

Enamel malformations associated with a defined dentin sialophosphoprotein mutation in two families

Shih-Kai Wang^{1,2}, Hui-Chen Chan¹,
Sudha Rajderkar², Rachel N.
Milkovich¹, Karen A. Uston³,
Jung-Wook Kim⁴, James P.
Simmer¹, Jan C-C. Hu¹

Wang S-K, Chan H-C, Rajderkar S, Milkovich RN, Uston KA, Kim J-W, Simmer JP, Hu JC-C. Enamel malformations associated with a defined dentin sialophosphoprotein mutation in two families.

Eur J Oral Sci 2011; 119 (Suppl. 1): 158–167. © 2011 Eur J Oral Sci

¹Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, MI, USA; ²Oral Health Sciences Program, University of Michigan School of Dentistry, Ann Arbor, MI, USA; ³Department of Orthodontics and Pediatric Dentistry, University of Michigan School of Dentistry, Ann Arbor, MI, USA, USA; ⁴Department of Pediatric Dentistry & Dental Research Institute, School of Dentistry, Seoul National University, Chongno-Gu, Seoul, Korea

Dentin sialophosphoprotein (*DSPP*) mutations cause dentin dysplasia type II (DD-II) and dentinogenesis imperfecta types II and III (DGI-II and DGI-III, respectively). We identified two kindreds with DGI-II who exhibited vertical bands of hypoplastic enamel. Both families had a previously reported *DSPP* mutation that segregated with the disease phenotype. Oral photographs and dental radiographs of four affected and one unaffected participant in one family and of the proband in the second family were used to document the dental phenotypes. We aligned the 33 unique allelic *DSPP* sequences showing variable patterns of insertions and deletions (indels), generated a merged dentin phosphoprotein (*DPP*) sequence that includes sequences from all *DSPP* length haplotypes, and mapped the known *DSPP* mutations in this context. Analyses of the *DSPP* sequence changes and their probable effects on protein expression, as well as published findings of the dental phenotype in *Dspp* null mice, support the hypothesis that all *DSPP* mutations cause pathology through dominant-negative effects. Noting that *Dspp* is transiently expressed by mouse pre-ameloblasts during formation of the dentino–enamel junction, we hypothesize that *DSPP* dominant-negative effects potentially cause cellular pathology in pre-ameloblasts that, in turn, causes enamel defects. We conclude that enamel defects can be part of the dental phenotype caused by *DSPP* mutations, although *DSPP* is not critical for dental enamel formation.

Jan C-C. Hu, Department of Biologic and Materials Sciences, University of Michigan Dental Research Laboratory, 1210 Eisenhower Place, Ann Arbor, MI 48108, USA

Telefax: +1-734-9759329
E-mail: janhu@umich.edu

Key words: enamel; dentin; *DSPP*; *dentinogenesis imperfecta*; genetics

Accepted for publication August 2011

The principal organic components in forming enamel are amelogenin, ameloblastin, and enamelin (1). In tooth dentin they are type I collagen and dentin sialophosphoprotein (*Dspp*) (2). During formation of the dentino–enamel junction (DEJ), when the initial dentin and enamel layers mineralize, there is transient expression of enamel proteins by odontoblasts and of dentin proteins by pre-ameloblasts (3). Dentin sialophosphoprotein is only transiently expressed by pre-ameloblasts, but is secreted by odontoblasts throughout the formation of dentin (4, 5). Despite the transient expression of dentin and enamel proteins by opposing cells during formation of the DEJ, it is clear that enamel proteins such as amelogenin and enamelin are not critical for dentinogenesis and that *Dspp* is not critical for amelogenesis. Patients with amelogenesis imperfecta (AI) caused by mutations in amelogenin, X isoform (*AMELX*) or enamelin (*ENAM*) genes do not exhibit a dentin phenotype (6). In addition, there is a loss of selection to maintain the integrity of enamel protein genes in vertebrates that have lost the ability to form dental enamel during evolution (7). The *DSPP* gene is not expressed by secretory-stage or maturation-stage ameloblasts, and enamel volume and density are normal in *Dspp* null mice

(8). In humans, mutations in *DSPP* cause inherited dentin malformations, such as dentin dysplasia type II (DD-II) and dentinogenesis imperfecta types II and III (DGI-II and DGI-III). Dental enamel often undergoes rapid attrition in patients with dentinogenesis imperfecta (9, 10), which is generally ascribed to a lack of support by the defective underlying dentin.

To date, 35 different *DSPP* mutations have been shown to cause inherited dentin defects in humans (11). All show an autosomal-dominant pattern of transmission, which means that only one *DSPP* allele is defective. This contrasts with the recessive pattern of transmission observed in mice following total disruption of the *Dspp* gene (12). Tell-tale characteristics of the reported human mutations led to the hypothesis that *all* of the disease-causing *DSPP* mutations lead to the production of a mutant protein which damages the expressing cell and/or the extracellular matrix (13). If this compelling hypothesis is true, *DSPP* mutations cause inherited dentin defects through a dominant-negative mechanism, and not by a loss of function or a reduction in the amount of normal *DSPP* protein (haploinsufficiency), which is the pathogenic mechanism at work in the *Dspp* knockout mice. We use the term ‘dominant-negative’ to indicate

that the phenotype is caused by the pathological effects of the mutant protein, although the actual pathological mechanism might be better described as a ‘gain of function’.

DSPP 5' mutations

Fifteen different disease-causing mutations have been reported in the 5' end of *DSPP* (Fig. 1). Five of the mutations are in exon 2, which encodes the signal peptide (amino acids 1–15) plus two amino acids from the N-terminus of DSPP (amino acids 16 and 17). The first two mutations are within the signal peptide and must

cause a problem with protein targeting, as the sequence of the secreted protein is identical to that of the wild-type protein. Five coding mutations alter the amino acids in the +2 and +3 positions relative to the signal peptide cleavage site. The remaining eight mutations cluster around the splice boundaries of exon 3. Where experimental evidence is available, the result of a defective splicing junction was the skipping of exon 3, rather than intron retention (14, 15). All of the *DSPP* 5' disease-causing mutations (either directly or by skipping of exon 3) alter the context of the signal peptide cleavage site. Six of the 15 mutations have been reported more than once, so many of the *DSPP* 5' mutations that potentially cause inherited dentin defects may have been identified.

