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FOCUS ON HEMATOLOGY

Unmutated Ig V_H Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia

By Terry J. Hamblin, Zaidie Davis, Anne Gardiner, David G. Oscier, and Freda K. Stevenson

Despite having several characteristics of naïve B cells, chronic lymphocytic leukemia (CLL) cells have been shown in some cases to have somatically mutated Ig variable region genes, indicating that the cell of origin has passed through the germinal center. A previous study of patients with CLL found an association between lack of somatic mutation and trisomy 12 and, therefore, possibly with a less favorable prognosis. We have sequenced the Ig V_H genes of the tumor cells of 84 patients with CLL and correlated our findings with clinical features. A total of 38 cases (45.2%) showed \geq 98% sequence homology with the nearest germline V_H gene; 46 cases (54.8%) showed $>$ 2% somatic mutation. Unmutated

V_H genes were significantly associated with V1-69 and D3-3 usage, with atypical morphology; isolated trisomy 12, advanced stage and progressive disease. Survival was significantly worse for patients with unmutated V_H genes irrespective of stage. Median survival for stage A patients with unmutated V_H genes was 95 months compared with 293 months for patients whose tumors had mutated V_H genes ($P = .0008$). The simplest explanation is that CLL comprises 2 different diseases with different clinical courses. One, arising from a memory B cell, has a benign course, the other, arising from a naïve B cell, is more malignant.

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CHRONIC LYMPHOCYTIC leukemia (CLL) is characterized by the relentless accumulation of monoclonal B cells with the appearance of small mature lymphocytes and with a characteristic immunophenotype. Typically, they are positive for CD5, CD23, and CD19 and negative for surface CD22 and FMC7.¹ Surface Ig (usually IgM plus IgD) is sparse and the immunoglobulin-associated molecule, CD79b, is low or absent.² Most cells are in the G₀ phase of the cell cycle and are unresponsive to mitogenic stimuli.³ The cells overexpress the Bcl-2 gene product and are resistant to apoptosis.⁴

Because CD5 positive B cells are found in the fetal spleen⁵ and surface IgD is a feature of cells that have not yet met antigen in the germinal center,⁶ it has been suggested that CLL is a tumor of naïve B cells possibly arising in the follicular mantle zone.⁷

A further means of establishing the stage of differentiation of a B lymphocyte is provided by examining the Ig variable domain genes. Although the selection and recombination of V_H, D, and J_H genes and the insertion of nontemplated nucleotides at the V_H-D and D-J_H junctions are early events occurring in the bone marrow, the process of somatic mutation, which tends to focus amino acid changes in the first and second complementarity determining regions (CDR1 and CDR2) of the molecule, occurs in the germinal center environment.⁸

Early sequences of the V_H genes of tumor cells from patients with CLL found them to be in germline configuration,⁹⁻¹¹ tending to confirm their origin from a naïve B cell. However, reports began to appear in the literature detailing cases with evidence of somatic mutation culminating in 1994 with a review of the literature by Schroeder and Dighiero,¹² which found that 36 of 75 reported cases had V_H genes with less than 98% sequence homology to the appropriate germline gene. The figure of 98% was chosen because polymorphisms, which are quite common in V_H genes, can account for that degree of disparity.¹³ Schroeder and Dighiero suspected that CLL might be a heterogeneous disorder, but were unable to cull from the literature the comprehensive clinical detail needed to establish this. They did report, however, that some of the cases with mutated V_H genes were CD5 negative and therefore not cases of classical CLL.

More recently, a multicenter study of 64 patients with surface IgM⁺, CD5⁺ CLL also found 2 groups of roughly equal numbers with respectively mutated and unmutated V_H genes.¹⁴ Although no clinical detail was available otherwise to distinguish the 2 subsets, the investigators were able to confirm the observation of Schroeder and Dighiero that the presence or absence of somatic mutations was associated with the use of particular V_H genes.

In 1997, our group examined the V_H genes of 22 patients with classical B-cell CLL segregated according to karyotype. Tumors with trisomy 12 had unmutated V_H genes, but those with 13q14 abnormalities detected by conventional cytogenetics had evidence of somatic mutations.¹⁵ Because it has been previously shown that CLL patients with trisomy 12 have a poorer survival than those with abnormalities at 13q14,¹⁶ this pointed to an association between clinical status and degree of somatic mutation.

The suspicion that the clinical heterogeneity of CLL might have a biological basis led us to extend this study. We have now examined the Ig V_H gene sequences in a series of 84 patients with classical B-cell CLL attending our hematology clinic and compared our results with various clinical characteristics of the patients and their survival. The striking finding to emerge is that the presence of V_H gene mutations places the CLL patient in a disease group with a clearly better prognosis.

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MATERIALS AND METHODS

Patients. Patients were selected for study from over 600 cases of CLL who have attended our hematology clinic in recent years. An attempt was made to include a representative selection of karyotypic disorders, but most patients were chosen simply because they happened to be attending the outpatient clinic for routine follow-up. Twenty-two of the patients were included in the series linking trisomy 12 with the absence of somatic mutation that we reported recently.¹⁵ Almost all of the patients studied came from the local area and included many whose diagnosis was made incidentally from a blood count ordered for another purpose and whose CLL has remained entirely asymptomatic. It has been our practice, nevertheless, to continue to observe such asymptomatic patients once or twice a year. They were staged at diagnosis according to the Binet Classification.¹⁷ Immunophenotyping was performed afresh during the course of the current investigation. All cases scored 4 or 5 according to the Royal Marsden scoring system for CLL,¹ were CD23 and CD5 positive, and had monotypic expression of weak surface Ig.

