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DETECTION OF AN *EDA* MUTATION CAUSING HYPOHIDROTIC ECTODERMAL DYSPLASIA IN A VIETNAMESE PATIENT

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SUMMARY

Hypohidrotic ectodermal dysplasia (HED) is a rare disease characterized by abnormal development of the structures derived from the ectoderm layer, including hair, teeth, nails, and sweat glands. The most observed form of HED, which is known as X-linked hypohidrotic ectodermal dysplasia (XLHED), is commonly attributed to genetic abnormalities in the ectodysplasin A (*EDA*) gene. We have successfully discovered a known mutation c.1045G>A (p.A349T) in a Vietnamese proband who displayed the main symptoms of XLHED using PCR and Sanger sequencing. Furthermore, the segregation of the mutation showed that the mother, who exhibited a normal phenotype, was a carrier of the mutation, while the father was hemizygous for the wild-type allele. The identification of the mutation c.1045G>A (p.A349T) contributes to HED research worldwide and can be used for genetic counseling in Vietnam.

Keywords: Ectodysplasin A, *EDA*, genetic counseling, hypohidrotic ectodermal dysplasia, Vietnam.

INTRODUCTION

Hypohidrotic ectodermal dysplasia (HED) is the most prevalent form of a syndrome called ectodermal dysplasia (Deshmukh, Prashanth, 2012). The estimated prevalence of HED is 1–9 in 100,000 individuals and 1 in 5,000–10,000 newborns (Albeik *et al.*, 2023). This disease is characterized by the abnormal development of ectoderm-derived organs, including the absence of sweat glands (anhidrosis or hypohidrosis), scant hair (hypotrichosis), and atypical or absent teeth (anodontia or hypodontia) (Cluzeau *et al.*, 2011; Albeik *et al.*, 2023). HED can be caused by mutations in several genes, which include ectodysplasin A (*EDA*), ectodysplasin A receptor (*EDAR*), ectodysplasin A receptor-associated death domain (*EDARADD*), *WNT10A, TRAF6, NEMO*, and *IKBKG* (Nguyen *et al.*, 2021). Notably, *EDA* mutations are responsible for 80% of HED cases (Cluzeau *et al.*, 2011; Keller *et al.*, 2011). The HED type caused by *EDA* mutations is X-linked hypohidrotic

ectodermal dysplasia (XLHED). The *EDA* gene is one member of the tumor necrosis factor (TNF) family. This gene is located on the short arm of chromosome X (Xq13.1). The XLHED-associated isoform of the EDA protein, called EDA-A1, is encoded by eight exons of the *EDA* gene and contains 391 amino acids, featuring several different domains such as a transmembrane domain, an intracellular domain, an extracellular domain, a furin subdomain, a collagen subdomain, and a TNF homology subdomain (Pääkkönen *et al.*, 2001). Among them, the furin subdomain is the site for proteolytic cleavage to release EDA ligand as a soluble protein, while the collagen and the TNF homology subdomain are responsible for the formation of the EDA-A1 homotrimer, the functional form of this protein (Li *et al.*, 2008; Liu *et al.*, 2023). The EDA-A1 ligand initiates the pathway involving EDAR and EDARADD, which regulates the expression of genes that control the development of ectoderm-derived organs by activating the transcription factor NF-κB (Cui, Schlessinger, 2006). Therefore, any abnormalities in the proteins EDA-A1, EDAR, or EDARADD, as well as their associated downstream pathways, can give rise to alterations in ectodermal development, which can lead to the expression of HED.

