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ABSTRACT

The proven candidate genes for amelogenesis imperfecta (AI) are *AMELX*, *ENAM*, *MMP20*, *KLK4*, *FAM83H*, and *WDR72*. We performed mutation analyses on seven families with hypomaturation AI. A novel *WDR72* dinucleotide deletion mutation (g.57,426_57,427delAT; c.1467_1468delAT; p.V491fsX497) was identified in both alleles of probands from Mexico and Turkey. Haplotype analyses showed that the mutations arose independently in the two families. The disease perfectly segregated with the genotype. Only persons with both copies of the mutant allele were affected. Their hypomineralized enamel suffered attrition and orange-brown staining following eruption. Expression of *WDR72* fused to green fluorescent protein showed a cytoplasmic localization exclusively and was absent from the nucleus. We conclude that *WDR72* is a cytoplasmic protein that is critical for dental enamel formation.

KEY WORDS: protein localization, enamel maturation, enamel, tooth, Amelogenesis imperfect.

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Novel *WDR72* Mutation and Cytoplasmic Localization

INTRODUCTION

Amelogenesis imperfecta (AI) is a heterogeneous group of inherited conditions featuring the pathological formation of dental enamel. One out of every 700 to 14,000 persons is affected, depending upon the population being studied (Witkop and Sauk, 1976; Chosack *et al.*, 1979; Backman and Holm, 1986; Dummer *et al.*, 1990). By the strict definition, the phenotype is limited to dental enamel and is not associated with more generalized defects (Witkop, 1971). The initial candidate genes for AI were those encoding enamel matrix proteins, and 4 have been implicated in its etiology: amelogenin (*AMELX*; MIM *300391) (Lagerström *et al.*, 1991), enamelin (*ENAM*; MIM *606585) (Rajpar *et al.*, 2001), kallikrein 4 (*KLK4*; MIM *603767) (Hart *et al.*, 2004), and enamelysin (*MMP20*; MIM *604629) (Kim *et al.*, 2005b).

In addition to candidate gene approaches, genome-wide scans combined with mutational analyses identified AI-causing genes not previously suspected to play a role in dental enamel formation. Family with sequence similarity 83 member H (*FAM83H*; MIM 611927) was shown to cause autosomal-dominant hypocalcified AI (ADHCAI) (Mendoza *et al.*, 2007; Kim *et al.*, 2008), a severe and common form of AI. The function of *FAM83H* is still obscure, but it is expressed by ameloblasts (Lee *et al.*, 2009) and is associated with intracellular vesicles (Ding *et al.*, 2009). The enamel of persons with *FAM83H* mutations is less mineralized than normal and undergoes extensive post-eruptive enamel loss, but sometimes retains focal areas of normal-looking enamel (El-Sayed *et al.*, 2010).

WD repeat-containing protein 72 (*WDR72*; MIM 613214) was recently linked to AI by homozygosity mapping with a single nucleotide polymorphism (SNP) microarray followed by mutational analyses (El-Sayed *et al.*, 2009). Three different AI-causing mutations in *WDR72* were identified, and all showed a recessive pattern of inheritance. At the time of tooth eruption, the enamel was relatively smooth and creamy-brown-colored, but suffered rapid attrition and staining that left the enamel surface irregular and dark brown or orange (El-Sayed *et al.*, 2009).

Hypomaturation AI is thought to be caused by malformations that specifically affect modulating ameloblasts in the late stages of enamel formation,

after the enamel layer has reached its final dimensions, but before it has fully mineralized and hardened (Smith, 2008). In this study, we report a *WDR72* mutation in two families with autosomal-recessive hypomaturation AI and test the hypothesis that *WDR72* functions inside the cell by expressing the protein fused to green-fluorescent protein (GFP) in HEK293T cells and localizing it by fluorescent microscopy.

MATERIALS & METHODS

Patients

Seven families with AI phenotypes resembling those associated with *WDR72* mutations were selected from the AI families we have recruited for genetic studies. Three families showed an autosomal-dominant pattern of inheritance, two were recessive, and two could not be determined. Clinical and radiological examinations were performed, and blood samples were collected with the understanding and written consent of each participant according to the Declaration of Helsinki. This study protocol was independently reviewed and approved by the Institutional Review Boards at Seoul National University Dental Hospital, the University of Istanbul, and the University of Michigan.

