JDR

JOURNAL OF DENTAL RESEARCH®
Featuring Critical Reviews in Oral Biology & Medicine

VOLUME 84 • NUMBER 11 • NOVEMBER 2005

***MMP20* Active-site Mutation in Hypomaturation Amelogenesis Imperfecta**

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J Dent Res 84(11):1031-1035, 2005

ABSTRACT

The Amelogenesis Imperfecta (AI) are a group of clinically and genetically heterogeneous disorders that affect enamel formation. To date, mutations in 4 genes have been reported in various types of AI. Mutations in the genes encoding the 2 enamel proteases, matrix metalloproteinase 20 (*MMP20*) and kallikrein 4 (*KLK4*), have each been reported in a single family segregating autosomal-recessive hypomaturation AI. To determine the frequency of mutations in these genes, we analyzed 15 Turkish probands with autosomal-recessive hypomaturation AI for *MMP20* and *KLK4* gene mutations. No *KLK4* mutations were found. A novel *MMP20* mutation (g.16250T>A) was found in one family. This missense mutation changed the conserved active-site His226 residue of the zinc catalytic domain to Gln (p.H226Q). Zymogram analysis demonstrated that this missense mutation abolished *MMP20* proteolytic activity. No *MMP20* mutations were found in the remaining 14 probands, underscoring the genetic heterogeneity of hypomaturation AI.

KEY WORDS: amelogenesis imperfecta, *MMP20*, proteolytic activity, hypomaturation, enamelysin.

MMP20 Active-site Mutation in Hypomaturation Amelogenesis Imperfecta

INTRODUCTION

The Amelogenesis Imperfecta (AI) are a group of clinically and genetically heterogeneous disorders that affect enamel development, resulting in abnormalities of amount, composition, and/or structure of enamel. Although enamel appearance (hypoplastic, hypomature, or hypocalcified) and mode of inheritance (autosomal-dominant, autosomal-recessive, or X-linked) have historically been used to classify AI into 14 subtypes (Witkop, 1988), current classifications of AI are problematic (Hart *et al.*, 2002a; Aldred *et al.*, 2003; Nusier *et al.*, 2004). Development of a more robust nosology for AI will require a greater understanding of how enamel development is altered. This will necessitate identification of the genetic etiology of various forms of AI, and an understanding, at the molecular level, of how mutations of these genes affect enamel development.

To date, mutations in 4 genes—*AMELX*, *ENAM*, *KLK4*, and *MMP20*—have been reported to cause AI in humans. Most cases of X-linked AI result from mutations in the *AMELX* gene that codes for amelogenin, the most abundant protein in enamel (Hart *et al.*, 2002a,b; Wright *et al.*, 2003; Kim *et al.*, 2004). Depending on the specific mutation, the phenotype associated with *AMELX* mutations can range from smooth hypoplastic to hypomineralized/hypomaturation (Hart *et al.*, 2002b; Wright *et al.*, 2003; Kim *et al.*, 2004). *ENAM* mutations are etiologic for some autosomal forms of hypoplastic AI, including both dominantly and recessively inherited forms (Rajpar *et al.*, 2001; Kida *et al.*, 2002; Mardh *et al.*, 2002; Hart PS *et al.*, 2003; Hart TC *et al.*, 2003; Kim *et al.*, 2005b). Only one mutation each has been reported in genes encoding the enamel proteinases, matrix metalloproteinase 20 (*MMP20*) and kallikrein 4 (*KLK4*). These both result in a hypomaturation AI phenotype that is transmitted as an autosomal-recessive trait (Hart *et al.*, 2004; Kim *et al.*, 2005a).

In this study, members of 15 Turkish families identified through ascertainment of probands with hypomaturation AI segregating as an autosomal-recessive trait were evaluated for mutations in the *MMP20* and *KLK4* genes.

