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Somatic Mutation in Immunoglobulin Gene Variable Region in Patients With Chronic Lymphoid Leukemia and Its Influence on Disease Prognosis

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Abstract

Background: Chronic lymphocytic leukemia (CLL) is a common blood cancer in people aged over 40. In addition to clinical and pathologic staging and blood tests, immunoglobulin variable heavy chain (IgVH) mutation analysis is a relevant prognostic factor for CLL. Finding the most prevalent mutation type and conducting a molecular analysis of immunoglobulin in the majority of the patients can contribute to identifying the disease pattern.
Objectives: In the present study, we used molecular detection methods to find the relationship between clinical and pathologic findings with immunoglobulin heavy chain mutations in CLL patients in Iran.
Patients and Methods: Patients with CLL were randomly selected from patients referred to Imam Khomeini hospital, Tehran, Iran. All patients underwent a clinical staging of the disease and had flow cytometric analysis performed on their blood samples. The panels of cell surface markers used for the diagnosis of chronic lymphoid leukemia include CD19, CD3, CD23, CD10, and CD5. The diagnosis confirmed a minimum of 20% positive expression of dual CD5 and CD19 markers. Genomic DNA was then extracted from the patients' blood and *IgVH* mutation analysis was conducted with pGEM-T (easy vector) cloning kit followed by *IgVH* sequencing.
Results: Study patients were 42 to 80 years old, with their mean age of 62 (SE = 1.87) years. About 73% of them were male. The mean white blood cell (WBC) count, lymphocytes percentage, average hemoglobin level, and platelet count were 56,000/μL, 85%, 12 g/dL, and 150,000/μL, respectively. According to their molecular analysis, 38.9% of patients were unmutated and 61.1% showed mutation in the variable heavy chain locus. The most common mutation had occurred in *IgVH3* allele (66.66%). The mean overall survival rate of patients, mutated and unmutated, was, respectively, 39 (95% CI, 32 to 46) and 31 (95% CI, 26 to 36) months (P = 0.4). Binet stage had statistically significant relationship with patients' survival (P = 0.02).
Conclusions: According to this study, *IgVH3* mutation was found to be prevalent (Although a correlation was found to exist between the patients' survival and *IgVH* mutation, it was not statistically significant). We can conclude that clinical methods are still valuable to predict the prognosis of patients with CLL. Given the high cost and need for specialized laboratory, determining the cost and value of examining immunoglobulin heavy chain mutations and types of mutation such as *IgVH3* are necessary in further studies.

Keywords: Chronic Lymphocytic Leukemia, Somatic Hypermutation, Prognostic Factor, Overall Survival, *Igvh* Mutation

1. Background

Chronic lymphocytic leukemia (CLL) is a common blood cancer in people aged over 40 (1). Its prevalence in the Western world, particularly across Europe, North America, and Australia, is 40% of all leukemia cases in people over 65. The prevalence of this cancer is reported to be lower in Asia and is rare in India, China, and Japan (2-5). According to the morphological evaluations, the dominant cancer cells in this type of leukemia are small mature B lymphocytes found in the blood, which exist in greater numbers in the bone marrow, lymph nodes, and spleen compared with normal cells. Most tumor cells spread through the lymphoid organs and blood circulation. As the disease progresses, the lymphoid organs and the lymph nodes gradually enlarge. This clinical symptom

is significant for determining the stage and grade of the cancer (6). Malignant cells presenting themselves in patients with CLL have certain markers that distinguish them from different types of lymphomas (7). The main cause of this disease is still unknown, but like many other cancers, it is greatly affected by genetic factors like chromosome abnormalities and genetic mutations as well as environmental factors such as radiation, magnetic field, viral factors, and chemicals such as insecticides and toxins (8, 9).

Immunophenotypic markers of CLL consist of B cell populations increase with the markers of CD19, CD5, and CD23; absence of FMC-7 marker; and low expression of CD22, CD79b, and surface immunoglobulin (10). Approximately 99% of tumor cells are in the G0 or the beginning of the G1 phase of the cell cycle; therefore, it is difficult to conduct cytogenetic tests on these cells in the S phase (11).

In addition to clinical markers based on the appropriate classification systems, detection of different molecular markers are of great value for the prognosis of the disease. Molecular markers consist of determining mutation in the variable region of immunoglobulin heavy chain (*IgVH*), Fluorescent in situ hybridization (FISH), cytogenetic analysis, and measuring ZAP70 and CD38 protein expression (12, 13).

CLL is a heterogeneous disease in which numerous genetic factors are involved. For example, the mean survival rate in groups with chromosomal deletion (which deletions) ranges between 32 and 133 months (14). The diagnosis and study of these abnormalities is conducted through Array Comparative Genomic Hybridization (aCGH) of the normal and cancer cells as well as through FISH study (15).

