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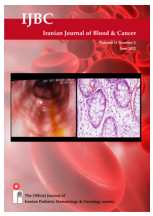

Original article

CD38 gene polymorphisms and genetic susceptibility to chronic lymphocytic leukemia

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Abstract

Introduction: CLL is one of the most common leukemias, which is categorized by the accumulation of mature CD5+ B-lymphocytes in the peripheral blood, bone marrow, and secondary lymphoid organs. In this study, the status of rs6449182 polymorphism of the CD38 gene and its association with clinical and laboratory parameters of CLL patients was evaluated.

Methods: Genomic DNA extraction was performed using the salting out method. The CD38 gene polymorphism (rs6449182) was studied in 70 patients with CLL and 70 healthy individuals using the PCR-RFLP method.

Results: The results of this study showed that the control group had 86% wild-type rs6449182 (CC), 12% heterozygous (CG), and 2% homozygous (GG) genotypes. In the case group, 62% had wild-type genotype (CC) 26% were heterozygous (CG), and 12% were homozygous (GG). Statistical analysis showed that the heterozygous genotype for the CD38 gene was significantly associated with CLL. It was also understood that this polymorphism had a significant relationship with hemoglobin, age, and organomegaly of patients.

Conclusions: The CD38 gene polymorphism of rs6449182 SNP G allele had the highest frequency. Moreover, based on the results, this polymorphism has a significant relationship with organomegaly, which indicates the importance of these markers in the pathogenesis and prognosis of the disease.

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1. Introduction:

Chronic Lymphocytic Leukemia (CLL) is a CD5 + B-cell malignancy that shows the proliferation of mature lymphocytes B. CLL is characterized by the presence of lymphocytes with a monoclonal population of B cells expressing CD19, CD5, and CD23, which also show decreased surface expression of IgM, superficial IgD, and CD79b. The tumor cells accumulate in the blood, bone marrow, spleen, and liver lymph nodes [1]. CLL is the most common leukemia in the adult population of Europe and the United States, which is most common in older adults

and is very rare in children. Specific genomic diagnoses can help the prognosis and making treatment choices [2, 3]. Regardless of the symptoms, most leukemic cells attack the bone marrow and are typical sites for disease recurrence. Recurrent and severe infections such as hypo gammaglobulins occur in at least 60% of CLL patients even in the early stages of the disease. Very variable periods are observed in CLL patients, which are divided into two subgroups. The first group of patients includes people who show an indolent state of the disease and do not need to start treatment. While in the second group, patients suffer from an aggressive state that requires immediate treatment [4]. An important marker for the diagnosis of CLL is the CD38 gene, CD38 is a type 2 membrane glycoprotein expressed by the short arm of chromosome 4. This protein is found on many immune cells in the human body, including leukocytes and Natural killer (NK) cells. The reaction of CD38 to monoclonal antibodies stimulates and differentiates B, T, and NK cells. CD38 acts both as a membrane surface coenzyme and receptor, and its main role is to regulate calcium ions (Ca++). Recently, researchers have addressed the association between CD38 and the pathogenesis of blood cell malignancies. CD38 plays an important role in the proliferation and survival of lymphocytes B [5-7]. Single Nucleotide Polymorphisms (SNPs) are useful in identifying candidate genes involved in disease and phenotypic traits. SNPs are used as a genetic markers in disease-related studies [8]. The CD38 gene has a single-allelic nucleotide polymorphism (SNP). Changes in this polymorphism at the 5' end position and intron 1 are in the genotypic position of 184C> G [rs6449182 184C>G] [9]. Considering that polymorphisms are clinically important and cause unfavorable prognosis in some cases, the aim of this study was to evaluate the importance of allele G in the rs6449182 polymorphism of CD38 gene in determining the prognosis and progression of B-CLL.

2. Materials and methods

2.1 Patient selection

This study was performed by descriptive method. The study population included 70 patients with B-CLL (B-Chronic Lymphocytic Leukemia), including 43 male patients (61%) and 27 female patients (39%) with a mean age of 58.78±10.95 years referred to Shariati Hospital in Tehran. The control subjects included 70 patients (45 men and 25 women) who had been referred to Shariati Hematology-Oncology Center in Tehran. The mean age of control subjects was 58.48±10.88. According to the Rai clinical staging system [10], 43 (61%) patients with CLL are in the advanced clinical stage of Rai (≥stage II), and 27 (39%) patients with a low clinical stage (<stage II). CLL patients with the age range of over 40 years that were able to sign informed consent, whose disease was confirmed by morphological and flow cytometric characteristics (CD5+, CD19+, CD23+) were included in this study. Exclusion criteria for CLL patients were the presence of an infectious, underlying disease (active or previously known hepatitis B or C, HIV positive, cardiopulmonary disease, myocardial infarction, diabetes) or treatment of cancer during the past 5 years. The inclusion criteria for healthy controls were the presence of any active infection and chronic disease, fever, inflammation or underlying disease, pregnancy or lactation, and the use of specific or immunosuppressive drugs. To detect the rs6449182 polymorphism of the CD38 gene the RFLP-PCR (restriction fragment length polymorphism-Polymerase chain reaction) and specific primers designed for this method were used. RFLP-PCR is one of the standard methods for detecting polymorphisms. This method is based on pattern digestion by specific endonucleases, each of which has its own identification and cleavage sites, and this technique is mostly common in the study of chromosomal variants [11, 12]. This study was approved by the ethics committee of Baqiyatallah University of Medical Sciences (Ethical ID: IRIR.BMSU.REC.1398.254) Tehran, Iran. The consent form was signed by all participants participating in the study.