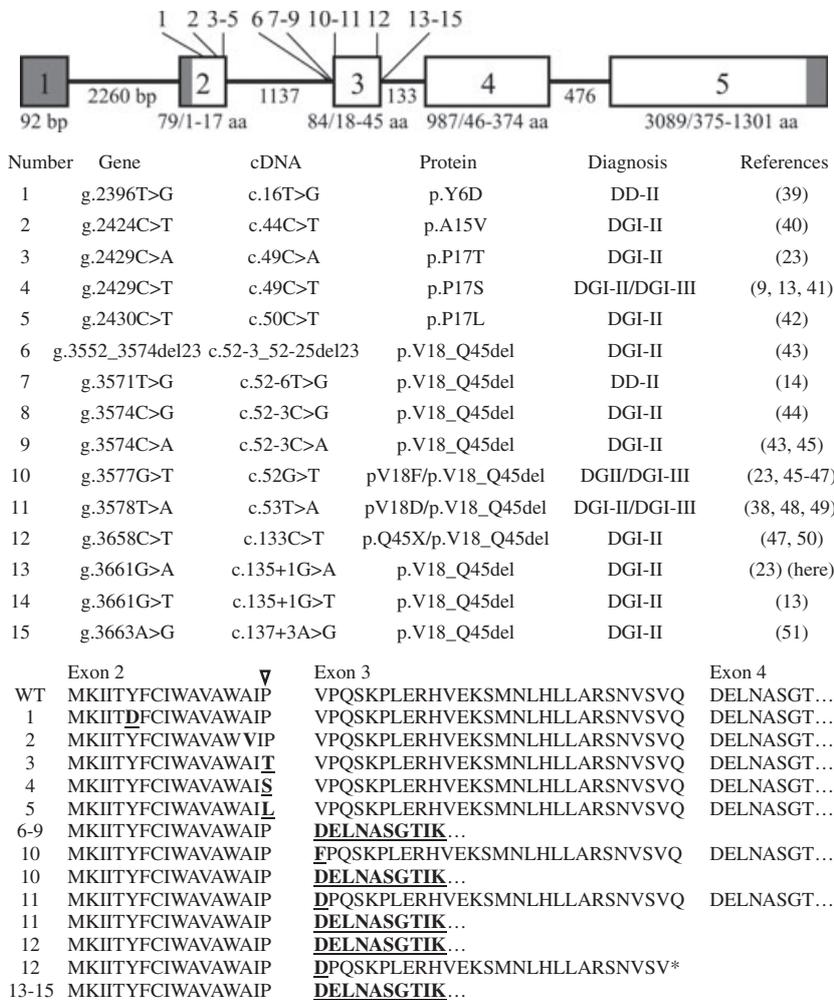


Fig. 1. Mutations in the 5' end of the human dentin sialophosphoprotein (*DSPP*) gene causing inherited dentin defects. Top: the intron/exon structure of *DSPP*. The five *DSPP* exons are indicated by numbered boxes; the four introns are the lines connecting the exons. The numbers below each intron represent the number of base pairs in that intron. The numbers below each exon show the number of base pairs in that exon followed by the amino acids (aa) encoded by it. The locations of the 15 reported *DSPP* 5' mutations are indicated by numbered lines, starting at the 5' end of *DSPP*. The first five mutations are in exon 2. We hypothesize that these mutations directly interfere with signal peptide function or cleavage. The other ten mutations cluster around the splice boundaries of exon 3. Skipping of exon 3 might affect cleavage or functioning of the DSPP signal peptide of (amino acids 1 to 15). Signal peptide malfunction is presumed to interfere with protein secretion and to cause cell pathology. Middle: list of the 15 reported *DSPP* 5' mutations and their dental phenotypes. Note that six of the 14 *DSPP* 5' mutations have been reported more than once and that the diagnoses of DGI-II or DGI-III have both been used to describe the phenotype of persons with the same *DSPP* mutation. Bottom: wild-type (WT) and mutant predicted amino acid sequences of human DSPP. Changed amino acids are in bold and underlined; the signal peptide cleavage site is indicated by an arrow.

None of the *DSPP* 5' mutations alter the protein at the many conserved sites that are involved in potentially important post-translational modifications (16), suggesting that heterozygous loss-of-function mutations in *DSPP* do not cause inherited dentin defects. A reasonable explanation for the dominant-negative effects is that the 5' mutations interfere with protein targeting (secretion) and cause cell pathology (13).

***DSPP* 3' mutations**

Twenty different disease-causing mutations have been reported at the 3' end of *DSPP* within the coding region for dentin phosphoprotein (DPP), the C-terminal cleavage product of *DSPP* (17–19). Dentin phosphoprotein is the most acidic protein known, having an isoelectric point near 1.1 (20). Its sequence is highly repetitive and the length of the repeat region varies greatly among different mammalian species and even within species, which is reflected in the size of the DPP protein (21). The first studies to successfully characterize the DPP coding region in patients with inherited dentin defects reported therein 22 (13) and 13 (22) different patterns of insertions and deletions (indels).

McKNIGHT *et al.* (13) identified 22 *DPP* indel patterns in 188 normal human chromosomes, while SONG *et al.* (22) reported 13 *DPP* indel patterns from 220 normal human chromosomes. Only two of the *DPP* indel patterns were identical between the two studies, so 33 unique indel patterns are known for the human *DPP*-coding region, with the deduced amino acid sequences ranging from 770 to 902 amino acids. The *DPP* region of the *DSPP* reference sequence (GB# NM_014208.3) has 839 amino acids. We carefully aligned the *DPP* coding region from each of the 33 haplotypes with novel indel patterns and noted the positions of the 20 disease-causing mutations in the *DSPP* 3' region (Fig. S1). This allowed us to construct a merged *DPP* coding region that includes all of the sequences found within the 33 known *DPP*-length haplotypes (Fig. S2). The currently known *DPP*-length haplotypes, and the locations of the 20 disease-causing mutations in the merged *DPP* coding region, are charted in Fig. 2. The 20, 3' disease-causing mutations and their associated phenotypes are listed in Fig. 3.

Because the *DPP* coding region is so repetitive, something unusual occurs when a genetic alteration causes a shift in its reading frame. A deletion or insertion that causes a shift to the –2 reading frame (i.e. a deletion of two nucleotides or the insertion of four) results in an in-frame translation termination codon after introducing only a few extraneous amino acids (Fig. S3). A deletion or insertion that causes a shift to the –1 reading frame, however, continues without hitting a translation termination signal until downstream of the natural *DPP* stop codon. Removing the first nucleotide at the beginning of the *DPP* coding region in the *DSPP* reference sequence replaces *DPP* with an 850 amino acid missense protein that is composed of 35% alanine, 23% valine, 20% threonine, and 12% isoleucine. Any –1

reading frameshift starting at 47 or fewer codons before the start of *DPP* in exon 5, or within the *DPP* coding region itself, will terminate translation at the same stop codon downstream of the normal *DSPP* termination codon. The length of the missense peptide in each case is a function of the location of the frameshift and of the *DPP* length haplotype in which it occurs. It seems that all 20 of the disease-causing mutations in the *DPP* coding region shift the reading frame to the –1 frame. Mutations that shift all or part of the *DPP* coding region into the –2 reading frame terminate translation without producing much of a missense peptide. Such changes reduce the amount of *DPP* protein (haploinsufficiency) or generate a truncated *DPP* protein (loss of function), but these types of mutations have not been observed, presumably because they do not cause inherited dentin defects. This analysis supports the hypothesis that disease-causing mutations in *DSPP* are the result of dominant-negative effects and that haploinsufficiency secondary to the loss of a single *DSPP* allele does not cause disease in the heterozygous condition.

