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ORIGINAL ARTICLE

IGHV MUTATIONAL STATUS IN A COHORT OF BULGARIAN CLL PATIENTS: HIGH UNMUTATED CLL PREVALENCE IN NORTH-EAST BULGARIA

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. One of the best established CLL prognostic markers is the somatic hypermutational status of the *IGHV* gene which is a part of the immunoglobulin heavy chain variable region. Technology for IGHV genotyping has been optimized and has been applied in routine diagnostics for the first time in Bulgaria. A total of 105 patients with CLL from different Bulgarian regions were tested. IGHV mutational status was determined by Sanger sequencing on total genomic DNA (gDNA) or RNA extracted from mononuclear cells. All sequencing profiles were analyzed with the IMGT/V-QUEST tool. Within the course of the analysis a high percentage of IGHV unmutated status was established in the Varna district on the Black Sea (Northeast Bulgaria). In addition, the IGHV genotyping performed on gDNA revealed a rare case with multiple rearrangements. The present data from IGHV genotyping will help in choosing the proper treatment for the benefit of Bulgarian CLL patients.

Keywords: CLL, IGHV mutational status, multiple rearrangements

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and is a remarkably heterogeneous disease. Some of the patients present with an indolent form and never require treatment, while others manifest rapidly progressive disease, despite therapy. One of the best established CLL prognostic markers is the somatic hypermutational status of IGHV gene, which is a part of the immunoglobulin heavy chain variable region [1].

B cell malignancies arise from clonal expansion of a single mature B cell. The rearrangement of immunoglobulin (IG) heavy chain genes is unique for all malignant B cells of a patient and is used as a powerful prognostic marker: IGHV mutational status. The individual IGHV mutational status is examined in the context of its existing prognostic values and the effect it has on personalized CLL therapy [2]. The malignant B cells all have certain B cell receptor (BCRs) signaling, mainly expressing certain IgM and IgD isotypes. An important factor is the existing BCR stereotypy among CLL patients with a possible effect on the disease pathogenesis [3].

The assembly of the variable region of the immunoglobulin heavy chain in the process of formation and maturation of the B-cells represents a chromosomal recombination of V (variable), D (diversity), and J (junctional) segments. The specificity of the antibody is determined by three main complementarity-determining regions (CDRs) and a relatively constant sequence called the framework region (FR). Every V segment encodes the first three framework regions (FR1, FR2 and FR3) along with the CDR1 and CDR2 regions, as well as a part of the CDR3 region. Each D segment covers CDR3 completely. The J segment begins with its own recombination signal and encodes the complete FR4, as well as a part of CDR3 [4,5] (Figure 1).

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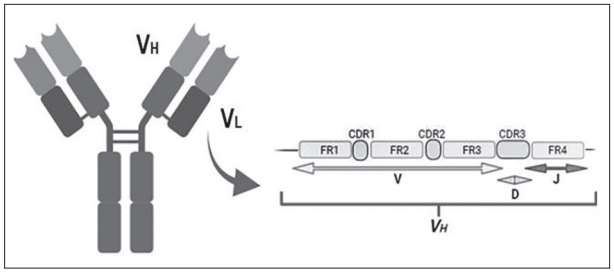

Figure 1. Structure of immunoglobulin heavy chain variable region.
VH – variable region of heavy chain; VL – variable region of light chain; V – variable segment; D – diversity segment; J – junctional segment; CDR – complementarity-determining region; FR – framework region

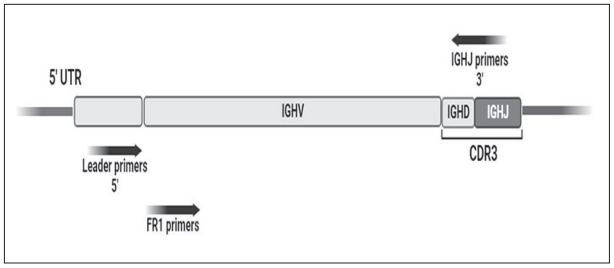

Figure 2. Locations of the primers for V-D-J gene rearrangements.
5' IGHV Leader primers are located upstream of the IGHV coding sequence; 5' IGHV FR1 (framework) primers are located within the rearranged IGHV gene; 3' IGHJ primers are located at the end of the rearranged IGHJ gene; UTR – untranslated region

This present study focusses on the implementation of the IGHV mutational status analysis in a cohort of Bulgarian CLL patients because of the high value of this marker in determining the disease outcome and selecting the appropriate treatment.

