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Phenotypic Variation in *FAM83H*-associated Amelogenesis Imperfecta

ABSTRACT

FAM83H gene mutations are associated with autosomal-dominant hypocalcified amelogenesis imperfecta (ADHCAI), which is typically characterized by enamel having normal thickness and a markedly decreased mineral content. This study tested the hypothesis that there are phenotype and genotype associations in families with *FAM83H*-associated ADHCAI. Seven families segregating ADHCAI (147 individuals) were evaluated. Phenotyping included clinical, radiographic, histological, and biochemical studies, and genotyping was by mutational analysis. Multiple novel *FAM83H* mutations were identified, including two 2-bp-deletion mutations, the first non-nonsense mutations identified. Craniofacial deviation from normal was more prevalent in the affected individuals. Affected individuals having truncating *FAMH3H* mutations of 677 or fewer amino acids presented a generalized ADHCAI phenotype, while those having mutations capable of producing a protein of at least 694 amino acids had a unique and previously unreported phenotype affecting primarily the cervical enamel. This investigation shows that unique phenotypes are associated with specific *FAM83H* mutations.

KEY WORDS: enamel, *FAM83H*, amelogenesis imperfecta, hypocalcified, phenotype, craniofacial.

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INTRODUCTION

Enamel formation is exquisitely regulated at the molecular level, with numerous genes being critical for normal development. Five known genes have mutations directly affecting the quantity and or quality of enamel formation, causing amelogenesis imperfecta (AI) while not causing other tissues defects (Wright, 2006; Kim *et al.*, 2008). Mutations in genes coding for the extracellular matrix proteins amelogenin and enamelin are associated with poorly mineralized and/or hypoplastic enamel (Hu and Yamakoshi, 2003; Wright *et al.*, 2003). Mutations in genes coding for critical proteinases involved in processing the enamel extracellular matrix, matrix metalloproteinase 20 and kallikrein 4, result in enamel of normal thickness with a decreased mineral content (Hart *et al.*, 2004; Kim *et al.*, 2005). Recently, the molecular basis of autosomal-dominant hypocalcified amelogenesis imperfecta (ADHCAI) was identified as mutations in the *FAM83H* gene (Kim *et al.*, 2008). While AI is a relatively rare condition (1 in 700 to 1 in 15,000 in different populations), ADHCAI is one of the more prevalent AI types in North America (Witkop, 1957; Chosack *et al.*, 1979; Backman and Holm, 1986; Sundell, 1986).

The ADHCAI phenotype typically presents yellow-brown teeth with poorly mineralized enamel that has increased retention of enamel proteins. The ADHCAI enamel protein content in mature teeth is about 5%, while that in normal enamel is less than 1% (Wright *et al.*, 1995). The ADHCAI enamel mineral content ranges from 45 to 80%, while that in normal enamel ranges from 70 to 98% mineral *per* volume (Wright *et al.*, 1995). This reduction in mineral and increase in protein cause the enamel to be easily abraded from the teeth and to serve as a poor insulator, causing extreme hypersensitivity in many affected individuals. ADHCAI is often associated with an anterior open bite and class III malocclusion (Ravassipour *et al.*, 2005).

The role of the *FAM83H* gene and protein (1179 amino acid) during enamel formation remains unknown. The gene is expressed in many tissues; however, all mutations reported to date result only in enamel abnormalities, suggesting that this gene is essential for enamel formation, but is not as critical in other tissues (Kim *et al.*, 2008; Lee *et al.*, 2008). A potential transactivation domain was identified *in silico*, suggesting that FAM83H protein could serve as a transcription factor (Kim *et al.,* 2008). This study tests the hypothesis that there are phenotype and genotype associations in families with *FAM83H*-associated ADHCAI.

MATERIALS & METHODS

The Institutional Review Board approved the study, and all participants provided written informed consent. Affected and unaffected individuals from families segregating for ADHCAI were recruited. All available individuals were examined by one of two investigators (TW, PC), and affection status was

assigned based on clinical phenotype.

