A Novel EDAR Missense Mutation Identified by Whole Exome Sequencing with Non-Syndromic Tooth Agenesis in a Chinese Family

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Abstract

To investigate novel mutations of pathogenic genes responsible for non-syndromic tooth agenesis (NSTA) and to provide a genetic basis for the study of its pathogenesis. Peripheral blood samples of four pedigrees with NSTA were collected for DNA extraction. The coding region of the EDA1 gene was amplified by PCR and sequenced to investigate new mutations. Whole exome sequencing and Sanger sequencing were then performed for Family 4. A novel mutation c.338G>A (Cys113Tyr) in the EDAR gene was identified in a pedigree. In addition, three EDA1 mutations were identified in three patients: c.865C>T (Arg289Cys), c.866G>A (Arg289His), and c.1013C>T (Thr338Met). Genotype-phenotype correlation analysis of EDAR gene mutation showed that NSTA patients were most likely to lose the maxillary lateral incisors, and the maxillary first premolars were the least affected. The phenotype of mutations at codon 289 of EDA1 in NSTA reported patients were characterized by lateral incisors loss, rarely affecting the maxillary first molars. A novel EDAR missense mutation, c.338G>A (Cys113Tyr), was identified in a pedigree with NSTA, extending the mutation spectrum of the EDAR gene. Genotype-phenotype correlation analyses of EDAR and EDA1 mutation could help to improve disease status prediction in NSTA families.

1 | INTRODUCTION

Tooth agenesis is a pathological condition involving the absence of teeth due to a developmental failure(De Coster, Marks, Martens, & Huysseune, 2009). Non-syndromic (selective) tooth agenesis is one of the most common dental anomalies and is known to be associated with variants of *MSX1* , *PAX9* ,*AXIN2* , *EDA1* , *EDAR* , *EDARADD* ,*WNT10A* (Arte, Parmanen, Pirinen, Alaluusua, & Nieminen, 2013),*WNT10B* (P. Yu et al., 2016),*LRP6* (Ockeloen et al., 2016; M. Yu, Wong, Han, & Cai, 2019) and*GREM2* (Kantaputra et al., 2015). Of these, *EDA* , *EDAR* , *EDARADD* , and *WNT10A* are candidate genes of both non-syndromic tooth agenesis (NSTA) and syndromic tooth agenesis (STA). Ectodysplasin-A1 (EDA1) has been shown to bind specifically to the ectodysplasin-A receptor (EDAR), a member of the TNF receptor superfamily, and activate the nuclear factor kappa B (NF-ϰB) (Yan et al., 2000). The EDA-EDAR-NF-ϰB signaling pathway crosstalks to the WNT and BMP pathways(Shen et al., 2016) and plays an important role in embryonic ectodermal development(Cluzeau et al., 2011; Koppinen, Pispa, Laurikkala, Thesleff, & Mikkola, 2001).

Ectodermal dysplasia caused by *EDAR* mutations have been widely reported; according to M. Yu et al.(M. Yu et al., 2019) about 58 *EDAR* mutations have been found in STA. However, only seven mutations of *EDAR* have been found in patients with NSTA(Arte et al., 2013; Jonsson et al., 2018; Mumtaz, Nalbant, Blükba, Huma, & Malik, 2020; Zeng et al., 2017). Similarly,*EDA1* is the only gene known to be associated with X-linked hypohidrotic ectodermal dysplasia (XLHED), which accounts for 95% of cases of hypohidrotic ectodermal dysplasia (HED). According to Trzeciak(Trzeciak & Koczorowski, 2016), there have been 345 reported cases of HED, of which 206 are due to*EDA1* mutations. As at 2017, the Human Gene Mutation

Database (HGMD Professional 2017.2) had registered 314 mutations in the*EDA1* gene(Reyes-Reali et al., 2018).