MATERIALS AND METHODS

The present prospective study includes a total of 105 newly diagnosed CLL patients (72 males and 33 females) at the ages of 38-80 years from different Bulgarian regions. To the best of our knowledge, all of them have previously tested negative by FISH for 17p13 deletion, and none of them have received treatment prior to IGHV testing. All patient samples were obtained after signing an informed consent form.

Samples collection and cells extraction: IGHV mutational status was determined by PCR/Sanger sequencing on genomic DNA (gDNA) or complementary DNA (cDNA) extracted from venous blood mononuclear cells collected in EDTA tubes. During the whole process of testing, ERIC Recommendations were strictly followed [6].

Mononuclear cells were obtained after density gradient separation by FiColl-Paque PLUS with some in-house modifications: after gradient centrifugation, the mononuclear cell pellet formed between the upper plasma layer and FiColl fraction was dissolved in Dulbecco's Phosphate-Buffered Saline (D-PBS 1x – calcium and magnesium free), instead of Roswell Park Memorial Institute (RPMI 1640 Medium 1x).

Total DNA/RNA was extracted from collected mononuclear cells. The preferred target for us is gDNA because there is no need for a reverse transcription step. High molecular weight gDNA was extracted by QIAGEN - QIAmp® DNA Blood kit following the manufacture's instructions. Total RNA (extracted by ZYMO Research - Quick-RNA Viral kit), followed by cDNA synthesis (SensiFAST cDNA Synthesis Kit) was used only in 9 problematic cases, in which gDNA amplification showed unproductive rearrangements or poor quality of the sequencing profile.

Following ERIC recommendations, the PCR amplification was performed with leader primers in a multiplex reaction. In rare cases (n=3 patients), the amplification of the clonotypic IG rearrangement was successful only by utilization of internal IGHV FR1 (framework) primers (Figure 2). In all 3 cases the amplification with the leader primers failed. Consensus primers targeting the IGHJ genes were used in reverse direction. These results were interpreted with caution, because FR1 primers do not amplify the entire V region [7].

The PCR amplification was performed with primers and protocols, according to ERIC recommendations and BIOMED2 [8,9].

The analysis of the rearranged IG sequences in FASTA format was performed with the IMGT/V-QUEST tool [10]. Cases with ≥98% identity were considered unmutated, while those with a homology less than 98% - mutant type, and cases were considered borderline when the homology is between 97-97.99% [11].

BCR stereotyped subsets were determined by ARResT/AssignSubsets online tool [12, 13].

Statistical analysis for correlation was performed by using Fisher's Exact Test.

RESULTS AND DISCUSSION

Following the 98% identity cut-off value, a total of 57 patients were genotyped as unmutated IGHV (U-CLL), 44 – as mutated (M-CLL), and 4 – as borderline (B-CLL) (Figure 3). Different BCR stereotyped subsets were found in 7 out of 105 cases (6.67%) (Table 1).

According to the published data, the expected ratio of unmutated and mutated cases at diagnosis are 40% vs. 60%, respectively [14,15]. Within the course of the analysis, a high prevalence of unmutated CLL patients was detected in the Varna district on the Black Sea (Northeast Bulgaria).

