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p53 p.Pro72Arg (rs1042522) and Mouse Double Minute 2 (MDM2) Single-Nucleotide Polymorphism (SNP) 309 Variants and Their Interaction in Chronic Lymphocytic Leukemia(CLL): A Survey in CLL Patients from Western Iran

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ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. The MDM2 and p53 are interacting proteins that play crucial roles in cell biology. Genetic variations of p53 and MDM2 have been identified in many cancers including CLL; among which are SNP309 in the promoter of MDM2 and SNP codon72 in p53.

Materials and Methods: In this study, we sought to find the impact of two SNPs of p53 and MDM2 in the pathogenesis of CLL. A total of 100 CLL patients and 102 healthy controls were recruited. Genomic DNA was extracted, and genotyping was performed using the PCR-RFLP method. The allele and genotype associations were analyzed using the $\chi 2$ test. The gene-gene interaction analysis was studied using GMDR v0.9.

Results: Our study found the absence of a significant difference between CLL patients and controls related to the allelic frequencies or genotypic distributions for both MDM2 SNP309 and p53 codon72. A significantly higher frequency of p53 C allele was found in patients with disease duration of more than 36 compared to those less than 36 months. However, GMDR analysis suggests genetic interaction between the genes under study.

Conclusion: Our findings indicated each polymorphism of p53 codon72 and MDM2 (SNP309) was not a risk factor for CLL but the p53 C allele could be associated with the disease duration. Besides, the interaction between p53/MDM2 genotypes may confer susceptibility to CLL. Our study could be useful in genetic association studies of CLL and the role of gene-gene interactions in the susceptibility to the disease.

Keywords: CLL, p53, Mouse double minute 2 (MDM2), gene polymorphism, gene-gene interaction

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia found in adults in western countries, with an incidence rate of 4-6 per 100,000 people annually. Actually, CLL is a lymphoproliferative disease with clonal expansion of CD5+CD23+ B cells in the marrow, blood, and secondary lymphoid tissues[1].

CLL has a variable clinical course, ranges from indolent to highly progressive disease. This variability may reflect a heterogeneous biological course; for example, CLL patients with cells expressing immunoglobulin (Ig) encoded by mutated Ig heavy-chain variable-region genes (IGHVs) generally have milder disease compared to those carrying unmutated IGHVs, showing a more aggressive disease[2, 3].

CLL International Prognostic Index model with a combination of genetic, biologic, and traditional clinical prognostic variables separates four distinct groups with significantly different prognosis and overall survival. This prognostic model uses five variables of age >65 years, serum β2-microglobulin >3.5 mg/L, clinical stage (Rai stage >I), and IGHV mutational status (unmutated IGHV), TP53 status (del (17p) and/or TP53 mutation deleted and/or mutated)[4].

To date, different genetic alterations have been identified in CLL, some of which can be considered as prognostic factors. These include mutation status of IGHV, as denoted above, or del17p13 that is associated with poor outcomes. However, this prognostic significance may also depend on the proportion of malignant cells with this aberration. In other words, a lower percentage of cells with del17p correlates with a more favorable prognosis. Besides, the presence of del11q is also associated with unfavorable clinical course, extensive lymphadenopathy, and shorter median survival, while del13q14 as a sole abnormality confers favorable outcomes. Trisomy 12 is also a frequent chromosomal aberration that is considered to be an intermediate prognostic factor and is categorized as a neutral prognostic factor according to NCCN guideline [5, 6].

Mutations in the guardian of the genome, p53, occur in almost 50% of all cancers[7]. In CLL, mutations of this gene have been found in about 4–37% of patients[8-10], some of which are associated with poor prognosis [11,12]. A single-nucleotide variation in the p53 which replaces Proline (P) by Arginine (Arg) at codon72, could affect apoptotic functions of the p53 protein. Actually, p.R72R homozygosis can induce apoptosis 15-fold higher compared with the presence of the Proline. On the other hand, the Pro allele is associated with significant induction of G1 arrest.