In the present study, we investigate a novel missense mutation (*EDAR* c.338G>A p. Cys113Tyr), as well as three previously-reported missense mutations of *EDA1* in Chinese Han families. The genotypes and phenotypes of all published *EDAR*mutant patients and mutations at codon 289 of *EDA1* in NSTA patients were analyzed. The aim of our study is to investigate the potentially pathogenic gene mutations for NSTA, to provide a genetic mechanism and a genotype-phenotype correlation for NSTA caused by mutations.

2 | MATERIALS AND METHODS

2.1 | Subjects and ethical approval

The study involved four non-consanguineous patients with NSTA who were referred to the Department of Prosthodontics, School and Hospital of Stomatology of Hebei Medical University, and 100 non-consanguineous controls. All participants were examined by prosthodontics specialists to determine the status of dentition. An oral examination and a dental treatment history were performed, and panoramic radiographs were taken to confirm the congenital absence of teeth (Figure 1). The study was conducted under the approval of the Ethics Committee of the School and Hospital of Stomatology, Hebei Medical University (NO: [2016] 004). All participants or their guardians signed written informed consent.

2.2 | DNA sample collection and extraction

The probands were patients at the School and Hospital of Stomatology, Hebei Medical University. Peripheral blood samples were collected using EDTA as anticoagulant. DNA was extracted from leukocytes using standard proteinase-K phenol chloroform methods (E.Z.N.A. Blood DNA Midi Kit, Omega, GA) and stored at -20°C.

2.3 | PCR amplification and mutation screening

The primers used to amplify the eight coding exons of the *EDA1*gene in PCR were based on those used by Song et al(Feng et al., 2018; Song et al., 2009). PCR reactions were elicited in a total volume of 50μl, each containing 100ng DNA, 4μl dNTPs, 5ml 10 × TransStart Taq Buffer, 0.2μl of each primer, and 1.25 U TransStart Taq DNA Polymerase (Thermo Fisher, USA). After denaturing at 95°C for 5 min, amplification was carried out as follows: 35 cycles at 95degC for 30 s, 60degC for 30 s, 72degC for 30 s, and finally 72degC for 7 min. Primers of exon 4 of the *EDAR* gene were as follows: F: 5'-GGCAAGAGTAGCTTCTGGAGAC-3'; R: 5'-GTTAATGGCCACTTAGGAGACAC-3'. Amplification was tested by agarose gel electrophoresis and DNA was sequenced by the Beijing Genomics Institute, Beijing, China. The nucleotide sequence was analyzed using the BLAST database of the National Center for Biotechnology Information (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). We identified the nucleotide variants in the *EDA1* and *EDAR*genes and 100 unrelated population-matched controls.

2.4 | Whole exome sequencing and Sanger sequencing

Whole exome sequencing was performed for the proband (Family 4), who did not have any *EDA1* mutations detected by PCR. Target enrichment and amplification were performed via liquid-phase capture method with testing kits from iGeneTech Bioscience (Beijing, China) Co., Ltd. Illumina NovaSeq 6000 Genome Analyzer platform (Illumina, San Diego, CA, USA) was used to sequence the exons from the targeted regions. With a sequencing yield of more than 17,550 Mb raw bases, the samples achieved a mean target depth of 138 x. Reads were aligned to the Genome Reference Consortium Human Build 37 (GRCh37/hg19) with the Burrows-Wheeler Aligner. Single-nucleotide variants and small indels were identified with SAMtools and Genome Analysis Toolkit (GATK) and then annotated by ANNOVAR. The candidate mutation of *EDAR* was verified with PCR, followed by Sanger sequencing. PCR was performed, and the PCR products were sequenced as described in Section 2.3. The reference sequence for*EDAR* was NM_022336.4. A cross-species alignment of the amino acid sequence of EDAR was performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo).

