V_H gene usage differs in germline and mutated B-cell chronic lymphocytic leukemia

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Background and Objectives. Given the prognostic relevance that the identification of mutated and germline subgroups of chronic lymphocytic leukemia (CLL) has recently acquired we set out to analyze in depth individual V_H gene usage rearrangements in patients with mutated and germline CLL.

Design and Methods. Using sequence analysis of FR1/J_H polymerase chain reaction products, the V_H immunoglobulin gene configuration was analyzed in 159 rearranged IgH alleles from 154 CLL patients. Having previously identified a spatial relationship between V_H gene usage and J_H proximity in patients with acute lymphocytic leukemia (ALL), we performed linear and Poisson regression analysis on patients with germline and mutated CLL against V_H rearrangements from normal peripheral blood.

Results. Sequence analysis showed that 102 patients (64%) had mutated sequences (>2% DNA base pair changes) while 57 (36%) had germline sequences. The germline CLL group showed J_H proximal overusage similar to that reported in ALL patients, while the mutated CLL group showed a pattern comparable to that of the control group (peripheral blood rearranged V_H sequences). The CDR3 region was statistically longer in the patients with germline CLL than in those with mutated CLL.

Interpretation and Conclusions. This study highlights differences in the VDJ profile in mutated and germline CLL, consistent with the suggestion that CLL comprises two subgroups. The interpretation of these differences is that the B-cell of CLL, particularly in the germline group, may derive from a pool that has been unable to follow or complete the normal pathway of B-cell differentiation.

Key words: CLL, immunoglobulin genes, V_H genes.

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B-cell chronic lymphocytic leukemia (CLL) is characterized by the clonal expansion of $CD5^+$ B cells but its etiology is, as yet, unknown. The cytogenetic features that have a prognostic significance within CLL are heterogeneous. The most frequently observed chromosomal abnormalities in CLL are trisomy 12 and deletions of 11q23 or 17p11, associated with poorer prognosis, and deletion of 13q14, which correlates with a more favorable clinical outcome.[1,2]

Further evidence for heterogeneity within CLL has emerged recently from investigations of the mutational status of immunoglobulin genes.[3-6] Patients with germline V_H genes (<2% mutations at the DNA level) show shorter survival than do patients with mutated V_H genes (>2% mutations).[4,5,7] Germline V_H genes, high CD38 expression and poor prognostic cytogenetic features can be used to define patients with CLL and an unfavorable prognosis.[2,3,8–10] There are 123 V_H segments,[11,12] 26 D segments[12,13] and 6 J_H segments[14] organized in a telomeric to centromeric orientation on chromosome 14 band q32.[11] Fifty-five (44.7%) of the 123 V_H segments are capable of undergoing rearrangement but only 42 are potentially functional.[12] V_H segments are classified into seven families (V_H1- V_H7) on the basis of amino acid sequence homology in the framework (FR1) region.[15] V_H3 is the largest family, followed by V_H4 and V_H1 (64, 32, and 19 members, respectively), whereas V_H2, V_H5 and V_H6 contain only four, two and one member(s), respectively.

As the production of functional IgH genes encoding high affinity antibodies is an ongoing process during B-cell development, normal B cells analyzed at different stages of differentiation show differences in IgH gene rearrangement patterns. This is reflected in their leukemic counterparts. For instance, analysis of V_H genes has suggested in some studies that mammalian fetal V_H-(D)-J_H repertoires are highly restricted. Only a few rearranged V_H3 or V_H5 genes and mostly V_H6 J_H proximal gene dominate[16-18] in agreement with murine studies.[19-22] Other studies, however, have failed to confirm these findings.[23–25]

The final repertoire in mature, adult B cells is thought to be unrestricted and unbiased, as shown by studies in peripheral blood, spleen or tonsil B cells.[26-29] Furthermore, adult life, particularly beyond 50 years of age, is associated with a reduction of V_H gene mutation frequencies, compared to those of cord blood B cells and of younger adults (aged 29-49 years).[30] This indicates a decrease in the ability to generate and maintain a heterogeneous B-cell population in older individuals. This may lead to the

emergence of the more immature B-cell disorders seen in adults than in children, in whom the type of rearrangement is indicative of a more mature B cell being involved.[31]

We and others[32-35] have demonstrated that V_H gene usage in acute lymphoblastic leukemia (ALL) follows a pattern that differs from that mathematically predicted by the normal V_H gene repertoire. In ALL, the pattern reflects the early stage of B-cell differentiation, resulting in an over-usage of the J_H-proximal genes as observed in immature mouse B cells.

Studies have been conducted in a variety of other B-cell disorders to investigate the relationship between IgH rearrangement and B-cell malignancies. In multiple myeloma[36] despite a V_H family usage largely reflecting the germline complexity, individual V_H genes were reported to be over-represented (V_H1-69, V_H3-9, V_H3-23 and V_H3-30) while others were totally absent (V_H3-49, V_H3-53 and V_H4-34).

No apparent restriction was detected among 87 B-cell lymphomas (follicular, lymphoplasmacytoid or large B cell)[37] while in prolymphocytic leukemia (PLL) cells a skewed repertoire is observed with predominant use of the V_H3 family (73%) and V_H3-23 (50%). The frequency of somatic mutations in PLL is high and indicates a post-germinal center origin of the PLL cell.[38]

In CLL over-representation of V_H1-69, V_H3-23, V_H5, V_H6 and V_H4-34 (associated with auto-immune symptoms) has been extensively reported.[3,37,39-44] The IgH repertoire in IgM$^+$ and IgM-CLL populations was found to deviate statistically from those observed in CD5$^+$ cells, considered the normal CLL counterpart.

Given the relevance that the identification of mutated and germline CLL subgroups has recently acquired we set out to analyze in depth individual V_H gene rearrangements in our cohort of patients.

