Identification of DSPP Novel Variants and Phenotype Analysis in dentinogenesis dysplasia shields type II Patients

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Abstract

Objectives: To investigate the genetic causes and characteristics of teeth of dentin dysplasia Shields Type-II(DD-II) in three Chinese families.

Materials and Methods: Three Chinese families affected with DD-II were collected. The whole-exome sequencing(WES) and whole-genome sequencing(WGS) were conducted to screen for variations, and sanger sequencing was used to verify mutation sites. The physical and chemical characteristics of the affected teeth including tooth structure, hardness, mineral content and ultrastructure were investigated.

Results: A novel frameshift deletion mutation c.1871_1874del(p.Ser624fs) in *DSPP* was found in family A and family B, while no pathogenic mutation was found in family C. The affected teeth's pulp cavities were obliterated, and the root canals were smaller than normal teeth and irregularly distributed comprising a network. The patients' teeth also had reduced dentin hardness and highly irregular dentinal tubules. The Mg content of the teeth was significantly higher than the controls but the Na content was obviously lower than the controls.

Conclusions: A novel frameshift deletion mutation c.1871_1874del(p.Ser624fs) in the *DPP* region of the *DSPP* gene caused DD-II. The DD-II teeth demonstrated compromised mechanical properties and changed ultrastructure, suggesting an impaired function of DPP. Our findings expanded the mutational spectrum of *DSPP* gene and strengthen the understanding of clinical phenotypes related to the frameshift deletion in the *DPP* region of the *DSPP* gene.

Clinical Relevance: *DSPP* mutation can cause the characteristics of the affected teeth including tooth structure, hardness, mineral content and ultrastructure.

Introduction

Hereditary Dentin Defects (HDD) show autosomal dominant transmission patterns, characterized by abnormal dentin development. The affected teeth were typically grey or yellow-brownish colored and the pulp chamber was early obliterated or delay developed. The enamel was easily to exfoliate, leading to severe attrition of the dentin. Due to the abnormal development of dentin, caries are more likely to occur than in normal teeth, which seriously affects the patient's aesthetics and chewing function. Shields et al. [1] classified hereditary dentin dysplasia into dentinogenesis imperfecta (DGI) and dentin dysplasia (DD) in 1973 based on clinical and radiographic features. Among them, DGI is divided into three types, and DD is classified as two types.

The dentin sialophosphoprotein (*DSPP*) gene plays an important role in dentin development and mineralization. Studies have found that the *DSPP* gene is the cause for DGI, shields type II(DGI-II) (OMIM 125490)[2, 3], DGI, shields type III(DGI-III) (OMIM 125500) [4], DD, shields type II (DD-II) (OMIM 125420)[5] and deafness with dentinogenesis (OMIM 605594). DGI-II, DGI-III and DD-II are now considered as a

spectrum of diseases[6]. Among them, DGI-III has the most severe clinical manifestations, followed by DGI-II, and DD-II is the mildest.

DSPP (NM_014208) is located on chromosome 4q21.3, including 5 exons. Exons 1–4 and the anterior part of exon 5 encode DSP, and the posterior part of exon 5 encodes DPP[7]. The known mutations are mainly in exons 2, 3, 4, 5 [8]. Previous studies[4, 8–10] indicated that mutation types in the *DSP* region included missense mutations, nonsense mutations, and splicing mutations, and the mutations in the *DSP* region mostly correlated to DGI-II. The mutations in the *DPP* region are mainly frameshift mutations, which in the anterior region of DPP were associated with the DD-II clinical phenotype, while mutations in the posterior region correlated with DGI-II. However, the underlying reason for the correlation between the frameshift mutation location and the phenotypes remains to be elucidated.

In this study, three Chinese families with phenotype of DD-II were recruited. By thorough sequence analysis, a novel frameshift deletion mutation c.1871_1874del(p.Ser624fs) in the *DPP* region of the *DSPP* gene was identified in family A and family B. The primary teeth of the patients were collected and the physical and chemical properties of the teeth were examined. The study provides a detailed understanding of the genotype–phenotype correlation in DD-II affected by the frameshift deletion in the *DPP* region of the *DSPP* gene.

