

# Immunoglobulin heavy chain variable region gene usage and mutational status of the leukemic B cells in Iranian patients with chronic lymphocytic leukemia

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(Received June 23, 2009/Revised August 18, 2009; August 23, 2009/Accepted August 23, 2009/Online publication October 12, 2009)

The mutational status of the immunoglobulin variable region heavy chain genes (IGHV) is an important prognostic marker in chronic lymphocytic leukemia (CLL). The data accumulated in the literature has largely been derived from studies conducted on Caucasian Western populations. Little is known about Asian CLL patients. In this study the IGHV genes usage and somatic hypermutation status have been investigated in 87 Iranian CLL patients. Based on a cut-off of 98% nucleotide sequence homology, 64.4% and 35.6% of the patients expressed mutated and unmutated IGHV genes, respectively, with most non-progressive patients being in the mutated group (35/44 vs 19/40;  $P = 0.009$ ). Progression-free survival (PFS) and time to first treatment (TTFT) were significantly higher in our mutated and non-progressive patients compared to unmutated and progressive subtypes, respectively. The most frequently used IGHV gene was IGHV3-7 (12.6%) followed by IGHV3-30 (11.4%), IGHV3-48 (9.2%), IGHV4-39 (6.9%), and IGHV1-8 (6.9%) genes, which taken together comprised nearly half of the IGHV genes expressed in the Iranian CLL patients. Of the IGHV genes, IGHV3-7 was significantly over-represented in non-progressive compared to progressive CLL patients ( $P = 0.036$ ), whereas IGHV1-69 and IGHV1-2 were expressed at a higher frequency in unmutated compared to mutated CLL patients ( $P < 0.03$ ). Comparison of IGHV gene usage in our patients with that of Western CLL patients revealed significant differences in expression of IGHV1-69, IGHV3-7, IGHV3-21, and IGHV4-34 genes. Analysis of the IGHV third complementary determining region (HCDR3) sequences revealed a high frequency use of certain HCDR3 motifs, such as YYGMDV, in our samples. These findings imply contribution of antigen selection and regional (ethnic/geographic) parameters in the leukomogenesis of CLL. (*Cancer Sci* 2009; 100: 2346–2353)

Leukemic cells of chronic lymphocytic leukemia (CLL) are derived from clonally expanded and phenotypically mature B cells ( $CD5^+/CD19^+/sIg^{low}$ ) which accumulate in the bone marrow, blood, and lymphoid tissues.<sup>(1)</sup> In recent years a number of biological prognostic factors have been identified in CLL.<sup>(2)</sup> CD38 expression was the first marker that was found to correlate with disease progression.<sup>(3)</sup> Intracytoplasmic detection of the ZAP-70 enzyme in leukemic B cells was subsequently revealed to be correlated with CLL prognosis.<sup>(4)</sup> Most patients with CD38 and ZAP-70 expression on their leukemic cells tend to have a more aggressive disease. In addition to these markers, chromosomal abnormalities have also been documented in the majority of CLL patients and found to be associated with disease prognosis.<sup>(5)</sup>

Among the prognostic biomarkers, analysis of the immunoglobulin (Ig) genes has been of considerable interest, particularly

in terms of somatic hypermutation status of Ig heavy chain variable region (IGHV) genes.<sup>(6–8)</sup> This helped to define two disease subsets, mutated and unmutated, based on nucleotide sequence homology to the closest germline gene. Patients with unmutated leukemic cells tend to have a more aggressive clinical course relative to those with mutated IGHV.<sup>(9–11)</sup> This categorization has led to the speculation that these two subtypes might represent two distinct entities: one derived from memory B cells that had undergone Ig somatic mutation, and the other derived from naïve or pre-germinal center B cells.<sup>(12)</sup>

The results obtained from most previous studies which have been conducted in Western populations suggest that IGHV genes usage among CLL patients is not random; besides, their frequencies differ in mutated and unmutated subsets of CLL patients.<sup>(3,6–8,13–19)</sup> Of the IGHV genes, IGHV1-69, which is associated with unmutated subtype of CLL patients, is over-represented in Western patients,<sup>(18,20)</sup> predominantly recombined with IGHV3-3 and IGHV4-39 genes, encoding longer than average heavy chain complementarity determining region 3 (HCDR3) compared to other IGHV members.<sup>(20–22)</sup> Other frequently used IGHV genes are IGHV3-23, IGHV4-34, and IGHV3-7<sup>(8,15,23,24)</sup> that usually belong to the mutated subtype.

In addition to the skewed representation of certain IGHV genes in mutated and unmutated subtypes of CLL, there is compelling evidence indicating differential expression of some IGHV genes in different geographic parts of Western countries. While IGHV3-21 is frequently expressed in northern European (Scandinavian) CLL patients,<sup>(20,25)</sup> there is no or a low frequency of usage of this gene in southern or central parts of European countries and the USA.<sup>(15,26,27)</sup>

All the findings of biased repertoire of IGHV genes, as well as similar amino acid motifs in HCDR3, have led to the hypothesis of involvement of an as yet undefined set of antigens (self or non-self) with a restricted set of epitopes in the pathogenesis of CLL, implicating the role of B-cell antigen receptor (BCR) in the etiopathogenesis of the disease.

As the current bulk of data on IGHV gene representation and mutational status in CLL is derived from studies conducted in Western populations with only two reports of Asian CLL patients from China and Japan, we investigated for the first time IGHV gene usage and mutational status of the leukemic B cells in an Iranian CLL population. Comparison of our results with those reported from the two other Asian populations, as well as Western CLL patients, strongly suggest contribution of regional parameters in shaping the IGHV repertoire of the leukemic CLL B cells.