We report here on the analysis of the IgH gene in 154 B-CLL patients (159 alleles) using polymerase chain reaction (PCR) and sequencing of rearranged V_H genes. We investigated the mutation status, the position of individual mutations (within the CDR or FR region) and the pattern of V_H, J_H and D_H gene usage and compared them to those of IgH rearranged alleles of normal B cells as described in peripheral blood B cells. We analyzed the frequency of V_H gene usage as a function of distance along the chromosomal locus.

Design and Methods

Patients' materials

The mononuclear cell suspensions from peripheral blood of 154 CLL patients were used as the source of DNA or RNA for investigation. The diagnosis of CLL was based on clinical history, lymphocyte morphology and immunophenotypic criteria. Twenty milliliters of peripheral blood were diluted 1:1 with sterile Hanks media and the mononuclear layer was separated using Ficoll-Hypaque gradient centrifugation. B-lymphocytes were then harvested as previously described,[45] and DNA was prepared from the mononuclear cell pellet (in 84 of 154 cases) using the Puregene DNA extraction kit (Gentra Systems, Lichfield, UK) following the manufacturer's instructions. RNA was extracted from CLL cells (in 70 of 154 cases) using Triazol reagent (Invitrogen, Paisley, UK) and 1 μg aliquots were reverse transcribed with M-MLV reverse transcriptase enzyme (Promega, Southampton, UK) and an oligo (dT) 15 primer.

Previously published data on the use of V_H genes in peripheral B cells are used as the controls in this study.[26]

PCR for V_H family assignment

For amplification from DNA, six PCR reactions were set up for each patient, using one each of six sense family-specific (V_H1-V_H6) leader or FR1 primers in combination with an antisense J_H primer, as previously described.[34,46,47] The V_H1 forward primer was designed to co-amplify also V_H7 sequences. The PCR reactions were performed using the following cycling conditions; one cycle of denaturing at 94°C for 5 mins followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 66°C for 1 min and extension at 72°C for 2 mins with a final extension step at 72°C for 5 mins. PCR products were then analyzed by gel electrophoresis on a 1.5% agarose gel and individually positive reactions selected for further analysis. A similar approach was used for amplification from RNA, except that a combination of three antisense primers derived from constant region sequences was used.

Cloning and DNA sequencing

Positive PCR products from DNA templates were purified through GFX columns (Amersham Pharmacia Biotech, Buckinghamshire, UK), following the manufacturer's recommendations, prior to their direct sequencing or cloning[46] using Bluescript plasmid KS$^+$ as a cloning vector (Stratagene Ltd., Cambridge, UK). DNA from recombinant colonies was prepared using the QIAprep Spin Plasmid Kit (Qiagen, West Sussex, UK) and the relevant DNA fragments were sequenced using an automated DNA Sequencer (ABI PRISM 377) both following the manufacturers' specifications (Applied Biosystems, Warrington, UK).

Sequence analysis

The V_H-(D)-J_H sequences were submitted to the ImMunoGeneTics (IMGT),[48] and Ig BLAST databases for analysis (http://www.ncbi.nlm.nih.gov/BLAST/), to identify the closest match with germline functional V_H, J_H and D_H segments. Mutations were measured by comparing the CLL and germline IgH gene at the DNA and protein levels. However, the assignment of a patient to the *germline* or *mutated group* was final-

ly based on the DNA changes. The human V_H locus, as published by Matsuda and colleagues,[12] was taken to be the main reference for comparison of sequences, their position and relative distances from each other and the J_H region.

Statistical analysis

Standard statistical tests were carried out using the statistical package STATA including χ^2 tests. To investigate the relationship between frequency of each V_H gene used and the distance along the locus from J_H, linear and Poisson regression analyses were carried out for normal and CLL subjects separately and then compared with results from ALL patients. The same analyses were performed for the use of D segments.

Results

V_H gene and mutation analysis

One hundred and fifty-nine alleles from 154 CLL patients were sequenced and compared to the IMGT and Ig BLAST databases, which identified the closest matching functional germline V_H, D and J_H segment as well as providing information on the incidence of IgH mutations (Table 1, see Appendix).

Based on DNA analysis, 102 of the 159 alleles (64%) were found to carry >2% mutations and were classified as mutated. The remaining 57 alleles (36%) showed <2% mutations and were classified as germline (Table 1, see Appendix). One IgH rearrangement was identified in all patients except five. All sequences were in frame.

Mutations were assessed in the FR1, 2 and 3 and CDR1 and 2 regions. These were more frequent in the CDR1 and CDR2 than in the FR3 and less frequent in the FR1 and FR2 regions. Overall CDR regions accumulated larger numbers of mutations than the FR regions (*data not shown*) in keeping with mutations being predominantly *antigen driven*.

The mutated and germline groups were compared, when available, for various clinical or biological characteristics. In our cohort of patients we found no differences in sex (male to female ratio; 2:1), or age (median 66.8 years) between the two groups. However, a difference was noted between the mutated and germline groups when considering white blood cell count (median 55 and 75.16, respectively) although this difference did not reach statistical significance (p=0.094). It is also noteworthy that in the mutated group the patients predominantly had stage A disease (32/45, 71%), while in the germline group stage C disease was most prevalent (7/13, 54%).

V_H family usage

We compared the mutated and germline groups to a previously published normal control data set (Figures 1A, 2A-D).[26] There was little variation in the overall V_H usage profile with a few exceptions (Figure 1A). As expected, the majority of V_H gene rearrangements involved members of the V_H3 family (77 of 159 clones; 48%), followed by V_H1 (40 of 159 clones; 25%) and V_H4 (25 of 159 clones; 16%). We observed that, if V_H1-69 (the most frequently used V_H1 gene in its family) was removed, V_H1 usage accounted for 14% (22 alleles) of overall gene usage, in line with the mathematical prediction[12] and previous observations.[49] Only V_H family usage which showed differences from the mathematically expected frequencies will be discussed below.