MATERIALS & METHODS

Pedigrees and Diagnosis

Individuals affected with hypomaturation AI were identified in the dental clinics at the School of Dentistry, Istanbul University. Informed consent was obtained according to the Declaration of Helsinki. Available family members received an oral examination, including dental radiographs, to determine if they had AI. Fifteen families were identified with hypomaturation AI, characterized by opaque, whitish, yellowish to brownish enamel with radiographically evident decreased contrast between dentin and enamel. The thickness of the enamel was not affected, but because the enamel was often softer than normal, it could be abraded on the incisal/occlusal surfaces. Eleven of the 15 families reported a

Figure 1. Pedigree, mutation, and biochemical analyses of family segregating autosomal-recessive hypomaturation AI. **(A)** Pedigree of family. Individuals from whom a blood sample was obtained are shown by an asterisk. Individuals clinically affected with hypomaturation AI are indicated by fully blackened symbols. These individuals are homozygous for the *MMP20* g.16250T>A mutation. Carriers of the *MMP20* g.16250T>A mutation are shown by partially blackened symbols. The enamel phenotype of all carriers is normal. **(B)** Mutational analysis of *MMP20*. The left panel shows the wild-type sequence for part of *MMP20* exon 5 (from II-3). The middle panel shows the sequence from the same region of exon 5 from a carrier (III-1), showing the heterozygous missense mutation (g.16250T>A), which results in a p.His226Gln substitution. The right panel shows the sequence from the proband (IV-1), showing the homozygous change. **(C)** Enzymography of recombinant human wild-type and mutant *MMP20*. Recombinant human wild-type and mutant *MMP20* cDNA were transiently transfected into the human kidney 293 cell line. The culture-conditioned media were collected, concentrated, and run on 4-16% SDS-polyacrylamide gradient gels containing casein. Following electrophoresis and washing, the gel was incubated at 37°C for 2 days in 50 mM Tris buffer (pH 7.4) containing 5 mM CaCl₂ and 1 mM MgCl₂. Lanes marked by WT indicate recombinant human wild-type *MMP20*, lanes marked by MT indicate recombinant human mutant *MMP20*, and EV indicates expression vector only.

history of consanguinity. Complete medical histories were reviewed to rule out syndromic disorders.

Mutation Analysis

Genomic DNA was isolated from whole blood with the QIAamp DNA Blood Maxi Kit (Qiagen Inc., Valencia, CA, USA). The oligonucleotide primers and conditions used to amplify and sequence all exons, including introns/exon splice sites for *KLK4* and *MMP20* amplification, were described previously (Hart *et al.*, 2004; Kim *et al.*, 2005a). Amplification products were sequenced on an ABI 3100 automated DNA sequencer. Sequence data were compared with reference sequences (*MMP20*: NT_033899) (*KLK4*: AF228497) using the Sequencer program (Gene Codes Corporation, Ann Arbor, MI, USA). One hundred healthy Turkish control individuals were also sequenced for *MMP20*.

Protein Expression

The full-length human *MMP20* cDNA containing open reading frame (ORF) was generated by PCR, with *MMP20* specific primers: 5'-CTA CTG TGA GGG GAT GAA GG-3' (SS) and 5'-TTT CTA TTT AGC AAC CAA TCC-3' (AS), AccuPrime™ *Pfx* polymerase (Invitrogen, Carlsbad, CA, USA), and human tooth cDNA library as a template. The PCR product was cloned into the TA vector (Invitrogen), sequence-verified, and subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen). The mutant *MMP20* cDNA (g.16250T>A) was generated with primers (5'-GTT TAC CGT TGC TGC TCA AGA ATT TGG CCA TGC C-3' [SS] and 5'-GGC TAG GCC AAA TTC TTG AGC AGC AAC GGT AAA C-3' [AS], where bold represents the T>A mutated sequence) and the QuikChange Site-Directed Mutagenesis kit (Stratagene Inc., La Jolla, CA, USA), according to the manufacturer's instructions. Clones were sequence-verified. The wild-type and mutant *MMP20* cDNAs in the pcDNA3.1 mammalian expression vector were transfected into HEK293 cells by means of the lipofectamine plus transfection kit (Invitrogen). HEK293 cells transiently transfected with wild-type and mutant human *MMP20* expression constructs were cultured in Dulbecco's Modified Eagle Media (DMEM), containing antibiotics without serum, for 48 hrs, to express human *MMP20* proteins. The conditioned media were harvested and concentrated by ultrafiltration through an Amicon YM10 membrane (Millipore Inc., Bedford, MA, USA) for the Western blot and zymogram analyses. The expression vector pcDNA3.1 alone was transfected into HEK293 cells to serve as a negative control.