Materials And Methods

Participants and clinical assessments

All procedures involving human participants in this study were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant. This study was approved by the Medical Ethics Committee of the Sichuan Provincial People's Hospital [No. 2020(02)].

Three Chinese families with phenotype of DD-II were recruited for this study. The probands and their relatives were recruited, and the detailed information of the participants was listed in Table S1. No affected individuals had symptoms of hearing loss or bone defects.

DNA extraction

Blood samples were collected from all participants, then genomic DNA was isolated from peripheral blood using TIANGEN Blood DNA Kit (TIANGEN, Beijing, China), according to standard procedures. Nanodrop was used to detect the concentration of extracted DNA. The DNA samples were stored at -20°C until use and DNA integrity was assessed by 1% agarose gel electrophoresis as previously described[11].

Next generation sequencing for mutation analysis

The WES of the proband's genomic DNA samples was performed. 200ng genomic DNA of each participant was sheared by Biorupter (Diagenode, Belgium) to acquire 150 ~ 200bp fragments. The ends of the DNA fragment were repaired, and Illumina Adaptor was added (Fast Library Prep Kit, iGeneTech,

Beijing, China). After sequencing, the library was constructed, and the whole exons were captured with AIExome Enrichment Kit V1 (iGeneTech, Beijing, China) and sequenced on the Illumina platform (Illumina, San Diego, CA) with 150 base-paired-end reads.

The WGS was conducted when WES results of the proband showed no pathogenic variants. 200ng genomic DNA of each individual was used to construct sequencing library with Enzyme Plus Library Prep Kit (iGeneTech, Beijing, China) according to the instructions. The WGS was performed on DNBSEQ-T7 sequencing instrument with 150 base paired-end reads as well as a median coverage of 35X.

Raw reads were filtered to remove low quality reads by using FastQC. Then clean reads were mapped to the reference genome GRCh37 by using Burrows-Wheeler Aligner (BWA). Variant calling was performed using GATK with Haplotype Caller, then SNV and InDel were annotated by dbSNP138 (https://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes Project (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp), ClinVar(https://www.ncbi.nlm.nih.gov/clinvar) and Gnomad (https://gnomad.broadinstitute.org/) and in-house database. Common variants (minor allele frequency, >0.1%) were excluded. Variants were classified in accordance with the interpretation guidelines of the American College of Medical Genetics and Genomics (ACMG), as potentially pathogenic variants, variants of unknown clinical significance, or benign variants[12].

Sanger sequencing

Candidate pathogenic variants were first validated using sanger sequencing. Specific primers were designed to amplify the corresponding regions. PCR products were sequenced using an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA) according to manuals for the BigDyeTM Terminator v3.1 Cycle Sequencing Kits. The primers are listed in Table S2

Micro-CT

Two primary molars extracted due to severe apical infection from the probands of family A and family B respectively were collected for Micro-CT. Teeth were scanned using a micro-CT system (μCT 100; SCANCO Medical AG, Bruttisellen, Switzerland) with an isotropic resolution of 22.5μm at 70kV/200uA with an exposure time of 400ms.

Microhardness

A total of four primary teeth from the patients were collected for the Microhardness test, one primary anterior tooth and one primary molar from family A, one primary molar from Family B, and one primary anterior tooth of from the proband of a DGI-II family we have reported [11]. Four primary teeth from age-matched normal individuals were used as control. The periodontal membrane and other debris were removed, the tooth samples were immersed in a 10% formaldehyde solution at 4℃ and crosscut into 2 parts by dental diamond burs and inlaid with epoxy resin. Teeth were polished with 600, 1,000, 2,000, 3,000, 5,000, 7,000 and 10,000 mesh water sandpaper (Starcke, Melle, Germany) in sequence and ultrasonically cleaned with distilled water. Three points of the enamel and five points from the dentin

were selected from each tooth, the measurement of the microhardness was performed three times and averaged at each point. Vickers microhardness was measured by using a microhardness tester (QNESS Q10A+, Austria), for which a 0.2 kgf load was applied for 12s to obtain the measurement.

