RESEARCH NOTE

A novel missense mutation in collagenous domain of *EDA* gene in a Chinese family with X-linked hypohidrotic ectodermal dysplasia

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In this study, we identified a novel missense mutation located at the collagenous domain of *EDA* gene in a Chinese family with X-linked hypohidrotic (or anhidrotic) ectodermal dysplasia (XLHED). Sequencing analysis was carried out in all family members, and the mutation was identified in the patients and five carrier women. Moreover, this alteration was not present in the healthy males and other females in this family, or in additional 200 healthy control individuals. The mutation caused a substitution of a proline with a leucine in EDA protein (p.P220L), which is located at the third position of the 13th Gly-X-Y repeat, and may affect the stability and multimerzation of EDA. Our finding expands the spectrum of EDA mutations and is benefit for genetic counselling.

Introduction

The ectodermal dysplasias (EDs) are a large and complex group of diseases characterized by a primary defect in at least one of the following tissues: nails, hair, teeth and sweat glands. They comprise more than 170 different clinical conditions, with an estimated incidence of seven in 10,000 births (Priolo *et al.* 2000). The syndrome of hypohidrotic (or anhidrotic) ectodermal dysplasia (HED or EDA) is the most frequent ED and can be inherited either in an autosomal dominant, autosomal recessive, or X-linked patterns. X-linked HED (XLHED) is the most common form of HEDs and the incidence is estimated to 1 in 100,000 births (Wright *et al.* 2009).

XLHED is characterized by sparse hair, abnormal or missing teeth and inability to sweat due to lack of sweat glands (Zonana 1993). Mutations of *EDA* gene were identified as causing XLHED, and this gene is located at the long arm of the X-chromosome (Xq12−13.1) (Monreal *et al.* 1998; Vincent *et al.* 2001). *EDA* gene undergoes extensive alternative splicing from 12 exons and forms eight transcripts encoding different ectodysplasin (EDA) isoforms. The longest splice form of *EDA* (*EDA1* or *EDA*) includes eight exons and encodes a protein composed of 391 amino acids. Mutations in this isoform were detected in 95% of families with XLHED. EDA is a type II transmembrane proteins and belong to the tumour necrosis factor (TNF) superfamily of ligands involved in the early epithelial–mesenchymal interaction that regulates ectodermal-derived appendage formation. The protein is comprised of a small N-terminal intracellular domain and a larger extracellular domain that contains a consensus furin cleavage site, a collagenous domain of 19 Gly-X-Y repeats with an interruption by two amino acids between repeats 11 and 12, a TNF homologous domain, and a cysteine-rich C-terminal domain (Monreal *et al.* 1998). TNF domain directly binds the extracellular region of death domain-containing receptor called the ectodysplasin-A receptor (EDAR). The EDAR interacts with an adaptor molecule, ectodysplasin-A receptor associated death domain (EDARADD), to activate NF-κB intracellular signalling pathway (Yan *et al.* 2002). With further involvement of TNF-receptor-associated factor 6 (TRAF6), the NF-κB essential modulator (NEMO) –IκB–NF-κB signalling cascade is activated. NF-κB factor translocate into the nucleus where

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it induces transcription of many genes that are necessary for initiation and differentiation of skin appendages such as hair, teeth and sweat glands. In the previous studies, mutations in *EDAR*, *EDARADD*, *TRAF6*, *NEMO* genes have been reported as associated with HED (Zonana *et al.* 2000; Wisniewski and Trzeciak 2012). The *WNT10A* gene was also reported to be associated with HED, and the interaction between *WNT10A* and *EDA* mutations were involved in the pathogenesis of tooth agenesis (He *et al.* 2013).

In this study, we genetically investigated a four-generation, 24-member Chinese family with XLHED and identified a novel missense mutation in collagenous domain of *EDA* gene which caused the substitution of a proline residue (CCA) by a leucine residue (CTA) in EDA protein.

Materials and methods

Enrollment of human subjects

This study was reviewed and approved by the genetic research ethics committees of Xi'an Jiaotong University School of Medicine. Written informed consent was obtained from all participants.

Clinical examination of subjects and isolation of genomic DNA

In this study, we investigated a four generation Han Chinese family with 24 family members (figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The proband (III-9) is a 21-year-old male. Oral examinations for all the affected and unaffected individuals from this family were completed by a prosthodontist, who determined the status of the dentition. A panoramic radiograph was taken to confirm the diagnosis of tooth agenesis for the proband and the other patients. Moreover, a healthy control group (100 males and 100 females) with no family history of XLHED was also recruited for the studies of EDA of X-chromosome.

