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Evaluation of *TP53* Codon 72, *P21* Codon 31, and *MDM2* SNP309 Polymorphisms in Iranian Patients with Acute Lymphocytic Leukemia

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Abstract

Background: The tumor suppressing protein p53 and its downstream effector p21 play important roles in cell cycle regulation. Deficiency or deactivation of these proteins as a result of gene alterations has been indicated in several cancers. Such genetic variations could be considered as susceptibility indicators in acute lymphocytic leukemia (ALL). Therefore, we investigated the associations between ALL risk and *TP53* codon 72, *p21* codon 31, and *MDM2* SNP309 polymorphisms in an Iranian population.

Methods: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the *MDM2* T309G (rs2279744), *TP53* codon Arg72Pro (rs1042522), and *p21* Ser31Arg (rs1801270) single nucleotide polymorphisms (SNPs). This study was performed in 115 ALL patients and 115 healthy controls in Khuzestan province in southwest Iran.

Results: In the control group and ALL patients, *p21* Ser/Arg, and *MDM2* TG and GG genotypes were associated with significant 1.81-fold (95% confidence interval CI= 1.008-3.267; $P < 0.05$), 11.07-fold (95% CI= 5.10-24.05; $P < 0.0001$), and 19.41-fold (95% CI= 8.56-43.99; $P < 0.0001$) increased risks for ALL, respectively. The *TP53* 72 Arg allele was significantly more prevalent in ALL patients (56.96%) than in control subjects (47.39%), and was significantly associated with ALL (OR= 1.47; 95% CI = 1.017-2.121, $P < 0.05$).

Conclusions: The *MDM2* T309G and the *p21* Ser31Arg SNPs indicate a significantly increased risk for developing ALL in Khuzestan province.

Keywords: Acute lymphocytic leukemia, Khuzestan, *MDM2*, *TP53*, *p21*.

Introduction

Leukemia is one of the most prevalent cancers, accounting for 3.2% of total cancer deaths worldwide in 2018 (1), with various incidences in different populations (2). In Iran, leukemia was reported to be increasing in recent years (3). Khuzestan province, in particular, has encountered an increase of leukemia incidence and mortality. The prevalence of leukemia in this region was 12.6 and 8.1 per 100,000 person-years in males and females, respectively during 2004-2008 (4), while during this time, the global prevalence of leukemia was reported as 5.8 and 4.3 per 100,000 person-years in males and females, respectively (5).

Leukemia is a heterogeneous group of hematopoietic malignancies broadly classified as acute or chronic according to the clinical course, and sub-classified as lymphoid or myeloid according to the predominant progenitor cell line (6). Acute lymphocytic leukemia (ALL) is a heterogeneous disease of lymphocyte precursor cells or lymphoblasts and is further characterized based on immunophenotyping and cytogenetic and molecular studies. Among adult malignancies, ALL remains a challenge, especially in respect to therapy (7). Approximately 20% of acute leukemias in adults are classified as ALL, and it is the most common

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type of leukemia in children (8).

Cells use several mechanisms to prevent carcinogenesis. Tumor suppressor protein p53 is a transcription factor encoded by the TP53 gene (*TP53*). The gene contains 11 exons and 10 introns and is found on chromosome 17p13. Multiple cellular processes, including maintenance of genome integrity via DNA repair, cell cycle arrest, and apoptotic cell death, are mediated via p53 activation (9). A key downstream effector in the p53 pathway is p21 (CDKN1A) encoded by the p21 gene (*p21*). This gene contains 3 exons and 2 introns, is found on chromosome 6p21.2. p21 is a subclass of cyclin-dependent kinase inhibitors that binds to various cyclin-CDK complexes and arrests the cell cycle at the G1/S and G2/M checkpoints (10). Therefore, alterations in expression or activity due to mutations in the p53 pathway proteins are evident in the development of many of human malignancies (11).

Single nucleotide polymorphisms (SNPs) in the human genome may influence susceptibility to different cancer types, including leukemia (12). Among various *TP53* and *p21* SNPs, functional polymorphisms in codon 72 of *p53* (rs1042522; in exon 4) and codon 31 of *p21* (rs1801270; in exon 2) are the most prominent. The *p53* codon 72 polymorphism, located in the proline-rich domain of the protein, encodes either proline (CCC; 72 Pro) or arginine (CGC; 72 Arg), and consequently changes the activity of p53 in inducing apoptosis (13). The 72 Arg variant increases the ability of p53 to locate mitochondria and induce apoptosis, whereas the 72 Pro allele is more effective in inducing cell cycle arrest in G1 and DNA repair capacity than the 72 Arg variant (14). Also, The *p21* Ser31Arg SNP leads to an amino acid change from serine (31 Ser) to arginine (31 Arg) in the protein's DNA-binding zinc finger domain (15). The polymorphism reduces *p21* transcription (16) and subsequently increases susceptibility to cancer (17). Another important SNP is located on the promoter murine double minute 2 gene (*MDM2*) at position 309. The variant G allele was shown to increase the affinity of the transcriptional activator SP1 resulting in *MDM2* up-regulation and consequent inactivation of p53 (14, 18). Moreover, MDM2 negatively regulates p21 protein stability via proteasome-mediated degradation (19).