All patients have been followed for at least a year since diagnosis, the longest follow-up being 25 years. Patients were designated as having stable or progressive disease on the basis of the following criteria: lymphocyte count doubling time of less than 1 year; progression to a more advanced Binet stage; development of systemic symptoms; development of Richter's syndrome; downward trend of hemoglobin (Hb) or platelet count to below the normal range (Hb < 13.5 g/dL for males and < 11.5 g/dL for females; platelet count < 150 × 10⁹/L) even when not meeting the criteria for stage C disease (Hb < 10 g/dL; platelet count < 100 × 10⁹/L). Possession of one of these characteristics was sufficient to qualify as progressive disease. Patients with progressive disease have generally been treated conventionally with chlorambucil as first line therapy and with fludarabine for those with resistant disease. Patients with stable disease were not offered chemotherapy.

Lymphocyte morphology was assessed on blood films stained with Jenner Geimsa. Atypical morphology was defined according to the criteria of Matutes et al¹⁸ as greater than 10% prolymphocytes or greater than 15% cells with cleaved nuclei and/or lymphoplasmacytoid cells in the blood of patients whose predominant cell type was a small lymphocyte with coarsely clumped chromatin.

Cytogenetics. Whole peripheral blood was cultured at 37°C in the presence of tetradecanoyl phorbol 12-myristate 13-acetate (TPA) (0.05 µg/mL) for 3 to 5 days. Cells were treated with colcemid (0.1 µg/mL) for 60 minutes at 37°C before harvesting. After hypotonic treatment (KCL 0.075 mol/L for 10 minutes at 37°C), cells were resuspended in fixative (methanol: glacial acetic acid 3:1). Standard cytogenetic preparations were made, G banded, and karyotyped according to the International System for Cytogenetic Nomenclature (1995).

Preparation of cDNA and DNA. Blood samples for testing were taken during the past 5 years. Some were tested immediately, while for others, the lymphocytes were cryopreserved and tested later. Because the V_H gene signature is believed not to change during the clinical course of CLL, it was deemed satisfactory to determine this at any stage of the disease whether treated or not. The preferred source material was RNA, as this reduces the possibility of amplifying an aberrantly rearranged V_H gene; cDNA was synthesized by reverse transcription using an oligo(dT) primer as previously described.¹⁹ Where RNA was not available, genomic DNA was extracted using the QIAmp blood kit (Qiagen, Hilden, Germany).

Amplification of V_H genes. One fifth to one third of a sample of cDNA was amplified by polymerase chain reaction (PCR) using a mixture of oligonucleotide 5' primers specific for each leader sequence of the V_H1 to V_H6 families,²⁰ together with either mixed 3' primers complementary to the germ line J_H regions²¹ or 3' primers complementary to the constant region.¹⁴ When there was failure to amplify, an

alternative mixture of 5' primers specific for framework 1 region of V_H1 to V_H6 was substituted.²¹ In our hands, the V_H1 leader primer also amplifies sequences from the closely related V_H7 family. In all cases, PCR was performed in a final volume of 50 µL with 20 pmol of each primer, 50 µmol deoxyribonucleotide triphosphates (dNTPs), and 2.5 U Taq DNA polymerase with reaction buffer (Boehringer, Lewes, E Sussex, UK). Amplification consisted of an initial denaturation step of 3 minutes at 94°C followed by 30 cycles of 94°C, 56°C and 72°C for 45 seconds each, with a final extension step of 10 minutes at 72°C. All PCR reactions were performed in duplicate. For each PCR, a control with no added template was used to check for contamination.

Sequencing and cloning of PCR products. Clonal sequences were determined by sequencing amplicons from at least 2 independent PCR reactions. The majority of samples were sequenced directly using an automated DNA sequencer (Applied Biosystems, Foster City, CA). However, for the first 35 cases, cloning of gel-purified products into pGEM-TA vector was performed.^{19,21} After transformation of JM109 competent cells, clones found to contain an insert of appropriate size by restriction analysis of plasmid DNA were sequenced.^{19,21} A minimum of 5 bacterial colonies were analyzed. In addition, in 3 later cases, direct sequencing was unsuccessful and the sequence was determined by cloning.

Analysis of Ig gene sequences. Nucleotide sequences were aligned to EMBL/GenBank and current databases (V-BASE sequence directory,²² using MacVector 4.0 sequence analysis software; International Biotechnologies Inc, New Haven, CT). Where there was >2% deviation from a germline V_H sequence, the Chang and Casali formula²³ was used to determine whether the replacement mutations had undergone antigenic selection. We have followed the criteria of Corbett et al²⁴ in assigning membership of the 2 longer D gene families (D2 and D3), but the requirement for 10 consecutive nucleotides of identity are probably too stringent for the shorter D gene families, and we have followed Fais et al¹⁴ in requiring only 7 consecutive nucleotides with no more than 2 differences. We have eliminated DIR segments and "minor" D segments from analysis.²⁴

Statistical analysis. The significance of associations between characteristics was determined using Fisher's exact test. Survival curves from date of diagnosis were plotted using GraphPad Prism (version 2) software (San Diego, CA). This program calculates survival fractions using the Kaplan-Meier method and compares survival curves using the log-rank test.

