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J Dent Res 92(10):899-904, 2013

ABSTRACT

Amelogenesis imperfecta (AI) can be either isolated or part of a larger syndrome. Junctional epidermolysis bullosa (JEB) is a collection of autosomal-recessive disorders featuring AI associated with skin fragility and other symptoms. JEB is a recessive syndrome usually caused by mutations in both alleles of *COL17A1*, *LAMA3*, *LAMB3*, or *LAMC2*. In rare cases, heterozygous carriers in JEB kindreds display enamel malformations in the absence of skin fragility (isolated AI). We recruited two kindreds with autosomal-dominant amelogenesis imperfecta (ADAI) characterized by generalized severe enamel hypoplasia with deep linear grooves and pits. Whole-exome sequencing of both probands identified novel heterozygous mutations in the last exon of *LAMB3* that likely truncated the protein. The mutations perfectly segregated with the enamel defects in both families. In Family 1, an 8-bp deletion (c.3446_3453del GACTGGAG) shifted the reading frame (p.Gly 1149Glufs*8). In Family 2, a single nucleotide substitution (c.C3431A) generated an in-frame translation termination codon (p.Ser1144*). We conclude that enamel formation is particularly sensitive to defects in hemidesmosome/basement-membrane complexes and that syndromic and non-syndromic forms of AI can be etiologically related.

KEY WORDS: genetic diseases, dental enamel, tooth, junctional epidermolysis bullosa, basement membrane.

DOI: 10.1177/0022034513502054

Received May 23, 2013; Last revision July 12, 2013; Accepted July 26, 2013

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LAMB3 Mutations Causing Autosomal-dominant Amelogenesis Imperfecta

INTRODUCTION

Amelogenesis imperfecta (AI), by the strict definition, refers to inherited malformations of dental enamel that occur in the absence of non-dental phenotypes (Witkop, 1989). Non-syndromic forms of autosomal-dominant AI can be caused by mutations in *ENAM* (Hu and Yamakoshi, 2003) and *FAM83H* (Kim *et al.*, 2008). *ENAM* is a tooth-specific gene (Meredith *et al.*, 2009), and the phenotype caused by *ENAM* mutations is limited to dental enamel (SG Simmer *et al.*, 2012). Sometimes the phenotype is mild when a single *ENAM* allele is mutated and is manifested by well-delineated enamel pits, whereas little or no enamel forms when both alleles are affected (Ozdemir *et al.*, 2005). *FAM83H* mutations cause autosomal-dominant hypocalcified amelogenesis imperfecta (ADHCAI). Patients with disease-causing *FAM83H* mutations have one wild-type allele and one allele with a truncation mutation that induces a dominant-negative pathogenic mechanism (Kweon *et al.*, 2013). Unlike enamelin, which is secreted, FAM83H is intracellular and expressed in tissues other than developing teeth. The tooth-specificity of the phenotype may relate to ameloblasts (the cells that make enamel) being the cell-type that is most severely affected by pathology resulting from a truncated FAM83H protein.

Amelogenesis imperfecta also describes the enamel phenotype in syndromes. Junctional epidermolysis bullosa (JEB) is a collection of recessive inherited disorders featuring skin fragility and AI caused by defects in genes encoding the components of hemidesmosome/basement-membrane complexes (Masunaga, 2006). JEB cases with well-described enamel defects have been reported for *COL17A1* (McGrath *et al.*, 1996; Pasmooij *et al.*, 2007), *ITGB4* (Pulkkinen *et al.*, 1998), and genes encoding laminin-332, such as *LAMB3* (Buchroithner *et al.*, 2004). The enamel is characterized by deep pits and grooves that might increase the risk for dental caries (Wright *et al.*, 1994). A single defective allele for a gene that causes JEB (when both alleles are defective) may be manifested as autosomal-dominant amelogenesis imperfecta (ADAI) with little or no apparent skin fragility. The first reported cases were two heterozygous daughters of a *COL17A1* compound heterozygous (g.3514ins25/p.G627V) proband with JEB. Both daughters inherited the same defective *COL17A1* allele (p.G627V) and showed extensive enamel defects with

