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Whole-exome Sequencing Identified a Novel Hemizygous Missense Variant in the EDA Gene in an Iranian Patient Causing Hypohidrotic Ectodermal Dysplasia

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Abstract

Ectodermal dysplasia (ED) is a congenital heterogenic disorder caused by the defect in the ectoderm and its derivatives. This complex disorder has different subtypes, the most common form of which is hypohidrotic ectodermal dysplasia (HED). Symptoms include spare hair, defects in sweat glands activity, abnormal teeth and dystrophic nails. In the present study, whole-exome sequencing was performed to identify disease-causing variants in an Iranian 4-year-old affected boy with sparse hairs, low eyelashes and eyebrows, reduced teeth, severe dry skin, and reduced sweat glands. We confirmed the pathogenicity and its novelty within silico tools. Identifying variant confirmation in the patient and segregation analysis in her family were performed using the Sanger sequencing method. A novel hemizygous missense variant [NM_001399.5: c.1001G>C; p. (Arg334Pro)] was identified within the EDA gene; and this is the third case of HED in Iran that is related to the EDA gene. The distinction between our patient's indications and those recorded for a few past subjects may be due to the differences in the mutations involved.

Key Words: Hemizygous Missense Variant, Hypohidrotic Ectodermal Dysplasia (HED), Mutation analysis, Sanger sequencing method, Segregation analysis, Whole-exome Sequencing.

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1- INTRODUCTION

Ectodermal dysplasia (ED) is a rare heterogeneous disorder characterized by defects of two or more ectodermal-derived structures with a prevalence of one in 100,000 live births. This complex congenital disorder has different inheritance patterns, including autosomal dominant, autosomal recessive, and X-linked recessive (1). It mainly affects hairs, sweat glands, teeth, nails, and skin. ED has a variety of subgroups; so far, about 200 types have been reported, and it is named after which parts (two or more) are involved. Many genes have been reported in this disorder, such as EDA, EDAR, NEMO, EDARADD, CDH3, WNT10A, HOXC13, TP63, EVC2, and NECTIN1 (2, 3). EDA is the truncation for ectodysplasin A, found on Xq13.1. EDA is communicated in numerous organs, counting salivary organs, adrenal, etc (4). EDA has five distinctive expressions, with EDA-A1 having the foremost expression with eight exons. This gene has a place in the TNF-linked ligand family. It has four critical regions, including the following: collagen-like domains, several domains similar to TNF, and the part that links between the extracellular domain and transmembrane and cleaves the furin fragment (5). The binding of EDA to the receptor activates pathways involved in the morphogenesis and maintenance of the evolution of ectoderm-derived organs, and within the repair and recovery of the skin and improving the recovery capacity of bone marrow mesenchymal stem cells (6). Defects in the gene cause anhidrotic ectodermal dysplasia (XLHED). The most common type is hypohidrosis ectodermal dysplasia (HED), which is reported as the autosomal dominant, autosomal recessive, and X –linked recessive; the X –linked recessive form is most commonly seen in males than females (7). Other genes involved in HED include EDAR, EDARADD, and WNT10A, responsible for the development of ectodermal-derived structures, but EDA is the major causative gene of HED. Hypotrichosis and hypohidrosis are two main HED skin properties (8). In this study, children with clinical manifestations of HED and their parents were investigated. One variant in EDA was identified and analyzed to determine their clinical significance and inheritance mode.

2- MATERIALS AND METHODS

2-1. Clinical Investigation and Blood Sampling

Proband was born into a family of seven members in a village in Zahedan, Sistan and Baluchistan, Iran, with non-consanguineous and no history of the HED who had an eight-year-old boy suspected of having ED. The affected has referred to a dermatologist with symptoms of spare hairs, abnormal teeth, dry skin and lack of sweat glands, that these signs were the inclusion criteria of the study. To accurately diagnose the type of disease, in addition to pedigree drawing, radiographic examination (The shapes and the sizes of the residual teeth were observed by the dentist) and genetic tests were candidates. For genetic tests, 5-10 ml of blood was taken from the proband, his parents, and normal people in tubes containing EDTA.

