Involvement of and Interaction between \textit{WNT10A} and \textit{EDA} Mutations in Tooth Agenesis Cases in the Chinese Population

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Abstract

\textbf{Background:} Dental agenesis is the most common, often heritable, developmental anomaly in humans. Although \textit{WNT10A} gene mutations are known to cause rare syndromes associated with tooth agenesis, including onycho-odontodermal dysplasia (OODD), Schönff-Schulz-Passarge syndrome (SSPS), hypohidrotic ectodermal dysplasia (HED), and more than half of the cases of isolated oligodontia recently, the genotype-phenotype correlations and the mode of inheritance of \textit{WNT10A} mutations remain unclear. The phenotypic expression with \textit{WNT10A} mutations shows a high degree of variability, suggesting that other genes might function with \textit{WNT10A} in regulating ectodermal organ development. Moreover, the involvement of mutations in other genes, such as \textit{EDA}, which is also associated with HED and isolated tooth agenesis, is not clear. Therefore, we hypothesized that \textit{EDA} mutations interact with \textit{WNT10A} mutations to play a role in tooth agenesis. Additionally, \textit{EDA}, \textit{EDAR}, and \textit{EDARADD} encode signaling molecules in the Eda/Edar/NF-kB signaling pathways, we also checked \textit{EDAR} and \textit{EDARADD} in this study.

\textbf{Methods:} \textit{WNT10A}, \textit{EDA}, \textit{EDAR} and \textit{EDARADD} were sequenced in 88 patients with isolated oligodontia and 26 patients with syndromic tooth agenesis. The structure of two mutated \textit{WNT10A} and two mutated \textit{EDA} proteins was analyzed.

\textbf{Results:} Digenic mutations of both \textit{WNT10A} and \textit{EDA} were identified in 2 of 88 (2.27\%) isolated oligodontia cases and 4 of 26 (15.38\%) syndromic tooth agenesis cases. No mutation in \textit{EDAR} or \textit{EDARADD} gene was found.

\textbf{Conclusions:} \textit{WNT10A} and \textit{EDA} digenic mutations could result in oligodontia and syndromic tooth agenesis in the Chinese population. Moreover, our results will greatly expand the genotypic spectrum of tooth agenesis.

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Introduction

Permanent tooth agenesis is the most common developmental dental anomaly in humans, with an incidence rate of 2.2–10.1\% in the general population (excluding anomalies of the third molars) [1]. Permanent tooth agenesis can occur as an isolated anomaly (non-syndromic) or as a part of multiple congenital anomalies (syndromic) [2–4], such as anhidrotic or hypohidrotic ectodermal dysplasia (HED), onycho-odontodermal dysplasia (OODD) and Schöpf-Schulz-Passarge syndrome (SSPS). Selective tooth agenesis is divided into 2 types: hypodontia, the agenesis of fewer than six teeth, and oligodontia, the agenesis of six or more permanent teeth.

Mutations of the \textit{WNT10A} gene are responsible for OODD, HED and SSPS [5–7]. OODD, a rare form of ectodermal dysplasia, is characterized mainly by abnormal teeth, nail dystrophy, palmoplantar keratoderma, hypotrichosis and various other associated ectodermal abnormalities, including neoplasms [5]. HED is characterized by a triad of signs comprising sparse hair (hypotrichosis), abnormal or missing teeth (anodontia or hypodontia), and inability to sweat (anhidrosis or hypohidrosis) [6]. SSPS is distinguished by the presence of multiple eyelid cysts, histologically corresponding to apocrine hidrocystomas [7].

\textit{WNT10A}, a 46.4-kDa protein with 10 putative \alpha-helices and seven putative \beta-strands, belongs to \textit{Wnt} proteins, a large family of secreted signaling proteins. \textit{Wnt10a} is expressed in embryonic limb [8], skin [9], teeth [10,11], and hair follicles [12,13] during embryonic development; it plays an important role in odontoblast differentiation and tooth morphogenesis [11]. Recently, \textit{WNT10A} mutations were identified in more than half of isolated hypodontia cases [14]. However, the phenotypic expression of \textit{WNT10A} mutations shows a high degree of variability, ranging from only isolated hypodontia to various symptoms of ectodermal dysplasia.
Moreover, the genotype-phenotype correlations and the mode of inheritance of WNT10A mutations remain unclear [6,14,15]. Therefore, other genetic factors or unidentified mutations may also influence phenotypic expression in patients with WNT10A mutations [6,14].

