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A Novel Missense Mutation in the Gene *EDARADD* Associated with an Unusual Phenotype of Hypohidrotic Ectodermal Dysplasia

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Hypohidrotic ectodermal dysplasia (HED) is a rare disorder characterized by deficient development of structures derived from the ectoderm including hair, nails, eccrine glands, and teeth. HED forms that are caused by mutations in the genes *EDA*, *EDAR*, or *EDARADD* may show almost identical phenotypes, explained by a common signaling pathway. Proper interaction of the proteins encoded by these three genes is important for the activation of the NF-κB signaling pathway and subsequent transcription of the target genes. Mutations in the gene *EDARADD* are most rarely implicated in HED. Here we describe a novel missense mutation, c.367G>A (p.Asp123Asn), in this gene which did not appear to influence the interaction between EDAR and EDARADD proteins, but led to an impaired ability to activate NF-κB signaling. Female members of the affected family showed either unilateral or bilateral amazia. In addition, an affected girl developed bilateral ovarian teratomas, possibly associated with her genetic condition. © 2015 Wiley Periodicals, Inc.

Key words: amazia; EDARADD; hypohidrotic ectodermal dysplasia; NF-κB signaling; teratoma

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INTRODUCTION

Hypohidrotic ectodermal dysplasia (HED), a rare congenital disorder affecting the normal development of ectodermal structures, is characterized by a triad of hypotrichosis, hypodontia and hypohidrosis, the latter of which can lead to life-threatening hyperthermia [Blüschke et al., 2010]. Other typical symptoms such as dryness of the eyes, skin, nose and airways are the result of a deficient development of exocrine glands and may lead to atrophic rhinitis, ocular problems and recurrent infections of the upper respiratory tract [Clarke et al., 1987; Dietz et al., 2013]. HED has been associated with a distinct facies and special clinical features, such as dark wrinkles under the eyes, frontal bossing, prominent lips, protruding ears, abnormal teeth, malformation of mammary glands and a reduced ability to breast-feed [Cluzeau et al., 2011; Lindfors et al., 2013; Burger et al., 2014; Al Marzouqi et al., 2014].

If HED is caused by mutations in the genes *EDA* (location: Xq13.1; MIM *300451; encoding ectodysplasin A), *EDAR* (location: 2q12.3; MIM *604095; encoding the ectodysplasin A receptor, EDAR) or *EDARADD* (location: 1q42-q43; MIM *606603; encoding the EDAR-associated death domain adapter, EDARADD), it may lead to an almost undistinguishable phenotype as the result of a common linear signaling pathway [Kere et al., 1996; Monreal et al., 1999; Headon et al., 2001]. This NF-κB pathway starts with the binding of ectodysplasin A to its receptor, leading to the recruitment of the adapter EDARADD via interaction of its death domain with that of EDAR [Thesleff and Mikkola, 2002]. The most common form of HED is an X-linked disorder caused by mutations in the gene *EDA* (MIM #305100). Autosomal recessive and autosomal dominant forms of classical HED are often due to mutations in either *EDAR* (MIM #129490 and #24900) or *EDARADD* (MIM #614940 and #614941). The gene *EDARADD* encodes two isoforms (A and B), which only differ in the N-terminus, but not in the highly conserved death domain. Our nomenclature of mutations refers to the longer transcript variant A (RefSeq NM_145861.2)

Conflict of interest: None.

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[Yan et al., 2002]. To date, six different HED-causing mutations in the gene *EDARADD* have been reported [Headon et al., 2001; Bal et al., 2007; Chassaing et al., 2010; Suda et al., 2010; Cluzeau et al., 2011; Koguchi-Yoshioka et al., 2014]. In this study, we identified the causative mutation in members of a German, non-consanguineous family with more than just the typical symptoms of HED.

SUBJECTS AND METHODS

The index patient, a 16-year-old girl, her mother and her grandfather all have very thin, brittle, sparse or even absent hair and a reduced sweating ability (Fig. 1A). Dental panoramic radiographs of the index patient confirmed the absence of a considerable number of permanent teeth (six in the upper jaw, eight in the lower jaw; Fig. 1D). Her mother and grandfather also have a reduced number of permanent teeth. Furthermore, the index patient suffers from a bilateral amazia, while unilateral amazia was evident in her mother. Most interestingly, the girl developed bilateral mature ovarian teratomas containing hair, sebaceous and sweat glands (Fig. 1E). After informed consent was obtained, genomic DNA was extracted from blood samples (QIAamp DNA Blood Mini Kit from Qiagen, Hilden, Germany) from the index patient, her brother, mother,