2-2. DNA Extraction

After filling in the consent form by the patient's parents, a DNA sample was extracted from a Microliter (μl) of mononuclear blood cells in the Kowsar kit (Cat. No. K1135). After DNA extraction, its concentration and purity were determined using a nanodrop device. The screening was completed on DNA extracted from whole blood.

2-3. Whole Exome sequencing

The qualified genomic DNA sample was randomly fragmented by Covaris, and the size of the library fragments was distributed predominantly between 150 and 200 base pairs (bp). Adapters were

then ligated to both ends of the resulting fragments. The adapter-ligated templates were purified by the Agencourt AMPure SPRI beads, and fragments with an insert size of about 176 bp were excised. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the SureSelect Biotinylated RNA Library (BAITS) for enrichment. Hybrid pieces were connected to streptavidin seeds, while non-hybrid parts were washed after 24 hours. Captured LM-PCR products were subjected to Agilent 2100 Bioanalyzer to estimate the magnitude of enrichment. Each captive library was then loaded on the HiSeq2000 platform, and high-throughput sequences were carried out for each captive library to ensure that each sample had the specified normal sequence depth. Raw image files were processed by Illumina Soft-ware 1.7 for base-calling with default parameters, and the sequences of each individual were generated as 90/100 bp paired-end reads. The bioinformatics examination was connected to the sequencing information (“raw data") produced from the Illumina pipeline. To begin with, the adapter sequence and low-quality reads were discarded. This stage created the “clean data". Secondly, Burrows-Wheeler Aligner (BWA) was used for alignment to the published human genome build GRCh37/UCSC hg19. Read groups were added, duplicates were marked, and the reads were sorted using Picard (http://broadinstitute.github.io/picard).
Following these processes, the ultimate BAM files were used for variant calling using the GATK haplotype caller to distinguish single nucleotide variants (SNVs) and indels.

2-4. In silico analysis of variants

Pathogenicity of candidate mutation was then checked by tools, based on several tools, including SIFT, PolyPhen2, MutationTaster, CADD, FATHAM, metaLR, and REVEL was determined and identified variants as deleterious. Also, the most relevant variants were manually assessed to detect those that matched clinical outcomes.

2-5. Sequencing and Co-segregation study

Sanger sequencing confirmation for the reported variant was done after data analysis. Forward and reverse primers were designed with Oligo 7 software to amplify variant sites. We amplified exon eight and then evaluated the sequence chromatograms with a codon code aligner. We then performed a co-segregation study to confirm the mutation in the patient’s parents and his sisters.

3- RESULTS

3-1. Clinical manifestation

After designing the pedigree and matching the apparent symptoms such as reduced teeth, hypohidrosis, and sparse hairs, both the X-linked recessive hereditary pattern and HED were identified (**Fig. 1**).

3-2. Genetic testing

After examining the clinical manifestations based on the patient phenotype and the initial diagnosis of a dermatologist and dentist, by performing the whole-exome sequencing, the analysis was done until the first step of the mutation: in the first phase, the total number of variants was 79112, then the Total Number of exonic and Splicing Variants filter was applied 20145, in the next step, with Remove Synonymous Variants, the number of variants decreased to 11852, then we did Remove Benign Variants (cadd_phred <20) and reached 5320 variants. In the final stage of the variants matching with phenotype, in Retain Homozygous 96 Variants, we reached to 15 variants; and finally, the gene that was in line with this phenotype was a homozygous mutation in EDA: NM_001399.5, exon 8, c.1001G>C (p. Arg334Pro). This mutation is inherited

in an X-linked recessive manner, and the genetic testing result confirms the clinical diagnosis of Ectodermal dysplasia 1, hypohidrosis, X-linked.

