λ with universal *IGLJ3* usage and a high incidence of LCDR3 homologous motifs suggest selective influences on the B-cell receptor of hairy cell leukemia. Ongoing mutations and isotype switching suggest that influences occur on the tumor B-cell receptor at ectopic sites.

Key words: hairy cell leukemia, immunoglobulin, *IGH*, *IGL*, *IGK*, heavy chain, light chain.

Citation: Forconi F, Sozzi E, Rossi D, Sahota SS, Amato T, Raspadori D, Trentin L, Leoncini L, Gaidano G, and Lauria F. Selective influences in the expressed immunoglobulin heavy and light chain gene repertoire in hairy cell leukemia. Haematologica 2008 May; 93(5):697-705. doi: 10.3324/haematol.12282

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Introduction

Hairy cell leukemia (HCL) is a rare, chronic B-cell neoplasm characterized by leukemic hairy cells present in blood, bone marrow, and splenic red pulp, with atrophy of white pulp. Lymph node involvement is infrequent. HCL is typically associated with markers of activation, which include expression of CD25, CD11c, FMC7, and CD103 at high intensity.[1] A distinctive feature of HCL is expression of multiple surface (s) immunoglobulin (Ig) isotypes, although their prevalence in HCL has not been fully mapped.[1,2]

B-cell tumors preserve the B-cell receptor (BCR) features of the originally transformed cell.[3] Consequently, immunoglobulin gene (*IG*) analysis delineates the critical events of clonal development and defines the *IG* heavy (*H*) and light κ (*K*) or λ (*L*) repertoire selected by specific tumor entities.[3] In some instances, *IG* analysis may also have prognostic value,[3,4] and the selected *IGH/IGL* or *IGH/IGK* pairs can associate with specific BCR structure and clinical behavior.[4] Selective stimuli to the tumor BCR may be of different types, including viral or bacterial antigens, or, in germinal center-derived lymphomas, stromal elements acting on N-glycosylated residues acquired by somatic mutation.[5,6] Analysis of the selective influences on the tumor BCR has often been hampered in HCL by the rarity of the disease, and only small series of cases have been analyzed to date.[1]

In small series of HCL, we and others have observed that most HCL carry mutated *IGH* variable region (*V*) genes, with low levels of intraclonal heterogeneity. Only a minor subset of HCL has unmutated *IGHV* genes.[2,7-9] Both mutated and unmutated subsets of HCL express multiple sIgH isotypes with no evidence of subpopulations.[2] Also, activation-induced cytidine-deaminase is expressed in HCL, and Ig sterile transcripts are produced prior to class switch deletional recombination.[2] However, hairy cells fail to express the germinal center markers CD38, CD10 and BCL6, or the memory B-cell marker CD27.[10,11] Most importantly, studies of gene expression profiling of B cells from discrete normal subsets or HCL have shown that hairy cells are related to memory cells, although with altered expression of genes controlling cell adhesion and chemokine-receptors.[10] This raises questions on the incidence of the BCR events and where they occur in HCL.

The observation that HCL biology may not be simply recapitulated by normal B-cell physiology raises several unresolved issues. These include the significance of ongoing somatic mutation and isotype switch events in HCL, and the role of selective influences in shaping the BCR repertoire of HCL. Here we addressed these issues by investigating the Ig heavy and light chains expressed by the tumor cells of a large series of patients with HCL.

Design and Methods

Patients

Peripheral blood samples were collected from 88 patients with HCL. In all instances, the specimens were collected at diagnosis before specific therapy. The diagnosis of typical HCL and variant HCL was based on peripheral blood morphology, flow cytometry and bone marrow immunohistochemistry according to the World Health Organization classification.[12] Informed consent was obtained in all cases and the study was approved by the local Institutional Review Board.

Phenotypic analysis

Peripheral blood mononuclear cells were obtained by Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient separation. Immunophenotypic studies were carried out on these cells by direct immunofluorescence techniques with a large panel of antibodies.[7] Expression of CD27, CD38 and sIgH isotypes on HCL was determined by three-color staining with F(ab')2 anti-sIgG, anti-sIgM, anti-sIgD, and anti-sIgA antibodies.[7] Expression of sIgκ/λ was determined by three color staining with fluorescein isothiocyanate (FITC)–conjugated F(ab')2 anti-sIgλ, phycoerythrin (PE)-conjugated F(ab')2 anti-sIgκ and peridinin-chlorophyll protein (PerCP)-conjugated anti-CD20. Previously described procedures were used to avoid non-specific binding.[7] Data were acquired and analyzed and antigen expression was defined as described elsewhere.[7]

Polymerase chian reaction amplification of IGHVDJ, IGKVJ and IGLVJ transcripts and sequence analysis