3 | RESULTS

3.1 | Clinical features

The clinical examination of four probands from the four NSTA families all exhibited congenital oligodontia, each missing 6-10 deciduous teeth and 8-18 permanent teeth (excluding the third molars, Figure 1). The loss of permanent teeth was confirmed by panoramic radiographs and found to be distributed extensively in both dentitions. However, the shapes and sizes of the residual teeth were normal. None of their parents (except the mother of proband 4) had congenitally missing teeth, and mothers did not have small or conical teeth. All participants reported normal sweating and lachrymal secretions. They had no complaints of dry mouth, intolerance to heat, or susceptibility to respiratory tract infections. The participants had hair on the body and scalp, and their facial features, skin, and nails were observed to be normal. (Figure 1).

3.2 | Mutation analysis of *EDAR* and *EDA1*

After diagnosis, we aimed to determine the causative gene. After screening the coding sequences of the *EDA1* gene by PCR, no mutation was found in Family 4. We found a new *EDAR* mutation c.338G>A (Cys113Tyr) by using whole exome sequencing technology by "MAF<1%" and "exonic" filtering. SIFT, Polyphen2 and MutationTaster predictions for the mutation were "deleterious" (0.00), "probably damaging" (0.996), and "disease-causing" (1.00), respectively, suggesting the variant was highly pathogenic. Candidate mutation was then confirmed for proband 4 and his mother by Sanger sequencing (Figure 2B), while his father and brother were wild type at this location. A cross-species amino acid sequence alignment of EDAR showed that the mutation site was highly conserved among humans (>NP_071731.1), cattle (>XP_005212787.1), zebrafish (>NP_001108536.2), rhesus monkeys (>XP_014968589.2), dogs (>XP_005626028.2), mice (>NP_034230.1), and chickens (>NP_001012629.1) (Figure 2C).

Three of the mutations found in oligodontia patients have previously been reported in *EDA1* exons: c.865C>T (Arg289Cys)(Song et al., 2009); c.866G>A(Arg289His)(Ruiz-Heiland et al., 2016); and c.1013C>T (Thr338Met)(Han et al., 2008) (Figure 3), all of which are located in the TNF domain. In patient 1, the p.Arg289Cys (c.865C>T) mutation occurs in exon 7 of*EDA1* , changing codon 289 from encoding Arg to Cys. A p.Arg289His (c.866G>A) mutation was found in exon 7 of *EDA1* of patient 2, changing codon 289 from encoding Arg to His. For patient 3, there was a p.Thr338Met (c.1013C>T) mutation in exon 8 of*EDA1* , changing codon 338 from encoding Thr to Met.

3.3 |Phenotypes of NSTA patients with *EDAR* mutations

We reviewed four published studies and summarized data from*EDAR* -related NSTA patients with a total of seven *EDAR*mutations(Arte et al., 2013; Jonsson et al., 2018; Mumtaz et al., 2020; Zeng et al., 2017). The mutation sites of the *EDAR* gene, protein changes, and the types of mutations are given in Table 1. The details of the missing teeth are listed in Table 2. In NSTA patients with *EDAR* mutations, the maxillary lateral incisor had the highest missing rate (83.3%), followed by the mandibular lateral incisor (43.3%), the mandibular central incisor (40.0%), and the second premolars (33.3%); although this does not include the third molars. The missing rate of the maxillary first premolars is lowest (6.7%), followed by the mandibular canines (10%). In all, the rate of loss of the molars is lower than that of anterior teeth (Figure 4).

3.4 | Phenotypes of NSTA patients with *EDA1*mutations at codon 289

Two *EDA1* mutations (c.865C>T and c.866G>A) at codon 289 were reported in our study as well as in previous studies (Table 3, (Lee et al., 2014; Ruiz-Heiland et al., 2016; Song et al., 2009)). The p.Arg289Leu and p.Arg289Cys mutations caused a change from a positively-charged residue to a non-polar residue. Although the Arg289His mutation did not cause a change in physicochemical properties, the volume of the side chain decreased. Phenotype analysis shows that reported mutations at codon 289 of *EDA1* affects the lateral incisors (100%, 91.7%), the mandibular central incisors (91.7%), and the mandibular second premolars (75.0%). However, the second molars (8.3%), mandibular first molars (16.7%), and maxillary central incisors (16.7%) are less affected. It is particularly interesting that maxillary first molars are present in all patients (Figure 5).