Scanning electron microscopy (SEM) investigation and Energy dispersive spectroscopy (EDS) investigation

After polishing, teeth were vacuum dried, the surfaces were coated with gold in a vacuum, and the cross-section surfaces were observed using SEM as previously described[11] (Inspect F50, FEI Co., Hillsboro, O, USA). EDS results were analyzed using an SEM (FEI Co.) equipped with an EDS INCA system (Oxford Instruments Analytical, Abingdon, UK). The EDS was conducted at a high accelerating voltage of 20 kV. Three points of the enamel and five points from the dentin were selected from each tooth, which is the same as for microhardness (Fig. 3A). Elemental measurements were repeated 3 times at each point, and the Ca/P ratio was calculated.

Statistical Analysis

All data were analyzed by SPSS 22.00. All data were expressed as mean ± SD. The student's t-test was used to compare the mean ± SD between two groups, and the one-way ANOVA analysis was used to compare the mean ± SD of multiple groups. 0.05 was seen to have a significant difference.

Results

Clinical and radiographic features

Family A was a 3-generation Chinese family, a total of four family members were collected including two affected individuals(II:1, III:1) and two unaffected members(I:2, II:2) (Fig. 1A). The proband (III:1) was a 4-year-old boy with a typical yellow-brownish translucent appearance of all teeth (Fig. 1F). The panoramic radiograph also showed a typical feature of obliterated pulp cavities (Fig. 1G). The mother of the proband (II:1) showed normal permanent teeth in both color and shape (Fig. 1H). However, the panoramic radiograph also revealed thistle-shaped pulp cavities (Fig. 1I).

Family B was a 3-generation Chinese family, three family members were collected including two affected ones(II:3, III:2) and one normal phenotypic individual(II:4), and the mother of the proband denied family history(Fig. 1C). The proband (III:2) was a 7-year-old boy with a typical yellow-brownish translucent appearance of primary teeth, but all the permanent teeth of the proband were normal both in color and shape (Fig. 1J). The periapical radiographs revealed obliterated pulp cavities in the primary teeth (Fig. 1K). The mother of the proband (II:3) showed normal permanent teeth in both color and shape (Fig. 1L). But the intraoral panoramic radiograph showed thistle-shaped pulp cavities in the permanent teeth of the entire mouth (Fig. 1M). Family A and Family B were from different provinces of China and both of them denied knowing each other.

Family C was a 4-generation Chinese family, and four family members were collected including three affected ones(III:23, IV:16, IV:17) and one unaffected member(III:24) (Fig. 1E). Both the proband (IV:16) and her younger brother (IV:17) showed the typical yellow-brownish translucent appearance of primary teeth and the newly erupted permanent teeth of the proband were normal both in color and shape (Fig. 1N). The panoramic radiograph of both (IV:16) and (IV:17) revealed obliterated pulp cavities in the primary teeth (Fig. 1O). The father of the proband (III:23) had relatively normal permanent teeth, but the canines showed a more brownish color than normal teeth (Fig. 1P). The panoramic radiograph also revealed thistle-shaped pulp cavities, especially for the anterior teeth (Fig. 1Q).

According to the clinical manifestations, Family A, B and C were diagnosed as dentin DD-II.

Genetic findings

A novel frameshift deletion mutation c.1871_1874del(p.Ser624fs) on exon 5 of the *DSPP* gene was identified by WES in the probands in both family A and B. The affected individuals were heterozygous for the variant, and the variant was not found in the unaffected family members. The variant was completely co-segregated with the phenotype in the family (Fig. 1B,D). The novel mutation was classified as a pathogenic variant according to the ACMG interpretation guidelines [12]. This variant caused a 3-bp deletion near the N-terminus, and a frameshift mutation starting from the serine position on position 624.

No pathogenic variant in the *DSPP* or other possible genes was found by WES and WGS in family C. Four polymorphisms were found in *DSPP* (Table.S3).

Micro CT

Micro CT results revealed that the teeth suffered from severe root obliteration, and root canals were smaller than normal teeth and irregularly distributed comprising of a network as reported before [13]. The macro photo of the tooth also showed that the surface of the affected teeth is milky white, the dentin is typical yellow-brownish, and translucent, most of the enamel still exists, and the root canal is very thin and almost invisible (Fig. 2).