Total RNA was isolated from peripheral blood mononuclear cells and cDNA prepared as described previously.[7] The full tumor *IGHVDJ* transcripts were amplified by PCR with a mixture of Leader-VH-mix primers and a constant-region primer.[7] The full tumor *IGKVDJ* or *IGLVJ* transcripts were identified by PCR using a mixture of 5' primers specific for known *IGK* or *IGL* leader sequences (IgKV- or IgLV-leader mix), together with a 3' primer specific for either the *IGK* or *IGL* constant-region.[13] Amplified products were run on agarose gel and purified with a Jet Quick Gel extraction kit (Celbio, Milan, Italy). The tumor *IGHVDJ*, *IGKVJ* and *IGLVJ* sequences were identified by direct sequencing and/or after cloning the purified band. Ligation and cloning were performed with pGEM®-T Easy Vector System II and JM109 competent cells (Promega, Milan, Italy). Sequencing was carried out using the v1.1 Big Dye Terminator Ready Reaction sequencing kit (AB Applied Biosystems, Applera Italia, Monza, Italy), on an ABI Prism 310 genetic analyzer (PerkinElmer, Warrington, UK). Direct sequencing was performed with the 3' primer on the constant region and the identified sequence was confirmed with the family specific leader 5' primer, which allowed identification of the full *V*-gene transcript. When cloning was performed, M13 forward and reverse primers were used to

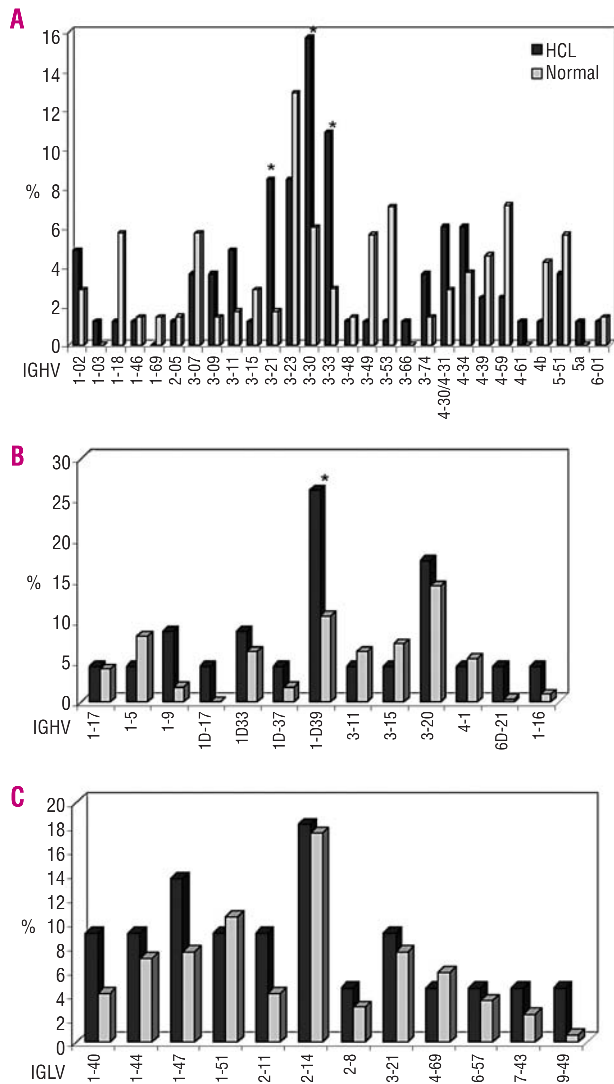

Figure 1. **Expressed *IGHV*, *IGHK* and *IGLV* region repertoire in HCL. The incidence of *IGHV* (A), *IGKV* (B) and *IGLV* (C) region transcripts expressed by HCL was compared to the repertoire of normal B cells from published data.[20,24,25] Dark gray columns: HCL; light gray columns: normal B-cell repertoire. Asterisks indicate *V* genes whose incidence was a significantly different between HCL and the normal B-cell repertoire.**

sequence in both directions. The data were analyzed using Chromas 1.51 software and aligned to the 2005 updated V-BASE and ImMunoGeneTics (IMGT) databases.[14,15]

IGHV, *IGKV* and *IGLV* gene usage and mutation patterns were analyzed as previously described.[7] IMGT nomenclature was used to assign *IG* gene use,[14] since this allowed comparison with data from previously used nomenclatures for *IGH*, *IGK* and *IGL* genes.[16,17] Lengths in the *IGH* and *IGK/LCDR3* (HCDR3, KCDR3 and LCDR3, respectively) were calculated according to IMGT criteria.[14] IMGT criteria and nomenclature were also used for *IGHD* determination, again allowing comparison to segments with other designations from the literature.[14] N-addition of G and C at the joining ends of the V(D)J junction [(Ngc) *Online Supplementary Table S1*] was performed to investigate TdT activity. Recurrent amino acid sequence motifs in HCDR3, KCDR3 and LCDR3 were sought using the ClustalW tool (*at http://www.ebi.ac.uk/clustalw/#*). Amino acid identity >60% in the HCDR3 or >80% in the K/LCDR3 was required for the inclusion of an IgH or an IgK/L chain in the same subset, respectively.[18] Intraclonal heterogeneity was assessed in the cloned products and was distinguished from Taq infidelity by an increased frequency compared to Taq error rate and by the finding of the same mutation in more than one clone.[7] If only direct sequencing was performed, the tumor *IGHV*, *IGKV* and *IGLV* sequences were confirmed by replicate RT-PCR and sequencing. The incidence of potential novel N-glycosylation sites in *IGHVDJ*, *IGKVJ* and *IGLVJ* transcript sequences was assessed as previously described.[6,19] Statistical analyses were performed using χ^2 or Fisher's exact tests. p values <0.05 were considered statistically significant.

