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ENAM Mutations with Incomplete Penetrance

ABSTRACT

Amelogenesis imperfecta (AI) is a genetic disease affecting tooth enamel formation. AI can be an isolated entity or a phenotype of syndromes. To date, more than 10 genes have been associated with various forms of AI. We have identified 2 unrelated Turkish families with hypoplastic AI and performed mutational analysis. Whole-exome sequencing identified 2 novel heterozygous nonsense mutations in the *ENAM* gene (c.454G>T p.Glu152* in family 1, c.358C>T p.Gln120* in family 2) in the probands. Affected individuals were heterozygous for the mutation in each family. Segregation analysis within each family revealed individuals with incomplete penetrance or extremely mild enamel phenotype, in spite of having the same mutation with the other affected individuals. We believe that these findings will broaden our understanding of the clinical phenotype of AI caused by *ENAM* mutations.

KEY WORDS: amelogenesis imperfecta, hypoplastic, enamel, tooth, enamelin, expressivity.

INTRODUCTION

The outer surface of the human tooth is covered by dental enamel, which provides a beautiful appearance with a luster and enables efficient feeding with its exceptional hardness. To achieve such fine characteristics, enamel formation should be well coordinated throughout all the stages, including the presecretion, secretion, transition, and maturation stages. This coordination includes not just components of the extracellular matrix secreted by ameloblasts but also other genetic factors influencing the function of ameloblasts themselves.

Amelogenesis imperfecta (AI) is a group of hereditary diseases affecting tooth enamel formation. AI is heterogeneous in etiology as well as in phenotypes (Witkop, 1988). Genes encoding enamel matrix proteins have been suspected as primary candidates of AI, and indeed, disease-causing mutations have been identified in AI patients. Mutations in amelogenin (*AMELX*; MIM *300391; Kim *et al.*, 2004), enamelin (*ENAM*; MIM *606585; Rajpar *et al.*, 2001; Mardh *et al.*, 2002), enamelysin (*MMP20*; MIM *604629; Kim *et al.*, 2005b), and kallikrein 4 (*KLK4*; MIM *603767; P.S. Hart *et al.*, 2004) have been identified by candidate gene approach. Genetic analysis approaches, such as linkage analysis, autozygosity mapping, and especially whole-exome sequencing, have identified new players in AI: family with sequence similarity 83 member H (*FAM83H*; MIM *611927; Kim *et al.*, 2008), WD repeat-containing protein 72 (*WDR72*; MIM *613214; El-Sayed *et al.*, 2009), family with sequence similarity 20 member A (*FAM20A*; MIM *611062; O'Sullivan *et al.*, 2011), chromosome 3 open reading frame 26 (*C4orf26*; MIM *614829; Parry *et al.*, 2012), solute carrier family 24 member 4 (*SLC24A4*; MIM *609840; Parry *et al.*, 2013), laminin beta 3 (LAMB3; MIM *150310; Poulter *et al.*, 2014b), and integrin beta 6 (ITGB6; MIM *147558; Poulter *et al.*, 2014a; Wang *et al.*, 2014).

Clinically, AI can be classified as hypoplastic, hypocalcification, and hypomaturation. Hypoplastic enamel is thin but hard in most cases. Hypocalcified enamel is very soft, and hypomatured enamel has reduced mineral density and brown discoloration. However, it is not possible or extremely difficult to determine the clinical phenotype exactly in some cases. Hypocalcified enamel is easily broken down after tooth eruption due to extreme softness, with the remaining surfaces being rough and discolored. In some cases, hypoplastic enamel occurs in combination with hypomaturation AI (Sundell and Valentin, 1986).

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Hypoplastic AI without other systemic conditions is caused as an X-linked pattern by mutations in the *AMELX* gene or as an autosomal pattern by mutations in the *ENAM, LAMB3*, and *ITGB6* genes. In this study, we recruited 2 hypoplastic AI families and identified 2 novel nonsense *ENAM* mutations. Most interesting, in both families, we identified incomplete penetrance or an extremely mild clinical phenotype even though these individuals carried the same mutations as each proband.

MATERIALS & METHODS

Enrollment of Human Subjects

Two unrelated Turkish families having hypoplastic AI were recruited for the genetic studies. The study protocol was independently reviewed and approved by the Institution Review Board at Seoul National University Dental Hospital and University of Istanbul. Clinical examinations were performed, and blood samples were collected with the understanding and written consent of each participant according to the Declaration of Helsinki.