We identified two DGI-II kindreds that show dentin defects typical of this condition, but also display pathological malformations of their dental enamel. We characterized the dental condition in these families and identified the same *DSPP* mutation in both, which was previously reported to cause DGI (23), but without documenting the dental phenotype. We hypothesize that transient expression of the mutated *DSPP* protein caused a dominant-negative effect in pre-ameloblasts that resulted in the observed enamel malformations.

Material and methods

The study protocol and subject consents were reviewed and approved by the Institution Review Board at the University of Michigan, and appropriate informed consent was obtained from all subjects. Two families were recruited. Medical and dental histories were obtained, and pedigrees spanning four generations were constructed. Oral photographs and radiographs were taken of five members in family 1 (III:7, III:8, IV:1, IV:2, and IV:3) and of the proband in family 2 (IV:2). Copies of previous dental radiographs were obtained for members IV:1, IV:2, and IV:3 of family 1 and for members II:4, III:7, III:8, and IV:3 of family 2. Dental phenotypes were assessed by oral examination and by inspection of dental radiographs. In total, 15 persons – 11 affected and four unaffected – were recruited. The subjects ranged in age from 9 years to adult.

DNA isolation

Ten millilitres of peripheral whole blood was obtained from participating family members. In family 1 we were able to obtain DNA from five affected [II:6, III:7, IV:1 (18 yr of age), IV:2 (17 yr of age), and IV:3 (16 yr of age)] and two unaffected (I:2 and III:8) members. In family 2 we were able to obtain DNA from six affected [II:3, III:4, III:7, IV:1, IV:2 (13 yr of age), and IV:3 (9 yr of age)] and two unaffected (II:4 and III:8) members. Genomic DNA was isolated using the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). Ages of adults were not specified.

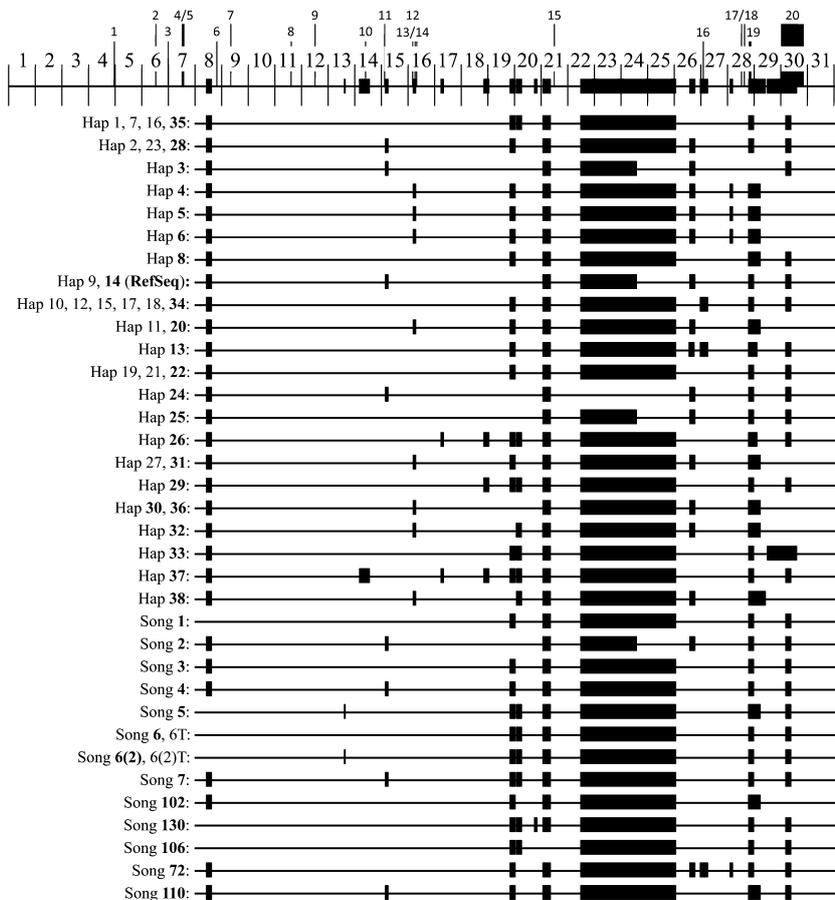


Fig. 2. Human dentin phosphoprotein (DPP) length haplotypes and positions of indels and disease-causing mutations in merged human DPP. Top: the merged DPP sequence (from Fig. S2) is shown divided into a little over 31 equal, numbered segments corresponding to 90 bp (30 amino acids) of DPP. The thin bar represents universal segments found on all DPP length haplotypes; the thick bars are variable regions found only on some DPP length haplotypes. The numbered lines above the merged DPP map indicate the locations of the 20 dentin sialophosphoprotein (*DSPP*) 3' disease-causing mutations (listed in Fig. 3). Note that most indels are located in the downstream half of the DPP code. Bottom: maps showing positions of the indels (thick bars) on each of the 33 novel length haplotypes. The first seven segments of DPP show no length polymorphisms and are not included in the diagrams for each length haplotype. There can be multiple DPP haplotypes for each length haplotype because of nucleotide substitutions (a polymorphism that does not affect length). The 22 length haplotypes from MCKNIGHT *et al.* (13) and the 13 length haplotypes from SONG *et al.* (22) are shown.

Polymerase chain reaction

All PCR amplifications were carried out using Platinum[®] PCR SuperMix (Invitrogen, Carlsbad, CA, USA), as follows: a 5-min denaturation at 94°C; 35 cycles of denaturation at 94°C for 90 s, primer annealing at 56–59°C for 60 s, and product extension at 72°C for 90 s; and an extension at 72°C for 7 min in the final cycle. PCR amplification products were purified using the QIAquick PCR Purification Kit and protocol (Qiagen).

Exons 2–4 were amplified along with their adjoining intron sequences. Exon 5 was characterized up to the repetitive region. Exon 2 was amplified with 5'-gatgtccc cataaccacacc (forward) and 5'-ctccatgacttctgggcatt (reverse) primers to generate a 596-bp product (annealing at 56°C and extension at 72°C/90 s). Exons 3 and 4 were amplified together as a 1,490-bp product using the 5'-caagccc tgtaagaagccact (forward) and 5'-acatggatgcttgcattgg (reverse) primers (annealing at 59°C and extension at 72°C/90 s). The 5' part of exon 5 was amplified using the 5'-cc

tatggcaacttttcccaagt (forward) and 5'-tgtcattgtcatcattcccatt (reverse) primers (annealing at 56°C and extension at 70°C/90 s) to generate a 589-bp product.