Results

Analysis of expressed IGHV *region transcripts and sIgH isotype proteins*

The expressed tumor *IGHV* sequences were identified in 83/88 HCL (*Online Supplementary Table S1*). The distribution of *IGHV* families was similar to that of normal B-cells, but use of individual *IGHV* gene segments showed differences from that of the normal B-cell repertoire (Figure 1A). The *IGHV* gene segments most frequently used in HCL were *IGHV3-30* (13/83, 16%), *IGHV3-33* (9/83, 11%), *IGHV3-23* (7/83, 8.5%), *IGHV3-21* (7/83, 8.5%), *IGHV4-30/4-31* (5/83, 6%) and *IGHV4-34* (5/83, 6%). Among these segments, the usage of *IGHV3-21*, *IGHV3-30* and *IGHV3-33* was significantly increased compared to the normal B-cell repertoire (p=0.001, p=0.003 and p=0.001, respectively).[20] Other *IGHV* segments were used less frequently in HCL than in normal B cells (Figure 1A), although the differences were not statistically significant, for any of the segments individually, likely due to the number of cases investigated. However, by combining results of this study with those of all published HCL *IGHV* gene sequences (n=164, *Online Supplementary Figure S1A*), we confirmed the overuse of *IGHV3-21*, *IGHV3-30* and *IGHV3-33*, and demonstrated the significantly reduced use of *IGHV1-18* (p=0.02) and *IGHV3-53* (p=0.02).[8,9,21,22]

Phenotypic analysis of surface IgM, IgD, IgG and IgA was performed in 56 HCL (*Online Supplementary Table S1*). The majority (46/56, 82%) co-expressed multiple pre- (IgM±D) and post-switch (IgG±A) isotypes, indicating that multiple isotype expression is a dominant feature in HCL.[2] Of 63 HCL tested, CD38 was negative in all cases, and the memory B-cell marker CD27 was negative in 58/63 cases. Interestingly the five CD27-positive cases (cases HCL8, HCL17, HCL72, HCL82, and HCL87) differed from all the remaining HCL investigated, as they had features consistent with the diagnosis of variant HCL, including high white blood cell counts with prolymphocytic morphology and CD25

negativity of the peripheral blood hairy cells.[12] These data confirm that lack of surface CD27 and CD38 is a feature of typical HCL.[11]

Analysis of HCDR3 junction

Sixty-nine cases of HCL were evaluable for the *HCDR3* junction (*Online Supplementary Table S1* and Figure 2). *IGHD* gene segment analysis revealed significantly increased use of *IGHD1* (11/69 cases, 16%) and *IGHD6* (12/69, 17%) families (*p*=0.001 and 0.02, respectively). Analysis of specific segments revealed additional notable biases. In fact, the *IGHD1* family used almost exclusively *IGHD1-26* (10/11 cases) and the *IGHD6* family frequently utilized *IGHD6-19* (5/12 cases). This observation is remarkable, since neither of these segments has been reported to be used in normal B cells.[20] In addition, significantly increased selection of *IGHD3-3* (8/69, *p*=0.00009), *IGHD3-9* (4/69, *p*=0.004), *IGHD3-10* (7/69, *p*=0.00005) and *IGHD4-17* (5/69, *p*=0.0009) was also documented. Again, *IGHD3-3*, *IGHD3-9*, and *IGHD4-17* are not reportedly selected in the functional normal B-cell repertoire, and only *IGHD3-10* is restricted to 0.5% of all D segments.[20] *IGHJ* gene segments used in the expressed HCL repertoire distributed similarly to those in the normal B-cell repertoire (*Online Supplementary Table S1*), HCDR3 length was variable (range 9-28 amino acids, median 16, mean 17), and analysis of recurrent amino acid sequence motifs could not identify significant similitudes of HCDR3. However, the number of sequences investigated (n=69) here and the few HCDR3 currently available in the literature (n=27) preclude any statistical clustering.[8,22,23] Thus, the significance of *IGHD* biases remains unresolved as yet.

Analysis of sIgκ and sIgλ protein expression and IGKV and IGLV region transcripts

Seventy of 83 HCL were characterized for sIgκ and sIgλ protein expression (*Online Supplementary Table S1*). Expression of sIgλ was observed in 41/70 (59%) HCL with an Igκ:Igλ ratio of 0.7:1, indicating preferential secondary rearrangement of Igλ. The expressed *IGK* and *IGL* tumor transcript sequences were investigated in 45/70 cases (23 HCL expressing sIgκ and 22 HCL expressing sIgλ). In two additional samples (HCL30 and HCL33), *IGL* transcripts from the non-functional allele were amplified and sequenced. Four HCL (HCL4, HCL42, HCL70, and HCL83) co-expressing double *IGK* or double *IGL*, or *IGK/IGL*, were excluded from the analysis. Among *IGKV* gene segments (*Online Supplementary Table S2* and Figure 1B), *IGKV1D-39 (012/02)* was the one most frequently used (6/23, 26%), and its usage was significantly higher than in normal B-cells (*p*=0.03).[24] The distal gene segments *IGKV1D-17* (case HCL63) and *IGKV6D-21* (case HCL11), which are not reported in the normal B-cell repertoire, were both used once. The other *IGKV* segments distributed similarly to normal B-cells.