Mutational Analysis

Genomic DNA was isolated from peripheral blood leukocytes or saliva with the QIAamp blood kit (Qiagen, Santa Clara, CA, USA) or the Oragene saliva kit (DNA Genotek, Ottawa, ON, Canada). All 5 exons of the *FAM83H* were amplified as previously described (Hart *et al.,* 2009). PCR products were subjected to electrophoresis through 1.8% agarose gels, and the amplicons were extracted and sequenced with ABI Big Dye terminator chemistry and an ABI 3730 DNA Analyzer (Foster City, CA, USA). PCR products were sequenced in both directions to minimize sequencing artifacts.

Histological Analysis

Teeth were evaluated by light microscopy and scanning and transmission electron microscopy as described previously (Hart *et al.*, 2004). Teeth were sectioned with a diamond blade for light microscopy and fractured for scanning electron microscopy. Enamel was embedded in resin, and ultrathin sections were cut with a microtome for evaluation of crystallite distribution and morphology.

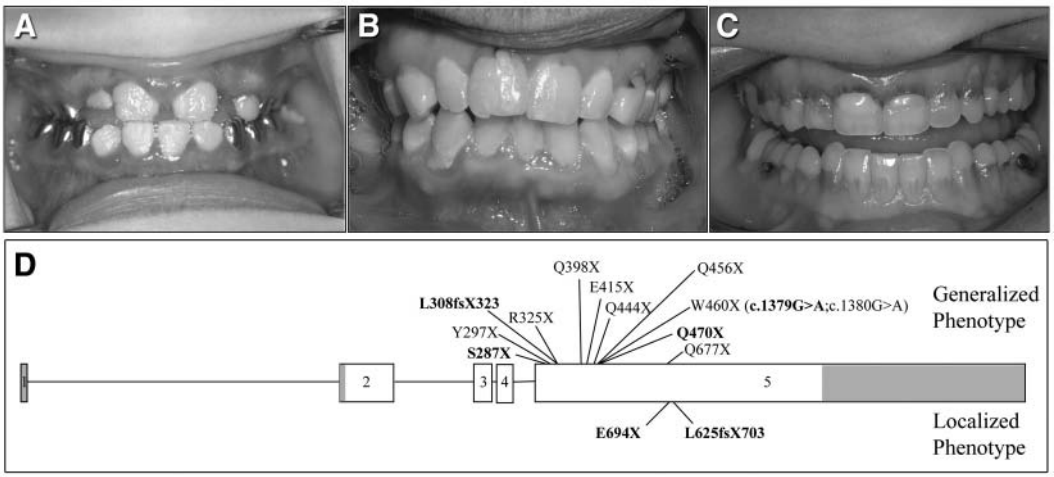

Figure 1. Nonsense mutations truncating the protein prior to AA 677 resulted in severe generalized ADHCAI with the characteristic yellow-brown enamel **(A)**, as seen in the proband of Family AIC5 (see Appendix Fig. 1), while frameshift and point mutations truncating the protein after AA 694 resulted in a localized ADHCAI that primarily affected the cervical areas **(B)** (p. L625fsX703) in Family AIC46 proband #254 and **(C)** (p.E694X) in Family AI10-002. **(D)** Diagram of the *FAM83H* gene depicting the exons (rectangles) and introns (lines). The UTRs are shown in grey. The mutations illustrated above the diagram result in a generalized phenotype, while those below result in a localized phenotype. The novel mutations identified by this study are indicated in bold. The frameshift mutations are indicated by the length of the predicted translated protein.