RESULTS

Clinical and cytogenetic features of patients studied. We studied 35 women and 49 men with classical B-cell CLL. The mean age at diagnosis was 63.3 years (median, 65; range, 33 to 92). A total of 62 were stage A, 9 stage B, and 13 stage C. A total of 34 had progressive disease and 50 stable disease. Lymphocyte morphology was typical in 58 cases and atypical in 26 cases.

Karyotypes are shown in Tables 1 and 2. Trisomy 12 was found as a single abnormality in 11 patients and in combination with other abnormalities in a further 15 patients. Nine patients had translocations or deletions at 13q14 as single abnormalities, and 7 had these in association with other abnormalities. Ten had translocations involving chromosomal regions close to Ig genes on chromosomes 14 and 22, mainly in association with other abnormalities. Deletions involving 11q23 were found in 8 patients. Twenty-three patients had a normal karyotype. In 2 patients, chromosomal analysis was not attempted, and in 2, no metaphases were obtained.

Use of V_H, D, and J_H genes. In common with several other series,^{9,12,14} we found an overuse of the V1-69 gene. Twenty-eight of the 51 translatable V_H genes were used in this series. Six genes accounted for 57% of cases. V1-69, V3-23, and

Table 1. Demographics and Clinical and Laboratory Findings in Patients With CLL Whose V_H Genes Showed ≥98% Sequence Homology With the Nearest Germline V_H Gene

ID	Age/Sex	VH Gene	Percent Sequence Homology	D Gene Segment	JH Gene	Stage	Pace of Disease	Cell Type	Partial Karyotype of Predominant Clone
3	61/M	V1-02	100	D3-22	JH4b	C	p	t	46,XY,t(13;20)(q14;q11)
18	77/M	V1-02	100	NA	JH4b	C	p	t	46,XY,del(11)(q23q25)
83	70/M	V1-02	100	D2-2	JH6b	A	s	at	47,XY,+12,del(13)(q12q14)
22	71/M	V1-03	99	D5-12	JH4b	A	p	t	46,XY,t(13;18)(q14;p11)
11	53/F	V1-18	100	NA	JH4b	B	p	at	46,XX,del(3)(p21)
52	79/M	V1-58	100	D6-13	JH3b	A	s	at	47,XY,+12
2	74/M	V1-69	100	NA	JH6c	B	p	at	46,XY,t(3;11)(q25;q25)
8	81/M	V1-69	100	D3-3	JH6b	A	p	at	46,XY,t(14;18)(q32;q22)
12	57/M	V1-69	100	D2-2	JH5b	A	p	t	46,XY
19	50/M	V1-69	100	D3-3	JH4b	B	p	at	47,XY,+12
21	72/F	V1-69	100	D3-22	JH4b	A	p	t	47,XX,+12
24	85/M	V1-69	100	D3-22	JH6c	A	p	at	47,XY,+12
32	67/M	V1-69	100	D3-9	JH4b	C	p	t	46,XY,t(2;6)(p24;p21)
64	55/M	V1-69	100	D3-10	JH6c	A	s	t	complex
74	82/F	V1-69	100	D2-15	JH6c	A	s	t	46,XX
29	70/M	V2-70	100	D2-21	JH4b	A	p	at	47,XY,+12
25	65/F	V2-70	99	D7-27	JH4b	A	p	at	47,XX,+12
31	56/M	V3-07	98	D6-13	JH5a	C	p	t	46,XY
23	88/M	V3-09	100	D3-3	JH6b	B	p	at	47,XY,+12
54	78/F	V3-09	100	D3-3	JH4b	A	s	at	47,XX,+12
28	73/M	V3-21	99	NA	JH6b	C	p	t	46,XY,del(6)(q21q25)
10	50/F	V3-23	98	D1-7	JH4b	C	p	at	46,XX,del(11)(q14q23)
53	74/M	V3-23	98	D4-17	JH4a	A	s	t	46,XY,del(7)(q?32q?34)
14	69/F	V3-30	100	D1-7	JH6b	B	p	at	Complex including +12
33	73/F	V3-30	100	D3-9	JH6b	A	p	at	46,XX
20	73/M	V3-30.3	100	D5-12	JH6b	A	p	at	47,XY,+12,t(14;19)(q32;q13)
26	71/M	V3-30.3	100	NA	JH4b	A	p	at	47,XY,+12,t(14;18)(q32;q21)
44	56/M	V3-30.3	100	NA	JH1	A	s	at	47,XY,+12,t(14;18)(q24;q21)
72	40/M	V3-30.3	98	D6-13	JH1	B	s	at	46,XY
27	64/F	V3-33	100	D3-3	JH4b	A	p	t	No metaphases
41	63/M	V3-48	98	NA	JH6b	A	s	t	46,XY,t(8;22)(q24;q11),del(11)(q13q23)
9	54/M	V3-73	100	D3-3	JH6b	C	p	at	47,XY,+12,del(14)(q11q24)
13	71/F	V3-74	100	D3-3	JH4b	A	p	at	46,XX,del(11)(q22q24)
1	49/F	V4-34	100	D2-2	JH3b	C	p	at	47,XX,+12
17	36/M	V4-59	100	D2-21	JH3b	B	p	t	46,XY,del(11)(q?15?21)
5	82/F	V4-61	100	D3-10	JH4b	A	p	t	47,XX,+12,t(14;18)(q32;q21)
38	55/M	V5-51	100	D6-19	JH4b	C	p	t	45,XY,der(15)t(15;17)(p11;q21)
15	50/F	V6-21	99	D3-3	JH6b	C	p	t	46,XX

