Immunoglobulin Mutational Status Detected through Single-Round Amplification of Partial V_H Region Represents a Good Prognostic Marker for Clinical Outcome in Chronic Lymphocytic Leukemia

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The immunoglobulin (Ig) mutational status in B-cell chronic lymphocytic leukemia (CLL) distinguishes two subsets of patients with different prognosis. Ig status detection is commonly performed with a panel of V_H family-specific primers. Although this method detects clonal VDJ rearrangement in virtually all cases, it is technically cumbersome and therefore not widely used clinically. Here, we describe a simple and rapid method to establish the mutational status of IgV_H in CLL. The method is based on a consensus V_H FR2 primer, used in both polymerase chain reaction (PCR) and sequencing reactions. Overall, monoclonal B-cell populations were detected in 163 of 189 CLL patients (86%). The prognostic value of IgV_H mutational status was then evaluated by analyzing survival in 146 CLL cases using different V_H homology cutoffs. CLL prognostic groups were best separated by the classical 98% cutoff: median survival was 127 and 206 months in unmutated and mutated CLL cases, respectively ($P = 0.0023$). V_H FR2 consensus and V_H family PCR were compared in 41 cases, correctly assigning all cases by both methods. Therefore, we suggest a sequential strategy to detect immunoglobulin mutational status in CLL patients by first using the approach described in this study followed by alternative V_H family-specific PCRs for negative cases. (*J Mol Diagn* 2005, 7:566–574)

The clinical outcome of B-cell chronic lymphocytic leukemia (B-CLL) is very variable: some patients experience a very stable condition and never require treatment,

whereas others become symptomatic very quickly and soon require cytostatic therapies.^{1,2} Therefore, although the median overall survival of CLL patients is ~10 years, individual patients present extremely heterogeneous prognosis that can vary from a very short to a normal life span. Clinical staging systems were developed almost 3 decades ago and have provided a foundation on which clinicians base their management and therapeutic decisions. Both Binet and colleagues³ and Rai and colleagues⁴ clinical staging systems are able to divide B-CLL patients on the basis of simple clinical parameters in low-, intermediate-, and high-risk groups with median survival ranking from 3 to more than 12 years. Nevertheless, clinical heterogeneity also appears within the three prognostic groups and staging systems cannot distinguish between patients at the early stages of the disease who are likely to progress through the disease, who are refractory to the treatment, who develop infectious or autoimmune complications and will have a shorter survival than expected, and those in whom the disease will remain stable for a long period.

Moreover, in the last decade, CLL has often diagnosed accidentally, due to the increasing practice of performing blood screening for minor reasons, leading to the identification of CLL disease in younger and asymptomatic patients at earlier stages of the disease. Furthermore, the recent progress in treatments using monoclonal antibodies in combination with chemotherapy^{5,6} and in the field of autologous and allogeneic stem cell transplantation,⁷ raises the necessity of accurate identification of good versus poor prognosis patients' groups.

In an attempt to give clinical stages a more important prognostic role, allowing the application of prompt anti-leukemic treatment also on asymptomatic patients whose condition is likely to progress, many other parameters have been evaluated in the management of CLL patients such as the histopathology of bone marrow,⁸ blood lym-

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phocyte counts and morphology,⁹ and lymphocyte doubling time.¹⁰ In addition, serum concentrations of lactate dehydrogenase, thymidine kinase, beta2-microglobulin, CD23 as well as the presence of different cytogenetic abnormalities and CD38 expression levels of leukemic cells have revealed their prognostic value in some studies.^{11–15} In particular, recurrent cytogenetic abnormalities are associated with different outcome: patients with del(13q) have an excellent prognosis whereas patients harboring del(11q) and especially del(17p) have a very poor survival.¹⁶ Moreover, patients with high CD38 expression are characterized by an unfavorable clinical course with a more advanced stage of disease at presentation, poor responsiveness to chemotherapy, and shorter survival.¹⁷

