



One missense mutation in exon 2 of the *PAX5* gene in Iran

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ABSTRACT. The *PAX5* gene, which encodes the B-cell specific activator protein, is one of the most important factors in determination of B-cell development. This gene is the main target of somatic mutations in acute B lymphoblastic leukemia (B-ALL). For example, point mutations, deletions, as well as other gene rearrangements may lead to several forms of B-cell malignancy. In this study, we obtained 50 blood samples from patients diagnosed with ALL, and screened for *PAX5* mutations using sequencing in exons 1, 2 and 3. We found a heterozygous germline variant, c.113G>A (p.Arg38His), which affects the paired domain of *PAX5*. It seems that this mutation is pathogenic, but is recessive. Our findings suggest that this mutation in a single allele of the *PAX5* gene is not sufficient to cause disease, and it is possible that other alleles are also involved in the onset of B-ALL.

Key words: Acute lymphoblastic leukemia; *PAX5*; Missense mutation; Exon 2; Iran

INTRODUCTION

Leukemia is a group of disorders with heterogeneous characters, which leads to neoplastic transformation during differentiation of blood cell precursors in the bone marrow. The most common leukemic cancer is acute lymphoblastic leukemia, which comprises 75% of all leukemia cases (Pahloosye et al., 2011). B-lineage acute lymphoblastic leukemia (B-ALL), which originates from an early B-cell progenitor, is a common subtype of acute leukemia in children (Sadakane et al., 2007). At the genetic level, B-ALL is determined by a relatively small series of frequent chromosomal alterations, from aneuploidy to chromosomal rearrangements (Campos-Sanchez et al., 2011). The most frequently mutated genetic targets in human lymphoid leukemias are transcription factors, which are essential for blood cell development (O'Neil and Look, 2007). During the transition from common lymphoid progenitors (CLPs) to pro-B cells, commitment to the lineage is dependent on the action of transcription factors such as E2A, EBF1 and PAX5 (Nutt and Kee, 2007). *PAX5* expression in the B-lymphocyte lineage is remarkably stable from the pro-B cell stage until its downregulation in plasma cells (Enver, 1999; Cobaleda et al., 2007; Nutt and Kee, 2007). B cells lacking the transcription factor PAX5 cannot complete all the stages of their differentiation. PAX5 is a paired-domain transcription factor that binds to DNA via the N-terminus, and can both positively and negatively regulate transcription (Enver, 1999; Nutt and Kee, 2007). Analysis of gene expression of wild-type and PAX5 knock-out pro-B cells revealed that 110 and 170 genes are repressed and activated, respectively, by PAX5 and that these genes encode essential proteins that participate in several important cell process. PAX5 regulates expression of these genes through induction or elimination of active chromatin (Revilla-i-Domingo et al., 2012). Importantly, *PAX5* has also been associated with human B-cell tumors. Frequent inactivation of one of the two *PAX5* alleles identified it as a haploinsufficient tumor suppressor gene in B-ALL. Moreover, chromosomal translocation in *PAX5* induces its participation in the development of a subset of B-ALL and non-Hodgkin lymphomas (McManus et al., 2011). Furthermore, direct involvement of *PAX5* in the development of B-ALLs has also been investigated (Campos-Sanchez et al., 2011). Its alteration, including deletions, point mutations, and amplifications, contributes to approximately 30% of B-ALL cases, and chromosomal rearrangements occur in 2-3% of the cases (Campos-Sanchez et al., 2011, Fortschegger et al., 2014). Genomic analysis of leukemic cells from pediatric ALL patients by Mullighan et al. (2007) showed several changes such as point mutations, deletions, and structural rearrangements in the genes which encode regulators for B lymphocyte development. Among those, *PAX5* was the most mutated somatic gene in children affected with B-ALL (31.7%). Familiades et al. (2009) examined adult B-ALL patients, and demonstrated that *PAX5* is mutated in 34% of adult B-ALL. These observations suggest that alteration of PAX5 function may contribute to leukemogenesis of B-lineage ALL via improper regulation of early B-cell differentiation (Sadakane et al., 2007). The human *PAX5* gene, which encode the protein PAX5, is situated at the 9p13 locus, and composed of 10 exons (Downing and Mullighan, 2006; Arseneau et al., 2009). The coding regions length of the exons is between 46 bp to 198 bp. In the present study, the prevalence of probable mutations among B-ALL patients in Khuzestan province of Iran was investigated in exons 1, 2 and 3 of *PAX5* gene.

