



Patients with chronic lymphocytic leukemia with mutated V_H genes presenting with Binet stage B or C form a subgroup with a poor outcome

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Background and Objectives. The immunoglobulin V_H gene mutation status is a strong prognostic indicator in B-cell chronic lymphocytic leukemia (CLL), since unmutated V_H genes are correlated with short survival. However, the *traditional* cut-off level dividing mutated and unmutated cases, i.e. more or less than 2% mutations, has been questioned and other cut-offs have been suggested. We investigated whether an alternative cut-off should be applied and the relation of mutational status to another prognostic marker, Binet staging.

Design and Methods. V_H gene mutation status was assessed in 332 CLL cases by polymerase chain reaction amplification and nucleotide sequencing and was further correlated with overall survival using different V_H mutation cut-offs (1-7%) and Binet stage.

Results. After testing different mutation borders, the 2% cut-off remained the best discriminative level for determining prognosis. Interestingly, prognostic stratification was improved by combining the information on V_H gene mutation status with that of Binet stage: unmutated cases (all stages, n=151, mutated cases with stage A (n=77), and mutated cases with stage B or C (n=37) had a median survival of 82, 179 and 74 months, respectively.

Interpretation and Conclusions. CLL cases displaying mutated V_H genes with Binet stage B or C had a survival similar to that of unmutated cases and significantly shorter than that of mutated stage A CLL. Our result reveals clinical heterogeneity within the V_H mutated CLL group by inclusion of Binet stage data, a finding which is of importance when considering surrogate marker(s) for V_H mutation status.

Key words: chronic lymphocytic leukemia, immunoglobulin V_H genes, somatic hypermutation status, Binet stage, prognosis.

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B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia in developed countries and is a clinically heterogeneous disease in that many patients have an indolent course while others succumb rapidly to their disease. Two prognostic scoring systems are currently used to divide the disease in clinical practice, the Rai (stage 0-IV) and Binet (stage A-C) systems,^{1,2} but neither of these is sufficient to predict aggressive disease in early stages of CLL. The finding that immunoglobulin variable heavy chain (IgV_H) genes and their degree of somatic hypermutation could subdivide CLL into two entities with different survivals was an important advance within CLL research, since CLL cases with mutated V_H genes, i.e. >2% somatic mutation of the corresponding germline V_H gene, had a considerably longer overall survival than unmutated (<2% mutation) cases. In the initial reports, the mutated cases had a

median survival of 24 years or a median survival that was not reached, whereas the unmutated cases had an overall survival of 9-10 years.^{3,4} Thereafter, we and others have confirmed the prognostic usefulness of V_H gene mutation status, both in indolent and progressive CLL, which has also proven to be one of the strongest independent predictors of survival in multivariate analysis.⁵⁻¹⁰

Recently, it has been discussed whether the 2% cut-off is appropriate since some groups have reported that a higher cut-off (3 or 5%) improves the discrimination of cases with a poor outcome.^{6,9} Furthermore, we recently showed that patients using V_H³⁻²¹ had a poor survival similar to that of unmutated cases despite the fact that two-thirds of them showed mutated V_H genes.^{8,11} This V_H³⁻²¹⁺ group did not fit the postulated division of CLL into mutated and unmutated cases and we suggested that the V_H³⁻²¹⁺ cases constitute an addi-

tional CLL entity.

In this study, we extended our V_H gene analyses to 332 CLL cases and tested different cut-offs (1-7%). We also studied the relationship of mutational status and Binet stage.

Design and Methods

Patients' material and clinical data

Tumor samples were collected from 332 patients with CLL from the frozen tissue specimen archives at the University Hospitals in Uppsala (n=155), Umeå (n=51), Linköping (n=76) and Huddinge (n=17), Sweden, and in Tampere (n=33), Finland, between 1981 and 2001. Frozen tumor material was mainly obtained from peripheral blood and bone marrow, but also from lymph nodes and spleen in a few cases. Morphology was classified according to the WHO classification and the tumors typically expressed CD5 and CD23 and showed weak Ig expression.¹² CD38 expression was determined in 137 tumors by flow cytometry as described earlier.¹³ The median age at diagnosis was 65 years with a male/female ratio of 2:1. Overall survival was available for all included cases from medical records and local Swedish cancer registries. The median follow-up was 65 months (range, 1-480 months), the median survival 92 months (quartile range, 47-144 months) and the 10-year mortality rate 71%. One hundred and forty-five cases were classified as Binet stage A, 71 as stage B and 49 as stage C.

