The Annals of Clinical & Analytical Medicine
Original Research

A novel missense mutation that may be associated with the polydactyly in the *HOXD13* gene: Q241H

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Abstract

Aim: *HOX* gene cluster which is termed as architectural genes and affects the expression of certain genes on DNA are effective in the development of limb. Therefore, mutations are observed in *HOX* genes, particularly *HOXD13*, lead to various congenital limb malformations. In this context, it was aimed to determine the expression level of *HOXD13* gene and screening of *HOXD13* mutations in patients with congenital lower/upper limb malformations who applied to Clinic of Plastic Reconstructive and Aesthetic Surgery of Meram Faculty of Medicine Hospital in this study. Material and Method: The case group of the study was composed of 20 unrelated patients with congenital lower/upper limb malformations and the control group was composed of 20 healthy individuals. Mutation analysis was performed using NGS and Sanger sequencing methods. The expression level of the *HOXD13* gene was determined by the qPCR. Results: According to the qPCR results, in the case group, a 3.43 fold decrease was observed in the expression of *HOXD13* gene when compared with the control group. However, this result was not statistically significant. According to NGS and Sanger sequencing results, a 723G> T variation that could lead to amino acid changes (Q241H) and could be defined as a missense mutation was detected in a patient. Discussion: 723G> T variation observed in a patient with a polydactyly anomaly was found in the patient's mother. However, more detailed studies are needed to assess this variation, which are not found in the literature, as a missense mutation in *HOXD13* associated with polydactyly.

Keywords

HOXD13; Missense Mutation; Next-Generation Sequencing; Polydactyly

DOI: 10.4328/ACAM.6205 Received: 08.02.2019 Accepted: 06.03.2019 Published Online: 27.03.2019 Printed: 01.03.2020 Ann Clin Anal Med 2020;11(2): 155-158
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Introduction

Limb malformations are one of the most frequent congenital malformations in infants with an occurrence of 1 in about 500 live births [1]. These malformations may be caused by environmental factors such as teratogens or may be caused by mutations that occur in the genes involved in embryonic development and differentiation of the limb [2,3].

Limb formation is initiated in the very early stages of pregnancy. The limb buds are observed on day 26 in the upper limb and day 28 in the lower limb [4,5]. The limb derives from the mesenchymal cells of the lateral mesodermal plate and develops along three major axes depending on the efficiency of the various signal centers [6]. Apical ectodermal ridge (AER), one of these centers, controls the limb growth along the proximal-distal axis via fibroblast growth factors (Fgfs). The development of limb along the anterior-posterior axis is provided by the zone of polarizing activity (ZPA) signaling center where the Sonic hedgehog (Shh) is effective. Finally, the third signal center, non-AER limb ectoderm, regulates the limb growth along the dorsal-ventral axis. At this stage, Wnt7A, which is expressed by the dorsal limb ectoderm and is a member of the Wnt family, plays an important role [6-9].

As well as the signaling centers, various transcription factors are also involved in the formation of the limb. The most known of these are the transcription factors encoded by the *HOX* gene clusters [3]. *HOX* genes, a subgroup of homeobox genes, encode highly conserved transcription factors contain a homeodomain which is a 60 amino acid helix-turn-helix DNA-binding motif [10]. The homeodomain binds to specific DNA sequences and it is known that such a binding regulates the expressions of the target genes [11]. In vertebrates, there are 39 *HOX* genes, key regulatory factors of morphogenesis and cell differentiation in embryonic development, organized into 4 clusters as follows: *HOXA*, *HOXB*, *HOXC* and *HOXD* [12]. *HOX* gene clusters are particularly important regulators in development along the anterior-posterior axis. Specifically, the 3' genes of a cluster are expressed in the anterior part of the developing embryo and in early stages of the development, while the 5' genes are more effective in the posterior part and in the later stages [13]. Expression errors in these genes are associated with limb malformations in connection with the efficiency of *HOX* genes in the developmental process. *HOXD13* is the first *HOX* gene known to be associated with developmental disorders in human [14].