Whole-exome Sequencing

Whole-exome sequencing was performed with DNA samples from the probands of the 2 AI families after exome capturing with NimblGen exome capture reagent (family 1) or Illumina TruSeq DNA sample prep kit (family 2); 75-bp (family 1) and 90-bp paired-end sequencing reads were obtained with Illumina HiSeq 2000 (Yale Center for Mendelian Genomics, West Haven, CT, USA; or Macrogen, Seoul, Korea). Sequencing reads were aligned to the NCBI human reference genome (NCBI build 37.2, hg19), and the sequence variations were annotated with dbSNP build 137.

Polymerase Chain Reaction and Sequencing

Identified variation in the *ENAM* gene was confirmed with Sanger sequencing, and segregation within each family was confirmed. DNA sequences of the primers and polymerase chain reaction (PCR) protocol were previously described (Kim *et al.*, 2005a). PCR amplifications were done with the HiPi DNA polymerase premix (Elpis Biotech, Taejeon, Korea), and PCR amplification products were purified with a PCR Purification Kit and protocol (Elpis Biotech). DNA sequencing was performed at a DNA sequencing center (Macrogen).

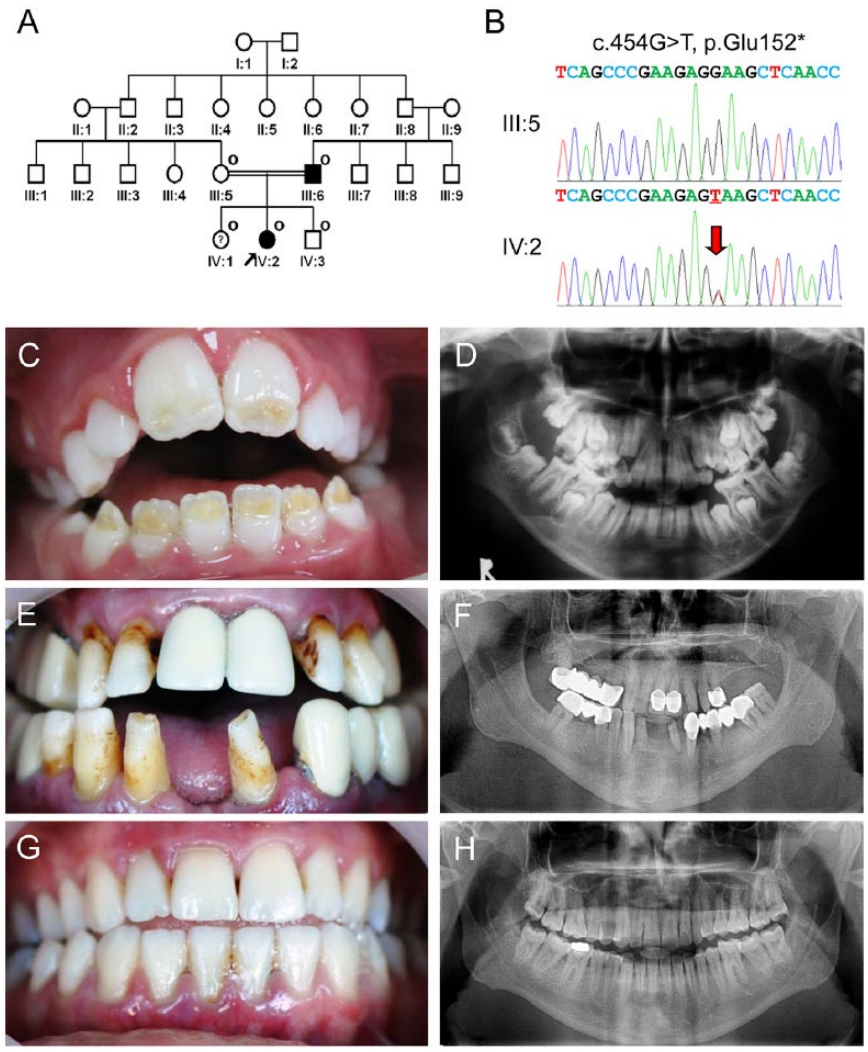

Figure 1. Clinical and mutational analysis of family 1. (**A**) Pedigree of family 1. The "O" symbol indicates members recruited for this study. The proband is indicated with a black arrow. The question symbol (?) indicates that this individual is clinically normal but has the mutation. (**B**) Comparison of the ENAM exon 7 sequencing chromatograms for the unaffected family member (III:5) with the wild-type (top) sequence, and the mutated allele in the proband (IV:2) reveals a G-to-T transversion: c.454G>T, p.Glu152*. A red arrow indicates the mutated nucleotide. (**C**) Frontal clinical photo of the proband. Yellow dentin color can be seen through the hypoplastic enamel located on the incisal half of the permanent anterior teeth. (**D**) Panoramic radiograph of the proband reveals unusual crown of the developing second permanent molars due to hypoplastic enamel. (**E**) Frontal clinical photo of the affected father shows the characteristic horizontal hypoplastic grooves. (**F**) Panoramic radiograph of the affected father shows multiple prosthetics and thin enamel in remaining teeth. (**G**) Frontal clinical photo of a sister of the proband. Despite having the mutation, her dentition has no enamel defects. (**H**) Panoramic radiograph of a sister of the proband reveals normal-looking tooth structures.

