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Original article

# Novel *EDA* mutation resulting in X-linked non-syndromic hypodontia and the pattern of *EDA*-associated isolated tooth agenesis

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**Abstract**

Familial non-syndromic hypodontia shows a wide phenotypic heterogeneity and inherits in an autosomal-dominant, autosomal-recessive or X-linked mode. Mutations in genes *PAX9*, *MSX1* and *AXIN2* have been determined to be associated with autosomal-dominant tooth agenesis. Recent studies in two families showed that X-linked non-syndromic hypodontia resulted from *EDA* mutations. In this study, a novel *EDA* mutation (Thr338Met) that results in X-linked non-syndromic hypodontia in a Chinese family was identified. The patterns of tooth agenesis in these related subjects with defined *EDA* mutation were analyzed using comparative statistical analysis of tooth agenesis in *EDA*, *MSX1* and *PAX9*. Statistically significant differences ($p < 0.001$) were observed at eight positions. The resulting data of congenital absence of maxillary and mandibular central incisors, lateral incisors and canines, with the high possibility of persistence of maxillary and

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mandibular first permanent molars, appears as a pattern of tooth agenesis, suggesting the presence of an *EDA* mutation.


*Keywords:* *EDA* gene; Hypodontia; Non-syndromic; X-linked

## 1. Introduction

Congenital agenesis of one or more permanent teeth (excluding the third molar agenesis) (Hypodontia/Oligodontia, [MIM 106600]) is the most prevalent abnormality of human dentition [20]. Numerous studies have been published on the prevalence of hypodontia (third molars excluded) in various populations. The reported hypodontia rates range from 3.2% to 10.1%, depending on racial background [4,8,19,21].

Hypodontia can occur as an isolated anomaly (non-syndromic) or as part of a multiple congenital anomaly (syndromic). Genes associated with several syndromic conditions that present hypodontia have been identified, including the *EDA* gene (MIM 300451), which underlies X-linked hypohidrotic ectodermal dysplasia (XLHED, [MIM 305100]). XLHED is a rare condition characterized by sparse hair growth, oligodontia or anodontia, and an inability to sweat [10]. Non-syndromic hypodontia is expressed in a variety of phenotypes and is classified as either sporadic or familial form, and is inherited either in an autosomal-dominant, autosomal-recessive or X-linked mode [1].

In most cases, familial non-syndromic hypodontia has been shown to be inherited as an autosomal-dominant trait [7]. Mutations in genes *PAX9*, *MSX1* and *AXIN2* have been determined to be associated with autosomal-dominant tooth agenesis [12,23,29]. In one report [31], a rare, heritable, permanent tooth agenesis was transmitted in an autosomal-dominant fashion with incomplete penetrance in a large Chinese family was described (MIM 610926). The gene locus for this deficiency was thereupon mapped to chromosome 10q11.2 [15]. Recently, mutations of the *EDA* gene (MIM 300606) have been reported in two X-linked hypodontia families [24,25]. Unlike patients previously described with XLHED, affected patients from both families did not have any clinical features except hypodontia, suggesting that X-linked non-syndromic hypodontia resulted from *EDA* mutations.

The present study was designed to screen an X-linked family with non-syndromic hypodontia for a mutation in the *EDA* gene. The pattern of tooth agenesis for *EDA*-associated non-syndromic hypodontia was analyzed and a three-dimensional structural analysis of the EDA protein was made.

## 2. Material and methods

### 2.1. Clinical details

Subjects of this study were of Chinese descent. The proband was a 7-year-old boy referred to the Department of Prosthodontics at Peking University School and Hospital of Stomatology for diagnostic evaluation and treatment of hypodontia. A pedigree construction was constructed by clinical examinations of available family members and through interviews. A total of 16 family members participated in this study. Tooth agenesis could be traced back to two generations.