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## Materials and Methods

**Patients and controls.** Twenty mL of heparinized peripheral blood was collected from 87 CLL patients attending the Hematology and Oncology Clinics of Vali-Asr and Firozgar Hospitals, affiliated to Tehran University of Medical Sciences and Iran University of Medical Sciences, respectively. All patients were Iranians, living in Tehran city. Written consent was obtained from all patients and the study was approved by the Ethical Committee of Tehran University of Medical Sciences. The diagnosis was based on immunophenotypic analysis, blood cell count, cell morphology, and clinical symptoms, according to the World Health Organization classification of tumors.<sup>(28)</sup>

Immunophenotyping of leukemic cells by flow cytometry revealed expression of CD19 and CD5 molecules in all cases and CD23 in 77 cases. For the following reasons CD23-negative patients were diagnosed as CLL and not mantle cell lymphoma (MCL): (a) as opposed to CLL, MCL is a very aggressive disease with a short overall survival and treatment free survival. Among these 10 patients, five patients displayed a non-progressive course, of whom four patients did not require chemotherapy and the fifth patient responded very well to chemotherapy. (b) CLL usually displays a better response to conventional chemotherapy than MCL, with most MCL cases being refractory to chemotherapy. Five of our 10 CD23-negative patients (four progressive and one non-progressive) underwent chemotherapy. With the exception of one patient who died due to unrelated complications (sepsis), the remaining four patients responded quite well to therapy. (c) Surface Ig is usually expressed weakly in CLL as compared to MCL. Indeed, this has been the case in all our five non-progressive patients (IgM+ leukemic cells, 7–26%; mean, 13.7%). In progressive patients, however, moderate expression of surface Ig was observed (IgM+ cells, 23–62%; mean, 44%).

The age range of patients was 39–84 years with a median of 63 years. Fifty-four patients were males and 33 were females. The patients were classified into non-progressive ( $n = 44$ ) and progressive ( $n = 40$ ) groups. Disease progression of three patients could not be identified. Disease progression was identified on the basis of either of the following criteria: lymphocyte count doubling time of <1 year; progression to a more advanced Rai stage; development of systemic symptoms; development of Richter's syndrome; downward trend of hemoglobin (Hb) concentration or platelet count to below the normal range (Hb < 13.5 g/dL for males and <11.5 g/dL for females; platelet count <  $150 \times 10^9/L$ ) even when not meeting the criteria for stage III or IV disease (Hb < 10 g/dL, platelet count <  $100 \times 10^9/L$ ). Possession of one of these characteristics was sufficient to qualify as progressive disease.<sup>(29)</sup> Of 87 CLL patients, 47, five, 18, five, and 11 patients were in Rai stages 0, I, II, III, and IV, respectively. Of 44 non-progressive CLL patients, 33, two, five, two, and one patient were in Rai stages 0, I, II, III, and IV, respectively. Staging of one non-progressive patient could not be defined. Of 40 progressive CLL patients, 12, three, 12, three, and 10 patients were in Rai stages 0, I, II, III, and IV, respectively.

Patients with one or more of the following manifestations were treated: splenomegaly, anemia, thrombocytopenia, disfiguring lymphadenopathy, and constitutive symptoms not related to other syndromes. Thirty-one (35.6%) patients were under therapy at the time of sample collection, whereas 48 (55.2%) patients received their first therapy after diagnosis. The remaining eight patients had stable disease and did not require treatment. The median time from diagnosis to first treatment (TTFT) for these 48 patients was 6 months with a minimum and maximum of 1 and 84 months, respectively. Of these 48 patients, 13 and 35 cases had non-progressive and progressive diseases, respectively. The patients' median TTFT time was 15 months

for non-progressive, 4 months for progressive, 12 months for mutated, and 4 months for unmutated groups of patients. The median follow-up of patients was 49 months (range, 8–204 months). During this study, nine patients died, seven of whom due to CLL complications.

Progression-free survival (PFS) was available for 33 patients with a median of 11 months. Median PFS for non-progressive and progressive CLL patients was 24 and 8.5 months, respectively, whereas that of the mutated and unmutated patients was 24 and 8 months, respectively. The TTFT and PFS in our series of patients are lower than Western patients<sup>(27,30)</sup> which could be due to genetic and/or environmental differences.

**DNA, RNA isolation, and cDNA synthesis.** Genomic DNA was extracted from heparinized peripheral blood leukocytes according to a standard protocol.<sup>(31)</sup> Total cellular RNA was isolated from three million PBMCs of CLL patients using RNA-Bee (BioSite, Taby, Sweden) based on the guanidine thiocyanate phenol chloroform extraction method. First strand cDNA was synthesized from 1–3  $\mu\text{g}$  of total RNA (unfolded at 65°C for 5 min) using M-MuLV reverse transcriptase (RT) enzyme (Fermentas Life Sciences, Ontario, Canada), random hexamer primers (20 pmol), and dNTPs (20 mM) (Roche, Mannheim, Germany). The mixture was incubated at 42°C for 45 min followed by 5 min at 90°C for inactivation of RT enzyme.

**Determination of IGHV family gene expression by polymerase chain reaction (PCR).** Serial dilutions of DNA and cDNA (1:10 to 1:2000) were prepared and PCR amplification was performed using IGHV family-specific degenerative primers. The 5' primers used for IGHV gene amplification were designed to anneal to the leader sequence of IGHV1–7 families, and one degenerative IGHJ or a C $\mu$  primer were used as 3' primers. PCR reactions were performed as described.<sup>(16)</sup>

**Cloning and sequencing of PCR products.** Clonal PCR products were purified by excision using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel purified PCR products were cloned into pGEM-T easy vector (Promega, Southampton, United Kingdom). Briefly, after transformation of chemically prepared JM109 competent cells, at least five colonies were selected randomly for sequencing. Sequencing was performed from both directions using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA), T7, and SP6 primers.