As shown in several studies, the *TP53* Arg72Pro, *p21* Ser31Arg, and *MDM2* T309G polymorphisms have been associated with increased tumorigenesis risk (20), but no studies have investigated their effect on ALL in Iranian patients. Therefore, we conducted a case-control study to investigate the possible role of these functional polymorphisms in the p53 pathway on ALL risk in a population in Khuzestan province, Iran.

Materials and methods

Subjects

This case-control study included 115 ALL patients aged 10 to 70 years and 115 healthy similarly-aged individuals as controls. Acute lymphocytic leukemia in patients was confirmed by pathologic examinations.

In this study, after obtaining the sampling permission and the ethics code IR.AJUMS.REC.1395.201 from Ahvaz Jundishapur University of Medical Sciences and completing the patient consent form, samples were obtained from Shafa Hospital patients in Ahvaz, Khuzestan, in southwest Iran, in 2015 and 2016.

Genotyping Assay

Genomic DNA was extracted from leukocytes by the salting-out method using proteinase K, and purified by isopropanol and ethanol precipitation (21). The quality and quantity of the extracted DNA were evaluated by agarose gel electrophoresis and spectrophotometry (Nanodrop, USA).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the *MDM2* 309 (rs2279744), *TP53* codon 72 (rs1042522), and *p21* codon 31 (rs1801270) genotypes. PCR with specific forward (F) and reverse (R) primers (Eurofins Genomics, Ebersberg, Germany) listed in Table 1, was conducted using Ampliqon master mix and 200 ng of genomic DNA. The PCR program included an initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C for 60 sec, 60-68 °C for 45 sec, and 72 °C for 45 sec, with a final extension at 72 °C for 7 min.

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The *MDM2*, *TP53*, and *p21* PCR products of 213, 199, and 221 bp were digested with MspA1I, BstUI, and BlpI enzymes (BioLabs, New England, USA) respectively, at 37°C for 2 h. The digestion fragments were then separated by electrophoresis on 3% agarose gels.

Table 1. Primers and restriction enzymes used to genotype the studied SNPs in the p53 pathway genes

Gene	Primers 5′- 3′	PCR products (bp)	Restriction enzymes	Fragment sizes (bp)
MDM2	F: GCGGGAGTTCAGGGTAAAGGTCACGG R: ACTCCTTTTACTGCAGTTTCGGAACG	213 bp	*Msp*A1I	TT: 213 TG: 213, 163, 50 GG: 50, 163
TP53	F: TTGCCGTCCCAAGCAATGGATGA R: TCTGGGAAGGGACAGAAGATGAC	199 bp	*Bst*UI	CC: 199 CG: 199, 113, 86 GG: 113, 86
p21	F: ACCAGGGCCTTCCTTGTATC R: GTCACCCTCCAGTGGTGTCT	221 bp	*Blp*I	CC: 98, 123 CA: 221, 123, 98 AA: 221

Statistical Analysis

Deviation of the genotype distribution from Hardy-Weinberg equilibrium and differences in allele frequencies between patient and control groups were analyzed by the chi-square test. The odds ratios (ORs) with 95% confidence intervals (CIs) were used to assess the associations between diseases and genotypes. P values < 0.05 were considered statistically significant. The results were analyzed using SPSS software version 17.00 (SPSS Inc, Chicago IL, USA).

Results

MDM2 T309G polymorphism

Genotypes were identified for the *MDM2* 309 polymorphism as TT (213 bp), TG (213, 163, and 50 bp), or GG (50 and 163 bp) (Fig. 1A). The MDM2 rs2279744 polymorphism genotypes, allele frequencies, and odds ratios for both groups are presented in Table 2. The *MDM2* 309 TG and GG genotype frequencies were significantly greater in ALL patients than in controls (42 and 48% vs. 22 and 15%, respectively). The MDM2 309 TG genotype was associated with an 11-fold greater risk for ALL, while the *MDM2* 309 GG genotype was associated with an approximately 19.4-fold greater risk for ALL. The MDM2 309 G allele was also more frequent in ALL patients than in healthy controls (69 vs. 26%) (Table 2). Genotype frequencies in patient and control groups agreed with the Hardy-Weinberg equilibrium. The distribution of the genotypes was significantly different between ALL patients and controls.