Age indicates age at diagnosis; stage indicates Binet stage; pace of disease distinguishes between stable and progressive disease. GenBank/EMBL/DDBJ accession numbers for sequences not previously reported in Tables 1 and 2 are AJ239330-239391.

V4-34 were each used on 10 occasions (each 11.9%) and V1-02, V3-30.3, and V3-07 were each used on 6 occasions (each 7.1%). However, the normal antibody repertoire does not use V_H genes randomly, and V3-23 (13.9%), V3-07 (5.6%), and V3-30.3 (8.3%) are the V_H genes most commonly used by normal CD5⁺ B cells.²⁵ In addition, we have previously shown that the V4-34 gene product is used by between 2.9% to 10.8% of normal B cells.²⁶ On the other hand, V1-69 is used by only 1.6% of normal B cells²⁷ and therefore appears overrepresented in this series.

The majority of the tumors used J_H4 (35 of 84; 41.7%) or J_H6 (27 of 84; 37.1%), which is similar to their use in normal CD5⁺ B cells (52% and 27%, respectively).²⁵

Because of extensive mutations or N additions or nucleotide loss, a D segment gene could not be assigned to the sequences in 31 cases. The most commonly used gene was D3-3 (D_{XP4}), which was used in 8 cases, all of which had unmutated V_H

genes. In all, 17 of the 27 different D-segment genes were used. D segments were not used preferentially with any V_H or J_H gene, but could not be assigned in the cases with the greatest numbers of somatic mutations.

Somatic mutations. Whereas the leukemic cells of 38 patients (45.2%) had V_H genes with ≥ 98% sequence homology with the nearest germline gene, the remaining 46 cases (54.8%) showed evidence of somatic hypermutation, dividing the series into 2 subsets.

There are clear differences between the 2 subsets. Patients with unmutated V_H genes (Table 1) had characteristics associated with a more malignant type of disease than those with evidence of somatic mutations (Table 2). Those lacking mutations were significantly more likely to have advanced stage disease, whereas those with mutations were more likely to have stage A disease ($P = .0009$). Lack of mutations was significantly associated with progressive disease and the presence of

Table 2. Demographics and Clinical and Laboratory Findings in Patients With CLL Whose V_H Genes Showed <98% Sequence Homology With the Nearest Germline V_H Gene

ID	Age/Sex	VH Gene	Percent Sequence Homology	D Gene Segment	JH Gene	Stage	Pace of Disease	Cell Type	Partial Karyotype of Predominant Clone
71	73/M	V1-e	95	NA	JH4b	A	s	t	ND
77	75/F	V1-e	94	D3-22	JH4b	A	s	t	46,XX,del(13)(q14q24)
39	68/M	V1-e	93	D4-23	JH4a	A	s	t	47,XY,+12,t(12;13)(p11;q24)
49	76/F	V1-02	93	D4-17	JH4b	A	s	t	46,XX,del(13)(q12q14)
35	66/F	V1-02	92	D5-5	JH3b	A	s	t	46,XX
46	70/F	V1-02	91	NA	JH4b	A	s	t	46,XX,t(13;17)(q14;q11),t(20;22)(q11;p11)
50	43/M	V1-69	93	NA	JH3c	A	s	t	46,XY,inv(7)(p22q32)del(13)(q12q14)
84	79/M	V2-05	91	NA	JH4b	A	s	t	No metaphases
79	55/M	V2-70	96	D3-9	JH3b	A	s	t	46,XY,del(1)(q21q?)
62	62/M	V3-07	94	D2-15	JH3b	A	s	t	49,XY,+12,+18,+19
82	39/M	V3-07	94	NA	JH4b	A	s	t	46,XY
78	92/F	V3-07	93	NA	JH6b	A	s	t	46,XX
60	55/F	V3-07	92	NA	JH5b	A	s	t	46,XX
30	70/F	V3-07	91	NA	JH6b	C	p	at	47,XX,+12,del(13)(q12q14)
66	50/M	V3-09	97	NA	JH6b	A	s	t	46,XY,t(2;7)(q32;p22)
59	57/F	V3-15	94	NA	JH6b	A	s	t	Complex
73	66/M	V3-15	94	NA	JH4b	A	s	t	46,XY
76	76/F	V3-20	95	D1-1	JH6b	A	s	at	47,XX,+12
81	78/F	V3-21	96	D3-10	JH4b	A	s	t	46,XX
70	69/M	V3-23	97	D3-22	JH4b	A	s	t	46,XY,t(5;6)(q35;q21)
75	76/F	V3-23	94	NA	JH5b	A	s	t	47,XX,+12,t(18;22)(q22;q12)
4	49/M	V3-23	92	D3-22	JH1	A	p	t	47,XY,+12,t(14;18)(q32;q21)
7	74/F	V3-23	92	NA	JH6b	A	p	t	46,XX,t(14;18)(q32;q21)
40	75/F	V3-23	92	D3-10	JH1	A	s	t	46,XX,t(13;13)(q14;q22)
65	69/F	V3-23	92	D3-10	JH3b	A	s	t	46,XX
67	67/M	V3-23	91	NA	JH6c	A	s	t	46,XY
69	49/M	V3-23	91	NA	JH4b	A	s	t	46,XY
61	62/M	V3-30	91	D3-22	JH4b	A	s	t	ND
56	57/F	V3-30	90	NA	JH4b	A	s	at	47,XX,+12
48	52/M	V3-30.3	95	D1-26	JH4b	A	s	t	46,XY,del(13)(q12q22)
51	65/F	V3-30.3	94	D2-15	JH3b	A	s	t	46,XX
55	70/M	V3-33	97	NA	JH1	A	s	at	49,XY,+12,+18,+19
63	58/F	V3-48	97	D2-2	JH1	A	s	t	46,XX
68	51/M	V3-48	97	D6-13	JH5b	A	s	t	46,XY,del(11)(q?13q?23)
16	60/M	V3-48	96	NA	JH6b	B	p	t	46,XY,t(9;13)(p11;q14)
6	64/M	V3-73	90	NA	JH4b	C	p	at	47,XY,+12,t(4;17;13)(p16;?:q14)
37	51/M	V4-30.4	96	NA	JH4b	A	s	t	46,XY
43	69/F	V4-34	97	D2-15	JH4b	B	s	t	Complex including del(11)(q13)
80	51/M	V4-34	95	NA	JH4b	A	s	t	46,XY
34	75/F	V4-34	94	D3-10	JH5a	A	s	t	47,XX,+12,del(13)(q12q14)
42	61/M	V4-34	94	NA	JH6b	A	s	t	46,XY,t(13;14)(q14;q25)
45	33/F	V4-34	94	D2-15	JH6b	C	s	t	46,XX,del(13)(q14q22)
47	51/M	V4-34	94	D5-5	JH6b	A	s	t	46,XY
36	44/M	V4-34	92	D6-13	JH6b	A	s	t	46,XY
57	55/F	V4-34	91	NA	JH5a	A	s	t	46,XX
58	42/F	V4-34	91	NA	JH6b	A	s	t	46,XX