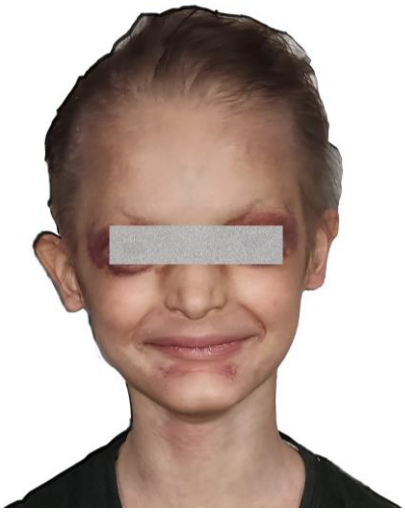

Fig. 1: Sparse eyebrows and eyelashes and clinical manifestations of HED

3-3. In silico analysis

Since the found mutation is novel and no study has reported the mutation (EDA: C.1001G> C) in terms of whether it is the pathogen or not, we examined it with bioinformatics tools. The Varsome Database (EDA: C.1001G> C) was introduced to the pathogen, in SIFT as Damaging; Polyphen2 referred to it as probably damaging; CADD score cited the number 25.5 in front of the mutation; MetalR was referred to as deleterious and was classified in Mutation Taster as damaging.

3-4. Co-segregation study

After bioinformatics tools identified the novel mutation. Finally, Sanger sequencing was performed to confirm mutations in the patient and co-segregation in his parents and his sisters. The result confirmed that the mother and sister were carriers and that the proband's father was healthy (**Fig. 2**).

4- DISCUSSION

Here, we report one patient with HED syndrome, genetically confirmed to have missense mutation, p.Arg334Pro, in the EDA gene. This is a novel mutation that has not been reported yet. The pattern and distribution of the affected areas and their phenotypic characteristics were similar to other patients with HED. In this patient, hypohidrosis, hypodontia, and sparse hair are visible. This study aimed to identify the cause of an eight-year-old boy suspected of having ED in an Iranian family. After the patient's initial evaluation by a dermatologist, dentist, and geneticist, he was nominated to determine the entire exome sequence. After analyzing the exome, we obtained the c.1001G>C mutation and confirmed the mutation with the sequencing trench in the patient's parents and sister. Since this is a novel mutation and has not been reported in any study, we have confirmed that it is a pathogen with bioinformatics tools.

Of the published studies on mutations in the EDA exon gene of eight nucleotides, 1001 was obtained in the study by Song et al., in 2009. In this study, they sequenced 15 men with HED who resulted from their non-consanguineous marriage.