**Analysis of Ig gene sequences.** For each sample IGHV, IGHD, and IGHJ genes were identified by matching to the closest known human germline gene using the ImMunoGene Tics (IMGT) Database (<http://imgt.cines.fr>) and the IgBLAST search (<http://www.ncbi.nlm.nih.gov/igblast/>). As the IMGT database is the most reliable and updated database, the results were reported based on the analysis of the sequences by this database. Classification of patients into mutated ( $n = 56$ ) and unmutated ( $n = 31$ ) subtypes was based on more than 98% homology in nucleotide sequence of immunoglobulin variable region heavy (IGHV) chain genes of the leukemic cells.<sup>(13)</sup> The IGHV third complementary determining region (HCDR3) was determined by the IMGT database.

**Antigenic selection and amino acid changes in IGHV.** Distribution of replacement (R) and silent (S) mutations in the IGHV sequence of mutated CLL patients was evaluated by the multinomial distribution model proposed by Lossos *et al.*<sup>(32)</sup>

**Statistical analysis.** Statistical analyses were performed using the Pearson  $\chi^2$ -test, Fisher's exact test, and Mann-Whitney *U*-test. Kaplan-Meier methods and log-rank tests were used to compare TTFT and PFS in mutated and unmutated as well as progressive and non-progressive CLL patients. Analyses were conducted using the SPSS statistical package (SPSS, Chicago, IL, USA). *P*-values of <0.05 were considered significant.

## Results

**IGHV mutation and disease outcome.** Based on a cut-off of 98% nucleotide sequence homology of isolated *IGHV* gene with that of the germline counterpart, 56 out of 87 (64.4%) *IGHV* sequences displayed somatic mutations, whereas the remaining 31 sequences (35.6%) were unmutated. In the mutated cases, the range of mutations varied from 2% to 19% with an average of 4.9%. The average of mutations was different among *IGHV* families with 6.1% for *IGHV4*, 5% for *IGHV3*, and 3.2% for *IGHV1* families.

Of 44 non-progressive patients, 35 cases had a mutated *IGHV* sequence, and from 40 progressive patients, 21 had an *IGHV* sequence with <98% homology to the corresponding germline *IGHV* sequence ( $P = 0.009$ ).

Comparison of TTFT means between our CLL subtypes revealed a significantly higher TTFT in our non-progressive and mutated (25.3 and 16.4 months) CLL patients compared to progressive and unmutated patients (6.6 and 7.4 months) ( $P = 0.001$  and  $P = 0.03$ , respectively). A higher PFS mean was also observed in non-progressive and mutated (44 and 37 months) CLL patients compared to progressive and unmutated (13 and 9 months) CLL patients ( $P = 0.006$  and  $P = 0.0001$ , respectively). Representative TTFT and PFS results of the mutated and unmutated groups are illustrated in Figure 1. Overall survival was significantly lower in *IGHV* unmutated CLL patients compared to mutated patients (98% vs 84%,  $P = 0.04$ ).

**IGHV gene usage in CLL patients.** The frequencies of the expressed *IGHV* gene families were determined in our CLL patients. Cumulative results are summarized in Table 1. Overall, the most frequently expressed *IGHV* family was *IGHV3* ( $n = 49$ , 56.3%), followed by *IGHV4* ( $n = 18$ , 20.7%), *IGHV1* ( $n = 16$ , 18.4%), *IGHV6* ( $n = 3$ , 3.4%), and *IGHV5* ( $n = 1$ , 1.1%), with no expression of *IGHV2* and *IGHV7* families.

The *IGHV3* family was more represented in non-progressive patients. Of the *IGHV3* family members, only *IGHV3-7* was preferentially expressed in mutated and non-progressive CLL subtypes, but significant difference was observed only between non-progressive and progressive groups of patients (20.5% vs 5%;  $P = 0.05$ ) (Table 2).

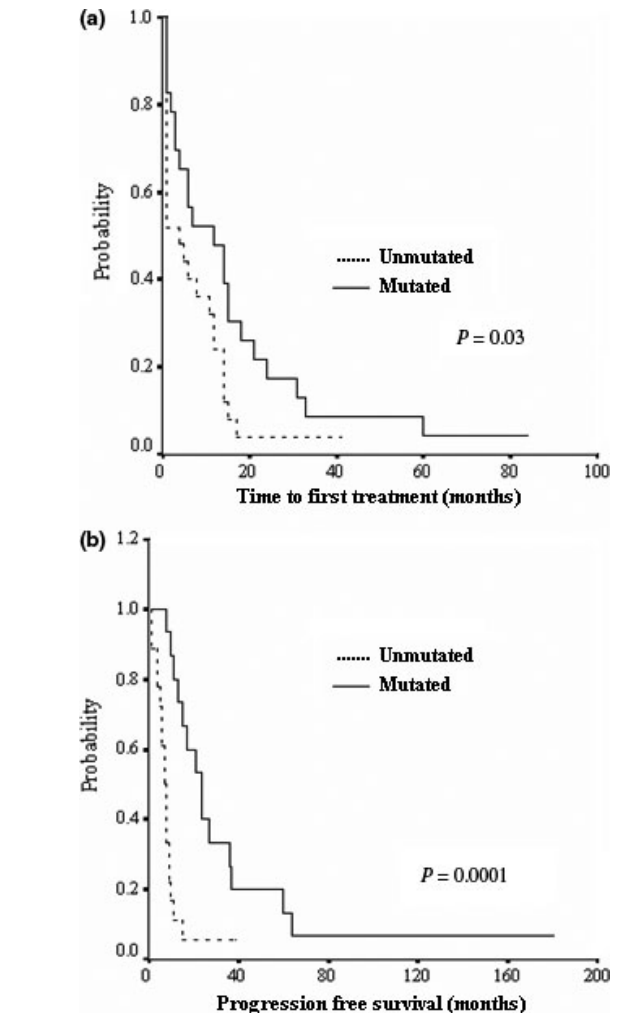
The expression of the *IGHV1* gene family was more represented in progressive and unmutated patients compared to non-progressive and mutated groups of patients, but the difference was significant only between mutated and unmutated patients (10.7% vs 32.3%;  $P = 0.02$ ) (Table 1). Among the *IGHV1* family members, *IGHV1-69* (12.9% vs 1.8%;  $P = 0.05$ ) and *IGHV1-2* (7.9% vs 0%;  $P = 0.04$ ) genes were expressed at significantly higher frequency in unmutated compared to mutated patients (Table 2).