The HOXD13 gene (OMIM:142989), located at the 5' end of the *HOXD* gene cluster localized on chromosome 2q31, is 1365 bp and its coding region is 1008 bp. The first exon located at the 5' end of the gene, comprising of two exons, contains the triple repeat sequences forming the chain of polyalanine, while the second exon located at the 3' end encodes a highly conserved homologous box domain [15]. *HOXD13* gene mutations are known to cause variable expression in a wide spectrum of clinical manifestations of limb malformations [16].

Based on the activity of *HOXD13* in the developmental process, in this study, it was aimed to screen the possible mutations of the *HOXD13* gene in unrelated 20 patients with congenital lower/upper limb malformations using next-generation sequencing (NGS) technology and to reveal the *HOXD13* gene expression level in the anomalies.

Material and Methods

Sample Collection

The case group of the study was composed of 20 unrelated patients with various congenital lower/upper limb malformations who applied to the Clinic of Plastic Reconstructive and Aesthetic Surgery of Meram Faculty of Medicine Hospital. For using qPCR analysis, the control group was composed of 20 healthy individuals without limb malformations who applied to the same department. The study was approved by the Ethics Committee of Necmettin Erbakan University, Meram Faculty of Medicine (2016/414) and was carried out in accordance with the principles of the declaration of Helsinki. Written informed consents were obtained from each individual or legal guardians. Peripheral blood was collected from the individuals in EDTA tubes for DNA and RNA isolation.

NGS analysis and Sanger validation for determination mutation in cases

Genomic DNA was isolated from peripheral blood lymphocytes using standard Phenol/Chloroform method. For each sample, a PCR mix containing primer mix, DNA (50ng), master mix, Enhancer master mix, and dH_2O was prepared as two reactions and PCR protocol was performed. After the reaction, non-specific PCR products were cleaned by magnetic beads. Resuspension buffer was added to the bead to obtain a purified PCR product. Adapters were attached to the ends of the DNA sequences for binding of barcodes used to identify samples. In this stage, the PCR products which were prepared in two tubes were combined into a single tube, and purification was repeated using beads. A reaction was prepared by combining the adapter-coupled purified DNA with the master mix and the barcode primer mix. Thus, a library was created and each sample was barcoded. The purification process was applied again to the samples and measurement was performed with a spectrophotometer. Finally, PCR products were assembled and an amplicon library was constructed. The library was prepared to be 4 nM. The gene sequencing was done on the Illumina MiSeq platform. Coverage of *HOXD13* was screened in Integrative Genomics Viewer software (IGV-2.3.91). For validation of results with Sanger sequencing, all samples were sequenced using ABI 3500 Genetic Analyser with both forward and reverse primers. The sequence data were analyzed with the BioEdit Sequence Alignment Editor.

RNA isolation, cDNA synthesis and qPCR analysis

For RNA isolation, 1X Red Blood Cell Lysis Buffer (RBCL; 1.68M NH_4Cl, 0.1M $KHCO_3$, 0.5 M EDTA) was added to 2 ml of the blood sample, and the supernatant was removed after centrifugation. After the same procedure was repeated several times, RNA isolation was performed by adding TRIzol Reagent on the pellet. cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The expression level of *HOXD13* was analyzed by qPCR using cDNAs from each of the control and case groups. The qPCR reaction was performed using EvaGreen Mastermix. Briefly, the qPCR mix was conducted in 10 µl volume containing 5 µl 2x EvaGreen Mastermix, 5 pMol forward primer, 5 pMol reverse primer, and 2 µl cDNA. The qPCR protocol was applied as denaturation at 95°C for 10 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The primers for *HOXD13* (Forward primer: 5'-GGAAGAAGAGAGTGCCTTACAC-3'; Reverse primer: 5'-GTCCTTCACTCTTCGGTTCTG-3') and *ACTB* (Forward primer: 5'-TGAACGGGAAGCTCACTG-3'; Reverse primer: 5'-TCCACCACCCTGTTGCTGTA-3') genes were designed using IDT PrimerQuest (https:// eu.idtdna.com/site) and *ACTB* was used in

the normalization of the results as housekeeping genes.

