Leukemia (2004) 18, 1941–1947

www.nature.com/leu

Evidence of biased immunoglobulin variable gene usage in highly stable B-cell chronic lymphocytic leukemia

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Recognition of biased immunoglobulin variable (IgV) gene usage in B-cell chronic lymphocytic leukemia (B-CLL) may yield insight into leukemogenesis and may help to refine prognostic categories. We explored Ig variable heavy (V_H) and light (V_L) chain gene usage in highly stable and indolent B-CLL ($n=25$) who never required treatment over 10 or more years. We observed an unexpectedly high usage of mutated V_H3-72 (6/25; 24.0%), a gene that was otherwise rare in B-CLL (7/805; 0.87%; $P<0.01$), including mutated cases (6/432; 1.39%; $P<0.01$) and was exceptional among indolent (1/230, 0.435%; $P<0.01$), and aggressive B-cell lymphomas (0/105; $P<0.01$). Three of six V_H3-72 B-CLL cases utilized the same V_L V_κ4-1 gene. Two V_H3-72 B-CLL cases had highly homologous V_H complementarity determining regions 3 (CDR3s), encoding Cys-XXXX-Cys domains, and utilized V_κ4-1 genes with homologous IgV_L CDR3s. An identical threonine to isoleucine change at codon 84 of V_H3-72 framework region 3 (FR3) recurred in four cases of highly stable V_H3-72 B-CLL. This mutation is expected to cause a conformational change of FR3 proximal to CDR3 that might critically affect high-affinity antigen binding. B-cell receptors encoded by V_H3-72 may identify a specific B-CLL group and be implicated in leukemogenesis through an antigen-driven expansion of B cells.

Leukemia (2004) **18,** 1941–1947. doi:10.1038/sj.leu.2403537
Published online 14 October 2004
Keywords: chronic lymphocytic leukemia; immunoglobulin genes; leukemogenesis

Introduction

B-cell chronic lymphocytic leukemia (B-CLL), the most common leukemia type in the Western hemisphere, may be dissected into at least two clinico-pathologic variants based on somatic hypermutation (SHM) of immunoglobulin variable (IgV) genes, the presence of mutations favoring prolonged survival.[1,2] The expression level of ZAP-70 and, to a lesser extent, of CD38 is correlated with the Ig SHM status.[1,3]

Within the two major categories of IgV mutated and unmutated B-CLL, disease heterogeneity may be further refined by usage of specific IgV heavy (V_H) and light (V_L) chain genes.[4–6] To date, several groups of B-CLL displaying a biased usage of IgV genes have been identified among both mutated and unmutated cases.[7–11] In some instances, restriction of B-cell receptor (BCR) usage in B-CLL may have prognostic significance independent of the IgV mutational status.[10] Remarkably, extensive analysis of complementarity determining regions 3 (CDR3s) of V_H and V_L chains has revealed that a fraction of B-CLL is characterized by a high degree of BCR homology.[12–15] As biased IgV usage and BCR homology suggest selection for a particular reactivity implicated in B-cell expansion, the recognition of these IgV features in some B-CLL groups may shed light on leukemogenesis.[4–6,16]

Recently, we reported on a series of highly stable and indolent B-CLL patients who never required treatment over prolonged follow-up.[17] Here, we address the issue of a potential V_H and V_L bias in these highly stable B-CLL. We report that a significant subset of highly stable B-CLL patients in our series utilize the V_H gene V_H3-72 and, in some cases, express V_H and V_L sequences with homologous CDR3s, suggesting recognition of a common antigen.

Materials and methods

Patients and database construction

Out of a consecutive series of B-CLL of Caucasian origin (Italy) followed at the Division of Hematology of 'La Sapienza' University, Rome, we studied highly stable cases ($n=25$) with indolent disease never requiring treatment over a 10–23 year follow-up period from diagnosis.[17] These 25 cases represented the B-CLL patients matching the criteria for highly stable B-CLL and consecutively seen at the outpatient clinic during a 3-month period.

For comparison, a total of 1140 IgV genes from various B-cell malignancies (805 B-CLL, 230 indolent B-cell lymphoproliferative disorders and 105 aggressive B-cell lymphomas) were included in a database. Initially, we collected information concerning VDJ usage and mutation frequency of 100 V_H productive rearrangements from B-CLL consecutively seen at the University of Eastern Piedmont. To exclude bias in our ability to amplify the B-CLL V_H spectrum, we compared our internal database to 705 V_H productive rearrangements from published B-CLL (see Table 1 for details). Since statistical analysis did not disclose differences, data were merged into a single database of 805 B-CLL. A database of indolent B-cell lymphoproliferative diseases (48 follicular lymphomas, nine lymphoplasmacytic lymphomas, 142 marginal zone lymphomas, 20 hairy cell leukemias, 11 prolymphocytic leukemias) and of aggressive lymphomas (90 diffuse large B-cell lymphomas and 15 Burkitt lymphomas) was constructed from unpublished cases in our files (21 indolent B-cell lymphoproliferative disorders and 105 aggressive lymphomas) and from the literature (209 indolent B-cell lymphoproliferative disorders; for details, see Table 1).

