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A novel splicing mutation of ectodysplasin A gene responsible for hypohidrotic ectodermal dysplasia

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Hypohidrotic ectodermal dysplasia (HED) is characterized by hypohidrosis, hypodontia, sparse hair, and characteristic facial features. This condition is caused by an ectodysplasin A (*EDA*) gene mutation. In this study, we examined two HED pedigrees and investigated the molecular genetics of the defect. Direct sequencing analysis revealed a previously unidentified mutation in the *EDA* splice donor site (c.526 + 1G>A). The function of the mutant *EDA* gene was predicted through online investigations and subsequently confirmed by splicing analysis in vitro. The mutation resulted in the production of a truncated EDA-A1 protein caused by complete omission of exon 3. This novel functional skipping–splicing EDA mutation was considered to be the cause of HED in the two pedigrees reported here. Our findings, combined with those reported elsewhere, provide an improved understanding of the pathogenic mechanism of HED as well as important information for a genetic diagnosis.

KEYWORDS
ectodysplasin A, hypohidrotic ectodermal dysplasia, mutation, splicing

1 | INTRODUCTION

Ectodermal dysplasia (ED) comprises a group more than 170 types of congenital disorders resulting from abnormal development of ectoderm-derived structures (Pinheiro & Freire-Maia, 1994; Priolo & Lagana, 2001). The most common type of ED, hypohidrotic ectodermal dysplasia (HED), occurs in 4.2 (cases of molecularly confirmed XLHED plus clinically diagnosed cases of HED) in 100,000 cases reported by Nguyen-Nielsen, Skovbo, Svaneby, Pedersen, and Fryzek (2013). The structures involved in ED can be teeth, the craniofacial skeleton, skin, and cutaneous appendages, such as hair, nails, and eccrine sweat glands, resulting in clinical features of hypodontia, hypohidrosis, sparse hair, abnormal nail development, and characteristic facial features including frontal bossing, chin prominence, saddle nose, maxillary hypoplasia, low-set ears, wrinkles, and periorbital hyperpigmentation.

Most HED patients carry mutations in the ectodysplasin A (*EDA*) gene (GenBank accession number: NM 001399), which is located within the X q13.1 region, causing an X-linked recessive pattern of inheritance (Deshmukh & Prashanth, 2012; Kere et al., 1996). The most severely affected patients are often hemizygous males who show the "classic" clinical features, while heterozygous females generally show normal or moderately affected features (Chassaing, Bourthoumieu, Cossee, Calvas, & Vincent, 2006; Clarke, Phillips, Brown, & Harper, 1987; Deshmukh & Prashanth, 2012; Itin & Fistarol, 2004; Kere et al., 1996).

The EDA-A1 protein, which is the largest and also the most common product encoded by the *EDA* gene, contains 391 amino acids (Podzus et al., 2017). It is a trimeric type II transmembrane protein composed of intracellular and transmembrane domains in addition to a cysteine-rich C-terminal domain and a 19 repeat Gly-X-Y collagenous domain as well as furin and tumor necrosis factor (TNF) homology subdomains (Monreal, Zonana, & Ferguson, 1998).

To date, more than 300 mutations of the *EDA* gene have been reported (Trzeciak & Koczorowski, 2016), including missense, nonsense, and splicing mutations as well as small deletions (Wohlfart,

Hammersen, & Schneider, 2016; Yin, Ye, & Bian, 2013). All 28 of the splicing mutations reported in the database of The Human Gene Mutation Database (HGMD) and the single nucleotide polymorphisms (SNP) of the National Center for Biotechnology Information (NCBI) are point mutations, although none has been validated in functional studies.

Here, we describe the clinical features and molecular characterization of a splicing mutation identified in two Chinese families with X-linked HED. We also performed minigene experiments to elucidate the molecular mechanisms underlying the effects of this mutation.

2 | MATERIALS AND METHODS

2.1 | Subjects

The probands were referred to the School and Hospital of Stomatology, Peking University (China), seeking a diagnosis and treatment of their oligodontia condition. After examination by pediatric dentistry specialists, the probands were diagnosed as HED. Clinical images and radiographs were obtained. All participants or their guardians provided informed consent to the genetic studies. This study has been approved by Peking University School of Stomatology Institutional Review Board (approval number: PKUSSIRB-201628059).