Since the first investigation on *EDA* mutations that cause XLHED in a male patient was conducted (Zonana *et al.*, 1993), many *EDA* mutations have been found worldwide. In China, researchers identified four different missense mutations in four unrelated patients by using Sanger sequencing to examine eight exons of *EDA* (Wang *et al.*, 2020). Another study in Italy using a similar method also detected a missense mutation (c.158T>A), which can be the causative factor of XLHED in the proband (Savasta *et al.*, 2017). In a recent study conducted in Vietnam, researchers identified a missense mutation c.2T>C (p.M1T) that may be implicated as a potential causal factor for XLHED in two patients who were closely related (Nguyen *et al.*, 2022). Overall, a total of 82 pathogenic variants in the *EDA* gene have been identified as being associated with XLHED (Han *et al.*, 2020). They include 41 (50%) missense, 13 (15.9%) deletion, 12 (14.6%) nonsense, 9 (11%) frameshift mutations, and only one (1%) intronic mutation (Han *et al.*, 2020). The study by Han *et al*. (2020) also pointed out that these mutations are distributed primarily on the TNF homology subdomain (37.8%) and collagen subdomain (22%) (Han *et al.*, 2020).

In this study, we detected an *EDA* variant c.1045G>A (p.A349T) in a proband with XLHED and screened for the presence of this variant in the other members of the family by using PCR in combination with Sanger sequencing. The determination of the disease-causing variant in the proband has the potential to provide valuable information for genetic counseling within this family while also making a significant contribution to the global understanding of XLHED, particularly in Vietnam, where the number of studies on this disease is currently limited.

MATERIALS AND METHODS

Study subjects and primer design

The study involved the analysis of three samples obtained from three individuals belonging to one Vietnamese family. The blood samples were collected in 2021 at the Human Genomics Laboratory, Institute of

Genome Research, Vietnam Academy of Science and Technology, and preserved at -20 ºC. Informed consent was obtained from all participants before the collection of blood samples. The research was approved by the Institutional Review Board (IRB) of the Institute of Genome Research, Vietnam Academy of Science and Technology, under the reference number 2-2019/NCHG-H.

Eight primer pairs were designed to amplify eight exons of the *EDA* gene. These sequences were designed based on the sequence of the *EDA* transcript available on GenBank (accession number NM_001399.4) (Table 1). To prevent the occurrence of self-dimers and hetero-dimers during PCR amplification, primers were rechecked using the OligoAnalyzer tool.

Table 1. Primer sequences for *EDA* amplification.

Exon	Primer	Sequence
1	Forward	5'-ACCCCTCGGAGTAGAGCTG-3'
	Reverse	5'-TGGTCCTGCCCTCTAAATTG-3'
2	Forward	5'-CTTAAGGTACAGGTAGACTGTC-3'
	Reverse	5'-CATGCCCTACCAAGAAGGTA-3'
3	Forward	5'-TCTTGGGGATCCCTCCTAGT-3'
	Reverse	5'-CAGACAGACAATGCTGAAAGAA-3'
4	Forward	5'-TTAGCAGGTCGCGGTGGCAC-3'
	Reverse	5'-GCTCTCAGGATCACCCACTC-3'
5	Forward	5'-GGTGAGGGGAAAAGGAAGTC-3'
	Reverse	5'-GCTGTGAGTGAAAACCGTCA-3'
6	Forward	5'-AGGATGGAAACATGGGACTG-3'
	Reverse	5'-GGCTGGGTGATTATTTGGAG-3'
7	Forward	5'-GGGGTTGTGAACTCCTTGGT-3'
	Reverse	5'-CCCCTATGTGGCCTGCACC-3'
8	Forward	5'-CCCATCCATGGGGTATACTAA-3'
	Reverse	5'-CTCACTCCACAGCAGCACTT-3'

Total DNA extraction

The blood samples of the patient and his parents were collected after receiving their informed consent for blood collection and genetic testing. These samples were then stored in EDTA tubes at -20 ºC before being subjected to total DNA extraction using the GeneJET Whole Blood Genomic DNA Purification Mini Kit manufactured by Thermo Fisher. The concentration and purity of the extracted DNA were determined by 1% agarose gel electrophoresis and a NanoDrop One/Onec spectrophotometer (Thermo Fisher Inc.).