The most predominantly used *IGHV4* members were *IGHV4-39* ( $n = 6$ ) and *IGHV4-34* ( $n = 5$ ) genes representing 61% (11/18) of the total *IGHV4* family. All these genes, with the exception of one, were mutated (Table 2).

Significant differences were observed neither for the frequency of *IGHV* family members, nor for their mutational status between male and female CLL patients (data not shown).

**Association of rearranged *IGHV* genes with disease outcome.** Analysis of the association of TTFT and PFS with the usage of *IGHV* families revealed no significant association with any of the *IGHV* gene families. Of the *IGHV* gene members a higher TTFT was observed in patients expressing *IGHV3-7* compared to those expressing other *IGHV* family members (29 vs 10 months,  $P = 0.08$ ).

**HCDR3 length and motifs.** The median length of the HCDR3 region was 17 amino acids (aa) in our CLL patients. There was a significantly higher mean length of HCDR3 in unmutated CLL patients compared to the mutated group (20 vs 15 aa,  $P < 0.0001$ ). Comparing non-progressive and progressive



**Fig. 1.** Kaplan-Meier plot comparing time from diagnosis to first treatment (a) and progression-free survival (b) between immunoglobulin variable region heavy chain (*IGHV*) mutated and unmutated chronic lymphocytic leukemia (CLL) patients.

patients, there was also a significantly higher mean length of HCDR3 in progressive CLL patients (18 vs 15 aa,  $P = 0.008$ ).

When HCDR3 mean length was analyzed in relation to the rearranged *IGHV* gene family, the length of *IGHV1*-expressing samples (19 aa) was greater than *IGHV3*- (16 aa) and *IGHV4*- (15 aa) expressing samples. When HCDR3 length was compared among the most frequently used gene members of these families, *IGHV3-7* was associated with an average length of 16 aa, *IGHV3-30* of 20, *IGHV3-48* of 19, *IGHV4-39* of 15, *IGHV1-8* of 19, *IGHV4-34* of 19, *IGHV3-33* of 20, and *IGHV1-69* of 21 amino acids.

Composition of the HCDR3 region expressed in our CLL samples revealed similarities between some samples. We found the motif (Y or H) *YYYGMDV* in 13 (15%) samples who all used *IGHJ6* gene in their HCDR3 together with *IGHD6-13* in six samples, *IGHD3-22* in two, and *IGHD1-26*, *IGHD5-5*, *IGHD4-17*, *IGHD3-3*, and *IGHD2-2* each in one sample. Seven of these patients used the *IGHV3-30* gene, while *IGHV4-34* and *IGHV3-48* were used each in two patients, and *IGHV3-49* and *IGHV1-69* were used each by one patient. Eight of these patients had an unmutated *IGHV* gene.

The motif '*ARDQDGGWGV**VDY*' was found in three (3.5%) patients with *IGHV3-64*, *IGHD6*, and *IGHJ4* rearrangement,



**Table 1. Frequency of *IGHV* gene families expressed in leukemic cells of Iranian CLL patients**

Patients	IGHV family							Total
	1	2	3	4	5	6	7	
Non-progressive	6 (13.6)	0	<b>30 (68.2)</b>	8 (18.2)	0	0	0	44
Progressive	9 (22.5)	0	<b>17 (42.5)</b>	10 (25)	1 (2.5)	3 (7.5)	0	40
Newly diagnosed	1 (33.3)	0	2 (66.7)	0	0	0	0	3
Mutated	<b>6 (10.7)</b>	0	32 (57.1)	14 (25)	1 (1.8)	3 (5.4)	0	56
Unmutated	<b>10 (32.3)</b>	0	17 (54.8)	4 (12.9)	0	0	0	31
Total	16 (18.4)	0	<b>49 (56.3)*</b>	18 (20.7)	1 (1.1)	3 (3.4)	0	87
†Normal adult B cells	778 (28.3)	104 (3.8)	971 (35.3)	770 (28)	94 (3.4)	33 (1.2)	0	2750
‡Normal CD5 <sup>+</sup> /IgM <sup>+</sup> B cells	27 (18.8)	3 (5.9)	81 (56.3)	26 (18.1)	2 (1.4)	2 (1.4)	3 (2.1)	144
¶Known functional genes per family	11 (21.6)	3 (5.9)	22 (43.1)	11 (21.6)	2 (3.9)	1 (2)	1 (2)	51

Data are presented as number (%) of patients expressing the corresponding immunoglobulin variable region heavy chain (IGHV) family; All significant differences between groups are shown in bold. \*Comparison between the frequency of expressed gene family in the Iranian chronic lymphocytic leukemia (CLL) population and the frequency of gene family expression in normal adult B cells ( $P < 0.0001$ ). †The frequency of *IGHV* gene family expression in normal adult B cells represents IGHV clones identified in IgM cDNA libraries of unstimulated peripheral blood lymphocytes from healthy adults.<sup>(41)</sup> ‡The frequency of expressed IGHV family genes in human peripheral CD5<sup>+</sup>/IgM<sup>+</sup> B cells from two healthy adult donors.<sup>(42)</sup> ¶The percent of functional *IGHV* genes per family observed as IGHV recombinants were obtained from the V BASE sequence directory.

