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SHORT REPORT

# Variants of the *ectodysplasin A1 receptor* gene underlying homozygous cases of autosomal recessive hypohidrotic ectodermal dysplasia

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Hypohidrotic ectodermal dysplasia (HED) is a rare genetic condition resulting from defective development of ectodermal derivatives, such as hair, teeth, and sweat glands. Autosomal recessive (AR) forms of HED may be caused by pathogenic variants of the *ectodysplasin A1 receptor (EDAR)* gene that encodes a receptor involved in the NF-κB signaling pathway. Here, we describe three cases of AR-HED in families of Turkish, Austrian, and German-American origin (with or without known consanguinity). In these cases, two out-of-frame deletions and a pathogenic missense variant of *EDAR* were found to be disease-causing due to reduced availability of the respective messenger RNA or impaired interaction of the encoded protein with its binding partner leading to diminished signal transduction. The same missense variant, c.1258C>T (p.Arg420Trp), has actually been reported to be restricted to the Icelandic population and to be associated with non-syndromic tooth agenesis but not HED. As our patient has no known relationship to Icelandic individuals and displays a rather severe HED phenotype, we suggest that EDAR-Arg420Trp is a more widespread variant, possibly with variable clinical expressivity.

**KEYWORDS**

autosomal recessive, EDAR, hypohidrotic ectodermal dysplasia, NF-κB

## 1 | INTRODUCTION

Hypohidrotic ectodermal dysplasia (HED), a rare genetic disorder affecting the development of ectodermal derivatives, is characterized by a triad of hypotrichosis, hypo- or anodontia, and hypo- or anhidrosis, which may lead to life-threatening hyperthermia.[1] Furthermore, the lack or malformation of several eccrine glands often evokes chronic skin issues, keratoconjunctivitis sicca, atrophic rhinitis, and recurrent airway infections.[2,3] In females, breast development may be impaired.[4] The canonical ectodysplasin signaling pathway starts with the binding of ectodysplasin A1 (EDA) to the ectodysplasin A1 receptor (EDAR), leading to the recruitment of the EDAR-associated death domain adapter (EDARADD) protein. Subsequent activation of specific signaling complexes entails the transnuclear migration of NF-κB and transcription of several target genes.[5,6] Most HED cases are caused by pathogenic variants of the X-chromosomal gene *EDA*, followed by variants of the gene *EDAR* (NM_022336.3).[7] The latter often underlie HED with AR inheritance; only patients homozygous or compound-heterozygous for the disease allele display the classic phenotype whereas the heterozygous state is associated with mild if any symptoms.

While the effect of a missense variant depends on its position and the kind of substitution, a frameshift mutation leading to a premature termination codon (PTC) usually results in nonsense-mediated messenger RNA (mRNA) decay (NMD).[8] In this report, we describe three familial cases of AR-HED caused by pathogenic variants of *EDAR*.

## 2 | SUBJECTS AND METHODS

Three individuals with HED and some of their relatives were investigated. All adults gave written informed consent to the use of their DNA for molecular analysis of HED-related genes and further research. In the case of minors, parental consent was obtained. The study was conducted according to the principles of the declaration of Helsinki.

### 2.1 | Assessment of sweat duct density and sweat production

Palmar sweat duct densities were determined by reflectance confocal laser-scanning microscopy with the VivaScope 1500 (Caliber Imaging & Diagnostics, New York, New York). Pilocarpine-induced sweating was quantified by sweat collection over 30 minutes from an area of 57 $mm^2$ of the forearm after standardized stimulation using the Wescor 3700 device (Wescor, Logan, Utah).

### 2.2 | DNA analysis

Genomic DNA extraction, polymerase chain reaction (PCR), and Sanger sequencing were performed as described previously.[7] The Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, Michigan) was used for analysis of the electropherograms. All detected variants were assessed with the mutation prediction tool Mutation Taster (Charite, Berlin, DE; Cardiff University, Cardiff, UK) and included in the Leiden Open Variation Database (https://databases.lovd.nl/shared/variants/EDAR/unique#object_id=VariantOnTranscriptUnique%2CVariantOnGenome&id=EDAR&order=owned_by_%2CASC&search_transcriptid=00006860&search_owned_by_=Sigrun%20Wohlfart&page_size=100&page=1).