Recently, the mutational status of immunoglobulin genes (IgV_H) expressed in the leukemic population has been identified as a very good prognostic marker of CLL clinical outcome.^{18,19} The mutated CLL cases have a more favorable prognosis and require less treatment than the unmutated ones.²⁰ Moreover, in multivariate analyses, the Ig mutational status appears to be the best prognostic marker of clinical outcome with respect to other parameters such as CD38 expression, genomic aberrations or thymidine kinase serum concentration.^{21,22} The assessment of IgV_H mutational status through V_H family polymerase chain reaction (PCR) is certainly a time consuming and elaborate practice to perform in most laboratories. During the last decade, surrogate genes such as CD38 and ZAP70 have been suggested as possible alternatives. Unfortunately, CD38 expression does not always correlate with the IgV_H mutational status^{23–25} rather it appears to be an independent prognostic marker in B-CLL.²⁶ In addition, expression of ZAP70 kinase was first recognized to correlate with Ig mutational status and prognosis,^{27,28} but recent studies have shown a discordance between ZAP70 and Ig mutational status,²⁹ although ZAP70 maintains an important prognostic independent value.³⁰ Here we describe a rapid and simple method to analyze the nucleotide sequence of the variable portion of Ig heavy chain gene using amplicons obtained with degenerate primers commonly used for diagnostic B-cell clonal detection purposes.

Materials and Methods

B-CLL Patients

Data from 189 random B-CLL patients, who were seen in the Hematology Division of Modena, and the Internal Division of Carpi and Sassuolo Hospitals, have been collected since 2001; 61% of cases have been diagnosed since 2001, 35% of cases were diagnosed between 1990 and 2000, and 4% were diagnosed before 1990. All patients fulfilled the recommended diagnostic criteria with dim surface immunoglobulins, CD5/CD19/CD23 expression pattern, and persistent lymphocytosis of more than $5.0 \times 10^9/L$. The median age at diagnosis was 66 years and it ranged from 31 to 87 years with the

Table 1. Main Clinical Parameters of B-CLL Patients

Gender (male/female)	122/67 (1.8/1)
Age at diagnosis	
Median	66 years
Range	31 to 87 years
Rai stage (n = 117)	
0	37 (31.6%)
I to II	54 (46.2%)
III to IV	26 (22.2%)
Treatment required (n = 122)	
No	58 (47.5%)
Yes	64 (52.5%)

male to female ratio of 1:8. According to the Rai clinical staging system,⁴ 31.6% of patients were in the low-risk stage, 46.2% in the intermediate stage, and 22.2% in the high-risk stage. Treatment data were available for 122 patients: 52.5% received conventional chemotherapy. Survival data were available for 146 patients, with a median follow-up of 43 months, ranging from 1 to 289 months. At present, 13 patients have died of causes related to B-CLL. There were no differences in follow-up between the subgroups analyzed. Table 1 summarizes the patients' characteristics.

Total RNA Isolation and cDNA Synthesis

Total RNA from mononuclear cells, obtained from anti-coagulated peripheral and bone marrow blood by density gradient centrifugation (Ficoll-Hypaque; Pharmacia LKB Biotechnology, Piscataway, NJ) was isolated by acid guanidinium thiocyanate phenol-chloroform extraction³¹ with a few modifications.³² RNA (1.5 µg) was reverse-transcribed to cDNA in a 30-µl reaction mixture containing 400 U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), 20 U of RNase Inhibitor (Roche, Basel, Switzerland), 20 pmol of random primer, 500 µmol/L dNTP each, 0.1 mg/ml of bovine serum albumin in 1× RT buffer (60 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.3, 3 mmol/L MgCl₂, 10 mmol/L dithiothreitol). After a denaturation step at 65°C for 15 minutes, reactions were performed at 42°C for 1 hour and then heated at 95°C for 3 minutes to inactivate the enzyme.

PCR Conditions for Partial IgV_H Region Amplification and Sequencing

The pattern of clonality of 189 samples was evaluated amplifying in a single round PCR only a portion of the V_H region using an upstream primer (FR2: 5'-TGG(A/G)TC-CG(C/A)CAG(G/C)C(T/C)(T/C)CNGG-3') complementary to the second framework region of IgV_H gene in association with a downstream primer directed to an outer conserved sequence of J_H region (JHE: 5'-ACCTGAG-GAGACGGTGACC-3'). The VDJ rearrangement amplifications were performed in a 50-µl reaction mixture containing 5 µl of cDNA in 1X PCR Buffer II (Applied Biosystems, Foster City, CA) with 2.25 mmol/L MgCl₂, 200 µmol/L of each dNTP, 1 µmol/L of each primer, and 2.5 U of Amplitaq Gold polymerase (Applied Biosys-