The murine double minute 2 (MDM2) is an important regulator of p53, it suppresses p53 transcriptional activity through binding to the p53 transactivation domain, it also promotes proteasome-mediated degradation of p53[13]. Genetic variations of MDM2 have been identified in many cancers, including CLL. Among which is a single nucleotide polymorphism in the first intron of core promoter region of MDM2 (IVS1+309). This variant known as SNP309 can affect p53 function through influencing MDM2 transcript and protein levels. Studies on the effects of the MDM2 SNP309 on clinical outcome in CLL have shown conflicting results. The clinical course and response to therapy in CLL patients is heterogeneous; for example, a patient with an indolent form of CLL (stages 0-II) may survive for years without treatment, while stages III-IV, the aggressive form of CLL, may be fatal within a short time period[14].

CLL is still incurable and highly demands robust diagnostic and prognostic indicators that can accurately determine individual risks. Regarding the importance of the p53 pathway in CLL, the interaction of MDM2 with p53 along with the relevance of the p53P72R and MDM2 SNP309 as prognostic markers, which remains arguable; in this study, we aimed to investigate the association of p53P72R (rs1042522) and MDM2 SNP309 with susceptibility to CLL in an ethnic-based manner, in Kermanshah Province of Iran.

MATERIALS AND METHODS

Study population

Totally, 100 (67 males and 33 females) unrelated CLL cases with the mean age of 61.6±11.1 years were recruited from Imam Reza Hospital of Kermanshah University of Medical Sciences, Kermanshah, Iran. All patients were diagnosed according to the guidelines for the diagnosis and treatment of chronic lymphocytic leukemia. The control group consisted of 102 unrelated healthy individuals who had medical checkups at mentioned hospital (72 males and 30 females, p=0.58) with the mean age of 56.7±7.6 years (p<0.001). All subjects signed the written consent forms. The study was approved by ethics committee of Kermanshah University of Medical Sciences and was under the principles of the Declaration of Helsinki II.

DNA extraction and genotyping

About 5 ml of peripheral blood was collected from each sample in EDTA containing tubes, and the DNA was extracted using standard phenol-chloroform protocol[15]. Genotyping was performed using the polymerase chain reaction (PCR)-restriction length polymorphism (RFLP) method. The forward primer of 5'-TCCCCCTTGCCGTCCCAA-3' and the reverse primer of 5'-CGTGCAAGTCACAGACTT-3' were used for amplification of a 279-bp fragment of the p53 codon 72 gene (C>G). The parameters of PCR thermal cycling were: 1 cycle at 94°C for 5 min, 40 cycles by 94°C for 45 sec, 58°C for 45 sec and 72°C for 1 min followed with final extension for 7 min at 72°C. The 279-bp PCR products were digested with BstUI restriction enzyme. In the presence of the C allele (Pro) the 279-bp fragment remained intact. However, in the presence of G allele (Arg) the 279-bp fragment digested to two fragments with 160- and 119-bp [16] (Figure 1). For amplification of MDM2 SNP 309 (T>G) pair of primers 5'-CGGGAGT-TCAGGGTAAAGGT-3' and 5'-AGCAAGTCGGTGCT-TACCTG-3' were used as forward and reverse primers, respectively. The thermal cycler conditions were 94°C for 1 min; 40 cycles by 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and one cycle at 72°C for 10 min. The amplified 352-bp fragment PCR products were digested using one unit of restriction enzyme MspA1I. The TT (233 – and 88-bp), TG (233- and 187- and 88-bp), and GG (187- and 88-bp) genotypes were detected using electrophoresis of digested products on 2.5% agarose gel (Figure 2). Both SNPs were stained with DNA stain [17].

