SELECTIVE INFLUENCES IN THE B-CELL RECEPTOR IMMUNOGLOBULIN HEAVY AND LIGHT CHAIN IN HAIRY CELL LEUKEMIA

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INTRODUCTION

Hairy cell leukemia (HCL) is a rare, chronic B-cell neoplasm characterized by leukemic hairy cells (HCs) 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, that include expression of CD25, CD11c, FMC7, and CD103 at high intensity (1). A distinctive feature of HCL is expression of multiple surface immunoglobulin (sIg) isotypes, although its prevalence in HCL is not fully mapped (1,2).

B-cell tumors preserve the B-cell receptor (BCR) features of the originally transformed cell (3). Consequently, immunoglobulin (Ig) gene analysis delineates the critical events of clonal development and defines the Ig heavy (H) and light kappa (K) or lambda (L) repertoire selected by specific tumor entities (3).

In some instances, Ig gene 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 behaviour (4). Selective stimuli on the tumor BCR may be of different types, including viral or bacterial antigens, or, in germinal center (GC) derived lymphomas, stromal elements acting on N-glycosylated residues acquired by somatic mutation (SM) (5,6). In HCL, analysis of the selective influences on the tumor BCR has often been hampered by the rarity of the disease, and only small series of cases have been analysed to date (1).

In small HCL panels, 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 have unmutated IgHV genes (2,7-9). Both mutated and unmutated HCL subsets express multiple surface (s) IgH isotypes with no evidence of subpopulations (2). Also, activation-induced-cytidine-deaminase (AID) is expressed in HCL, and Ig sterile transcripts are produced prior to class switch deletional recombination (2). However, HCs fail to express the GC markers CD38, CD10 and bcl6, as well as 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 HCs are related to memory cells, although with altered expression of genes controlling cell adhesion and chemokine-receptors (10). This raises questions on 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 poses several unresolved issues. These include the significance of ongoing SM and class switch recombination (CSR) in HCL, and the role of selective influences in shaping the BCR repertoire of HCL. Here we addressed these issues by investigating the IgH and Ig light chains expressed by the tumor cells of a large series of HCL.

MATERIALS AND METHODS

Patients. Peripheral blood samples were collected from 88 HCL. In all instances, the specimens were collected at diagnosis before specific therapy. 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).

Phenotypic analysis. Peripheral blood mononuclear cells (PBMC) were obtained by Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient separation. Immunophenotypic studies were carried out on PBMC by direct immunofluorescence techniques with a large panel of antibodies (7). Expression of CD27, CD38 and sIgH isotypes on HCL was determined by 3-color staining with F(ab')2 anti-sIgG, anti-sIgM, anti-sIgD, anti-sIgA antibodies (7). Expression of sIgK/L was determined by 3 color staining with fluorescein isothiocyanate (FITC)–conjugated F(ab')2 anti-sIgL, phycoerythrin (PE)-conjugated F(ab')2 anti-sIgK and peridinin-chlorophyll protein (PerCP)-conjugated anti-CD20, using our previously described procedures (7).

PCR amplification of IgHVJ, IgKVJ and IGLVJ transcripts and sequence analysis

Total RNA isolation from PBMC, preparation of cDNA and amplification of the full tumor IgHVJ, IgKVJ or IgLVJ transcripts were performed by polymerase chain reaction (PCR) with a mixture of Leader-VH-mix