4 | DISCUSSION

Mutations of the *EDAR* gene can result in HED(A.K. et al., 2017; Feng et al., 2018). The Human Gene Mutation Database (HGMD Professional, 2018.3) has 62 registered pathogenic variants of *EDAR* , of which 50 have the HED phenotype(Parveen et al., 2019). In recent years, several *EDAR* mutations in NSTA patients have been identified(Arte et al., 2013; Jonsson et al., 2018; Mumtaz et al., 2020; Zeng et al., 2017). In our study, a new*EDAR* missense mutation c.338 G>A(Cys113Tyr) was detected by whole exome sequencing in Patient 4 and his mother. A cross-species alignment of amino acid sequences of *EDAR* showed that p.C113 was conserved across seven species, implying the high pathogenicity of the variant. Our result expands the mutation spectrum of *EDAR* .

EDAR is a type I transmembrane protein and a member of the TNF receptor superfamily. It has a cysteine-rich domain in the extracellular region (encoded by exons 3, 4 and 5), as well as a death domain in its intracellular region (encoded by exon 12). EDAR interacts with extracellular EDA1 and intracellular EDARADD via the extra- and intracellular regions to form a complex. This in turn activates downstream nuclear factor kappa B (NF-ϰB) to mediate transcription of the target gene(Kumar, Eby, Sinha, Jasmin, & Chaudhary, 2001; Masui et al., 2011; Okita, Asano, Yasuno, & Shimomura, 2019; Outi et al., 2001; Parveen et al., 2019). The p.Cys113Tyr (c.338G>A) mutation in exon 4 occurs in a region that encodes the cysteine-rich domain that binds to EDA1. We speculated that the p.Cys113Tyr substitution of EDAR might alter its affinity with EDA1, so that it instead binds to EDARADD, ultimately affecting the activation of downstream nuclear factor (NF)-ϰB.

By studying the information of all NSTA patients with *EDAR*mutations, we found that more than half (5/8) of *EDAR* mutations in NSTA patients were concentrated in the death domain encoded by exon 12, and the majority of mutations (6/8) were missense mutations (Table 1). We also observed that the most common missing teeth were the maxillary lateral incisors, followed by the mandibular lateral incisors and the mandibular central incisors. The maxillary first premolars and mandibular canines were the least likely to be affected (Table 2; Figure 4). It seems that anterior teeth, especially the incisors, are sensitive to mutations in the *EDAR* gene. Arte et al. (Arte et al., 2013)and Mumtaz et al. (Mumtaz et al., 2020) found that both deciduous and permanent incisors were involved most commonly in those EDAR-related TA cases, is similar to our finding. In particular, the number of permanent teeth lost in patients with *EDAR* -related NSTA ranged from 2 to 10. In the present study, the proband and his mother had 18 and 17 permanent teeth missing, respectively. This differs from previous studies, but our participants presented with no signs of other ectodermal hypoplasias, such as in the hair or skin. We hypothesized that the differences in the number of missing teeth might be associated with single nucleotide polymorphisms or epigenetic factors.

According to M. Yu et al.(M. Yu et al., 2019), over the past two decades 198 different mutations had been detected that are responsible for NSTA, of which 27 are derived from*EDA1* . Previous studies revealed a clear link between the genotype and phenotype for congenital tooth deficiency(Han et al., 2008; He, Liu, Han, Liu, & Feng, 2016; Wong et al., 2018; Zhang et al., 2011). Han et al.(Han et al., 2008) studied 24 NSTA patients with *EDA1* mutations and conducted statistical analysis on the number of missing teeth in each position of dentition. They found that the most likely missing teeth were the lateral incisors followed by the mandibular central incisors; and the least likely missing teeth were the maxillary central incisors and first permanent molars. The results of He et al.(He et al., 2016) also confirmed that these characteristics were specific phenotypes of NSTA caused by *EDA1* mutations.