EDA is an important gene associated with tooth agenesis, it is located on chromosome Xq12-q13.1 and encodes for ectodysplasin-A (EDA) (MIM 300451), a member of the tumor necrosis factor (TNF) family [16]. EDA is a type II transmembrane protein with a C-terminal TNF homology domain and a signaling molecule [17]. EDAR and EDARADD encode the protein EDAR and EDARADD, belonging to the EDA/Edar/TNF-κB signaling pathway. The binding of Eda-Edar (complex formed by EDA and its receptor) to the downstream adaptor Edaradd leads to activation of the transcription factor NF-κB and is essential for the development of hair follicles, teeth, exocrine glands and other ectodermal derivatives [17–19]. EDA gene mutations have been detected in 63–95% of X-Linked HED (XLHED) patients [16,20–23]. Moreover, EDA mutations are also associated with cases of isolated tooth agenesis [24,25]. We therefore hypothesized that EDA mutations may interact with WNT10A mutations and play a role in the development of tooth agenesis.

In this study, we investigated the contribution of WNT10A, EDA, EDAR and EDARADD mutations in patients with isolated or syndromic tooth agenesis. Our results suggest that WNT10A and EDA digenic mutations could result in tooth agenesis. This is the first time such mutations have been reported in patients with tooth agenesis.

**Materials and Methods**

**Participants**

Written informed consent for DNA analysis and reproduction of the photographs was obtained from all the participants and the parents on the behalf of the minors or children participants. This study was conducted with the approval of the Ethics Committee of Peking University Health Science Center. The study participants were 88 non-consanguineous patients with isolated tooth agenesis, 26 non-consanguineous patients with syndromic tooth agenesis (24 with HED and 2 with OODD), and 451 non-consanguineous normal controls, who were referred to the Department of Prosthodontics, Peking University School and Hospital of Stomatology, or the Department of Prosthodontics, Beijing Stomatological Hospital. Oral examinations for all participants were performed by a prosthodontist, who determined the status of the dentition. A panoramic radiograph was taken to confirm the diagnosis of tooth agenesis for these participants. The shape and size of the residual teeth were also observed.

The patients with isolated dental agenesis reported normal sweating and lachrymal secretions. They had no complaints about dry mouth, intolerance to heat, or susceptibility to respiratory tract infections. The patients had hair on their body and scalp, and their facial features, skin, and nails were normal on observation.

Patients with HED presented abnormalities of at least two of the three following ectodermal structures: teeth, hair and sweat glands. The OODD patients had abnormal teeth, nail dystrophy, palmoplantar keratoderma and other associated ectodermal abnormalities.

**DNA extraction**

Genomic DNA of the participants and their relatives was extracted from peripheral blood lymphocytes using a QIAamp DNA Blood Midi Kit (Qiagen). DNA samples of the normal participants recruited from the general population were extracted from buccal epithelial cells using the Chelex-100 (Sigma) method.

**Detection of mutations**

Screening of the WNT10A, EDA, EDAR, and EDARADD genes was performed by direct sequencing of five PCR fragments for WNT10A, eight PCR fragments for EDA, ten PCR fragments for EDAR, and eight PCR fragments for EDARADD, which cover the entire cDNA including exons and intron-exon junctions of more than 100 base pairs. We compared all primer sequences to the whole-genome assembly in the ENSEMBL database to verify their uniqueness against gene families. Primer sequences are available upon request.

**Protein structure analysis**

We performed protein structure analysis on the two WNT10A mutations (p.R171C and p.G213S) and two novel EDA mutations (p.G257R and p.I312M) that were identified in this study. For WNT10A, the conservation of residues in sequences was determined to predict the influence of the two mutations. The sequences of orthologs of human WNT10A protein were retrieved from the KEGG database [26]; these sequences were used to perform sequence alignment with ClustalX2.0.12 [27]. PsiPred 3.0 [28] was used to predict the 2D structure of human WNT10A protein.

We used the crystal structure of EDA-A1 (PDB id 1RJ7 [29]) as a scaffold to predict the influence of the mutations. The structures were analyzed using the Insight II (2000) software package (Accelrys Inc., San Diego, CA, USA). Images were produced by PyMOL (DeLano WL., 2002. The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org) from PDB file 1RJ7.

**Results**

Digenic mutations of both EDA and WNT10A were identified in 2 of 88 (2.27%) isolated oligodontia cases and 4 of 26 (15.38%) syndromic tooth agenesis cases. The patients in all four syndromic cases had HED (Fig. 1). No mutation in EDAR or EDARADD gene was found, however, some single nucleotide polymorphism (SNP) loci were detected (Table S1).