In 1999, Hamblin et al. (16) and Damle et al. (17) demonstrated that patients with CLL can be divided into 2 subgroups based on *IgVH* mutation of BB-cell receptor (BCR) of leukemic cells (18). In fact, CLL is called a naive B lymphocyte (a mature lymphocyte which has not yet been exposed to an antigen in lymph nodes). During the pathogenicity process, the naive $CD5^+$ B cells might undergo somatic mutations in *IgVH* upon entering the germinal center of lymph nodes (18, 19). Meanwhile, another group of patients have a population of intact B lymphocytes tumor cells that have not entered the germinal center of the lymph nodes (and are still in the pre-germinal center stage), cells that have not been mutated yet and are thus called *IgVH* unmutated cells (19). The findings of the studies show that prognosis is poorer for patients whose cancer cells have not been somatically mutated.

2. Objectives

This study aimed to determine the frequency of heavy chain mutation in patients with chronic lymphocytic leukemia and examine its relationship with clinical reports and flow cytometric analysis. Through sampling and collecting genomic DNA using the primers defined for *IgVH* mutation, we detected the genomic DNA mutation by PCR and gene sequencing.

3. Patients and Methods

The study population comprised all CLL patients referred to the central clinic and Valiasr center of Imam Khomeini hospital in Tehran from 2009 to 2013. All patients with CLL disease were included and exclusion criteria were having allergic or other inflammatory diseases. The study was approved by local ethics committee.

Upon registering at the hospital and undergoing clinical examinations, those CLL patients who had no record of chemotherapy signed informed written consents for CBC test (peripheral blood cell count) and participation in the research. Next, their blood samples were collected using 2 heparin tubes marked by each patient's confidential code.

3.1. Disease Staging Based on Rai and Binet Systems

The patients were followed up by means of further referrals and periodic visits to the hospital or clinic every 3 or 6 months for at least 3 years after the beginning of the research or their demise. CBC and lactate dehydrogenase (LDH) tests were carried out and analyzed during each examination session.

3.1.1. Flow Cytometry Analysis

Upon lysing the peripheral red blood cells, specific monoclonal antibody conjugated with different fluorescent dyes were used against CD23, CD3, and CD10 markers and a dual dye to distinguish CD19 and CD5 markers as well as control antibodies (Dako, Denmark) (1).

3.1.2. Isolation of Peripheral Mononuclear Blood Cells

Patients PBMC were isolated from 5 mL of the peripheral blood by Ficoll (Lymphodex, Inno-Train, Kronberg, Germany) (1).

3.1.3. Chemotherapy Protocols

Some patients in this study have received anti-cancer chemotherapy protocols such as FC: fludarabine and cyclophosphamide; FCR: fludarabine, cyclophosphamide, and rituximab; and FR: fludarabine and rituximab (1). Table 1 shows the percentages of these patients.

Table 1. Chemotherapy Protocols in Patients With Chronic Lymphocytic Leukemia[a]

Chemotherapy Protocols	No Drug	FC	FCR	Chlorambucil	FR
Patients percentage	60	8	11	6	15

Abbreviations: FC: fludarabine, cyclophosphamide; FCR: fludarabine, cyclophosphamide, rituximab; FR: fludarabine, rituximab.
[a]Values are expressed as percentage.

3.1.4. Freezing PBMC

Ficoll separated PBMCs stained by Trypan blue (SIGMA®, Taufkirchen, Germany) and counted by Neubauer slide and resuspended in the culture medium used for freezing viable cells, which is RPMI-1640 (GIBCO, Life® technology, USA), FBS 10% (GIBCO, Life® technology, USA), and DMSO 10% (SIGMA®, Taufkirchen, Germany). The mononuclear cells were stored at -70°C freezer until all samples were successfully collected (1).

3.1.5. Genomic DNA Extraction

Upon transferring the cells from -70°C freezer to 37°C water bath, they were washed with PBS, and upon centrifuging, DNA was extracted on a plate using Genet Bio Kit (Genet bio, Korea). Concentration of the obtained DNAs was measured using a NanoDrop Thermo (Thermo, Thermo Fisher Scientific, and USA) (1, 20).