Primer Design, Polymerase Chain-reaction (PCR), and DNA Sequencing

Genomic DNA was extracted from peripheral whole blood. *WDR72* exons and adjacent intron boundaries were amplified as described previously (El-Sayed *et al.*, 2009). Primer pairs for exon 1 (sense, 5'-CGGTACCCGTGACCCTAAG; antisense, 5'-GGCCAGCTGTATCCAAG) were designed with Primer 3 on the Web (<http://frodo.wi.mit.edu/primer3/>). PCR amplifications were performed with the HiPi DNA polymerase premix (Elpis Biotech, Taejeon, Korea) and purified with a PCR Purification Kit (Elpis Biotech). DNA sequencing was performed at the DNA sequencing center (Macrogen, Seoul, Korea). Sequences were analyzed with Mutation Surveyor (Softgenetics, Ann Arbor, MI, USA).

Expression Vector Construction

WDR72 coding sequence was amplified with synthetic oligonucleotide primers (sense, 5'-ggctcgagAAATGAGGACTTCCCTGCAGG; antisense, 5'-ggctctagaCACCTTGCAGGGGAGACCT) with a human cDNA clone (BC101614.1; Thermo Scientific Open Biosystems, Huntsville, AL, USA) as template. The amplification product was purified and subcloned into the pEGFP-C1 vector (Clontech, Mountainview, CA, USA) after both the plasmid and amplification product were double-digested with *Xho*I and *Xba*I restriction endonucleases. The engineering strategy placed the *WDR72* translation initiation codon in-frame with an N-terminal green fluorescent protein (GFP) domain. The entire construct was characterized by DNA sequencing.

Analysis of Intracellular Localization

The pEGFP-C1-based plasmids that express green fluorescent protein (GFP) alone and *WDR72* fused to GFP were purified with the AccuPrep Nano-Plus Plasmid Midi Extraction Kit

(Bioneer, Taejeon, Korea). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS (GIBCO-BRL, Carlsbad, CA, USA) and Antibiotic-Antimycotic liquid (GIBCO-BRL) at 37°C under 5% CO₂. For transfection, cells were grown on 6-well plates and transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). After 24 hrs, transfected cells were trypsinized and seeded onto poly-L-lysine-coated cover glass in 6-well plates. The next day, the cells were fixed with 4% paraformaldehyde, and nuclear DNA was stained in 20 µM bisBenzimide H 33342 trihydrochloride (Sigma, St. Louis, MO, USA) for 10 min. The samples were washed in PBS and mounted with Fluorescent Mounting Medium (DAKO, Glostrup, Denmark). Confocal laser scanning was performed with an OLYMPUS-FV300 fluorescence microscope.

RESULTS

Mutation Results

Mutational analysis of probands from families 1 and 2 revealed the same dinucleotide deletion mutation in exon 12 in both alleles of *WDR72* (Appendix Fig. 1). The standardized description of this mutation according to published nomenclature recommendations (den Dunnen and Antonarakis, 2003) for the cDNA sequence is c.1467_1468delAT, based upon the NCBI reference sequence NM_182758.2, where the A of the ATG translation initiation codon is nucleotide 1. The genomic description is g.57,426_57,427delAT and is based upon the NCBI reference sequence NC_000015.9, with the first nucleotide of the reference sequence (not the translation initiation codon) being nucleotide 1. The dinucleotide deletion leads to a predictable change in the translation product, which is described as p.V491fsX497. The normal *WDR72* amino acid sequence in this region (GDLSDC VILWDIF...) is changed to GDLSDCLVG^{*}YLY*. Instead of a *WDR72* translation product having 1102 amino acids, the protein is correctly translated through Cys⁴⁹⁰, 7 random amino acids are added, and the mutant protein terminates after Tyr⁴⁹⁷. Despite certainty in predicting the effect of the mutation on translation, no evidence indicates that the mutant mRNA is translated at all, rather than being degraded by nonsense-mediated decay. The mutation segregated perfectly with the AI phenotype in both families, indicating that it is disease-causing. A summary of the *WDR72* gene structure is provided in Appendix Fig. 2. If translated, the p.V491fsX497 mutation would abbreviate the protein in the sixth of seven WD40 repeat domains on the N-terminal side of *WDR72* (Appendix Fig. 3).