Among functional *IGLV* gene segments, *IGLV2-14* was most frequently used (4/22, 18%), followed by *IGLV1-47* (3/22, 14%), *IGLV1-40*, *IGLV1-44*, *IGLV1-51*, *IGLV2-11* and *IGLV3-21* (2/22, 9% each) (*Online Supplementary Table S2* and Figure 1C), with an overall distribution similar to that of the normal B-cell repertoire.[25]

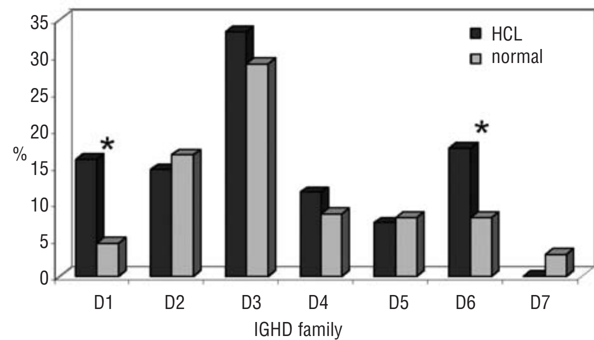

Figure 2. Expressed *IGHD* families in HCL. Incidences of *IGD* families expressed by HCL were compared to the repertoire of normal B-cells from published data.[20] Dark gray columns: HCL; light gray columns: normal B-cell repertoire. Asterisks indicate regions with identified significant differences between HCL and the normal B-cell repertoire. The D1 family was represented by D1-26 in 10/11 cases. The D6 family was represented by D6-19 in 5/12 cases. D1-26 and D6-19 are not reportedly used by normal B cells.[20]

Analysis of KCDR3 and LCDR3 junctions

Twenty-one HCL were evaluable for the KCDR3 junction. *IGKJ* genes were used by HCL in a fashion similar to that in the normal B-cell repertoire (*Online Supplementary Table S2*).[24] KCDR3 length ranged from 7 to 11 amino acids (median 9) and 11/21 KCDR3 had identical pI (range 6.5-13, median 13). Overall analysis of KCDR3 amino acid sequences reflected pI similarities and identified three subsets, all with 88.8% sequence identity (*Online Supplementary Table S2*). Subset 1K (HCL28 and HCL2) harbored *IGKV1D-33-J1/4* rearrangements (QQYDNLP[L/R]T), which associated with *IGHV3-23* (HCL28) or *IGHV1-02* (HCL2). Subset 2K (HCL7/330 and HCL67) harbored *IGKV3-20-J1/2* rearrangements (QQYGRSP[Q/Y]T), which associated with *IGHV2-05* (HCL7/330) or *IGHV4-34* (HCL67). Subset 3K (HCL19 and HCL63) harbored *IGKV1-17/1D-17-J1/2* rearrangements (LQHNSYP[R/Y]T), which associated with *IgHV3-21* (HCL19) or *IgHV4-34* (HCL63).

Twenty-two HCL were evaluable for the functional LCDR3 junction. Remarkably, among *IGLJ* segments, virtually all cases used *IGLJ3* (21/22, 95.5%) (*Online Supplementary Table S2* and Figure 3). The universal use of *IGLJ3* was higher than expected by chance alone (50%), and significantly higher than its frequency (34%) in normal B cells (*p*=0.000000001).[26] Remarkably, 2/2 HCL with non-functional LCDR3 junctions (HCL30 and HCL33) failed to use *IGLJ3*, further indicating strikingly selective influences on the functional repertoire. In particular, HCL30 used *IGLJ1* with a TAG stop at joining codon 115, whereas HCL33 used *IGLJ6* with and out-of-frame rearrangement in codon 115 (*Online Supplementary Table S2*). LCDR3 length ranged from 9 to 13 amino acids (median 11) and the pI ranged from 4.4 to 13 (median 13), with 13/22 LCDR3 having an

identical pI.

Analysis of LCDR3 amino acid sequences identified three HCL subsets (Figure 4). Subset 1L (HCL35, HCL22, HCL44, HCL49, and HCL40) harbored *IGLV1-44/47-J3* rearrangements with ≥85% homologous LCDR3 (Figure 4). Of note, these HCL paired only with the highly identical *IGH3-30* or *IGHV3-33* segments in four out of five cases, the exception being case HCL22, which used *IGHV3-21*. Remarkably, the selective clustering of *IGHV3-21/30/33* segments (5/5, 100%) within this subset was significant, when compared to pairing of *IGHV3-21/30/33* segments with the remaining CDR3 investigated for *IGLV-J* (8/17, 47%, p=0.03) or the total *IGL/KV-J* (13/38, 34%, p=0.005) rearrangements (*Online Supplementary Table S2*). Subset 2L (HCL27 and HCL32) harbored *IGLV3-21-J3* rearrangements with 91% homologous LCDR3 (QVWDSSSDH[W/V]V), and associated with either *IGHV3-30* or *IGHV3-23*. Subset 3L (HCL64 and HCL23) harbored *IGLV1-40-J3* rearrangements with 81.8% homologous LCD3 (QSYD[S/N]SL[SG/TR]SGV), and associated with *IGHV4-34* or *IGHV3-21*. Overall, the overused *IGHV3-30*, *IGHV3-33* and *IGHV-3-21* genes clustered within the identified LCDR3 sets (7/9 cases; 78%), and not with the CDR3 from the remaining *IGK/LV-J* rearrangements investigated (9/34, 26%, p=0.008). Together with Igλ light chain preference and universal *IGLJ3* use, these data further suggest that selection events are dominant in the Igλ light chain of HCL. TdT activity was absent in almost 50% light chain rearrangements (20/41 light chain junctions). Since TdT activity decreases during B-cell ontogeny,[27] lack of N-addition/deletion of G and C at the ends of V-J junction (*Online Supplementary Table S2*) is suggestive of rearrangements having occurred late (in the periphery) as a result of receptor revision in the light chain, after downregulation of the TdT enzyme.[14]