**Table 2. Frequency of *IGHV* genes expressed in leukemic cells of Iranian CLL patients**

No	IGHV	Total	Mutated (n = 56)	Unmutated (n = 31)	Non-progressive (n = 44)	Progressive (n = 40)
1	<b>IGHV3-7</b>	11 (12.6)	9 (16.1)	2 (6.5)	<b>9 (20.5)</b>	<b>2 (5)</b>
2	<b>IGHV3-30</b>	10 (11.5)	5 (8.9)	5 (16.1)	5 (11.4)	5 (12.5)
3	<b>IGHV3-48</b>	8 (9.2)	6 (10.7)	2 (6.5)	5 (11.4)	3 (7.5)
4	<b>IGHV4-39</b>	6 (6.9)	5 (8.9)	1 (3.2)	3 (6.8)	3 (7.5)
5	<b>IGHV1-8</b>	6 (6.9)	5 (8.9)	1 (3.2)	2 (4.5)	3 (7.5)
6	<b>IGHV4-34</b>	5 (5.7)	5 (8.9)	0	3 (6.8)	2 (5)
7	<b>IGHV3-33</b>	5 (5.7)	2 (3.6)	3 (9.7)	3 (6.8)	2 (5)
8	<b>IGHV1-69</b>	5 (5.7)	<b>1 (1.8)</b>	<b>4 (12.9)</b>	3 (6.8)	2 (5)
9	<b>IGHV3-23</b>	4 (4.6)	3 (5.4)	1 (3.2)	4 (9.1)	0
10	<b>IGHV1-2</b>	3 (3.4)	<b>0</b>	<b>3 (9.7)</b>	0	3 (7.5)
11	<b>IGHV3-11</b>	3 (3.4)	2 (3.6)	1 (3.2)	1 (2.3)	2 (5)
12	<b>IGHV3-64</b>	3 (3.4)	3 (5.4)	0	2 (4.5)	0
13	<b>IGHV6-1</b>	3 (3.4)	3 (5.4)	0	0	3 (7.5)
14	<b>IGHV1-46</b>	2 (2.3)	0	2 (6.5)	1 (2.3)	1 (2.5)
15	<b>IGHV3-15</b>	2 (2.3)	2 (3.6)	0	0	1 (2.5)
16	<b>IGHV3-49</b>	2 (2.3)	0	2 (6.5)	0	2 (5)
17	<b>IGHV4-30</b>	2 (2.3)	1 (1.8)	1 (3.2)	0	2 (5)
18	<b>IGHV4-4</b>	2 (2.3)	2 (3.6)	0	1 (2.3)	1 (2.5)
19	<b>IGHV4-b</b>	2 (2.3)	0	2 (6.5)	1 (2.3)	1 (2.5)
20	<b>*Others</b>	3 (3.4)	2 (3.6)	1 (3.2)	1 (2.3)	2 (5)

Data are presented as number (%) of patients expressing the corresponding immunoglobulin variable region heavy chain (IGHV) member; All significant ( $P < 0.05$ ) differences between groups are shown in bold. \*Others represent *IGHV3-66*, *IGHV4-59*, and *IGHV5-a* genes, each expressed in one patient. CLL, chronic lymphocytic leukemia.

all with mutated IGHV sequence. The HCDR3 sequence 'GRVIYSSERNLDF' was also observed in two (2.3%) patients, both with *IGHV3-48/IGHD6/IGHJ4* genes.

## Discussion

The observation of highly significant differences in disease outcome between mutated and unmutated CLL subtypes has prompted extensive investigations on the mutational status and VH/VL gene usage in CLL over the last few years. These studies have demonstrated a biased use of certain *IGHV* gene members, preferentially higher mutation rate in certain IGHV gene members as well as higher frequency of closely related (stereotyped) HCDR3 sequences in patients sharing the same or homologous *IGHV* gene members.<sup>(33,34)</sup> These findings imply selection and clonal expansion of leukemic B cells by certain

unidentified foreign or self antigens. As such, environmental and/or ethnic variations may affect *IGHV* gene usage in CLL.

Most previous studies have been conducted in Western populations with similar geographic and/or ethnic backgrounds. Nevertheless, such variations have been reported; perhaps the most frequent one is the use of *IGHV3-21* with a frequency of approximately 11.7% in the Scandinavian region,<sup>(20)</sup> 7.9% in Ireland,<sup>(35)</sup> and 2–3% in the Mediterranean region.<sup>(6)</sup> Such analysis in CLL patients from disparate ethnic and geographic origins could expand our understanding of CLL and deserves special consideration.