tems). Thirty-five cycles of amplification were performed at the following conditions: 30 seconds at 95°C, 30 seconds at 50°C, 30 seconds at 72°C with an initial denaturation/activation step at 95°C for 10 minutes and a final extension step at 72°C for 7 minutes. For each PCR, a control with no added template was used to check for contamination. In 163 patients one discrete band of predicted size (240 to 280 bp) (monoclonal pattern) was detected and, after its excision from a 1.5% agarose gel, it was then purified with QIAquick gel extraction kit (Qiagen, Valencia, CA) and directly sequenced using the BigDye Terminator cycle sequencing kit and an automated sequencer (ABI Prism 310; Applied Biosystems). Four pmol of primer were used for each cycle-sequencing reaction. Both strands were sequenced using the same sense and anti-sense primers used in PCR VDJ rearrangement amplification. In 41 patients the limited sequence identified with FR2 amplification was confirmed amplifying the entire V_H region with separate mixtures in which an oligonucleotide 5' primer specific for each leader sequence of the VH1 to VH6 families together with a 3' primer complementary to the germ line J_H region were used.³³

Analysis of V_H, D, J_H Sequence and CDR3 Length

Both sense and anti-sense V_H sequences obtained from each patient were compared to each other using the Sequence Navigator 1.0.1 aligning software (Applied Biosystems). Then, the correct patient-specific VDJ rearrangement sequence was aligned to NCBI/GenBank using the IgBLAST software (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD; URL: <http://www.ncbi.nlm.nih.gov/>) to define V_H, D, and J_H segments used, the percentage of mutations, and the framework status. The results were also compared to IMGT immunoglobulin database using the IMGT/V-QUEST tool (International ImMunoGeneTics information system, Marie-Paule Lefranc, University of Montpellier, CNRS, France; URL: <http://imgt.cines.fr>) to determine the length of CDR3, which was established by counting the number of amino acids between position 105 at the end of FR3 (usually one amino acid downstream the conserved cystidine) and position 117 at the beginning of FR4 (one amino acid upstream the conserved tryptophan). We considered a V_H gene sequence to be mutated if it had equal to or more than 2% sequence alterations when compared with the published germ line sequence. Patients with a percentage of mutation between 2% and 5% were included in a mutated subgroup with low-level mutations (LM-CLL).

Statistical Analysis

Data were analyzed using SPSS for Windows Version 11.0 (SPSS, Chicago, IL). To determine significant differences in the representation of V_H, D, and J_H segments as well as in the distribution of gender and chemotherapy requirements between the unmutated and mutated

groups, the Pearson χ^2 test was used. Instead, comparisons of quantitative variables such as CDR3 length, follow-up time, and age at diagnosis between groups, were performed with Mann-Whitney nonparametric test. All were two-sided. The nonparametric Spearman's rank order coefficient for estimating correlations between quantitative parameters was used. An effect was considered statistically significant at $P = 0.05$. Survival function was estimated using the product-limit (Kaplan-Meier) method and the curves have been compared between groups using the log-rank test.

Results

IgV_H Sequence Analysis and Mutational Status

The mutational status of IgV_H genes was examined in 189 CLL cases by a single round PCR using, as upstream primer, a degenerate oligonucleotide specific for the FR2 region of V_H genes coupled with an anti-sense primer, specific for a highly conserved J_H sequence. To easily obtain a single band representative of the neoplastic clones, the cDNAs derived from total RNA obtained from mononuclear peripheral or bone marrow blood cells were used. A single band of the expected nucleotide length was obtained in 163 samples, equal to 85.8% of the cases examined. Direct sequencing analysis of the amplicons was performed using as primers the same sense and anti-sense oligonucleotides used in the amplification reactions. Clear nucleotide sequencing data were obtained from all amplicons examined (Figure 1).