### 2.3 | Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) was conducted with the commercial kits SALSA MLPA P183 EDA-EDAR-EDARADD probemix version C1 and SALSA MLPA EK1 reagent kit-Cy5 (MRC-Holland, Amsterdam, The Netherlands). Data were analyzed as described previously.[7]

### 2.4 | Vectors, site-directed mutagenesis, and transfection

For functional studies, the following gene expression vectors were used: pGL3 basic, NF-κB-Luc containing a luciferase gene under the control of the NF-κB promoter, pCR3 with the full-length wild-type cDNA of EDAR (provided by Pascal Schneider), pcDNA3.1 containing the wild-type cDNA of the hemagglutinin (HA)-tagged murine death domain region of EDAR (highly homologous between mouse and human; provided by Paul Overbeek), and pcDNA3.1 containing the wild-type EDARADD cDNA with a Myc epitope tag (provided by Asma Smahi). Site-directed mutagenesis was performed as described previously.[9]

AD293 cells were cultured in Dulbecco's Modified Eagle`s Medium with penicillin/streptomycin and 10% fetal calf serum (FCS) at 37°C and 5% $CO_2$. Vectors were (co-)transfected into 2 × $10^6$ AD293 cells using the K2 Transfection System (Biontex Laboratories GmbH, Munich/Laim, Germany), followed by incubation of the cells for 24 to 48 hours.

### 2.5 | RNA isolation, reverse transcription, and quantitative PCR

Total RNA was extracted from 1 × $10^6$ AD293 cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts) and a standard protocol for RNA isolation. Reverse transcription (RT)-PCR for cDNA synthesis was performed with the Moloney Murine Leukemia Virus Reverse Transcriptase system (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. The iQ SYBR Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) and exon-exon spanning primers to avoid genomic DNA amplification were used for quantitative real-time PCR (qPCR; primer sequences available upon request). Relative gene expression levels were calculated by the delta-delta Ct method.

### 2.6 | Co-immunoprecipitation and immunoblotting

The EDAR-EDARADD interaction was investigated by co-immunoprecipitation and subsequent immunoblotting as described previously.[9] Briefly, cell lysates containing both Myc-tagged EDARADD and another gene product of interest were incubated with anti-Myc antibodies (host: mouse), followed by precipitation of immunoglobulins with A/G agarose beads. Proteins were separated by SDS-acrylamide gel electrophoresis and analyzed by Western blotting with anti-HA antibodies (host: rabbit). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were applied as secondary antibodies to visualize bound immunoglobulins.

### 2.7 | Luciferase assays

After co-transfection of wild-type or mutant gene expression vectors together with the NF-κB-dependent reporter gene vector NF-κB-Luc into 3 × $10^5$ AD293 cells, luciferase activities in the cell lysates were determined (combined data from six experiments, each sample measured in duplicate). Vector pGL3 basic served as transcriptionally independent control.

## 3 | RESULTS

### 3.1 | Case reports

The 26-year-old female index patient of family #1 showed the classic phenotype of HED with thin and sparse hair, missing eyebrows, everted lips, saddle nose, and oligodontia (only two teeth and two additional tooth buds in the mandible, seven teeth in the maxilla; Figure 1A). She also suffered from ozaena, photosensitivity, severe underweight (body mass index < 15 $kg/m^2$), and bilateral amazia. In her early childhood, she had experienced several episodes of severe hyperthermia. A skin biopsy at that time had revealed a complete absence of sweat glands as the reason for anhidrosis. Her Turkish first-cousin parents had two more children without any sign of HED. A sister of the mother died at the age of 9 months due to hyperpyrexia. The index patient was found to carry the *EDAR* variant c.486del (p.Ser163ArgfsX26) homozygously,[7] while her parents are heterozygous (Figure 1B).