Little is known about Asian CLL patients. Analysis of *IGHV* gene family usage in Asian CLL patients has so far been reported only in small cohorts of Japanese,<sup>(7,36)</sup> Chinese,<sup>(37)</sup> and Iranian<sup>(38)</sup> patients. This could partly be due to the low incidence of CLL in Asian populations<sup>(39,40)</sup> compared to the

Western countries. Our previous study, which was performed in a small number of Iranian CLL patients to determine the frequency of the expressed IGHV gene family, revealed significant differences in the expression of IGHV1 and IGHV4 gene families between Iranian and Western patients.<sup>(38)</sup>

In the present study we investigated the frequency of the expressed IGHV gene family members and the prognostic significance of somatic hypermutation of IGHV genes in Iranian CLL patients. We also analysed the HCDR3 sequence in leukemic B cells to find out homologous motifs in our CLL patients. In general, the most frequently used IGHV family by the leukemic B cells of our CLL patients was IGHV3 followed by IGHV4, IGHV1, IGHV6, and IGHV5, with no expression of the IGHV2 and IGHV7 families. Although the IGHV3 family was predominantly expressed in non-progressive (30/49, 61.2%) and mutated CLL subtypes (32/49, 65.3%), the IGHV1 family was more represented in progressive (9/16, 56.3%) and unmutated (10/16, 62.5%) CLL patients (Table 1). Our results on the frequency of IGHV families are similar to those obtained in Japanese<sup>(7,36)</sup> and Chinese<sup>(37)</sup> CLL patients. Comparing the frequency of IGHV family expression in CLL patients with normal adult B cells<sup>(41)</sup> revealed a higher representation of the IGHV3 family in our CLL patients, but no differences with their normal CD5<sup>+</sup>/IgM<sup>+</sup> B cell counterparts,<sup>(42)</sup> which is also in agreement with the Japanese CLL patients.<sup>(7,36)</sup> Our results did not confirm the results of Fais *et al.*,<sup>(13)</sup> who reported a significant decrease in IGHV3 family expression in their CLL patients compared to normal CD5<sup>+</sup>/IgM<sup>+</sup> B cells. The IGHV1 family is predominantly used after the IGHV3 family in Western CLL patients,<sup>(15,16,25,43)</sup> while the use of IGHV4 family is more predominant after IGHV3 in our patients and those from China<sup>(37)</sup> and Japan.<sup>(7,36)</sup>

The most frequently represented IGHV genes in our cohort of CLL patients were IGHV3-7 (12.6%), IGHV3-30 (11.4%), IGHV3-48 (9.2%), IGHV4-39 (6.9%), and IGHV1-8 (6.9%). Collectively, these five IGHV genes represent nearly half (47%) of all Iranian CLL cases, whereas they are expressed in about 20% of Western, 21.3% of Japanese,<sup>(36)</sup> and 14.3% of Chinese<sup>(37)</sup> CLL patients. While IGHV3-7 is the most frequent IGHV gene in Iranian CLL patients, IGHV4-34 is most frequently

expressed in Chinese<sup>(37)</sup> and Japanese<sup>(36)</sup> CLL patients. IGHV3-7 was predominantly expressed in our mutated and non-progressive CLL patients. Higher frequency usage of this gene in mutated CLL patients has also been observed in other studies.<sup>(16,19,25)</sup> Comparing the expression frequency of IGHV3-7 in Iranian CLL patients with Western CLL patients revealed a significantly higher representation of this gene in Iranian CLL patients (Iranian vs Western,  $P = 0.005$ ; Iranian vs American,  $P = 0.03$ ; Iranian vs European,  $P = 0.004$ ), but no differences with Chinese and Japanese CLL patients as Asian populations (Table 3).

IGHV1-69 is the most frequently used IGHV gene in Western CLL patients reported by many investigators, generally with unmutated phenotype (Table 3). The frequency of this gene varies in different populations based on their geographic origin. The most frequent usage of this gene has been reported in Ukrainian<sup>(44)</sup> and Spanish<sup>(45)</sup> CLL patients (~21.5%) of whom 98% displayed an unmutated sequence. The frequency of this gene is significantly higher in our unmutated CLL patients compared to mutated ones (12.9% vs 1.8%,  $P = 0.03$ ) (Table 2) confirming the results obtained in Western patients.<sup>(4,6,8)</sup> However, the frequency of this gene in Iranian patients is significantly lower than that in Western CLL patients (Iranian vs Western,  $P = 0.02$ ; Iranian vs American,  $P = 0.04$ ; Iranian vs European,  $P = 0.02$ ), with no significant difference compared to other Asian (Japanese and Chinese) CLL populations. Taken together, representation of IGHV1-69 is significantly lower in Asian CLL patients (Asian vs Western,  $P < 0.00001$ , Table 3). This difference is the most striking feature of Asian compared to Western CLL patients.

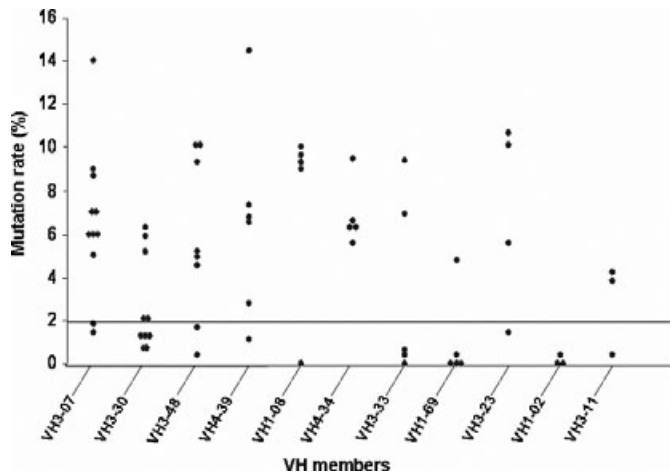
While IGHV1-2 and IGHV1-69 genes in our patients carry a few or no mutations, IGHV1-8, IGHV3-7, IGHV3-23, and IGHV3-48 genes showed a high load of mutations in their sequences. Our results are in agreement with other studies,<sup>(1,6,13)</sup> showing a few mutations in the IGHV1-69 gene and a higher frequency of mutations in IGHV3-23 and IGHV3-7 genes (Fig. 2).