Somatic mutation analysis of IGHV, IGKV *and* IGLV

The mutation status of *IGHV* tumor transcripts was evaluated in 83 HCL (*Online Supplementary Table S1*). Overall, *IGHV* genes carried variable tiers of mutations (range 80.95-100%; median 96.56%, mean 95.97%). Three of 83 HCL had completely unmutated *IGHV* genes (100% homology to germline). However, by using the arbitrary 2% cut-off that defines mutational status in other lymphoproliferative disorders and that is being used for clinical correlates in ongoing clinical studies,[28] the unmutated HCL subgroup expanded to 17/83 HCL (20.5%).

The distribution of individual *IGHV* genes varied among mutated and unmutated cases of HCL (*Online Supplementary Table S2* and Figure 5A). In particular, *IGHV3-30*, the segment most frequently used in HCL, showed a selective tendency to distribute in unmutated rather than mutated HCL cases (6/17, 35% unmutated HCL *vs.* 7/66, 10% mutated HCL, p=0.01). *IGHV4-34* was also more frequently used in unmutated HCL (3/17, 18%) than in mutated cases (2/66, 3%) (p=0.02). *IGKV* mutation status (n=23) (*Online Supplementary Table S2* and Figure 5B) was variable (homology range 93.61%-100%, median 97.26%, mean 97.31%), and

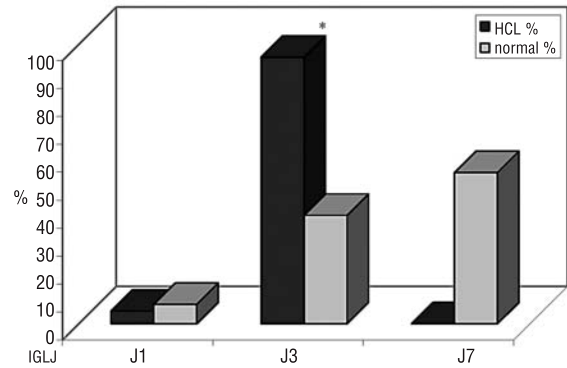

Figure 3. ***IGLJ*** **segment usage in Igλ HCL. The incidence of IgLJ segments in the functional** ***IGLVJ*** **transcripts expressed by HCL was compared to the functional repertoire of normal B cells from published data.[26] Dark gray columns: HCL; light gray columns: normal B-cell repertoire.** ****IGLJ3 segment use in HCL was universal (>95% cases) and significantly superior to IGLJ3 use in normal B cells.***

	IGLV	LCDR3	IGLJ	Paired IGHV	Probability of IGHV3-21, 3-30 or 3-33 associating with LCDR3 subsets (*)	(**)	(***)
Subset 1L							
HCL35	1-44	CAAWDASLNGV VF	3	3-30	pΔ=0.034	pΔ=0.005	pΔ=0.008
HCL22	1-44	.....D....HY ..	1	3-21			
HCL44/42	1-47	........S.W ..	3	3-33			
HCL49	1-47	........S.G ..	3	3-33			
HCL40v	1-47	........R. ..	3	3-30			
Subset 2L							
HCL27	3-21	CQVWDSSSDHW VF^	3	3-23			
HCL32	3-21	..........V ..°	3	3-30			
Subset 3L							
HCL64	1-40	CQSYDSSLSGSG VF	3	4-34			
HCL23	1-40	.....N....TR ..	3	3-21			

Figure 4. Subsets of highly similar LCDR3 in HCL. Amino acid sequences are shown for all HCL cases within a LCDR3 subset. The rearranged IGLV and IGLJ and the associated ***IGHV*** **gene segment are indicated together with the amino acid sequence of each case. Amino acid homology to the first LCDR3 from each subset is indicated by dots. HCL belonging to these subsets preferentially utilized the most frequent** ***IGHV3-21*****,** ***3-30*** **or** ***3-33*** **gene segments. (*, **, ***): *selective distribution of** ***IGHV3-21*****,** ***3-30*** **or** ***3-33*** **in HCL from subset 1L compared to Igλ-positive HCL not belonging to subset 1L; **selective distribution of** ***IGHV3-21*****,** ***3-30*** **or** ***3-33*** **in HCL from subset 1L compared to Igλ-positive or Igκ-positive HCL with light chain CDR3 not belonging to subset 1L; ***selective distribution of** ***IGHV3-21*****,** ***3-30*** **or** ***3-33*** **in HCL from subsets 1L, 2L and 3L compared to HCL with light chain CDR3 not belonging to these subsets. ^An identical LCDR3 was reported in 2/368 normal or autoreactive B cells (0.5%), with no indication of antigen reactivity. °Identical LCDR3 in 5/368 normal or autoreactive B-cells (1.3%), that identified anti-platelet $\alpha IIb\beta_3$ integrin (EBA11) or anti-La (SS-B)SLE autoantibodies.[46]**