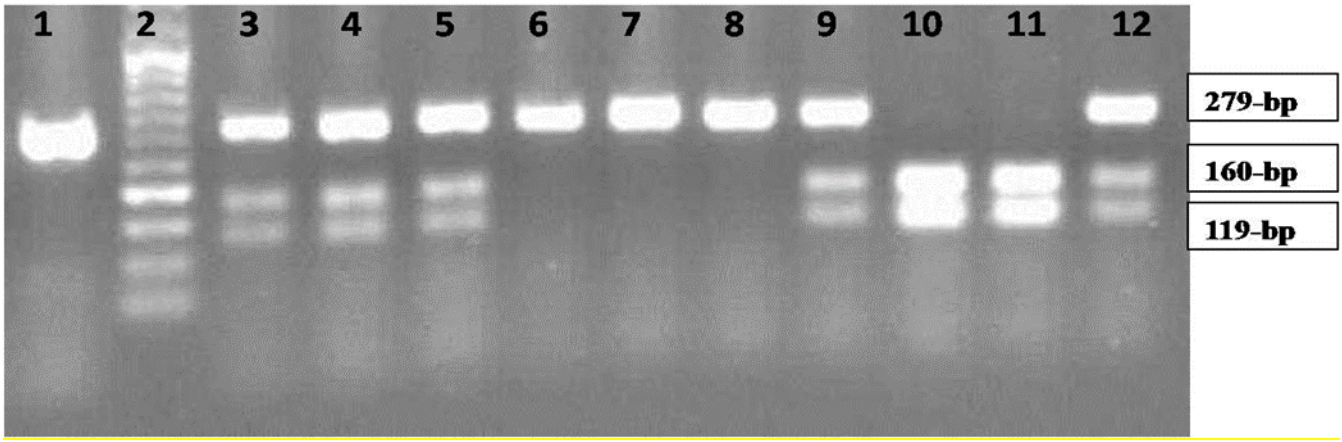

Figure 1. Agarose gel electrophoresis pattern of RFLP products of p53 C>G polymorphism. From left to right, lane 1 depicts undigested PCR-product with 279-bp. Lane 2 is a 50-bp DNA molecular weight marker. Lanes 3-5, and 9&12 demonstrate heterozygous individuals with CG genotype. Lanes 6-8 indicate individuals with CC genotype. Lanes 10-11 show homozygous individuals with GG genotype and lane 9 & 12 is a subject with heterozygous CG genotype.

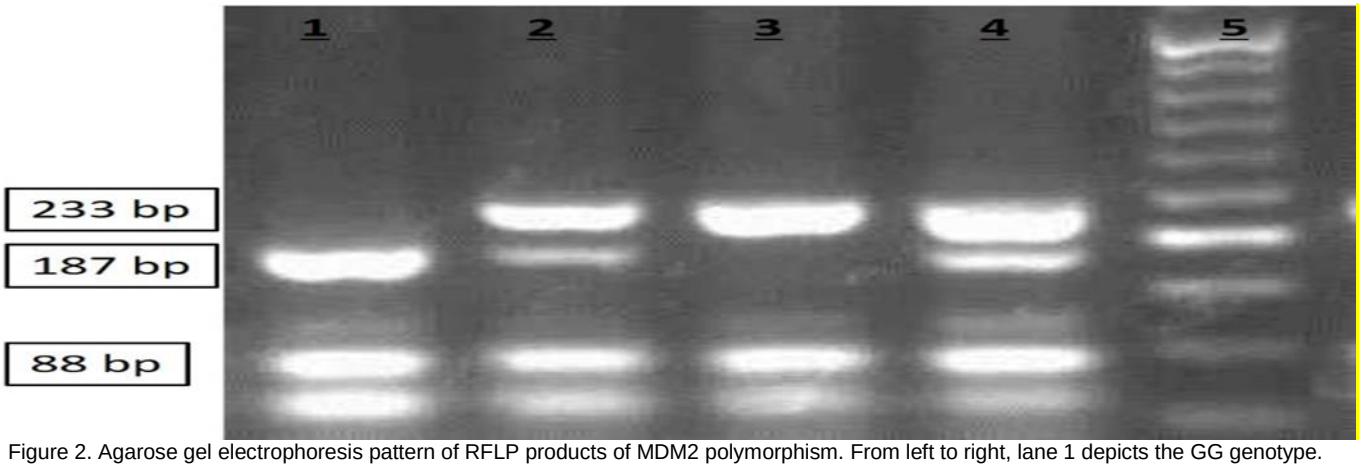

Figure 2. Agarose gel electrophoresis pattern of RFLP products of MDM2 polymorphism. From left to right, lane 1 depicts the GG genotype. Lanes 2 and 4 demonstrate TG genotype. Lane 3 indicates TT genotype. Lane 5 is a 50-bp DNA molecular weight marker.