Besides the relative limited length of V_H sequence obtainable, which includes the 3' portion of FR2, the entirety of CDR2, FR3, CDR3, and a small portion of FR4 region, and which corresponds to 56% of the complete V_H sequence, V_H and J_H segments were observed in all cases. Moreover, in all 163 cases the sequence of the CDR3 region was accurately determined, whereas a D segment was identified in 119 cases, which corresponds to 73% of the cases. Thus, homology to V_H germline sequence was determined using the classical homology cutoff value of 98%; 94 patients (57.7%) had mutated V_H genes (M-CLL group) and 69 (42.3%) had unmutated V_H sequences (UM-CLL group). The germline homology rate in the mutated group ranged from 83 to 98% (median, 93%). Among the unmutated group, 38 cases had 100% homology, whereas in 31 patients, a nucleotide homology ranging from 98 to 100% was observed. All patients, with the exception of only five cases, expressed V_H1 ($n = 33$, 20.2% of total cases), V_H3 ($n = 89$, 54.6%), or V_H4 ($n = 36$, 22.1%) segments. Analysis of the different immunoglobulin gene segments revealed a biased usage related to the mutational immunoglobulin V_H gene status with a preferential usage of V_H1 family in unmutated V_H genes group ($n = 29$, 87.9% of V_H1 total cases). On the contrary, V_H3 family members were in the mutated configuration in 61 of 89 cases (68.5%) as well as the V_H4 family that exhibited somatic mutations in 27 of 36 cases (75%) ($P < 0.0001$) (Table 2). In particular, the V_H1-69 segment was used in the unmutated configuration in 18 of 19

Figure 1. Comparison between nucleotide sequences obtained with upstream FR2 and downstream JHE primers in a single case. Sequence alignment was performed using Clustal tool of Sequence Navigator 1.0.1 software. Sense and complementary reverted anti-sense (*) nucleotide sequences were aligned together (above); correspondent sense and anti-sense electropherograms relative to CDR3 region were shown below.

V_H1-69-positive cases. On the contrary, V_H4-34 segment was mutated in 15 cases, while expressed in only 2 patients in a germline configuration. V_H3-23 and V_H3-30 were the most frequently used V_H3 segments, in 14 and 12 cases, respectively, with slight prevalence in mutated CLL subgroup. Moreover, V_H3-21 segment, which is thought to be related to CLL cases with poor prognosis, was used in nine cases in both mutated (four cases) and unmutated (five cases) configuration.

The majority of B-CLL studied used D3 (53 of 119, 44.5%), in particular D3-22 and D3-3 ($n = 13$ and $n = 19$,

respectively), and J_H4, J_H6 segments ($n = 66$, 40.5% and $n = 46$, 28.2%, respectively). There was not a differential usage of D segments between M-CLL and UM-CLL cases ($P = 0.11$), whereas significant difference in J_H region distribution was shown ($P = 0.015$) between the two groups, with the unmutated group revealing a consistent representation of J_H6 region (43.9% of unmutated cases and 63% of J_H6 total cases), and the mutated group showing a major expression of J_H4 and J_H5 (62.1% and 73.9% of J_H4 and J_H5 total cases, respectively). Again, V_H, D, and J_H genes usage in the LM-CLL group

Table 2. Representation of V_H Family Genes in all 163 Cases Analyzed and in Mutated and Unmutated IgV_H Groups

V _H family gene	All cases*	Mutated IgV _H group†	Unmutated IgV _H group†
V _H 1	33 (20.2%)	4 (4.3%)	29 (42.0%)
V _H 3	89 (54.6%)	61 (64.9%)	28 (40.6%)
V _H 4	36 (22.1%)	27 (28.7%)	9 (13.0%)
V _H 5	2 (1.2%)	1 (1.1%)	1 (1.4%)
V _H 6	2 (1.2%)	0 (0%)	2 (2.9%)
V _H 7	1 (0.6%)	1 (1.1%)	0 (0%)
Total	163	94 (57.7%)*	69 (42.3%)*

Pearson χ^2 test revealed a statistically significant difference in distribution of V_H family genes between groups with $P < 0.0001$.

*Number of cases (percent total 163 cases).

†Number of cases (percent inside group).

Figure 2. Differential CDR3 length between three groups of B-CLL patients with different mutational rate in IgV_H region expressed by leukemic population. The **horizontal line** inside **boxes** indicates the median value. LM-CLL patients have a percentage of mutations ranging from 2 to 5%, HM-CLL patients have a percentage of mutations greater than or equal to 5%, and UM-CLL patients have mutations in a percentage inferior to 2%. A statistically significant difference is shown between M-CLL (LM-CLL + HM-CLL) and UM-CLL groups using a nonparametric Mann-Whitney test ($P < 0.0001$).

was not dissimilar to the patients with a percentage of mutation greater than 5%.