V_H1 gene usage

Differences in V_H gene usage were almost entirely restricted to the V_H1-69 overusage in the germline CLL (13 cases; 68%) compared to normal (one case; 6%; p=0.0326) and mutated CLL (five patients; 26%). The overusage of V_H1-69 in the germline group was statistically significant and consistent with previous observations.[37,41,50]

V_H3 gene usage

The most commonly used genes in the mutated groups were V_H3-7, V_H3-30, and V_H3-23 (Figure 2C). The V_H3-23 was used in only one (4%) patient in the germline group compared to 12 (44%) in the mutated group and 14 (52%) in the controls (p=0.0415). There appeared to be significant overusage of both V_H3-48 and V_H3-21 among CLL patients compared to the control group (p=0.0025 and p=0.0267, respectively). The multiple significance tests performed comparing V_H3-48 to the rest of the V_H3 family showed statistically significant differences between the germline and control groups (p=0.001) and the germline and mutated groups (p=0.015). Differences were also observed in the use of V_H3-21 between the germline and the control groups (p=0.0059).

V_H4 gene usage

V_H4-34 usage was predominant in the mutated group (9 patients; 64%) while only 3 patients (21%) in the control group and two patients (14%) in the germline group used these genes. The overusage in the mutated group was statistically significant p=0.0120 (Figure 2D) compared to in the control group.

D_H segment usage

D_H segments were identified in 95% of sequenced alleles. The largest number of segments identified were D_H3 (52 of 159 clones; 33%), D_H2 (31 of 159 clones; 20%) and D_H6 (21 of 159 clones; 13%) (Table 1; Figure 1B) The D_H3-3-segment was found to be the most frequently used gene in the D_H3 subfamily (18/52)(p=0.0035) followed by D_H 3-22 (15/52). The D_H 3.3-segment showed underusage in the mutated group (17%) compared to the germline CLL (43%) (p=0.0008) and normal controls (40%)(p=0.011).

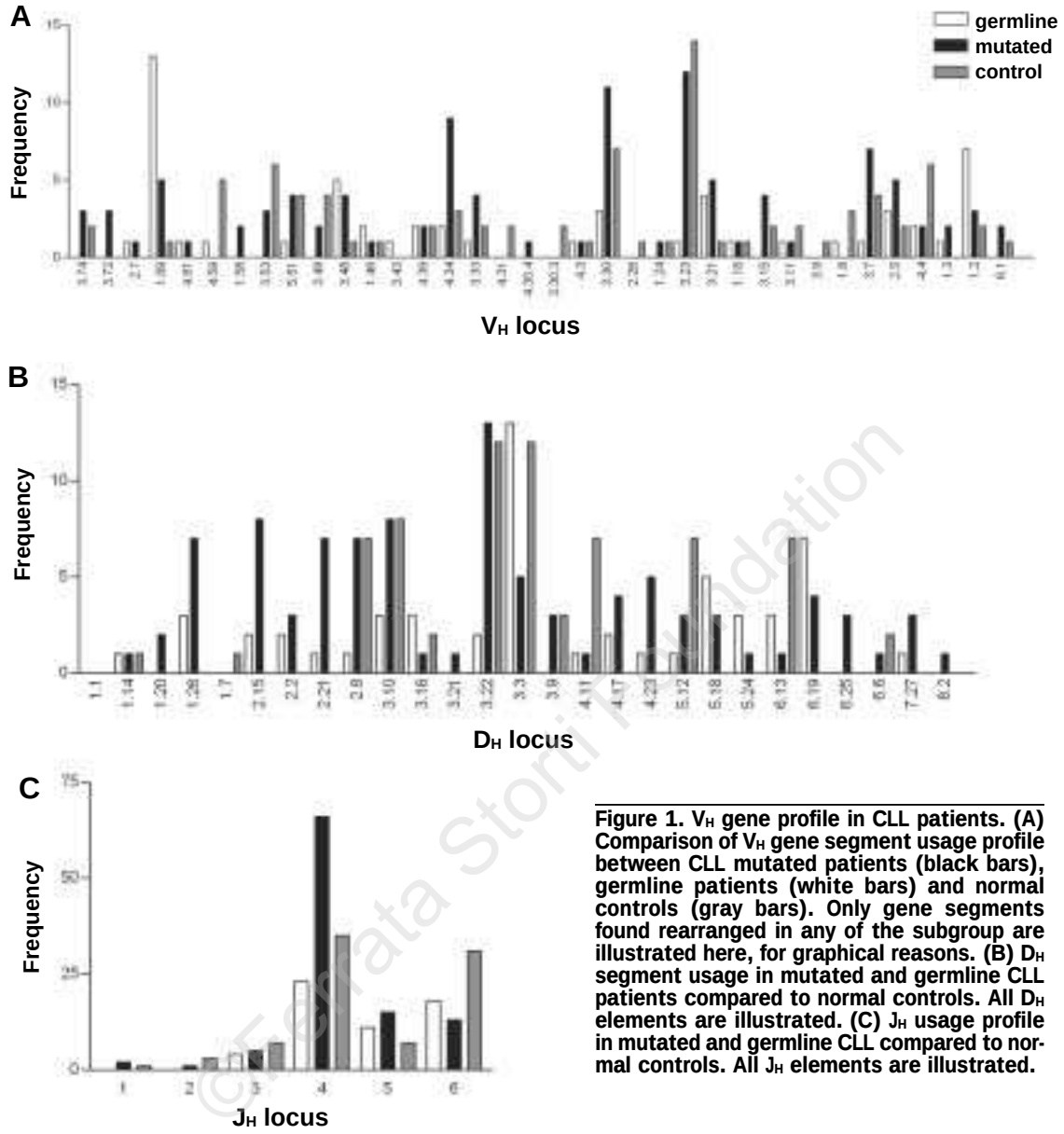

Figure 1. V_H gene profile in CLL patients. (A) Comparison of V_H gene segment usage profile between CLL mutated patients (black bars), germline patients (white bars) and normal controls (gray bars). Only gene segments found rearranged in any of the subgroup are illustrated here, for graphical reasons. (B) D_H segment usage in mutated and germline CLL patients compared to normal controls. All D_H elements are illustrated. (C) J_H usage profile in mutated and germline CLL compared to normal controls. All J_H elements are illustrated.