TP53 codon 72 polymorphism

The *TP53* codon 72 polymorphisms were identified as homozygous Arg/Arg (GG; 113 and 86 bp) and Pro/Pro genotypes (CC; uncut 199 bp), and heterozygous Pro/Arg genotype (CG, 199, 113, and 86 bp) (Fig. 1B).

The genotypes and allele frequencies of the *TP53* codon 72 polymorphism and their odds ratios in both groups are presented in Table 2. Genotype frequencies in the patient and control groups agreed with the Hardy-Weinberg equilibrium. The genotype distributions were not significantly different between the ALL patients and normal controls.

We also examined the 72 Arg allele frequency between ALL patients and normal controls; a significant association was found between the 72 Arg allele and ALL risk (Table 2). No increased risk was found in ALL patients for Arg/Arg versus Pro/Pro (Table 2). Additionally, in both the patients and controls, subjects with the heterozygous genotype Arg/Pro had no significantly different risk for developing ALL than those with the Pro/Pro genotype (Table 2).

p21 Ser31Arg polymorphism

The *p21* Ser31Arg analysis identified homozygous Ser/Ser (CC; 98 and 123 bp) and Arg/Arg (AA; undigested 221 bp) genotypes, and a heterozygote Ser/Arg genotype (CA; 221, 123, and 98 bp) (Fig. 1 C).

The genotypes and allele frequencies of the *p21* Ser31Arg polymorphism are presented in

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Table 2. The genotype distributions were not significantly different between the ALL patients and normal controls.

We compared *p21* Arg and Ser allele frequencies between ALL patients and normal controls and found a significant association between the 21 Arg allele and ALL risk (Table 2).

In both the ALL patients and normal controls, subjects with the heterozygous CA genotype (Ser/Arg) had a significantly greater risk of developing ALL than those with the CC (Ser/Ser) genotype. Additionally, no significant difference was found between ALL patients with the AA (Arg/Arg) and CC (Ser/Ser) genotypes (Table 2).

Table 2. *MDM2*, *P53*, and *p21* genotypes and alleles, and their genotype-based OR in ALL patients and healthy controls

Genotype	ALL (N= 115) n (%)	Controls (N= 115) n (%)	OR (95% CI)	P value
MDM2 T309G				
TT	12 (10)	72 (63)	1.00 (reference)	-
TG	48 (42)	26 (22)	11.07 (5.10-24.05)	P < 0.0001
GG	55 (48)	17 (15)	19.41 (8.56-43.99)	P < 0.0001
T allele	72 (31)	170 (74)	1.00 (reference)	-
G allele	158 (69)	60 (26)	6.21 (4.14-9.32)	P < 0.0001
P53 Pro72Arg				
Pro/Pro	30 (26)	35 (30.43)	1.00 (reference)	-
Arg/Pro	39 (34)	51 (44.35)	0.892 (0.47-1.695)	0.727
Arg/Arg	46 (40)	29 (25.22)	1.851 (0.944-3.63)	0.072
72 Pro allele	99 (43.04)	121(52.61)	1.00 (reference)	-
72 Arg allele	131 (56.96)	109 (47.39)	1.469 (1.017-2.121)	0.04
P21 Ser31Arg				
Ser/ Ser	54 (46.96)	70 (60.87)	1.00 (reference)	-
Ser/Arg	42 (36.52)	30 (26.09)	1.815 (1.008-3.267)	0.046
Arg/Arg	19 (16.52)	15 (13.04)	1.642 (0.765-3.526)	0.201
31 Ser allele	150 (65.22)	170 (73.91)	1.00 (reference)	-
31 Arg allele	80 (34.78)	60 (26.09)	1.511 (1.013-2.255)	0.043