DNA sequence analysis

DNA sequencing was performed at the University of Michigan DNA sequencing core. The original PCR primers used to generate each amplicon were used in the sequencing reaction. In addition, for the larger amplification product of exon 3–4, two sense primers (5'-ggaccatgggaaagaagatg and 5'-gcattccagggacaagtaagc) and three antisense oligonucleotides (5'-cattcccttcccttctgtga, 5'-cctcgtttctacaggaattctca, and 5'-tggaggctgtgtctccatca) were used for sequencing reactions.

Alignment of 33 DPP indel patterns

Haplotype sequences from MCKNIGHT *et al.* (13) were imported from GenBank and provided the haplotype

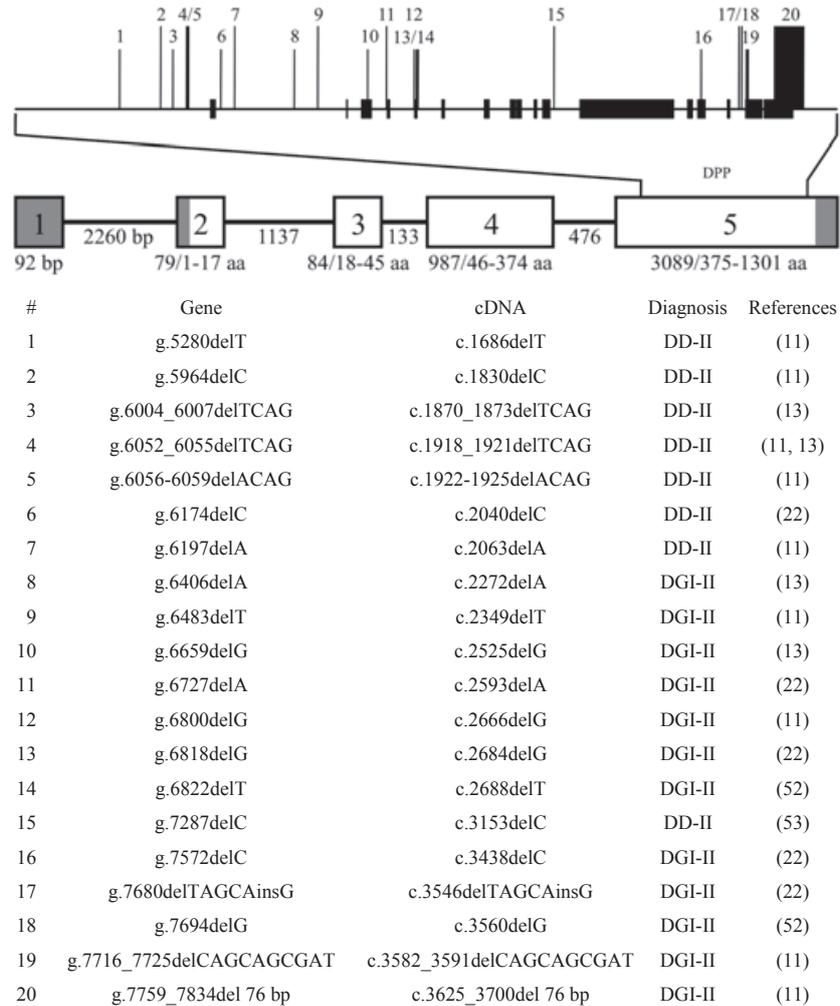


Fig. 3. Top: dentin sialophosphoprotein (*DSPP*) intron/exon structure with the dentin phosphoprotein (*DPP*) region expanded to show the positions of indels (thick bars) and the sites of 20 disease-causing frameshift mutations (numbered). Bottom: list of the 20 reported *DSPP* 3' mutations, their designations, and their associated dental phenotypes. All 20 disease-causing mutations shift the downstream sequence into the -1 reading frame. DD-II, dentin dysplasia type II; DGI-II, dentinogenesis imperfecta type II.

sequences starting with *DSPP* nucleotide 2572 encoding Arg396. Each sequence can be obtained by searching the National Center for Biotechnology Information (NCBI) database with 'DSPP haplotype #'. For the purpose of this analysis, the reference sequence (NM_014208.3) was used to complete the *DPP* sequences 5' to this position, with the exception of clone 37, where the short sequence 5' to nucleotide 2572 was obtained from Hap37A to ensure the correct positioning of indel 1. The haplotype sequences given in SONG *et al.* (22) were not available in GenBank. These sequences were obtained by altering the *DSPP* cDNA reference sequence (NM_014208.3) according to Tables 2 and 3 in SONG *et al.* (22). SONG *et al.* reported 15 haplotypes that showed 13 different indel patterns. The SONG *et al.* haplotype designations are in bold. One Song haplotype from each of the 13 groups was used in the alignment: 1 (803 amino acids), 2 (839 amino acids), 3 (788 amino acids), 4 (788 amino acids), 5 (790 amino acids), 6, 6T (797 amino acids), 6(2), 6(2)T (796 amino acids), 7 (782 amino acids), 102 (791 amino acids), 130 (794 amino acids), 106 (806 amino acids), 72 (785 amino acids), and 110 (782 amino acids). Song haplotype 2 has the same indel pattern as

McKnight haplotypes 9 and 14 as well as the reference sequence. Song haplotype 3 has the same indel pattern as McKnight haplotypes 19, 21, and 22.

Results

Two apparently unrelated Caucasian families of northern European ancestry with inherited defects of dentin were examined at a University of Michigan dental clinic. There was no history of any unusual bone brittleness or unexplained hearing loss in either family. Affection status (the presence of inherited dentin defects) was ascertained by routine oral examination, and pedigrees of the two kindreds going back four generations were constructed following interviews with multiple family members (Fig. 4). Mutational analyses of the *DSPP* 5' region identified the same disease-causing mutation in both families: IVS3+1G>A; c.135+1G>A. This transition mutation changes the first nucleotide of

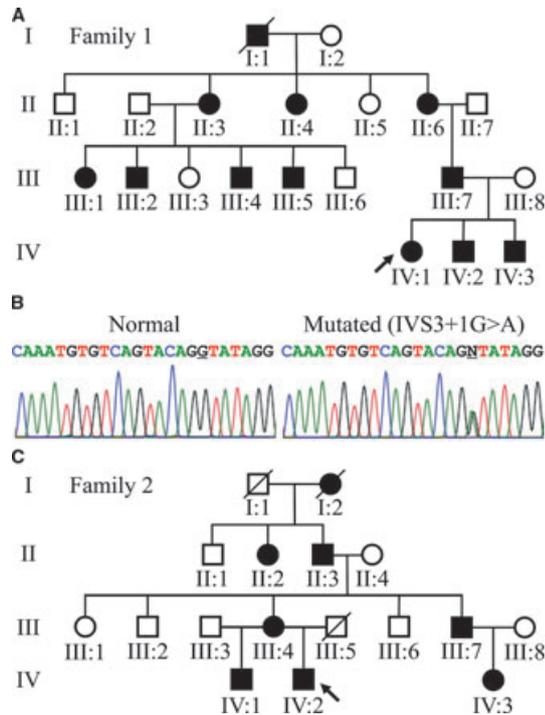


Fig. 4. (A,C) Pedigrees of two Caucasian families with dentinogenesis imperfecta type II (DGI-II). The diseases of these two families segregated as an autosomal-dominant trait. (B) DNA sequencing chromatograms identified the same reported disease-causing mutation (IVS3 + 1G>A) in both families. This mutation changes the first nucleotide of intron 3 and is predicted to disturb normal mRNA splicing, presumably by causing the skipping of exon 3 during RNA splicing.

intron 3, which is absolutely required for proper mRNA splicing. The mutation was identified in all 10 of the affected members who donated samples for mutation analyses. The enamel surfaces of the probands, particularly in the anteriors, appeared shriveled, with vertical lines of hypoplastic enamel (Fig. 5).