Complementary Determining Region 3 Size

The median CDR3 length in all patients analyzed was of 15 amino acids. A comparison of CDR3 region size between the mutated and unmutated CLL populations was performed: CLL mutated group had a significantly shorter CDR3 region than the group with unmutated V_H genes (13.2 versus 17.3 amino acids, respectively; $P < 0.0001$). Finally, CDR3 length in the LM-CLL group was not dissimilar to the patients with a percentage of mutation greater than 5% (Figure 2).

Median Survival Data

Survival curves were plotted according to the Kaplan-Meier method. The impact of IgV_H mutational status on the survival rate is shown in Figure 3. The median survival of patients with mutated IgV_H genes was 206 months, whereas patients with unmutated IgV_H genes had a median survival of 127 months, which shows that patients with a percentage of somatic mutation inferior to 2% in the FR2-CDR3 segment analyzed have a worse prognosis ($P = 0.0023$). Moreover, because in this study we decided to determine the nucleotide sequence of fragments representative of only a little more than one-half of

Figure 3. Kaplan-Meier survival curve comparing 146 CLL patients with mutated and unmutated V_H gene (homology level cutoff = 98%). Median survival for 63 unmutated CLL, 127 months; median survival for 83 mutated CLL, 206 months. The difference is significant at the $P = 0.0023$ level (log-rank test).

the whole IgV_H sequence, overall survival analysis was performed using different cutoffs harboring 96 to 98.5% homology rate with germ line sequence. Comparing Kaplan-Meier overall survival curves, the better value able to separate CLL patients into good or poor prognosis groups remained the classical 98% homology cutoff (Figure 4).

Figure 4. Log-rank statistics were performed for the V_H homology rate to test for a possible cutoff value separating two groups with different survival distributions. V_H germ line homology rate from 96 to 98.5% is shown on the x axes. Corresponding P values calculated by log-rank test for the distinction of two groups with different survival probability are shown on the y axes. Classical 98% homology rate is able to best separate prognostic groups between B-CLL patients.

Comparison of Immunoglobulin Mutational Status Determined by Partial V_H FR2 and V_H Family-Specific PCR

To confirm the data obtained, 41 cases were subjected to nucleotide sequence analysis using amplification fragments representative of the entire neoplastic IgV_H sequence obtained by V_H family-specific leader region primers.³³ As expected, some differences in the rate of mutations between the complete V_H sequence obtained by V_H family-specific leader primers and the partial V_H sequence obtained by FR2 degenerate primer in the same patient were detected (median gap, 0.3%; range, 0 to 3.8%), in particular in mutated CLL cases (Figure 5A). In a high number of cases (30 of 41 cases), the short V_H FR2 sequence presented a higher percentage of IgV_H mutations than the complete IgV_H sequence. However, all cases were assigned to the same prognostic group (mutated and unmutated CLL) by both methods. Moreover, a large group of CLL IgV_H sequences (set 1, $n = 40$;¹⁸ set 2, $n = 40$ ³⁴) were obtained from the literature and analyzed comparing the percentage of somatic mutations between complete and partial IgV_H sequences. Similarly, we found slight differences (median gap, 0.85%; range, 0 to 3.7% in set 1 and median gap, 0.4%; range, 0.1 to 3.4% in set 2) (Figure 5B), but no misclassifications of CLL patients were observed.

Discussion

B-CLL was historically believed to represent a leukemic transformation of naïve B lymphocytes that had not been exposed to an antigen, had not taken part of the germinal center reaction, and as consequence, did not carry somatic mutations in their IgV_H-rearranged sequences.^{35–37} Several studies have now demonstrated that more than half of CLL cases exhibit somatic mutations in the immunoglobulin genes expressed by the leukemic cellular clones, suggesting the presence of two types of disease arising, either from the postgerminal center memory cells or from naïve B cells.^{1,2,34,38} Further studies by microarray expression analysis showed that CLL must still be considered a unique pathology, of which expression pattern is very similar to memory B cells, irrespective of the IgV_H mutational status.^{39,40} Although the biological significance of IgV_H gene mutation status in B-CLL is substantially unclear, division of CLL into two subsets, with and without mutations of the IgV_H genes, has important prognostic significance. In 1999, two studies simultaneously reported the prognostic role of IgV_H mutational status in CLL, observing a shorter median survival of 8 years, in patients with unmutated leukemic cells, compared to somatically mutated ones, whose survival could exceed 24 years.^{18,19} Subsequently, the prognostic significance of the IgV_H mutational status was confirmed by several publications and it is currently considered the best marker able to predict the disease progression in CLL patients.^{20–22} All these studies were performed analyzing the entire V_H gene sequences, obtained by direct sequencing of fragments amplified using six or seven