Proband 1 was a 13-year-old boy who presented with the absence of several teeth and a history of delayed dental eruption (upper anterior teeth erupted when he was 13 months old). He showed mild hypohidrosis with no obvious heat intolerance. Any history of tooth extraction or loss was denied. Clinical examination showed slightly sparse hair and eyebrows as well as periorbital hyperpigmentation. However, facial features including frontal bossing, chin prominence, maxillary hypoplasia, and protuberant lips were not obvious in this patient. The development of the middle- and lower-third regions of his face appeared essentially normal. His skin was soft and slightly dry, although the palmar skin appeared to be normal. Intraoral examination revealed congenital absence of most permanent anterior and premolar teeth, a lack of alveolar bone development, normal-shaped existing teeth, and ordinary salivary flow. Moreover, his maternal grandfather had hypodontia and sparse hair and his mother and younger sister also had hypodontia. The phenotypic features of proband 1 and his family members are shown in Figure 1.

Proband 2 was a boy aged 3 years and 10 months who presented with delayed dental eruption (upper anterior teeth erupted at 13 months, and only two upper anterior and four molar teeth had erupted at the time of attendance). He had several family members who appeared to exhibit an absence of teeth, sparse hair, and dry skin. Due to privacy requirements of the guardian, only the pedigree information is shown in this report (Figure 2).

2.2 | Mutation analysis

Blood samples were obtained from both probands and members of their families. Genomic DNA was extracted from peripheral blood leukocytes using TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. DNA sequences were amplified by polymerase chain reaction (PCR) using specific primers covering the exons and intron–exon boundaries of the *EDA* gene as described previously (Bayes et al., 1998; Fan et al., 2008; Kere et al., 1996). Sanger sequences of individual