Dental phenotyping: family 1

In family 1, the proband was an 18-yr-old teenager (IV:1) exhibiting translucent and chalk-colored crowns with vertical grooves of hypoplastic enamel and spaces between the maxillary and mandibular anterior teeth (Fig. S4). On radiographs, the enamel was more opaque than the dentin. The pulp chambers and many of the root canals were already obliterated. The roots of many teeth were noticeably shorter than normal, particularly in the anteriors. Some of the molar crowns had a bulbous morphology. Attrition was apparent along the incisal edges of the anteriors and on the buccal cusp tips of the mandibular cuspids and premolars.

The proband's youngest brother (IV:3) at 16 years of age already had extensive attrition of all anterior teeth and detectable attrition on the buccal cusp tips of the mandibular premolars (Fig. S5). The crowns were translucent or chalky, with some vertical grooves still evident in cervical areas that had not yet abraded, such as

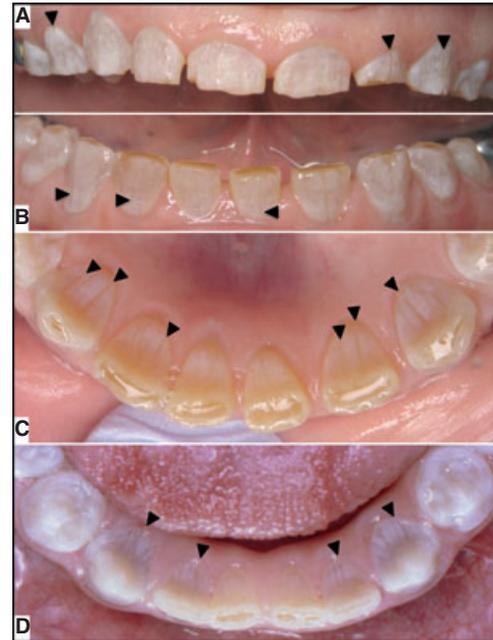


Fig. 5. Oral photographs of the family 1 proband (A–C) and the family 2 proband (D). Arrowheads mark the most conspicuous vertical bands of hypoplastic enamel. More complete oral photographic surveys of the probands are provided in Figs S4 and S9.

the buccals of teeth 12 and 13. The pulp chambers and root canals were obliterated and the molar and premolar crowns appeared bulbous. The enamel was more radiopaque than dentin. The proband's other brother (IV:2) at 17 years of age had extensive attrition of the anterior teeth, and less attrition on the buccal cusp tips of the mandibular premolars and second molars (Fig. S6), presumably worsened by failure of the restorations on three of the first molars. Like his siblings, the crowns of his teeth were translucent and chalky, the pulp chambers and root canals were obliterated, the molar and premolar crowns were bulbous (which appeared to play a role in the impaction of the lower left third molar), and the enamel was more radiopaque than dentin. Vertical grooves were evident on the buccal cervical areas of the premolars, which were not yet destroyed by attrition. The father (III:7) had a very similar dentition, although the crowns of his teeth were mostly translucent rather than chalky (Fig. S7). Attrition was most severe in the maxillary anteriors, but was not as bad as his sons' condition. The radiographs showed obliterated pulp chambers and root canals, bulbous crowns, and enamel that was more radiopaque than dentin. The enamel surfaces had a polished appearance. The mother (III:8) was not affected and had a normal and healthy dentition (Fig. S8). The expression of the DGI-II phenotype in the four affected members of this family was remarkably consistent.

Dental phenotyping: family 2

The proband of family 2 (IV:2) was, at 12 years of age, the youngest affected person to be characterized. Like

family 1, the proband of family 2 had classic DGI-II (Fig. S9). The crowns alternated between chalky and translucent in color and had vertical grooves in the enamel. The pulp chambers and most of the root canals in the incisors and first molars had been obliterated. The crowns of the posterior teeth were bulbous. The enamel was more radiopaque than dentin. Attrition was evident on the incisal edges of the anterior teeth. The affected mother had complete maxillary and mandibular dentures. Taking into account the differences in age between the subjects in the two families, the dental phenotypes of these patients with the IVS3+1G>A; c.143+1G>A mutation in *DSPP* were very similar.

Discussion

We characterized the dental phenotype of two families with a classic DGI-II phenotype and observed enamel defects, in the form of vertical grooves, in both probands. Both families had the same *DSPP* transition mutation (IVS3+1G>A). This mutation might cause the retention of intron 2 or the skipping of exon 3. Retention of intron 2 would lead to premature translation termination and delete all but two amino acids of the DSPP secreted protein. Such a transcript would probably be degraded by nonsense-mediated decay (24), potentially causing haploinsufficiency but not dominant-negative effects. Skipping of exon 3 would delete amino acids 18 to 45 and potentially alter the context of the signal peptide cleavage site after Ala15. This is the most likely predicted scenario and has experimental support (14, 15). Ten of the disease-causing *DSPP* 5' mutations localize along the splice junctions of exon 3. We hypothesize that during RNA splicing of transcripts expressed from the mutant *DSPP* allele, exon 3 is skipped all or part of the time, and the resulting protein is not targeted properly and causes cell pathology.

Dentin sialophosphoprotein has a signal peptide and is translated into the endoplasmic reticulum (ER) for secretion. Following translocation into the ER, the DSPP signal peptide is cleaved by the ER signal peptidase complex (SPC). Secretory proteins have a distinct secondary structure requirement at their cleavage site for processing by the ER SPC (25–28). The flanking amino acid residues around the signal peptide cleavage site significantly influence this cleavage process (28). The *DSPP* IVS3+1G>A mutation, which alters the consensus sequence of a splice donor site, presumably leads to skipping of exon 3. Although skipping of *DSPP* exon 3 does not cause a frameshift or premature termination, it significantly changes the C-terminal flanking amino acid residues of the signal peptide cleavage site, which could disturb signal peptide cleavage and interfere with normal DSPP folding and secretion. Mutations in or near the signal peptide coding region of other genes are known to cause diseases by inducing ER stress and cell pathology (29–32). Different proteins have different specific requirements for targeting. Recently it has been shown that a proline in the +2 position, which is specifically altered in three of the *DSPP* 5' mutations, is

critical for the export of nucleobindin 1 (33). A mouse amelogenin missense mutation (p.Y64H) induced extensive ER stress and ameloblast cell pathology (34).