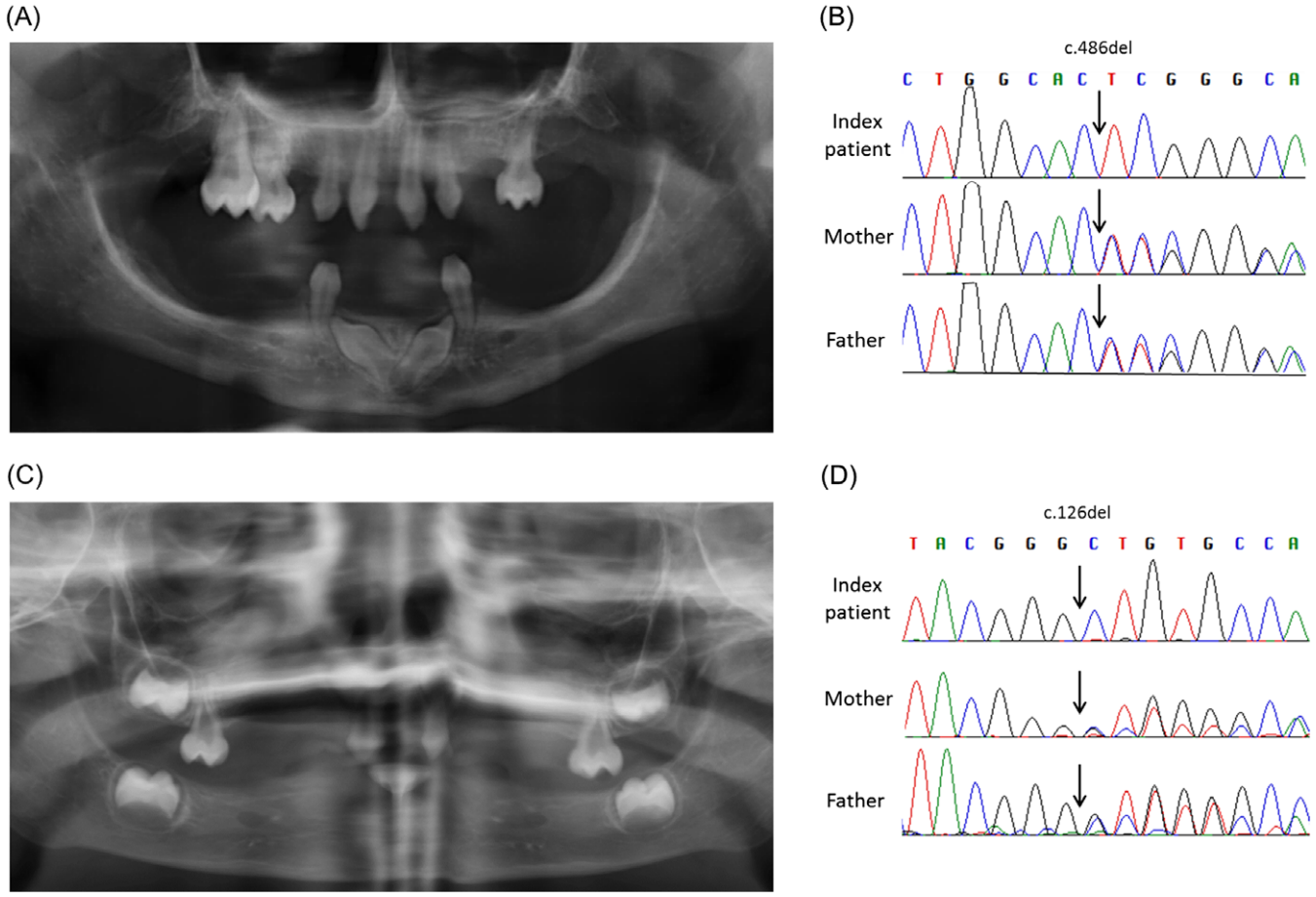


**FIGURE 1** Oligodontia and genotypes of index patients #1 and #2. A, Panoramic radiograph of patient #1 at 10 years of age. B, DNA sequencing showed the *EDAR* variant c.486del. C, Panoramic radiograph of patient #2 at the age of 3 years and D, DNA sequence chromatograms showing the *EDAR* variant c.126del [Colour figure can be viewed at wileyonlinelibrary.com]

The 5-year-old patient #2 is the firstborn of an Austrian couple displaying mild signs of HED. He had suffered from several episodes of unexplained fever during his first years of life due to negligible sweat production (<1 μL). At the age of three, two conical teeth erupted. These were followed later by two rather normal molars but no further primary teeth. Secondary dentition will most probably be restricted to four teeth in total (Figure 1C). Additional HED-related features included scanty hair, dry and eczematous skin, and chronic nasal congestion. The parents had grown up in the same geographic region, but were not closely related. In this family, the *EDAR* variant c.126del (p.Leu43CysfsX60)[7] was detected heterozygously in the parents and homozygously in their son (Figure 1D).