Conversely, D_H3-22 showed overusage in the mutated group (48%) compared to in germline CLL (7%)(p=0.0502) while its usage was comparable to that in normal controls (44%). Finally, there appeared to be a statistically significant (p=0.0089) underusage of D_H4-11 in the CLL group compared to controls.

J_H segment usage

J_H4, J_H5 and J_H6 were greatly used in the CLL group (Figure 1C). There was a statistically significant overusage of J_H4 and J_H6 (p=0.0017 and p= 0.0004, respectively) when compared to the rest of the J_H genes used. There was a particularly striking overusage of J_H4 in the mutated CLL patients (53%) compared to the germline patients (19%) (p=0.0047).

Spatial relationship of V_H genes in the IgH locus

We and other investigators had previously identified a spatial relationship between the frequency of individual V_H genes used and the J_H locus in ALL patients.[16,34] We performed the same linear and Poisson regression analyses on the control, mutated and germline groups of patients (Figure 3A-C). This analysis failed to identify any significant relationship between gene usage and distance along the J_H locus for the CLL group as a whole. However, when the

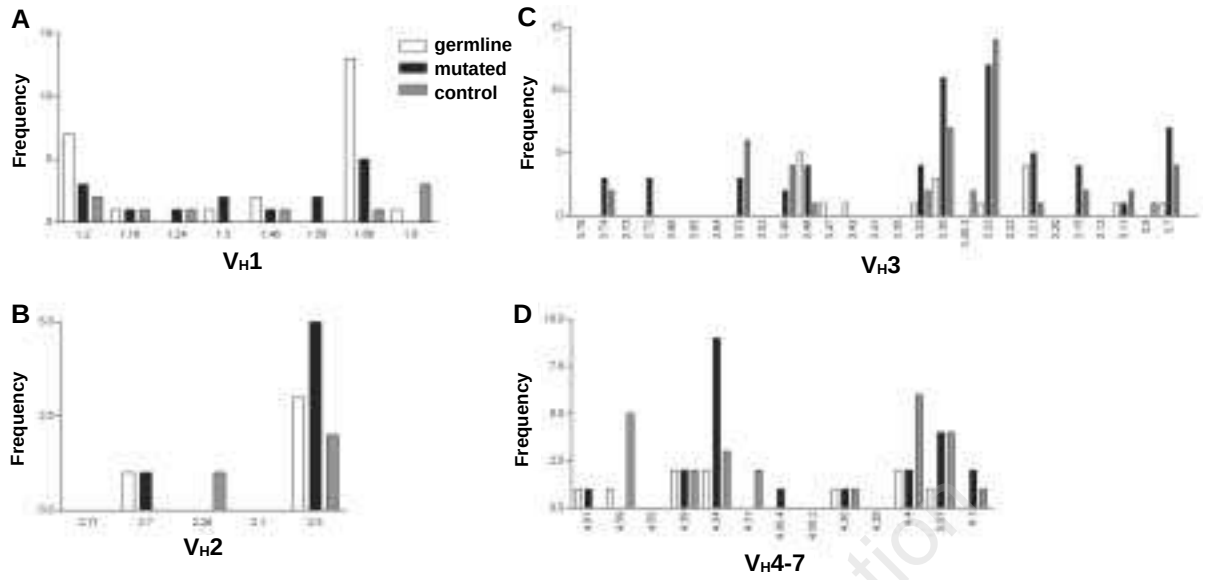

Figure 2. Individual V_H gene family members usage. A) V_H1 family; B) V_H2 family; C) V_H3 family; D) V_H4-7 families. Comparison of normal controls (gray bars) mutated (black bars) and germline (white bars) individual V_H gene families.

germline CLL group was analyzed separately a negative relationship between frequency of gene usage and distance from the J_H segments was observed (p=0.026), particularly when V_H1-69 positive cases were removed from the analysis (Figure 3D). This was done since the V_H1-69 usage is clearly skewed in CLL patients, as previously reported,[41,50,51] most significantly in the germline CLL. Similarly to the V_H genes, the frequency of D_H usage as a function of distance along its locus revealed a positive relationship in the mutated CLL and control groups (p=0.004 and p=0.006, respectively) due to the high usage of D_H3-22, but not in the germline group, further highlighting differences in IgH gene rearrangements in the CLL subgroups.

Complementarity determining region size (CDR3)

The size of the CDR3 region has been previously described to reflect the stage of maturity of the B cell analyzed.[30,52] We found that the CDR3 region was significantly shorter in our cohort of patients, as a whole, than in the control group (p<0.0001) (Figure 4A). When analyzed separately, however, the CLL mutated group had significantly shorter CDR3 regions than the germline group (p=0.0035)(Figure 4B). This difference was not due to selection of germline D_H elements since, for instance, D_H3-3 (overused in the germline group) and D_H3-22 (over used in the mutated group) are both 31 nucleotides long. Furthermore, in the germline group rearrangements involving the overused V_H1-69 gene were associated with a longer CDR3 region (average 37.9 bp) than those of the V_H1-69 gene used in the CLL mutated group (25.4 bp) and this difference was statistically significant (p=0.0332).

We also examined CDR3 length in association with V_H gene usage. We found that the average CDR3 length in V_H4 and V_H1 expressing cells was identical (30.2 bp) but we confirm that it is shortest in V_H3 expressing cells (26.9 bp), as previously described.

These data are in keeping with the more immature nature of the V_H gene rearrangements occurring in the germline CLL patients than in the mutated CLL patients.