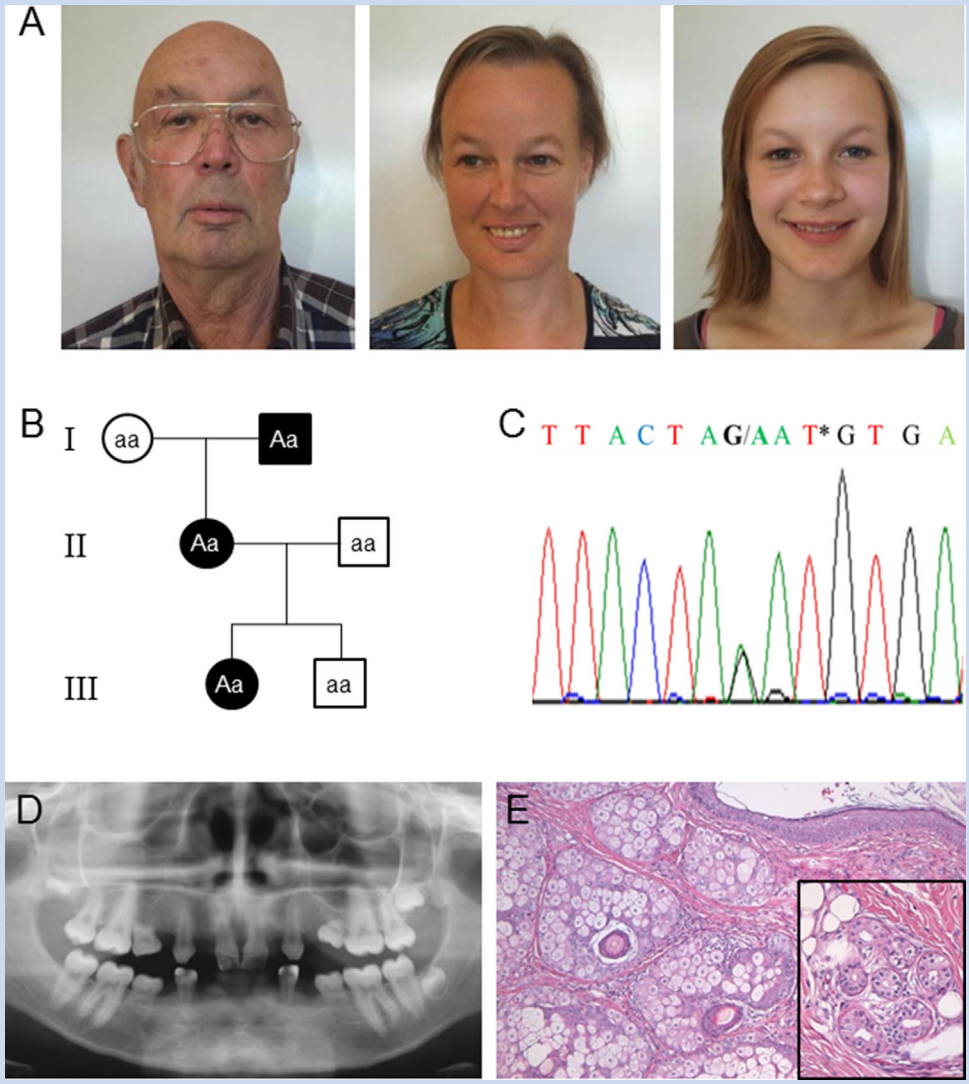

FIG. 1. Phenotypic and genetic features of affected family members. A: Portrait pictures of the grandfather, the mother and the index patient (from left to right) showing their facial characteristics like periorbital wrinkles, prominent lips and their sparse/absent hair. B: Pedigree of the patient's family with an autosomal dominant inheritance pattern. Affected individuals are represented by filled circles (females) or squares (males). Upper case *A* stands for the mutant, lower case a for the wild-type allele. C: Sequencing chromatogram of the heterozygous missense mutation c.367G>A (p.Asp123Asn) in the gene *EDARADD*. The polymorphism rs604070 is indicated by an asterisk. D: Panoramic radiograph of the index patient showing her reduced number of permanent teeth. E: Histologic section of the first teratoma (magnification 1:10) showing ectodermal structures such as keratinized epithelium, hair and sebaceous glands as well as sweat glands (insert, magnification 1:16).

father and grandfather. Polymerase chain reaction and subsequent Sanger sequencing was performed using specific primers covering the exons and the intron-exon boundaries of the genes *EDA*, *EDAR*, and *EDARADD* (sequences available upon request).

Further functional studies were performed using the expression vector pcDNA3.1 containing either the wild-type cDNA of the gene *EDAR* with a HA epitope tag or the wild-type cDNA of the gene *EDARADD* with a Myc epitope tag. Site-directed mutagenesis was performed in order to introduce the relevant mutation into cDNA of the gene *EDARADD* (QuikChange II Site-directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA), followed by Sanger sequencing for verification of the constructs.