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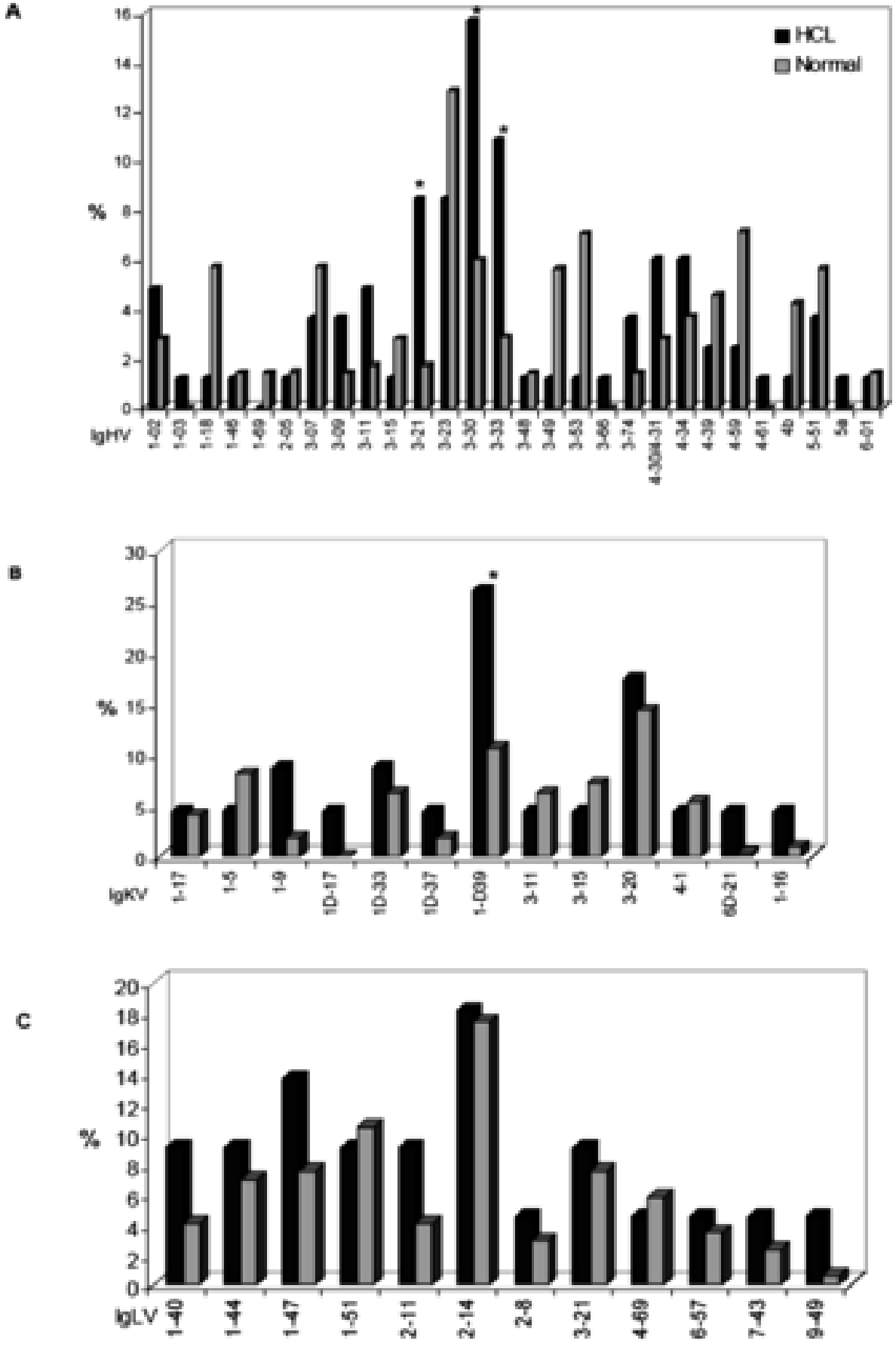

Figure 1.
Expressed IgHV, IgHK and IgLV region repertoire in HCL. The incidence of IgHV (1A), IgKV (1B) and IgLV (1C) region transcripts expressed by HCL was compared to the repertoire of normal B-cells from published data.[20,24,25]
Dark grey columns: HCL; light grey columns: normal B-cell repertoire. Asterisks indicate V genes whose incidence showed a significant difference between HCL and normal B-cell repertoire.

primers and a constant-region primer (7,13). Amplified products were run on agarose gel and purified with the Jet Quick Gel extraction kit (Celbio, Milano, Italy). Identification of the tumor IgHVJ, IgKVJ and IgLVJ sequences was performed by direct sequencing and/or after cloning of the purified band. Ligation and cloning was performed with pGEM®-T Easy Vector System II and JM109 competent cells (Promega, Milano, Italy). Sequencing was performed with the v1.1 Big Dye Terminator Ready Reaction sequencing kit (AB Applied Biosystems, Applera Italia, Monza, Italy), on an ABI Prism 310 genetic analyser (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, that allowed identification of the full V-gene transcript. When cloning was performed, M13 forward and reverse primers were used to sequence in both directions. The data were analyzed by means of Chromas 1.51 software and aligned to the 2005 updated V-BASE and ImMunoGeneTics (IMGT) databases (14,15).