Microhardness

Three points of the enamel and five points from the dentin were selected from each tooth (Fig. 3A). The Vicker's hardness of the DD-II enamel showed no significant difference with the control (287.83 ± 14.74 vs. 302.00 ± 3.61, $P = 0.067$, Fig. 3B, Table S4). The Vicker's hardness of the DD-II dentin was similar with the DGI dentin (29.31 ± 5.28 vs. 29.76 ± 0.55, $P = 0.99$, Fig. 3C Table S5), and both were significantly lower than the control (60.99 ± 4.27, $P = 0.000$).

SEM and EDS results

The SEM showed that the dentinal tubules of the patient teeth were highly irregular in both size and shape, and reduced in number (Fig. 3D,E), while the normal dentinal tubules were highly regular in shape and size (Fig. 3F,G). The EDS results showed that the Mg content of the DD dentin was significantly lower than that of the normal dentin (0.36 ± 0.16 vs. 0.79 ± 0.11, $P = 0.000$). The Na content of the DD dentin

was significantly higher than that of the normal dentin (0.64 ± 0.09 vs. 0.50 ± 0.07, $P = 0.007$). However, there were no significant differences in other elements of the DD teeth and controls (Table S6, Fig. 3H). The EDS result of the enamel also showed that the mineral content of the DD-II's enamel was similar to controls (Table S7, Fig. 3I).

Discussion

DSPP belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of secreted phosphoproteins, which are involved in bone mineralization. Full-length nascent DSPP is cleaved to yield dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) by bone morphogenetic protein 1(BMP1)[14]. DSP and DPP, 2 principal noncollagenous proteins of mature dentin matrices, play essential but distinct roles in dentinogenesis. DSP regulates initiation of dentin mineralization, and DPP is involved in the maturation of mineralized dentin[15, 16].

Veis and Perry reported DPP in 1967 for the first time[17]. DPP, a highly acidic protein, is the major non-collagenous protein in the dentin matrix. It is synthesized and secreted by mature odontoblasts[18–20] and then is transported to the mineralization front, which binds calcium ions with high affinity[21, 22], aggregates collagen fibrils[23] and assumes a structure promoting the formation of initial apatite crystals. As predentin is converted to dentin, the mineral crystals turn to be an oriented fashion. DPP and other proteins bind to the growing hydroxyapatite faces to modulate crystal growth[24].

In this study, three DD-II families in total were collected. In family A and B, through WES sequencing method, a novel frameshift deletion mutation of *DSPP*: c.1871-1874del was identified, which shows complete co-segregation with the disease phenotype in two families. In family C, through WES and WGS sequencing methods, no pathogenic mutations were identified. However, four polymorphisms were found in the *DPP* region. Previous studies had shown that there were no pathogenic mutations in *DSPP* or *Col1A2* in some patients diagnosed with DGI-III or DD-II, which is consistent with our results. Song et al [10] reported eight families with mutations in the *DSP* region. They found *DPP* mutations in five families, while no pathogenic mutations were confirmed in the remaining three families. Wang et al (2012) found a mutation in *COL1A2* in a DGI family without bone defects, and they suggested that *COL1A1* and *COL1A2* should be considered as candidate genes in isolated DGI cases without a *DSPP* mutation. Li et al[8] reported seven families diagnosed as DGI/DD, one of which had no pathogenic variants, only several polymorphisms. We speculated that there were other pathogenic genes for the dentin defects. Polymorphisms may mitigate a defect when they appear together, and copy number variation may lead to the occurrence and development of disease to some degree[8, 25]. In previous studies, mutations related to DD-II were found only in signal peptide region and *DPP* region [8, 9, 26], but not found in *DSP* region, which may be related to the different functions of DSP and DPP. Further research is required to investigate the similarities and differences between DSP and DPP protein.

Micro-CT and macro-structure photos showed that the primary molars had severe pulp obliteration, and the root canals were fine and reticular, indicating early-stage pulp obliteration. Taleb et al[27] collected

three Danish DGI-II families, whose mutations were located in the *DSP* region. CT of primary molars showed an enlarged pulp cavity and root canal, which was different from the primary molars in this study. Park et al[28] analyzed a premolar with a mutation in the *DPP* region (c.2688delT), and found that the morphology of the pulp cavity did not change significantly. The DGI-II family (c.53T > G) found in Lee et al(c.53T > A, p.Val18Asp)[29]and our previous study[11], the teeth were characterized by congenital enamel defects. These studies suggest that the corresponding mutation mechanisms of different *DSPP* mutation sites are different, and the molecular mechanism remains to be further studied.