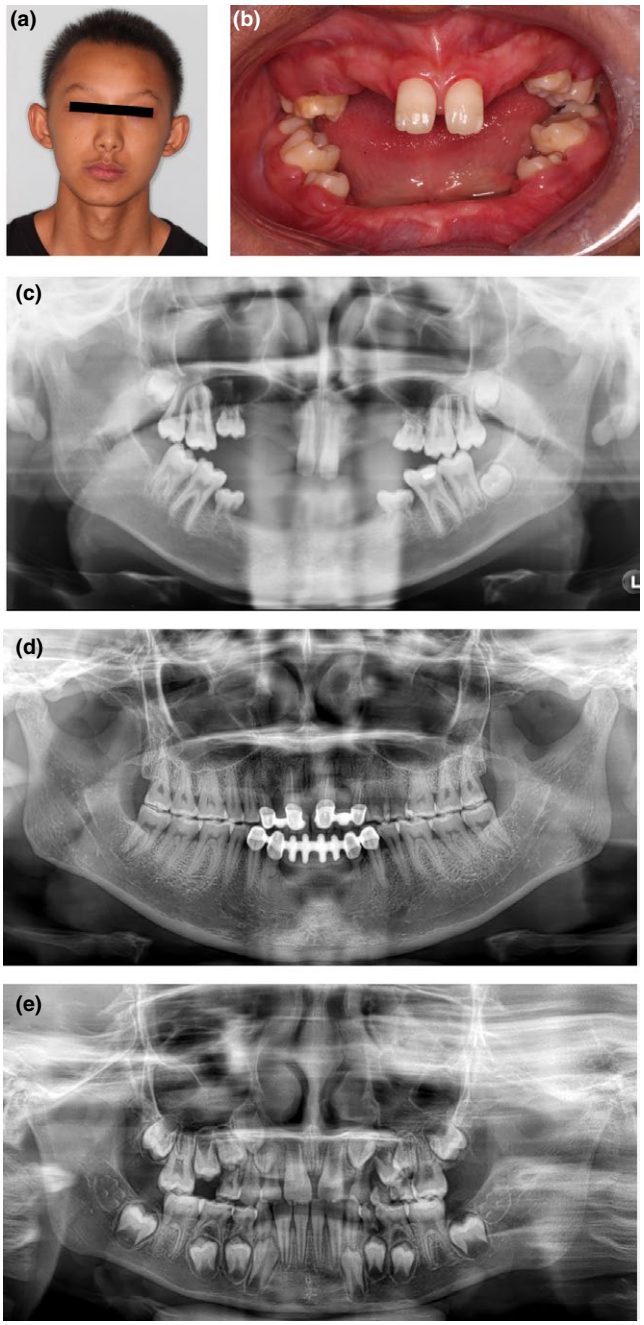


FIGURE 1 Phenotypic features of members of pedigree 1. (a) Photograph of proband 1 showing mild facial features including slightly sparse hair and eyebrows, and periorbital hyperpigmentation. (b and c) Intraoral photograph and panoramic radiograph of proband 1 showing the reduced number of permanent teeth. (d and e) Panoramic radiograph of the mother and younger sister of proband 1 showing the absence of permanent incisors [Colour figure can be viewed at wileyonlinelibrary.com]

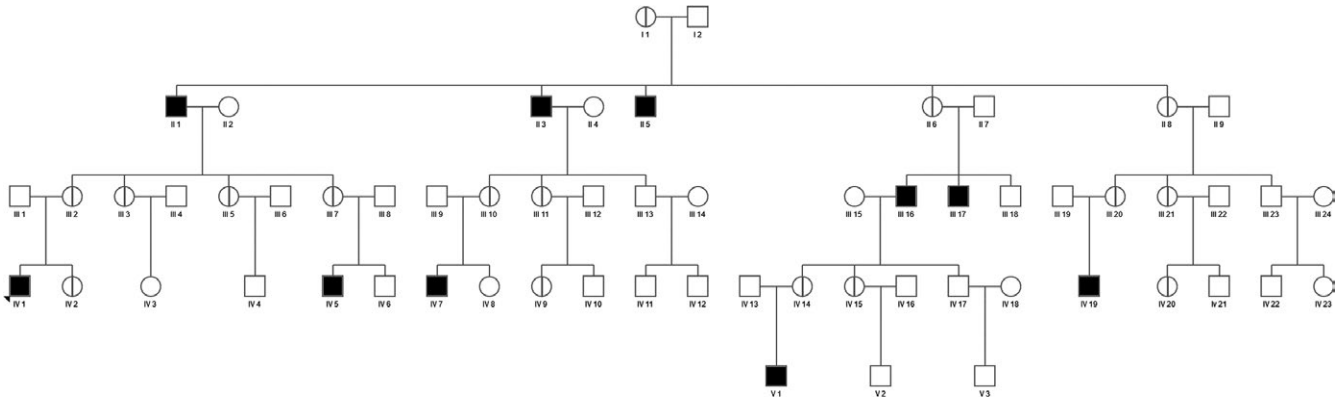


FIGURE 2 Pedigree of the family of proband 2 with an X-linked recessive inheritance pattern. Affected individuals are represented by filled squares (males), while non-penetrant carriers are represented by circles with a vertical line (females)

genes were analyzed using the Chromas chromatogram viewer (http://technelysium.com.au/wp/chromas/) and the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The detected variant was assessed with the mutation prediction tool Mutation Taster (http://www.mutationtaster.org). The splice site mutation was analyzed with Human Splice Finder (HSF, http://www.umd.be/HSF3/).

2.3 | Minigene construct generation, transfection, and RT–PCR

To investigate the effect of the splice site mutation located in intron 3 (c.526 + 1G>A), we designed a minigene including exon 3 and part of introns 2 and 3 of the *EDA* gene using the exon trapping pSPL3 plasmids (Invitrogen Corporation, Carlsbad, CA, USA; Zhao et al., 2016; Kimani et al., 2015). Fragments encoding the wild-type or mutant alleles involving exon 3 (24 bp) and flanked by the upstream (144 bp) and downstream (141 bp) intronic sequences were cloned into the pSPL3 splicing vector using specific primers incorporating the 5′ *Eco*R I and 3′ *Sac* I restriction enzyme sites. Construction of the wild-type (WT) and mutant-type (MT) expression vectors was performed by Genewiz, Suzhou, China. All constructs were verified by direct sequencing.