In our research, three mutations were found in the *EDA1* gene of oligodontia Patient 1-3. The probands shared the *EDA1* mutations with their mother (Figure 3), indicating that the mutant alleles were inherited from the maternal line in Family 1, 2, and 3. Two of the mutations (c.865C>T and c.866G>A) observed in this study were located in the TNF domain of *EDA1* . The TNF homology domain forms a homotrimer, which is required for interaction with the receptor at the monomer–monomer interface(Hymowitz et al., 2000). Song et al.(Song et al., 2009) carried out structural analysis on the EDA1 protein and found that in the wild-type 3D EDA1 structure, the Arg289 residue is located at the outer surface of the homotrimer and makes structural hydrogen bonds with Asn272. In addition, Arg289 forms hydrogen bonds and electrostatic

interactions with Glu308 of the adjacent homotrimer(s) to stabilize the multi-trimer asymmetric unit(Lee et al., 2014; Ruiz-Heiland et al., 2016; Song et al., 2009). Variants at the p.289 location would abolish these interactions and reduce protein stability. Therefore, codon 289 mutations in the TNF domain partly impact on the binding of EDA protein to EDA receptor.

However, it is worth noting that although all changes occurred at p.289 of *EDA1* , the phenotypes of patients were slightly different. We performed a genotype-phenotype analysis and found that all maxillary lateral incisors are affected, followed by mandibular incisors and mandibular second premolars. In contrast, the second molars, mandibular first molars and maxillary central incisors were less affected. It is particularly interesting that the maxillary first molars were present in all patients. Our phenotypic analysis of the mutations at codon 289 of*EDA1* is consistent with the typical phenotype resulting from*EDA1* gene mutations. However, the mechanism that causes the slight changes to the phenotype of teeth loss due to protein mutations remains to be studied.

5 | CONCLUSION

The study identified a new *EDAR* mutation c.338G>A (Cys113Tyr), and both conservative and pathogenic analyses suggest that this mutation is highly pathogenic. In addition, we studied previously-reported *EDAR* mutations and summarized their genotype-phenotype correlation in NSTA patients. This allowed us to expand the *EDAR* mutation spectrum as well as providing a genetic basis for the pathogenesis of congenital tooth agenesis. This research could help in preconception genetic counseling, prenatal screening and fetus diagnosis, which contribute to disease status prediction in NSTA families.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Web resources

National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo)

Conflict of interest:

The authors declare no conflict of interest.

Figure legends

FIGURE 1 Characteristics of the four probands with non-syndromic tooth agenesis (NSTA).

(A) Intraoral image of probands; (B) Legend of intraoral missing teeth of probands; (C) Panoramic radiography of probands. Black squares indicate missing teeth; Max: maxillary; Mand: mandibular.

FIGURE 2 Identification of a missense mutation of the EDAR gene in NSTA Family 4.

(A) The pedigree of NSTA Family 4; the black arrow points to the proband. (B) DNA sequencing chromatograms of the proband and his mother. (C) Sequence alignment results show that the other six residues are conserved across seven species. The mutant allele is marked in the red box. *, indicate a completely conserved column of amino acids.

FIGURE 3 Pedigrees and mutation information in NSTA Families 1-3.

Black arrows point to the probands. Red arrows indicate the mutated bases in DNA sequencing chromatograms. Three reported EDA1 mutations c.865C>T (Arg289Cys), c.866G>A (Arg289His), and c.1013C>T (Thr338Met) were identified in the probands of NSTA families 1-3. Mothers of patients are all heterozygotes, fathers are all unaffected.

FIGURE 4 Tooth missing rate of NSTA patients with EDAR mutations (excluding the third molars).

Max, maxillary; Mand, mandibular; CI, central incisor; LI, lateral incisor; Ca, canine; PM, premolar; Mo, molar. Light stripes indicate tooth missing rate of maxillary dentition. Dark stripes indicate tooth missing rate of mandibular dentition.

FIGURE 5 Tooth missing rate of NSTA patients with EDA1 mutations at codon 289 (excluding the third molars).