During enamel formation, *DSPP* is transiently expressed in pre-ameloblasts. To explain the enamel defects in our patients, we hypothesize that the *DSPP* IVS3+1G>A mutation interferes with DSPP secretion and causes ER stress and cell pathology in pre-ameloblasts, leading to developmental enamel defects. However, this pathogenic concept does not suggest that *DSPP* plays an important role in enamel formation. Any genetic or environmental aberration that induces ameloblast pathology will potentially cause developmental enamel defects. Dentin sialophosphoprotein is transiently expressed at a sensitive transition period in ameloblast differentiation when the basal lamina is being degraded, enamel proteins are starting to be expressed, and cell–matrix interactions are in a state of flux (1). The pathology of secretory stage ameloblasts could explain the thin enamel. The vertical grooves might relate to buckling of the ameloblast layer during transition/early maturation because of ameloblast overcrowding on the pathologically small enamel surface. The scarcity of reports of enamel defects in persons with *DSPP*-related dentin malformations might be caused by the rapid attrition of enamel in DGI patients, so the enamel defects are seldom observed.

The finding that disease-causing mutations cluster at the borders of exon 3 and that improper splicing can potentially be caused by mutations at the edges of the exon (i.e. in the coding sequence) or six nucleotides into the intron suggests that this exon might be particularly susceptible to skipping, despite the fact that inclusion of this exon is critical to avoid pathological consequences. Perhaps this exon has undergone recent changes during evolution. The *DSPP* gene belongs to the secretory calcium binding phosphoprotein (SCPP) family of genes that all evolved from *SPARCLI* (secreted protein, acidic, cysteine-rich related) (35). In humans there are seventeen *SCPP* genes in the enamel/milk/saliva group and five in the evolutionarily older dentin/bone group, which includes *DSPP* (36). The *DSPP* exon 3 is unusual in that it is longer than the exon 3 of its homologs and does not encode a Golgi casein kinase phosphorylation site.

We assembled data concerning natural and pathological variations in *DSPP*. Alignment of the 33 known DPP indel patterns (Fig. S1) allowed us to produce a merged sequence and to chart the 20 frameshift mutations (Fig. 2). Such a merged sequence should prove helpful in localizing the positions of indels in as-yet-undiscovered length variations in the DPP code. As only two of the DPP indel patterns were the same in the two studies that reported them, there are likely to be many more DPP indel patterns in the population as a whole.

Recently reported *DSPP* mutations and their associated phenotypes strengthen our previously stated hypothesis that autosomal-dominant dentin defects caused by *DSPP* mutations should be considered to be a single disease, with a continuous spectrum of phenotypes that can be subclassified, according to their severity, into DD-II, DGI-II, and DGI-III, with DD-II being the least

severe and DGI-III being the most severe (37). Patients with the 15 disease-causing 5' *DSPP* mutations have been diagnosed as having DD-II, DGI-II, and DGI-III. The p.P17S, p.PV18F, and p.V18D mutations have been identified in persons diagnosed with DGI-II and DGI-III. The 20 disease-causing 5' *DSPP* -1 frameshift mutations have been identified in patients diagnosed with DD-II and DGI-II. All 35 characterized disease-causing *DSPP* mutations show a dominant pattern of inheritance. The pathogenesis in all cases (which is currently based upon logic and not on scientific experiments) seems to occur by a similar mechanism: dominant-negative effects induced by the mutant DSPP protein.

In this study we provided a detailed summary and analysis of the normal polymorphic structures of *DSPP* and of the 35 *DSPP* mutations that cause DD-II, DGI-II, and DGI-III. Current evidence supports the interpretation that *DSPP* mutations cause these dominant forms of inherited dentin defects by a dominant-negative mechanism, and that the three disease designations reflect varying levels of severity of a common disease. We characterized the dental phenotypes, and the mutation that causes them, in two DGI-II families. The dental phenotypes in these two families were similar, and both showed linear vertical defects in the dental enamel. We interpreted these enamel defects as being developmental in origin, rather than being caused by damage to the weakened structure during or following eruption. In a recent case, a 5' *DSPP* mutation (c.53T > A, p.V18D) caused enamel defects that were evident on radiographs even before tooth eruption (38).

If enamel defects can occur when *DSPP* is mutated, is *DSPP* necessary for proper dental enamel formation? Because *Dspp* is not expressed by ameloblasts during dental enamel formation and no enamel defects are observed in *Dspp* null mice, the answer is almost certainly no. No dental phenotype is observed in *Dspp* heterozygous mice (where only one *Dspp* allele is deleted) (8). In contrast, all of the 35 reported disease-causing *DSPP* mutations show a dominant pattern of inheritance. Analyses of these mutations strongly suggest that the resulting dental phenotype is not caused by a loss of *DSPP* function in mouse, but rather is caused by poorly understood pathological mechanisms that we have referred to simply as 'dominant-negative effects'. As *Dspp* is transiently expressed by pre-ameloblasts, we propose that enamel defects associated with *DSPP* mutations are probably the result of cell pathology induced by this transient expression.

Acknowledgements – We thank the family for their participation, and the Pediatric Dental Clinic at the University of Michigan for their cooperation. This investigation was supported in part by USPHS Research Grant DE015846 from the National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health (NIH), Bethesda, MD 29892, and a Science Research Center grant to the Bone Metabolism Research Center (2010-0001741) funded by the Ministry of Education, Science, and Technology (MEST) of the Republic of Korea.

Conflicts of interest – The authors declare no conflict of interest.