The index patient of family #3, a 19-year-old man of asthenic build with German parents and an US-American maternal grandfather, had been diagnosed with HED after numerous events of hyperthermia during childhood. His hair and eyelashes were sparse and light-colored; he had no eyebrows but normal beard growth, displayed further facial characteristics of HED (Figure 2A), and had only three teeth (Figure 2B). Quantification of pilocarpine-induced sweating and confocal laser-scanning microscopy of his skin showed anhidrosis because of a complete absence of sweat glands (Figure 2C). The variant c.1258C>T (p.Arg420Trp) in the region encoding the death domain of EDAR was found to be present heterozygously in his grandmother, mother, one of two uncles, and a cousin (Figure 2D). The index patient himself proved to be homozygous for this trait (Figure 2E). Hemizygosity because of an exon deletion was excluded by MLPA.

## 3.2 | Functional studies

The deletions c.126del (in exon 3 of *EDAR*) and c.486del (in exon 6) lead to frameshift-induced PTCs in exons 4 and 7, respectively. Hence, the mutant mRNAs were expected to be degraded by NMD. Sequencing of amplified cDNA obtained after transfection of each variant into AD293 cells revealed the presence of both mutant and wild-type transcripts (endogenous expression of *EDAR* in AD293 cells; data not shown). Determination of relative gene expression levels (Figure 3A), however, showed that the amount of mRNA with the deletion was much lower than that of the wild-type transcript (both including the full-length endogenous mRNA). Even if the deletions did not cause complete degradation of mutant transcript, any shortened EDAR proteins which lack the death domain would be unable to bind to EDARADD.

The impact of the missense variant c.1258C>T on the interaction of EDAR (HA-conjugated) with its binding partner EDARADD (Myc-tagged) was investigated by co-immunoprecipitation using anti-Myc antibodies and subsequent Western blotting with anti-HA antibodies. As expected, wild-type HA-EDAR could be detected by Western blot