Pedigree analysis showed an X-linked pattern of inheritance (Fig. 1A). Oral examinations for all participants were completed by a prosthodontist, who determined the status of the dentition. The proband had several deciduous teeth missing, but the shape of the residual teeth appeared normal (Fig. 1B). A panoramic radiograph was taken to confirm the diagnosis of tooth agenesis for the proband (Fig. 1C). Four male and one female participant showed congenital absence of teeth (Fig. 1D). Although, the manifestation of hypodontia was not consistent in this family, the

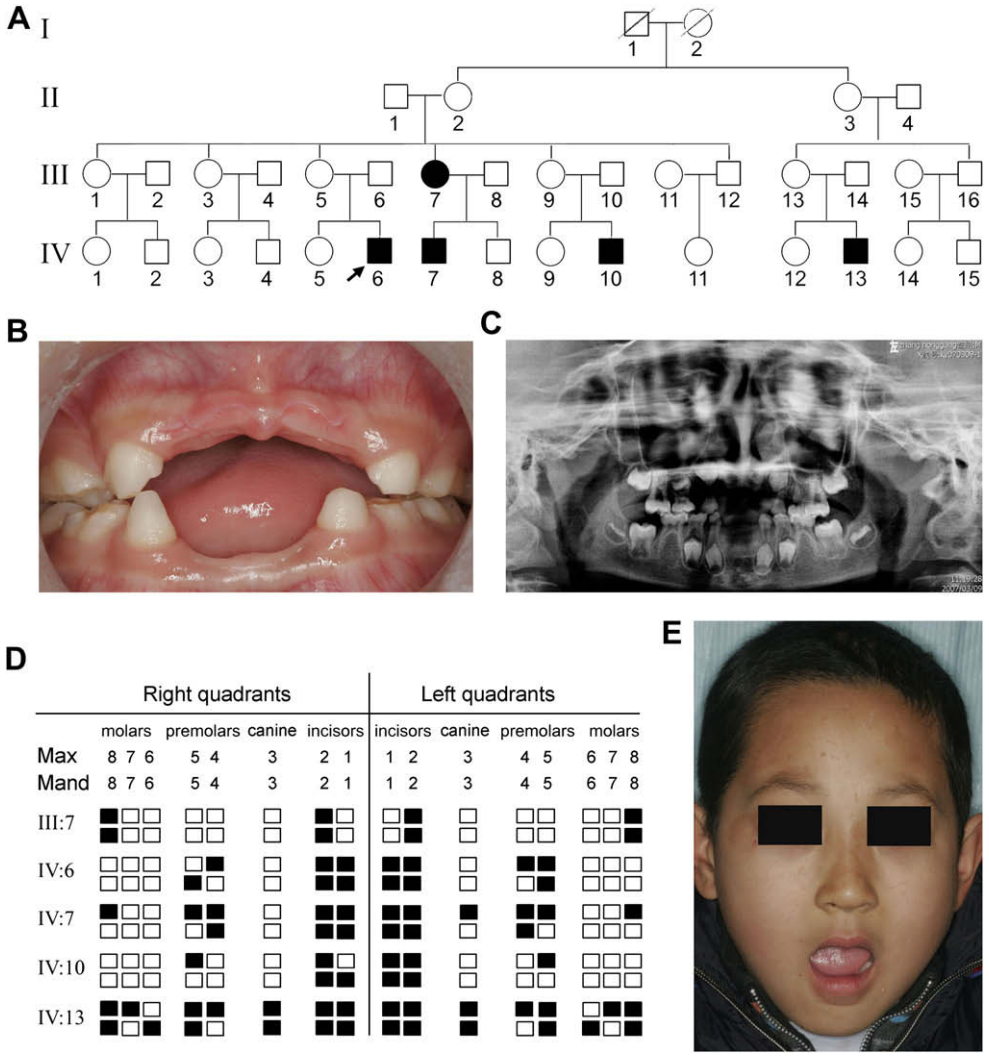


Fig. 1. A X-linked family affected with non-syndromic hypodontia. (A) Pedigree of the family shows X-linked inheritance. Affected males and female are indicated by filled squares and circle, respectively. (B) Clinical phenotype of the proband (IV-6) shows several deciduous teeth missing, but the shape of the residual teeth appeared normal. (C) Panoramic radiograph of the proband shows congenital absence of maxillary and mandibular central and lateral incisors, maxillary first premolars of both sides, left maxillary second premolars and mandibular second premolars of both sides. (D) Schematic presentation of congenitally missed teeth among four affected males and one female carrier. Filled squares present the missing teeth. (E) The proband's facial features, skin, and hair appear normal.

