PROGNOSTIC VALUE OF IMMUNOGLOBULIN VARIABLE HEAVY CHAIN GENE MUTATION STATUS: LONG TERM FOLLOW-UP IN A SERIES OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

Panovska-Stavridis I., Cevreska L., Stojanovic A., Efremov D.

Department of Hematology, Clinical Centre, Medical Faculty, Ss. Cyril and Methodius University, Skopje, R. Macedonia

Abstract: B-cell chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease, with many patients surviving for decades with minimal or no treatment, whereas others succumb rapidly to their disease despite therapy. Classical staging systems and laboratory features help predict survival in CLL, but they do not distinguish patients who will progress from those whose disease will remain indolent. In recent years, new molecular prognostic factors have emerged that have significantly improved prediction of the risk for disease progression. The mutational status of the immunoglobulin variable heavy chain genes (V_H) is one of the major molecular prognostic factors. In this study we evaluated the association between the immunoglobulin V_H gene mutation status and the clinical characteristics and outcome in 65 CLL patients that had been followed for a considerably long period at our institution. At diagnosis, patients with unmutated V_H genes had higher median lymphocyte counts (P = 0.001), higher total tumor mass score (P = 0.001) and more often presented at an advance clinical stage (P = 0.005) compared to patients utilizing mutated V_H genes. Moreover, the median survival of patients with unmutated V_H genes was considerably shorter (V_H unmutated, 56 months, V_H mutated, 125 months; P < 0.001). These data confirmed the prognostic value of immunoglobulin V_H genes mutational status in CLL, which divides the disease in two prognostic subsets in terms of overall survival and clinical characteristics of the disease. Analysis of the mutational status of the immunoglobulin V_H genes may allow for an individualized approach to CLL treatment in the near future.

Key words: chronic lymphocytic leukemia, prognostic factors, Immunoglobulin variable region genes, V_H gene mutation status, survival.
Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia in developed countries. The disease is more common in males than in females, with a median age of diagnosis of approximately 65 years [1].

CLL is characterized by the uncontrolled proliferation and accumulation of monoclonal B cells with the appearance of small mature lymphocytes and with a characteristic immunophenotype. Typically, the leukemic cells express CD19, CD5, CD23, and low levels of membrane IgM, IgD and CD79b, a phenotype of mature, activated B lymphocytes [2–3].

CLL is clinically a heterogeneous disease. Some patients with CLL survive for many years without therapy and eventually die, from unrelated diseases, whereas others have a rapidly fatal disease in spite of aggressive therapy [1–3]. Two prognostic scoring systems are currently used in clinical practice, the Rai (stage 0–4) and Binet (stage A–C) systems. These systems are based on clinical and hematological parameters of the disease. Although effective in classifying CLL patients into broad prognostic subgroups, neither of these staging systems is sufficient to predict the course of the disease in individual patients, particularly to predict aggressive disease in those with early stage CLL [3–5]. However, within the past decade, molecular and genetic studies have provided new insights in the clinical course and biology of the disease. Novel genetic and biological parameters, such as cytogenetic abnormalities, immunoglobulin (Ig) variable heavy chain (V\textsubscript{H}) gene mutation status, and CD38 and ZAP–70 expression, are increasingly being used to predict the risk for disease progression [6–18]. Until now, no single genetic abnormality has been identified in all CLL patients, although a number of aberrations are evident in up to 80% of the cases. The most common aberrations define different prognostic groups, where poor risk groups include cases with deletion (del) 11q (the ATM gene), del 17 (the p53 gene) and trisomy of chromosome 12, while del13q is associated with better prognosis. However, it is important to note that these changes may not be present initially and may only be acquired during disease progression [6–7].

Currently, the mutational status of Ig V\textsubscript{H} genes represents one of the strongest and most important prognostic markers in CLL [3, 10]. Until recently, CLL had been considered as a single entity with a variable clinical course, but analysis of the Ig V\textsubscript{H} gene mutation status by Hamblin et al. and Damle, et al. in 1999 showed that it consists of 2 subsets, distinguished by the presence or absence of somatic mutations [8–9]. The prognosis of the patients belonging to the 2 subsets is markedly different, since cases with mutated V\textsubscript{H} genes show significantly longer survival than unmutated CLL cases [8]. Moreover, these observations showed that CLL cells are derived from antigen experienced B...
lymphocytes that differ in the level of immunoglobulin variable gene mutations and indicate the possible association between the clinical heterogeneity of CLL and the biology of the disease [3, 21–22].