References

1. SIMMER JP, PAPAGERAKIS P, SMITH CE, FISHER DC, ROUNTREY AN, ZHENG L, HU JC. Regulation of dental enamel shape and hardness. *J Dent Res* 2010; **89**: 1024–1038.
2. NANJI A. Dentin-pulp complex. In: NANJI A, ed. *Ten Cate's Oral Histology Development, Structure, and Function*. St Louis, MO, USA: Mosby, 2008; 191–238.
3. NAGANO T, OIDA S, ANDO H, GOMI K, ARAI T, FUKAE M. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J Dent Res* 2003; **82**: 982–986.
4. BRONCKERS AL, D'SOUZA RN, BUTLER WT, LYARUU DM, VAN DIJK S, GAY S, WOLTGENS JH. Dentin sialoprotein: biosynthesis and developmental appearance in rat tooth germs in comparison with amelogenins, osteocalcin and collagen type-I. *Cell Tissue Res* 1993; **272**: 237–247.
5. BEGUE-KIRN C, KREBSBACH PH, BARTLETT JD, BUTLER WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J Oral Sci* 1998; **106**: 963–970.
6. WRIGHT JT, HART TC, HART PS, SIMMONS D, SUGGS C, DALEY B, SIMMER J, HU J, BARTLETT JD, LI Y, YUAN ZA, SEOW WK, GIBSON CW. Human and mouse enamel phenotypes resulting from mutation or altered expression of AMEL, ENAM, MMP20 and KLK4. *Cells Tissues Organs* 2009; **189**: 224–229.
7. MEREDITH RW, GATESY J, CHENG J, SPRINGER MS. Pseudogenization of the tooth gene enamelysin (MMP20) in the common ancestor of extant baleen whales. *Proc Biol Sci* 2011; **278**: 993–1002.
8. SUZUKI S, SREENATH T, HARUYAMA N, HONEYCUTT C, TERSE A, CHO A, KOHLER T, MULLER R, GOLDBERG M, KULKARNI AB. Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. *Matrix Biol* 2009; **28**: 221–229.
9. HART PS, HART TC. Disorders of human dentin. *Cells Tissues Organs* 2007; **186**: 70–77.
10. KIM JW, SIMMER JP. Hereditary dentin defects. *J Dent Res* 2007; **86**: 392–399.
11. NIEMINEN P, PAPAGIANNOULIS-LASCARIDES L, WALTIMO-SIREN J, OLLILA P, KARIAJAINEN S, ARTE S, VEERKAMP J, WALTON VT, KUSTNER EC, SILTANEN T, HOLAPPA H, LUKINMAA PL, ALALUUSUA S. Frameshift mutations in dentin phosphoprotein and dependence of dentin disease phenotype on mutation location. *J Bone Miner Res* 2011; **26**: 873–880.
12. SREENATH T, THYAGARAJAN T, HALL B, LONGENECKER G, D'SOUZA R, HONG S, WRIGHT JT, MACDOUGALL M, SAUK J, KULKARNI AB. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. *J Biol Chem* 2003; **278**: 24874–24880.
13. MCKNIGHT DA, SUZANNE HART P, HART TC, HARTSFIELD JK, WILSON A, WRIGHT JT, FISHER LW. A comprehensive analysis of normal variation and disease-causing mutations in the human DSPP gene. *Hum Mutat* 2008; **29**: 1392–1404.
14. LEE SK, HU JC, LEE KE, SIMMER JP, KIM JW. A dentin sialophosphoprotein mutation that partially disrupts a splice acceptor site causes type II dentin dysplasia. *J Endod* 2008; **34**: 1470–1473.
15. LEE KE, LEE SK, JUNG SE, LEE Z, KIM JW. Functional splicing assay of DSPP mutations in hereditary dentin defects. *Oral Dis* 2011; **17**: 690–5 [Epub ahead of print].
16. YAMAKOSHI Y, NAGANO T, HU JC, YAMAKOSHI F, SIMMER JP. Porcine dentin sialoprotein glycosylation and glycosaminoglycan attachments. *BMC Biochem* 2011; **12**: 1–6.
17. SUN Y, LU Y, CHEN S, PRASAD M, WANG X, ZHU Q, ZHANG J, BALL H, FENG J, BUTLER WT, QIN C. Key proteolytic cleavage site and full-length form of DSPP. *J Dent Res* 2010; **89**: 498–503.
18. VON MARSCHALL Z, FISHER LW. Dentin sialophosphoprotein (DSPP) is cleaved into its two natural dentin matrix products by three isoforms of bone morphogenetic protein-1 (BMP1). *Matrix Biol* 2010; **29**: 295–303.