Analysis of IgHV, IgKV and IgLV gene usage and mutation pattern was performed as previously described.[7] IMGT nomenclature was used to assign Ig gene use,[14] since it allowed comparison with data from previously used nomenclatures for IgH, IgK and IgL genes (16,17). Length in the IgH and IgK/L□CDR3 (HCDR3, KCDR3 and LCDR3, respectively) were calculated according to IMGT criteria (14). For IgHD determination, IMGT criteria and nomenclature were used that allowed comparison to segments with other designations from the literature (14). N-addition of G and C at the joining ends of V(D)J junction (Ngc) was performed to investigate TdT activity. Recurrent amino acid sequence motifs in HCDR3, KCDR3 and LCDR3 were sought using the ClastalW tool (at http://www.ebi.ac.uk/clustalw/#). Amino acid identity >60% in the HCR3 or >80% in the K/LCDR3 was required for the inclusion of a IgH or a 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 (NGS) in IgHVJ, IgKVJ and IgLVJ transcript sequences was assessed as previously described (6,19).

Statistical analyses were performed using X2 or Fisher's exact tests. P-values <0.05 were considered significant.

RESULTS

Analysis of expressed IgHV region transcripts and sIgH isotype proteins

The expressed tumor IgHV sequences were identified in 83/88 HCL. The distribution of IgHV families was similar to that of normal B-cells, but use of individual IgHV gene segments showed differences from the normal B-cell repertoire (figure 1A). The IgHV gene segments most frequently used by HCL were IgHV3-30 (13/83, 16%), IgHV3-33 (9/83, 11%), IgHV3-23 (7/83, 8.5%), IgHV 3-21 (7/83, 8.5%), IgHV4-30/4-31 (5/83, 6%) and IgHV4-34 (5/83, 6%). Among these segments, 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 none reached significant differences individually, likely due to the number of cases investigated. In fact, by assembling results of this study with all published HCL IgHV gene sequences (n=164), we could confirm the overuse of IgHV3-21, IgHV3-30 and IgHV3-33, and the significantly reduced use of IgHV1-18 (p=0.02) and IgHV3-53 (p=0.02) could be demonstrated (8,9,21,22).

Phenotype of surface IgM, IgD, IgG and IgA was available from 56 HCL. 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 restricted to all 5/63 variant HCL (cases HCL8, HCL17, HCL28, HCL72, HCL82, HCL87). These data confirm that lack of surface CD27 and CD38 is a feature of typical HCL (11).

Analysis of HCDR3 junction

Sixty-nine HCL were evaluable for HCDR3 junction (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 use of neither of these segments has been reported 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 represented in 0.5% of all D segments (20). IgHJ gene segments used in the expressed HCL repertoire distributed similarly to the normal B-cell repertoire, 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 from the literature (n=27) have precluded any statistical clustering(8,22,23). Thus significance of IgHD biases remains yet unresolved.