no signs of skin fragility (McGrath *et al.*, 1996). In the next generation, a single copy of the *COL17A1* p.G627V allele was passed on to the proband's granddaughter, who showed enamel defects and mild skin fragility, being the first reported case of autosomal-dominant JEB (Almaani *et al.*, 2009). The *COL17A1* p.G627V substitution lies within the collagen triple-helical domain and causes abnormal folding of the COL17A1 trimer, making it susceptible to degradation (Tasanen *et al.*, 2000). In another case, a *COL17A1* p.T239fs*52 mutation that caused ADAI in the heterozygous condition reduced the amount of wild-type COL17A1, since the mutant mRNA was subjected to nonsense-mediated decay and degraded (Murrell *et al.*, 2007).

ADAI in heterozygous carriers of JEB have also been reported for *LAMA3* and *LAMB3* mutations. A JEB proband was a *LAMA3* compound heterozygote (p.G163Dfs*30/p.A1495V), while his mother and brother carried only a single defective *LAMA3* allele (p.G163Dfs*30) and had ADAI manifested as pitted enamel defects (Yuen *et al.*, 2012). More recently, a p.E1133Gfs*27 mutation in a single *LAMB3* allele segregated perfectly with the enamel phenotype in a four-generation ADAI family (Poulter *et al.*, 2013). In this case, the defect was in the last exon, so the mRNA transcript likely escaped nonsense-mediated decay and allowed for translation of the truncated protein. Here, we report two novel *LAMB3* mutations causing ADAI, confirming that heterozygous defects in *LAMB3* can cause ADAI and providing new insight into the types of mutations that cause dominant, non-syndromic AI.

MATERIALS & METHODS

Patients

Two unrelated families, of Turkish and Iranian descent, with irregular hypoplastic ADAI phenotypes were selected from the AI families we have recruited for genetic studies. Clinical examinations were performed and blood samples were collected with the understanding and written consent of each participant according to the Declaration of Helsinki. The 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.

Whole-exome Sequencing

Whole-exome sequencing was performed with genomic DNA isolated from the probands of the two AI families to identify the disease-causing mutation(s). Exomes were captured with Illumina TruSeq DNA sample prep kit, and 90-bp paired-end sequencing data were obtained with Illumina HiSeq 2000 (Macrogen, Seoul, Korea; Theragen Bio Institute, Suwon, Korea). Alignment of the sequencing data to the NCBI human reference genome (NCBI build 37.2, hg19) was performed, and the sequence variations were annotated with the dbSNP build 135.

Polymerase Chain-reaction and DNA Sequence Analyses

Primer pairs for the last exon of the *LAMB3* gene (sense, 5'-CTGGAGAGGCATGAAGCTG; antisense, 5'-GCTGCAGC

TCAGGGTAATCT) 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).

RESULTS

Clinical Findings

The proband of Family 1 was a 6.5-year-old girl presenting with sensitive teeth. Her mother, who was the source of information concerning the pedigree and affection status of unexamined individuals, reported that the biological father had dental problems similar to those of the proband (Fig. 1A). The mother's dentition was within normal limits (data not shown). The proband's enamel for both the primary and secondary dentition was thin (hypoplastic), with deep grooves and pitting defects (Fig. 1B). Radiographic examination revealed severe generalized enamel hypoplasia. The enamel was generally more radiopaque than dentin, but not in all places. Taurodontism was evident in both the primary and secondary molars (Fig. 1D).

The proband of Family 2 also presented with enamel hypoplasia in all teeth, a trait she inherited from her mother (Fig. 2). The proband complained of thermal hypersensitivity and displayed a distinctive pattern of enamel defects characterized by deep grooves and pits that led us to consider that the enamel phenotype in these two families might be caused by defects in a common gene. In both probands, the linear grooves in the incisors tended to run vertically, with the most pronounced grooves extending from between the mamelons and delineating regions where differentiating ameloblasts derived from adjacent growth centers met during tooth formation. In both families, the pattern of inheritance was autosomal-dominant, and neither family reported a medical history of skin fragility. Differences between the phenotypes were minor: The Family 2 proband displayed hypertrophic gingiva in the maxillary anterior, and her radiographs showed little or no taurodontism.