Human genomic DNA was extracted from the peripheral blood using the DNA Isolation Kit for Mammalian Blood (Tiangen Biotech, Beijing, China).

DNA sequencing and mutation detection

Mutation screening was performed using direct DNA sequencing for *EDA* gene. The entire coding regions and exon–intron boundaries of EDA (GenBank ID: NM 001399) were amplified by polymerase chain reaction (PCR) method with the primers in the table 1 in electronic supplementary material. PCR was performed in 25 μL of standard PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μL of each primer, 1 unit of *Taq* DNA polymerase, and 25 ng of human genomic DNA. The PCR thermal cycle programme was as follows: one cycle of 2 min for denaturation at 95°C, 35 cycles of 30 s at 95°C, 35 s at 59–61°C depending on the primers, 45 s at 72°C, and one 7 min extension step at 72°C. PCR products were analysed using 1.5% agarose gel electrophoresis. Purified PCR products were sequenced bidirectionally using PCR primers as sequencing primers and the Applied Biosystems Prism BigDye terminator cycle sequencing reaction kit. The products were evaluated on an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystem, San Diego, USA).

Results

Clinical features and genetic model

A four-generation Han Chinese family with XLHED was investigated in this study. The pedigree consists of 24 members is shown in figure 1 in electronic supplementary material. The proband is a 21-year-old male. He displays the characteristics of missing and misshapen teeth, sparse hair and dry and thin skin. Similar clinical features of teeth agenesis and less impaired appearance in hair and skin were detected in three other affected members in his family. All

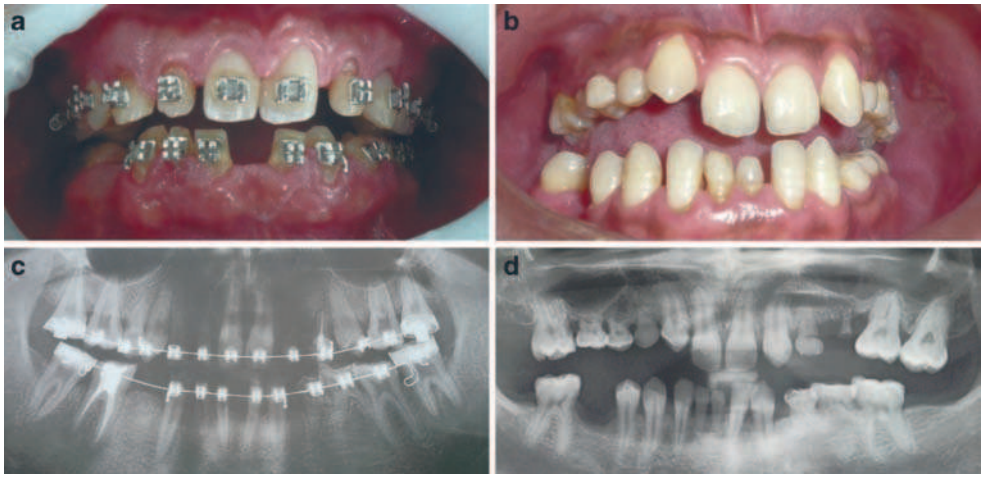

Figure 1. Clinical phenotype of XLHED. (A and B) Clinical photographs and radiographs of the proband (III-9), a 21 years old male. (C and D) Clinical photographs and radiographs from a patient (III-11), a 19 years old male.

the four patients are males, and all the females showed normal appearance. The disease in this family is identified as X-linked recessive HED and the five females are considered to be carriers. The clinical photographs and oral radiographs of the proband (III-9) and his affected male cousin (III-11) are shown in figure 1.

Mutation analysis

The eight coding exons and exon–intron boundaries of *EDA* were amplified by PCR method. After purification, the products were sequenced directly. Sequencing in all the four affected male detected one nucleotide transition in exon 5, c.659C>T. This mutation, located at the collagenous domain in the third amino acid of the 13th Gly-X-Y repeat, results in a substitution of a proline residue (CCA) with a leucine residue (CTA) in EDA protein (p.P220L). Further sequencing analysis was performed in all the family members. The alteration was present in the five carrier women. They are heterozygous carriers of c.659C>T transition. This alteration was not found in the healthy males and other females in this family (figure 2). Moreover, this nucleotide transition was not found in 200 healthy control individuals (300 alleles), demonstrating that this alteration was not a polymorphic variants but a mutation of *EDA* gene.