Clinical Findings

Family 1 is a Hispanic family from Mexico. Nine family members spanning three generations were recruited and contributed DNA. Haplotype analysis revealed that the two grandmothers (I:2 and I:4) are related and carriers of the same disease allele. All three of the grandchildren are homozygous for the c.1467_1468delAT mutation and display the AI phenotype. The parents of the proband have normal dentitions (Appendix Fig. 4); the pattern of inheritance is autosomal-recessive.

The permanent dentition of the proband showed orange-brown staining throughout (Fig. 1). Enamel roughness was

Figure 1. Family 1 pedigree and haplotypes; proband's (III:1) oral photos and radiograph. **(A)** Pedigree of family 1. The haplotype of each individual is shown below the symbol. Red boxes indicate alleles harboring the mutation (key: T, thymine; C, cytosine; W, wild-type; M, mutant). **(B)** Frontal and lateral photographs of the proband (III:1) taken at age 10 yrs, 7 mos. **(C)** Dental panoramic radiograph of the proband (III:1) taken at age 6 yrs, 6 mos.

localized and more likely to be observed in the occlusal or incisal half of the tooth and is apparently due to attrition following eruption. Indeed, progressive deterioration of the dentition was documented photographically in the proband's younger sister (III:2; Fig. 2). It is evident that the true phenotype of the *WDR72* mutation is best observed during tooth eruption, because of the occurrence of rapid and extensive post-eruptive changes. The enamel layer has reduced radiodensity compared with normal enamel, an indication that the enamel layer is hypomineralized.

Figure 2. Family 1 affected sister (III:2). **(A)** Oral photo taken at age 5 yrs, 6 mos. **(B)** Oral photo taken at age 6 yrs, 6 mos. **(C)** Oral photos taken at age 8 yrs, 7 mos. **(D)** Panoramic radiograph taken at age 5 yrs, 6 mos. Note the progressive deterioration of the enamel crowns, especially in the occlusal and incisal regions following eruption into function.

Family 2 is from Turkey. Six family members spanning two generations were recruited and contributed DNA. The pattern of inheritance is autosomal-recessive; only the two sons who are homozygous for the c.1467_1468delAT mutation display the AI phenotype (Fig. 3A). The enamel phenotype appears to be more severe in family 1 compared with family 2 (Fig. 3B), although the genetic etiology is the same. This suggests that habits and diet might influence the pattern of degeneration (attrition and staining) following tooth eruption; it also suggests the possibility of a genetic modifier. Radiologically, the phenotype is similar between the two families, with the enamel layer showing reduced radiodensity (Fig. 3C).

An SNP in exon 12 of *WDR72* (c.1407T>C, p.T469T, rs6416452) immediately adjacent to the AI-causing mutation site is a C in the diseased allele of family 1 and a T in the diseased allele of family 2. This finding demonstrates that the identical disease-causing mutation in the two families is not identical because of common ancestry (Figs. 1A, 3A), but arose independently in the two families. This confirms what is obvious from the various ethnic backgrounds and raises the possibility that this is a mutational hotspot in *WDR72*.

Analysis of Intracellular Localization

The wild-type *WDR72* fused to GFP localized exclusively in the cytoplasm of the transfected HEK293T cells, in contrast to the widespread localization of pEGFP-C1 control plasmid (Fig. 4).

DISCUSSION

The WD40 or WD-repeat domain family (pfam00400) includes 609 human genes (Speicher *et al.*, 2010). The naming of human genes based upon the presence of WD-repeat domains extends up to *WDR94*. The WD40 designation reflects the approximate

Figure 3. Family 2 pedigree and haplotypes; proband's (IV:3) oral photo and radiographs. (A) Pedigree of family 2. Haplotype of each individual is shown below the symbol. Red boxes indicate alleles harboring the mutation (key: T, thymine; C, cytosine; W, wild-type; M, mutant). (B) Frontal photograph of the proband (IV:3) at age 11 yrs. (C) Dental panoramic and intra-oral radiographs of the proband (IV:3) at age 11 yrs.