Human epithelial kidney 293 T (HEK 293 T) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, MA, USA), penicillin (100 U/L), and streptomycin (100 mg/L) at 37°C under 5% CO_2. One day prior to transfection, cells were transferred to 6-well culture plates and cultured to approximately 70%–80% confluence in serum-free medium. Cells were transfected with the WT, MT, and empty control vectors cells using Lipofectamine® 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were harvested at 24 hr post-transfection, and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) for reverse transcriptase PCR (RT–PCR; Primerscript RT reagent; TaKaRa). The pattern of transcripts generated from the transfected minigenes was evaluated by PCR amplification using the following vector-specific primers: a forward primer SD-SA F (5′-TCTGAGTCACCTGGACAACC-3′) and a reverse primer SD-SA R (5′-ATCTCAGTGGTATTTGTGAGC-3′). The thermocycling conditions were as follows: 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, and 70°C for 30 s, followed by a final elongation step at 70°C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis and sequencing.

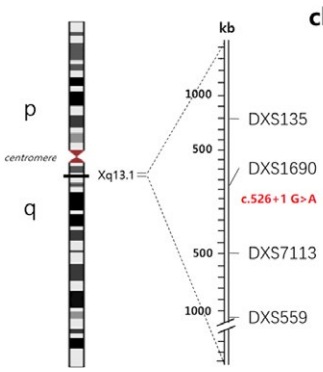
2.4 | Short tandem repeat (STR) marker genotyping

To ascertain whether the c.526 + 1G>A mutant allele of *EDA* has a founder effect in these two families, four STR markers near or within the *EDA* gene were analyzed (Figure 3). Primers for these four STR markers were generated based on the sequences available in the UCSC Genome Browser (http://genome.ucsc.edu/). The sequences of the primer pairs for the detection of the microsatellite markers were as follows: DXS135 (5′-TCAGACACAGGAAGCAGTAG-3′) and (5′- ACTGAATGGGTTTCTGTCAT-3′); DXS1690 (5′-AGACTGGATTTGTACGATGC-3′) and (5′-GGACAGAAAGATGATAAGGG-3′); DXS7113 (5′-CTTCTATTGGTAGCATATCC-3′) and (5′-ATGTGAAGAGACAGCTAAGA-3′); DXS559 (5′-ATCCTTCACCACTGCCTCCA-3′) and (5′-CTCCCTGCTCCCATCGCCAA-3′). The CA repeats of the STR markers were analyzed by PCR and Sanger sequencing.

3 | RESULTS

Direct sequencing revealed the presence of a previously unidentified mutation in the splice donor site of intron 3 (c.526 + 1 G>A) in both probands. The mother, younger sister, and maternal grandfather of proband 1 and the mother and aunt of proband 2 carried the same mutation (Figure 4). This mutation was not found in the healthy members of the two families. The online HSF predicted a score of 94.19 for the WT splice donor site in *EDA* intron 3, while a lower score of 67.35 was predicted for the c.526 + 1 G>A splicing mutation.

RT–PCR analysis of the product of the WT EDA minigene revealed a single band of 287 bp containing the expected SD, exon 3 (24 bp), and SA regions. The mutant RT–PCR product consisted



Position	STR Markers
chrX:68866020-68866247	DXS135
chrX:69895264-69895509	DXS1690
chrX:70592925-70593225	DXS7113
chrX:71661540-71661775	DXS559

FIGURE 3 Location of c.526 + 1 G>A of *EDA* and STR markers linked to the c.526 + 1 G>A mutation on chromosome Xq13.1. Four STR markers were located within or near the *EDA* gene (chrX:69616067-70039472) [Colour figure can be viewed at wileyonlinelibrary.com]