Table 1. ADHCAI Families and *FAM83H* Mutations

Family	N	cDNA	Protein	Phenotype
AIC5	1	c.1408C>T	p.Q470X	Generalized hypocalcified
AIC33	77	c.1408C>T	p.Q470X	Generalized hypocalcified
AIC19	33	c.860C>A	p.S287X	Generalized hypocalcified
AIC38	5	c.923_924delTC	p.L308fsX323	Generalized hypocalcified
AIC40	20	c.1379G>A	p.W460X	Generalized hypocalcified
AIC46	8	c.1872_1873delCC	P.L625fsX703	Localized hypocalcified
AI10-002	3	c.2080G>T	p.E694X	Localized hypocalcified

Enamel Protein Analysis

Enamel was dissected from thin sections, weighed, and hydrolyzed in concentrated HCl for 24 hrs under a nitrogen atmosphere. Hydrolyzed samples were analyzed by amino acid analysis and traditional ninhydrin derivatization. Amino acid profiles for each sample and matched normal enamel were compared and presented as residues *per* thousand and graphically illustrated as rose diagrams (Wright *et al.*, 1997). Protein amount was calculated as residue weight of analyzed material, and the total protein was expressed as percent yield (wt/vol).

Cephalometric Analysis

Cranial morphology was examined by cephalometric analyses. Lateral cephalographs were scanned as previously described (Cartwright *et al.*, 1999). Cephalographs were analyzed with Dolphin, version 9.0 (Dolphin Imaging Systems, Chatsworth, CA, USA). We used 67 cephalometric variables to characterize the craniofacial phenotype and to make comparisons between affected and unaffected individuals and published normative data. Among the measurements calculated, 38 were linear, 25 angular, and 4 proportional. Multivariate Analysis of Variance (MANOVA) was used to distinguish the variations within the Class III phenotype between families. Affected family members within each family were analyzed by the MANOVA based on their z-scores to identify statistically significant differences between the means of the variables between and among families.

RESULTS

In total, 147 (74 affected, 73 unaffected) individuals from seven Caucasian families were evaluated. The phenotype segregated as an autosomal-dominant condition and positive linkage was identified to the chromosome 8q23 locus in several of the families (data not shown). The phenotype was similar in five families, with all affected individuals having generalized yellow-brown teeth in both the primary and/or permanent dentitions (Fig. 1A). Two families had a unique ADHCAI phenotype, with the affected enamel being localized to the cervical 1/3 of the teeth in most affected individuals (Figs. 1B, 1C). The severity varied among individuals in families with localized ADHCAI. Some had localized enamel defects of a few teeth, while others had more extensive and generalized defects.