**Clinical details and DNA sequence analysis of six nuclear families**

“N1” in the non-syndromic group (Table 1) represents a pair of male monozygotic twins (4 years old) who were considered as one participant. The number of missing teeth in both boys was 26. The nucleotide sequence showed a G to C transition at nucleotide 769 (c.769G>C) of the coding sequence in exon 7 of EDA, which results in the substitution of Gly at residue 257 to Arg. Additionally, the nucleotide sequence showed a monoallelic C to T transition at nucleotide 511 (c.511C>T) of the coding sequence in exon 3 of WNT10A, which results in the substitution of Arg at residue 171 to Cys. DNA sequencing of the parents’ genome revealed that both mutant alleles were from their mother (Fig. 2A), who carried a heterozygous ED4 mutation (c.769G>C) and a heterozygous WNT10A c.511C>T mutation, and showed absence of only the left upper lateral incisor without other clinical abnormalities. No mutations in these genes were found in the father.

“N2” is a 6-year-old boy who was normal except for the absence of 24 teeth (Table 1). The p.G312Met (c.936C>G) mutation in EDA and heterozygous p.Arg171Cys (c.511C>T) mutation in WNT10A were detected. The coding sequence in exon
9 of *EDA* showed a C to G transition, which results in the substitution of Ile at residue 312 to Met; also, the coding sequence in exon 3 of *WNT10A* showed a C to T transition at nucleotide 511, which results in the substitution of Arg at residue 171 to Cys. Analyses of his parents' genome revealed that the mutant alleles were from his mother, who carried digenic heterozygous *EDA* and *WNT10A* mutations at the same locus as that of N2 (Fig. 2B). Clinical examination showed that maxillary lateral incisors on both sides and the left mandibular second molar were missing in the mother, but there were no anomalies in other organs. The father did not have any mutations for these genes.

“S1” is a 14-year-old boy who had 21 permanent teeth missing (Table 1). The nucleotide sequence showed a T deletion at nucleotide 252 (c.252DelT) of the coding sequence in exon 1 of *EDA*; this leads to a frame shift from residue 84 and a premature termination at residue 90. Additionally, a monoallelic C to T transition at nucleotide 511 (c.511C>T) of the coding sequence in exon 3 of *WNT10A* was detected, this leads to the substitution of Arg at residue 171 to Cys. Sequence analyses revealed that both mutant alleles were from his mother (Fig. 2C), who only had microdontia of the upper lateral

“S2” is a 17-year-old boy who had curly hair, 17 missing permanent teeth and hypohidrosis, his skin and nails were normal (Fig. 1 and Table 1). The p.Arg153Cys (c.457C>T) mutation was found in exon 3 of *EDA*, it results in the substitution of Gly at residue 153 to Cys. Moreover, a heterozygous p.Gly213Ser (c.637G>A) mutation was detected in exon 3 of *WNT10A*, it leads to the substitution of Gly at residue 213 to Ser. Sequence analyses revealed that both mutant alleles were from his mother (Fig. 2D), who had a very mild phenotype of isolated tooth agenesis. His father did not have mutations in either of these genes.

“S3” is a 14-year-old girl who had the typical clinical characteristics of HED: sparse hair, 26 missing permanent teeth, hypohidrosis, dry skin, and eczema on her body, but no plantar hyperkeratosis or nail abnormalities (Table 1). The heterozygous p.Arg156Cys (c.466C>T) mutation was found in exon 3 of *EDA*, it results in the substitution of Arg at residue 156 to Cys. Additionally, the monoallelic p.Gly213Ser (c.637G>A) mutation was also detected in exon 3 of *WNT10A*, it results in the substitution of Gly at residue 213 to Ser. Sequence analyses of her parents' genome revealed that the mutant alleles were from her mother (Fig. 2E), who only had microdontia of the upper lateral

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**Figure 1. Clinical characteristics of patients with tooth agenesis with digenic mutations in both WNT10A and EDA.** (A) Clinical phenotype of patient S2 showing congenital tooth agenesis. (B) Panoramic radiograph of the dentition of patient S2. (C) Facial profile of patient S2. (D) Schematic presentation of congenitally missing teeth of the patients with digenic mutations, two with non-syndromic oligodontia and four with syndromic tooth agenesis. The missing tooth is represented by a filled square; △, tapered tooth; Max, maxillary; Mand, mandibular.

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incisors. Her father did not carry mutations for either of these genes.