Mutation Results

We obtained potential disease-causing sequence variations from the whole-exome sequence data by combining the annotation files of the two probands and searching for genes with potentially deleterious sequence variations in both probands. Heterozygous, destructive sequence variations in *LAMB3* were ascertained that are not listed in the 1000 Genomes Project. The *LAMB3* mutation identified in Family 1 (Fig. 1C) was an 8-bp deletion in the last exon (c.3446_3453delGACTGGAG) that shifted the reading frame and caused premature translation termination following the addition of 7 extraneous amino acids (p.Gly1149Glufs*8). The proband of Family 2 had a *LAMB3* nonsense mutation (c.C3431A, p.Ser1144*) in the last exon (Fig. 2C).

For clarity, the cDNA designation for the disease-causing mutation in Family 2 requires explanation. The three affected members of Family 2 (III:4, III:5, IV:1) differed from the *LAMB3* cDNA reference sequence (NM_000228.2) at two

Figure 1. Clinical and mutational analysis of Family 1. **(A)** Pedigree of Family 1. The “plus” symbol indicates members recruited for this study. **(B)** Frontal and maxillary occlusal clinical photographs of the proband. The enamel surface has many hypoplastic pits and linear grooves, mostly in a vertical orientation, which are highlighted by disclosing solution applied to the maxillary labial surfaces. There are no signs of gingival hypertrophy. **(C)** Comparison of the *LAMB3* exon 23 sequencing chromatograms for the unaffected family member (II:2) with the wild-type (top) sequence and the mutated allele in the proband (III:2) reveals an 8-bp deletional mutation: c.3446_3453delGACTGGAG, p.Gly1149Glufs*8. **(D)** Panoramic and bitewing radiographs of the proband show generally thin enamel that is only sometimes in contrast to dentin. The maxillary and mandibular first molars show signs of taurodontism.

adjacent positions (c.C3431A and c.A3432G). The two unaffected members (III:6, IV:2) showed the reference sequence at both positions. Therefore, we considered the designation c.3431_3432delinsAG. However, the G at c.3432 is listed as a single nucleotide polymorphism (SNP; rs1049607), so the disease-causing mutation was likely a single nucleotide change in *LAMB3* that preceded this SNP and resulted in a nonsense mutation and should be designated c.C3431A.

Distribution of the putative disease-causing mutations among the recruited members of both families was analyzed by Sanger sequencing of exon 23 PCR amplification products and demonstrated complete segregation of the *LAMB3* mutations with the disease phenotype. There are now 3 known *LAMB3* mutations associated with ADAI, all causing premature translation termination in the last coding exon (Fig. 3A). An alignment of the exon 23-encoded protein sequence at the *LAMB3* C-terminus

shows strong conservation of sequence length and 5 perfectly conserved amino acids between fish and humans that are lost in the mutant *LAMB3* alleles (Fig. 3B).

DISCUSSION

Laminin-332 (formerly laminin V) is a component of basement membranes and comprised of 3 subunits. Laminin is anchored to epithelial cells by collagen XVII and can activate integrin signaling through $\alpha 6/\beta 4$ receptors. The C-terminal regions of LAMA, LAMB1, and LAMB2 and other possible variants form the long arm of Laminin V, which contains sites for receptor-mediated cell attachment and promotion of neurite outgrowth (Beck *et al.*, 1990). Defects in both alleles of *LAMA3*, *LAMB3*, *LAMC2*, and *COL17A1* cause JEB, while defects in both alleles of *ITGA6* and *ITGB4* cause JEB with pyloric atresia. All of these

Figure 2. Clinical and mutational analysis of Family 2. (A) Frontal and lateral clinical photographs of the proband, showing maxillary anterior gingival hypertrophy and generalized severe enamel hypoplasia with deep pits and grooves, often oriented vertically. (B) Pedigree of Family 2. The “plus” symbol indicates members recruited for this study. (C) *LAMB3* exon 23 sequencing chromatogram for the unaffected family member (IV:2) with the wild-type sequence (top) compared with the heterozygous mutated allele in the proband (IV:1) with a 2-bp substitution of CA with AG (underlined; c.C3431A, p.Ser1144*). (D) Radiographs of the posterior teeth showing severe enamel hypoplasia. The molar root form is within normal limits, with only a trace of taurodontism.