Discussion

The results of this study show that, when the much overused V_H1-69 gene is excluded, there is a significant negative relationship between V_H usage and distance from the J_H locus (p=0.026) in the group of patients with germline IgH genes. This is reflected in the linear regression analysis giving a plot (Figure 3D) similar to that seen in ALL patients and in immature normal B cells.[16,17,27,49,53-56] In B-precursor ALL, V_H rearrangement progresses from J_H-proximal V_H genes to distal genes via V_H-V_H replacement as observed in oligoclonal B lineage ALL cases[57] or normal B cells[58] and in agreement with observations in the normal repertoire in human mice and rabbit.[17,22,59–61] In support of this finding, the size of CDR3 region differs between the two groups of patients. CDR3 length is

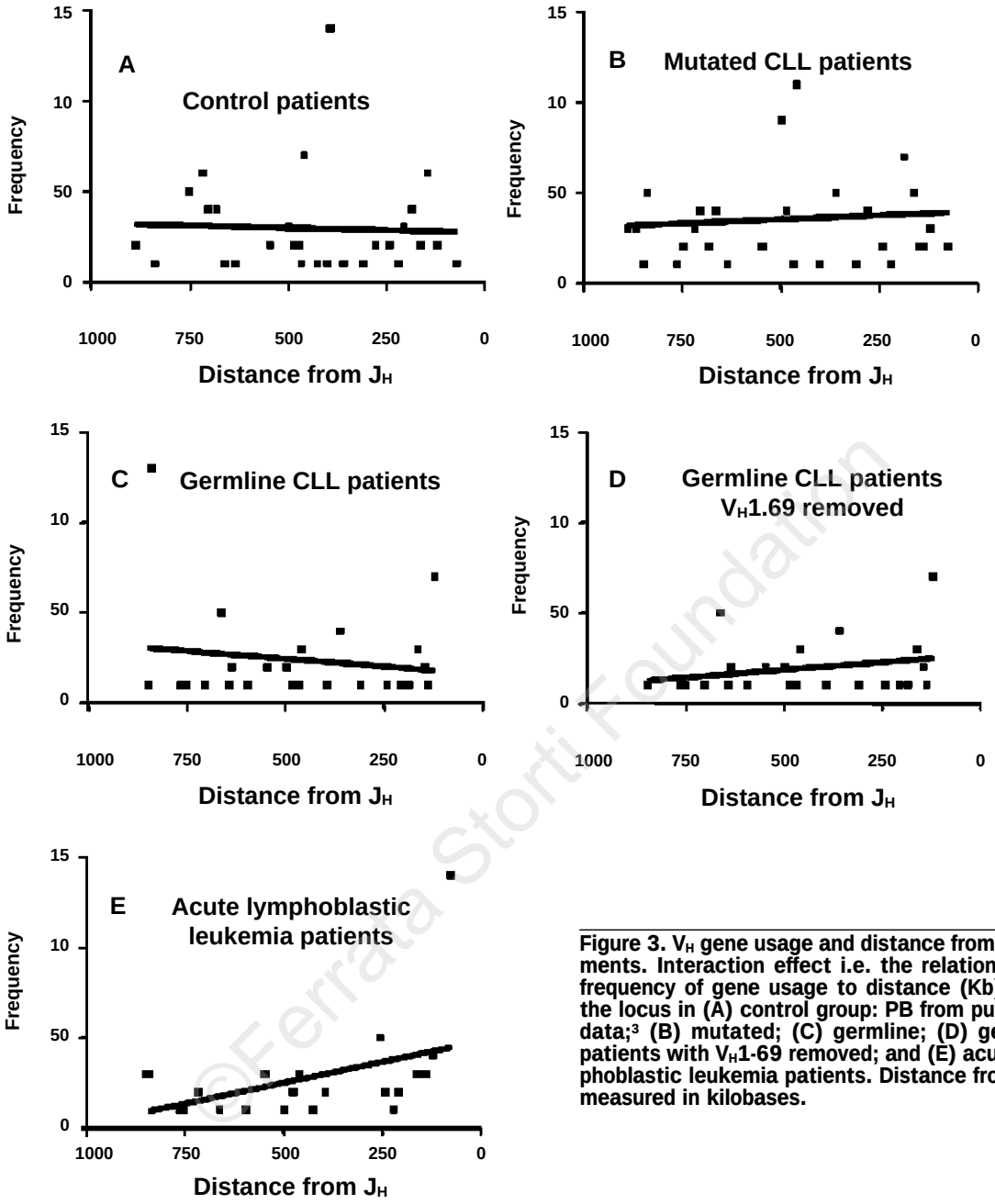

Figure 3. V_H gene usage and distance from J_H segments. Interaction effect i.e. the relationship of frequency of gene usage to distance (Kb) along the locus in (A) control group: PB from published data;[3] (B) mutated; (C) germline; (D) germline patients with V_H1-69 removed; and (E) acute lymphoblastic leukemia patients. Distance from J_H is measured in kilobases.

known to vary according to age and hypermutation status of the V_H gene. The length of the CDR3 region increases continuously during fetal life until birth in mice and humans. This increase does not continue into adult life. CDR3s of old people are the same size as those of young adults.[62,63] However, mutation status has been shown to influence CDR3 length. Mutated antibodies have shorter CDR3 regions than non-mutated antibodies. The length of V_H heavy chains in mice and humans decreases as the B cell matures.[64] In our cohort of patients the CDR3 regions were found to be longer in germline CLL than in mutated. This was also observed in the CDR3 regions of germline V_H1-69 rearrangements but not their mutated counterparts (p=0.0332). These data support the concept that the germline CLL cell derives from a more immature B cell than the B cell of mutated CLL. Our study also reveals a significant underusage of various genes in the unmutated group compared to the mutated CLL group and normal subjects (V_H3-30, V_H3-7, V_H3-23 and V_H4-34) with V_H3-48 and V_H3-21 significantly overused in CLL as a whole compared to normal. We found overrepresen-