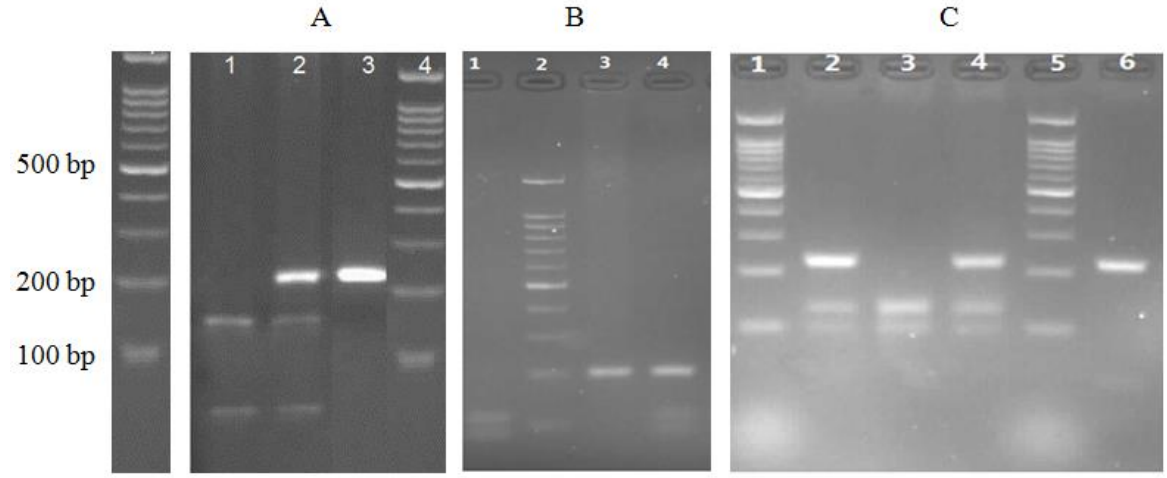

Fig. 1. PCR-RFLP analysis of the polymorphisms by 3% agarose gel electrophoresis. A) *MDM2* T309G polymorphism after digestion with *Msp*A1I; lane 1: GG genotype, lane 2: TG genotype, and lane 3: TT genotype; B) *P53* codon 72 polymorphism after digestion with *Bst*UI; Lane 1: GG genotype, lane 3: CC genotype, lane 4: CG genotype; C) *P21* Ser31Arg polymorphism after restriction digestion with *Blp*I; lanes 2 and 4: CA genotype, lane 3: CC genotype, and lane 6: AA genotype.

Discussion

Determining particular alterations in key genes involved in cell cycle regulation in different populations could aid in prognoses. The MDM2/p53/p21 axis is a main pathway in genomic stability and regulation of cell cycle progression. The SNPs in *MDM2* rs2279744, *p53*

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rs1042522, and *p21* rs1801270, which are involved in the P53 activation pathway, were reported to be associated with leukemia risk. To our knowledge, no studies have been reported investigating the effect of these polymorphisms on susceptibility to leukemia in an Iranian population. The increasing leukemia incidence and mortality in Khuzestan province in recent years motivated us to conduct this case-control study to assess the prevalence and association between these SNPs and ALL risk in this population.

We found significantly increased adult ALL risk associated with the homozygous *MDM2* 309 GG and heterozygous 309 TG genotypes. A previous study in a Chinese population reported significantly increased risk for individuals with the *MDM2* T309G genotype to develop ALL (22)**.** However, another study on Chinese patients reported that the *MDM2* 309 G allele was associated with reduced leukemia risk (23). Despite contrary reports, our results agree with a meta-analysis report, which found that the *MDM2* 309 GG and TG genotypes were significantly associated with increased leukemia risk in an Asian population (24). The *MDM2* 309 G variant allele was found in 69% of ALL patients and 26% of controls, suggesting that this polymorphism may be a marker of genetic susceptibility to ALL in the Iranian population.

No significant association was found between the *TP53* Pro72Arg polymorphism and ALL risk, although a significant association was seen between the *TP53* Arg-encoding allele and ALL. The 72 Arg allele carriers were found to have a 1.47-fold greater risk for ALL than the 72 Pro allele carriers. The *TP53* 72 Pro variant is more efficient in activating DNA repair target genes than the 72 Arg variant (25), and also more efficient in inducing cell-cycle arrest (26); therefore, 72 Pro could prevent the propagation of genome mutations. One previous study reported significantly increased risk for the 72 Arg genotype to develop ALL (26), although a recent meta-analysis found a significant association between *TP53* Arg72Pro polymorphism and ALL risk under the recessive model (27).

In addition, another well-studied sequence polymorphism in the p53 pathway is the *p21* Ser31Arg SNP. This variation results in the loss of p21 expression, which has been implicated in many malignancies (16), although reports conflict regarding associations of the *p21* 31 Arg allele in various cancers (16). Moreover, the allelic frequencies of *p21* 31 Ser and 31 Arg differ greatly among populations (28). Few studies have examined the effect of this polymorphism in ALL (29); however, a previous study conducted in Japan indicated that alterations in *p21* may not be associated with the pathogenesis of childhood T cell ALL (29). Our study found a significant association between the *p21* Ser31Arg SNP and ALL risk; subjects with the heterozygous CA Ser/Arg variant had a 1.81-fold greater risk of developing ALL than those with wild-type CC (Ser/Ser) genotype

In our study, *MDM2* SNP309 was closely associated with ALL. We also demonstrated that the G allele frequency in *MDM2* SNP309 was greater in the ALL patients than in controls, and this might be a prognostic indicator.

These results also suggest that the *p21* Ser31Arg polymorphism might be involved in ALL susceptibility in the Khuzestan province population.

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