absence of maxillary and mandibular lateral incisors were observed in all affected members. Especially the affected female (III-7) only had all of the lateral incisors missing, besides the absence of the third molars. Furthermore, tooth malformation, such as conical tooth, was not observed in any family member. Phenotypic characteristics of scalp and body hair, skin, and nails, tolerance to heat and ability to sweat were examined by a practitioner. All of the individuals reported normal levels of sweating, lachrymal and salivary secretions. There were also no complaints about intolerance to heat and susceptibility to respiratory tract infections. Overall, their facial features, skin, hair and nails appeared normal (Fig. 1E). This study was conducted with the approval of the Ethics Committee of Peking University Health Science Center.

### 2.2. Genetic linkage analysis

After informed consent, venous blood samples were obtained from 16 individuals, including accessible affected and unaffected members of this family. Genomic DNA was isolated from peripheral blood lymphocytes by a standard high-salt method. Affected and unaffected family members were genotyped for microsatellite marker DXS1689. A two-point LOD score was calculated using the MLINK program of the LINKAGE version 5.1 software packages.

### 2.3. Mutation detection

Screening of the *EDA* gene was performed by direct sequencing of eight PCR fragments that cover the entire cDNA of eight exons and intron–exon junctions. Fifty nanograms of genomic DNA templates were suspended in a total volume of 50 μl PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 nM of each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP (TaKaRa. Bio. Inc., Dalian, China), and 1.0 unit of TaKaRa LA Taq DNA polymerase (TaKaRa. Bio. Inc., Dalian, China). Amplification conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 45 s, 63 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Gel-purified PCR fragments were sequenced on an Applied Biosystem 377 automated sequencer using an ABI PRISM fluorescent dye terminator system (Perkin Elmer, Foster City, CA).

### 2.4. Protein structure analysis

The crystal structure of EDA, PDB coordinate 1RJ7 [9], was used as a scaffold for the protein structure analysis. The structures were analyzed with an Insight II (2000) software package (Accelrys Inc., USA) and figures were created with PyMOL (DeLano WL., 2002. The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org).

### 2.5. Statistical analysis

Including the subjects studied in this paper, there are now 24 patients with defined *EDA* mutations with known patterns of tooth agenesis in their permanent dentitions [24,25]. The number of missing teeth at each position in the four quadrants of the mouth for the 24 patients were compiled. The number of missing teeth in each site of left and right side were combined and chi-square tests were performed. In order to determine statistically significant differences in the numbers of missing teeth between the maxilla and mandible, the data of the eight sites of teeth in the maxilla and mandible were combined separately, and chi-square tests were

performed. In order to identify an *EDA*-associated pattern of tooth agenesis, the data for three *EDA*-deficient family members was analyzed, and compared with the data for patients with *MSX1* and *PAX9* mutations compiled by Kim et al. [11] (Fig. 2A). Chi-square tests were performed according to the previous study that was conducted by Kim et al. [11].