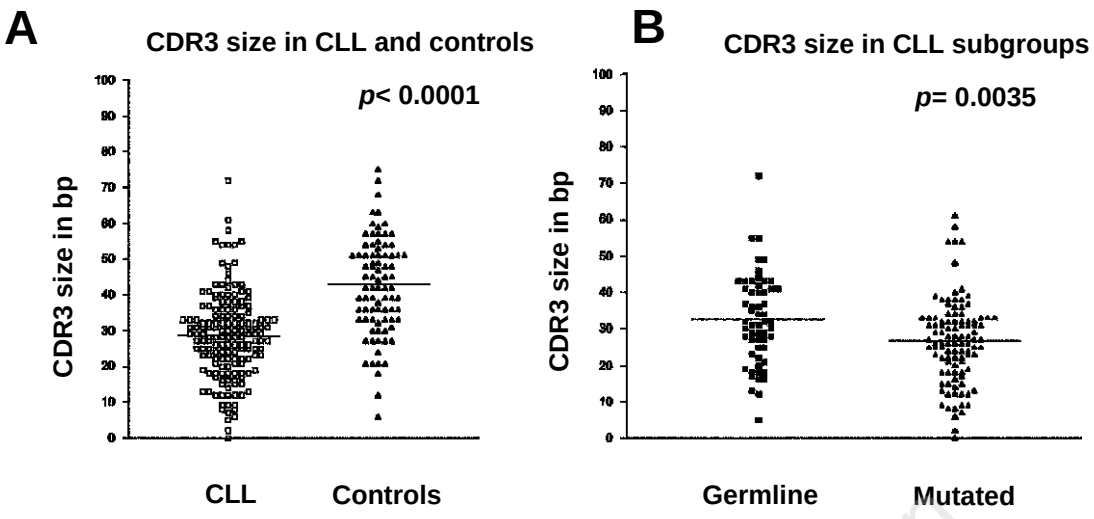

Figure 4. CDR3 size. (A) CDR3 size in bp in CLL and controls; (B) CDR3 size in bp in germline and mutated B-CLL patients.

tation of V_H1-69 usage (p=0.0326) in the germline group as previously described by some[3,41,65] but not all investigators.[66] We could, however, confirm the association of V_H1-69 with the use of J_H6, D_H3-3, D_H3-10 or D_H2-2 genes as reported by others.[62,67]

It is noteworthy that V_H4-34 together with V_H3-07 had previously been described as being the most frequently encountered gene in CLL patients, in addition to V_H1-69. Our study fails to corroborate these findings.[3] V_H4-34 was significantly overused in the mutated group as observed by Kraj *et al*.[66] in normal adult human peripheral blood B cells. We and others,[2,3,5,68] failed to confirm the overusage of V_H3-21 in mutated CLL patients,[44] although this segment was highly used in CLL patients, as a whole.

The mutation frequency has been found to vary according to which V_H family is utilized. For example 77% of all V_H3 genes expressed in our cohort of patients were mutated, as opposed to only 38% of all V_H1 genes. This is in agreement with another study[3] although the mechanism for this imbalance remains unclear. In the V_H1 family the lack of mutations is clearly skewed by the heavy usage of the V_H1-69 in the germline group, as described above.

In our cohort of patients we found no differences in the male to female ratio in the mutated and unmutated groups. This differs from the findings of others[4,7] who report a much higher proportion of males in the unmutated CLL group. They suggest that gender may indirectly influence B-cell maturation, differentiation and clinical outcome.

This study highlights differences in the VDJ profile in CLL patients with mutated and unmutated IgH rearrangements, consistent with the suggestion that CLL comprises two subgroups. Our study substantially expands on data previously presented on smaller cohorts of patients.[3] These differences underlie the fact that leukemic cells in CLL patients, particularly in the germline group, may derive from a pool of B cells that have been unable to follow or complete the normal pathway of B-cell differentiation. On the other hand, the better outcome of the group with mutated IgH rearrangements could be due to somatic mutations which can trigger a cytotoxic T-cell response, as recently demonstrated by some human and mouse models.[69-72] This may result in the leukemic cells being killed unless an additional event (such as a deletion on chromosome 6q21, 11q23, 13q14 or 17p13) provides a growth advantage. This mechanism underlies novel therapeutic approaches for the treatment of lymphomas[73,74] and myelomas.[75]

Finally, a link between germline sequences and inability to generate somatic mutations due to defects in the DNA repair machinery has been proposed. The lack of somatic mutations would then be just a phenotypic marker of some other event with biological consequences on clinical outcome as demonstrated in other disorders carrying DNA repair genes defects.[76,77] In this scenario IgH gene status may have no intrinsic pathogenetic role but its mutation versus germline status would be a marker for the presence or absence of another abnormality with major clinical consequences.

Appendix.

Table 1. Properties of sequenced VDJ rearrangements from 159 B-CLL clones.