Abbreviations: NA, not assigned; p, progressive; s, stable; at, atypical; t, typical; ND, not done.

mutations with stable disease ($P < .0001$). Atypical morphology was similarly associated with lack of mutations and typical morphology with their presence ($P < .0001$). Trisomy 12, as an isolated karyotypic abnormality, was significantly associated with a lack of somatic mutations ($P = .0019$), and deletions or translocations at 13q14 were significantly associated with their presence ($P = .023$).

There was a significant tendency for V_H1 family genes ($P = .0142$) and especially V1-69 ($P = .0038$) to be used by the subset that lacked mutations. A similar tendency towards the use of the D3-3 gene segment was found in this subset ($P = .0058$),

although there was no tendency for these gene segments to be used together.

For patients with unmutated V_H genes, the mean age at diagnosis was 64.9 years (median, 66; range, 36 to 88). There were 13 women and 25 men. For patients with mutated V_H genes, the mean age was slightly younger at 61.9 years (median, 63; range, 33 to 92), and the gender ratio was closer to unity with 22 women and 24 men, although these differences did not reach statistical significance.

Antigenic selection. Analysis of the distribution of replacement and silent mutations²³ showed a clustering of replacement

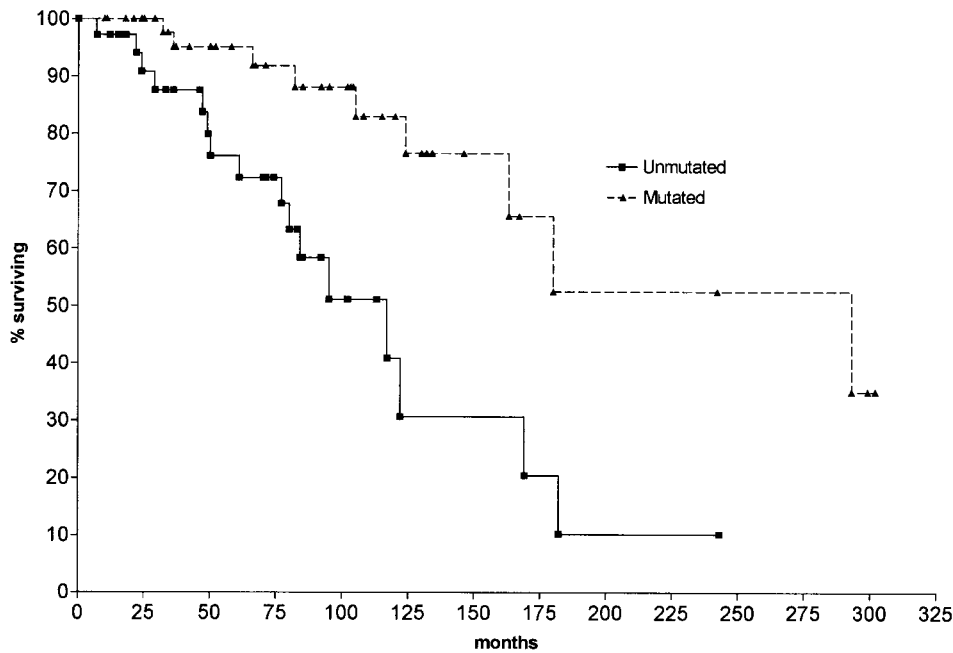


Fig 1. Kaplan-Meier survival curve comparing CLL patients with mutated and unmutated V_H genes. Median survival for unmutated CLL: 117 months; median survival for mutated CLL: 293 months. The difference is significant at the $P = .001$ level (log-rank test).