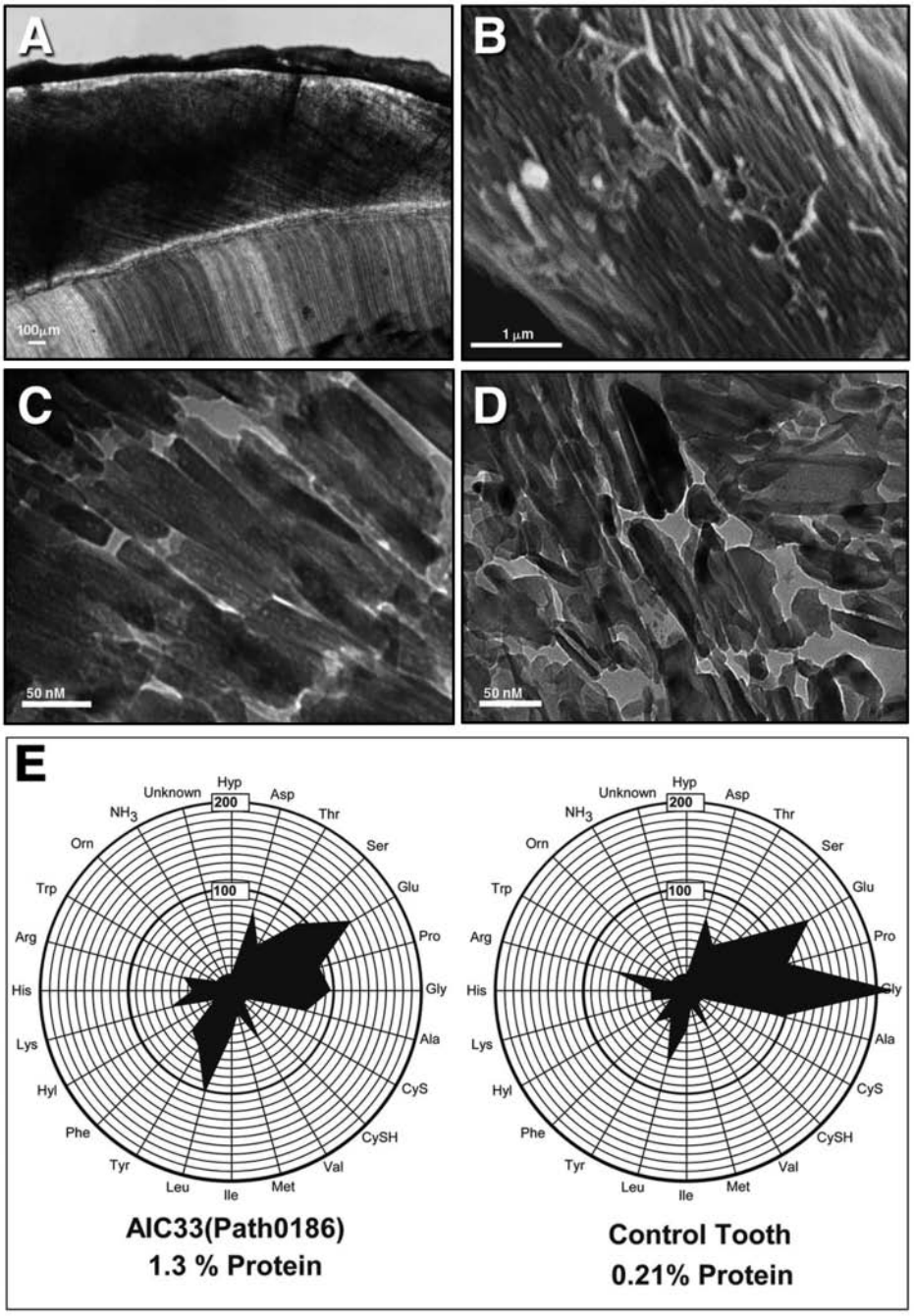

Figure 2. The ADHCAI enamel was of normal thickness, but typically was opaque when viewed in mineralized thin sections **(A)** by light microscopy. There was a prismatic architecture **(B)** that often had areas of non-crystallite material interspersed, as seen on this prism (SEM). The ADHCAI crystallites **(C)** appeared relatively normal in shape, size, and orientation, but appeared to have increased porosity and were more beam-labile compared with the normal highly dense normal crystallites **(D)** when viewed with TEM. The ADHCAI enamel **(E)** protein content was increased and had an amino acid composition that differed from that of the control enamel, as viewed in these Rose diagrams. All AI samples are from the proband of family AIC33.

Molecular Studies

Sequencing of the *FAM83H* gene revealed 6 novel mutations in these seven families, including 4 nonsense mutations and 2 2-bp-deletion mutations (Table 1, Appendix Fig. 1). Only one mutation, c.1408C>T (p.Q470X), was found in more than one family. Based upon the presence of a novel SNP, c.1407C>G, immediately adjacent to the mutation, it appears that the c.1408C>T mutations in AIC5 and AIC33 are identical by descent. One mutation, c.1379G>A, was predicted to cause a nonsense mutation at codon 460 (p.Q460X). A nonsense mutation at this same codon was reported (Lee *et al*., 2008) to be due to a transition mutation at the adjacent nucleotide, c.1380G>A. Two mutations, c.2080G>T and c.1872_1873delCC, were associated with a localized phenotype. Interestingly, these 2 mutations would produce the longest proteins of any known *FAM83H* mutations. If the c.1872_1873delCC mutant gene produced a protein, it would contain 78 novel amino acids with no homology to any known protein. The other frameshift mutation, c.923_924delTC, would produce a chimeric protein containing 18 novel amino acids, again without homology to any known proteins.