Statistical analysis

The gene expression analysis was performed by using the $2^{-\Delta\Delta CT}$ method with RT² Profiles™ PCR Array Data Analysis. A p-value <0.05 was considered to be statistically significant.

Results

According to NGS analysis results, a variation that can be defined as a novel missense mutation (723G> T) was found in a patient. This variation in the 1st exon of the *HOXD13* gene was confirmed by the Sanger sequencing method. It was observed that the patient who possesses an extra finger in his right hand has the heterozygous genotype for this variation. The Sanger sequencing analysis with the mother and the father of the patient revealed that the patient's mother had the same mutation and also had the heterozygous genotype for this mutation (Figure 1). Normally, glutamine is encoded in the region where the variation is located, but G-T transversion causes histidine to be involved in the protein structure. Accordingly, this variation observed in the *HOXD13* gene is thought to be regarded as a missense mutation.

The expression level of the *HOXD13* gene was evaluated with the qPCR analysis in control and case groups. According to the qPCR results, a decrease was observed in the expression of *HOXD13* gene in case of group as 3.43 fold when compared with the control group. However, this decrease was not statistically significant (p>0.05) (Figure 2).

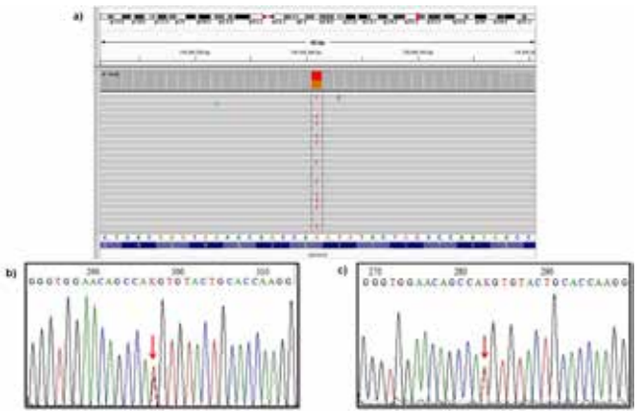

Figure 1. The novel missense mutation detected in the HOXD13 gene. a) The NGS result of the patient was visualized with IGV-2.3.91 and G-T transversion was observed at position 723 (723G>T) in the 1st exon of the *HOXD13* gene (Total count:17922; A=0%, C=0%, G=50%, T=50%). b) The patient's electropherogram confirmed the result of NGS and showed that he was heterozygous for the 723G>T mutation. c) The electropherogram of the patient's mother revealed that she also had the heterozygous genotype for the same mutation.

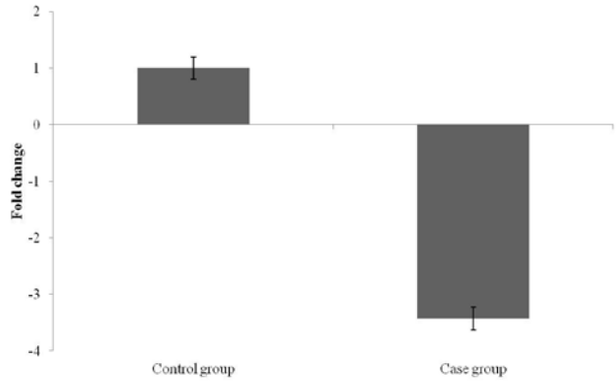

Figure 2. The expression level of *HOXD13* gene in control and case groups

Discussion

In epidemiological studies, it has been shown that *HOX* gene groups play a role in a wide variety of extremity anomalies. Mutations in *HOXD13* gene, a member of *HOX* gene groups, are also associated with congenital limb malformations. Mutations observed in the *HOXD13* gene are classified into three groups as loss of function mutations, increase in the N-terminal polyalanine tract and missense mutations [17]. It is thought that nonsense and frameshift mutations lead to haploinsufficiency in the *HOXD13* protein, but this has not yet been proven experimentally [18].

The expansion in trinucleotide repeat, which locates on the 1st exon of *HOXD13* gene and encodes a 15-residue polyalanine tract, is associated with synpolydactyly [15]. Although the addition of six or fewer alanine does not lead to pathological results, the increase as 7-14 alanine may result in limb malformations that are reflections of different phenotypes [19]. It is known that such mutations disrupt protein stabilization. It has also been shown that the expansion of polyalanine has caused settlement of the protein which is normally located in the nucleus as a transcription factor, into the cytoplasm [20].