PCR and Sanger sequencing

Eight exons of *EDA* were amplified from the extracted total DNA of the proband using the specific primers. The reaction was conducted on an Eppendorf Vapo Protect

Mastercycler® Pro with a total volume of 30 µL, which contained 19.85 µL of nuclease-free water, 3 µL of DreamTaq buffer, 1.8 µL of dNTPs, 0.6 µL of forward primer, 0.6µL of reverse primer, 0.15 µL of Taq polymerase, and 4 µL of DNA template. Eight negative controls were used to check for contamination of the amplification reactions of eight exons. These negative controls contained all the components of PCR but no DNA template. The size and quality of the PCR products were then checked by using 1.5% agarose gel electrophoresis. After that, these products were subjected to purification using the Thermo Fisher GeneJET PCR Purification Kit. The protocol for DNA purification was followed by the user guide to the kit. The purified PCR products were sequenced using the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems in Carlsbad, CA, USA) and the BigDye Terminator v3.1 Cycle Sequencing Kit. The sequencing results were visualized on SnapGene Viewer and compared to the reference sequence, NM_001399.4.

In silico prediction of the effect of the variant

Four different *in silico* tools - MutationTaster (Ruiz-Heiland *et al.*, 2016), SIFT (He *et al.*, 2018), PolyPhen-2 (Ruiz-Heiland *et al.*, 2016; He *et al.*, 2018), and CADD (Xing-Yu Liu *et al.*, 2022) —were used to predict the effect of the detected variant. In addition, the preservation of the amino acid at position 349 of the EDA-A1 protein was determined based on the data from the UCSC Genome Browser.

RESULTS

Detection of the variant in the *EDA* gene of the family members

The patient had the main symptoms of X-linked hypohidrotic ectodermal dysplasia (XLHED), particularly the absence of sweat glands, cone-shaped or missing teeth, and sparse hair. To detect a disease-causing variant, the amplification of eight exons of the *EDA* gene was performed on the patient. The agarose gel electrophoresis results of the PCR products showed that only one specific band was obtained for each exon (Figure 1). Subsequently, the amplified products of all *EDA* exons were subjected to purification for Sanger sequencing.

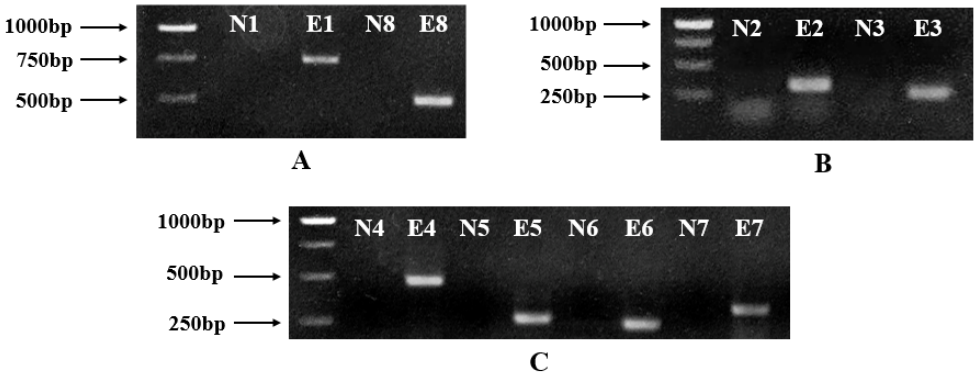

Figure 1. Gel electrophoresis results for PCR products of eight *EDA* exons. E1-E8: Exon 1 to exon 8; N1-N8: Negative controls of exon 1 to exon 8.