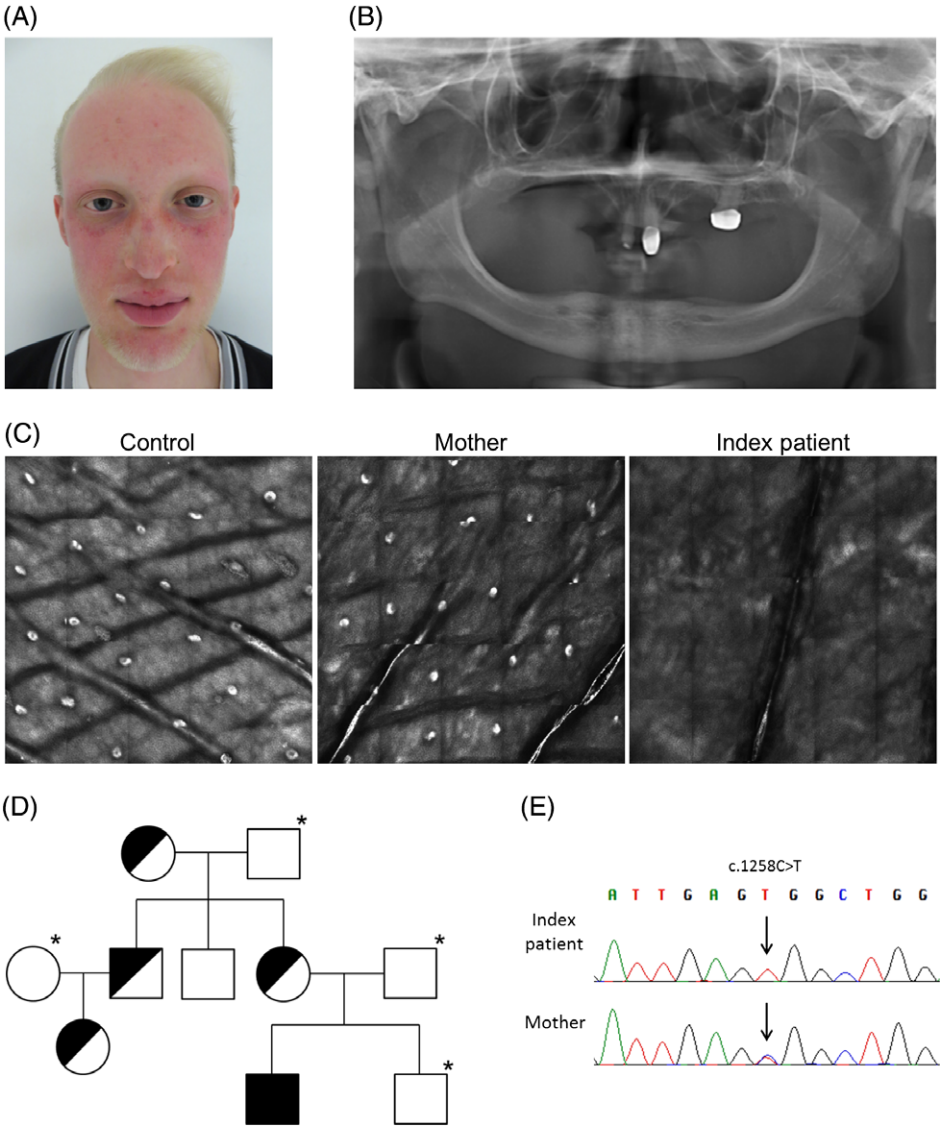


**FIGURE 2** Phenotypic and genetic characteristics of family #3. A. Portrait picture of the index patient depicting hypohidrotic ectodermal dysplasia-related facial features, such as sparse hair, absence of eyebrows, thick lips, and periorbital wrinkling. B, Panoramic radiograph of the index patient showing only three permanent teeth. C, Laser scanning microscopy images indicating normal number and distribution of sweat ducts in the skin of the patient's mother but complete absence of sweat ducts in the patient. D, Pedigree: Females are represented by circles, males by squares, heterozygous carriers by half-shaded symbols, and subjects homozygous for the mutant allele by filled symbols. Family members marked with an asterisk had been unavailable for genotyping. E, DNA sequence chromatograms of the *EDAR* variant c.1258C>T [Colour figure can be viewed at wileyonlinelibrary.com]

confirming a normal interaction between the proteins (Figure 3B, first row), while HA-EDAR with the variant p.Arg420Trp was not detectable (but present in the lysates). This indicates an abolished ability to bind to EDARADD-Myc. Although NF-κB-dependent luciferase reporter assays showed some residual NF-κB activation despite the mutation, the Arg420Trp substitution led to a significant reduction of luciferase activity compared with the wild-type (Figure 3C).

## 4 | DISCUSSION

In case of very rare conditions, such as AR-HED, identical pathogenic variants of a given gene on both homologous chromosomes are often identical by descent, either because of consanguineous marriages or geographic proximity.[10] The homozygous index patients of this study all displayed distinct HED-associated symptoms, whereas their heterozygous relatives were either asymptomatic or very mildly affected (thin hair, slightly reduced sweat production, lack of a few permanent teeth). The *EDAR* variants in families #1 and #2 are most probably identical by descent. The affected individuals carry out-of-frame deletions predicted to evoke NMD, which may occur incompletely in a system of massive overexpression as used in this study. *EDAR* variant c.1258C>T (p.Arg420Trp) has so far been reported only in a recent Icelandic publication referring to a genome-wide association study for tooth agenesis.[11] Our protein binding assays elucidated that this variant caused a complete abrogation of the interaction between the death domains of EDAR and EDARADD. Downstream NF-κB signaling was found to be reduced. There is one more published variant at