Histological Analysis

While the dentin generally appeared histologically normal, some affected teeth demonstrated areas of interglobular dentin. The enamel thickness appeared normal in areas where it had not fractured from the underlying dentin. Enamel loss was visible on many of the erupted teeth. The enamel had a brown discoloration throughout the full thickness of enamel (Fig. 2A). The enamel showed a prismatic architecture when evaluated by both LM and SEM, with the AI enamel prisms being similar in size and morphology to normal prisms (Figs. 2A, 2B). Fractured SEM samples often showed areas of amorphous non-crystalline material, presumed to be protein (Fig. 2C). The AI enamel crystallites had a morphology similar to that of normal enamel crystallites, but were more easily damaged by the TEM beam, suggesting a decreased or more labile mineral content compared with normal enamel crystallites (Fig. 2D).

Enamel Protein Composition

There was an increased amount of protein in the AI enamel samples, ranging from 1.3 to 5.5% protein (vol/wt), with most samples having closer to 5% protein (teeth from four kindreds evaluated). The amino acid composition was similar for all hypocalcified AI samples, with enriched levels of serine,

leucine, tyrosine, phenylalanine, and lysine, and diminished levels of glycine, alanine, and arginine (Fig. 2E).

Cephalometric Analysis

We examined the relationship between ADHCAI and craniofacial relationship in three families and 108 individuals (49 evaluated with cephalograms). The Class III trait appeared to segregate in an autosomal-dominant fashion, although not exclusively associated with the AI trait. For better understanding of the relationship between Class III malocclusion and HCAI, we computed a relative risk for Class III in two AI families (N = 23 individuals with available cephalometric radiographs in families AIC19 and AIC33) by estimating the number of expected individuals with the Class III phenotype if the presence of Class III was strictly related to population rates alone (using the general population incidence of 1% for Class III). We then compared the expected number with the observed number to estimate the relative risk for skeletal Class III. We found that the relative risk of developing the Class III phenotype within two AI families, AIC19 and AIC33, respectively, to be [29.03, Confidence Interval (CI) 10.1; 48.0 and 12.99, CI 4.9; 21.0], indicating that these ADHCAI families had a markedly increased risk of Class III malocclusion compared with the general population (1-5%) (Proffit *et al.*, 1998). Unaffected family members did not exhibit the markedly increased propensity of Class III malocclusion compared with affecteds.

Further characterization revealed an overall decrease in ANB angle (see Table 2) and overjet. We found that family #19 had a decreased maxillary unit length (Z score = -0.27), while mandibular unit length was significantly increased (Z score = 3.3). Indicators of vertical relationships were normal SN to GoGn (Z score = 0.02), but LFH was slightly increased (Appendix Table). Family #33 was similar to family #19, with an even more significant decrease in maxillary unit length (Z score = -1.66), and increased mandibular unit length (Z score =1.88) (Appendix Fig. 2). Vertical proportions were slightly increased in family #33 (SN to GoGn - 0.28).

Table 2. Cephalometric Summary of Craniofacial Changes within AI Families 19 and 33

Variable	AI
Chin angle	decreased
Mx* unit length	slightly decreased
Mn unit length	moderately increased
B to N perp.	normal
LFH	slightly decreased
PFH	moderately increased
Upper lip to E plane	very decreased
Lower lip to E plane	very decreased
Midface length	slightly decreased
Anterior cranial base	increased

* Mx, maxillary; Mn, mandibular; B, basion; N, nasion; LFH, lower facial height; PFH, posterior facial height; E plane, Ricketts' E plane.

DISCUSSION

Evaluation of seven kindreds with ADHCAI revealed 6 different *FAM83H* mutations, including 4 novel nonsense mutations and 2 deletion mutations, resulting in a frameshift and premature truncation, bringing the total number of mutations reported in *FAM83H* to 14. The current findings support and extend previous findings (Kim *et al.,* 2008) indicating that normal ameloblast function and enamel mineralization are critically dependent on the presence of sufficient amounts of the FAM83H protein. It has been suggested (Witkop and Sauk, 1977) that ADHCAI was due to a defect in the initial mineralization of the enamel crystallites, resulting in sustained defective mineralization. If *FAM83H* indeed functions as a transcription factor, then it must regulate the expression of genes critical for mineralization and/or protein processing during amelogenesis. Interestingly, the enamel proteins present in fully developed ADHCAI enamel have a different amino acid profile compared with those present in hypomaturation AI types, where a proline-rich amelogenin-like profile is observed (Wright *et al.*, 2006). This suggests that *FAM83H* likely has a role in amelogenesis other than, or at least in addition to, regulating the enamel proteinases.