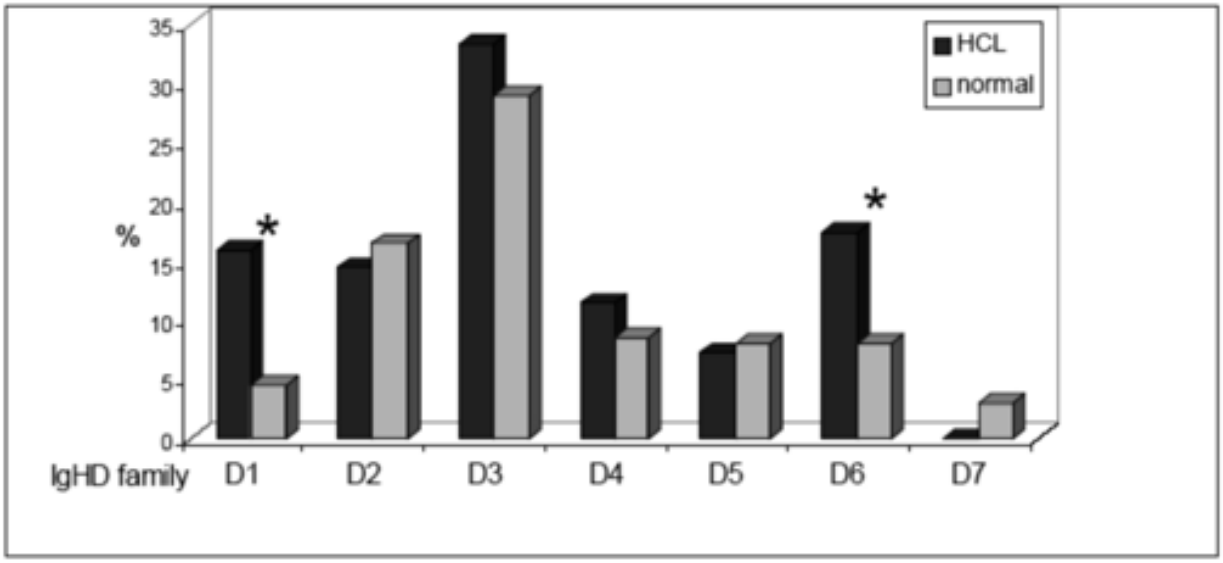

Figure 2.
Expressed IgHD families in HCL. Incidences of IgD families expressed by HCL were compared to the repertoire of normal B-cells from published data.[20]
Dark grey columns: HCL; light grey columns: normal B-cell repertoire. Asterisks indicate regions with identified significant difference between HCL and 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. D1-26 and D6-19 are not reportedly used by normal B-cells.[20]

Analysis of sIgk and sIgλ protein expression and IgKV and IgLV region transcripts

Seventy of 83 HCL were characterized for sIgK and sIgL protein expression. Expression of sIgL was observed in 41/70 (59%) HCL with a IgK:IgL ratio of 0.7:1, indicating preferential secondary rearrangement of IgL.

The expressed IgK and IgL tumor transcript sequences were investigated in 45/70 cases (23 HCL expressing sIgK and 22 HCL expressing sIgL□□ In two additional samples (HCL30 and HCL33), IgL transcripts from the non-functional allele were amplified and sequenced. Four HCL (HCL4/163, HCL42, HCL70, HCL83) co-expressing double IgK or double IgL, or IgK/IgL, were excluded from the analysis.

Among IgKV gene segments (figure 1B), IgKV1D-39(012/02) was most frequently used (6/23, 26%), and showed significant increase compared to normal B-cells (p= 0,03) (24). The distal gene segments IgKV1D-17 (case HCL63) and IgKV6D-21 (HCL11), that 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) (figure 1C), with an overall distribution similar to the normal B-cell repertoire (205).

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 the normal B-cell repertoire (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 acidic sequences reflected pI similarities and identified three subsets, all with 88.8% sequence identity. Subset 1K (HCL28 and HCL2) harbored IgKV1D-33-J1/4 rearrangements (QQYDNLP[L/R]T), that 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), that 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), that 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%) (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 TAG stop at joining codon 115, whereas HCL33 used IgLJ6 with out-of frame rearrangement in codon 115. LCDR3 length ranged from 9 to 13 amino acids (median 11) and pI from 4.4 to 13 (median 13), with 13/22 LCDR3 having identical pI. Analysis of LCDR3 amino acidic sequences identified 3 HCL sub-