2/23 cases had completely unmutated *IGKV* genes. Using the arbitrary 2% cut-off value, 9/23 (39.1%) *IGKV* were considered unmutated. *IGLV* mutation status was evaluable in 22 cases with functional *IGLV* rearrangements (*Online Supplementary Table S2* and Figure 5C). *IGLV* homology to germline ranged from 89.9% to 100% (median 97.12%, mean 96.83%), and 1/22 HCL had completely unmutated *IGLV* genes. Using the arbitrary 2% cut-off, 8/22 (36.4%) *IGLV* were unmutated.

Parallel assessment of *IGHV* and *IGKV/IGLV* mutation status was feasible in 40 cases of HCL (*Online Supplementary Table S2*). The *IGHV-IGKV/IGLV* mutation status was concordant in 22/40 HCL. Eighteen cases were mutated in both *IGHV* and *IGKV/IGLV*.

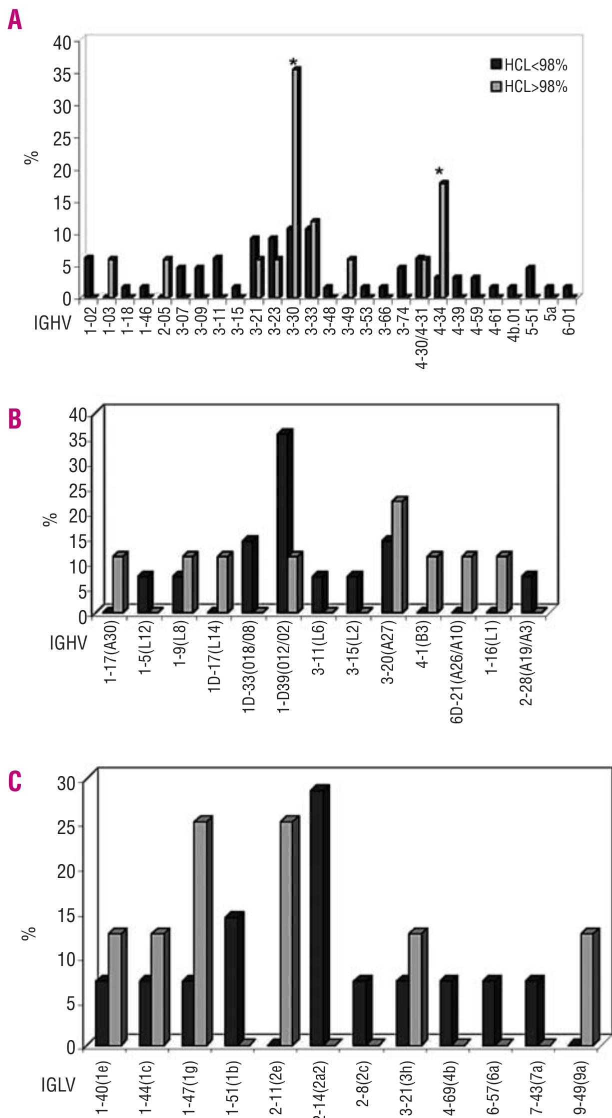

Figure 5. Distribution of mutated and unmutated *IGHV* (*A*), *IGKV* (*B*) and *IGLV* (*C*) segments in HCL. Incidence of tumor *IGHV*, *IGKV* and *IGLV* with homology to germline inferior (mutated) or superior (unmutated) to 98% in HCL. Segments with <98% homology are indicated by dark gray columns; segments with >98% homology to germline are indicated by light gray columns. Asterisks indicate segments with significantly different distribution between mutated and unmutated cases of HCL.

Four were unmutated in both *IGHV* and *IGKV/IGLV*. Of these, two cases displayed both *IGHV* and *IGKV/IGLV* rearrangements with a 100% homology to closest germline, confirming the existence of a very minor subset of HCL with completely unmutated *IG* genes.[2]

The *IGHV-IGKV/IGLV* mutation status appeared discordant in 18 cases of HCL (12 *IGHV* mutated-*IGKV/IGLV* unmutated cases and six *IGHV* unmutated-*IGKV/IGLV* mutated cases). However, 16/18 discordant cases carried some level of mutation (homology to germline <100%) in both *IGHV* and *IGKV/IGLV*, suggesting that the variations likely represent true mutations and not polymorphisms.[29] Only two discordant cases (HCL37 and HCL81) carried heavily mutated *IGHV* (94.79% homology) or *IGKV* (94.98%) genes coupled to completely unmutated (100%) *IGKV* or *IGHV* genes, respectively. These two particular cases might be representative of an antigen experienced BCR (with mutated *IGH* or *IGK*) rescued after secondary recombination of the *IGK* or *IGH* chain on the second allele, and suppression of the first functionally rearranged allele (receptor revision).[30,31]