The impact of the mutation on the EDARADD protein's ability to bind to EDAR was investigated by co-immunoprecipitation and subsequent immunoblotting. For this purpose the constructs mentioned above were co-transfected into 2×10^6 Ad293 cells. After incubation for at least 24 hr the medium was discarded and cells were washed twice with ice-cold PBS, followed by treatment with a lysis buffer. Portions of each cell lysate were first incubated with anti-Myc antibodies (Cell Signaling Technology, Leiden, The Netherlands) binding to wild-type and mutated EDARADD constructs due to their Myc-tags, then A/G agarose beads (Santa Cruz Biotechnology, Dallas, TX) were added for antibody precipitation. The precipitated antibody-bound proteins were washed, re-dissolved in sample buffer by boiling, separated by SDS-acrylamide gel electrophoresis, and analyzed by Western blotting with anti-HA as primary antibody, detecting only EDAR proteins that were able to bind to EDARADD. Western blot analysis of the untreated original cell lysates with anti-HA or anti-Myc antibodies, respectively (dilutions 1:1,000), revealed the relative expression levels of HA-EDAR and EDARADD-Myc proteins. GAPDH antibodies were used for normalization. HRP-conjugated anti-mouse (for Myc-tags) and anti-rabbit (for HA-tags) immunoglobulins were applied as secondary antibodies to visualize bound antibodies using the enhanced chemiluminescence (ECL) detection system for immunoblots.

The impact of the mutation on downstream activation of NF-κB was investigated with an NF-κB-dependent reporter gene construct. Therefore, a co-transfection of the pBIIxLuc plasmid containing a luciferase gene under the control of an NF-κB promoter and the expression vector pcDNA3.1 containing either wild-type *EDARADD* cDNA or the mutated *EDARADD* cDNA into 3×10^5 Ad293 cells was performed (K2 Transfection System, Biontex, Munich/Laim, Germany) and luciferase activity in the cell lysates was determined after 24 hr in duplicates. Data were collected from three independent experiments.

RESULTS

We detected a novel missense mutation c.367G>A (p.Asp123Asn) in the death domain of the gene *EDARADD*, which co-segregated

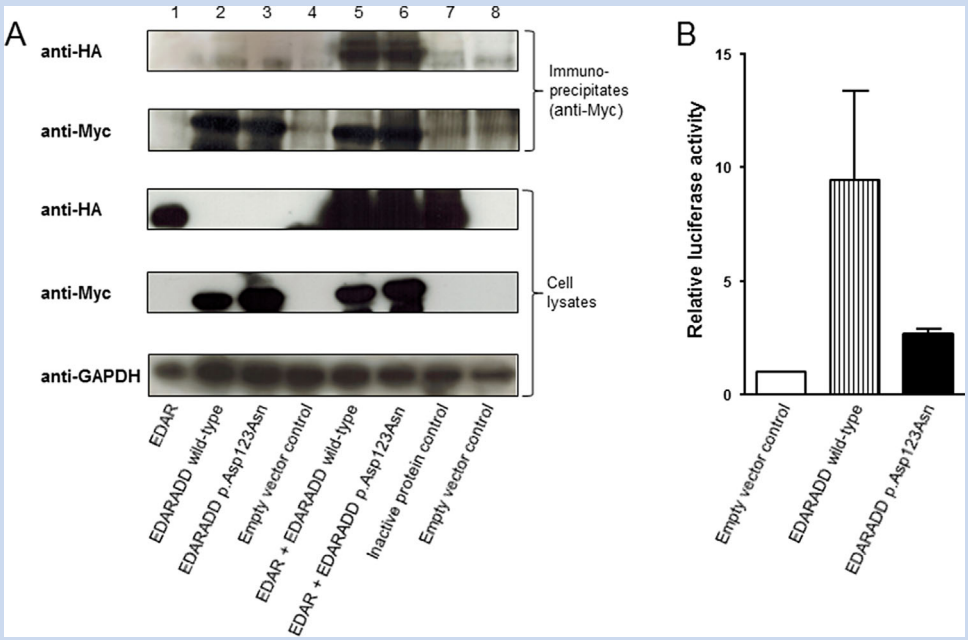

FIG. 2. Functional studies of the impact of the *EDARADD* mutation p.Asp123Asn. A: Co-immunoprecipitation with anti-Myc antibodies and subsequent immunoblotting with anti-HA antibodies revealed that Myc-tagged wild-type and mutated EDARADD proteins bind to EDAR protein with similar intensity (first row, lanes 5 and 6). Further immunoblots of the same immunoprecipitates with anti-Myc antibodies (second row) and of the original cell lysates with either anti-HA (third row) or anti-Myc antibodies (fourth row) were used to demonstrate the presence and similar expression levels of the proteins. GAPDH antibodies were used for normalization (fifth row). B: NF-κB luciferase assays showed a marked reduction of NF-κB activity indicated by relative luciferase activity in comparison to the wild-type. Data are expressed as mean of the relative luciferase activity (+/−SEM) of three independent experiments, each performed in duplicate.