Figure 5. Scatter diagram of IgV_H mutation percentage determined analyzing both complete IgV_H sequence obtained by V_H family-specific leader primers (x axes) and partial sequence obtained by FR2 degenerate primer (y axes). **A:** Comparisons were performed in 41 cases of our cohort; median gap in percentage of mutations was 0.3%, ranging from 0 to 3.8%. These two parameters were positively correlated with Spearman's rank order correlation coefficient of 0.96 ($P < 0.0001$). **B:** Comparisons were also performed on 80 cases selected from literature (set 1,¹⁸ $n = 40$, circle; set 2,³⁴ $n = 40$, triangle) considering only the portion of V_H sequence 3' downstream to the annealing site of the FR2 primer. Cases with 100% homology to germ line of the entire V_H sequence were obviously excluded from the analysis. Median gap in percentage of mutations was 0.85% (range, 0 to 3.7%) in set 1 and 0.4% (range, 0.1 to 3.4%) in set 2. These two parameters were positively correlated with Spearman's rank order correlation coefficient of 0.96 ($P < 0.0001$) and 0.95 ($P < 0.0001$) in sets 1 and 2, respectively. The vertical and horizontal lines indicate the mutation cutoff point of 2% commonly used to distinguish B-CLL patients into mutated and unmutated subsets. All cases were assigned to the same prognostic group (mutated and unmutated CLL) by both methods.

different oligonucleotides, each one specifically designed to recognize different V_H family members. Unfortunately, this IgV_H mutational status detection is still con-

sidered cumbersome, expensive, technically difficult, and therefore not widely available for clinical use. For these reasons, a marker closely related to Ig mutational status, able to substitute sequence analysis of IgV_H genes with a more practical and readily available laboratory technique, is required.⁹ Damle and colleagues¹⁹ first observed that >30% of the expression of CD38 on CD5+/CD19+ CLL cells was strongly associated with unmutated V_H genes, whereas all cases with mutated V_H genes had <30% CD38 expression. Many subsequent studies have revealed discordant results (CD38-positive in mutated cases or CD38-negative in unmutated cases) in 28 to 43% of patients.^{23–25} At the moment, the best surrogate marker of Ig mutational status appears to be ZAP70 protein levels ascertained by flow cytometry.^{27,28} Nevertheless, ZAP70 expression does not always correlate with the mutational status of Ig genes; in fact it does not correlate with more than 90% of cases.²⁹ Very recently, Rassenti and colleagues³⁰ better defined the correlation between Ig mutational status and ZAP70 expression levels. They showed that ZAP70 was highly expressed in only 71% of UM-CLL cases and in 17% of M-CLL cases with an overall concordance of 77%. In this series, ZAP70 was revealed to be a prognostic marker that appeared to be independent of the IgH mutational status, identifying CLL cases that started therapy shortly after diagnosis. Moreover, IgH status still identified different prognostic subgroups among ZAP70-negative patients. As a consequence, the assessment of the mutational status of Ig heavy chain genes still maintains its biological discriminative capacity able to distinguish between high- or low-risk CLL patients.

In this study we describe a simple and rapid method to identify the mutational status as well as the V_H, D, J_H usage in the CLL samples. The method is based on the single degenerate FR2-specific primer, that can be used in both PCR and sequencing reactions. FR2 primer is the classic degenerate and widely used primer for diagnostic purposes, specific for the FR2 region, initially used by De Re and colleagues.⁴¹ It consists of a 20-mer degenerate primer in which differences are present in six nucleotide positions leading to a mix of 128 different oligonucleotides. Using this primer coupled with a single primer recognizing a consensus sequence present in all six J_H segments, a monoclonal band was obtained in the great majority of CLL patients (85.8%). The clonality detection rate of V_H FR2 consensus primer was previously reported between 58% and 85% in B-cell malignancies.^{41–46} Most studies, using PCR assay to detect clonality, examined heterogeneous collections of B-cell neoplasms, and thus did not specify the actual monoclonality detection rates in the different subtypes of B-cell lymphoma or leukemia. The sensitivity of PCR assay can vary among different categories of B-cell malignancies due to a different rate of somatic mutations and to preferential IgV_H usage; in particular B-CLL seems more amenable to PCR clonality detection than other B-cell neoplasms. In a study in which diagnostic B-cell malignancy categories were specified, albeit composed of relatively few CLL samples (*n* = 21), a clonal detection rate of 81% in B-CLL cases was shown, using V_H FR2 primer.⁴⁷ Therefore, in this case the