2.2. Isolation of peripheral blood mononuclear cells (PBMC)

About 5-10 ml of blood sample was collected in EDTA anticoagulant tubes from CLL patients and the control group and peripheral blood mononuclear cells were isolated in both patient and control groups using the gradient method and Ficoll-Hypaqe solution (Gibco, UK).

2.3. Genomic DNA extraction

Genomic DNA extraction in these patients was performed by the salting out method. In this method, NaOH buffer (5M) was used for red blood cells lysis and then 10% SDS and 20 µl of proteinase K were

added simultaneously. SDS was added and incubated overnight at 65°C. Saturated salt was then added to the microtube and incubated on ice for 5 minutes. After centrifugation, the supernatant was removed and the tube was dried in the laboratory. Depending on the size of the formed mass, some TE was added to it and placed in the laboratory for 15 minutes so that the precipitate would dissolve. Finally, the obtained product was stored in the freezer at -80° [11].

2.4. Analysis of CD38 gene polymorphism

To perform RFLP- PCR, first using specific primers and temperature program, the CD38 gene fragment containing the polymorphism region related to rs6449182 polymorphism was proliferated and then electrophoresed on an agarose gel to confirm PCR accuracy. The amplified DNA product was then incubated in the presence of restriction enzyme (Pvu II) at 37°C. Finally, the product of enzymatic digestion was electrophoresed on 4% agarose gel and polymorphism was detected by considering the fracture pattern of DNA fragments. The primary product of PCR in individuals with a common allele lacks a cleavage site for the enzyme. However, in individuals carrying the 184 C> G allele, replacement of the C nucleotide with a G causes an enzyme cleavage site in this region and causes the primary product of proliferation with the length of 128 bp to be cut into two pieces 63 and 65 bp under the influence of enzyme. The presence of 63 and 56 bp fragments after the enzyme effect indicates the presence of 184C>G in the patient.

2.5. Statistical analysis

For quantitative data, (numerical values) was presented as the mean ± SD of the statistical difference between the data set and the T-student test. Fisher's exact test was used to estimate the risk from the odds ratio (OR) with a 95% confidence interval (CI). The SPSS16 (Chicago, IL) SPSS software was used for statistical analysis. P<0.05 was considered statistically significant.

3. Results

This study examined the rs6449182 polymorphism of the CD38 gene in 140 samples, including 70 patients with CLL diagnosis and 70 healthy control samples. Clinical parameters and laboratory characteristics of patients with CLL and healthy control groups are given in (**Table 1**). Based on the results of the present study, the frequency of the C allele was 62.8 and 87.1, and that of the G allele was 11.5 and 1.4% in the patient and control groups respectively. There was a significant relationship between the frequency of genotypes and C and G alleles with CLL in the studied population (P<0.001). In the genotypic distribution for healthy persons, 8 samples were heterozygous CG (11.5%) and 61 samples were homozygous CC (87.1%). In the genotypic distribution for patients, 18 samples were heterozygous CT (25.8%), 44 samples were Homozygous CC (62.8%) and 8 sample patients were homozygous GG (11.5%). In individuals with both alleles as heterozygous, the simultaneous presence of uncut 128 bp long fragments and 63 and 65 bp long fragments from enzyme digestion is observed (**Fig. 1**). Performing independent chi-square at a 95% confidence interval showed that the heterozygous genotype for the CD38 gene (rs 6449182) was significantly associated with the CLL disease (P=0.001) (**Table. 2**). Statistical analyses showed that this polymorphism has a significant relationship with the age (**Fig. 2**) and hemoglobin levels (P≤0.001, r = -0.478) (**Fig. 3**). The results of our study showed that organomegaly was significantly higher in rs6449182 CG CLL patients compared to the healthy control group (P≤0.022 r= -0.324).