In contrast to Western CLL patients, especially of those Scandinavian origin,<sup>(20,25)</sup> leukemic B cells of our CLL patients did not use the IGHV3-21 gene. Over-representation of IGHV3-21 has also been reported in other Western CLL populations from

**Table 3. Comparison of IGHV gene usage in Iranian CLL patients with other studies**

Country	No of patients	IGHV member										Reference
		1-2	1-8	1-18	1-69	3-7	3-21	3-23	3-30	4-34	3-48	
USA	83	6	0	4.8	7.1	12.1	0	4.8	3.6	18.1	2.5	13
USA	34	3	3	0	5.9	3	5.9	8.8	5.9	11.8	0	26
USA	172	6.4	0.6	2.3	10.5	8.2	1.2	6.4	4.7	14.5	2.9	17
USA	356	4.8	0.5	2.4	18	4.3	1.6	7	4.8	7	4.3	19
USA	190	4.7	NI	NI	11	4.7	NI	5.8	4.7	14.2	7.9	45
England	84	7.1	0	1.2	11.9	7.1	1.2	11.9	11.9	11.9	4.8	15
England	154	6.3	0	1.3	11.3	5	3.5	8.2	8.8	7.1	5.7	16
Sweden	346	4	NI	NI	14	3	9	5	6	7	NI	25
Sweden	265	NI	NI	NI	17.4	3.8	11.7	4.9	5.7	NI	NI	20
Italy	85	5.7	5.7	NI	9.1	5.7	1.1	8	9.1	8	NI	8,43
Spain	56	1.8	0	3.6	21.5	1.8	1.8	5.4	3.6	7.1	3.6	4
Mediterranean area	553	4.2	1	2.8	10.7	5.4	3	9.2	6.7	10.7	2.2	6
Ukraine	189	5.3	NI	NI	21.7	4.8	5.8	1.6	3.7	7.4	4.2	44
Ireland	228	7.9	NI	NI	12.3	NI	7.9	NI	NI	13.5	3.5	35
Japan	44	NI	NI	NI	2.3	NI	7	14	7	20.9	NI	7
Japan	80	1.25	3.75	2.5	1.25	5	7.5	8.75	6.25	27.5	3.75	36
China	65	0	0	0	1.5	4.6	3	7.7	6.2	12.3	1.5	37
Iran	87	3.4	6.9	0	5.7	12.6	0	4.6	11.4	5.7	9.2	Present study

The results represent the percent of each immunoglobulin variable region heavy chain (IGHV) member. CLL, chronic lymphocytic leukemia; NI, not identified.



**Fig. 2.** Distribution of mutations in the most frequently expressed immunoglobulin variable region heavy chain (IGHV) genes in chronic lymphocytic leukemia (CLL) patients. Baseline denotes a cut-off mutation value (2%) assigned to differentiate between mutated and unmutated IGHV sequences.

the USA,<sup>(13)</sup> Italy,<sup>(8,43)</sup> and England,<sup>(15)</sup> though contradicting results have also been reported.<sup>(6,17,19,26)</sup> The differences in the frequency of this gene in patients with the same ethnic origin might be due to the small number of patients studied or more importantly because of their geographic origin. The important role of geographic origin of patients for the expression of *IGHV3-21* by leukemic B cells of CLL patients has been verified in Scandinavian patients.<sup>(20,25)</sup> This possibility is highlighted by differential usage of *IGHV3-21* and *IGHV1-69* genes between Asian (especially Iranian) and Western CLL patients. For further insight into this issue, the normal IGHV repertoire should also be investigated in healthy subjects of the same ethnicity living in the same geographic areas, to be able to find out associations, if any, between the over-expressed *IGHV* genes and ethnic/geographic parameters.

The frequency of *IGHV4-34* has been reported to be high in Americans<sup>(13,17,26,45)</sup> and the two Asian, Japanese and Chinese,<sup>(7,36,37)</sup> CLL patients, but only a small number (5.7%) of our Iranian CLL patients expressed this gene. The frequency of *IGHV4-34* gene usage in our patients is comparable to that of European patients, but significantly lower than the American CLL patients ( $P = 0.003$ ). Over-representation of this gene has also been observed in Japanese and Chinese patients (Japanese–Chinese vs Iranian,  $P = 0.003$ ; Japanese–Chinese vs European,  $P < 0.0001$ ). Together with some similarities between Asian populations, the lower frequency of the *IGHV4-34* gene in Iranian patients compared to that of Japanese and Chinese CLL patients may also reflect the differences in genetic background or environmental factors between these two distinct populations.

Collectively four *IGHV* genes, including *IGHV1-69*, *IGHV3-7*, *IGHV3-21*, and *IGHV4-34* among all other *IGHV* genes showed significant differences in different ethnic/geographic CLL populations. This is a very important finding for our understanding of CLL etiopathogenesis. The apparent geographic/ethnic bias for the frequency of defined *IGHV* genes shown in our study and by others in different ethnic populations may theoretically be due to specific genetic background, depending on variations in germline composition of the *IGHV* locus, or to the effects of a potential environmental variable less frequently encountered in different regions as proposed by Ghia *et al.*<sup>(6)</sup> The lack of information for the frequency of *IGHV* genes in normal B cells and especially CD5<sup>+</sup> B cells in Iranian and also other ethnic populations such as Scandinavian and Asian populations

is the main gap in determining the exact biased expression of *IGHV* families and members in our CLL patients. The biased usage of *IGHV* genes and the presence of shared antigen-binding motifs in HCDR3 regions of neoplastic B cells in our series of CLL patients is important evidence indicating that CLL may be sustained by an antigen-driven process.<sup>(1)</sup> It has been shown recently that BCR stimulation of B cells from CLL patients results in over-expression of genes involved in signal transduction and cell cycle regulation, especially in unmutated CLL patients which resulted in extended survival of CLL cells.<sup>(46)</sup> These results imply that antigenic stimulation of BCR by a self or foreign antigen plays a pivotal role in disease progression and/or initiation of malignant transformation of B cells.