length (40 amino acids) of the conserved repeat domain and the high frequency of a tryptophan-aspartate (WD) dipeptide. WD40 domains are always found in a cluster of 4 to 16 repeats (Li and Roberts, 2001). The sequence repeat contains no absolutely conserved amino acids, but the basic pattern can be recognized by computer algorithms, such as REPV1.1 (Andrade *et al.*, 2000). Analysis of WDR72 by this software identified 7 WD repeats, all on the N-terminal side of the protein. WDR7, the closest homologue of WDR72, showed the same 7 repeats, plus 2 additional repeats near its C-terminus (which is a part of the protein that is not homologous to WDR72). WD-repeats form highly symmetrical folds that combine into a circular β

Figure 4. Analysis of intracellular localization. HEK293T cells transfected with pEGFP-C1 vectors expressing green fluorescent protein (GFP) alone and WDR72 fused to GFP (green). (A) Widespread GFP expression. (B) Nuclear Hoechst 33342 staining. (C) Overlapping image of (A) and (B). (D) Wild-type WDR72 localized in the cytoplasm. (E) Nuclear Hoechst 33342 staining. (F) Overlapping image of (D) and (E).

propeller structure that has no catalytic activity, but serves as a platform upon which protein complexes reversibly assemble (Smith, 2008). This structural element is comprised of the N-terminal 600 amino acids of WDR72, and folds into a domain that is presumed to engage in protein-protein complexes with unknown protein partners. WDR72 does not contain a signal peptide, and the WD40 domains start only 15 amino acids from the N-terminus. Taken together with the results of our GFP fusion study, it is unlikely that these associations occur in the nucleus or extracellular space.

A key aspect of WD-repeat proteins is that the β propeller structure that mediates protein-protein interactions is combined with a variable domain that conveys novel functions to individual members of the family. Among the 106 human proteins predicted to have 7 WD-repeats, 104 contain at least one significant additional non-WD domain (Smith, 2008). WDR7 shares strong homology with WDR72 up to Gly⁸⁵⁰ (Gly⁸⁴⁵ in WDR72), and then the two sequences diverge. WDR7 (from Thr⁸⁶³ to Leu⁹⁷⁴) has a conserved homology domain that resembles a PKC-like catalytic subunit. WDR72 has no recognizable homology domains in its C-terminal region.

Why mutations in both *WDR72* alleles cause isolated enamel defects is puzzling. The expressed sequence tag profile for *WDR72* indicates that *WDR72* is expressed in multiple tissues, with the healthy tissues showing the highest number of transcripts *per million* being the bladder (165), kidney (145), and mouth (74). Genes that are specific for enamel formation, such as amelogenin, amelotin, and ameloblastin, tend to degenerate when tooth development is discarded during evolution (Deméré et al., 2008; Sire et al., 2008; Meredith et al., 2009). Even when both *WDR72* alleles are defective, the only phenotype observed is in enamel, suggesting that *WDR72* expression is only critical for dental enamel formation. *WDR72*, however, is well-conserved in birds, although it is shorter (927 vs. 1102 amino acids) than in mammals, due to a deletion of ~175 amino acids from its C-terminus. The selective pressures that maintain *WDR72* in chickens are unclear. Perhaps *WDR72* is important for other processes besides enamel formation, and the reported *WDR72* mutations cause a subtle non-dental phenotype that has so far escaped detection. It seems that, among WD40 family members in non-dental tissues, there is functional redundancy which is not compensated for in ameloblasts.

Family 1 in this study was one of the original 24 families with AI whom we recruited for genetic studies (Kim et al., 2006). We have now identified two *AMELX* (Kim et al., 2004), two *ENAM* (Kim et al., 2005a, 2006), one *MMP20* (Kim et al., 2005b), five *FAM83H* (Lee et al., 2008; Ding et al., 2009), and one *WDR72* mutation in this group, bringing to 12 the number of families having the genetic etiology of their AI determined. None of the 24 families had a *KLK4* mutation. The number of genes that cause amelogenesis imperfecta is not known, but the 6 proven candidate genes cause AI in half of the first 24 kindreds we characterized.

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