The aim of our study was to determine the immunoglobulin V_H gene sequences in a series of CLL patients with a long follow-up, diagnosed and treated at our institution, and to compare our results with the various clinical characteristics of the patients and their outcome.

Materials and Methods

Patients

Blood samples were collected from 65 consecutive CLL patients that had been diagnosed at our institution, between 1977 and 2000. Diagnosis of CLL was based on standard morphologic and immunophenotypical features according to the World Health Organization classification [23].

All patients were staged at diagnosis according to the Rai classification and have been followed at least 5 years since diagnosis with the longest follow-up of 29 years. Patients were designated as having low or high total tumor mass score (TTM) on the basis of the sum of: the square root of the number of peripheral blood lymphocytes per ml, the diameter of the largest palpable lymph node in centimeters (cm) and the enlargement of the spleen below left costal margin in cm; high TTM was define as a sum equal to or greater than 9.0 [24].

Treatment was initiated for symptomatic or progressive disease. Treated patients received between 1 and 3 lines of therapy. First line therapy consisted of Chlorambucil in 48 patients (73.8%) and Fludarabine in 9 patients (13.8%). Refractory disease was treated with Fludarabine as a single agent or in combination with cyclophosphamide. Eight patients (12.4%) with early stage and stable disease were not treated at all [25].

Identification and sequencing of Ig V_H genes

RNA was isolated using the Trizol reagent from peripheral blood mononuclear cells (PBMC) separated by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden).

The PCR amplification, cloning and sequencing of V_H region genes have been described in detail elsewhere [6]. Briefly, RNA was reverse transcribed using random hexamers and then PCR amplified with a degenerate V_H FWR1 primer in combination with C_μ, C_γ and C_α reverse primers. Samples that failed to amplify with these combinations were amplified with a mixture of forward primers complementary to leader sequences of V_H families 1 to 6. PCR
products were purified with the QIAquick PCR purification kit (Qiagen) and ether sequenced directly or cloned with the PCR cloningplus kit (Qiagen). Sequencing was done with the BigDye Terminator v3.1 Cycle Sequencing kit and ABI 3100 genetic analyzer (Applied Biosystems).

Candidate germline genes were assigned by searching the VBASE directory. Percentage homology was calculated by counting the number of mutations between the 5' end of FR1 and the 3' end of FR3. Sequences with less than 2% differences from germline VH genes were considered unmutated [26].

Statistical analysis
Correlations between the different CLL subsets were made with the use of the t-test, Wilcoxon rank-sum test and Fisher exact test. The median time to treatment and survival were estimated by the method of Kaplan and Meier and assessed by the log-rank test. Data for patients that had not received treatment were regarded as censored. Statistical analyses were performed using the SigmaStat 3.1 program (Systat Software Inc., Richmond, CA, USA) [27].

Results
We studied 65 consecutive patients with CLL that had been diagnosed at our institution between 1977 and 2000. The median age of the patients was 61.4 year (range 29 to 79); 42 (64.6%) were male and 23 (35.4%) were female. At presentation 10 (15.3%) were Rai low risk group, 42 (64.6%) intermediate risk group and 13 (20.1%) high risk group. Analysis of the Ig V H gene sequence showed that it was unmutated in 44 (67.6%), mutated in 14 (21.6%), and could not be determined in 7 (10.8%) cases.

Comparison of the two patient groups stratified according to the V H gene mutation status showed that they do not differ with respect to the age and gender, but median Ly counts (51×10^6/ml for unmutated V H and 29.8×10^6/ml for mutated V H, P = 0.001) and total tumor mass score (TTM) at diagnosis (11.92 for unmutated V H and 6.03 for mutated V H, P = 0.001) showed statistically significant differences. The percentage of cases presenting at an advanced stage (intermediate + high risk) was significantly higher in the unmutated V H (93.18% of cases) than in the subgroup with mutated V H genes (57.14% of cases, P = 0.0048). Also, median time from diagnosis to initial therapy was significantly shorter for unmutated patients, P = 0.001 (Table 1).
Table 1 – Tabela 1

Clinical and laboratory features of CLL patients stratified according to $V_H$ mutational status