RESULTS

Family 1

The proband was a ten-year-old girl presenting a localized form of hypoplastic AI from a consanguineous family (Fig. 1A).

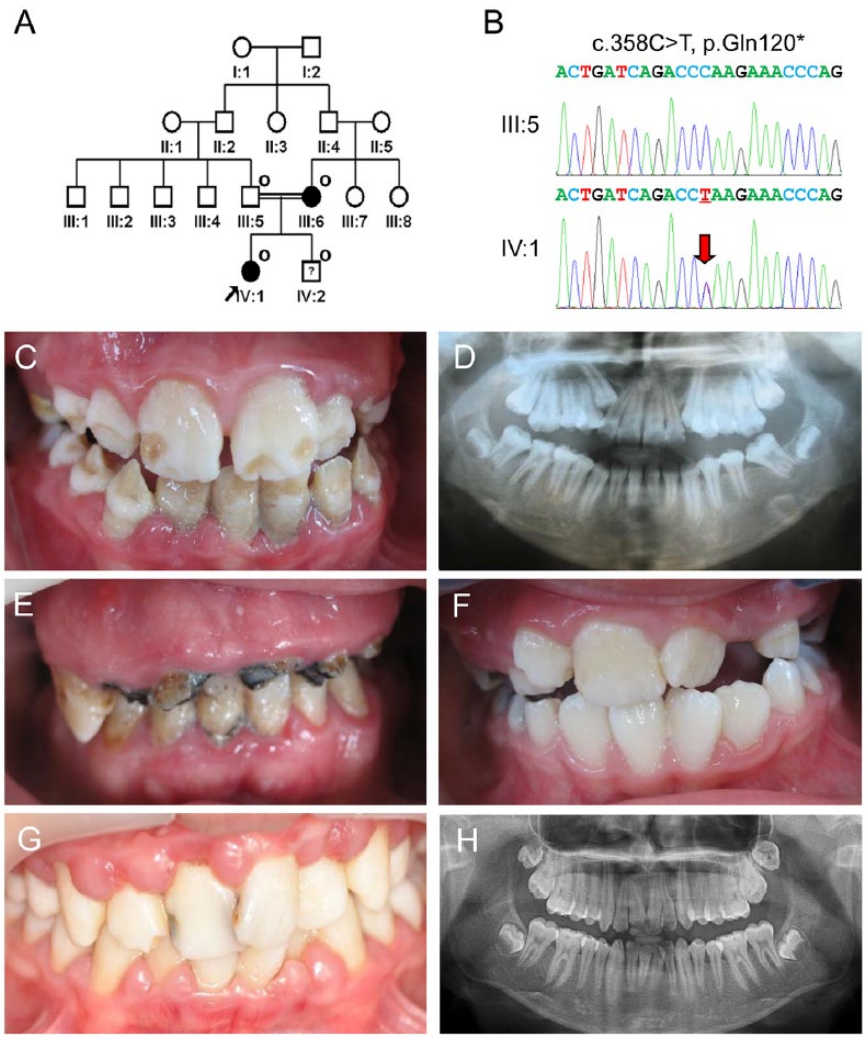

Figure 2. Clinical and mutational analysis of family 2. (**A**) Pedigree of family 2. The "O" symbol indicates members recruited for this study. The proband is indicated with a black arrow. The question symbol (?) indicates that this individual is clinically normal but has the mutation. (**B**) Comparison of the ENAM exon 7 sequencing chromatograms for the unaffected family member (III:5) with the wild-type (top) sequence, and the mutated allele in the proband (IV:1) reveals a C-to-T transition: c.358C>T, p.Gln120*. (**C**) Frontal clinical photo of the proband shows a localized form of enamel regions with some normal-looking areas. (**D**) Panoramic radiograph of the proband shows an irregular form of crowns. (**E**) Frontal clinical photo of the affected mother. She has several remaining teeth with gross destruction. (**F**) Frontal clinical photo of a brother of the proband at age 8 yr. He has normal-looking teeth, even though he also has the mutation. However, he has minor enamel defects, resembling an enamel fracture or dental caries, on the incisal corners of the maxillary right lateral incisor. (**G**) Frontal clinical photo of a brother of the proband at age 14 yr. Dental caries of maxillary central incisors and gingivitis can be seen. (**H**) Panoramic radiograph of a brother of the proband at age 14 yr.