Analysis of intraclonal heterogeneity of IGHV, IGKV *and* IGLV

Cloning and sequencing of *IGHV* and *IGKV* or *IGLV* transcripts was performed in 12 cases of HCL (Table 1). In all cases, cloning confirmed the results of direct sequencing. Cloning of *IGHV* transcripts revealed intraclonal variations within the same or different tumor isotypes in 11/12 cases, including 2/2 cases with >98% and <100% *IGHV* homology to germline (cases HCL7 and HCL35). Cloning of the paired *IGK/L* tumor sequence confirmed intraclonal variations in the light chain of the same 11/12 cases. Using stringent criteria for ongoing activity (i.e. ≥2 identical variations repeated in separate clones), intraclonal heterogeneity was restricted to 3/11 cases. Lack of repetitions in the remaining 8/11 cases may be due to the mutational frequencies in the light chain genes, which, in normal individuals, are generally lower than in the *IGHV* genes.[32] Only HCL38, having both *IGHV* and *IGLV* genes with 100% homology to germline, did not display intraclonal mutations in either *IGHV* or *IGLV* genes.

Discussion

Our immunogenetic analysis of the expressed *IGH* and *IGK/L* repertoire in a large cohort of patients has implications for the origin of HCL, and indicates that *IG* selection may play an important role in the pathogenesis of this disease. *IGHV* analysis shows that HCL is characterized by biased usage of *IGHV3-21*, *IGHV3-30* and *IGHV3-33*, in agreement with prior analyses on smaller HCL groups.[9,21] *IGHV3-30* and *IGHV3-33* are highly homologous segments with a single amino acid difference in the CDR2, are recognized by the same anti-CDR1 (B6) monoclonal antibody, and thus may share the ability to bind identical antigens.[33] Indeed, IgHV3 family members, including *IGHV3-30* and *IGHV3-33*, react with common bacterial superantigens, such as modified staphylococcal protein A,[34] or with the natural staphylococcal enterotoxin A, which is sufficient to induce survival of IGHV3-expressing B cells by low-affinity binding.[35] Furthermore, *IGHV3-30* is reportedly involved in the immune response against *Toxoplasma* infection, providing additional clues to potential antigens sustaining HCL.[36] Clearly, the selective influences active in HCL appear to follow routes that are different from those of other B-cell neoplasms, in particular from the extensively investigated IgM-positive chronic lymphocytic leukemia (CLL).[19] For example, *IGHV1-69* was totally absent in HCL from

Table 1. Intraclonal variability in hairy cell leukemia.

Code*	IGHV	Homology (%)	Intraclonal heterogeneity in in IGHV	IGK/LV	Homology (%)	Tumor/total IGL	Intraclonal heterogenity in IGK/LV	
							Unique	Repeats
HCL9	3-07	92.45	+	KV1-D39(012/02)	97.49	11/11	3	0
HCL25/206	3-23	91.31	+	KV3-11(L6)	97.13	6/11	1	0
HCL7/330	2-05	98.96	+	KV3-20(A27)	96.90	9/12	3	0
HCL35	3-30	98.49	+	LV1-44(1c)	97.16	4/12	4	1
HCL44/42	3-33	93.50	+	LV1-47(1g)	94.68	7/7	2	0
HCL40	3-30	97.61	+	LV1-47(1g)	98.93	11/12	2	0
HCL38/283	3-30	100.00	-	LV2-11(2e)	100.00	10/10	0	0
HCL3/266	1-02	96.18	+	LV2-14(2a2)	95.43	4/12	0	2
HCL68	4-39	93.84	+	LV2-8(2c)	96.84	8/8	5	2
HCL27	3-23	96.52	+	LV3-21(3h)	98.18	9/11	3	0
HCL32	3-30	95.83	+	LV3-21(3h)	96.01	7/12	4	0
HCL26/216	3-23	91.66	+	LV4-69(4b)	95.87	11/11	4	0

*Intraclonal heterogeneity in the cloned IgHV products was defined positive when the same mutation was present in more than one clone from different isotype transcripts.[2,7,29] *The HCL cases with previously published details on intraclonal variation in tumor IgH isotypes are listed with the original code after the slash.[2,7,29]*

our and previously published series (total 164 IgHV sequences),[2,7,8,21,22,37] while it dominates the unmutated subset in CLL (*Online Supplementary Figures S1A and S1B*).[19] Similarly, *IGHV4-34* is used predominantly in a mutated conformation by CLL, but was preferentially *unmutated* in the HCL cases in our series (Figure 5A), and even more significantly when all published HCL sequences were pooled (p=0.0002).[2,7,8,21,22,37] Lack of apparent HCDR3 stereotypy in HCL is another feature of variance with CLL, which, conversely, frequently groups cases with shared HCDR3 structural features.[4,18,38] This also indicates that antigen drive may not rely on HCDR3-mediated interactions in HCL. Conversely, the low mutational rate of *IGHV* genes expressed in HCL, particularly of *IGHV3-30* and *IGHV3-33* segments, which represent almost 50% of all *unmutated* HCL, suggests that selective influences may be related to the *IGHV* segment itself.[34,35]

Analysis of Ig light chains provides novel evidence that HCL is characterized by selection events in the tumor BCR. In fact, HCL display: (i) an inverted Igκ:Igλ ratio (0.7:1); (ii) universal usage of *IGLJ3* in the functional sIgλ expressors; and (iii) subsets with highly homologous KCDR3 and LCDR3. The preferential usage of Igλ light chain in our large series is consistent with prior independent studies,[39-42] and can be considered a unique feature of HCL. In the normal B-cell repertoire and in other B-cell neoplasms, Igκ is the most frequently used light chain.[43-48] On these bases, our results suggest that HCL requires selective usage of Igλ. The functional implications of Igλ selection in HCL remain speculative. The observations that (i) almost 50% HCL expressing sIgλ utilize IgHV3-21, IgHV3-30 or IgHV3-33 only; ii) virtually all HCL expressing Igλ utilize IgLJ3; and (iii) 40% HCL expressing Igλ display LCDR3 sets with shared structural features, suggest that HCL expressing Igλ may recognize common antigens requiring homologous LCDR3-J3 stretches.