<table>
<thead>
<tr>
<th></th>
<th>Unmutated $V_H$ genes</th>
<th>Mutated $V_H$ gene</th>
<th>Unm $V_H$ vs. mutated $V_H$ genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male : Female</td>
<td>34 : 10</td>
<td>8 : 6</td>
<td>$P = \text{n. s.},$ Fisher exact test</td>
</tr>
<tr>
<td>Median age</td>
<td>60 y.</td>
<td>62 y.</td>
<td>$P = \text{n. s.},$ t-test</td>
</tr>
<tr>
<td>Median follow-up (range)</td>
<td>58 months (5 to 109)</td>
<td>77 months (11 to 351)</td>
<td>$P = \text{n. s.},$ t-test</td>
</tr>
<tr>
<td>Median Ly count at dg. ($\times 10^6/ml$)</td>
<td>51.0</td>
<td>29.8</td>
<td>$P = 0.001,$ t-test</td>
</tr>
<tr>
<td>Total Tumor Mass score at dg.</td>
<td>11.92</td>
<td>6.03</td>
<td>$P = 0.001,$ t-test</td>
</tr>
<tr>
<td>Median time from diagnosis to initial therapy (range)</td>
<td>5(1–9) months</td>
<td>64 (1–343) months</td>
<td>$P = 0.001,$ t-test</td>
</tr>
<tr>
<td>Rai stage:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>– low risk</td>
<td>3</td>
<td>6</td>
<td>Unmut. (int. + high risk) versus Mut (int. + high risk) $P = 0.0048,$ Fisher exact test</td>
</tr>
<tr>
<td>– intermediate risk</td>
<td>31</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>– high risk</td>
<td>10</td>
<td>2</td>
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</tbody>
</table>

Survival curves were plotted according to the Kaplan-Meier method. Only 4 patients were censored as lost to follow-up. The graph comparing the survival of the patients with mutated and unmutated $V_H$ genes is plotted in Figure 1. The median survival for patients with unmutated $V_H$ genes was 56 months and for those with mutations 125 months. The difference is very significant ($P < 0.001$) (Figure 1).
**Discussion**

This study confirms previous findings that the presence or absence of somatic mutations in the Ig V\_H genes identifies two disease subsets and thus confers important prognostic information. Our study is especially relevant because of the long follow-up of our patients, considering that only patients that were diagnosed prior to 2000 were included.

The obtained results do not differ for the previous studies regarding the overall survival of the patients, median Ly counts at diagnosis, median TTM at diagnosis, and median time from diagnosis to initial therapy. However, there is marked difference between the initial Rai stage of our patients and patients from the other studies. At presentation most of our patients (93.18%) had advanced stage disease, and most of the patients (> 70%) from the recent studies are in low Rai stage. This difference is not unusual, because the majority of our patients were diagnosed more than 10 years ago, when patients with isolated lymphocytosis were rarely diagnosis as CLL [8–18].

There is also a difference between the percentage of unmutated CLL cases in our study group compared with previously published reports [8], which can again be explained by the fact that in past, the diagnosis of CLL was mainly...
made in patients with clinically overt disease, and cases with unmutated Ig $V_H$ genes, often present with more advanced stages at diagnosis.

In spite of these differences our results confirm that the most important prognostic molecular predictor for CLL is the mutational status of the Ig $V_H$ genes, which divides the disease in two prognostic subsets in terms of overall survival and clinical characteristics of the disease [10–11]. However, sequencing of rearranged $V_H$ genes is rather cumbersome and expensive, and therefore has not been adopted as a routine laboratory investigation. Searches for rapid and technically simple ways to determine the Ig$V_H$ mutational status of individual patients are still proceeding. Moreover, several surrogate markers for the mutational status have been suggested. Initially, CD38 was thought to be a good surrogate, but its measurement gives discordant results in about 30% of cases. Regardless, CD38 still serves as an independent prognostic factor [12–14].

Recently, comparative gene-expression profiling showed that ZAP–70, a tyrosine kinase which is predominantly expressed in $V_H$ umutated CLL cells, can serve as a more promising surrogate for the $V_H$ gene mutational status [15]. Although there is some discordance, the results from several studies show a strong correlation between the $V_H$ gene mutation status and the level of expression of ZAP–70. However, standardization of a valid ZAP–70 protocol is still required, considering that the most current assays show significant interlaboratory variability [16–18].