V_H gene analysis

DNA was prepared using standard protocols and V_H family PCR amplification was performed using consensus V_H/J_H primers as detailed previously.¹⁴ Clonal PCR products were sequenced using a BigDye Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer, ABI, Foster City, CA, USA) or a DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Piscataway, NJ, USA) and the sequence reactions were analyzed by an automated DNA sequencer (ABI377, Applied Biosystems, or MegaBACE 500 DNA Analysis System, Amersham Biosciences). Sequences were aligned to Ig sequences in the GenBank, V-Base and IMGT databases.

Statistical analyses

Kaplan-Meier survival analysis, log-rank tests and Cox's proportional hazard analysis were performed using Statistica 6.0 software (Stat Soft Inc, USA). To analyze different mutation borders for prognosis the Youden index was calculated in 1% intervals up to 7% mutation. The sensitivity and specificity were calculated from 10-year mortality rates as described

by Oscier *et al.*⁷ The best cut-off was selected as that having the highest index (Youden index = sensitivity + specificity - 1).

Results

V_H gene mutation status

In this CLL cohort, 390 V_H gene rearrangements were amplified and sequenced in 332 CLL cases, including 52 cases with double and 3 cases with triple rearrangements. Of the 52 cases with double V_H gene rearrangements, 11 cases had two mutated rearrangements (regarded as mutated [2% cut-off]), 32 cases had two unmutated rearrangements (considered unmutated) and nine cases had one unmutated and one mutated rearrangement (judged as mutated). In cases with triple V_H gene rearrangements, two cases showed three mutated rearrangements (regarded as mutated) and one case two unmutated and one mutated rearrangements (judged as mutated). Using the *traditional* 2% mutation cut-off, 141 (42%) cases were considered mutated, with a mean mutation frequency of 5.7% (range 2.1-13%), and 191 (58%) as unmutated; with this cut-off the mutated and unmutated subgroup had a median survival of 122 and 71 months, respectively (log-rank test, $p < 0.001$, Figure 1). The 10-year mortality rate was 86% and 51% in the unmutated and mutated subset, respectively. In the mutated group, 77 (68%), 17 (15%) and 20 (17%) cases were in stage A, B and C, whereas 68 (45%), 54 (36%) and 29 (19%) cases were classified as stage A, B and C in the unmutated group. In accordance with our previous studies,^{8,11} the V_H3-21⁺ cases (36 cases; 25 mutated and 11 unmutated) had a worse outcome with a median overall survival of 85 months.

Outcome in patients showing V_H genes with 2-5% mutation

Considering that Lin *et al.* recently reported poor outcome in CLL cases with 2-5% mutation, we were interested to investigate whether using a mutation interval rather just a single cut-off could provide a better prognostic subdivision than the current 2% definition.⁹ Accordingly, we subdivided the mutated cases into two groups with 2-5% mutation (65 cases) or >5% mutation (76 cases), in addition to the unmutated cases (191 cases); this strategy did indeed improve discrimination of outcome with median survivals of 96, 148 and 71 months, respectively. However, within patients with Binet stage A, this division showed only a trend to discrimination and no significant difference in overall survival was found between stage A cases with 2-5% and >5% mutation ($p = 0.336$). The percentage of stage B/C cases was somewhat higher among cases with 2-5% mutation

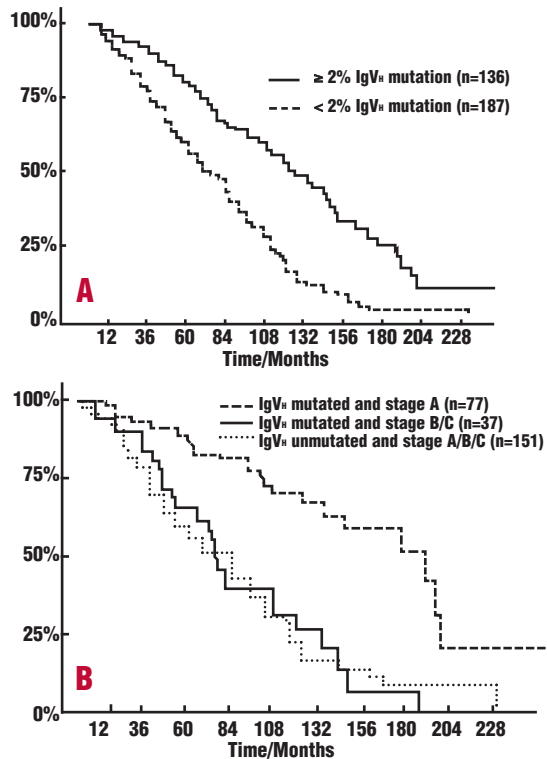


Figure 1. Kaplan Meier survival analysis in our CLL cohort. **A.** CLL with unmutated (n=191) and mutated (n=141) V_H genes using the 2% mutation cut-off (log-rank test, $p < 0.001$). **B.** CLL with unmutated (all stages, n=151) or mutated V_H genes with either stage A (n=77) or stage B/C (n=37) disease. The log-rank test revealed significant differences both when comparing mutated stage A vs. unmutated cases as well as mutated stage A vs mutated stage B/C cases ($p < 0.001$ and $p < 0.001$, respectively).