Max, maxillary; Mand, mandibular; CI, central incisor; LI, lateral incisor; Ca, canine; PM, premolar; Mo, molar. Light stripes indicate tooth missing rate of maxillary dentition. Dark stripes indicate tooth missing rate of mandibular dentition.

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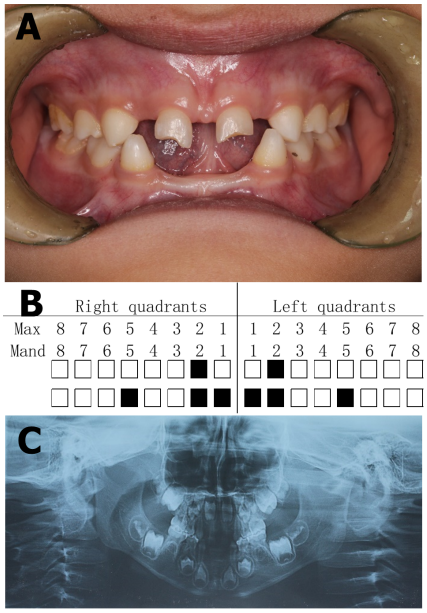
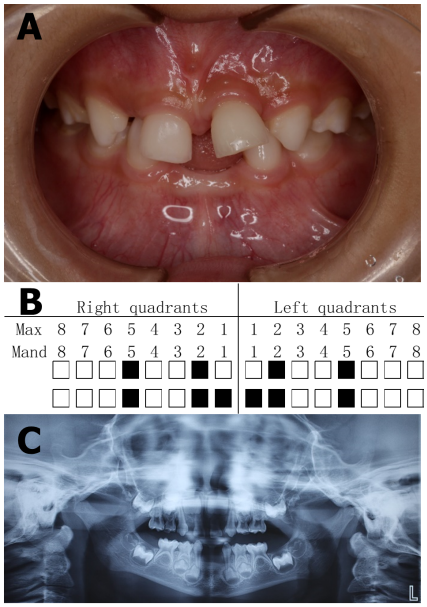


Patient 1 (Family 1 , II:1)



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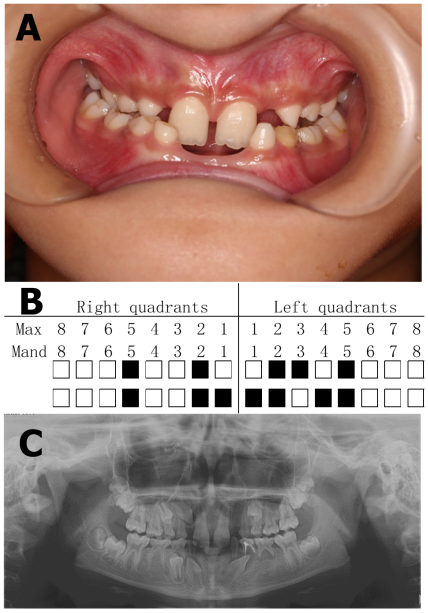

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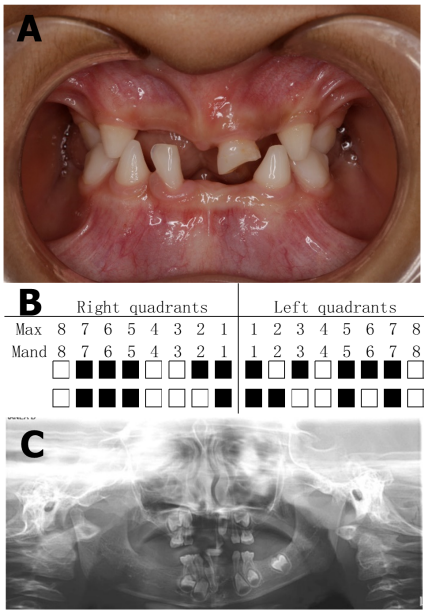

Patient 4 (Family 4 , III:6)