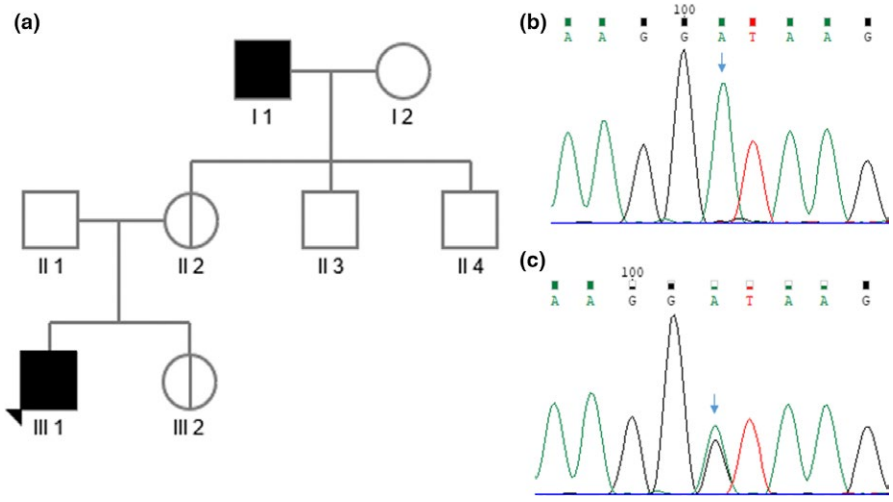


FIGURE 4 (a) Pedigree of the family of proband 1 with an X-linked recessive inheritance pattern. Affected individuals are represented by filled squares (males), while non-penetrant carriers are represented by circles with a vertical line (females). (b) Sequencing chromatogram of the splicing mutation c.526 + 1 G>A in the *EDA* gene of the affected males. (c) Sequencing chromatogram of the heterozygous mutation c.526 + 1 G>A in the *EDA* gene of the female carriers [Colour figure can be viewed at wileyonlinelibrary.com]

of a shorter band of 263 bp and a longer band of 541 bp (Figure 5). Complete omission of exon 3 was indicated by sequencing of the 263-bp product, while the 541-bp product showed an inclusion of 141 bp from intron 3, 113 bp of the pSPL3 introns alongside the pSPL3 exons and *EDA* exon 3.

The STR marker genotyping showed that the CA repeats of DXS1690, DXS7113, and DXS559 of the two probands were coincident, while that of the marker DXS135 was different in the two probands (Table 1).

4 | DISCUSSION

The transmembrane protein ectodysplasin A is expressed by keratinocytes, hair follicles, and sweat glands. The TNF-related ectodysplasin A pathway plays an important role in embryonic development and ectodermal structure formation (Mikkola & Thesleff, 2003). As a TNF-related ligand, it has been confirmed that *EDA* mutations are responsible for X-linked HED. The human *EDA* gene contains 12 exons, with five different transcripts of the EDA protein produced by alternative splicing according to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Among them, the EDA-A1 transcript, which contains eight exons, is the longest and most abundant product generated by transcription of the *EDA* gene.

Here, we identified two pedigrees with HED carrying a novel mutation in the splice donor site of intron 3 c.526 + 1 G>A in *EDA* gene. Online splicing prediction by HSF indicated that this mutation destroys the normal splice donor site, resulting in abnormal gene splicing. We failed to detect *EDA* transcripts in peripheral blood samples by RT–PCR; therefore, we performed in vitro splicing analysis using the exon trapping vector pSPL3 containing the functional SD and SA exons. Two splicing transcripts of different lengths were detected by RT–PCR and sequencing. The shorter transcript revealed skipping of exon 3 in *EDA*, causing a deletion of eight amino acids (169P to 176G) in the EDA-A1 protein, although the open-reading frame remained in-frame. The Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org/seq_submit.php) score was −8.186, and the mutation was predicted to be "deleterious." The longer transcript may have resulted from the activation of a cryptic site in the intronic sequence of pSPL3, causing retention of a downstream intronic fragment. However, due to the length of intron 3 of *EDA* (4,615 bp), it is difficult to carry out analogous in vitro splicing experiments to confirm the actual influence on intron 3.

To date, there are 28 *EDA* splicing mutations in the HGMD database, and none has been validated in functional studies. These include only two records of splicing mutations in exon 3, one synonymous mutation (rs727504537) and one missense mutation with no description of clinical significance (rs777604851). The