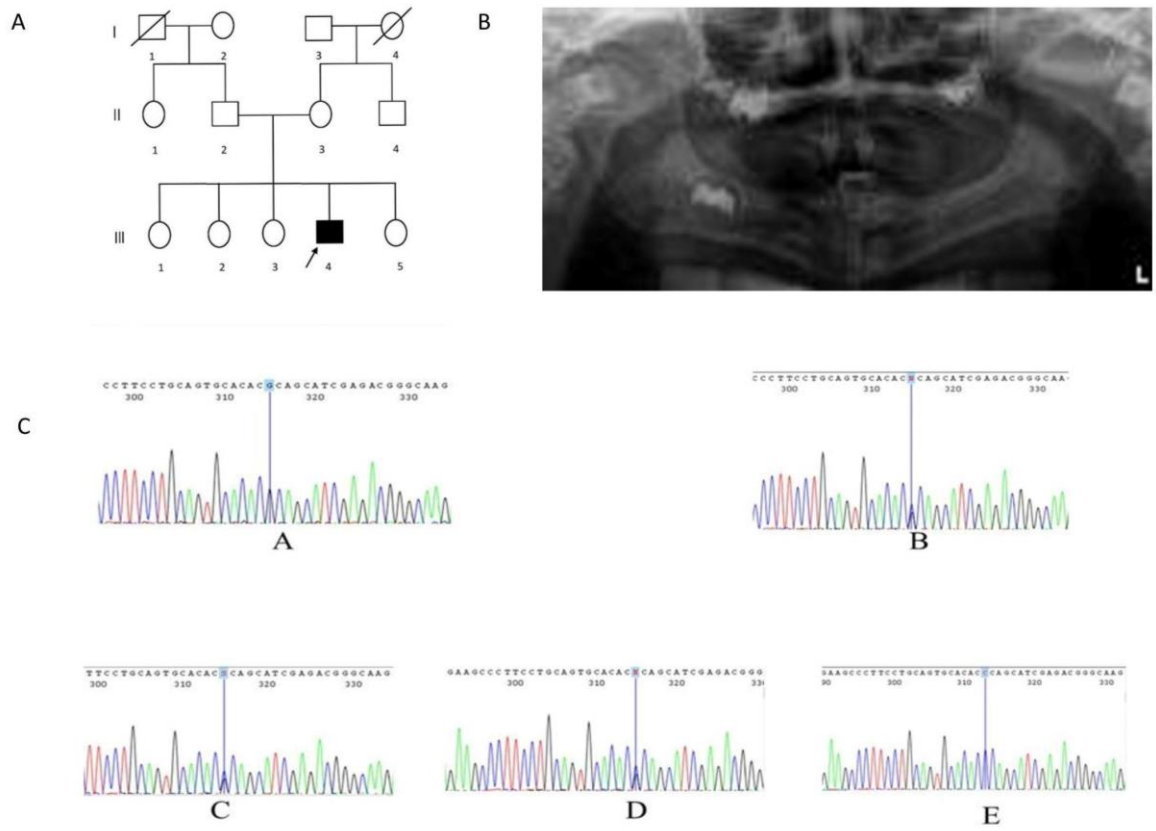

Fig. 2: A) Pedigree of the HED patient in a family with non-consanguinity marriage, B) Underdeveloped and malformed alveolar ridges, C) Sanger sequencing revealed a homozygous missense variant with potentially damaging effects on EDA:c.1001G>C, p.Arg334Pro. Sanger sequencing revealed a hemizygous missense variant with potentially damaging effects in EDA: c.1001G>C, p.Arg334Pro. C-A (II-2) normal, B (II-3), C-C (III-2), and C-D (III-3 show the carriers (heterozygous), and C-E (III-4) (hemizygous) show the affected.

Only one patient identified a mutation in the 1001 nucleotide exon 8 of the EDA gene, which resulted in the G nucleotide becoming A (arg334His) (9). Zhao Kai et al. in 2019, in the other study, eight Chinese patients with ectodermal dysplasia were determined by the whole-exome sequencing. After the analysis, three missense mutations were found in the EDA gene, including c.959A>G, c.1073A>G, and c.1001G>A (10). Shen et al. in 2019, in a study of children suspected of HED, determined the whole-exome sequencing after the analysis. Interestingly, some phenotypes of the mother appeared asymmetrically between the right and left sides of the body that were not reported in previous studies (11). Out of 25 patients reported in Iran until January 2022, 13 patients had three different mutations in EDAR (c.730-2 A > G, c.338G > A, c.278C > G) (12–14), three patients with a mutation in CDH3 (c.830delG) (15), two patients with two mutations in EDA (c.898_924 + 8del35ins4CTTA, c.467 G>A) (16,17), and two patients with a mutation in HOXC13 (c.837_838 ins28) (18), and one patient with a mutation in any of the genes UBR1 (c.4188C > A) (19), NEMO (c.932 A > G) (20), MSX1 (c. 3'-UTR, 6C > T) (21), EVC2 (c.942G > A) (22), and PORCN (c.611 T > C) (23) genes were reported. Among the mutations of ED in

Iran, only one case was related to the EDA gene, and therefore our case is the third case of HED in Iran with a mutation in the EDA gene. In the patient we studied, arginine was converted to proline in 334 positions (**Fig. 3** and **Fig. 4**), which is located in the TNF binding domain, resulting in dysfunction of the domain, resulting in defective signaling, and causing clinical manifestations to include involvement of sweat glands, hair, teeth, and nails. This gene has a place in the TNF-linked ligand family.