MATERIAL AND METHODS

The present study was carried out in 50 patients with B-Lineage ALL (31 males, 19 females), who were referred to Shafa hospital in Ahvaz between November 2013 and February 2014. The age of these patients ranged between 1 to 27 years with a mean of 9.4 years. Initial diagnosis of

ALL was based on clinical phenotypes. After obtaining information of the patients, peripheral blood (5 mL) was collected into 0.5M EDTA solution and kept at -70°C . Genomic DNA was extracted from white blood cells via the DNA extraction kit from Gen Fanavaran. The forward and reverse primers for exons 1, 2, and 3 on the *PAX5* gene were designed with the following sequences: 1F (5'-attattccgacctgtgagcg-3') and 1R (5'-gtgtcccggagtttgac-3'), 2F (5'agcgggtcttctctatgtg-3') and 2R (5'-acagctgctgggtcatgtt-3'), 3F (5'-tagcccgtattttgttcc-3') and 3R (5'-atccctccaaatccccaac-3'). PCR reactions were performed in a total volume of 25 μL with the following cycling parameters: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 30 s, followed by one cycle of 72°C for 3 min. The PCR products were run on 1.5% agarose gels to verify the fidelity of PCR reactions. Direct sequencing of exons 1, 2 and 3 was carried out with the big dye terminator cycle sequencing ready reaction kit (Applied Biosystems) on an ABI prism 3500 automated genetic analyzer (Applied Biosystems). Sequencing reactions were accomplished by the same primers that were used for exons amplification. Sequencing analyses were performed using Chromas, and the results were compared with the reported gene sequences. Estimation of the functional outcomes of mutations on protein structure was accomplished via Predict SNP and Mutation Taster.

RESULTS

The occurrence of *PAX5* mutations were examined in a group of 50 B-ALL patients. Direct sequencing analysis of the patients, after comparison with the *PAX5* reference sequence, revealed a heterozygous missense germline variant CGC > CAC substitution in exon 2. This mutation was found in codon 38 of the *PAX5* gene in one of the patients, an 18-years old male with B-ALL. At the protein level, this mutation causes an amino acid substitution from arginine to histidine. Sequencing of the *PAX5* gene in the family members of patient revealed that his mother also was heterozygous for the mentioned mutation but his father and brother did not show any mutations (Figure 1).

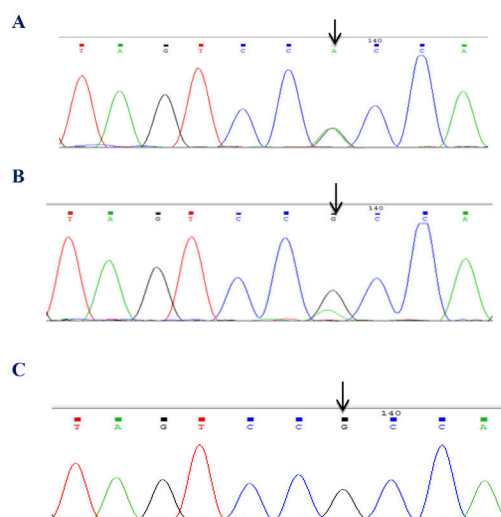


Figure 1. Sequencing results from **A.** the child with ALL, who was heterozygous for the mentioned mutation; **B.** the mother of the affected boy, who was heterozygous for the mentioned mutation; and **C.** the father of the affected boy, showing a normal sequence (result of the sequencing of the patient's brother was similar as his father) (sequencing with reverse primer).

The missense mutation (p.R38H) declared in this patient, was analyzed by Predict SNP and Mutation Taster. Accordingly, Predict SNP suggests that the missense mutation has a disease-causing probability of 87%. The R38H variant was also forecasted as disease causing by Mutation Taster with a 99% probability.