amino acids in CDR1 and CDR2 in 17 of 46 (37.0%) of the cases with somatic mutations and conservation of amino acid sequence in framework regions in 28 of 46 (60.9%).

Intraclonal heterogeneity. In 38 cases, at least 5 separate clones were analyzed. No intraclonal heterogeneity was found. This is in contrast to the finding in follicular lymphoma and implies that cells showing somatic mutations were no longer under the influence of mutator enzymes and had therefore passed through the germinal center. In 3 cases, a second blood sample was analyzed respectively 5 years, 3 years, and 18 months after the first. In each case, the clonal sequence was identical with the original sequence (with respectively 0, 2, and 19 mutations).

Prognostic significance of V_H gene mutations. Survival curves were plotted according to the Kaplan-Meier method. Only 2 patients were censored as lost to follow-up. Graphs comparing the survival of patients with mutated and unmutated V_H genes are plotted in Fig 1. The median survival for patients with unmutated V_H genes was 117 months and for those with mutations, 293 months. The difference is very significant ($P = .001$). Because the unmutated group contains more patients with advanced stage disease, we plotted the survival curve for stage A patients only (Fig 2). Median survival was 95 months for the patients without mutations and 293 months for those with mutations. Again, the difference in survival was very significant ($P = .0008$).

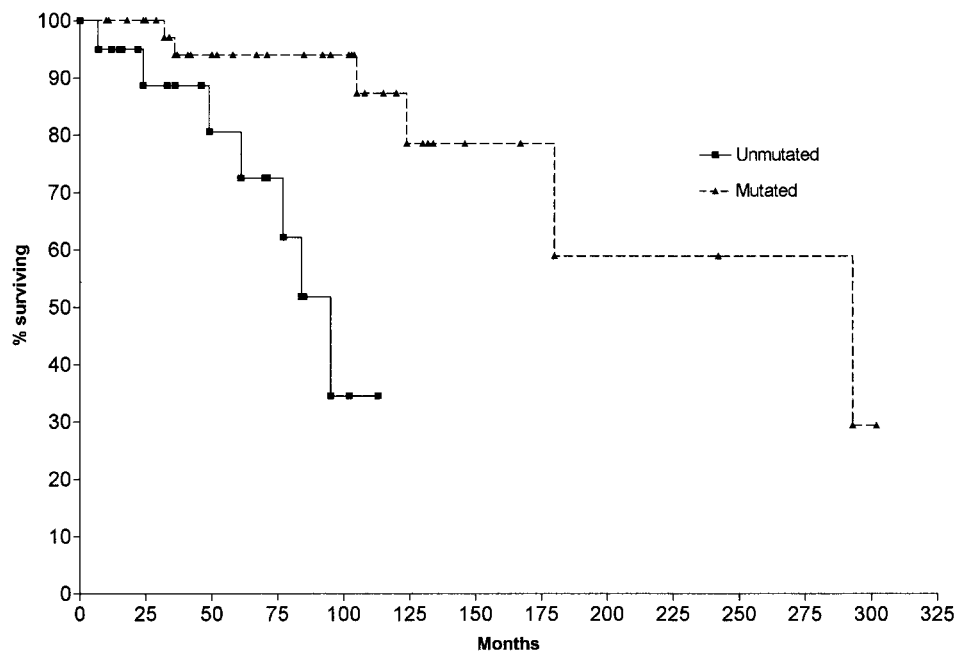


Fig 2. Kaplan-Meier survival curve comparing stage A CLL patients with mutated and unmutated V_H genes. Median survival for unmutated CLL: 95 months; median survival for mutated CLL: 293 months. The difference is significant at the $P = .0008$ level (log-rank test).

DISCUSSION

Somatic mutation is believed to occur in germinal centers or similar structures.⁸ Thus, the presence of such mutations in over half of our cases of CLL and the statistical evidence supporting the occurrence of antigenic selection in some of the cases argues strongly that these are tumors of memory B cells and not of naïve B cells.

In any study of CLL, it is important to establish that the series is not contaminated by other kinds of lymphoma. It is particularly important to exclude mantle cell lymphoma, which is also a tumor of CD5⁺ B cells and which has a much worse prognosis than CLL. Other diseases that must be excluded are splenic lymphoma with villous lymphocytes, of which a small proportion are reportedly CD5⁺,²⁸ and follicular lymphoma in leukemic phase. In this series, we have tried very hard to exclude all such cases. All had a Royal Marsden score of 4 or 5. No case had t(11;14)(q13;q32) translocations. Although 7 had t(14;18)(q32;q21) or t(18;22)(q21;q11) translocations, it is important to stress that such structural abnormalities have been previously reported in CLL and are not confined to follicular lymphoma.²⁹ None of these cases had cellular morphology or immunophenotype typical of follicular lymphoma, and none showed the intraclonal variation of immunoglobulin V_H genes seen in that condition.³⁰

Patients with unmutated V_H genes had a distinctly more malignant disease and a much shorter survival than those with somatic mutations. They were more likely to have atypical morphology, advanced stage, and progressive disease. Such a distinct clinical course argues in favor of CLL comprising 2 separate types of tumor arising at different stages of B-cell maturation: 1 a pregerminal center naïve B cell and 1 a postgerminal center memory B cell.