3.1.6. IgVH Mutational Analysis

For measuring the *IgVH* mutations, polymerase chain reaction (PCR) was performed by Taq DNA polymerase enzyme using the standard primers defined for *IgVH* gene families (21). The yielded PCR product was run on electrophoresis agar gel and those with a positive band were isolated with a scalpel blade and their DNA content was isolated from the gel using a Qiagen Kit (QIAGEN, USA). The PCR products were ligated into the pGEM®-T vector (Promega, Wisconsin, USA) and cloned in competent bacteria produced by JM109 high efficiency competent cells through fusion and freezing by T4 DNA Ligase. Next, bacterial suspensions were grown on X-gal medium and white colonies were selected and passaged in an LB culture medium containing antibiotics. Finally, the plasmids of the intended colonies were extracted by Plasmid Midi Kit 100 (QIAGEN, USA) and the specific plasmids were sequenced by ABI PRISM 7700 sequence detection system (Applied Biosystems, USA). After the sequences were determined, the gene bank was searched for the acquired sequence (BLAST) and its mutation rate was studied against somatic cells and germ line normal sequences (1)

3.2. List of Primers Used in Heavy Chain Mutation Analysis

3.2.1. Statistical Analysis

Data analysis was done using SPSS 20 (IBM, SPSS, statistics software). Demographic results were compared and studied using the Chi-square test and the survival data was estimated by Kaplan Meier estimator and Log-Rank test.

4. Results

4.1. Clinical Results

The initial sample included 30 patients. Four of them were excluded due to failure to revisit, their personal request, or improper care of the initial sample. Data of the remaining 26 patients were analyzed. There were 7 female and 19 male patients with CLL, with the mean age of 62 (standard error of 1.84) years and the median age of 64 (standard deviation of 9.14) years. The patients were not under any medications up until sampling started, but 10 of them later underwent chemotherapy during the follow-up period. Table 2 shows the medications they received.

4.2. Flow Cytometry Results

According to the cell surface marker screening panel, the CD markers, including CD5, CD10, CD23, CD3, and CD19 were analyzed in the patients' blood samples by flow cytometry to verify their chronic lymphoid leukemia. In this regard, the conformity of the patients to the flow cytometry panel and the mean percentage of the markers were assessed (Table 3).

4.3. IgVH Mutation Analysis Results

A sample of 20 DNAs from 26 patients was sufficient and had good quality for the analysis of IgVH gene mutations. Of 20 samples that were referred to the immunology laboratory of Semnan University of Medical Sciences, 7 received a negative result in the amplification, replication, or the PCR test, and 6 of them were in a heavy chain unmutated status. It was then determined that the most prevalent heavy chain gene pertained to, in descending order, *IgVH3* (66.66%), IgVH4 (16.1%), and IgVH1 (11.11%) alleles, but the least occurrence percentage was related to *IgVH2* gene. Then, the patients were assessed concerning their Rai stage

Table 2. Stages of Chronic Lymphocytic Leukemia Based on Rai and Binet Systems[a]

Staging	Stage 1	Stage 2	Stage 3	Stage 4
Rai	40.7	29.6	14.8	11.11
Binet	62.9	18.5	14.8	-

[a]Values are expressed as percentage.

Table 3. CD Markers Frequencies in Patients With Chronic Lymphocytic Leukemia

CD Markers	CD19	CD23	CD5	CD3	CD10	CD5/CD19
Percentage of patients	76.12 ± 23.172	59.48 ± 26.587	51 ± 28.479	10.72 ± 11.942	0.2 ± 0.5	39.08 ± 26.587

and their *IgVH* mutation status, and it was found that out of 7 cases in an *IgVH* unmutated status, 3 were in stage 1, 2 in stage 2, and 1 in stage 4 of the disease. Table 4 shows the comparison between the patients' *IgVH* genes.

On the follow-up period, 4 patients died. Using Kaplan Meier estimator and Log-Rank test, the mean survival rate of patients based on the presence or absence of *IgVH* gene mutation was estimated as 39 (32 - 46) and 31 (26 - 36) months, respectively (P = 0.407). The correlation between survival rate and *IgVH* gene mutation and Binet staging are shown in Figures 1 and 2.