The reason for the diverse prognosis between the CLL groups with different mutational status is still unknown [3, 10–11]. Several lines of evidence indicate that the survival differences reflect a distinct biological behavior of the leukemic clone [3, 10–11]. First, gene expression profiling of CLL samples using DNA microarrays demonstrated differences in the expression patterns of several hundred genes in independent studies [28]. Many of the differentially expressed genes were also modulated in normal B cells that have been activated by B-cell receptor (BCR) signaling, indicating that BCR-derived signals play a major role in promoting the growth and survival of the malignant clone. This notion is further substantiated by observations that the two subsets differ in their capacity to transmit BCR-derived signal, and by the apparently different antigen reactivity of the BCRs encoded by mutated and unmutated $V_H$ genes. Altogether, these data suggests that the two CLL subsets may rely on different stimuli or different signaling pathway for the growth and survival [3, 10–11, 21, 29–31].

Conclusion

Analysis of the Ig $V_H$ gene mutation status in CLL has resulted in new insights on the biology and the clinical course of the disease. The finding that
CLL consist of 2 clinical subsets, distinguished by the presence or absence of somatic mutations in the Ig variable region genes, has clearly allied prognosis to biology. Nevertheless, the pathogenesis of the disease and the true biological nature of the differing prognosis between the Ig $V_H$ gene unmutated and mutated CLL cases is still relatively unknown and future studies are necessary to be clarified. Although new prognostic markers, such as ZAP–70, are emerging as independent prognostic factors and as surrogate marker for the Ig $V_H$ gene mutation status, the latter still remains the most important prognostic predictor in CLL.

REFERENCES


**Резъме**

**ПРОГНОСТИЧКАТА ВРЕДНОСТ НА МУТАЦИОННИЯТ СТАТУС НА ГЕНИТЕ ЗА ИМУНОГЛОБУЛİNСКИТЕ ВАРИАБИЛНИ ТЕШКИ ЛАНЦI: ДОЛГОГОДИШНА СТУДИЈА НА СЕРИЈА НА ПАЦИЕНТИ СО ХРОНИЧНА ЛИМФОЦИТНА ЛЕУКЕМИЈА**

**Пановска-Ставридис И., Чевреска Л., Стојановиќ А., Ефремов Д.**

**Клиника за хематологија, Клинички центар, Медицински факултет, Универзитет „Св. Кирил и Методиј“, Скопје, Р. Македонија**

Б-клеточната хронична лимфатична левкемија (Б-ХЛП) претставува клинички хетерогена болест; голем број од пациентите преживуваат со децении без да имаат потреба од третман, додека останатите, покрај интензивниот третман многу бргу подлегнуваат на болеста. Класичните прогностички системи за ХЛП и лабораториските карактеристики на болеста овозможуваат да се предвиди вкупното преживување на пациентите со ХЛП, но не и да се одделуваат пациенти со прогресивна болест од оните што...
прогностички фактори кое значајно ја подобруваат
могносот да се предвиди ризикот од прогресија на болеста. Мутациониот
статус на гените за имуноглобулинските варијабилни тешки ланци (VH) е
еден од главните молекуларни прогностички фактори. Во оваа студија е
евалуирана асоциицијата помеѓу мутациониот статус на VH гените и клиничките
карактеристики и исходот од болеста на 65 пациенти со ХЛЛ, кои се
следени подолг период во нашата институција. При поставување на дијаг-
нозата, пациентите со немутирани VH гени имале повисоки средни лимфо-
цитни вредности (P = 0,001), поголема вкупна туморска маса (P = 0,001) и нај-
често биле со клинички напреднала болест (P = 0,005), во споредено со
пациентите со мутирани VH гени. Исто така и средното време на преживу-
вање на пациентите со немутирани VH гени е статистички сиграфикантно
пократко (VH немутирани – 56 месеци, VH мутирани – 125 месеци; P < 0,001).
Нашите резултати ја потврдуваат прогностичката вредност на имуноглобу-
линското VH генски мутационен статус кај ХЛЛ, кој овозможува издојување
на две прогностички подгрупи на ХЛЛ, во погled на вкупното преживување
на пациентите и клиничките карактеристики на болеста. Во блиска идина,
анализирањето на мутациониот статус на имуноглобулинските VH гени ќе
овозможи индивидуализиран тераписки пристап кај секој пациенти со ХЛЛ.

Ключни зборови: hroni-na limfocitna leukemija, prognosti-ki faktori, varijabilniot region na imunoglobuliniske geni, mutacionen status na genite za tejite varijabilni
imunoglobulinski lanci, pre`uvuvawe.

Corresponding Author:
Panovska-Stavridis Irina
Department of Hematology
Faculty of Medicine
Vodnjaska 17
1000 Skopje
Republic of Macedonia
Tel: +389 2 3 147782
Fax: +389 2 3093 610
E-mail: dr_irina@yahoo.com