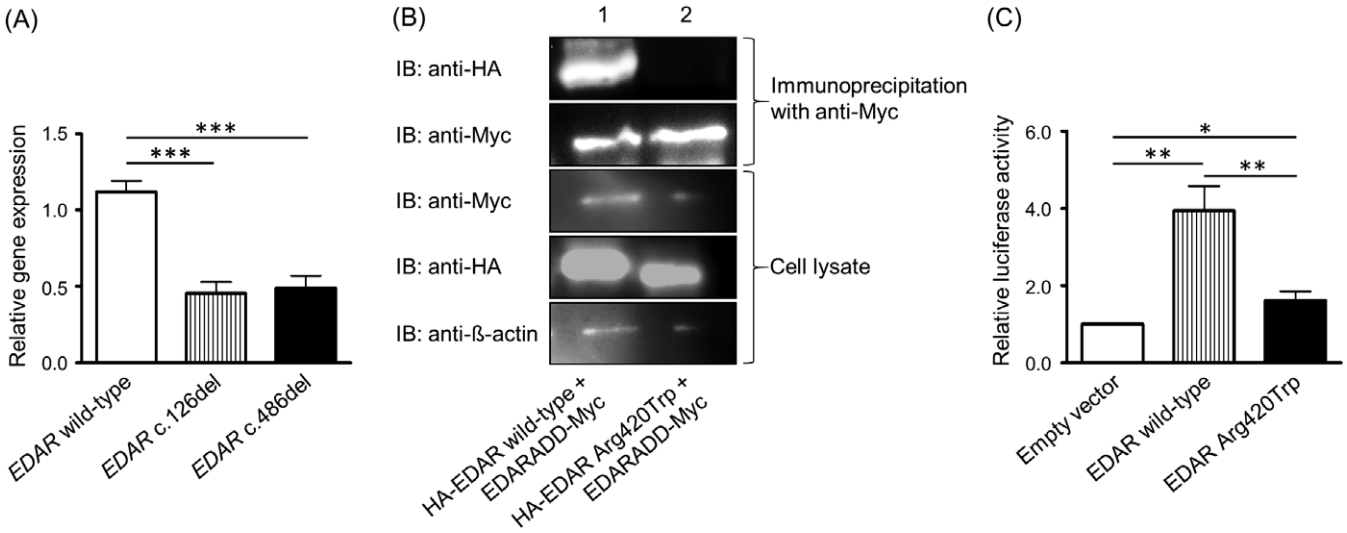


**FIGURE 3** Functional impact of the different *EDAR* variants. A, quantitative polymerase chain reaction analysis showed significantly lower gene expression for the variants c.126del and c.486del in comparison with wild-type *EDAR*. Data were normalized to *GAPDH* and displayed ±SEM; ***$P \leq 0.001$. B, Lysates of AD293 cells containing both Myc-tagged EDARADD and another gene product of interest were incubated with anti-Myc antibodies, followed by immunoprecipitation. Proteins were separated by sodium dodecyl sulfate-acrylamide gel electrophoresis and analyzed by Western blotting with anti-hemagglutinin (HA) antibodies, detecting only EDAR protein that was able to bind to EDARADD. Wild-type HA-tagged EDAR interacted normally with Myc-tagged EDARADD, while HA-EDAR with the Arg420Trp substitution failed to bind to EDARADD-Myc (first row). Western blots of the same immunoprecipitates with anti-Myc antibodies (second row) and of the original cell lysates with either anti-Myc (third row) or anti-HA antibodies (fourth row) showed the presence of comparable amounts of the respective proteins. Anti-ß-actin antibodies were used for normalization (fifth row). C, NF-κB-dependent luciferase reporter assays indicated that the Arg420Trp substitution is associated with a significant reduction of NF-κB activity. Data are shown ±SEM; *$P \leq 0.05$; **$P \leq 0.01$

the same amino acid position of EDAR (p.Arg420Gln) which has been considered as causative for AD-HED.[12] The authors of the Icelandic paper described Arg420Trp as a very rare variant associated with a high risk of tooth agenesis and presumed to be restricted to the Icelandic population — because of its absence from The Genome Aggregation Database (gnomAD).

Family #3 has no known genetic relationship to the Icelandic population. Thus, the homozygous state of the index patient is striking and leads us to hypothesize that EDAR-Arg420Trp may be an underestimated variant, not limited to the Icelandic ethnicity. The unlikely possibility of uniparental isodisomy, an alternative explanation for the homozygosity of the index patient, was not further investigated. Substitution of an amino acid in the death domain has been reported to underlie impairment of EDAR function "that causes tooth agenesis with incomplete penetrance."[13] In case of such pathogenic missense variants, however, non-syndromic tooth agenesis and AR-HED are probably not separate disease entities but may reflect the phenotypes of a heterozygous carrier and a homozygous individual, respectively, or may indicate variable clinical expressivity as observed for variant EDAR-Arg420Trp also in our family #3.

Because the most frequent form of HED appears to be amenable to prenatal therapy with a recombinant EDA protein,[14] further research into the biology of EDAR and on variants affecting its function is certainly warranted.

## ACKNOWLEDGEMENTS

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## CONFLICTS OF INTEREST

Nothing to declare.

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