Statistical analysis

Data were analyzed using SPSS statistical software (SPSS for Windows version 22.0, IBM SPSS Inc., USA). The allelic frequencies of p53 codon72 and MDM2 SNP309 were calculated by the direct counting method; subsequently, the frequency of genotypes and alleles were compared between patients and controls using chi-square test. Also, chi-square test was used to compare the frequency of gender between groups. Using student's T test, the mean age of both groups was compared. The $P<0.05$ level was considered statistically significant.

GMDR Analysis

In this study, we tested the effects of interaction among the two SNPs using Generalized Multifactor Dimensionality Reduction v0.9 (GMDR); an extended version of MDR. Considering two SNPs with 3 corresponding genotypes, it will generate 8 combinations of genotypes, which MDR can reduce this multidimensional set of multi-locus genotypes to one dimension by categorizing them into the 'high' or 'low' risk genotype[18]. In this study, MDR analyses for different SNPs combinations were correlated for the risk of CLL.

Protein-Protein Interaction Analysis

To further evaluate the networking of the genes, the p53 and MDM2 protein interaction studies were performed using GeneMania online tool (http://genemania.org).

RESULTS

The characteristics of the patients are demonstrated in Table 1. Duration of disease was 55.8±34.8 (1-144) months. In this study, the duration of the disease was defined as the average time that the cases had the disease. The available disease stages of 86 patients according to the Rai classification [14] are indicated in Table 1. There were 48 patients (55.8%) with stages 0-II and 38 patients (44.2%) with stages III-IV. No significant difference was found between two groups regarding gender distribution (P=0.58).

Analysis of MDM2 SNP309, revealed the allelic frequencies of 46% for G allele and 54% for T allele among CLL patients and 51% (G) and 49% (T) in controls, which the differences were not statistically significant. In the case of genotypic frequencies, there was no significant difference in genotype distribution among cases and controls (Table 2). The frequency of MDM2 TG genotype was higher (55.3%) in patients with CLL stages of III-IV compared to those with stages 0-II (41.7%, P=0.44). In the presence of GG genotype lower levels of hemoglobin (127.5±20.7 g/L), and lactate dehydrogenase activity (298.5±69.8 U/L) was observed compared to TT genotype [(129.6±15.8 g/L, P=0.26), and (401.7±117.8 U/L, P=0.1), respectively].

In the case of p53 codon72 polymorphism the allelic frequencies were 61% and 39% for C and G alleles, respectively in both cases and controls. Statistical analysis did not show any significant difference in the

frequency of the p53 codon72 polymorphism, allelic and genotypic frequencies between CLL patients and normal controls (Table 2). In patients with duration of disease of more than 36 months a significantly higher frequency of mutant allele of C (90.2%) was detected compared to those with duration of disease of less than 36 month (64.3%, P=0.003). The frequency of p53 CC genotype was higher (52.6%) in patients with CLL stages of III-IV compared to those with stages 0-II (31.3%, P=0.12). Also, in the presence of CC genotype significantly lower WBC count (35±27.1 count/mm^3) was detected compared to GG genotype (68.3±62 count/mm^3, P=0.036). Also, in the presence of CC genotype lower levels of hemoglobin (121.9±22.9 g/L), platelets count (156.3±59.5 count/mm^3) and lactate dehydrogenase activity (364.6±122.2 U/L) was observed compared to CC genotype [(124.3±23 g/L, P=0.28), (199.9±92.9 count/mm^3, P=0.09) and (385.4±128.9 U/L, P=0.7), respectively]. Allelic frequencies and genotype distributions for the two SNPs have been compared between CLL patients and control group that are shown in Table 2. The genotype distributions in patients and control subjects were within the values expected from Hardy-Weinberg equilibrium. Distribution of MDM2 SNP309genotypes was in Hardy-Weinberg equilibrium in patients (χ^2=1.31, p>0.1) and in controls (χ^2=0.01, p>0.1). Also, distribution of p53 codon72 genotypes was in Hardy-Weinberg equilibrium in patients (χ^2=1.38, p>0.1) and in controls (χ^2=0.02, p>0.1).

Table 1: Characteristics of the CLL patients in the present study.