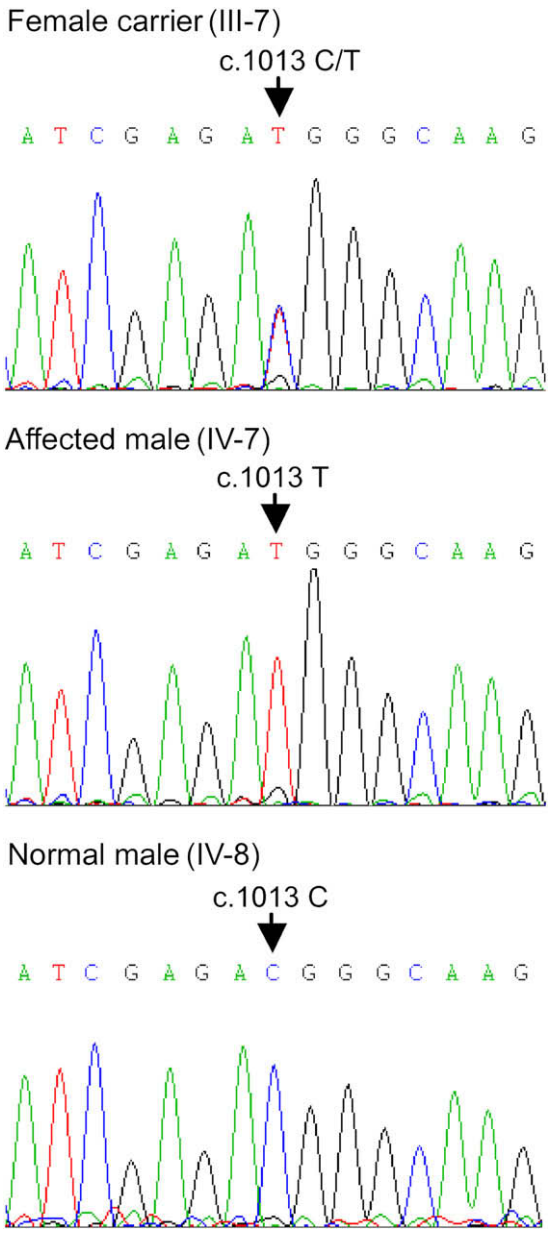


Fig. 2. A novel mutation c.1013C > T that results in Thr338Met is identified. Direct sequencing identifies a novel mutation c.1013C > T. This mutation is found in the mother (III-7; heterozygote 1013C/T) and her affected son (IV-7; hemizygote 1013 T); but not in her unaffected son (IV-8, hemizygote 1013C). Mutated nucleotide is indicated with an arrow.

## 3. Results

### 3.1. Linkage analysis supports a EDA defect

The X chromosome region harboring the *EDA* gene was investigated using microsatellite marker DXS1689 mapping of this region. A common allele was seen in all affected male individuals (IV-6, IV-7, IV-10, IV-13) and all female carriers (II-2, III-5, III-7, III-9, III-13, IV-9), but not in unaffected individuals (II-4, III-3, III-12, IV-5, IV-8, IV-11). A two-point LOD score of 2.38 at $\theta = 0$ was obtained, suggesting the involvement of the *EDA* gene in this family (data not shown).

### 3.2. A novel mutation identified in the family

Direct sequencing of PCR products derived from the proband revealed a novel C to T transition at the nucleotide 1013 (c.1013C > T, the nucleotide was numbered from the first base of the translation–initiation ATG codon) of the coding sequence in exon 8 of *EDA*, changing the 338 codon from encoding Thr (ACG) to Met (ATG). Sequencing analyses of the Thr338Met mutation was performed on all participants. The Thr338Met mutation was present in all affected male individuals (IV-6, IV-7, IV-10, IV-13) and all female carriers (II-2, III-5, III-7, III-9, III-13, IV-9), but not in unaffected individuals (II-4, III-3, III-12, IV-5, IV-8, IV-11) (Fig. 2). The mutant allele was not detected in 120 control chromosomes.

### 3.3. Three-dimensional EDA structure analysis

In the wild-type of three-dimensional EDA structure, residue Thr338 is located at the corner of a loop on the outer surface (Fig. 3), making it possible to form hydrogen bond with Tyr311 and Asn313 at the adjacent β-strand. When the Thr338Met mutation occurs, the side chain volume is enlarged, and the residue property is changed from hydrophilic to hydrophobic, likely resulting in a conformational change of the homotrimer.