"S4" is an 8-year-old boy who also had the typical characteristics and facial features of HED and was missing 28 permanent teeth, but he did not have plantar hyperkeratosis or nail abnormalities (Table 1). The p.Ala349Thr (c.1045G>A) mutation in exon 9 of ED1 and heterozygous p.Arg171Cys (c.511G>T) mutation in exon 3 of WNT10A were detected. These mutations were not found in his father’s genome, but because his mother’s DNA sample was unavailable, the origin of the mutant alleles was not clear (Fig. 2F).

All novel mutations that were identified in this study were not found in the normal controls.

Protein structure analysis

The results of protein structure analyses of WNT10A are shown in Figure 3. R171 and G213 are conserved residues throughout these organisms and located on conserved 2D fragments. Mutations of the residues could affect the function of the human WNT10A protein. In the case of R171C mutations, the substitution of Cys, a hydroxylic amino acid with a side chain shorter than Arg, might eliminate the electrostatic interaction of R171 with adjacent residues. The mutation G213S is expected to abolish the hydrophobic interaction of G213 with adjacent residues.

The 3D structure of EDA is shown in Figure 4. The G257 residue is located at the interface of two trimers. When G257R mutation happened, the side chain volume significantly enlarged, making it possible to form interaction with the R289 in adjacent trimer and abolish the stabilization of EDA. I312 is located at the outer surface of the three monomers. An I312M mutation could affect the interactions of EDA with its receptors.

Discussion

This is the first study to show that simultaneous WNT10A and ED1 mutations could lead to tooth agenesis in the Chinese population. We found that six participants harbored digenic mutations in both WNT10A and ED1: two of them had isolated oligodontia and the others had syndromic tooth agenesis. Two WNT10A and six ED1 mutations were identified in these six patients. Two of the ED1 mutations, p.G257R and p.I312M, which were identified in isolated oligodontia patients, are novel mutations. Based on the results of three-dimensional EDA structure analysis, we think that the two novel ED1 mutations cause functional abnormalities in the EDA protein; therefore, we consider these two ED1 mutations to be pathogenic. We used the sequence alignment of the orthologs to predict the 2D structure of human WNT10A protein; based on the results, we think that the two WNT10A mutations are highly conserved residues and that these mutations could affect the function of human WNT10A protein. The other four ED1 mutations identified in HED patients and the two WNT10A mutations have been reported in tooth agenesis patients previously [20,30,31].

Using phenotype-genotype analysis, we found that although patient S3 and her mother had the same mutation (heterozygous ED1 p.Arg156Cys mutation and heterozygous WNT10A p.Gly213Ser mutation), patient S3 showed typical phenotypic expression of HED while her mother only had microdontia. Because the ED1 mutation is the most frequent pathogenic cause of XLHED and the ED1 gene is located on chromosome X, most female carriers of ED1 mutations present with a normal or very mild phenotype [32,33]. Therefore, one possible explanation for the differential expression of the phenotypes in patient S3 and her mother could be random X chromosome inactivation.

| Table 1 | Clinical features and mutations in the six patients. |

| Group | Non-syndromic tooth agenesis | Syndromic tooth agenesis |
|-------|------------------------------|--------------------------|
| Proband | Gender | Age | Number of missing teeth | Hair | Dry skin | Hypohidrosis/anhidrosis | Plantar hyperkeratosis | Nails | Other signs |
| N1 | male | 4 | 26 | curl | - | - | - | rough | - | |
| N2 | male | 6 | 24 | - | + | - | - | - | - | |
| S1 | male | 14 | 21 | - | + | - | - | + | - | |
| S2 | male | 17 | 26 | - | + | - | - | + | - | |
| S3 | female | 14 | 26 | - | + | + | - | - | Eczema | |
| S4 | male | 8 | 28 | + | + | + | - | - | Dry eyes | |
| N | M, male; F, female; +, present; - , absent; ++, very mild; *, Novel mutations identified in the present study. |

| Number of missing teeth | Hair | Dry skin | Hypohidrosis/anhidrosis | Plantar hyperkeratosis | Nails | Other signs |
|--------------------------|------|----------|------------------------|----------------------|-------|-------------|
| 26 | curl | - | - | rough | - | - |
| 24 | - | + | - | - | - | - |
| 21 | - | + | - | + | - | - |
| 17 | - | + | - | + | - | - |
| 26 | - | + | + | - | - | - |
| 28 | + | + | + | - | - | - |

WNT10A and ED1 Digenic Mutations in Tooth Agenesis

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In this study, patients N1, N2 and S2 had hemizygous EDA mutations and heterozygous WNT10A mutations; their mutations were from their mothers, who harbored composite heterozygous mutations of both EDA and WNT10A. Therefore, the heterozygous WNT10A mutation was identical between the patient and mother, but the EDA mutation was not. If we consider this and the observation of the more severe phenotype in the patients together, it seems that the differences in the EDA mutation may have been responsible for the phenotype differences. This also means that heterozygous WNT10A mutations may result in a very mild phenotype. Moreover, the phenotype of these patients was not more distinctly severe than those of patient with single EDA mutations reported previously [6,32], which further supports our speculation. However, further studies are definitely required to confirm these speculations, possibly in a bigger patient group.