A database of mutated V_H3-72 sequences ($n=22$) derived from non-neoplastic B cells was also constructed (GenBank accession numbers AF103057, AF103194, AF103257, AF129753, AF471540, AF471541, AF471542, AF471543, AF471545, AJ008218, AJ009524, AJ231563, AJ275377, AJ275422, AJ407996, AJ415013, AY206989, D83679, S76904, Z11950, Z37300, Z80700).

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Received 8 May 2004; accepted 16 August 2004; Published online 14 October 2004

Table 1 References and other sources used for the construction of the IgV database

First author	*Reference*	*Year*	*No. of cases used for database construction*		
			Mutated IgV genes	*Unmutated IgV genes*	*Total*
B-CLL					
Chang C-C *et al*	*Blood* **100**: 4671	2002	14	8	22
Dono M *et al*	*Blood* **87**: 1586	1996	3	2	5
Efremov DG *et al*	*Blood* **87**: 3869	1996	10	14	24
Fais F *et al*	*J Clin Invest* **102**: 1515	1998	47	36	83
Fais F *et al*	Genbank Nos. AJ555251–AJ555275	2003	14	11	25
Gurrieri C *et al*	*J Exp Med* **196**: 629	2002	7	11	18
Hamblin TJ *et al*	*Blood* **94**: 1848	1999	46	38	84
Isobe K *et al*	*Leuk Lymphoma* **42**: 499	2001	3	1	4
Klein U *et al*	*J Exp Med* **194**: 1625	2001	19	17	36
Korganow AS *et al*	Genbank Nos. S73953, S73955, S73957, S73959, S73961, S73963	1994	2	4	6
Lanham S *et al*	*Blood* **101**: 1087	2003	25	15	40
Maloum K *et al*	*Blood* **96**: 377	2000	27	12	39
Matolcsy A *et al*	*Blood* **89**: 1732	1997	4	1	5
Mc Carty H *et al*	*Blood* **101**: 4903	2003	13	7	20
Nakamura N *et al*	*Pathol Int* **49**: 595	1999	8	4	12
Oscier DG *et al*	*Blood* **89**: 4153	1997	10	12	22
Pasqualucci L *et al*	*Cancer Res* **60**: 5644	2000	26	11	37
Pritsch O *et al*	*Br J Haematol* **107**: 616	1999	18	7	25
Ramsland PA *et al*	Genbank Nos. AF099197–AF099201	1999	0	5	5
Rassenti L *et al*	*J Exp Med* **185**: 1435	1997	6	7	13
Rosenwald A *et al*	*J Exp Med* **194**: 1639	2001	12	16	28
Sahota S *et al*	*Blood* **95**: 3534	2000	0	2	2
Sakai A *et al*	*Blood* **95**: 1413	2000	9	14	23
Schettino EW *et al*	*J Immunol* **160**: 820	1998	1	2	3
Schroeder HW and Dighiero G	*Immunol Today* **15**: 288	1994	36	39	75
Stankovic T *et al*	*Blood* **99**: 300	2002	22	27	49
Indolent lymphoproliferative disorders					
Aarts WM *et al*	*Blood* **95**: 2922	2000	25	0	25
Aarts WM *et al*	*Blood* **92**: 3857	1998	7	0	7
Bahler DW *et al*	*Blood* **89**: 3335	1997	5	0	5
Bende RJ *et al*	*Am J Pathol* **162**: 105	2003	3	0	3
Davi F *et al*	*Blood* **88**: 3953	1996	11	0	11
Dunn-Walters DK *et al*	*Hum Pathol* **29**: 585	1998	4	0	4
Forconi F *et al*	*Blood* **98**: 1174	2001	5	0	5
Garand R *et al*	*Br J Haematol* **109**: 71	2000	8	0	8
Hara Y *et al*	*Invest Ophtalmol Vis Sci* **42**: 2450	2001	19	1	20
Maloum K *et al*	*Br J Haematol* **101**: 171	1998	7	0	7
Marasca R *et al*	*Am J Pathol* **159**: 253	2001	10	0	10
Miranda RN *et al*	*Hum Pathol* **30**: 306	1999	16	0	16
Qin Y *et al*	*Blood* **86**: 3528	1995	4	0	4
Tierens A *et al*	*Blood* **91**: 2381	1998	14	0	14
Tierens A *et al*	*Am J Pathol* **162**: 681	2003	14	9	23
Zhu D *et al*	*Br J Haematol* **120**: 217	2003	34	0	34
Zhu D *et al*	*Blood* **99**: 2562	2002	13	0	13

Analysis of V_H and V_L genes

V_H and V_L rearrangements were amplified from genomic DNA using *Taq* polymerase with family-specific primers hybridizing to leader or framework (FR) 1 sequences and J_H, J_κ or J_λ degenerate primers.[8,18–21] For IgV sequencing, a DNA direct sequencing approach was utilized in all cases.[21] Sequences were analyzed as reported[21] and considered mutated if deviation from the closest germline gene was $\geqslant$2%. Criteria for D element identification in CDR3 were as reported.[22] DIR segments and 'minor' D segments were not considered.[23] V_H CDR3 length was determined according to Kabat *et al*[24] by counting the amino-acid (aa) number between position 94 at the end of FR3 (usually two aa downstream of the conserved cysteine) and position 102 at the beginning of FR4 (a conserved tryptophan in all J_H segments). The length of V_L CDR3 was determined by counting the number of aa between position 88 at the end of FR3 and position 97 at the beginning of FR4 (a conserved phenylalanine in all J_L segments). IgV CDR3 charge, defined by an estimated isoelectric point (pI), was determined using the MacVector software.