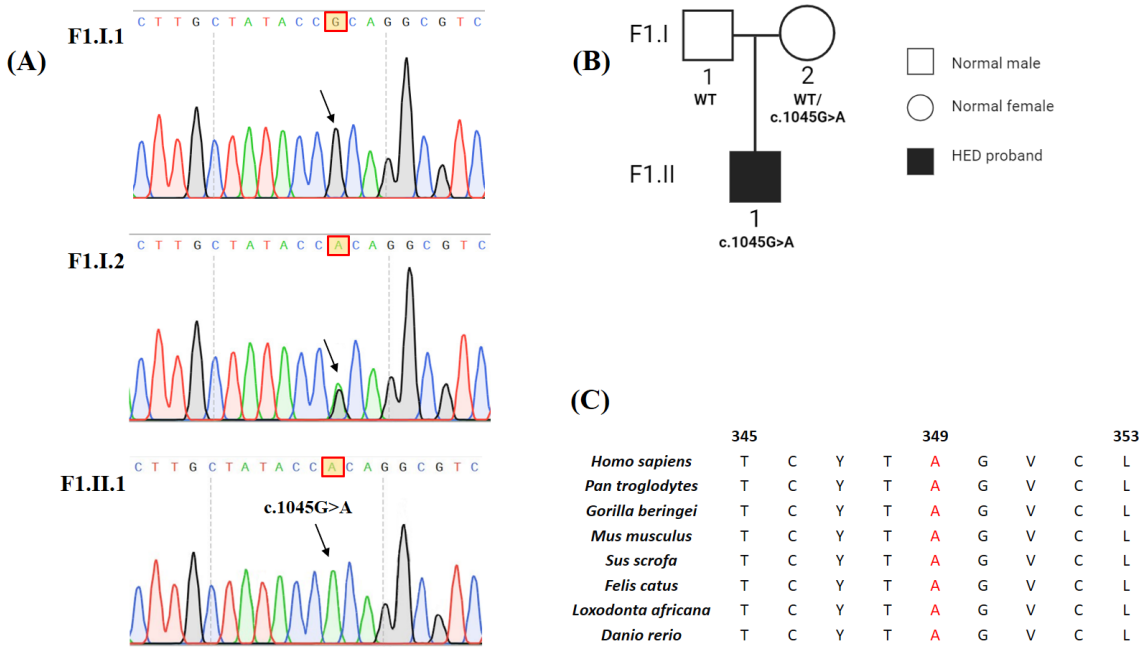

Figure 2. (A) Sanger sequencing results of the examined family. (B) The pedigree of the family showed the phenotype-genotype correlation. (C) Conservation of the amino acid alanine at position 349 of EDA-A1.

The result of Sanger sequencing revealed a hemizygous missense variant c.1045G>A (p.A349T) in exon 8 of the *EDA* gene of the proband (Figure 2A). The presence of the variant c.1045G>A (p.A349T) in the other family members was then determined using similar methods. The sequencing analysis showed that the father of the patient was hemizygous for the wild-type allele, while the mother was the carrier for the variant (Figure 2A and B). In addition, the variant occurred in alanine 349, which is a highly conserved region in the EDA-A1 protein. According to the data from the UCSC Genome Browser, the amino acid sequence containing alanine 349 was the same between humans and seven other species (Figure 2C).

Table 2. Effect prediction of c.1045G>A (p.A349T)

Tool	Predicted effect
MutationTaster	disease-causing
SIFT	damaging
PolyPhen-2	probably damaging (score 1.000)
CADD score	26.2 (deleterious)

***In silico* prediction of the effect of the variant c.1045G>A (p.A349T)**

The effect of the variant c.1045G>A (p.A349T) was predicted using four different tools: MutationTaster, SIFT, PolyPhen-2, and CADD. The prediction results from all tools indicated that this variant was potentially pathogenic (Table 2**Error! Reference source not found.**).