Prior to this report, all 10 previously described *FAM83H* mutations were nonsense mutations (Kim *et al.*, 2008; Lee *et al.*, 2008). The occurrence of only truncating mutations suggests either haploinsufficiency or a dominant-negative effect as the underlying molecular mechanism in ADHCAI. Although the *FAM83H* mutations all occur in the terminal exon, some terminal exon mutations can trigger nonsense-mediated decay (Tan *et al.*, 2008). Even if the mRNA transcript is not degraded prior to translation, it is possible that the improperly folded protein would be targeted for degradation. If haploinsufficiency is the underlying mechanism, then persons with terminal or interstitial 8q24.3 deletions would be expected to manifest hypocalcified AI. A search of the literature and the A Band Apart database (http://tomcat.esat.kuleuven.be/abandapart/) failed to identify persons with terminal or interstitial deletions of 8q24.3. Given that mutations in terminal exons may escape nonsense-mediated decay, the more likely explanation for the molecular etiology appears to be a dominant-negative effect. The unique localized AI phenotype resulting from 2 novel mutations, p.E694X and p.L625fsX703, suggests that the putative truncated proteins function differently than do shorter proteins, allowing the coronal enamel to form more normally. The mutations p.L625fsX703 and p.E694X would putatively produce proteins slightly less than half the length of wild-type FAM83H. These truncated proteins, of approximately 700 amino acids, appear to attenuate the more severe phenotype that results from the mutations that truncate the protein at 677 or fewer amino acids. If indeed the protein products are translated and not degraded, there would appear to be a functional difference in FAM83H proteins that are 677 *vs*. 694 amino acids or longer. Identification of additional mutations and phenotype characterization will help resolve our understanding of FAM83H function and variable AI phenotypes.

Several studies recognize the association of Class III malocclusions and/or open bites with AI, regardless of the molecular basis (Cartwright *et al.*, 1999; Ravassipour *et al.*, 2005). As has been reported in other families with *FAM83H* mutations, there is a predilection for dental open bites (Kim *et al.*, 2008) and, as

shown in the present study, for Class III malocclusion. Although the FAM83H protein appears to be expressed in a wide variety of tissues (Kim *et al.*, 2008), it remains to be determined whether abnormal expression of the gene or secondary factors associated with the primary dental changes—such as attrition, dental sensitivity, or bite force—contribute to the craniofacial changes observed in this population. Class III malocclusion is known to have a strong genetic predisposition and to be more prevalent in certain families (*e.g.*, Hapsburg) (El-Gheriani *et al.*, 2003; Cruz *et al.*, 2008). The present study shows individuals having the AI trait to have a greater frequency of Class III malocclusion compared with the non-AI-trait family members.

This investigation broadens our understanding of the AI conditions by identifying multiple allelic *FAM83H* mutations that cause ADHCAI, a relatively common form of AI in North America. Two of these mutations are associated with a unique localized cervical ADHCAI not previously described, suggesting that there are unique phenotypes associated with some *FAM83H* mutations. The roles of the *FAM83H* gene and its protein in enamel formation remain unclear; however, this study shows that amelogenesis occurring with inadequate FAM83H functional protein results in a severe diminution of mineralization, coupled with marked retention of protein that lacks an amelogenin-like composition. The enamel crystallites form in a relatively normal habit and are directionally ordered, producing a prismatic architecture that is poorly mineralized. When a truncated FAM83H protein of approximately 700 amino acids is potentially produced, there is an attenuated phenotype, where primarily the cervical enamel is affected and the coronal enamel forms normally. This suggests that the truncated protein has some activity and is able to sustain normal amelogenesis in the coronal areas, but not throughout the duration of crown formation. Taken together, these findings suggest a unique role for the *FAM83H* gene in, and clearly illustrate the complex nature of, enamel formation and its molecular regulation.

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