Statistical analysis

Data of V_H and V_L rearrangements, VDJ usage, mutation frequencies, CDR3 length and pI were handled in Excel spreadsheet format (Microsoft Corp, Redmond, WA, USA). SPSS

software was used for statistical elaboration. Fisher's exact test with two-tailed P and χ^2 test, with Bonferroni adjustment for multiple comparisons, was used to estimate differences in V_H, V_L, D and J_H use among various groups of lymphoid malignancies. The parametric *t*-test and the nonparametric Mann–Whitney test were used to estimate differences in CDR3 length and mutation frequency among different B-CLL groups. Mutation distribution between CDR and FR was evaluated by the Chang–Casali binomial and multinomial distribution models.[25,26]

Results

Biased usage of V_H3-72 and V_H2-05 genes in highly stable B-CLL

A total of 27 V_H rearrangements were amplified and sequenced from 25 highly stable B-CLL. Two cases carried both productive and nonproductive rearrangements. Only productive rearrangements were further analyzed (Table 2). Using a cutoff value of $\geqslant$2% mutation rate, all but one case was scored as somatically hypermutated, with a mean mutation frequency of 6.61$\pm$2.87%, median 6.46%, range 2.32–13.7%. Overall, the V_H family most frequently used was V_H3 (10/25; 40.0%), followed by V_H4 (5/25; 20.0%), V_H1 and V_H2 (4/25; 16.0% for both families), V_H5 and V_H6 (1/25; 4.00% for both families). Statistical analysis showed a significant over-representation of the V_H2 family in highly stable mutated B-CLL (4/24; 20.4%) compared to mutated B-CLL from the database (20/432; 4.63%) ($P<0.05$).

The single V_H gene most frequently encountered in highly stable B-CLL was V_H3-72 (6/24; 25.0%). In contrast, V_H3-72 was rarely utilized by B-CLL from the database (7/805; 0.87%; $P<0.01$), including somatically mutated cases (6/432; 1.39%; $P<0.01$) and was absent or rare among other indolent (1/230; 0.435%; $P<0.01$) and aggressive (0/105; $P<0.01$) B-cell lymphoproliferative disorders. Extensive analysis of published IgV data revealed that, in normal B-cells, the V_H3-72 allele accounts for <2% V_H rearrangements.[27–32] The complete nucleotide and aa sequence of V_H3-72 genes utilized by highly stable B-CLL are represented in Figure 1.

Highly stable B-CLL also displayed a biased usage of V_H2-05, occurring in 3/25 (12.0%) cases *vs* 21/805 (2.61%) B-CLL from the database ($P<0.05$), of which 15/432 (3.47%) were mutated. V_H2-05 was restricted to 2/230 (0.91%) indolent ($P<0.01$) and 1/105 (0.952%) aggressive lymphomas ($P<0.01$). In normal B cells, all three V_H2 family alleles account for <2.5% rearrangements.[29] Usage of V_H1-02, although occurring in three highly stable B-CLL, did not statistically differ from that of B-CLL cases from the database.

Nonrandom V_L rearrangements in highly stable B-CLL

A clonal productive V_L rearrangement could be identified in all but one highly stable B-CLL (Table 2). In one case (case 17), only the rearrangement of a nonfunctional allele of the V_κ2-29 locus was found. Overall, 19/25 (76.0%) highly stable B-CLL

Table 2 General characteristics of V_H and V_L productive rearrangements in highly stable B-CLL