DISCUSSION

In this study, PCR and Sanger sequencing techniques were employed to specifically focus on the *EDA* gene variants, as they were well recognized as the primary causative factor of HED. We identified a variant c.1045G>A (p.A349T) in the *EDA* gene in the patient. The c.1045G>A could lead to the substitution of amino acid alanine for threonine at position 349 of the TNF homology subdomain of the EDA-A1 protein. The amino acid alanine at this position was highly conserved among different species (Figure 2C), indicating that this amino acid was important for the function of EDA-A1. Furthermore, the variant was predicted to be deleterious using MutationTaster, SIFT, PolyPhen-2, and CADD (Table 2). Specifically, the PolyPhen-2 score of c.1045G>A (p.A349T) was 1, indicating that the effect of the variant was anticipated with a high level of confidence. Moreover, the score of CADD for this variant was higher than 20, signifying that it was one of the top 1% most damaging substitutions in the human genome.

The variant c.1045G>A (p.A349T) was detected in the patient who expressed the main symptoms of XLHED, including no sweat gland, cone-shaped or absent teeth, and scant hair. The mother of the patient was the carrier for this variant, while the father was hemizygous for the wild-type allele. The variant c.1045G>A (p.A349T), also known as rs132630317, was found in the Genome Aggregation Database (gnomAD) with the alternative allele frequency of 0.000002186 (https://gnomad.broadinstitute.org/). In addition, this variant has been reported as a pathogenic variant in several patients with different ethnicities. The first investigation conducted in the USA detected the mutation c.1045G>A (p.A349T) in two unrelated patients with XLHED (Monreal *et al.*, 1998). The mutation was determined to be *de novo* in one of the two patients (Monreal *et al.*, 1998). The other research performed in Finland found the mutation c.1045G>A (p.A349T) in a proband with XLHED and his healthy mother, who was a carrier for the mutation (Pääkkönen *et al.*, 2001). In 2004, a separate study in Korea also identified the mutation in a male proband, while the other members of this family were homozygous for the normal allele (Na *et al.*, 2004). Interestingly, the variant c.1045G>A (p.A349T) was found to cause XLHED in a Chinese female carrier who exhibited XLHED traits such as lack of teeth, sparse eyebrows, hypohidrosis, and some other facial abnormalities (Haochen Liu *et al.*, 2022). This female patient was determined to have significant skewed X-chromosome inactivation (XCI) with a ratio of 98:2. However, the particular mechanism of skewed XCI to cause XLHED in female patients was not clear, as its consequences vary among different female carriers (Haochen Liu *et al.*, 2022). Since the variant c.1045G>A (p.A349T) has been detected recurrently in several distinct patients with different ethnicities, it might be a mutational hot spot. Regarding the possible mechanism of the mutation c.1045G>A (p.A349T) to cause XLHED, it was suggested that the substitution of alanine 349 could result in the

destabilization of the overall structure of the EDA-A1 homotrimer by destabilizing the bonds among the monomers, which could be attributed to the difference in the size and the chemical characteristics of alanine with the substituted amino acid (Ruiz-Heiland *et al.*, 2016; Haghighi *et al.*, 2013; Conte *et al.*, 2008). Particularly, the substitution of alanine 349 with threonine could introduce a bulky group with several hydrogen bonds to the interface of EDA-A1 and disrupt the interactions involving aromatic residues located at this position (Conte *et al.*, 2008). In addition, p.A349T occurred in the TNF homology subdomain, an essential domain for the formation of the EDA-A1 homotrimer, which played a crucial role in receptor interaction (Li *et al.*, 2008). Thus, it was hypothesized that the mutation p.A349T could lead to a disruption in the interaction of the EDA-A1 ligand with its receptor, EDAR (Na *et al.*, 2004).

CONCLUSION

In this study, a known mutation c.1045G>A (p.A349T) was detected in the *EDA* gene in a Vietnamese patient who displayed the typical symptoms of XLHED. The segregation of the mutation was confirmed in the family. This mutation might interfere with the EDA-A1 pathway, thereby leading to the abnormal development of hair, nails, teeth, and sweat glands in XLHED patients. The identification of the mutation can contribute to the worldwide knowledge of XLHED as well as provide information for genetic counseling within the examined family.

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