DISCUSSION

One of the malignant disorders of immature lymphoid cells that affect both children and adults is acute lymphoblastic leukemia (Pui et al., 2008). In children 1 to 4 years of age, the incidence of this disease is 10 times higher than that in 20 to 24 year old adults (Rodriguez-Abreu et al., 2007). Out of the 50 subjects that we recruited for the study, 10 patients were between 1 and 5 years of age, 18 patients were between 5 and 10 years old, 11 patients were between 10 and 15 years old, 10 patients were between 15 and 20 years old, and one patient was 20-27 years old. Worldwide incidence for this disease is projected to be 1-4.75 per 100,000 people (Redaelli et al., 2005). Annual incidence of leukemia in some parts of Iran is approximately 3.3 to 5.6 per 100,000 people (Dastgiri et al., 2011). It is believed that the etiology of ALL is multifactorial, and is likely contributed by interactions between environmental and genetic variables (Ellinghaus et al., 2012). Therefore, it is important to examine the genetic components in different populations due to differences in genetic background and other factors that are specific for a certain population. Submicroscopic genetic alterations such as changes in B-lymphoid transcription factors have been recognized in ALL as important determinants of leukemia (Holmfeldt et al., 2013). The most common alterations would be in transcription regulators of B-lymphoid developments such as *PAX5*, *EBF1*, and *IKZF1*, which accounts for more than 60% of the B-ALL cases (Inaba et al., 2013). *PAX5* is the main target of somatic mutations in B-ALL, and mutations in this gene is estimated to account for one-third of B-ALL patients (Mullighan et al., 2009; Mullighan, 2012), indicating its importance in B-ALL pathophysiology. In spite of studies on somatic mutations in *PAX5*, few studies were conducted on the inherited mutations of the *PAX5* gene (Shah et al., 2013). In the present work, probable mutations in exons 1, 2 and 3 of the *PAX5* gene in patients with B-ALL were studied in the Khuzestan province of Iran. For this screening, following PCR, the exons were directly sequenced, and one heterozygous germline variant, c.113G > A encoding (p.Arg38His), was found in one of the patients. It was a missense mutation G > A (R38H) located in exon 2 of the *PAX5* gene. The known missense variant of p.Arg38Gly, is located in a conserved residue in the paired domain of *PAX5* (Figure 2).

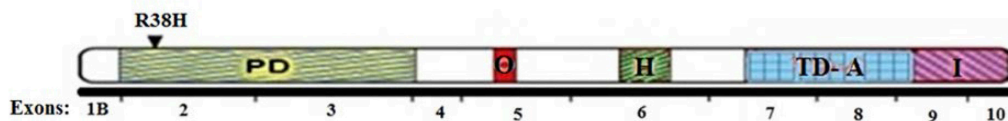


Figure 2. Schematic of exons that encode various domains of *PAX5*, and the mutation site which was found. In the domains, we can see the paired domain, octapeptide domain, homeodomain, transactivating domain, activating domain, and inhibitory domain, which are denoted with PD, O, H, TD, A, and I, respectively.

In the past few years, the R38H variant has been identified in several studies. Bernardi et al. (2013) found this variant in an Italian patient who suffered from B-ALL and Alagille syndrome. They identified damaging mutations in genes such as *PAX5* (R38H) and *NOTCH1* (K1821N) which may be strongly related to the observed disease (Bernardi et al., 2013). In addition, Santoro et al.

(2009) also recognized the R38H variant in B-ALL patients. They declared that point mutations of the *PAX5* gene along with other changes such as alternative splicing of *PAX5*, may be involved in the pathogenesis of B-ALL in both adults and children (Santoro et al., 2009). Furthermore, Miller et al. (2008) examined the *PAX5* gene in murine models and proposed that the R38H variant causes defective binding of *PAX5* to DNA. These studies all suggest that *PAX5* contributes to leukemogenesis.

According to accomplished software analyses by Mutation Taster, location of the reported mutation, codon 38, is highly conserved between species, and substitution of arginine by histidine leads to the mutated form of the protein (Table 1). In addition, as illustrated below, even regions surrounding codon 38 show highly conserved sequences, which further suggests that in this area may have profound effects on the protein structure and function.

Table 1. Conservation of codon 38 of the *PAX5* gene in different species.

| Species | Gene | Amino acids | Alignment |
|-----------------------|--------------------|-------------|-------------------------|
| Human | ENSG00000196092 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| Mutated | | 38 | FVNGRPLPDVVHQRIVELAHQGV |
| <i>P. troglodytes</i> | ENSPTRG00000020936 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>M. mulatta</i> | ENSMUG00000019639 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>F. catus</i> | ENFCAG0000001628 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>M. musculus</i> | ENSMUSG00000014030 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>G. gallus</i> | ENSGALG00000016431 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>T. rubripes</i> | ENSTRUG0000003227 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>D. rerio</i> | ENSDARG00000037383 | 38 | FVNGRPLPDVVRQRIVELAHQGV |
| <i>X. tropicalis</i> | ENSXETG0000006547 | 38 | FVNGRPLPDVVRQRIVELAHQGV |

Arginine is a positively charged amino acid. Analysis of proteins which interface with DNA indicates that these proteins are highly enriched in positive charges and are almost devoid of negative charges. On the DNA side, negatively charged phosphate residues represent approximately 43% of the surface area that is in contact with the proteins (Nadassy., 1999). Histidine is an ionizable amino acid with an ionization constant, or pKa value, of approximately 6.5 (Liao et al., 2013). Since arginine has a positive charge and histidine has a nearly neutral charge, substitution between the two amino acids may significantly affect protein affinity to DNA.