FR2 assay sensitivity appears to be in accordance with our results obtained on a larger group of CLL patients. Moreover, VDJ rearrangement was assessed using FR2 primer in CLL cases in two other very small series (*n* = 4, *n* = 11) obtaining clonal amplification in all cases;^{44,45} these data probably cannot be considered comparable to our larger group of CLL patients (*n* = 189). We did not consider other consensus, degenerate or not degenerate, specific primers for FR1 or FR3 IgV_H regions suitable for mutational analysis for diagnostic purposes. Although the clonal detection rate of FR3 degenerate primer is considered comparable or even better than FR2 primer, it generates an amplification segment representative of the entire CDR3 region in which a minimal portion of V_H segment is present and not sufficient to determine the V_H mutation rate; on the other hand, the FR1 degenerate primer, although it amplifies the majority of the V_H sequence, does not appear to have a higher sensitivity than FR2.

The amplicons obtained with V_H FR2 consensus PCR can be easily direct sequenced using a commercial kit with an automatic sequencer. All of the equipment used is normally present in molecular diagnostic laboratories. Moreover, despite using oligonucleotides usually considered unsuitable for sequencing reactions, a clear readable sequence was obtained in all V_H-D-J_H amplifiable samples, using the same sense degenerate primer in the sequencing reactions. Analysis of the nucleotide sequences of individual patients obtained from upstream and downstream primers was extremely useful to determine the whole correct sequence.

Here, we demonstrate that the analysis of a shorter portion of the V_H segments enables to distinguish patients into two groups with different survival rates (median survival for unmutated CLL, 127 months; median survival for mutated CLL, 206 months). The unmutated or mutated IgV_H status with different prognosis, is frequently defined using 98% homology to the closest IgV_H germline sequence, based on the observation that nucleotide sequence polymorphisms may lead to 2% difference in the DNA sequence of alleles within the immunoglobulin loci.⁴⁸ Several studies looking at the entire IgV_H coding sequence have used this cutoff value to assign CLL cases to the two subsets. The analysis described in this study was performed on only a portion of the V_H gene sequence excluding the FR1 and CDR1 regions. Because these regions generally carry a minor rate of somatic mutations, a cutoff value of 98% of homology might be unsuitable. For this reason, we evaluated the overall survival of CLL patients using, as a discriminator, different levels of V_H germ line homology, concluding that, also in this case, the better homology cutoff level is still 98%. Moreover, to confirm the data obtained, in 41 cases of our cohort and in 80 cases selected from published studies, the mutational analysis was also performed using nucleotide sequences representative of the entire neoplastic IgV_H gene obtained by V_H family-specific leader region primers. All cases were assigned to the same subsets (mutated and unmutated CLL) by both methods confirming the reliability of a sequence mutation analysis performed on a partial IgV_H gene in CLL patients.

In conclusion, the mutational analysis of IgV_H genes for diagnostic purposes, usually performed by seven PCR reactions using a set of sense primers specific for the leader family sequences, can be obtained in the great majority of patients by a single PCR reaction, using a degenerate FR2 primer. The prognostic significance of IgV_H mutational status remained unchanged considering the classical V_H homology cutoff value of 98%. Therefore, to detect immunoglobulin mutational status in CLL patients a sequential strategy is suggested: an approach with a single set of primers and partial IgV_H gene sequencing can be attempted first; if a monoclonal band is not obtained, the sample can be tested with multiple standard primers. Moreover, the method here described, is able to accurately define V_H, D, and J_H usage as well as CDR3 length and nucleotide sequence of immunoglobulin gene expressed by leukemic clone. These data, as in the case of V_H3-21 B-CLL subset, could allow a more accurate risk classification of CLL cases irrespective of the IgV_H mutational status.^{49,50}

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