Parameters	Value
Gender	
Male, n	67
Female, n	33
Age (Years) Mean±SD (range)	61.6±11.1 (31-84)
Duration of the diseases (months) Mean±SD (range)	55.8±34.8 (1-144)
WBC (count/mm^3)(range) Mean±SD (range)	43.8±42.5 (2-236)
Platelet (count/mm^3) Mean±SD (range)	173.5±69.9 (60-477)
Hb(g/L) Mean±SD (range)	125±21 (70-170)
LDH (IU/L) Mean±SD (range)	383.3±152.9 (50-980)
Stage	
0, n (%)	25 (29.1)
I, n (%)	15 (17.4)
II, n (%)	8 (9.3)
III, n (%)	21 (24.4)
IV, n (%)	17 (19.8)

Table 2: Allele and genotypic distribution of p53 codon72 and MDM2 SNP309 in CLL patients and controls

SNP	dbSNP	Alleles/ Genotypes	CLL (N=100) N (%)	Controls (N=102) N (%)	OR (95%CI, p)	p
		C	122 (61)	124 (61)	1.0091 (0.6766-1.5049)	0.96
		G	78 (39)	80 (39)		
P53 codon 72	rs1042522	CC	40 (40)	38 (37.3)		
		CG	42 (42)	48 (47.1)	0.77 (0.35-1.72)	0.53
		GG	18 (18)	16 (15.7)	0.96 (0.64-1.45)	0.87
Stage				-		
0-II		CC	15 (31.3)	-		
		CG	24 (50)	-		
		GG	9 (18.8)			0.12
III-IV						
		CC	20 (52.6)	-		
		CG	12 (31.6)	-		
		GG	6 (15.8)	-		
		G	92 (46)	107 (51)	0.82 (0.55-1.2, 0.31)	0.31
		T	108(54)	103 (49)		
MDM2	rs2279744	TT	32 (32)	25 (23.8)		
SNP309		TG	44 (44)	52 (49.5)	0.65 (0.33-1.25, 0.19)	0.19
		GG	24 (24)	28 (26.2)	0.83 (0.57-1.2, 0.34)	0.34
Stage				-		
0-II		TT	16 (33.3)	-		
		TG	20 (41.7)	-		
		GG	12 (25)			0.44
III-IV						
		TT	9 (23.7)	-		
		TG	21 (55.3)	-		
		GG	8 (21.1)	-		

MDR analysis for different SNPs

The summary of gene-gene interaction analysis is depicted in Figure 3. Combinations of the genotypes are categorized as 'high' (darker shaded cells) and 'low' (light shaded cells) risk for the SNPs models. In each cell, positive and negative scores are indicated above the columns. Genotypes combinations are regarded as high risk when the sum of these two values is above zero and low risk when the sum of the score is below zero. For example, combination of SNP309 TG genotype and codon72 GG genotype is considered as high risk because the sum of the score is above zero. Summary of GMDR analysis is also provided in Table 3.

The protein-protein interaction map generated by GENEMANIA demonstrated that p53 and MDM2 have both genetic and physical interactions and are co-localized in the nucleus (Figure 4).

Table 3. The best model of the MDR-multi locus.

Model	Training balanced accuracy	Testing balanced accuracy	CV consistency
[SNP 309]	0.5596	0.5432	10/10
[Codon 72, SNP 309]	0.6047	0.5736	10/10