Case	*V_H*						*V_L*				
	V_H	*Mutation (%)*	*D*[a]	*J_H*	*P*[b]		*V_L*	*Mutation (%)*	*J_L*	*P*[b]	
					FR P_B/P_M	*CDR P_B/P_M*				*FR P_B/P_M*	*CDR P_B/P_M*
1	3-72	2.32	1-7	4b	0.29/0.51	0.26/0.26	V_κ1-16	3.52	κ1	**<0.01/<0.01**	**<0.001/<0.001**
2	3-72	3.65	NA	6b	**0.010/<0.01**	0.26/0.77	V_κ1-5	5.94	κ4	**0.02/0.025**	**0.012/0.010**
3	3-72	4.98	NA	5b	0.064/0.070	0.23/0.72	V_κ4-1	3.50	κ2	**0.040/0.026**	0.25/0.32
4	3-72	3.67	NA	4b	**0.01/<0.01**	0.099/0 .091	V_κ1-5	4.06	κ3	0.082/0.076	**0.011/<0.01**
5	3-72	6.97	2-2	3b	**<0.01/<0.01**	0.11/0.14	V_κ4-1	2.11	κ1	0.187/0.136	0.312/0.325
6	3-72	3.56	2-2	3b	0.11/0.30	0.21/0.95	V_κ4-1	1.18	κ2	—	—
7	2-05	6.04	5-24	4b	**0.021/0.02**	**0.022/0.02**	V_κ1-5	2.66	κ1	**0.038/0.019**	**0.002/0.001**
8	2-05	8.00	1-1	5b	**0.040**/0.054	0.18/0.67	V_κ1-5	1.87	κ2	—	—
9	2-05	3.65	7-27	3b	0.20/0.71	0.29/0.51	V_κ1-5	4.92	κ2	**<0.01/<0.01**	**0.026/0.021**
10	1-02	6.10	3-10	6c	0.15/0.25	0.061/0.063	V_λ3-21	1.27	λ2/3a	—	—
11	1-02	8.44	7-27	2	**<0.01/<0.01**	**<0.01/<0.01**	V_κ1-5	5.38	κ1	0.23/0.25	0.35/0.64
12	1-02	7.09	6-6	5b	**0.013/0.013**	0.097/0.12	V_κ1-6	3.57	κ2	0.19/0.20	0.066/0.051
13	4-34	7.71	3-22+3-16	4b	0.16/0.40	0.053/0.059	V_λ8-61	4.77	λ3b	**0.020/0.014**	0.053/**0.045**
14	4-34	7.51	2-15	4b	**0.024/0.025**	0.10/0.13	V_λ1-51	5.78	λ2/3a	**0.027/0.025**	0.077/0.072
15	4-39	6.82	NA	5b	**0.035/0.025**	**<0.01/<0.01**	V_κ1-5	3.64	κ2	0.053/**0.039**	0.110/0.099
16	4-39	7.05	NA	1	**0.022/0.014**	0.11/0.14	V_κ1-5	6.16	κ2	**<0.01/<0.01**	**0.027/0.021**
17	1-18	4.10	4-17+1-26	4b	0.083/0.087	0.28/0.56	V_κ2-29	0	κ2	—	—
18	2-70	4.68	4-23	3b	0.12/0.16	0.15/0.18	V_κ2-28	1.74	κ2	—	—
19	3-23	10.7	NA	5b	**0.011/0.012**	**0.025/0.029**	V_κ3-15	1.95	κ2	—	—
20	3-48	10.8	5-12	4b	**0.023/0.029**	0.15/0.30	V_κ1-8	3.49	κ1	0.21/0.26	0.30/0.41
21	3-49	13.7	4-17	4b	**<0.01/<0.01**	**0.012/0.014**	V_κ3-20	6.84	κ1	**0.024/0.021**	**<0.01/<0.01**
22	3-74	11.5	NA	5b	**<0.01/<0.01**	0.07/0.10	V_κ1-9	6.59	κ1	**<0.01/<0.01**	**0.046/0.049**
23	4-30.1	4.68	NA	6c	0.13/0.17	0.071/0.068	V_κ3-15	4.68	κ1	0.20/0.66	0.16/0.19
24	5-51	1.35	3-10+3-22	4b	—	—	V_κ3-20	4.71	κ1	0.14/0.17	0.082/0.076
25	6-01	4.95	7-27	4b	**0.032/0.029**	**0.011/<0.01**	V_κ2-28	2.68	κ4	0.19/0.14	0.15/0.09

[a]NA, not assignable.
[b]CDR, complementarity determining region; FR, framework region; *P* is the probability calculated to evaluate whether the excess or the scarcity of R mutations in CDR and FR, respectively, is due to chance alone; P_B, *P*-value calculated according to the binomial distribution model; P_M, *P*-value calculated according to the multinomial distribution model; Bold values represent *P*-values <0.05.