Analysis of the *PAX5* mutations with respect to their locations within the DNA-binding domains suggested that these alterations may change or eliminate its binding to the DNA (Figure 3).

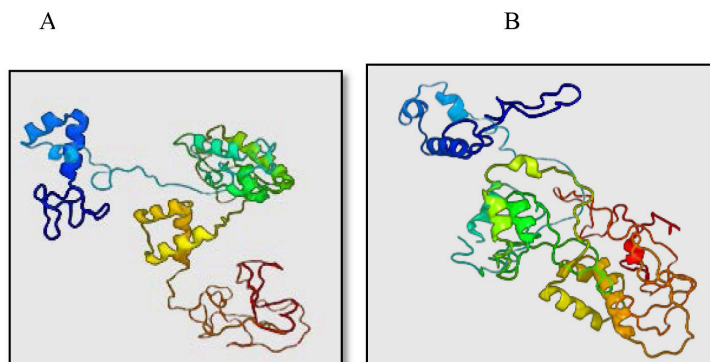


Figure 3. Three-dimensional structure of the *PAX5* protein. **A.** Normal protein. **B.** Mutant protein.

The function of a protein is intimately linked to its structural properties. Because of the role of *PAX5* in many cellular processes such as transcription control, receptor signaling, adhesion, migration, and immune function, change in protein structure may cause defects in many of these processes. The location of the R38H variant is in the paired domain of *PAX5*. The paired domain is a conserved DNA-binding motif, and can act as transcriptional repressors or activators. It is possible that alterations to the paired domain may affect its binding to DNA. However, to determine the exact effect of the R38H variant, further functional analyses are required.

Here we present separate images of the region of exons 1, 2, and 3 that make up the paired domain *PAX5*, and show the changes in structure as a result of the mutation (Figure 4). According to prediction of "Predict SNP" and "Mutation Taster", the probability of this mutation in causing disease is high.

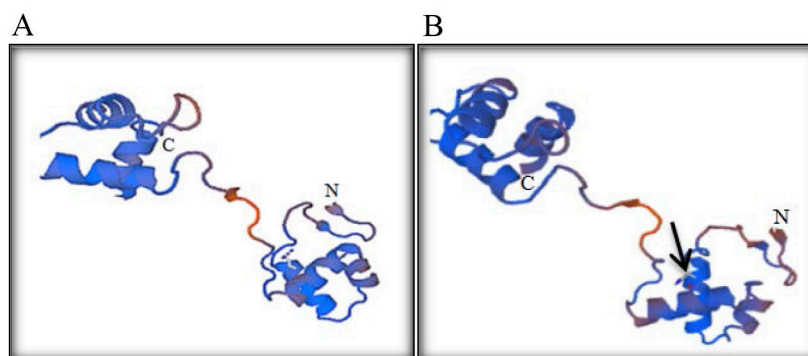


Figure 4. Picture of the paired domain of *PAX5* and location of its mutation. **A.** Normal. **B.** mutant. Arrow indicates the position of the mutation.

After observing the mutation in our patient, sequencing of the *PAX5* gene in exon 2 have been performed on all of the family members, including the father, mother, and brother of the patient, who didn't show any clinical feature of the disease. Based on the sequencing results, the mutation was present in the patient's mother in the heterozygous form, but not in either the father or the brother. This indicates that the boy inherited the allele from his mother. Interestingly, despite having the mutation, the mother is healthy, which suggests that the mutation is of a recessive origin. Therefore, the other allele on the mother's gene must be normal to achieve proper gene function. As recessive diseases require changes in both copies of the gene, the mutated allele from the patient was inherited from the mother as a germline mutation. However, since this defective allele is not sufficient to cause disease, it is possible that genetic defect on another allele of this gene may have occurred. As a result, normal gene function is lost. The origin of the other mutation is unknown; however, the new change in the other allele may be a somatic one. Therefore, while we have found one heritable mutation, it seems that more molecular and cytogenetic analyses are needed in order to explore every probable change in the alleles of *PAX5* gene.

Conflicts of interest

The authors declare no conflict of interests.

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