### 3.4. Frequency of tooth missing with EDA mutations

Among the patients with defined *EDA* mutations, the teeth with the highest probability of absence were the maxillary and mandibular lateral incisors (92%) and the mandibular central incisors (83%) (Fig. 4A). No statistically significant differences were observed for the absence of a specific type of tooth on the left and right sides. A statistically significant difference ($p < 0.001$) was observed between the maxillary and mandibular arches at the central incisor position (Fig. 4B). This suggests that patients with an *EDA* defect were more likely to miss mandibular central incisors than maxillary central incisors.

The comparative analysis between the data of patients with *EDA* mutations and of patients with *MSX1/PAX9* mutations indicate statistically significant differences ($p < 0.001$) were observed at eight positions (Fig. 4A and B). Patients with *EDA* mutations were more likely to be missing maxillary and mandibular central incisors, lateral incisors, and canines, but maxillary and mandibular first permanent molars were more likely to be present.

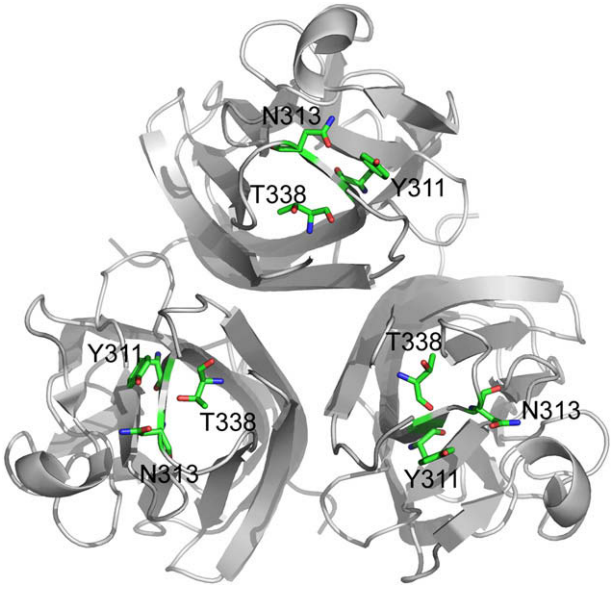


Fig. 3. Mutant residue in the three-dimensional EDA trimer. Residue Thr338 is located at the corner of a loop on the outer surface, making it possible to form hydrogen bonds with residues Tyr311 (Y311) and Asn313 (N313) at the adjacent β-strand in an EDA trimer. The relevant side chains were rendered by sticks.

## 4. Discussion

A novel mutation in the *EDA* gene was attributed to non-syndromic hypodontia in patients of Chinese descent. The patterns of missing teeth and X-linked inheritance in this family were similar to both of previously described cases, in which hypodontia was associated with *EDA* mutations [24,25]. Unlike the characteristics of XLHED patient, affected patients in our study do not have any other deformities, except tooth agenesis. In particular, all affected males exhibited isolated oligodontia, while almost all of female carriers showed normal or milder phenotype. Female carriers in *EDA* mutation − XLHED families also displayed partial penetrance and minimal to none clinical signs [2,18]. The reduced penetrance and phenotypic expression in female carriers may be explained by random X chromosome inactivation [16].

Although a number of mutations of the *EDA* gene that result in XLHED have been reported, there is no obvious correlation between the type of mutation and the severity of XLHED [6,30,32]. Compare with the tooth agenesis of the male patients in this study, the affected males with XLHED were more seriously affected missing more permanent teeth and showing more tooth malformations, such as a conical shape of maxillary central incisors [14]. Including the present report, three causative *EDA* gene mutations have been identified in X-linked pedigrees with non-syndromic hypodontia [24,25]. All of the mutations identified from these non-syndromic hypodontia families are novel, indicating that there is a noticeable link between the *EDA* mutation site (genotype) and the clinical phenotype (XLHED or isolated tooth agenesis), but more evidence is still needed for confirmation.