LCDR3 identical motifs were documented within *IGHV3-21*, *IGHV3-30* or *IGHV3-21* HCL (Table 1). In public databases of normal or autoreactive B-cells, we identified motifs identical to LCDR3 from HCL sets 1L or 2L, although specific antigen reactivities were found only in set L2.[46] The molecular triggers of the Igλ bias and LCDR3-J3 selection in HCL are unknown. One possibility of IgL selection is that the Igκ-to-Igλ shift may derive from secondary rearrangements with rescue of a new light chain in the periphery. Secondary rearrangements of Igλ after Igκ deletion have been observed in cases of Igλ-positive B-cell neoplasms.[45-47,49] The observation of absent Ngc incorporations due to absent TdT activity in almost 50% HCL light chain rearrangements favors the hypothesis that secondary rearrangements occur in the periphery.[27] We have observed the potential ability of HCL to revise light chains in cases co-expressing mutated double light chain transcripts and/or protein with peripheral up-regulation of RAG-1.[50] Also, double productive and functional Ig light chain expression has been described in one independent case of HCL and is putatively the consequence of peripheral receptor revision.[51]

Classically, mutational status of *IGHV* genes distinguishes whether the B-cell has encountered antigen in the germinal center with a T-cell-dependent reaction or whether it derives from antigen-naïve B cells.[7] However, there is evidence that the BCR can also interact with antigen in a T-independent pathway and accumulate a low level of somatic mutations ectopically, likely in the marginal zones.[21,52] Several observations from both Ig heavy and light chain rearrangements indicate that HCL cells have experienced antigen stimulation. First, the majority of HCL are characterized by somatic mutations. Second, cloning of *IGV* region transcripts confirms the existence of low levels of intraclonal heterogeneity also in cases with 98% ≤ homology <100% to germline.[19] Third, the vast majority (82%) of HCL co-express multiple pre-switch (IgM±D) and post-switch (IgG±A) sIgH, independently of mutational status and while activation-induced cytidine deaminase is expressed.[2] Histological findings and lack of CD27 and CD38 suggest that, in typical HCL, the mutational and switch events are unlikely to occur in the germinal cen-

ter.[11] Additionally, in the present large series of HCL, we observed an irrelevant incidence of novel N-glycosylation sites introduced by somatic mutation in the tumor IgV region (<6%), similar to that observed in normal memory B cells or in transformed memory and marginal zone B cells (*Online Supplementary Tables S1 and S2*).[5,6,19,53] Since N-glycosylation sites are specifically introduced in the IgV region of germinal center-derived lymphomas, while they are rare in marginal zone or post-germinal center B-cell neoplasms,[5,6,19,53] our results provide further support to the hypothesis that HCL originates from non-germinal center derived B cells.

CD27-negative memory B cells or marginal zone B cells are the candidate counterparts of HCL.[54] Comparison of the gene expression profiles of 14 HCL with the profiles of normal B cells from the naïve, germinal center, memory and plasma cell compartments showed that hairy cells were related to memory B cells.[10] However, the data were limited by the unavailability of a marginal zone B-cell compartment.[1,10] Indeed, HCL and marginal zone B cells share several immunogenetic features, including variable tiers of IGHV mutations, evidence of ongoing somatic hypermutation and lack of novel N-glycosylation sites.[19,36,55] The morphology and phenotype of hairy cells also resemble those of marginal zone-derived CD23-negative, CD27-negative, CD38-negative interfollicular B cells. Overall, efforts should be directed at identifying the normal counterpart within these categories.[1,36,55] Knowledge of *IGV* gene use in these B-cell populations will be informative and perhaps more relevant when available from both normal and transformed B-cells.

In conclusion, the present largest immunogenetic survey identifies the *IG* repertoire selected in HCL. The remarkable preference for Igλ with universal *IGLJ3* use and a high incidence of LCDR3 homologous motifs further clarifies the selective influences present in the BCR of HCL. Whether this distribution of repertoire in HCL reflects that of a postulated normal B-cell counterpart remains to be clarified, and may follow from a more accurate characterization of normal B-cell subsets.

Authorship and Disclosures

FF designed the study, collected and processed the samples, performed experiments, analysed results and wrote the manuscript; ES, DR, SSS, TA, DR, FT and LL collected and processed the samples, performed experiments, and analyzed results; LT provided tumor samples, performed experiments and analyzed data; GG designed the study, provided tumor samples, analyzed data and wrote the manuscript; FL provided tumor samples, supervised the project and manuscript writing. The authors reported no potential conflicts of interest.

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