In this series of patients, the influence of karyotype on prognosis is less than in our previous report,¹⁵ perhaps because we have included here 5 patients with both trisomy 12 and abnormalities at 13q14. As more sensitive techniques such as fluorescent in situ hybridization and comparative genomic hybridization are applied, it is apparent that leukemic chromosomes are more complicated than has been appreciated using conventional cytogenetics. It is clear that much more detailed work is required to establish the influence of chromosomal abnormalities on the 2 types of CLL.

We confirm in this study the biased use of certain V_H genes in CLL. Six genes accounted for over half of the cases. However, when Brezinschek et al²⁵ analyzed the normal B-cell immunoglobulin repertoire by single cell PCR of DNA from B cells of 2 individuals, they found that many of the same genes were similarly overused. An exception is V1-69, which apparently not overused by normal B cells,²⁷ yet has been reported consistently as the most commonly used V_H gene in CLL, usually in unmutated form.^{9,14,31} V1-69 occurs in several polymorphic forms and there is variation in the number of copies of V1-69 between different individuals.^{32,33} It would be helpful, therefore, to investigate the normal use of V_H genes from a wider range of normal individuals, including some from the same age range as patients with CLL, before concluding that this gene is used more commonly in CLL than in normals. Why the V1-69 gene is seen so commonly in CLL in unmutated form remains unanswered. None of our patients used the V4-39 gene,

but this gene is also usually unmutated in the reports in the literature, even in cases expressing surface IgG.^{12,14} Explanations for these phenomena are available,¹⁴ but lack certainty at present.

The suggestion that there might be 2 types of CLL pursuing distinct clinical courses and definable by the stage of differentiation of the cell of origin is independently supported by the work of Damle et al.³⁴ It raises many pathophysiological questions, but clinicians will be interested in the prognostic implications. Thirty-three of our 84 patients (39.3%) were diagnosed below the age of 60 and might today be considered as candidates for aggressive chemotherapy and stem cell autograft for this incurable disease. Knowledge that 19 of them belonged to a subtype with a median survival of 25 years and that the other 14 belonged to a subtype with a median survival of only 8 years would be valuable.

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REFERENCES

1. Matutes E, Owusu-Ankomah K, Morilla R, Garcia-Marco J, Houlihan A, Que TH, Catovsky D: The immunological profile of B cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* 8:1640, 1994
2. Zomas AP, Matutes E, Morilla R, Owusu-Ankomah K, Seon BK, Catovsky D: Expression of the immunoglobulin associated protein B29 in B cell disorders with the monoclonal antibody SN8 (CD79b). *Leukemia* 10:97, 1996
3. Andreef M, Darzynkiewicz Z, Sharpless TK, Clarkson BD, Melamed MR: Discrimination of human leukemia subtypes by flow cytometric analysis of cellular DNA and RNA. *Blood* 55:282, 1980
4. Pezella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY: Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. *Am J Pathol* 137:225, 1990
5. Antin JH, Emerson SG, Martin P, Gadol N, Ault KA: Leu-1+ (CD5+) B cells. A major lymphoid subpopulation in human fetal spleen. *J Immunol* 136:505, 1986
6. Nicholson IC, Brisco MJ, Zola H: Memory B lymphocytes in human tonsil do not express surface IgD. *J Immunol* 154:1105, 1995
7. Caligaris-Cappio F: B-chronic lymphocytic leukemia: A malignancy of anti-self B cells. *Blood* 87:2615, 1996
8. Berek C, Milstein C: Mutational drift and repertoire shift in the maturation of the immune response. *Immunol Rev* 96:23, 1987
9. Kipps TJ, Tomhave E, Pratt LF, Duffey S, Chen PP, Carson DA: Developmentally restricted immunoglobulin heavy chain variable region gene expressed at high frequency in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 86:5913, 1989
10. Deane M, Norton JD: Preferential rearrangement of developmentally regulated immunoglobulin V_{H1} genes in human B-lineage leukemias. *Leukemia* 5:646, 1991
11. Ebeling SB, Schutte MEM, Akkermans-Koolhaas KE, Bloem AC, Gmelig-Meyling FHJ, Logtenberg T: Expression of members of the immunoglobulin V_{H3} gene families is not restricted at the level of individual genes in human chronic lymphocytic leukemia. *Int Immunol* 4:313, 1992
12. Schroeder HW Jr, Dighiero G: The pathogenesis of chronic lymphocytic leukemia: Analysis of the antibody repertoire. *Immunol Today* 15:288, 1994
13. Matsuda F, Shin EK, Nagaoka H, Matsumura R, Haino M, Fukita