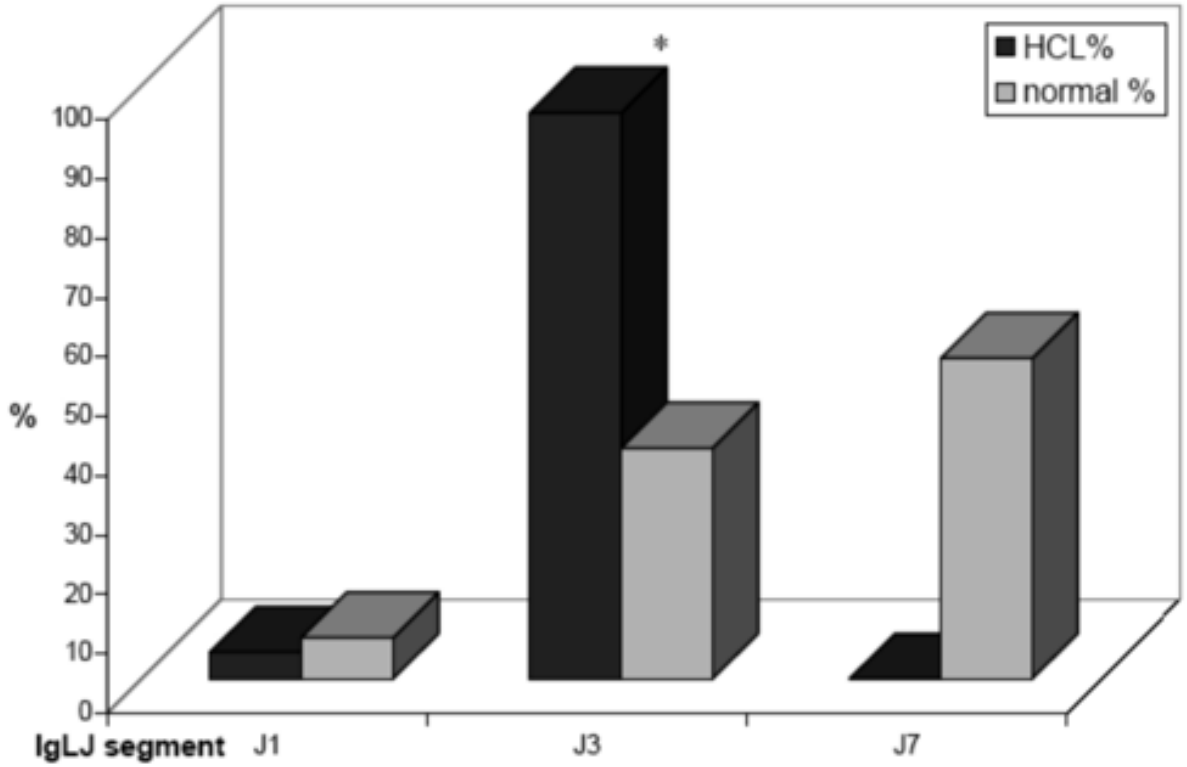

Figure 3.
IgLJ segment usage in IgL HCL. The incidence of IgLJ segment in the functional IgLVJ transcripts expressed by HCL was compared to the functional repertoire of normal B-cells from published data.[26]
Dark grey columns: HCL; light grey columns: normal B-cell repertoire. (*) IgLJ3 segment use in HCL was universal (>95% cases) and significantly superior to IgLJ3 use in normal B-cells.

Figure 4.
Subsets of highly similar LCDR3s 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 IgL+ HCL not belonging to subset 1L; (**) selective distribution of IgHV3-21, 3-30 or 3-33 in HCL from subset 1L compared to HCL with light chain CDR3s 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 CDR3s not belonging to these subsets. ^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-PLT alfa(IIb)beta(3) integrin (EBA11) or anti-La (SS-B)SLE autoAb.[46]

	IgLV	LCDR3	IgLJ	Paired IgHV	Probability of IgHV3-21, 3-30 or 3-33 to associate 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			

sets (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 4/5 cases, with the exception of case HCL22, that 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 from IgLV-J (8/17, 47%, p=0.03) or the total IgL/KV-J (13/38, 34%, p=0.005) rearrangements. 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 IgL/KV-J rearrangements investigated (9/34, 26%, p=0.008). Together with IgL light chain preference and universal IgLJ3 use, these data further suggest that selection events are dominant in the IgL light chain of HCL.

Interestingly, 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 (Ngc) is suggestive of rearrangements having occurred late (in the periphery) as a result of receptor revision in the light chain, after downregulation of TdT enzyme (14).

Somatic mutation analysis of IgHV, IgKV and IgLV

Mutation status of IgHV tumor transcripts was evaluated in 83 HCL. 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 extended to 17/83 HCL (20.5%).

The distribution of individual IgHV genes varied among mutated and unmutated HCL. In particular, IgHV3-30, the segment most frequently used in HCL, showed 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). Also, IgHV4-34 was more frequently used in unmutated (3/17, 18%) than in mutated HCL (2/66, 3%) (p=0.02).