The microhardness results revealed that the dentin hardness of DD-II and DGI-II teeth was significantly lower than that of normal teeth. This result is consistent with previous studies. For example, Wieczorek et al[30] found that the hardness of DI was significantly lower than that of normal teeth. Nutchoey et al[31] found that the hardness of OI patients' teeth was significantly lower than that of controls. The reason may be that *DSPP* mutation leads to the disorder of dentin formation, resulting in dysplastic dentin with defects in both structure and hardness. In this study, the enamel hardness of DD-II showed not significantly different from that of the normal teeth, indicating that the mutation of c.1871-1874del did not affect the formation of enamel.

SEM results showed that the teeth had a reduced number of dentin tubules, and dentin tubules were irregular. Large gaps and ectopic calcification masses can be seen in the dentin. The EDS results of dentin showed that Ca, P, C, and O had no significant change, while Mg decreased significantly, and Na increased significantly. The decrease in Mg is consistent with some previous studies[28, 32, 33]. Mg^{2+} is essential for maintaining the physiological homeostasis of tissues and organs, and promotes the proliferation of osteoblast and osteogenesis[34]. The decrease of Mg in DD-II teeth may be related to the structural disorder and reduced hardness of dentin. The Na content was significantly increased, which is consistent with the research of park et al[28] and Sabel et al[33]. The high content of Na is typical for extracellular tissue in preventing and may reflect impaired mineralization of DD-II dentine[35]. The EDS results of enamel showed no significant difference with controls, which is consistent with the results of a previous study by Intarak et al on enamel analysis of OI patients [36]. This result also suggests that the mutation of the *DPP* region does not affect enamel development, and the early enamel exfoliation is the result of a reduction of dentin hardness under long-term chewing pressure[37].

Analysis of teeth hard tissue showed that DD-II teeth had abnormal dentin structure and element content, which often led to early tooth fraction and periapical infections. The abnormal pulp morphology further increases the difficulty of dental treatment[38, 39]. Thus, whole-life-cycle dental health management is of great importance to such kinds of patients.

In this study, we collected three DD-II families in total and identified a novel *DSPP* mutation in family A and family B. Morphological observation showed that the enamel of DD-II primary teeth was relatively intact, the pulp cavity was partially obliterated, and the root canal was fine and reticular. The microhardness of dentin was significantly lower than that of normal teeth. The element test showed that

the content of Mg was significantly decreased, and the content of Na was significantly increased. Our study expands the phenotypic and genotypic spectra of DD-II.

Declarations

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Ethics Approval and Consent to Participate

All procedures involving human participants in this study were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant. This study was approved by the Medical Ethics Committee of the Sichuan Provincial People's Hospital

Author Contribution

Conceptualization: Jing Zou, Jiyun Yang, Qin Du; Li Cao; Methodology: Qin Du, Li Cao; Formal analysis and investigation: Nana Yan, Sujun Kang, Mu Lin, Peilin Cao, Ran Jia, Chenyang Wang, Hanyu Qi, Yue Yu; Writing - original draft preparation: Qin Du, Li Cao; Writing - review and editing: Jing Zou, Jiyun Yang; Funding acquisition: Qin Du, Jiyun Yang; Supervision: Li Cao, Jiyun Yang.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