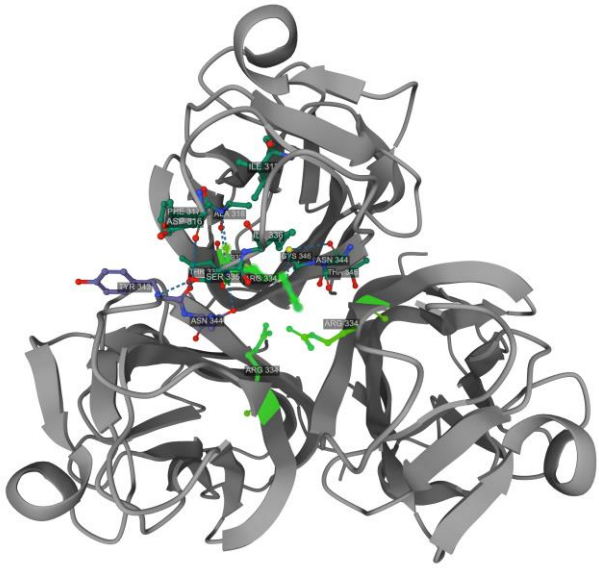

Fig. 3: X-ray structure of ectodysplasin a dimer visualized by Swiss- PDB viewer with some editing. The PDB of the EDA crystal structure is downloaded from uniport (www.uniport.org). The relative position of each domain showed in the EDA dimer.

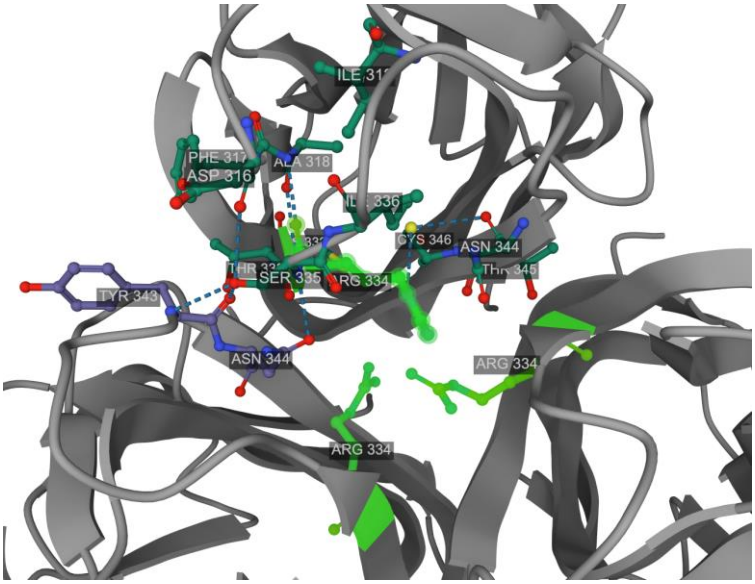

Fig. 4: X-ray structure of ectodysplasin a subunit dimer visualized by Swiss-PDB viewer with some editing. The PDB of ectodysplasin a crystal structure is downloaded from Uniport (www.uniport.org). Arg334 location within ASN-ARG-THR-ARG was shown. In the mutated EDA gene, Arg334 is converted to proline.

It has four critical regions, including the following: collagen-like domains, several domains similar to TNF, and the part that links between the extracellular domain and transmembrane and cleaves the furin fragment (5). The binding of EDA to the receptor activates pathways involved in the morphogenesis and maintenance of the evolution of ectoderm-derived organs, and within the repair and recovery of the skin and improving the recovery capacity of bone marrow mesenchymal stem cells (6). Since consanguineous marriage is common in the context of the study (24), we mostly expect the manifestation of diseases with autosomal recessive inheritance patterns. Still, our patient's inheritance pattern is X-linked recessive, so genetic counseling and sex determination of the fetus can help prevent the birth of another affected child by performing amniocentesis or chorionic Villus Sampling.

5- CONCLUSION

After doing WES, we reached a novel mutation in nucleotide 1001, the exon 8 of the EDA gene. In terms of protein change, the conversion of the amino acid arginine to proline at position 334 alters the structure and function of the protein and is prone to pathogenicity. Since this family had a non-consanguinity marriage and no history of ED, it can be said that by doing next-generation sequencing techniques, the cause of many diseases, especially diseases of unknown origin, can be understood.

6- ETHICAL CONSIDERATIONS

This study was approved by the Ethics Committee of Golestan University of Medical Sciences with the Ethics Code of IR.GOUMS.REC.1399.381 and Grant Number of 111485. All subjects in the study, including parents, siblings, and the proband, were informed, and a consent form was obtained before the initiation of the study.

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