4. Discussion

In the present study, the frequency of CD38 gene polymorphism in CLL patients and healthy controls and the relationship between polymorphism and several clinical and laboratory parameters were investigated. According to the results, in rs6449182 polymorphism, the control group had 86% wild-type (CC), 12% heterozygous (CG), and 2% homozygous (GG) genotypes. In the case group, 62% had wild-type rs6449182 (CC) 26% were heterozygous (CG), and 12% were homozygous (GG). The frequency of C and G alleles in the patient and control groups was 75%, 25%, 92%, and 8%, respectively. Previous studies have shown that about 99% of tumor cells are in the G0 stage or early G1 stage of the cell cycle. Defects in apoptosis lead to the accumulation of tumor cells and the progress of the disease. Besides the lack of apoptosis, the new findings also indicate the proliferation of tumor cells [13]. The clinical course of the disease varies from person to person. In some people, the condition is stable and does not require treatment, but in other cases, it progresses rapidly and the patients

Table 1. Characteristics of patients with CLL and healthy control groups

Groups	CLL patients N=70	Control N=70	P value
Age	58.78 ± 10.95	58.48 ± 10.88	0.038
Gender (Male/Female)	27-43	25-45	0.839
WBC (10^3/µl)	7.4 ± 0.69	7 ± 1	0.629
Hemoglobin (g/dl)	11.6 ± 2.8	13.7 ± 1.6	0.001
Platlet (10^3/µl)	120 ± 63	221 ± 56	0.347
Organomegaly	1.38 ± 0.03	2 ± 00	0.022
Rai stage	≥II (61%) <II (39%)		

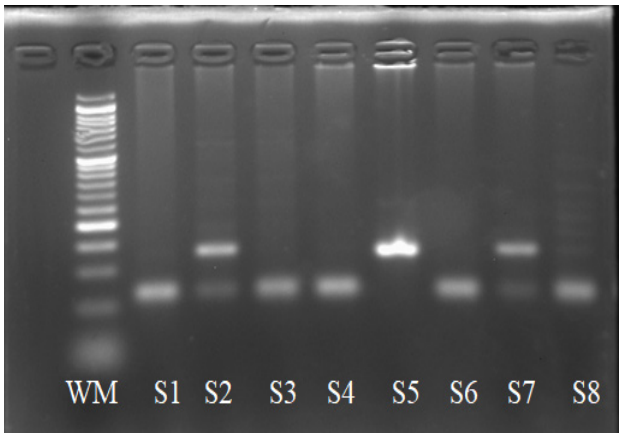

Figure 1: Gel electrophoresis showing CD38 (184 C>G) SNP. WM: DNA marker. S1, S3, S4, S6, S7: homozygous mutant genotype (GG) showing only one band at 128 bp. S5: homozygous genotype (allele without mutation). S2, S8: heterozygous mutant genotype (CG) showing three bands at 128, 65, and 63 bp.

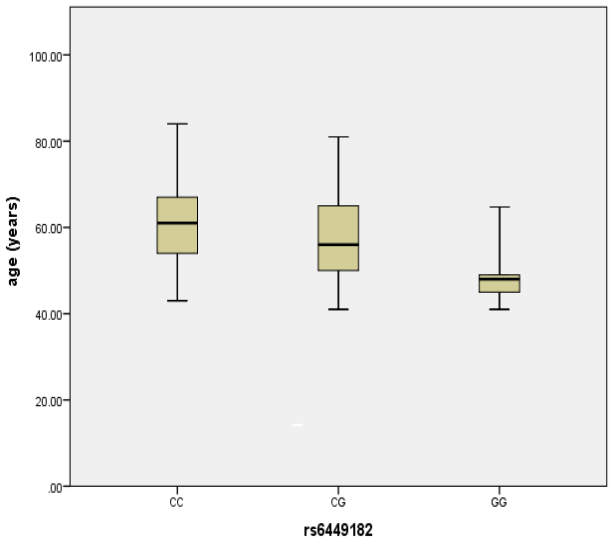

Figure 2: Associations between patients age and CD38 genotype (rs6449182). Results showed that this polymorphism has a significant relationship with the CLL patients age (p≤0.038, r = -0.294)

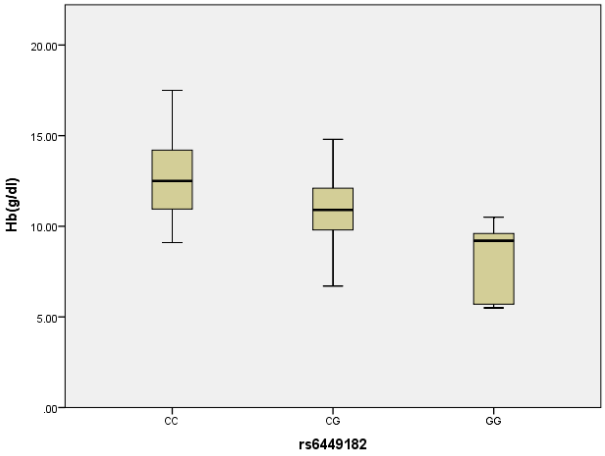

Figure 3: Associations between patients hemoglobin levels (g/dl) and CD38 genotype (rs6449182). There are significant relationship with the patients hemoglobin levels and rs6449182 polymorphism (p≤0.001, r = -0.478)