Table 1. Clinico-biological features and genetic characteristics of mutated and unmutated cases

Patients	Leukocytes count *(4.5 to 10x10⁹/L)**	General condition (Fit)	Treatment	17p deletion	Common IGHV genes	BCR stereotyped subsets (n)
44 mutated	15-180	Good condition	Newly diagnosed; Before first treatment	Negative	IGHV1-2 (4) IGHV3-7 (6) IGHV3-23 (4) IGHV4-34 (4) IGHV4-59 (4)	CLL#77 (1)
57 unmutated	17-328				IGHV1-69 (17) IGHV3-23(4) IGHV5-51 (5)	CLL#2 (1) CLL#5 (1) CLL#6 (2) CLL#8 (1) CLL#99 (1)

* White blood cell (WBC) count range
n – number of patients

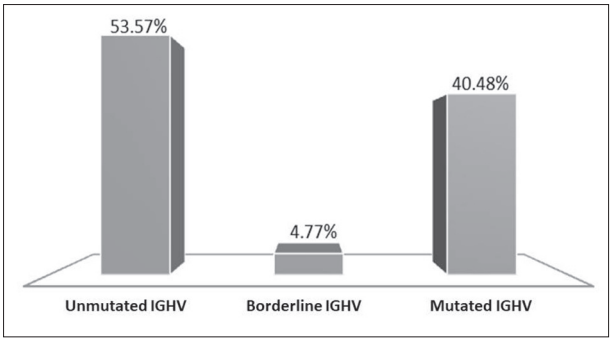

Figure 3. Distribution of Bulgarian CLL patients by their IGHV mutational status.
57 patients – unmutated IGHV; 44 patients – mutated IGHV;
4 patients – Borderline IGHV

From a total of 24 patients from the Varna region, 17 (75%) showed an unmutated status, hence more aggressive CLL, which we hypothesize might be related to the regional industrial activities. For the rest of the 81 patients originating from different regions in Bulgaria, the unmutated patients were 41 (51%). Fisher's Exact Test showed a statistically significant correlation between the region of origin of the patients and their IGHV mutational status (p=0.028, two-tailed Fisher's Exact Test), but this finding might be biased by the small number of patients tested (Table 2).

Furthermore, a difficult to categorize case with multiple rearrangements (triple productive rearrangements) was detected (Table 3). For diagnostic purposes, an analysis was performed on gDNA, and the obtained quality of the sequencing profiles was highly satisfactory, therefore RNA transcripts

Table 2. Fisher's Exact test for correlation between the region of origin of the patients and their IGHV mutational status.

	Origin of samples	
IGHV mutational status	**Varna (N)**	**Others (N)**
U-CLL*	17	41
M-CLL*	5	38
B-CLL*	2	2
two-tailed p value (α = 0.05)		0.028

*U-CLL – unmutated; M-CLL – mutated; B-CLL - borderline

were not tested. This case was interpreted and reported as unmutated, based on the published data showing a shift in favor of unmutated IGHV in a majority of the discordant cases [16]. In such cases, when discordant multiple productive rearrangements were detected, the resulting prognosis is inconclusive, and it is recommended to be considered and treated as a more aggressive unmutated status [16]. Following the current recommendations, cases with discordant multiple rearrangements, should be re-tested after six months [16].

In conclusion, the present data from IGHV genotyping could aid in estimating the disease's course and how to choose optimal initial treatment for Bulgarian CLL patients. Patients with unmutated IGHV CLL tend to relapse earlier due to the more aggressive course of the disease [17,18]. These patients have also demonstrated less benefit from treatment with chemoimmunotherapy and BCL2 inhibitors compared to patients with mutated IGHV, while Bruton Tyrosine Kinase (BTK) inhibitors have the same efficacy irrespective of the IGHV mutational status.

Table 3. Discordant mutational status in case with multiple IGHV rearrangements

IGHV gene	IGHV status	Identity %	CDR3 amino-acid length	Results
IGHV2-70	unmutated	100	19	Unmutated
IGHV3-30	mutated	96,53	16	
IGHV4-4	mutated	91,32	14	

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Conflict of Interest: The authors report there are no competing interests to declare.

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