IgKV mutation status (n=23) was variable (homology range 93.61%-100%, median 97.26%, mean 97.31%), and 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. IgLV homology to germline ranged from 89.9% to 100% (median 97.12%, mean 96.83%), and 1/22 HCL had completely unmutated IgLV. 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 HCL. The IgHV-IgKV/IgLV mutation status was concordant in 22/40 HCL. Eighteen cases were mutated in both IgHV and IgKV/IgLV. 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 HCL (12 IgHV mutated-IgKV/IgLV unmutated cases and 6 IgHV unmutated-IgKV/IgLV mutated cases). However, 16/18 discordant HCL carried some levels of mutation (homology to germline <100%) in both IgHV and IgKV/IgLV, suggesting that the variations very likely represent true mutations and not polymorphisms (29). Only 2 discordant cases (HCL37 and HCL 81) 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 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 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 the 2/2 cases with IgHV homology >98% and <100% 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 chains, that 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 provides implications for HCL origin, and indicates that Ig selection may play an important role in disease pathogenesis.

IgHV analysis shows that HCL is characterized by bi-

Table 1. intraclonal variability in HCL.

Code	IgHV	Homology	Intraclonal heterogenity in IgHV	IgK/LV	homology	tumor/total IgL	intraclonal heterogeneity 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	-	LV2-11(2e)	100	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, as described.[2,7,29] The HCL cases, with previously published details on intraclonal variation in the tumor IgH isotypes, were added the original code after the slash.[2,7,29]

ased 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 (SpA) (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 other B-cell neoplasms, in particular from the deeply investigated IgM+ve CLL.[19] For example, IgHV1-69 was totally absent in HCL from our and previously published series (total 164 IgHV sequences) (2,7,8,21,22,37), while it dominates the unmutated subset in CLL.[19] Similarly, IgHV4-34 is used predominantly in a mutated conformation by CLL, but is preferentially "unmutated" in the HCL of our series (figure 5A), and even more significantly if all HCL published sequences are integrated (p=0.0002) (2,7,8,21, 22,37). Lack of apparent HCDR3 stereotypy in HCL is another contrasting feature with CLL, that conversely frequently groups cases with shared HCDR3 structural features (4,18,38). This also indicates that antigen drive does may not rest on HCDR3-mediated interactions in HCL. Conversely, the low mutational rate of IgHV genes expressed in HCL, and particularly of IgHV3-30 and IgHV3-33 segments that 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) inverted IgK:IgL ratio (0.7:1); ii) universal usage of IgLJ3 in the functional sIgL expressors; and iii) subsets with highly homologous KCDR3 and LCDR3. The preferential usage of IgL 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, IgK is the most frequently used light chain (43-48). On these bases, our results suggest that HCL requires selective usage of IgL. The functional implications of IgL selection in HCL remain speculative. The observations that i) almost 50% HCL expressing sIgL utilize IgHV3-21, IgHV3-30 or IgHV3-33 only; ii) virtually all HCL expressing IgL utilize IgLJ3; and iii) 40% HCL expressing IgL display LCDR3 sets with shared structural features, suggest that HCL expressing IgL may recognize common antigens requiring homologous LCDR3-J3 stretch. 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 could identify 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 IgL bias and LCDR3-J3 selection in HCL are unknown. One possibility of IgL selection is that the IgK-to-IgL shift may derive from secondary rearrangements with rescue of a new light chain in the periphery. Secondary rearrangements of IgL after IgK

deletion have been observed in cases of IgL+ve 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 GC 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 have experienced antigen stimulation. First, the majority of HCL are characterized by SM. 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- (IgM+/-D) and post-switch (IgG+/-A) sIgH, independently of mutational status and while AID is expressed.[2] Histology and lack of CD27 and CD38 suggest that, in typical HCL, the mutational and switch events unlikely occur in GC (11). Additionally, in the present large series of HCL, we observe an irrelevant incidence of novel N-glycosylation sites (NGS) introduced by somatic mutation in the tumor IgV region (<6%), similar to that observed in normal memory B-cells or transformed memory and marginal zone B-cells (tables S1 and S2) (5,6,19,53). Since NGS are specifically introduced in the IgV region of GC-derived lymphomas, while are rare in marginal zone or post-GC B-cell neoplasms (5,6,19,53), our results provide further support to assign the origin of HCL from non-GC derived B-cells.

CD27-ve memory B-cells or marginal zone B-cells are the candidate counterpart of HCL (54). The comparison of 14 HCL with the gene expression profiles of normal B-cells from the naïve, GC, 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 SHM and lack of novel NGS (19,36,55). Morphology and phenotype also resemble marginal zone derived CD23-ve, CD27-ve, CD38-ve interfollicular B-cells. Overall, efforts should be directed at identifying the normal counterpart within these categories (1,36,55).

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