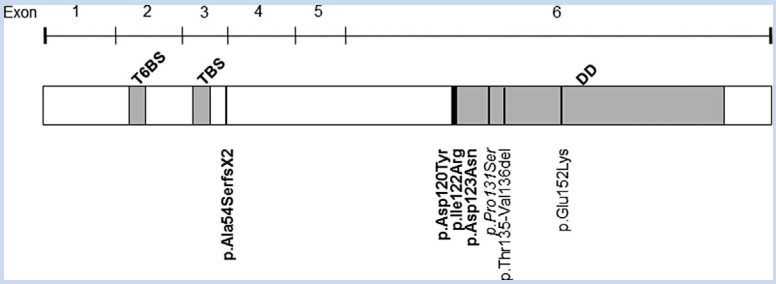

FIG. 3. Scheme of EDARADD with its functional domains and distribution of known mutations in the corresponding gene. The six exons of the gene *EDARADD* in relation to functional domains of the EDARADD protein are shown. EDARADD contains binding sites for both TRAF6 (T6BS) and other TRAF proteins (TBS), and a death domain (DD), known to be responsible for multimerization and interaction with EDAR. Published mutation sites in EDARADD (including the one reported here) are indicated (bold, autosomal dominant mode of inheritance; italics, unknown inheritance pattern; regular style, autosomal recessive mode of inheritance). The nomenclature of all mutations was adjusted to the transcript variant A of *EDARADD* (RefSeq NM_145861.2).

with the disease, as only the affected members of the family were heterozygous carriers of this variant (Fig. 1B and C). The mutation prediction tool Mutation Taster calculated a probability of 0.95 for it to be disease-causing (Charité, Berlin, Germany; Cardiff University, Cardiff, UK). The missense variant is absent from the Exome Aggregation Consortium (ExAC) control cohort and has been included in the Leiden Open Variation Database (http://databases.lovd.nl/shared/variants/0000071780#06861).

Myc-tagged EDARADD proteins and their potential binding partners were isolated by co-immunoprecipitation with anti-Myc antibodies and analyzed by Western blotting with anti-HA antibodies to detect any bound HA-tagged EDAR proteins. There was no noticeable difference between the EDAR protein bands in the EDARADD wild-type/EDAR and the EDARADD-Asp123Asn/EDAR lanes, indicating that the mutation does not severely reduce the binding ability of these two proteins via their respective death domains (Fig. 2A, first row, lanes 5 and 6). Both the mutated and the wild-type EDARADD were able to activate NF-κB in a dose-dependent manner (data not shown), but the *EDARADD* p.Asp123Asn mutation led to a severely impaired activity (Fig. 2B).

DISCUSSION

Mutations in the gene *EDARADD* are rarely implicated in cases of ectodermal dysplasia and can cause either autosomal recessive or autosomal dominant HED with clinically indistinguishable phenotypes. In this study, we detected a heterozygous missense mutation that, due to its segregation pattern and effect on NF-κB activity, is assumed to be the HED-causing variant in this family. This mutation has not been described in the literature yet and represents the seventh published HED-causing variant in the gene *EDARADD*. Interestingly, six of these mutations were found in exon 6 within or very close to the region encoding the death domain. Only one mutation is situated elsewhere, but also alters the death domain due to premature termination of translation (Fig. 3).

Protein binding studies revealed no significant restraint of EDARADD binding to EDAR. This indicates that even a marginally reduced binding ability (not detectable by Western blot), resulting from a minor deviation of the highly conserved death domain, could have a relevant effect on downstream signaling. As the EDARADD protein, like other death domain proteins, is known to self-associate via its death domain, the mutation may disturb EDARADD multimerization and thereby reduce signal transduction [Headon et al., 2001]. This impaired ability to activate NF-κB signaling probably affects transcription of developmentally relevant genes and explains the phenotype observed in this family. To our knowledge, this is the first reported case of ovarian teratomas in a patient with HED, although one young female suffering from both congenital hypodontia and ovarian teratomas has been described [Navale et al., 2014]. An association between tooth agenesis and the development of ovarian cysts or cancer has been discussed repeatedly [Chalothorn et al., 2008; Bonds et al., 2014]. It is still unclear whether there are common genetic factors affecting both tooth development and susceptibility to the formation of ovarian tumors, although in half of the dually affected patients an independent cause of the two conditions was found [Bonds et al., 2014]. Systematic mapping of HED-causing mutations and assessment of genotype–phenotype correlations will enable a deeper understanding of the pathogenesis of HED and is a prerequisite for the evaluation of future medical treatments.

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