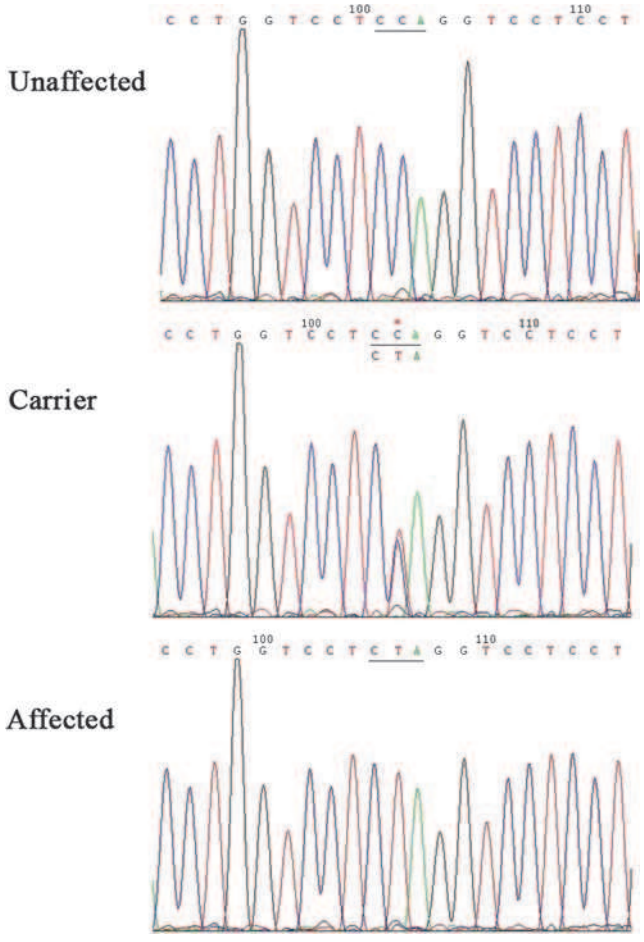

Figure 2. Identification of a novel mutation, c.659C>T (p.P220L), in *EDA* in a Chinese family with XLHED. DNA sequences are for a normal family member (above), a normal carrier (middle), and the proband III-9 (below). The sequence of codon 220 where the mutation occurs is marked. The C to T change in the proband results in the substitution of a proline residue (CCA) by a leucine residue (CTA) in the EDA protein.

Multiple alignments of amino acid sequence of *EDA* gene were performed by the program ClustalW (http://www.ebi.ac.uk/clustalw). The results showed that the P220 of EDA is highly conserved in other 14 vertebrate species (zebrafish, rabbit, chick, pig, sheep, dog, cow, dolphin, rat, mouse, baboon, macaque, gorilla, and chimpanzee) at this position (figure 2 in electronic supplementary material). The mutation p.P220L of EDA responsible for XLHED affects the evolutionarily highly conserved P220 residue. The multiple alignments of 19 Gly-X-Y repeats are all highly conserved in the 14 vertebrate species (rabbit, chick, pig, sheep, dog, cow, dolphin, rat, mouse, baboon, macaque, gorilla, chimpanzee, and human). The available nonsynonymous variants prediction program, PolyPhen-2, was used to predict the structural and functional effects of this mutation on EDA, and the prediction for this alteration suggested probably damaging with the maximum score 1.000 (table 2 in electronic supplementary material).

Discussion

In the current study, we identified a novel missense mutation located at the collagenous domain in exon 5 of *EDA* gene in a Chinese family with XLHED. The mutation caused a substitution of a proline with a leucine in EDA protein (p.P220L). This substitution mutation was found only in the affected males and the carrier females with normal phenotypes, not in the other family members and the 200 control individuals of the same ethnic background, which strongly suggests that this is the causative mutation for the XLHED in this family.