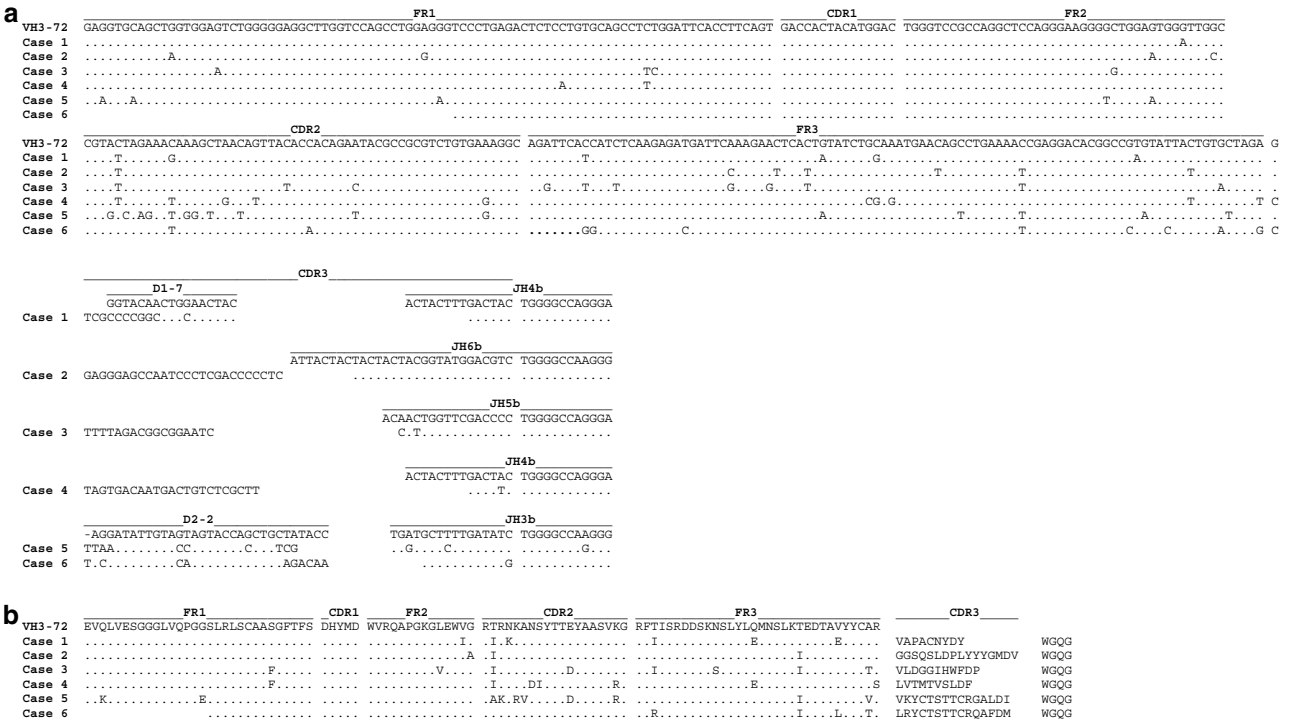

Figure 1 Nucleotide and deduced aa sequences of rearranged V_H3-72 genes derived from highly stable B-CLL. Nucleotide (a) and deduced aa sequence (b) alignments of the V_H3-72 rearrangements derived from highly stable B-CLL (cases 1, 2, 3, 4, 5, 6). The sequences of the V_H3-72 rearrangements were aligned and compared with the most homologous germline V_H, D and J_H sequences. Identity with the most homologous germline sequence is indicated by dots. Each nucleotide mutation and each aa replacement are indicated, respectively, by the appropriate nucleotide and aa. CDR, complementarity determining region; FR, framework region.

displayed mutations in V_L genes, with an average mutation frequency, among mutated cases, of $4.47 \pm 1.39\%$, median 4.68%, range 2.11–6.84%. A total of 22 cases rearranged a V_κ family gene (Table 2). Three cases rearranged a V_λ family gene (cases 10, 13, 14). Among cases rearranging a V_κ gene, the V_κ family most frequently used was V_κ1 (13/22; 59.1%), followed by V_κ3 (4/22; 18.20%), V_κ4 (3/22; 13.6%) and V_κ2 (3/22; 13.6%). The most frequently used V_κ gene was V_κ1-5 (8/22; 36.4%), followed by V_κ4-1 (3/22; 13.6%), V_κ2-28, V_κ3-15 and V_κ3-20 (2/19; 10.5% for each gene). Usage of V_κ1-5 in highly stable B-CLL appeared to be significantly higher compared to that of B-CLL from the database and of normal B cells ($P<0.01$).[33,34]

All V_H3-72 B-CLL used V_κ family genes (Table 2 and Figure 2). Remarkably, three of six V_H3-72 B-CLL (cases 3, 5, 6) utilized the same V_κ gene (V_κ4-1), which did not occur in the other highly stable cases. Two additional cases (cases 2 and 4) of V_H3-72 B-CLL used the V_κ1-5 gene.

Mutational hotspots in V_H3-72 genes of highly stable B-CLL

V_H3-72 genes of highly stable B-CLL exhibited a C>T mutational hotspot at codon 84 of FR3 causing the substitution of isoleucine for threonine in 4/6 (66.7%) cases (Figure 1). This change was restricted to 2/22 (9.09%; $P<0.01$) mutated V_H3-72 rearrangements of non-neoplastic B cells derived from public databases, despite the location of codon 84 within a WRCY hotspot. Since this mutation substitutes a hydrophobic for a polar residue, it is expected to cause a conformational change of FR3 proximal to CDR3 that might critically affect high-affinity antigen binding. V_H3-72 genes of highly stable B-CLL displayed another mutational hotspot at codon 51, also C>T, again causing the substitution of isoleucine for threonine in 4/6 (66.7%) cases (Figure 1). This substitution was not selective for highly stable B-CLL, being found in 9/22 (40.9%) V_H3-72 sequences of non-neoplastic B cells derived from public databases. These recurrent mutations are unlikely to be germline polymorphisms, because no allelic variation was seen in three independent sequences of V_H3-72 germline genes (GenBank accession numbers X92206, AF538057, NG_001019).[35,36] Moreover, the somatic origin of mutations was formally demonstrated in one case of V_H3-72 B-CLL, which, by DNA direct sequencing, displayed the mutations in the lymphocyte DNA, but not in the corresponding granulocyte DNA.