conditions are recessive and associated with enamel malformations. With the 2 ADAI mutations reported here, there are now 3 single-allele *LAMB3* mutations associated with ADAI in the absence of other signs of JEB. All 3 mutations prematurely terminate translation in the last coding exon. The wild-type *LAMB3* protein has 1172 amino acids. The truncated forms have 1131, 1143, and 1148 amino acids of normal *LAMB3* sequence, so the sizes of the C-terminal deletions are only 41, 29, and 24 amino acids, respectively. Since the defect in Family 2 is a simple nonsense mutation, it appears that the absence of the normal C-terminal amino acids from the *LAMB3* protein is sufficient to cause the enamel phenotype, even without the short (7- and 28-amino-acid) extraneous sequences translated downstream from the reading frame shifts. Truncation mutations in one allele, however, are not sufficient to cause skin fragility or the other non-dental phenotypes associated with JEB. But why should enamel formation be so sensitive to defects in hemidesmosome/basement-membrane complexes?

Although some reports suggest that laminin is expressed by secretory ameloblasts, it has long been known that the basement membrane of the inner enamel epithelia fenestrates and disappears under differentiating ameloblasts (Reith, 1967). Laminin

epitopes are lost as pre-ameloblasts initiate amelogenin secretion (Inai *et al.*, 1991) and do not appear again until ameloblasts transition to the maturation stage (Nanci *et al.*, 1993). In place of the basement membrane under secretory-stage ameloblasts is a mineralization front apparatus that initiates and elongates thin ribbons of amorphous calcium phosphate (Beniash *et al.*, 2009; JP Simmer *et al.*, 2012). Secretory-stage ameloblasts are not strongly attached to the extracellular matrix, and migrate past each other to give the enamel layer a decussating pattern of enamel rods.

Lama3 null mice do not show defects in the inner enamel epithelium, which are attached by hemidesmosomes to a Laminin-332 basement membrane. Dental abnormalities in *Lama3* null mice first appear during ameloblast differentiation, downstream of adhesion, when the basement membrane is being degraded and replaced by the mineralization front (Ryan *et al.*, 1999). Part of the reason for the downstream effects is the dysregulation of enamel protein genes. Laminin-332 activates integrin signaling primarily via $\alpha 6 \beta 4$ receptors (Chiang *et al.*, 2011). Amelogenin (*Amelx*) and enamelin (*Enam*) are specifically and strongly up-regulated in the enamel organs of *Itgb6* null mice (Mohazab *et al.*, 2013), suggesting that degradation of

the hemidesmosome/basement-membrane complexes de-represses enamel protein expression, synchronizing replacement of the basement membrane complex by the mineralization front apparatus and presumably establishing new matrix-cell interactions. Ameloblast cell pathology also occurs in *Enam* null and *Ambn* (ameloblastin) defective mice, both of which fail to form the enamel mineralization front (Fukumoto *et al.*, 2004; Hu *et al.*, 2008).

The onset of enamel formation involves fenestration of the basement membrane, extension of ameloblast processes into irregularities on the pre-dentin surface, expression of enamel proteins, and the initial formation of enamel ribbons. The nearly synchronous ameloblast cell pathology in *Lama3*, *Itgb6*, *Enam*, and *Ambn* defective mice suggests that coordinated replacement of hemidesmosome/basement-membrane complexes with the mineralization front apparatus is critical for ameloblast physiology and presumably places special demands upon these complexes beyond that normally required for cell adhesion. This likely explains why single-allele defects in *COL17A1*, *LAMA3*, *LAMB3*, and potentially other genes involved in the etiology of JEB can be manifested by severe enamel malformations, while both alleles of these genes must be mutated to induce the skin fragility and other manifestations in JEB patients.