To date, more than 100 missense mutations in *EDA* gene have been reported to be associated with XLHED in different population. The mutation hot spots were located in the four conserved domain containing a transmembrane domain, a furin protease recognition sequence, a collagenous domain of interrupt 19 Gly-X-Y repeats and a TNF homologous domain (Monreal *et al.* 1998). The collagenous domain is mainly in exon 5 and only the last Gly-X-Y repeat in exon 6. In this study, the mutation p.P220L of EDA was located at the third position of the 13th Gly-X-Y repeat. The highly evolutionary conservation of P220 residue in EDA and the result of the maximum score of the mutation suggest that this amino acid may play a key role in the function of the collagenous domain and the EDA protein. Moreover, the 19 Gly-X-Y repeats are also fully conserved among 14 species (rabbit, chick, pig, sheep, dog, cow, dolphin, rat, mouse, baboon, macaque, gorilla, chimpanzee, and human) which shows that collagenous domain is very important in the function of EDA protein.

Although the collagenous domain in EDA protein is among the smallest group, it still can form trimers like other TNF-related ligands. The third-position proline can affect

the stability of collagen helix (Brodsky and Shah 1995). The activity of soluble trimers in the TNF family can be dramatically increased by multimerzation (Schneider *et al.* 1998). The collagenous domain induces multimerization of EDA trimers and the missense mutations in this region could interrupt the collagenous triple helix to form multimers. In the anti-EDA antibodies experiment, Schneider *et al.* (2001) found that a naturally occurring point mutation p.G207R in the collagen domain completely abolished the bundle effect of EDA. The p.P220L mutation in this study may affect the stability and the multimerzation of EDA at the same time.

Including our novel mutation, nine missense mutations in the collagenous domain responsible for XLHED disease have been reported in five independent studies (Monreal *et al.* 1998; Schneider *et al.* 2001; Vincent *et al.* 2002; Zhao *et al.* 2008; Clauss *et al.* 2010). They are the mutation p.G189E, p.G195E, p.G198A, p.G207R, p.P209L, p.G218D, p.P220L, p.G224A and p.Q232X which distribute between Gly-X-Y repeat 4 and 17 in exon 5 (table 2 in electronic supplementary material). Among them, six mutations are at the first position of Gly-X-Y repeat and three in the third position. The eight mutations are highly conversed and reach maximum score 1.000 of probably damaging as predicted by the program PolyPhen-2. Meanwhile, the mutation p.Q232X creates a stop codon in exon 5 and cannot be analysed by PolyPhen-2 prediction program (Li *et al.* 2012). The structure and the function of collagenous domain indicate that it plays an important role in EDA protein underlying the molecular mechanism for XLHED. However, the deletion mutations in this region causing XLHED were predicted to truncate the collagen domain of the protein but otherwise left the protein intact and the deletions in frame did not affect the multimerization of collagenous triple helix *in vitro* (Monreal *et al.* 1998; Schneider *et al.* 2001). Thus, the integrity of the collagenous domain appeared to be essential for the function of EDA protein.

It was well established that the TNF homologous domain in EDA directly binds to the acceptor and EDA protein activated NF-κB signalling pathway through EDA-EDAR-EDARADD cascade reaction to regulate the ectodermal appendage formation (Schneider *et al.* 2001). Recently, a study in mouse model demonstrated that the target genes downstream NF-κB factor included four pathways: Shh, Wnt (Dkk4), BMP (Sostdc1) and LTβ/RelB, and when EDA is defective, failure of LTβ activation can impair the developing hair follicles and account for part of the HED phenotype (Cui *et al.* 2006). In different population, missense mutations in TNF homologous domain in EDA were responsible for both XLHED and X-linked nonsyndromic hypodontia involving only tooth agenesis, and there was no evident relationship between phenotype and the mutation (Vincent *et al.* 2001; Liu *et al.* 2012). In addition, one mutation associated with X-linked nonsyndromic hypodontia in the transmembrane domain of *EDA* gene was also reported (Tao *et al.* 2006). Whereas, all the missense mutations in the collagenous domain were associated with XLHED. Therefore, the complex molecular mechanism of EDA protein for the tooth agenesis is still obscure and the function of collagenous domain in EDA underlying the pathogenesis of XLHED is required to be further studied.

In summary, we identified a novel missense mutation p.P220L in the collagenous domain of EDA in a Chinese family with XLHED. Our finding expands the spectrum of EDA mutations and is benefit to genetic counselling. Further research is needed to clarify the molecular mechanism of EDA for XLHED and the particular effect of collagenous domain in the disease.

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