Distribution of IgV mutations in highly stable B-CLL

Mutation distribution between CDR and FR in both V_H and V_L utilized by highly stable B-CLL was evaluated by the Chang–Casali binomial and multinomial distribution models.[25,26] Cases were scored as positive for clustering of silent mutations in FR if the *P*-value was significant in either V_H or V_L genes or both. Similarly, cases were scored as positive for clustering of replacement mutations in CDR if the *P*-value was significant in either V_H or V_L genes or both. Results are summarized in Table 2: the *P*-values calculated by the Chang–Casali distribution model (for both FR and CDR) are indicated as P_B; *P*-values calculated by the multinomial distribution model (for both FR and CDR) are indicated as P_M. Combined analysis of available

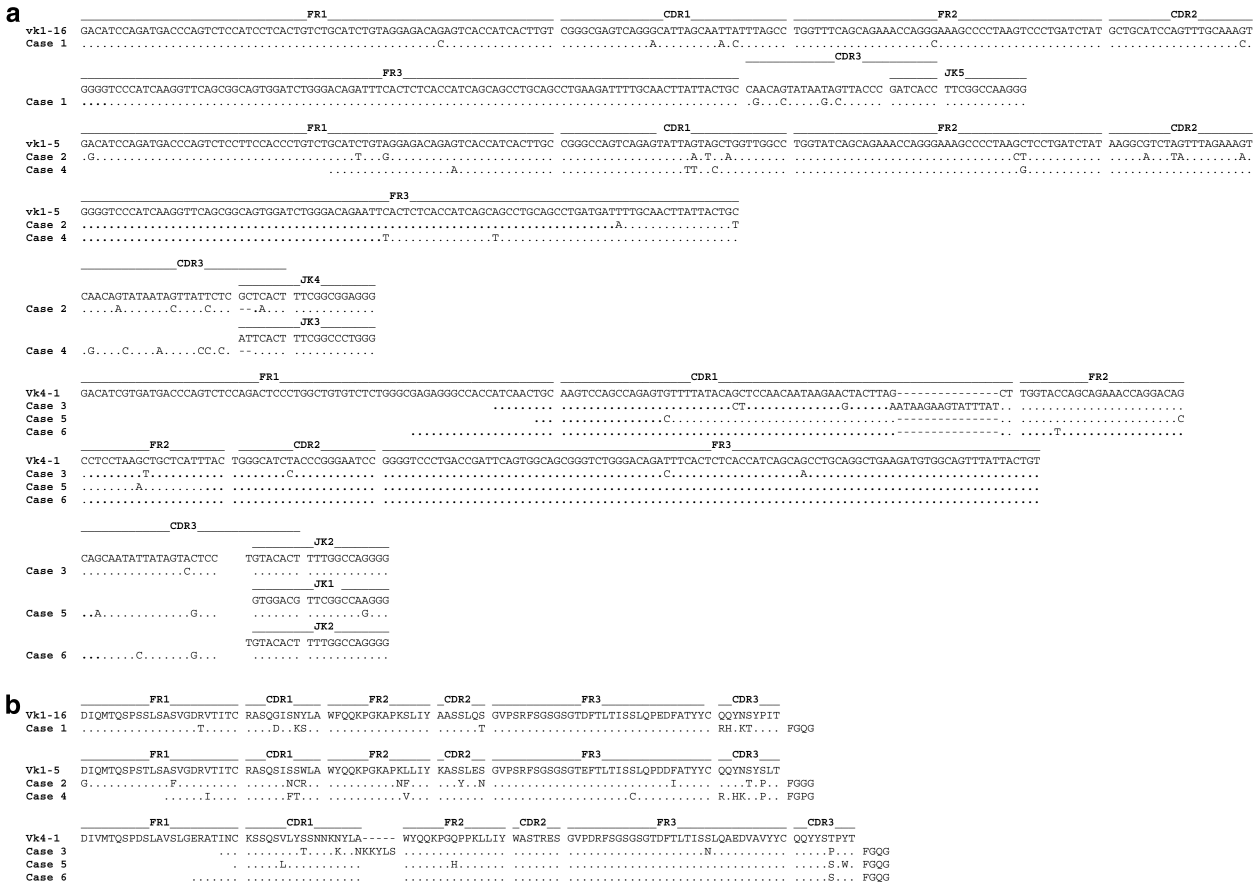

Figure 2 Nucleotide and deduced aa sequences of rearranged V_L genes derived from V_H3-72 highly stable B-CLL. Nucleotide (a) and deduced aa sequence (b) alignments of the V_L rearrangements derived from V_H3-72 highly stable B-CLL (cases 1, 2, 3, 4, 5, 6). The sequences of the V_L rearrangements were aligned and compared with the most homologous germline V_L and J_L sequences. Identity with the most homologous germline sequence is indicated by dots. Each nucleotide mutation and each aa replacement are indicated, respectively, by the appropriate nucleotide and aa. CDR, complementarity determining region; FR, framework region.