than cases with $> 5\%$ mutation (38% vs. 27%) and a majority of mutated V_H3-21 cases (22 of 25 mutated cases) was found in the 2-5% mutated group, which probably explains the finding of poor outcome in the 2-5% group when including all cases.

V_H gene mutation status and Binet stage

By combining the V_H gene mutation status and Binet staging data in 265 CLL cases (for which we had both V_H gene and stage data), we found that CLL cases in the V_H mutated group that presented with stage B or C (37 cases, median survival 75 months) had a worse outcome than mutated stage A patients (77 cases, median survival 179 months, Figure 1B), while no difference in overall survival was evident for unmutated stage A vs. stage B/C cases. Hence, the combination of V_H mutation status and Binet stage provided a more accurate predictor of outcome for the V_H mutated group. When examining the effect of

Table 1. Youden index in our cohort of 265 CLL cases.

Mutation cut-off	Youden index, all cases	% mutated, all cases	Youden index, stage A cases	% mutated, stage A cases
1%	37	46	32	57
2%	42	43	38	53
3%	47	37	37	48
4%	42	30	32	37
5%	44	22	32	31
6%	33	18	26	24
7%	28	15	16	18

the poor prognostic V_H3-21 group in the combined division of mutation status and stage the V_H mutated stage B/C cases still showed poor survival compared to the mutated stage A cases when excluding the V_H3-21⁺ cases, 9 of which were mutated and presented with stage B or C.

The best cut-off to determine prognosis in CLL

To define which mutation level best distinguished survival, we calculated the Youden index for different cut-offs ranging from 1% to 7% mutation (with 1% intervals). This analysis revealed that the 3% cut-off gave the highest Youden index in all patients, whereas for stage A cases the 2% border was the best discriminator of poor or good outcome (Table 1). Considering the poor outcome for mutated cases with stage B/C or V_H3-21 usage we believe that this is the explanation for getting a higher index for 3% than 2% in all patients than in the stage A cases. Further analysis of the patients displaying between 2-3% mutations revealed that these are a heterogeneous group comprising both good and poor-risk patients, in whom 6 of 14 cases with stage data presented with stage B/C and 4 cases utilized the V_H3-21 gene whereas 8 cases were in stage A. The median survival for the 2-3% mutation group was 87 months, but stage B/C or V_H3-21⁺ cases had a considerably shorter median survival (54 and 67 months, respectively) than stage A patients in whom the median survival was not reached. Thus, we believe that the 2% cut-off remains the best level for predicting prognosis in CLL.

Multivariate analysis

By employing a univariate Cox regression analysis, V_H gene mutation status combined with Binet stage (using the new division described above), Binet stage only, age, gender and CD38 expression were significant variables. When the material was divided into mutated and unmutated cells, Binet stage was statistically significant by univariate analysis only in the mutated group. Concerning CD38 expression, the

best prognostic division in this cohort of CLL patients was demonstrated using a 20% cut-off; 64 cases showed >20% and 73 cases <20% CD38 expression with median survivals of 67 vs. 93 months, respectively ($p=0.022$, data not shown). In multivariate analysis, age and V_H gene mutation status combined with Binet stage retained their prognostic importance ($p<0.001$ and $p<0.001$, respectively).

Discussion

The definition of the cut-off level of somatic mutation can be employed from at least two aspects: as the best statistical discriminator of outcome in CLL or the biological border between unmutated and mutated V_H genes. The initial cut-off for determining mutated and unmutated V_H genes was empirically set at 2% as a compromise in order to avoid counting polymorphisms as somatic mutations.¹⁵ This level has subsequently been applied by many groups worldwide, who have published their data on V_H gene mutation status and survival and confirmed the best prognostic use of V_H gene analysis at this particular mutation level.^{3,4,7,8} However, some groups have questioned this border and suggested a 3% or 5% level as the best cutoff to separate two subgroups with different clinical outcomes.^{6,14} The true biological demarcation between unmutated and hypermutated CLL is unknown. Since the 2% level corresponds to at least 5 mutations within the V_H gene segment, we and others recently analyzed some CLL cases with a low frequency of mutation (~1-3%) and unequivocally demonstrated that these mutated V_H gene rearrangements carried *true* somatic mutation by sequencing the unrearranged germline genes.^{11,16} Thus, it is likely that CLL cases with even 1-2% mutations have also undergone somatic mutation. However, this procedure is time-consuming and requires cell sorting of non-malignant cells and cannot, therefore, be considered for routine clinical use. Furthermore, the clinical utility is limited since cases with 0.1-2% mutation ($n=44$) show a clinical course similar to cases with germline V_H genes ($n=147$) (median survival 88 and 68 months, respectively, $p=0.052$).