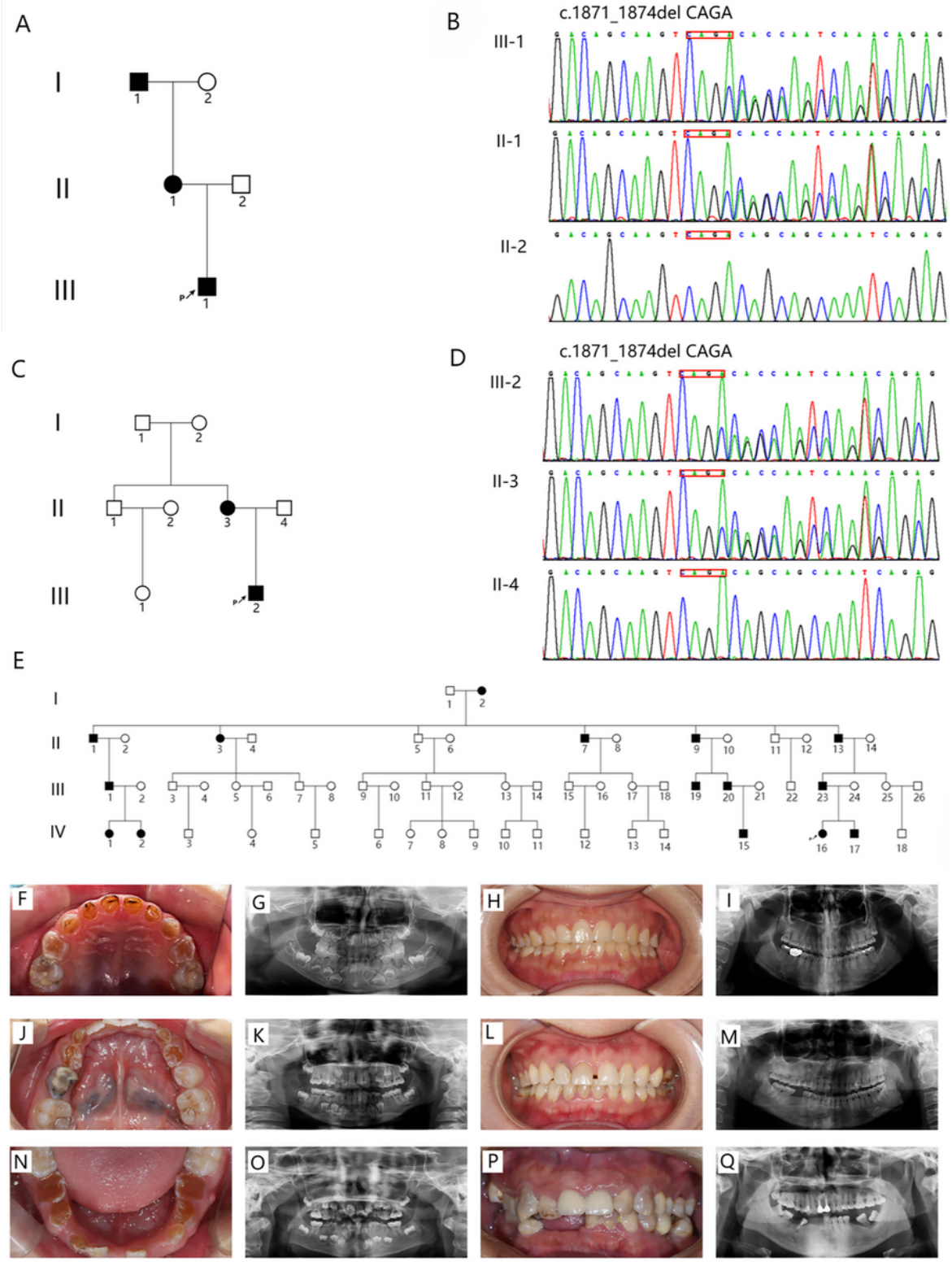

Figure 1

Pedigrees of three DD-II families, *DSPP* (NM_014208) variations identification by Sanger sequencing in participants, phenotype of the patients. **A** Pedigree of family A. **B** Sanger sequencing of the c1871-1874delCAGA variant in *DSPP*. **C** Pedigree of family B. **D** Sanger sequencing of the c1871-1874delCAGA variant in *DSPP*. **E** Pedigree of family C. **F** Intraoral examination of family A, III:1. The teeth were yellow-brownish colored. **G** Oral panoramic radiograph of family A, III:1. The pulp cavities of the primary teeth