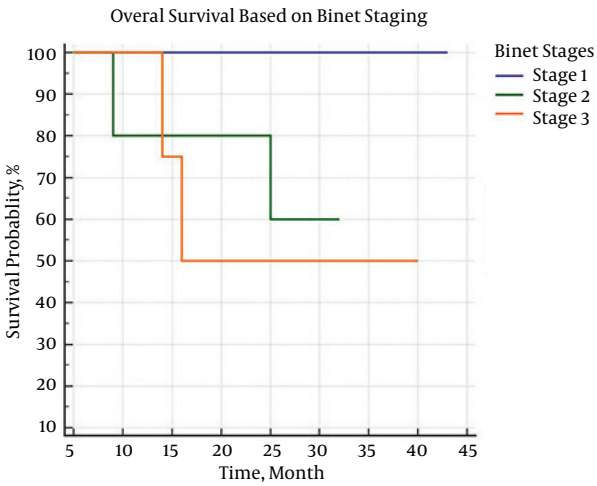

Figure 1. Overall Survival Rate of Patients Based on Binet Staging

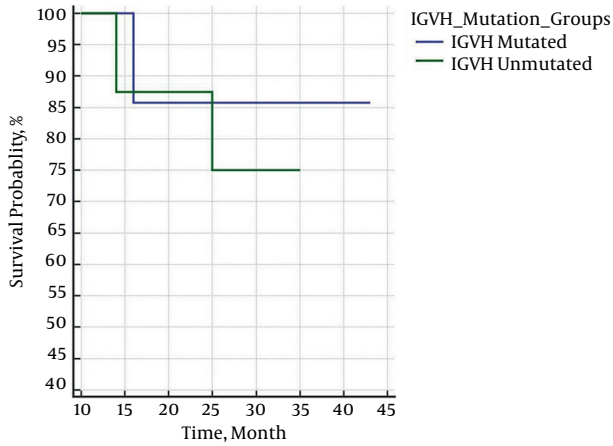

Figure 2. Overall Survival Rate of Patients Based on Mutation Status

5. Discussion

According to the results of the *IgVH* mutation analysis, 66.66% of the patients had mutations in *IgVH3* alleles with different heterogeneities. Based on a similar study conducted in 1998 on Burkitt lymphoma in Iran, the highest percentage of *IgVH* mutations in these patients occurred in *IgVH3*. In the study conducted by Chapman et al. in northwestern Iran, upon accomplishing clinical studies and the other tests, including *IgVH* analysis, mutation pertaining to the special sequences of Burkitt lymphoma was also studied as a complementary test. By comparing the Chapman et al. study and the present research, we can plan a phylogenic study based on the type of *IgVH* involved in malignancies (22). In the study conducted in 2007 on patients with CLL, the *IgVH* of 59 patients were analyzed and the patients' *IgVH* mutation percentages were reported to be 45.8% (*VH3*), 18.6% (*VH1*), 32.2% (*VH4*), 1.7% (*VH6*), and 1.7% (*VH5*). Similar to the findings of other studies discussed, the highest mutation percentage in this study related to *IgVH3* (23). According to the results of the study conducted in 2009 on 87 patients with CLL, *IgVH* mutations pertaining to *IgVH3* were more prevalent (56.4%) while the mutation percentages pertaining to the other types of *IgVH* were

Table 4. *IgVH* Mutation in Patients With Chronic Lymphocytic Leukemia[a]

	Total	*IgVH1*	*IgVH2*	*IgVH3*	*IgVH4*
Mutated	38.90	0.00	5.00	46.00	11.11
Unmutated	61.10	11.00	0.00	22.00	5.00

Abbreviation: IgVH, immunoglobulin variable heavy chain.
[a]Values are expressed as percentage.

20.7% (*IgVH4*), 18.4% (*IgVH1*), 3.4% (*IgVH6*), and 1.1% (*IgVH5*). Similar to other studies, the highest percentage of the *IgVH* unmutated samples in this study (17%) pertained to patients with *IgVH3* mutation.

The prognosis of patients with IgVH3 mutation is reported to be at a critical *IgVH* unmutated status (21). According to the study conducted in 1993, *IgVH3* mutation frequency is also related to factors causing multiple myeloma (24). In a study conducted in 2000 by Gharagozloo et al. on patients with multiple myeloma in Iran, a significant relationship was found between *IgVH3* mutations and disease severity. The frequency of B cell subtypes was also examined concerning the *IgVH* mutation type, and it was found that *IgVH3* is significant in this case (25). Therefore, based on the findings of this study and similar studies in Iran, there is a direct relationship between *IgVH3* and disease severity, and the percentage of patients who have a poor prognosis due to being *IgVH* unmutated is higher in this group. Because of the high cost of *IgVH* mutation analysis, the sampling was performed in a small number of patients, which is a major limitation for statistical analysis.

In both the present and other similar studies, mutation is more prevalent in *IgVH3*. Although the presence or absence of mutation affects the patients' survival rate, this effect might be statistically insignificant because of the small sample size. Nevertheless, there was a significant relationship between survival rate and the clinical stage of the disease in the present study. We can conclude that clinical methods are still valuable in determining the prognosis of patients with CLL. Given the high costs and the need for special laboratory, it is necessary to determine the cost-effectiveness and value of examining *IgVH* mutations and determining the types of mutation, such as *IgVH3*, in more extensive studies.

Limitation of this study was small sample size due to the lack of adequate budget.

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Footnote

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