Considering the recent finding of worse survival in 2-5% mutated cases,⁹ we first analyzed the utility of adding a mutation interval to predict survival and showed that low-mutated cases (2-5%) had a shorter survival than high-mutated (>5%) cases. However, when combined with Binet stage data this division of low- and high-mutated cases was not significant in stage A cases. We believe that there are two explanations for this discrepancy; first, the slightly higher

number of B/C cases and, second, a large proportion of mutated V_H3-21 cases in the low-mutated group, both of which are associated with worse outcome. Thereafter, we combined Binet staging with mutation status, which showed that mutated stage B or C cases had significantly poorer survival than mutated stage A cases. Thus, a poor prognostic group was identified among the mutated cases which would have been masked without inclusion of the stage data. This finding is in line with the poorer survival reported for patients with mutated stage B and C CLL in a previous study.¹⁰ While there were no differences in survival in unmutated cases depending on mutational load (germline vs 0.1-2% mutations) or Binet stage, the present study highlights the clinical heterogeneity among V_H mutated CLL cases. However, the biological reason for this heterogeneity within the mutated group is unknown; one possible explanation could be differences in poor risk genetic aberrations such as p53 mutations/deletions for which data were not available in this study.

Expression profiling of mutated and unmutated CLL cases recently revealed different levels of expression of the tyrosine kinase, ZAP-70, which was considerably higher in unmutated cases.¹⁷ The association with V_H gene mutation status was then confirmed in several studies, revealing that ZAP-70 can predict the V_H gene mutation status with a high accuracy in CLL.¹⁸⁻²¹ These findings suggest that ZAP-70 could function as a surrogate marker for V_H gene mutation status in CLL, which would enable much faster and cheaper prediction of prognosis than that afforded by V_H gene analysis. However, in two recent reports 12% and 23% of analyzed cases showed discordant results for ZAP-70/mutation status.^{21,22} Future studies must address the question of whether ZAP-70 expression levels can identify poor prognostic groups such as mutated cases presenting with Binet stage B or C.

We also examined which mutation cut-off is best for predicting outcome in our CLL patients, using V_H gene analysis by calculating the Youden index for different mutation levels (1-7%). When including all patients in the analysis the 3% border gave the highest Youden index, whereas the 2% cut-off had the best predictive value for stage A cases only. Considering that our mutated group contains subsets with more aggressive clinical course, eg. V_H mutated cases presenting with stage B/C and mutated V_H3-21^+ cases, we believe that this explains why the 3% border rendered the highest Youden index. In addition, cases with 2-3% mutations were shown to be a heterogeneous group that consisted of cases with good (stage A patients) and poor outcome (stage B/C and

V_H3-21⁺ cases). Thus, we still consider the 2% border to be the most appropriate to apply in CLL, but that stage data as well as V_H3-21 usage must be taken into account when using the V_H gene mutation status as a prognostic marker.

All in all, we confirm the 2% cut-off as the best discriminator of outcome and also suggest an improved division in CLL based on V_H gene data and Binet staging, which revealed additional prognostic information concerning survival; unmutated CLL with no or few mutations, mutated cases with Binet stage A, and mutated cases with Binet stage B or C. The finding of subgroups with different prognoses among mutated CLL cases highlights the heterogeneity in this group of patients, which may be important when investigating surrogate markers such as ZAP-70 expression levels.

GT and UT participated in the design of the study and analysis and interpretation of V_H gene data; AL, AA, MM and JV participated in the collection, analysis and interpretation of clinical data; KK participated in the collection, analysis and interpretation of clinical as well as V_H gene data; KW and OS participated in the analysis and interpretation of V_H gene data; MH participated in the statistical analysis and interpretation of flow cytometry data; CS and GR participated in the collection of tumor samples and interpretation of data; RR participated in the design of study, interpretation of data and was responsible for the study; GT, UT and RR drafted the manuscript, whereas all other co-authors participated in the critical reviewing of the paper.

All authors contributed to the intellectual content and approved the final version to be published.

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