V_H and V_L sequences showed a tendency to conserve FR sequences and maintain antigen binding in 19/25 (76.0%) highly stable cases of B-CLL, including 5/6 V_H3-72 cases (Table 2). A higher than expected number of CDR replacement mutations, suggesting selection for high-affinity antigen binding, occurred in 13/25 (52.0%) highly stable B-CLL, including 3/6 V_H3-72 cases.

CDR3 analysis in highly stable B-CLL

Use of J_H and J_κ genes in highly stable B-CLL was consistent with that found in B-CLL from the database and in non-neoplastic B cells.[29,33,34] (See also Table 2.) The most J-proximal D7-27 (DHQ52) D element was over-represented ($P<0.05$) in highly stable mutated B-CLL (3/13; 23.1%) compared to mutated B-CLL from the database (1/148; 0.676%) and non-neoplastic B cells.[29,37,38] The mean CDR3 length of highly stable B-CLL (13.3 ± 3.91 codons) was not different from that of mutated B-CLL from the database (13.6 ± 2.26 codons) (Table 3).

Cluster analysis revealed that two V_H3-72 B-CLL (cases 5 and 6 in Table 3 and Figure 1) had highly homologous V_H CDR3 aa sequences, differing only by conservative substitutions. Both cases rearranged the same D segment (D2-2, in the same reading frame) and the same J_H gene (J_H3). Both these V_H3-72 cases also utilized V_κ4-1 genes and displayed a highly homologous V_L CDR3, differing only by one conservative change (Table 3 and Figure 2).

Consistent with previous reports in B-CLL,[8] the estimated pI value of V_H CDR3 was acidic in the majority (19/25; 76.0%) of highly stable B-CLL (Table 3). The estimated pI value was basic in 4/25 (16.0%) cases, including two V_H3-72 B-CLL with highly homologous CDR3 (cases 5 and 6 in Table 3).

Discussion

In this study, we describe the biased usage of specific V_H and V_L genes and the CDR3 features in a series of clinically highly stable B-CLL cases, which did not progress or require treatment during a follow up of $\geqslant$10 years. The data reveal that usage of V_H3-72 and V_H2-05 genes is significantly higher in highly stable B-CLL patients compared to B-CLL from published databases, non-neoplastic B cells or other B-cell lymphoproliferative disorders. Among V_L genes, highly stable B-CLL display a favored usage of V_κ1-5 and V_κ4-1 genes compared to B-CLL from the database and non-neoplastic B-cells. Notably, two cases of highly stable B-CLL displayed highly homologous CDR3s both in V_H and V_L genes. These data expand on the emerging knowledge of a BCR restriction in B-CLL and document that this phenomenon is a characteristic of the disease also in highly stable cases.

Table 3 Analysis of V_H and V_L CDR3 diversity in highly stable B-CLL

Case	V_H					V_L				
	CDR3 length	CDR3 sequence[a]	No of charged residues		Estimated pI	CDR3 length	CDR3 sequence[a]	No of charged residues		Estimated pI
			+	−				+	−	
1	9	VAPACNY**D**Y	0	1	3.43	9	*R*H*K*TYPIT	3	0	9.70
2	15	GGSQSL**D**PLYYYGM**D**	0	2	3.22	9	QQYNTYPLT	0	0	5.50
3	11	VL**D**GGI*H*WF**D**P	1	2	3.94	9	QQYYSPPYT	0	0	5.50
4	10	LVTMTVSL**D**F	0	1	3.43	9	*R*Q*H*KSYPLT	3	0	9.96
5	15	V*K*YCTSTTC*R*GAL**D**I	2	1	8.80	9	QQYYSSPWT	0	0	5.50
6	15	L*R*YCTSTTC*R*QAF**D**M	2	1	9.05	9	QQYYSSPYT	0	0	5.50
7	14	IS*RR***D**GTNFVGF**D**Y	2	2	6.10	9	Q*H*YNSYPWT	1	0	7.00
8	15	R**E**WNTNWN**E**GWF**D**P	1	3	3.88	10	QQYNTYS*R*YT	1	0	8.79
9	16	*RH*TLPQANW**D**SSAF**D**I	2	2	5.11	8	QQYNSYST	0	0	5.50
10	15	AYGSG*R*STN*H*YYL**D**F	2	1	7.00	11	QVW**D**SSS**D***H*VV	1	2	3.94
11	14	**DE***K***D**VGAQL*R*FF**D**L	2	4	4.00	8	QQYNSFPT	0	0	5.50
12	13	**D**V**E**L*R*YG**E**GW**D**P	1	4	3.66	9	LQ**D**Y**D**YPYT	0	2	3.22
13	12	Y**D**S*R***D**N*R***E**GPGY	2	3	4.33	10	LLFMGSGIWV	0	0	5.50
14	18	GPP*R*G**D**CAGGSCYS**D**F**D**F	1	3	3.67	11	GTW**D**SSLSAVL	0	1	3.43
15	16	*HA***E**NPSPN**D**PQGWL**D**P	1	3	3.77	10	QQY*K*SYSPYT	1	0	8.67
16	13	LATSGL**D***R*FYFQ*R*	2	1	9.05	10	QQY**E**SYTPYT	0	1	3.62
17	12	G**D**YG**D**YSYYF**D**Y	0	3	3.10	—	—	—	—	
18	13	M*R*AVVGG*H***D**AF**D**I	2	2	5.11	9	M**E**AL*H*VPYT	1	1	5.14
19	12	Y**D**ANNG**E***R*WFGP	1	2	4.12	10	QQYNNWPPYT	0	0	5.50
20	13	GASGYSGYGG*R*GL	1	0	8.89	9	QQYY**D**YPWA	0	1	3.43
21	10	**E**MWSPYYF**D**Y	0	2	3.32	10	QLY**D**TFPPWT	0	1	3.43
22	15	**E**VS**D***R*SSYA*K*GWF**E**D	2	3	4.09	9	QQL**DD**Y*PR*T	1	2	3.95
23	14	**D***K*PGPGFFYYYL**D**V	1	2	3.95	9	QQYN**D**WP*R*T	1	1	5.96
24	15	*H*L*R*YY**D**NSG*HY***D**F**D**Y	3	3	5.04	9	QQY*H*TSPGT	1	0	7.00
25	10	**D**PVNG**D**NF**D**Y	0	3	3.10	9	MQALQAPNT	0	0	5.50