Whole-exome sequencing identified a novel guanine to thymine transversion in exon 7 of the *ENAM* gene (c.454G>T; Fig. 1B). There was no other rare or novel variant in other AI candidate genes, including *AMELX, AMBN, MMP20, KLK4, FAM83H, C4orf26, WDR72, SLC24A4, CNNM4*, and *FAM20A*. This mutation changes glutamic acid (GAA) to the ochre termination codon (TAA) at codon position 152 (p.Glu152*). Because this mutation introduces an early termination codon that is not located in the last exon, the mutant mRNA would be degraded by the nonsense-mediated decay system so that no truncated protein could be generated. Sanger sequencing of the participating family members confirmed the existence of this mutation in the father (III:6), proband (IV:2), and sister of the proband (IV:1). All participating family members had uneventful pregnancy and delivery, and there was no systemic condition that could influence amelogenesis. Clinical examination revealed that the proband had hypoplastic regions located on the incisal half of the anterior permanent teeth with anterior open bite occlusion (Fig. 1C). A panoramic radiograph showed that developing molars had hypoplastic enamel (Fig. 1D). The affected father had many crowns and bridges, but the remaining teeth showed horizontal hypoplastic enamel regions, which are considered as a characteristic feature of *ENAM* mutations (Fig. 1E, 1F). However, clinical examination could not detect any enamel defect related to the mutation in individual IV:1, who had the same *ENAM* mutation, even though she had several initial dental caries (both maxillary first molars and left mandibular first molar) and a restoration (right mandibular first molar; Fig. 1G, 1H).

Family 2

The proband was an 11-year-old girl from a consanguineous marriage, who presented a localized form of hypoplastic AI (Fig. 2A). All participating family members had uneventful pregnancy and delivery, and there was no systemic condition. Whole-exome sequencing identified a novel cytosine-to-thymine transition in exon 7 of the *ENAM* gene (c.358C>T; Fig. 2B). There was no other rare or novel variant in other AI candidate genes, including *AMELX, AMBN, MMP20, KLK4, FAM83H, C4orf26, WDR72, SLC24A4, CNNM4*, and *FAM20A*. This mutation changes glutamine (CAA) to the ochre termination codon (TAA) at codon position 120 (p.Gln120*). This nonsense mutation is also predicted to cause degradation of the mutant transcript, as in the case of family 1. The mutation was identified in the mother (III:6) as well as the brother of the proband (IV:2). The proband had a generalized thin enamel with some thick enamel in the middle of the mandibular permanent teeth and localized hypoplastic regions on the incisal half of the maxillary permanent teeth (Fig. 2C, 2D). The affected mother had only

Table. Disease-causing Mutations in *ENAM* Gene

Location	cDNA	Protein	Inheritance Mode	References
Exon 4	c.107delA	p.Asn36Ilefs*22	AD	Simmer *et al.*, 2013
Exon 5	c.157A>T	p.Lys53*	AD	Mardh *et al.*, 2002; Kim *et al.*, 2006
Intron 6	c.211-2A>C	p.Met71-Gln157del	AD	Kim *et al.*, 2005a
Exon 7	c.358C>T	p.Gln120*	AD	This report
Exon 7	c.454G>T	p.Glu152*	AD	This report
Intron 8	c.534+1G>A	p.Ala158-Gln178del	AD	Rajpar *et al.*, 2001; Song *et al.*, 2012
Exon 9	c.536G>T	p.Arg179Met	AD	Gutierrez *et al.*, 2007
Intron 9	c.588+1delG	p.Asn197Ilefs*81	AD	Kida *et al.*, 2002; P.S. Hart *et al.*, 2003; Kim *et al.*, 2005a; Pavlic *et al.*, 2007
Exon 10	c.647C>T	p.Ser216Leu	AR/AD	Chan *et al.*, 2010
Exon 10	c.737C>A	p.Ser246*	AD	Ozdemir *et al.*, 2005
Exon 10	c.1020-1021ins AGTCAGTACC AGTACTGTGTC	p.Val340-Met341insSer GlnTyrGlnTyrCysVal	AR/AD	Ozdemir *et al.*, 2005
Exon 10	c.1259-1260insAG	p.Pro422Valfs*27	AR/AD	T.C. Hart *et al.*, 2003; Ozdemir *et al.*, 2005; Pavlic *et al.*, 2007; Kang *et al.*, 2009; Chan *et al.*, 2010; Lindemeyer *et al.*, 2010
Exon 10	c.2991delT	p.Leu998Trpfs*65	AD	Kang *et al.*, 2009