Previous studies have indicated that digenic mutations in one patient could cause two different diseases. For example, Wang et al. reported the case of a 5-year-old boy who had dentin defects caused by a COL1A2 mutation and hypodontia caused by a PAX9 mutation [34]. On the other hand, two different gene mutations could influence each other and result in one disease: for example, Desmoglein-2 and Desmocollin-2 mutations have been reported to cause arrhythmogenic right ventricular dysplasia/cardiomyopathy [35]; a OGG1 mutation in combination with a MUTYH mutation reportedly led to hereditary colorectal cancer [36]; and NEK7 and D1NC2H1 mutations resulted in short rib polydactyly, Majewski type [37]. In this study, digenic mutations of both WNT10A and EDA caused isolated and syndromic tooth agenesis, and the protein structure analyses indicated that all the WNT10A and EDA mutations may affect protein function. Therefore, we think that interaction between two mutated proteins may cause tooth developmental anomalies.

Wnt10a functions through the canonical Wnt/B-catenin pathway, which plays crucial roles at multiple stages of tooth development, including osteogenesis and osteoclastogenesis.
development. Interruption of the Wnt/β-catenin pathway results in severe tooth agenesis [38]. Eda functions via the Eda/Edar/NF-κB signaling pathways. Reciprocal interaction between the Wnt/β-catenin and Eda/Edar/NF-κB signaling pathways continues throughout the process of ectodermal organ development. In developing skin appendages, Dkk4, which acts as an inhibitor of Wnt signaling, is the direct transcriptional target of Eda/Edar signaling during placode formation [39]. Zhang et al. recently showed a sequential interdependency between the Wnt and Eda pathways in developing hair follicles: Wnt/β-catenin signaling is essential for NF-κB activation, whereas Edar/NF-κB is thereafter required to strengthen and maintain Wnt/β-catenin activity [13]. Moreover, during tooth morphogenesis, Wnt10a and Edar were expressed at the same locations before the cap stage, that is, in the dental epithelium at initiation and bud stages and in the enamel knot during the cap stage [11]. Wnt signals regulate ectodysplasin expression in the oral ectoderm, and the expression of Edar in the epithelial signaling centers is responsive to Wnt-induced ectodysplasin from the nearby ectoderm [40]. Therefore, mutations in WNT10A and EDA proteins might affect these pathways and cause abnormalities in tooth development. However, further studies are required to elucidate the interaction between Eda/Edar/NF-κB and Wnt/β-catenin signaling in normal and abnormal tooth development.

In conclusion, this is the first study to report two simultaneous gene mutations of EDA and WNT10A in HED and isolated tooth agenesis patients. Our results suggest that WNT10A and EDA digenic mutations could result in oligodontia and syndromic tooth agenesis in the Chinese population. The results further confirm that other genetic factors influence the phenotypic expression in

Figure 3. Sequences of orthologs and predicted 2D structure of human WNT10A protein. (A) The alignment of orthologs of the human WNT10A protein. The R171 and G213 residues are represented by arrowheads. (B) The predicted 2D structure of human WNT10A protein. The R171 and G213 residues are in yellow.
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severe tooth agenesis patients with heterozygous WNT10A mutations. We believe that this study will greatly expand the genotypic spectrum of tooth agenesis.

Supporting Information

Table S1 Single nucleotide polymorphisms in EDAR and EDARADD of 114 patients.

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Author Contributions

Conceived and designed the experiments: HH DH HF. Performed the experiments: HH DH SS. Analyzed the data: HH DH HQ. Contributed reagents/materials/analysis tools: BB ZZ. Wrote the paper: HH DH HF.

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Figure 4. Structure analysis of mutant residues in the three-dimensional EDA trimer. The EDA trimer is shown as a ribbon with relevant side chains rendered in spheres. The G257 and I312 residues are in yellow and blue, respectively. The side chain of the R289 residue is represented by a colored stick. (A) The planform of the EDA trimer. (B) The side view of the EDA trimer.

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