*EDA* gene encodes a 391-residue protein, ectodysplasin-A (EDA). EDA is a trimeric type II membrane protein that belongs to the TNF-related ligand family, which includes an interrupted collagenous domain of 19 Gly-X−Y repeats and a domain similar to the tumor necrosis factor

A

| Gene | Quad-rant | Tooth position 1 | 2 | 3 | 4 | 5 | 6 | 7 | Refer-ence |
|---|---|---|---|---|---|---|---|---|---|
| *EDA* | Max R | 9/24 | 21/24 | 11/24 | 14/24 | 9/24 | 0/24 | 4/24 | This study; Tao et al 2006; Tarpey et al 2007 |
| | Max L | 8/24 | 23/24 | 10/24 | 14/24 | 10/24 | 0/24 | 4/24 | |
| | Mand R | 20/24 | 22/24 | 9/24 | 8/24 | 7/24 | 1/24 | 3/24 | |
| | Mand L | 20/24 | 22/24 | 9/24 | 8/24 | 7/24 | 1/24 | 3/24 | |
| | Max | 17/48(35) | 44/48(92) | 21/48(44) | 28/48(58) | 19/48(40) | 0/48(0) | 8/48(17) | |
| | Mand | 40/48(83) | 44/48(92) | 18/48(38) | 16/48(33) | 14/48(29) | 2/48(4) | 6/48(13) | |
| *MSX1* | Max | 0/76(0) | 23/76(29) | 4/75 (5) | 57/76 (75) | 74/76 (97) | 23/76 (30) | 10/76 (13) | Kim et al 2006 |
| | Mand | 16/78(21) | 17/78(22) | 6/77(8) | 12/78(15) | 71/78(91) | 40/78(51) | 18/78(23) | |
| *PAX9* | Max | 2/100(2) | 28/100(28) | 14/100 (14) | 20/100 (20) | 66/100 (66) | 79/100 (79) | 89/100 (89) | Kim et al 2006 |
| | Mand | 52/100(52) | 7/100(7) | 4/100(4) | 6/100(6) | 39/100(39) | 32/100(32) | 83/100(83) | |

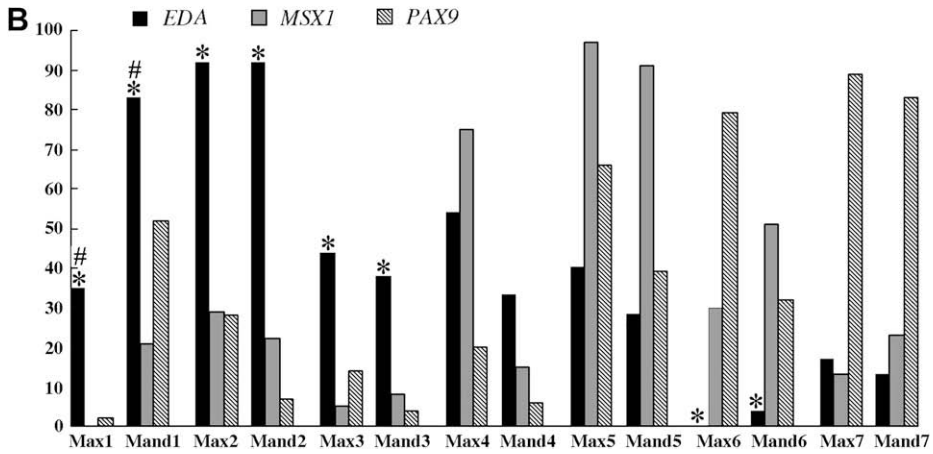


Fig. 4. The pattern of *EDA*-associated isolated tooth agenesis. (A) The number of missing teeth in 24 patients with defined *EDA* mutations is compiled for each position in the human dentition (28 teeth) based on the data from this report and the two previous reports [24, 25]. The numerators being the number of missing teeth, the denominators being the summation of the teeth that these patients should have at each position. The data for equivalent teeth on the left and right are combined at bottom. The number indicated between brackets denote the percentage of missing teeth. Under the data are the previously reported data obtained from patients with *MSX1* and *PAX9* mutations. (B) We plot the percentage of missing teeth at each maxillary and mandibular position for people with defined *EDA*, *MSX1* and *PAX9* mutations. Statistically significant differences ($p < 0.001$) between *EDA* and *MSX1*/*PAX9* are marked with *. Statistically significant difference ($p < 0.001$) between the maxillary and mandibular central incisors of *EDA*-associated tooth agenesis is indicated with #. *Key*: 1, central incisor; 2, lateral incisor; 3, canine; 4, first premolar; 5, second premolar; 6, first molar; 7, second molar; $x$ axis = tooth position; $y$ axis = missing percent.