Pat. no.	*Mutated/ Germline*	*V_H family*	*V_H gene*	*Distance of V_H from J_H (kb)*	*J_H segment*	*D_H segment*	*CDR3 (bp)*
26	M	V_H6	V6-1	74.31	J_H5	2.15	18
Ps35	M	V_H6	V6-1	74.31	J_H4	6.25	19
AB	G	V_H1	V1-2	121.36	J_H4	3.10	72
K	M	V_H1	V1-2	121.36	J_H4	3.10	28
20	M	V_H1	V1-2	121.36	J_H4	2.21	32
23	G	V_H1	V1-2	121.36	J_H3	4.17	32
27	G	V_H1	V1-2	121.36	J_H4	5.24	41
39	G	V_H1	V1-2	121.36	J_H5	4.17	30
N64	G	V_H1	V1-2	121.36	J_H4	6.19	17
Ps30	G	V_H1	V1-2	121.36	J_H4	6.19	25
Ps37	M	V_H1	V1-2	121.36	J_H5	2.8	33
Ps52	G	V_H1	V1-2	121.36	J_H6	3.16	16
7	G	V_H1	V1-3	139.94	J_H5	2.2	21
33	M	V_H1	V1-3	139.94	J_H5	2.2in	33
Ps74	M	V_H1	V1-3	139.94	J_H4	3.22	18
LG	M	V_H4	V4-4	146.79	J_H5	2.15	31
RB	G	V_H4	V4-4	146.79	J_H4	6.13	13
Ps6	M	V_H4	V4-4	146.79	J_H4	2.21	15
41	M	V_H2	V2-5	162.83	J_H4	7.27	26
L67	G	V_H2	V2-5	162.83	J_H4	6.19	37
Ps13	M	V_H2	V2-5	162.83	J_H4	5.24	34
Ps25	G	V_H2	V2-5	162.83	J_H5	3.3	37
Ps26	M	V_H2	V2-5	162.83	J_H6	2.21	54
Ps44	G	V_H2	V2-5	162.83	J_H6	5.18	25
Ps59	M	V_H2	V2-5	162.83	J_H4	1.26	32
Ps66	M	V_H2	V2-5	162.83	J_H4	4.17	24
RS	M	V_H3	V3-7	187.11	J_H4	2.21	23
Ze	M	V_H3	V3-7	187.11	J_H4	2.8	22
16	M	V_H3	V3-7	187.11	J_H5	2.15	31
35	M	V_H3	V3-7	187.11	J_H4	4.23in	26
47	M	V_H3	V3-7	187.11	J_H5	2.2	29
49	M	V_H3	V3-7	187.11	J_H5	1.26	27
I6	M	V_H3	V3-7	187.11	J_H4	noDH	8
Ps69	G	V_H3	V3-7	187.11	J_H5	3.3	36
Ps72	G	V_H1	V1-8	207.77	J_H6	1.26	28
AB	G	V_H3	V3-11	241.93	J_H4	6.13	18
MW	M	V_H3	V3-11	241.93	J_H4	6.6	33
BC	M	V_H3	V3-15	279.03	J_H4	2.21	22
Ps10	M	V_H3	V3-15	279.03	J_H4	2.15	29
Ps31	M	V_H3	V3-15	279.03	J_H4	3.22	25
Ps60	M	V_H3	V3-15	279.03	J_H4	4.23	25
Ps24	M	V_H1	V1-18	310.25	J_H4	noDH	2
Ps79	G	V_H1	V1-18	310.25	J_H4	6.13in	18

21	G	V_H3	V3-21	360.38	J_H4	3.3	12
25	G	V_H3	V3-21	360.38	J_H4	3.3	55
I2	M	V_H3	V3-21	360.38	J_H6	3.21	9
Ps7	G	V_H3	V3-21	360.38	J_H6	3.22	43
Ps8	G	V_H3	V3-21	360.38	J_H6	5	5
Ps33	M	V_H3	V3-21	360.38	J_H6	noDH	7
Ps49	M	V_H3	V3-21	360.38	J_H6	3.3	37
Ps50	M	V_H3	V3-21	360.38	J_H4	4.17	37
Ps78	M	V_H3	V3-21	360.38	J_H4	3.10	38
12	M	V_H3	V3-23	393.91	J_H4	5.18in	12
13	M	V_H3	V3-23	393.91	J_H4	5.18in	12
19	M	V_H3	V3-23	393.91	J_H4	4.23in	14
32	G	V_H3	V3-23	393.91	J_H5	2.21	34
I3	M	V_H3	V3-23	393.91	J_H4	7.27	28
46	M	V_H3	V3-23	393.91	J_H1	3.22	41
50	M	V_H3	V3-23	393.91	J_H1	3.22	38
52	M	V_H3	V3-23	393.91	J_H4	7.27	13
Ps1	M	V_H3	V3-23	393.91	J_H4	3.22	21
Ps5	M	V_H3	V3-23	393.91	J_H3	1.26	25
Ps29	M	V_H3	V3-23	393.91	J_H6	3.22	32
Ps47	M	V_H3	V3-23	393.91	J_H4	2.2	36
Ps73	M	V_H3	V3-23	393.91	J_H6	5.18	30
LD	M	V_H1	V1-24	401.83	J_H5	3.10	24
HD	M	V_H3	V3-30	459.71	J_H4	5.12	21
EM	G	V_H3	V3-30	459.71	J_H6	3.3	31
11	M	V_H3	V3-30	459.71	J_H4	2.8	31
30	M	V_H3	V3-30	459.71	J_H2	1.14in	16
38	M	V_H3	V3-30	459.71	J_H4	4.23	31
I1	M	V_H3	V3-30	459.71	J_H5	4.17	58
51	M	V_H3	V3-30	459.71	J_H4	5.12	40
L66	M	V_H3	V3-30	459.71	J_H4	4.23	30
Ps16	G	V_H3	V3-30	459.71	J_H3	5.24	29
Ps28	M	V_H3	V3-30	459.71	J_H3	1.2	9
Ps42	G	V_H3	V3-30	459.71	J_H6	5.18	29
Ps53	M	V_H3	V3-30	459.71	J_H3	3.9	39
Ps63	M	V_H3	V3-30	459.71	J_H4	6.13	61
Ps71	M	V_H3	V3-30	459.71	J_H3	2.8	54
PO	M	V_H4	V4-31	473.9	J_H5	3.10	48
Ps9	G	V_H4	V4-31	473.9	J_H5	2.15	46
Ps51	M	V_H4	V4-30.4	467.1	J_H4	6.19	18
10	M	V_H3	V3-33	484.42	J_H6	noDH	
54	M	V_H3	V3-33	484.42	J_H4	2.8	26
N58	M	V_H3	V3-33	484.42	J_H4	2.15	27
N63	M	V_H3	V3-33	484.42	J_H4	6.19	31
Ps38	G	V_H3	V3-33	484.42	J_H6	5.18	29
Sh	G	V_H4	V4-34	498.28	J_H4	3.22	43
JB	M	V_H4	V4-34	498.28	J_H6	2.21	28
17	M	V_H4	V4-34	498.28	J_H4	2.15in	54
Ps17	M	V_H4	V4-34	498.28	J_H5	3.22	24