19. TSUCHIYA S, SIMMER JP, HU JC, RICHARDSON AS, YAMAKOSHI F, YAMAKOSHI Y. Astacin proteases cleave dentin sialophosphoprotein (Dspp) to generate dentin phosphoprotein (Dpp). *J Bone Miner Res* 2011; **26**: 220–228.
20. JONSSON M, FREDRIKSSON S, JONTELL M, LINDE A. Isoelectric focusing of the phosphoprotein of rat-incisor dentin in ampholine and acid pH gradients. Evidence for carrier ampholyte-protein complexes. *J Chromatogr* 1978; **157**: 234–242.
21. YAMAKOSHI Y, LU Y, HU JC, KIM JW, IWATA T, KOBAYASHI K, NAGANO T, YAMAKOSHI F, HU Y, FUKAE M, SIMMER JP. Porcine dentin sialophosphoprotein: length polymorphisms, glycosylation, phosphorylation, and stability. *J Biol Chem* 2008; **283**: 14835–14844.
22. SONG YL, WANG CN, FAN MW, SU B, BIAN Z. Dentin phosphoprotein frameshift mutations in hereditary dentin disorders and their variation patterns in normal human population. *J Med Genet* 2008; **45**: 457–464.
23. XIAO S, YU C, CHOU X, YUAN W, WANG Y, BU L, FU G, QIAN M, YANG J, SHI Y, HU L, HAN B, WANG Z, HUANG W, LIU J, CHEN Z, ZHAO G, KONG X. Dentinogenesis imperfecta I with or without progressive hearing loss is associated with distinct mutations in DSPP. *Nat Genet* 2001; **27**: 201–204.
24. BHUVANAGIRI M, SCHLITZER AM, HENTZE MW, KULOZIK AE. NMD: RNA biology meets human genetic medicine. *Biochem J* 2010; **430**: 365–377.
25. DUFFAUD G, INOUE M. Signal peptidases recognize a structural feature at the cleavage site of secretory proteins. *J Biol Chem* 1988; **263**: 10224–10228.
26. PAETZEL M, KARLA A, STRYNADKA NC, DALBEY RE. Signal peptidases. *Chem Rev* 2002; **102**: 4549–4580.
27. HON LS, ZHANG Y, KAMINKER JS, ZHANG Z. Computational prediction of the functional effects of amino acid substitutions in signal peptides using a model-based approach. *Hum Mutat* 2009; **30**: 99–106.
28. CHOO KH, RANGANATHAN S. Flanking signal and mature peptide residues influence signal peptide cleavage. *BMC Bioinformatics* 2008; **9**: S15.
29. HUGHES AE, RALSTON SH, MARKEN J, BELL C, MACPHERSON H, WALLACE RG, VAN HUL W, WHYTE MP, NAKATSUKA K, HOVY L, ANDERSON DM. Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. *Nat Genet* 2000; **24**: 45–48.
30. KUTZ WE, WANG LW, DAGONEAU N, ODRIC KJ, CORMIER-DAIRE V, TRABOULSI EI, APTE SS. Functional analysis of an ADAMTS10 signal peptide mutation in Weill-Marchesani syndrome demonstrates a long-range effect on secretion of the full-length enzyme. *Hum Mutat* 2008; **29**: 1425–1434.
31. VIJAYASARATHY C, SUI R, ZENG Y, YANG G, XU F, CARUSO RC, LEWIS RA, ZICCARDI L, SIEVING PA. Molecular mechanisms leading to null-protein product from retinoschisin (RS1) signal-sequence mutants in X-linked retinoschisis (XLR5) disease. *Hum Mutat* 2010; **31**: 1251–1260.
32. BLEYER AJ, ZIVNA M, HULKOVA H, HODANOVA K, VYLETAL P, SIKORA J, ZIVNY J, SOVOVA J, HART TC, ADAMS JN, ELLEDER M, KAPP K, HAWS R, CORNELL LD, KMOCH S, HART PS. Clinical and molecular characterization of a family with a dominant renin gene mutation and response to treatment with fludrocortisone. *Clin Nephrol* 2010; **74**: 411–422.
33. TSUKUMO Y, TSUKAHARA S, SAITO S, TSURUO T, TOMIDA A. A novel endoplasmic reticulum export signal: proline at the +2-position from the signal peptide cleavage site. *J Biol Chem* 2009; **284**: 27500–27510.
34. BARRON MJ, BROOKES SJ, KIRKHAM J, SHORE RC, HUNT C, MIRONOV A, KINGSWELL NJ, MAYCOCK J, SHUTTLEWORTH CA, DIXON MJ. A mutation in the mouse Amelx tri-tyrosyl domain results in impaired secretion of amelogenin and phenocopies human X-linked amelogenesis imperfecta. *Hum Mol Genet* 2010; **19**: 1230–1247.
35. KAWASAKI K, WEISS KM. Mineralized tissue and vertebrate evolution: the secretory calcium-binding phosphoprotein gene cluster. *Proc Natl Acad Sci U S A* 2003; **100**: 4060–4065.
36. KAWASAKI K, WEISS KM. SPP gene evolution and the dental mineralization continuum. *J Dent Res* 2008; **87**: 520–531.
37. BEATTIE ML, KIM JW, GONG SG, MURDOCH-KINCH CA, SIMMER JP, HU JC. Phenotypic variation in dentinogenesis imperfecta/dentin dysplasia linked to 4q21. *J Dent Res* 2006; **85**: 329–333.
38. LEE SK, LEE KE, HWANG YH, KIDA M, TSUTSUMI T, ARIGA T, PARK JC, KIM JW. Identification of the DSPP mutation in a new kindred and phenotype-genotype correlation. *Oral Dis* 2011; **17**: 314–319.
39. RAJPAR MH, KOCH MJ, DAVIES RM, MELLODY KT, KIELTY CM, DIXON MJ. Mutation of the signal peptide region of the bicistronic gene DSPP affects translocation to the endoplasmic reticulum and results in defective dentine biomineralization. *Hum Mol Genet* 2002; **11**: 2559–2565.
40. MALMGREN B, LINDSKOG S, ELGADI A, NORNGREN S. Clinical, histopathologic, and genetic investigation in two large families with dentinogenesis imperfecta type II. *Hum Genet* 2004; **114**: 491–498.
41. ZHANG X, CHEN L, LIU J, ZHAO Z, QU E, WANG X, CHANG W, XU C, WANG QK, LIU M. A novel DSPP mutation is associated with type II dentinogenesis imperfecta in a Chinese family. *BMC Med Genet* 2007; **8**: 52.
42. LEE S-K, LEE K-E, CHO I-K, HYUN H-K, LEE S-H, KIM J-W. Novel DSPP mutation and characterization of the mutational effect. *IADR 2011 Abstract* 820. San Diego, CA, USA.
43. WANG H, HOU Y, CUI Y, HUANG Y, SHI Y, XIA X, LU H, WANG Y, LI X. A novel splice site mutation in the dentin sialophosphoprotein gene in a Chinese family with dentinogenesis imperfecta type II. *Mutat Res* 2009; **662**: 22–27.
44. KIM JW, NAM SH, JANG KT, LEE SH, KIM CC, HAHN SH, HU JC, SIMMER JP. A novel splice acceptor mutation in the DSPP gene causing dentinogenesis imperfecta type II. *Hum Genet* 2004; **115**: 248–254.
45. HOLAPPA H, NIEMINEN P, TOLVA L, LUKINMAA PL, ALALUUSUA S. Splicing site mutations in dentin sialophosphoprotein causing dentinogenesis imperfecta type II. *Eur J Oral Sci* 2006; **114**: 381–384.
46. KIM JW, HU JC, LEE JI, MOON SK, KIM YJ, JANG KT, LEE SH, KIM CC, HAHN SH, SIMMER JP. Mutational hot spot in the DSPP gene causing dentinogenesis imperfecta type II. *Hum Genet* 2005; **116**: 186–191.
47. SONG Y, WANG C, PENG B, YE X, ZHAO G, FAN M, FU Q, BIAN Z. Phenotypes and genotypes in 2 DGI families with different DSPP mutations. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006; **102**: 360–374.
48. KIDA M, TSUTSUMI T, SHINDOH M, IKEDA H, ARIGA T. De novo mutation in the DSPP gene associated with dentinogenesis imperfecta type II in a Japanese family. *Eur J Oral Sci* 2009; **117**: 691–694.
49. LEE SK, LEE KE, JEON D, LEE G, LEE H, SHIN CU, JUNG YJ, LEE SH, HAHN SH, KIM JW. A novel mutation in the DSPP gene associated with dentinogenesis imperfecta type II. *J Dent Res* 2009; **88**: 51–55.
50. ZHANG X, ZHAO J, LI C, GAO S, QIU C, LIU P, WU G, QIANG B, LO WH, SHEN Y. DSPP mutation in dentinogenesis imperfecta Shields type II. *Nat Genet* 2001; **27**: 151–152.
51. BAI H, AGULA H, WU Q, ZHOU W, SUN Y, QI Y, LATU S, CHEN Y, MUTU J, QIU C. A novel DSPP mutation causes dentinogenesis imperfecta type II in a large Mongolian family. *BMC Med Genet* 2010; **11**: 23.
52. LEE KE, KANG HY, LEE SK, YOO SH, LEE JC, HWANG YH, NAM KH, KIM JS, PARK JC, KIM JW. Novel dentin phosphoprotein frameshift mutations in dentinogenesis imperfecta type II. *Clin Genet* 2011; **79**: 378–384.
53. MCKNIGHT DA, SIMMER JP, HART PS, HART TC, FISHER LW. Overlapping DSPP mutations cause dentin dysplasia and dentinogenesis imperfecta. *J Dent Res* 2008; **87**: 1108–1111.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of human dentin phosphoprotein (DPP) length haplotypes noting the positions of disease-causing mutations.

Fig. S2. The merged DPP sequence and *DSPP* 5-prime disease-causing mutations.

Fig. S3. Human DPP reference sequence translated in three reading frames.

Fig. S4. Oral photographs and dental radiographs of the family 1 proband (IV:1).

Fig. S5. Oral photographs and dental radiographs of family 1 proband's affected youngest brother (IV:3).

Fig. S6. Oral photographs and dental radiographs of family 1 proband's other affected brother (IV:2).

Fig. S7. Oral photographs and dental radiographs of the family 1 affected father (III:7).

Fig. S8. Oral photographs and dental radiographs of the family 1 unaffected mother (III:8).

Fig. S9. Oral photographs and dental radiographs of family 2 proband (IV:2).

Please note: Wiley-Blackwell Publishing is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.