Sequences based on the reference sequence for mRNA (NM_031889.2) and protein (NP_114095.2), where the A of the ATG translation initiation codon is nucleotide 1.

several remaining teeth with gross destruction of the tooth structures (Fig. 2E). The brother of the proband had completely normal-looking mandibular permanent teeth; therefore, he was considered as a normal individual at the initial screening. The only abnormal enamel found was the regions on the incisal corners of the maxillary right lateral incisor, which resembled enamel fractures or dental caries (Fig. 2F). At age 14 yr, poor oral hygiene resulted in dental caries in the maxillary central incisors and gingivitis (Fig. 2G), but panoramic radiograph revealed normal-looking tooth structures (Fig. 2H).

DISCUSSION

Enamelin, one of the major proteins in the enamel matrix, is essential for proper enamel formation. A knockout mouse model demonstrated deficient enamel mineralization, resulting in no true enamel but an irregularly mineralized thin layer covering normal dentin (Hu *et al.*, 2008). The dosage effect of enamelin was noted with the recessive *ENAM* mutations (T.C. Hart *et al.*, 2003) and was further demonstrated by enamelin overexpressing transgenic mouse on the knockout background, indicating that an adequate quantity of enamelin is essential for normal enamel formation (Hu *et al.*, 2014).

Enamelin gene has been associated with dental caries susceptibility and recently identified as one of the genes contributing molar-incisor hypomineralization (Jeremias *et al.*, 2013; Chaussain *et al.*, 2014). Even though specific structural change(s) or genetic element(s) has not been identified yet, it seems obvious that sequence variations and allelic predilection are related to caries susceptibility or molar-incisor hypomineralization.

Unlike other genes involved in AI, *ENAM* mutations frequently exhibit wide variable expressivity (even within a family), from minor enamel pits to severe enamel loss, where virtually no enamel is remaining (Kida *et al.*, 2002; Chan *et al.*, 2011). It has been also noted that the same *ENAM* mutation results in different clinical phenotypes among families (P.S. Hart *et al.*, 2003). Phenotypic variation within family and between families is also shown in this study in spite of the similar nature of the mutations. A characteristic clinical feature frequently associated with *ENAM* mutations is horizontal hypoplastic grooves on the labial surface of the crown. The affected father of family 1 and the proband of family 2 also exhibit horizontal hypoplastic grooves.

Through the identification of recessive *ENAM* mutations, localized small enamel pits have been recognized as a milder phenotype of AI in heterozygous individuals (Table). Individuals with *ENAM* mutations in both alleles exhibited severe generalized hypoplastic AI with open bite, indicating a dosage-dependent effect of the mutation (T.C. Hart *et al.*, 2003). Interestingly, the same mutation has been identified in other autosomal-dominant AI families with characteristic horizontal hypoplastic grooves, confirming the variable expressivity of *ENAM* mutations (Pavlic *et al.*, 2007).

Most striking, a sibling had normal-looking enamel despite having the same mutation (c.211-2A>C) with other affected family members (Kim *et al.*, 2005a). In this study, we identified additional cases of incomplete penetrance or extremely mild clinical phenotype with 2 different nonsense mutations. Recently, *LAMB3* mutations have been identified to cause non-syndromic AI by a dominant negative effect (Kim *et al.*, 2013; Poulter *et al.*, 2014b), and a case with very mild expressivity has been noted in a truncating mutation with a prediction of a minimal dominant negative effect (Lee *et al.*, 2014).

The possibility of the hypoplastic AI with incomplete penetrance was noted in an epidemiologic study of Swedish population (Sundell and Valentin, 1986). It is highly possible that among them, there were individuals having less harmful mutations in genes such as *LAMB3, ENAM, ITGB6*, or other yet

unidentified genes; therefore, the phenotypes were variable and very mild in some cases. However, *ENAM* mutations with incomplete penetrance in a previous study and this study caused severe hypoplastic AI in other affected individuals.

Generally, mutations in the *ENAM* gene cause hypoplastic enamel, but the severity of the enamel defects is sometimes highly variable, even among individuals with the same mutation. At this moment, there is no direct explanation or mechanism to support the lack of penetrance or extremely mild clinical phenotype in an individual. Other genetic factors, such as yet unidentified cis- or trans-acting elements, could regulate and influence the expression of enamelin or ENAM protein function. Further genetic and functional studies are needed to understand the dynamic and variable nature of enamel formation.

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