were severely obliterated. **H** Intraoral examination of family A, II:1. The permanent teeth had a normal appearance in both color and shape. **I** Intraoral panoramic radiograph of family A, II:2 showed thistle-shaped pulp chambers of the permanent dentition. **J** Intraoral examination of family B. III:2. The primary teeth are translucent and amber in color whereas the erupting permanent teeth were of normal appearance. **K** Oral panoramic radiograph of family B, III:2. The pulp cavities of the primary teeth were severely obliterated. **L** Intraoral examination of family B, II:3. The permanent teeth had a normal appearance in both color and shape. **M** Oral panoramic radiograph of family B, II:3. Showed thistle-shaped pulp cavities in the permanent dentition. **N** Intraoral examination of family C, IV:16. The primary teeth are translucent and amber in color, and with severe attrition. The permanent teeth were of normal appearance. **O** Oral panoramic radiograph of family C, IV:16. The pulp cavities of the primary teeth were severely obliterated. **P** Intraoral examination of family C, III:23. The permanent teeth had a normal appearance in both color and shape. **Q** Oral panoramic radiograph of family C, III:23, showing thistle-shaped pulp cavities in the permanent dentition.

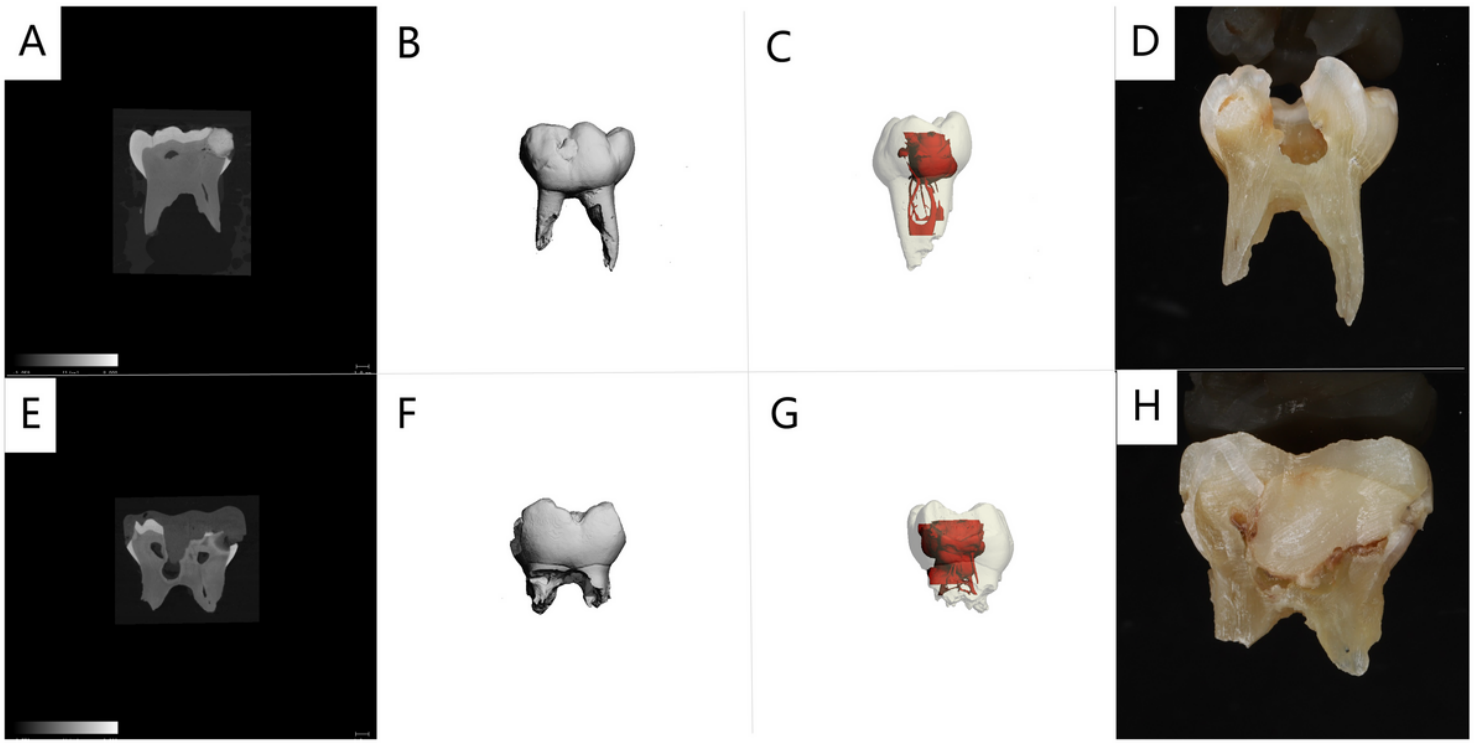

Figure 2

The Images of the DD-II teeth. **A-C** Micro-CT of the primary molar of family A, III:1. The red portion showed that the root canals were smaller than normal teeth and irregularly distributed. **D** The Macro photo of the tooth showed obliterated root canals. **E-G** Micro-CT of the primary molar of family B, II:2. The red portion showed that the root canals were also smaller than normal teeth and irregularly distributed. **H** The Macro photo of the tooth showed obliterated root canals.

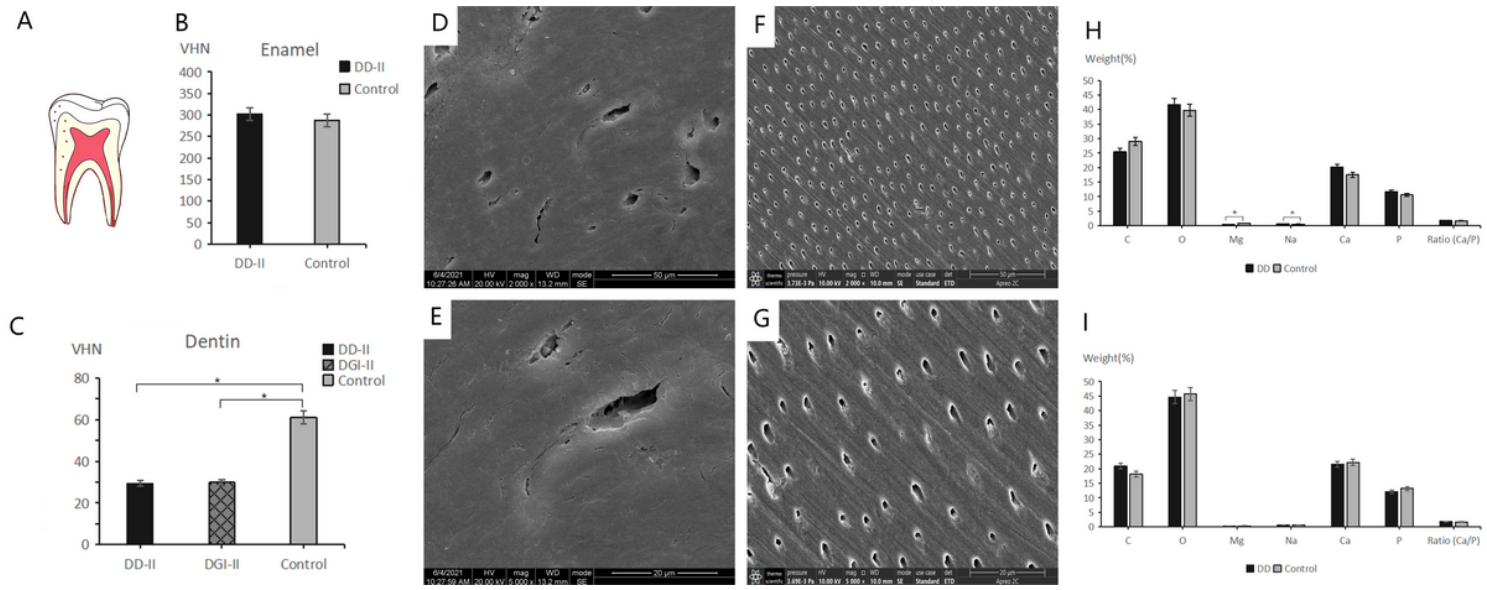

Figure 3

A Measurement points are indicated with light red spots in the tooth image. **B** Microhardness values of the DD enamel. **C** Microhardness values of the DD and DGI dentin. **D** SEM image of the DD tooth (2,000×). **E** SEM image of the DD tooth (5,000×). **F** SEM image of the normal tooth (2,000×). **G** SEM image of the normal tooth (5,000×). **H** EDS results of the DD-II dentin and the control. **I** EDS results of the DD-II enamel and the control. (*: P<0.05).

Supplementary Files

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- SupplementaryMaterials.docx