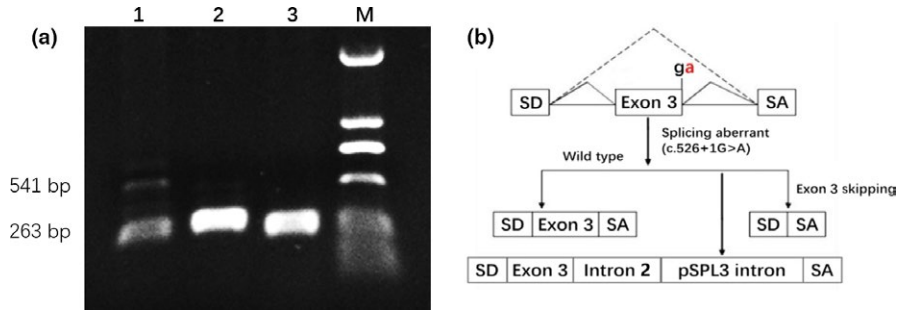


FIGURE 5 (a) RT–PCR products of the c.526 + 1 G>A fragment in pSPL3 minigene constructs, Lane 1: The 263-bp band indicates loss of exon 3 and the 541-bp band indicates inclusion of 24 bp of exon 3, 141 bp from intron 3 and 113 bp of the pSPL3 introns. Lane 2: The correctly spliced wild-type sequence (287 bp). Lane 3: Empty vector (263 bp). Lane M: 2,000-bp marker. (b) Schematic representation of splicing showing normal splicing for the wild-type, complete skipping of exon 3 in the mutant-type, and another splicing transcript comprising exon 3, part of intron 3, and part of the pSPL3 intron [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 STR marker genotyping results

	CA repeats		
STR markers	Proband 1	Proband 2	UCSC reference sequence
DXS135	17	15	15
DXS1690	13	13	19
DXS7113	19	19	19
DXS559	20	20	21

The CA repeats of DXS1690 and DXS559 of the two probands were coincident, but differed from the UCSC Reference Sequence. The CA repeats of DXS7113 of both probands were the same as the UCSC Reference Sequence. The CA repeats of the marker DXS135 were different in the two probands.

24-bp exon 3 of *EDA* encodes eight amino acids that are not located in any of the four functional regions of EDA-A1. However, it can be speculated that the loss of any of these amino acids could influence the connection of the upstream furin subdomain and downstream collagenous domain, leading to impaired function of the mutant protein.

Given the fact that more than 300 different mutations of *EDA* have been found according to HGMD and NCBI SNP, it seems a little unusual that the same splicing mutation was found in two different families in the same ethnic group. We next investigated the existence of the "founder effect," which would suggest a shared allele originating from the same ancestor within an isolated population. For this, we sequenced an upstream 2-KB fragment of *EDA* (X: 69614880–X: 69615240) containing 33 SNPs (according to the NCBI SNP database) to determine whether the two probands shared the same multiple genetic marker variants. However, the results showed that neither of the probands carried any variation in these SNPs. To find further evidence, we analyzed the following STR markers near or within the *EDA* gene: the internal marker DXS1690, an upstream marker DXS135, and two downstream markers, DXS7113 and DXS559. The results showed that the CA repeats of the two probands were coincident in DXS1690, DXS7113, and DXS559 but differed in the marker DXS135, indicating linkage of the c.526 + 1 G>A mutant allele between the two families to a certain extent and that the mutation may have arisen from a common founder. This finding could be helpful in identifying more affected individuals within the population or the same geographic location.

In addition, proband 1 displayed a very mild phenotype (Figure 1a). In 2012, Cluzeau et al. (2012) reported that the SNP rs3827760 (c.1109T>C, p.Val370Ala) may attenuate the severity of X-linked HED-related symptoms. According to the NCBI SNP database, the gain-of-function allele occurs with a frequency of between 10% and 15% in the Chinese population. Sequencing of the *EDAR* gene revealed that proband 1 was actually a carrier of the SNP rs3827760, which may account for his mild hypotrichosis phenotype.

In conclusion, we identified a novel mutation in the *EDA* splice donor site (c.526 + 1 A>G) and demonstrated its role in altering gene transcription (skipping of exon 3). Thus, we hypothesize that this mutation is the cause of HED in the two pedigrees described here.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTION

Liu Guannan designed study, completed experiments, analysed data and drafted the paper. Wang Xin gave specific instruction on experiments designing and operation. Qin Man reviewed and gave advice on the study scheme and the manuscript. Sun Lisha gave important advice on experiments operation and data analysation; Zhu Junxia was in charge of reviewing the study scheme and paper drafting.

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