[a]Positively charged residues are italicized and underlined; negatively charged residues are represented in bold type.

Recent studies have shown that a restricted usage of specific V_H and V_L genes is a frequent feature of B-CLL, and that B-CLL cells utilizing a particular V_H gene preferentially associate with specific V_L genes.[7–15] Although most examples of BCR restriction have been described in IgV unmutated B-CLL,[7–15] this and other reports document that BCR restriction may be a feature also of IgV-mutated B-CLL.[10,12,14] Whereas past examples of BCR restriction in B-CLL have all been associated with poor prognosis,[10,13] our study points to IgV genes that are preferentially utilized by B-CLL with a very good outcome.

Remarkably, highly stable B-CLL patients display a biased usage of V_H3-72 even when the comparison is restricted to the group of IgV-mutated B-CLL from the database, to which highly stable B-CLL belong. In this respect, it should be noted that highly stable B-CLL are patients who never required any treatment over a follow-up period of $\geqslant$10 years and for whom, consequently, the definition of highly stable B-CLL is more restrictive than that of IgV-mutated B-CLL. Indeed, only a fraction of mutated cases display a highly stable clinical behavior matching the criteria adopted in our study.

Alignment analysis of V_H and V_L CDR3s revealed the presence of two V_H3-72 B-CLL with highly homologous V_H CDR3s differing only by conservative substitutions. The CDR3 of these two cases included a Cys-XXXX-Cys domain, allowing for the formation of a disulfide bridge. Also, both these V_H3-72 cases utilized V_κ4-1 genes displaying highly homologous V_L CDR3s. The highly restricted and homologous structure of BCR strongly suggests selection for a specific reactivity in these cases of highly stable B-CLL. This hypothesis is also supported by selection of hotspot aa substitutions located in the FR3, which recurred in both cases of V_H3-72 B-CLL displaying homologous CDR3s. The nonrandom nature of the CDR3 homology observed in V_H3-72 B-CLL is suggested by the very low probability of finding two clones with highly homologous V_H and V_L rearrangements by chance alone in the same disease subset. Based on the potential number of V_H, D, J_H, V_L and J_L genes that could combine to form a functional BCR, one would expect that B cells would randomly express the same V_H and V_L genes at a probability lower than 10^{-6}. This estimate does not take into account junctional diversity, which would decrease the probability to less than 10^{-12}.

The biased usage of V_H and V_L genes in highly stable B-CLL may be the consequence of neoplastic expansion of B cells derived from a normal B-cell subset characterized by reduced BCR heterogeneity before transformation, or, alternatively, expansion of B cells selected by antigen at some stage of disease development. Independent of the mechanism leading to biased V_H and V_L usage in highly stable B-CLL, it is conceivable that selection of specific BCR structures may affect the growth and evolution of the leukemic clone.

A large-scale analysis of IgV gene rearrangements in highly stable B-CLL patients is required to completely exclude the possibility that the BCR restriction observed in our series might reflect a geographical bias. Finally, if our finding that V_H3-72 is largely restricted to highly stable cases proves true in a broader patient population, its detection may contribute to the prognostic stratification at the time of disease presentation.

Acknowledgements

This study has been supported by Progetto Strategico Oncologia, CNR-MIUR, Rome, Italy; Cofin 2002 – MIUR, Rome, Italy; Ricerca Finalizzata 2002, Ministero della Salute, Rome, Italy;

Regione Piemonte, Torino, Italy; Novara-AIL Onlus, Novara, Italy; and Associazione Italiana per la Ricerca sul Cancro (AIRC), Milano, Italy. EB has been supported by Fondazione 'Piera Pietro Giovanni Ferrero', Alba, Italy.

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