die despite the treatment. Therefore, the introduction of clinical factors determining the prognosis of the disease is necessary [14]. According to recent results on the importance of CD38 in B-CLL, the polymorphism of molecules involved in the CD38 signaling pathway may be considered determinants of B-CLL affinity [15]. Changes at the 5' end and intron 1 may alter the functional properties of the protein. Interestingly, this SNP was associated with a 50% reduction in CD38 activity as ADP-ribosyl

Table 2. Allele frequencies of study CD38 gene SNP (rs6449182) in B-CLL patients and controls and risk of B-CLL

Genotype	CLL patients N (%)	Healthy control N (%)	P value	OR **	95% CI ***	HWE ****
CC*	44 (62.8)	61 (87.1)	1.00			0.001
CG	18 (25.7)	8 (11.5)	0.015	3.11	1.24-7.81	
GG	8 (11.5)	1 (1.4)	0.01	11.09	1.33-91.91	
Allele						
C	106	130	1.00			
G	34	10	0.001	4.16	1.96-8.83	
Allele frequency (%)	C: 75.7 % G: 24.3 %	C: 92.8 % G: 7.2 %				

Group reference*, odds ratio**, Interval confidence ***, Hardy-Weinberg equilibrium**** (Fisher's exact test)

cyclase and cyclic ADP-ribose hydroxylase under the in vitro conditions [16]. According to statistical analysis, there is a significant relationship between the G allele of polymorphism rs6449182 and people with CLL, organomegaly, lower hemoglobin levels, and younger age. According to the results, this allele is much more common in people with poor response to the treatment, involvement of organs, and lymphatic organs. Several factors are involved in determining the pathogenesis, prognosis, and response to treatment in CLL patients. According to the Rai classification system, there are five stages of CLL patients factors such as the severity of lymphocytosis, lymphadenopathy, hepatomegaly, splenomegaly, anemia, and thrombocytopenia are involved in determining the stage of the disease [17]. Therefore, based on the authors' information, among rs6449182 G allele carriers, the B-CLL spreads more rapidly, has a more significant clinical course, and patient management is easily done with the attitude that the disease is aggressive. Gene and protein expression was increased in B-CLL cells that carry rs6449182 G alleles [18]. Moreover, a recent report showed that in vitro stimulation of B-CLL cells with interleukin-2 resulted in a significant increase in CD38 in cells carrying the rs6449182 G allele compared to the rs6449182 CC (wild) carrier [18]. Therefore, these findings may indicate that the stronger activation of the CD38 pathway increases the risk of B-CLL malignancy in patients. The strengths of the present research included designing a study that confirms the results of this research in a validation study and is consistent with the applied findings of other studies on patients [19]. A study by Krzysztof Jamroziak (2009) showed that the G allele of this polymorphism occurs in younger people [20], which confirms the present study that these people suffer from advanced disease and organomegaly. It should also be emphasized that this study used a selected gene approach in causing the disease and it only focused on SNPs with functional effects. As suggested by previous reports, it is not possible to rule out the possibility that SNPs could be associated with B-CLL risk other than the cases mentioned in the present study. Based on recent findings on the importance of CD38 in B-CLL, it is understood that the polymorphism of other molecules involved in the CD38 signaling pathway may be examined as a determinant of susceptibility to B-CLL disease. However, in combining the clinical, hematological, and molecular results of patients, in some cases, the expected results consistent with the studies of others were not achieved [21, 7]. However, differences in techniques and methods, patients' race, type of treatment, and the number of patients can be reasons for differences. The proven effects of genetic, environmental, nutrition, stress, and behavioral habits on epigenetics on the one hand and living conditions, treatment management on the part of patients and their families, and health conditions in the country, on the other hand, differentiate the patient community of Iran compared to the European patients [22]. However, other studies have been performed to describe invasive B-CLL phenotypes in the rs6449182 G allele carrier,

which are consistent with the findings of this study [23]. The results showed that the presence of rs6449182 polymorphism in the CD38 gene increases the risk of chronic lymphocytic leukemia. The results also showed that this polymorphism was significantly associated with hemoglobin levels and organomegaly. These factors can be used in staging and patient survival. The significant relationship between polymorphism and organomegaly can be considered as one of the factors of poor prognosis in patients. It is suggested to address the mRNA expression level of the CD38 gene in all studied patients regardless of the association with gene polymorphisms.

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Conflict of Interest

The authors report no conflicts of interest.

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Ethical Statement:

The study protocol was reviewed and approved by the ethics committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1398.254).

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