(TNF) at the C terminus [5,33]. The mutation Thr338Met identified in this study is spatially near the mutation Tyr343Cys, which has been found to cause XLHED in a previously reported study. Residue Tyr343 is located adjacent to the receptor specificity switch Glu308 and is predicted, based on its structure, to participate in a formation of active sites [9]. The Tyr343Cys mutation may severely affect EDA function, even leading to a loss of EDA function [22]. In comparing the mutation Thr338Met, which results in a non-syndromic hypodontia phenotype, to the mutation Tyr343Cys, which leads to a full XLHED phenotype, the mutant residue Thr338 identified in this study is located on the protein surface, away from the active site

Glu308. Accordingly, we speculate that the change of the residue would only influence the stability of homotrimers and partially affect EDA function. In the study of the mouse model, Eda-regulated proteins are involved in four signaling pathways. These include Shh in the hedgehog signaling pathway, Dkk4 in the Wnt signaling pathway, Sostdc1 in the BMP pathway, and Ltb in the NFKB signaling pathway [3]. Wnt and BMP signaling pathways are important in early tooth germ development [13], while Ltb in the NFKB signaling pathway regulates the form of hair in developing hair follicles [3]. Therefore, we propose that the mutations resulting in non-syndromic tooth agenesis may have altered EDA function in the Wnt and BMP signaling pathways, which are correlated with tooth development, but not with hair and skin growth.

*MSX1* and *PAX9* mutations have been determined to underlie non-syndromic tooth agenesis in some families inherited in autosomal-dominant mode. According to previous reports, there are three criteria based on dental phenotype used to accurately distinguish between *MSX1* and *PAX9* mutations. *MSX1*-associated hypodontia typically includes absent maxillary and mandibular second premolars and absent maxillary first premolars [11]. In contrast, patients with *PAX9* mutations typically show agenesis of almost all of their molars, with the absence of second molars best distinguishing them from persons with *MSX1* defects [11]. Our data shows that congenital absence of central incisors, lateral incisors and canines of maxilla and mandible, with the high possibility of persistence of maxillary and mandibular first permanent molars, is a pattern of tooth agenesis, and suggests the presence of an *EDA* mutation. Unlike the high frequency absence of the first molar in patients with *MSX1* or *PAX9* mutations, the first molar appears to be permanent in *EDA*-associated tooth agenesis. Similar to *MSX1* and *PAX9*-associated hypodontia, *EDA*-associated hypodontia shows bilaterally symmetric absence of a particular type of tooth, and statistically significant differences between the maxillae and mandible. In contrast, the most distinguishing feature of *EDA*-associated hypodontia is the frequent absence of maxillary and mandibular lateral incisors (92%).

In the study of the mouse model, Eda is expressed in the epithelium starting from the epithelial thickening to the bud stage, and then continually expressed in the mesenchyme until the end of the cap stage [27]. However, Pax9 and Msx1 are expressed only in the mesenchyme at the early stage of tooth development [26,17]. Moreover, interactions of Eda and its receptor Edar may regulate enamel knot formation [27]. Enamel knot is thought to act as a signaling center in the tooth, controlling proliferation and apoptosis, and leading to the development of cusps [13,28]. We presume that the difference of gene expression and function may explain the difference between the characteristics of *EDA*-associated hypodontia and *MSX1*/*PAX9*-associated hypodontia.

In conclusion, this study confirms the finding that X-linked non-syndromic hypodontia results from *EDA* mutations. It also provides evidence that *EDA* could be a good gene candidate in the specific pattern of tooth agenesis. Our findings can benefit further genetic studies of the *EDA* gene in patients with non-syndromic hypodontia based on the dental phenotype.

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