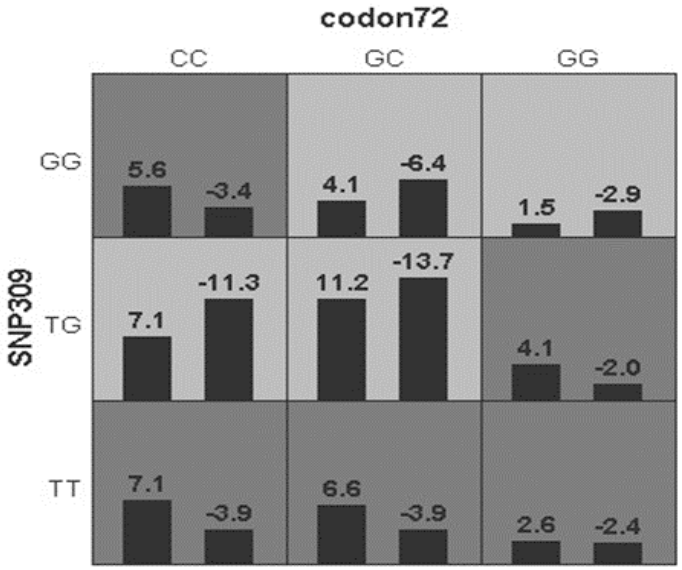

Figure 3. Summary of gene-gene interaction analysis

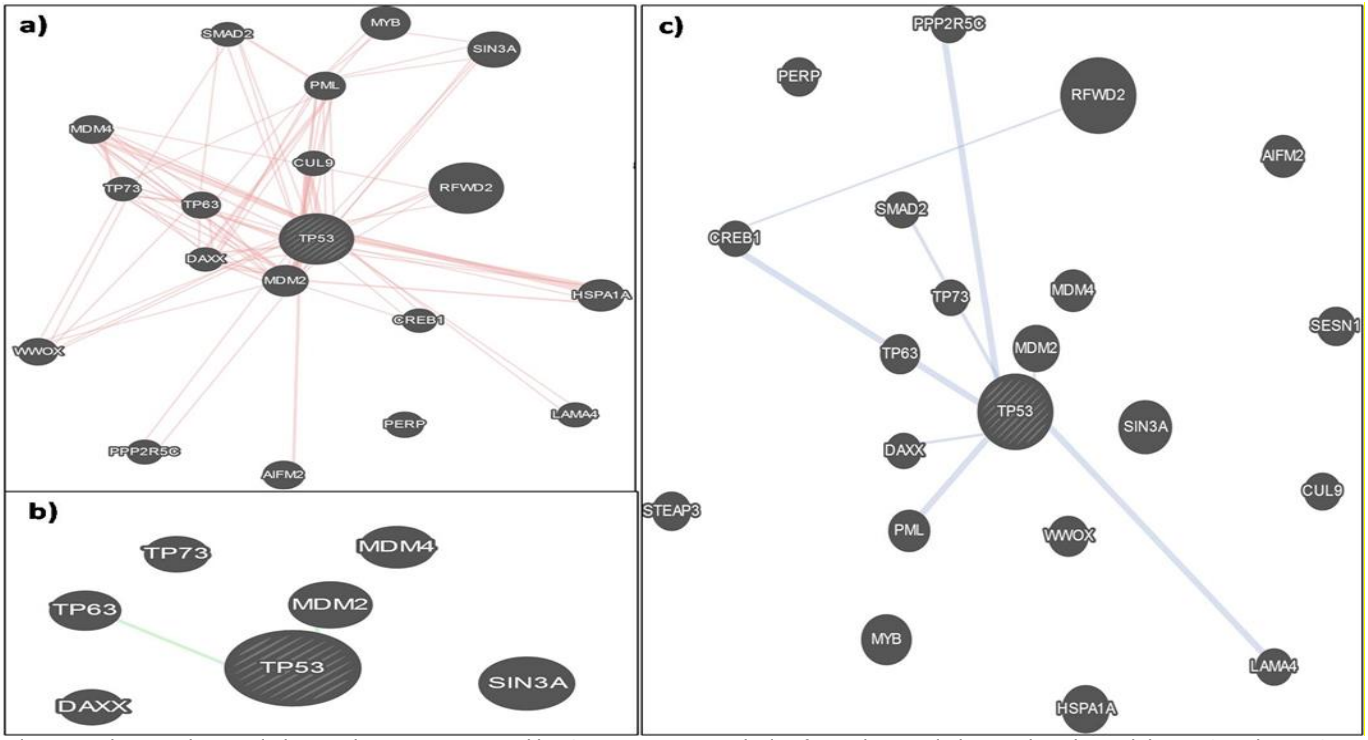

Figure 4. The protein-protein interaction map generated by GENEMANIA. Analysis of protein-protein interaction showed that p53 and MDM2 have both genetic and physical interactions and are co-localized in the nucleus; a) analysis of protein-protein interaction, b) analysis of genetic interaction, c) co-localization of p53 and MDM2.