Our Family 1 is one (AI#23) of the first AI families we recruited for genetic studies (Kim *et al.*, 2006). With this report, 14 of the AI-causing mutations have been identified in these 24 families. Among the 11 discernible autosomal-dominant cases, we identified 1 *LAMB3*, 3 *ENAM*, and 5 *FAM83H* disease-causing mutations. The proven candidate genes for ADAI should be expanded to include *FAM83H*, *ENAM*, *LAMA3*, *LAMB3*, and *COL17A1*. X-linked AI is caused by defects in *AMELX*. The current list of proven candidate genes for non-syndromic autosomal-recessive AI includes *MMP20*, *KLK4*, *WDR72*, *C4orf26*, and *SLC24A4*. There are also many genes, such as *CNNM4* and *FAM20A*, that have been proven to cause recessive syndromes where AI can be the predominant phenotype at the time of initial diagnosis. The complexity of the potential genetic etiology in a patient presenting with inherited enamel defects makes mutational analyses of target genes based upon genotype-phenotype correlations largely impractical. Whole-exome analyses are currently the best way to discover the disease-causing mutation in patients with AI (Wang *et al.*, 2013).

Non-syndromic AI-causing mutations have now been found in genes encoding secreted enamel matrix proteins and proteases (*AMELX*, *ENAM*, *C4orf26*, *MMP20*, *KLK4*), intracellular (*FAM83H*, *WDR72*), transmembrane (*SLC24A4*, *COL17A1*), and basement membrane (*LAMA3*, *LAMB3*) proteins. These genes, however, account for only about half of all cases, so more causative genes await discovery. Suspected candidate genes include *AMBN*, *ATN*, *ODAM*, *LAMC2*, *ITGA6*, *ITGB4*, and *ITGB6*.

ACKNOWLEDGMENTS

This work was supported by grants from the Bio & Medical Technology Development Program (2011-0027790), the Science Research Center grant to Bone Metabolism Research Center (2012-0000487), the Korea Research Foundation Grant funded

A

Poulter:	c.3394_3395insG;	p.Glu1132Glyfs*28
Family 1:	c.3446_3453delGACTGGAG	p.Gly1149Glufs*8
Family 2:	c.C3431A	p.Ser1144*

B

Wild-type:	MELELLRGSQAIMLRSDLTGLEKRVQIRDHNGRVLYYATCK*
Poulter:	MEL GAAAGQPGHHAALSGPDR TGEACGADP*
Fam 1:	MELELLRGSQAIMLRSDLT EACGADP *
Fam 2:	MELELLRGSQAIMLR*
Human	MELELLRGSQAIMLRSDLTGLEKRVQIRDHNGRVLYYATCK*
Mouse	MESELLRGSQAIMLRSDLTSGLEKRVQIRSYINGRVLYYATCK*
Opossum	MEAE LL GSQAITLRSDLTGLEKHVEKIRDHINKRVLYYATCQ*
Turtle	TE ME TQKSNNALITKSASLAGLEEQVEKISSHIERIAYYTGCS*
Chicken	LETEIQESNRALVAKSARLSGLQE Q VGRI DAI HKRVYYESCS*
Frog	IE TE LQ EG RETLLMKFSQLEGLEEAKLIRDNISERALFYATCK*
Fish	LERKFNKNEQKM Q QQRDELTDLEKNVTGIREYIRSKVLAYNNCQ*

Figure 3. Analysis of ADAI-causing *LAMB3* mutations. (A) Listing of the *LAMB3* mutation reported previously (Poulter *et al.*, 2013) and the 2 reported here. The designation for the first mutation includes a minor correction. (B) Alignment of the human wild-type exon 23-encoded sequence for the *LAMB3* C-terminus with the predicted truncations in the proteins expressed from the mutant alleles and *LAMB3* homologues going back to fish. Changes from the human wild-type sequence are in bold. Arrowheads mark amino acids strictly conserved between fish and humans and include a Cysteine thought to engage in intermolecular disulfide bridging. The GenBank sequences used in the alignment are human (NM_000228), mouse (AAA85255), opossum (XP_001373738), turtle (EMP35962), chicken (XP_425827), frog (XP_002933550), and fish (XP_692838).

by the Korean Government (MEST), and the NIDCR of the National Institutes of Health (Grant DE015846). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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