Up to now, five missense mutations (R298W, R298Q, S308C, I314L, and Q317R) which are associated with limb malformations in different phenotypes have been identified in the homeodomain region of *HOXD13* gene [21]. One of these, R298W, is associated with synpolydactyly and there is a C-T transition in position 892 of *HOXD13* gene in affected individuals. This transition causes the change of arginine, in position 31 of the homeodomain, to tryptophan, thus it has been suggested that the stability of homeodomain-DNA complex is impaired [22]. R298Q, another missense mutation associated with the syndactyly, also affects amino acid residue at position 31 like R298W mutation and causes arginine to glutamine substitution in homeodomain. It is thought that this alteration disrupts the role of *HOXD13* in the regulation of transcription and affects the formation of the limbs by acting as a partial loss of function mutation [23].

S308C and I314L missense mutations are associated with the brachydactyly phenotype. In the S308C mutation, there is C-G transversion at position 923 in the second exon of *HOXD13*. This transversion causes cysteine replacing with serine which is the 41st amino acid of the homeodomain. In the I314L mutation, the 47th amino acid found in the homeodomain structure is leucine instead of isoleucine. The binding activities of two proteins resulting from these mutations have been examined and it has been determined that the S308C mutation has not led to a change in binding of the protein to DNA. However, in the I314L mutation, it has been shown that the mutant protein has higher affinity to the target sequence containing the 5'-TTAC-3 recognition region and lower affinity to the target sequence containing the 5'-TTAT-3 'recognition region compared with the wild protein [24]. The Q317R mutation causes arginine replacing with glutamine which is highly conserved amino acid at the 50th position of the homeodomain. It is known that this region is important for DNA binding and specificity. This mutation, which has been found to disrupt transcription from the EPHA7 promoter that has a binding region for *HOXD13*, is associated with syndactyly [25].

In this study, it was aimed to identify possible mutations in *HOXD13* gene which plays a role in congenital lower/upper limb malformations and to investigate the efficiency of *HOXD13* gene expression. For this purpose, the *HOXD13* gene was screened in

20 unrelated patients with syndactyly or polydactyly in the upper or lower limbs; and the expression level of *HOXD13* gene in the anomalies was determined by comparing with 20 control individuals.

According to the qPCR results, when compared with the control group, a 3.43 fold decrease was detected in *HOXD13* gene expression in the case group, but this decrease was not statistically significant. On the other hand, NGS and Sanger sequencing results revealed the presence of a novel missense mutation in a 3-year-old patient with polydactyly anomaly with an extra finger in his right hand. The mutation is a G-T transversion and is located at position 723 (723G>T) in the 1st exon of *HOXD13* gene. This region encodes glutamine which is the amino acid 241 of the protein. 723G>T will lead to the presence of histidine instead of glutamine in the region involved in the construction of the protein (Q241H). This may also result in loss of protein function. The patient was heterozygous for the Q241H mutation and in terms of this mutation, the mother and the father of the patient have also been investigated and it has been shown that the mother has the same mutation for *HOXD13* with heterozygous genotype and polydactyly phenotype. However, there is no information about Q241H mutation which is considered to be a missense mutation in the *HOXD13* gene in the literature. When considered in terms of genes, polydactyly is a disease in genetically and clinically complex. Identifying the underlying causes of congenital diseases requires accurate interpretation of genetic variants. Therefore, this finding should be supported by more comprehensive studies in order to evaluate the Q241H mutation as a novel missense mutation that may be associated with congenital lower/upper limb malformations in the *HOXD13* gene. Furthermore, this finding is thought to be effective in evaluating the wide range of limb malformations when considering the developmental activity of *HOXD13*.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: None

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:

Vural H, Avcı E, Eroğlu C, Çınar İ, Yarar S, Gündeşlioğlu Ö. A novel missense mutation that may be associated with the polydactyly in the HOXD13 gene: Q241H. Ann Clin Anal Med 2020;11(2): 155-158