DISCUSSION

Chronic lymphocytic leukemia is a heterogeneous disorder characterized by progressive accumulation of functionally incompetent lymphocytes. The underlying basis of the disease is not yet fully understood. However, studies suggest a positive family history in almost 10% of CLL patients, which implies the genetic predisposition as an important risk factor for CLL[19]. Various studies have yielded a significant number of SNPs to be involved in susceptibility to CLL; among which are polymorphisms of p53 and MDM2 [20, 21].

The frequency of the p53 codon72 polymorphism was not significantly different comparing CLL patients and controls. However, in patients with disease duration of more than 36 months, the frequency of mutant allele C was significantly higher compared to those with disease duration of fewer than 36 months. Also, in the presence of CC genotype, a significantly lower WBC count was observed compared to GG genotype.

The nucleotide substitution at the p53 codon72 encoding either Arg or Pro can result in an altered biochemical and biological function of p53 in-vitro; where the Arg coding allele appeared to trigger an increased apoptosis response compared to the Pro allele[22]. Accordingly, the clinical significance of this alteration was tested in several studies. There, the Pro allele showed to be associated with thyroid[23], prostate[24], and cutaneous melanoma [25], whereas no significant association was found with the risk of glioma [26], sarcoma [27], and ovarian cancers [28].

Concerning CLL, different studies have questioned the relevance of p53 codon72 on conferring susceptibility to the disease. Sturm et al. examined whether the codon72 allele can affect cell death following administration of γ-irradiation and cytotoxic drugs. This hypothesis, tested on 138 B-CLL patients, failed to reveal any clinical relevance for patient survival and apoptosis induction in B-CLL[29]. In another study, the joint effect of p53 codon72 variant and p53 mutations on CLL prognosis was examined. Based on the results obtained, the Pro/Pro genotype was associated with increased p53 mutations/deletion, yet, it may not significantly influence the clinical response [30]. By contrast, we failed to observe a significant association between the p53 codon72 variant and the risk of CLL. This may add to the controversy of the clinical significance of codon72 on CLL and demands further studies.

The frequency of MDM2 SNP309 polymorphism was not significantly different comparing patients and controls. Although, in the presence of GG genotype, lower levels of hemoglobin and lactate dehydrogenase activity were observed compared to TT genotype, it did not reach a statistically significant level.

MDM2 SNP309 is known to be a poor prognostic factor in CLL[21]. However, there is no general agreement in this regard. Dong et al. genotyped SNP309 in 173 patients affected with CLL and in 260 healthy controls. They detected a possible association between the G allele and the GG genotype with CLL in China [30]. However, in the present study, we did not find a significant difference between the case and control groups for SNP309 frequency. Similar to our findings, Kaderi et al. failed to detect such association in a Swedish population of 418 CLL patients [13].

The interaction between p53 and MDM2 plays a vital role in the biology of the cells. The MDM2 has a key function in the regulation of p53, at least in early development[31]. On the other hand, expression of MDM2 is induced by p53[32]. In other words, these two proteins are linked through an autoregulatory negative feedback loop. In the present study, we aimed to make a comprehensive visual demonstration of different levels of interaction between the two genes as well as other interacting molecules. This sort of analysis is in line with gene-gene interaction analysis using GMDR and emphasizes the need to consider genes and proteins as interacting molecules in pathways. We observed genotypic combinations of p53/MDM2 which possibly confer susceptibility to CLL, where none of the individual genes show to be likely associated with the disease. These findings imply the importance of running gene-gene interaction analysis in genetic association studies. This issue will be more important when it comes to knowing that the impacts of

individual SNPs are often too small to explain the genetic basis of complex disease [32,33]. In this way, it is strongly recommended to search for gene-gene interactions to further elucidate whether they can complement the small effects of SNPs [34].

Briefly, in the present study, our findings could not support a genetic association between polymorphisms of p53 codon72 and MDM2 (SNP309) and the risk of CLL in the Iranian population with Kurdish background. This might be partly due to the small sample size. However, results of GMDR suggest a combination of p53/MDM2 genotypes may confer susceptibility to CLL. To the best of our knowledge, this is the first study that simultaneously evaluates SNPs of p53 and MDM2 and their combination in association with CLL. However, to confirm our findings studies with a larger sample size are required.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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