Y, Taka-ishi S, Imai T, Riley JH, Anand R: Structural and physical map of the 64 variable segments in the 3' 0.8 megabase region of the human immunoglobulin heavy chain locus. *Nat Genet* 3:88, 1993

14. Fais F, Ghiotto F, Hashimoto S, Sellers B, Valetto A, Schulman P, Vinciguerra VP, Rai K, Rassenti LZ, Kipps TJ, Dighiero G, Schroeder HW Jr, Ferrarini M, Chiorazzi N: Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 102:1515, 1998
15. Oscier DG, Thompssett A, Zhu D, Stevenson FK: Differential rates of somatic hypermutation in V_H genes among subsets of chronic lymphocytic leukemia defined by chromosomal abnormalities. *Blood* 89:4153, 1997
16. Juliusson G, Oscier D, Fitchett M, Ross FM, Stockdill G, Mackie MJ, Parker AC, Castoldi GM, Gunee A, Knuutila S: Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. *N Engl J Med* 323:720, 1990
17. Binet J-L, Lepoprier M, Dighiero G, Charron D, D'Athis P, Vaugier G, Beral HM, Natali JC, Raphael M, Nizet B, Follezuou JY: A clinical staging system for chronic lymphocytic leukemia. *Cancer* 40:855, 1977
18. Matutes E, Oscier DG, Garcia-Marco J, Ellis J, Copplestone A, Gillingham R, Hamblin T, Lens D, Swansbury GJ, Catovsky D: Trisomy 12 defines a group of CLL with atypical morphology: Correlation between cytogenetic, clinical and laboratory features. *Br J Haematol* 92:382, 1996
19. Sahota S, Hamblin TJ, Oscier DG, Stevenson FK: Assessment of the role of clonal B lymphocytes in the pathogenesis of multiple myeloma. *Leukemia* 8:1285, 1994
20. Campbell MJ, Zelenetz AD, Levy S, Levy R: Use of family specific leader region primers for PCR amplification of the human heavy chain variable region repertoire. *Mol Immunol* 29:193, 1992
21. Hawkins RE, Zhu D, Ovecka M, Winter G, Hamblin TJ, Long A, Stevenson FK: Idiotypic vaccination against human B-cell lymphoma. Rescue of variable region gene sequences from biopsy material for assembly as single chain F_v personal vaccines. *Blood* 83:3279, 1994
22. Cook GP, Tomlinson IM: The human immunoglobulin V_H repertoire. *Immunol Today* 16:237, 1995
23. Chang B, Casali P: The CDR1 sequences of a major proportion of human germline Ig V_H genes are inherently susceptible to amino acid replacement. *Immunol Today* 15:367, 1994
24. Corbett SJ, Tomlinson IM, Sonhammer ELL, Buck D, Winter G: Sequence of the human diversity (D) segment locus: A systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol* 270:587, 1997
25. Brezinschek H-P, Foster SJ, Brezinschek RI, Dorner T, Domiati-Saad R, Lipsky PE: Analysis of the human V_H gene repertoire. Differential effects of selection and somatic hypermutation on the human peripheral CD5+/IgM+ and CD5-/IgM+ B cells. *J Clin Invest* 99:2488, 1997
26. Stevenson FK, Smith GJ, North J, Hamblin TJ, Glennie MJ: Identification of normal B cell counterparts of neoplastic cells which secrete cold agglutinins of anti-I and anti-i specificity. *Br J Haematol* 72:9, 1989
27. Brezinschek H-P, Brezinschek RI, Dorner T, Lipsky P: Similar characteristics of the CDR3 of the $V_H1-69/DP10$ rearrangements in the normal human peripheral blood and chronic lymphocytic leukaemia B cells. *Br J Haematol* 102:516, 1998
28. Matutes E, Morilla R, Owsusu-Ankomah K, Houlihan A, Catovsky D: The immunophenotype of splenic lymphoma with villous lymphocytes and its relevance to the differential diagnosis with other B-cell disorders. *Blood* 83:1558, 1994
29. Dyer MJ, Zani VJ, Lu WZ, O'Byrne A, Mould S, Chapman R, Heward JM, Kayano ST, Jadayel D, Matutes E, Catovsky D, Oscier D: BCL2 translocations in leukemias of mature B cells. *Blood* 83:3682, 1994
30. Zhu D, Hawkins RE, Hamblin TJ, Stevenson FK: Clonal history of a follicular lymphoma as revealed in the immunoglobulin variable region genes. *Br J Haematol* 86:505, 1994
31. Johnson TA, Rassenti LZ, Kipps TJ: Ig V_H1 genes expressed in B cell chronic lymphocytic leukemia exhibit distinctive molecular features. *J Immunol* 158:235, 1997
32. Sasso EH, Willems van Dijk K, Bull AP, Milner EC: A fetally expressed immunoglobulin V_H1 gene belongs to a complex set of alleles. *J Clin Invest* 91:2358, 1993
33. Sasso EH, Johnson T, Kipps TJ: Expression of the immunoglobulin V_H gene 51p1 is proportional to its germline gene copy number. *J Clin Invest* 97:2074, 1996
34. Dame RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, Buchbinder A, Budman D, Dittmar K, Kolitz J, Lichtman SM, Schulman P, Vinciguerra VP, Rai KR, Ferrarini M, Chiorazzi N: Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 94:1840, 1999