Ps22	G	V_H4	V4-34	498.28	J_H4	5.24	40
Ps48	M	V_H4	V4-34	498.28	J_H4	4.17	33
Ps57	M	V_H4	V4-34	498.28	J_H4	3.22in	8
Ps61	M	V_H4	V4-34	498.28	J_H5	6.19	18
Ps62	M	V_H4	V4-34	498.28	J_H3	2.15	34
Ps64	M	V_H4	V4-34	498.28	J_H4	6.25	33
Ps65	M	V_H4	V4-34	498.28	J_H6	2.8	29
L65	M	V_H4	V4-39	546.31	J_H5	noDH	15
Ps12	G	V_H4	V4-39	546.31	J_H3	3.3	36
Ps23	M	V_H4	V4-39	546.31	J_H4	6.19	31
Ps43	G	V_H4	V4-39	546.31	J_H4	3.10	20
Ps54	G	V_H4	V4-39	546.31	J_H4	2.2	41
2	G	V_H4	V3-43	594.9	J_H4	5.18	27
Th	M	V_H1	V1-46	635.74	J_H4	2.15	39
Ps36	G	V_H1	V1-46	635.74	J_H5	noDH	19
Ps75	G	V_H1	V1-46	635.74	J_H6	5.18	18
MR	G	V_H3	V3-47	643.21	J_H6	1.26	40
LM	M	V_H3	V3-48	662.52	J_H6		6
I5	M	V_H3	V3-48	662.52	J_H4	3.10	17
Ps2	G	V_H3	V3-48	662.52	J_H5	1.26	27
Ps32	G	V_H3	V3-48	662.52	J_H5	2.8	55
Ps34	G	V_H3	V3-48	662.52	J_H5	1.14in	32
Ps39	G	V_H3	V3-48	662.52	J_H6	3.3	28
Ps46	M	V_H3	V3-48	662.52	J_H4	1.26	32
Ps55	G	V_H3	V3-48	662.52	J_H6	2.15	30
Ps58	M	V_H3	V3-48	662.52	J_H4	3.1	38
6	M	V_H3	V3-49	681.65	J_H4	2.21	34
Ps56	M	V_H3	V3-49	681.65	J_H6	5.12	23
GS	M	V_H5	V5-51	703.42	J_H4	3.10	31
1	M	V_H5	V5-51	703.42	J_H4	8.2	36
9	G	V_H5	V5-51	703.42	J_H4	6.19	27
N59	M	V_H5	V5-51	703.42	J_H4	2.8	32
RB	M	V_H5	V5-51	703.42	J_H4	3.16	26
LD	M	V_H3	V3-53	717.38	J_H5	3.3	
I7	M	V_H3	V3-53	717.38	J_H5	3.9in	13
Ps77	M	V_H3	V3-53	717.38	J_H6	3.10in	24
CT	M	V_H1	V1-58	747.06	J_H4	1.26	20
HD	M	V_H1	V1-58	747.06	J_H4	1.26	
Ps21	G	V_H4	V4-59	751.94	J_H4	3.16	35
Ps11	M	V_H4	V4-61	763.82	J_H4	3.22in	32
Ps27	G	V_H4	V4-61	763.82	J_H6	3.3	31
MR	G	V_H1	V1-69	838.62	J_H5	4.11	40
RM	G	V_H1	V1-69	838.62	J_H3	3.16	49
3	G	V_H1	V1-69	838.62	J_H4	6.19	16
14	M	V_H1	V1-69	838.62	J_H4	6.25	22
15	G	V_H1	V1-69	838.62	J_H4	6.19	22
24	G	V_H1	V1-69	838.62	J_H6	3.3	49
40	G	V_H1	V1-69	838.62	J_H4	7.27	32
I4	G	V_H1	V1-69	838.62			

43	M	V_H1	V1-69	838.62	J_H4	3.22	12
44	M	V_H1	V1-69	838.62	J_H4	1.20in	27
45	G	V_H1	V1-69	838.62	J_H6	3.3	41
57	M	V_H1	V1-69	838.62	J_H4	3.22	28
N62	G	V_H1	V1-69	838.62	J_H4	3.3	44
Ps14	M	V_H1	V1-69	838.62	J_H4	3.3	38
Ps18	G	V_H1	V1-69	838.62	J_H6	3.3	34
Ps40	G	V_H1	V1-69	838.62	J_H4	3.3	42
Ps41	G	V_H1	V1-69	838.62	J_H6	3.1	43
Ru	G	V_H1	V1-69	838.62	J_H6	6.19	43
Ps3	G	V_H2	V2-70	847.52	J_H4	4.23	23
Ps4	M	V_H2	V2-70	847.52	J_H4	4.11	25
KO	M	V_H3	V3-72	867.65	J_H6	3.3	23
N57	M	V_H3	V3-72	867.65	J_H4	3.3	25
Ps76	M	V_H3	V3-72	867.65	J_H4	3.9	9
AK	M	V_H3	V3-74	887.39	J_H4	3.22	26
37	M	V_H3	V3-74	887.39	J_H4	1.26in	26
48	M	V_H3	V3-74	887.39	J_H4	3.22	15

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Pre-publication Report & Outcomes of Peer Review

Contributions

VMD, DG were involved in the sequencing and analysis of all data presented. PDS and KL where involved in the analysis of V_H genes collected from the Liverpool Collaborative center; BH, PA, ABM and AVH where involved in providing material from individual patient and collecting clinical information. LF designed, co-ordinated and analyzed data throughout this whole project.

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Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 11, 2002; accepted October 2, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Recent studies suggest that there are two types of B–cell chronic lymphocytic leukemia according to the mutational pattern of IgVH genes: a) one arises from relatively less differentiated (immunologically naive) B-cells with unmutated heavy chain genes and has a poor prognosis; b) the other evolves from more differentiated B cells (memory B cells) with somatically mutated heavy chain genes and has a good prognosis.

What this study adds

This study highlights differences in the VDJ profile in mutated and germline CLL, consistent with the suggestion that CLL does indeed comprise two subgroups.