The sequence ([Y or H] YYYGMDV) was the most predominant HCDR3 motif found in 13 (15%) of our patients. This sequence is largely encoded by the *IGHJ6* gene which was found to be rearranged in all these patients. The motif 'ARD-QDGGWGVYD' was found in three patients with the *IGHV3-64/IGHD6/IGHJ4* combination, all with mutated *IGHV* sequences, and 'GRVIYSSERNLDF' was used in two patients with *IGHV3-48/IGHD6/IGHJ4* genes. This information is exclusive to our report with no previous precedent.

The motifs 'DIVVVA', 'DFWSGY', 'RFLEWL', and 'YYYGMDV', which have previously been reported in CLL patients,<sup>(8,18,22,47)</sup> were each found in HCDR3 of only one of our patients.

Comparison of the HCDR3 sequences of our CLL patients with archetypes clustering of HCDR3 recently reported by Messmer *et al.*<sup>(33)</sup> revealed that only one of our patients has the same sequence as the archetype no. 31 (CARD-QYYDSSGYSGYFDYW) rearranging the same *IGHV1-46/IGHD3-22/IGHJ4* combination with no mutation in the *IGHV* sequence. Based on either 70% or 60% sequence homology with 67 represented HCDR3 archetypes clustered by Messmer *et al.*<sup>(33)</sup> seven and 31 patients within our samples could be classified within 12 and 27 archetypes, respectively. The small sample size of our patients and the fact that these archetypes are based on HCDR3 sequences from a large cohort of Western CLL patients, may explain the low level of similarities of HCDR3 observed in our patients compared to those reported by others.<sup>(33,48)</sup> To determine the real archetypes of Iranian or Asian CLL patients, analysis of a large series of CLL patients is necessary.

The length of HCDR3 in our unmutated CLL patients was significantly longer than that in mutated cases ( $P < 0.0001$ ). This finding was also reported by others<sup>(8,18,22,49)</sup> and confirms that the *IGHV* sequence of unmutated leukemic B cells in the germline configuration might be derived from a more immature B cell than that of mutated CLL patients.<sup>(8,18)</sup> The differences in HCDR3 length was not due to the selected germline *IGHD* genes, since for instance *IGHD3-3* (overused in unmutated group) and *IGHD3-22* (overused in mutated group) are both 31 nucleotides long. Furthermore, in the mutated group rearrangements involving the overused *IGHV3-7* gene were associated with a shorter HCDR3 (average 16 aa) than those of the *IGHV3-7* gene used in the unmutated group (18 aa). Our results are in agreement with the study of Duke *et al.*<sup>(16)</sup> who reported a significantly longer HCDR3 length in the unmutated group with overused *IGHV1-69* gene compared to the *IGHV1-69* gene used in the mutated CLL patients.

The present data on the similarity of HCDR3 in a subset of CLL patients strongly suggests the role of antigen selection in these patients. Since the ratio of R/S mutations in Ig CDRs may reflect the role of antigenic selection in the mutated CLL patients, nearly half of our mutated CLL patients (46.5%) displayed this pattern, in agreement with other studies.<sup>(50,51)</sup> Together, the presence of specific HCDR3 motifs, biased expression of certain *IGHV* genes, and the ratio of R/S



mutations in leukemic cells of our CLL patients, suggest involvement of an as yet undefined pathogen or autoantigen in the pathogenesis or initiation of CLL.

Although it seems that patients with CLL have some defects in their immune responses (especially T-cell defects),<sup>(52)</sup> the better prognosis of patients with mutated *IGHV* genes could be due to induction of autologous T-cell response to the mutated epitopes (idiotypes) located on the *IGHV* region of the leukemic cells. We are currently embarking on elucidation of this issue by analysis of the autologous T-cell response to peptides resembling the mutated epitopes of the *IGHV* region of our CLL patients.

In conclusion, it seems that skewed representation of *IGHV* genes and the presence of similar HCDR3 sequences in leukemic B cells of mutated and unmutated CLL patients from different ethnic populations suggest a role for an as yet undefined set of pathogens or autoantigens in the initiation of CLL. Some types of lymphomas such as marginal zone lymphoma have been proposed to be associated with chronic stimulation by persistent microbial antigens and/or autoanti-

gens.<sup>(53)</sup> The role of pathogens in malignancies may be direct through their oncogenes or indirect by promoting favorable conditions for lymphocyte transformation, such as increased proliferation or decreased apoptosis of lymphoid cells.<sup>(53,54)</sup> Regional differences observed between our patients and those from other ethnic and/or geographic origins may imply contribution of different antigenic stimuli for selection of the neoplastic B cells. Extension of this study in a large cohort of Iranian and other Asian populations, particularly Chinese and Japanese patients with completely diverse ethnic and geographic backgrounds, as well as other ethnic populations, especially African and Australian CLL patients, may reveal some new aspects of the etiopathogenesis of CLL.

## Acknowledgments

This study was supported by a grant from the Nanotechnology Network of the Ministry of Health and Medical Education of Iran. We are grateful to Joseph Lawrence for his technical assistance in nucleotide sequencing.

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