Western Blotting

The concentrated media were mixed with 4x NuPAGE SDS sample loading buffer (Invitrogen) containing β-mercaptoethanol, boiled for 5 min, and run in 12% NuPAGE Bis-Tris gel with MOPS-SDS running buffer (Invitrogen). Following electrophoresis, the gel was trans-blotted onto a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by Western blotting with an *MMP20*-specific polyclonal antibody (provided by Dr. James Simmer), at 1:1000 dilution, and HRP-conjugated anti-chicken IgG antibody (Sigma-Aldrich Co., St. Louis, MO, USA) at 1:3000 dilution.

Zymography

Zymograms were performed on 4-16% SDS-polyacrylamide gradient gels containing casein. The concentrated media were mixed with 2 vol of zymogram sample loading buffer (Bio-Rad Laboratories) and incubated at 37°C for 10 min. Protein samples were run on a zymogram gel at a constant current of 20 mA. Following electrophoresis, the gel was washed (x3) for 15 min in 50 mL of 2.5% Triton X-100 solution (2.5% Triton X-100 in 50 mM Tris-HCl buffer [pH 7.4]). Gels were incubated at 37°C for 2 days in 50 mM Tris buffer (pH 7.4) containing 5 mM CaCl₂ and 1 mM MgCl₂.

RESULTS

Clinical Findings

Probands from 15 families were identified with hypomaturation AI. Examination of the probands' first-degree relatives (n = 65) identified hypomaturation AI in five siblings and one parent. The pedigree of one family with a mutation is shown in Fig. 1. The proband from this family had hypomaturation AI and an

Figure 2. Clinical photographs and radiograph of proband (IV-1 in Fig. 1). (A) Frontal view. Proband is in mixed dentition, showing anterior open bite and hypomaturation phenotype. (B) Maxillary occlusal view. Caries is evident on the central incisors and posterior teeth. (C) Mandibular occlusal view. Note extensive attrition of posterior teeth, and caries is evident on the molars. (D) Panoramic radiograph showing mixed dentition and reduced mineralization of the enamel.

anterior open bite (Fig. 2). Caries lesions were evident on the maxillary permanent central incisors as well as in primary and permanent molars. His affected sibling also had hypomaturation AI, an anterior open bite, and caries on primary and permanent posterior teeth (Fig. 3).

Mutation Results

No *KLK4* gene mutations were found in any probands. One family (Fig. 1) was found to have 4 *MMP20* sequence alterations: g.18661T>C, g.18679C>A, g.31605G>A, and g.16250T>A. The first 3 genetic variants were found in the SNP database, indicating that they are polymorphisms present in at least 1% in the population, and therefore are unlikely to have any pathological effects (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp>): rs1784423, rs1784424, and rs1784440. The last alteration, an exon 5 single-base substitution (g.16250T>A), changes the DNA codon for the amino acid histidine (CAT) to glutamine (CAA) (p.H226Q; Fig. 1B). His226 is an important catalytic site component of MMP20. The proband and his affected sister were both homozygous for this mutation, consistent with consanguinity. The parents, paternal grandfather, and maternal grandfather were all carriers of the mutation. Sequence analysis of 100 healthy Turkish controls (200 chromosomes) did not reveal this sequence alteration, indicating that this g.16250T>A mutation is not a common variant in the Turkish population.

Functional Studies

Recombinant human wild-type and mutant *MMP20* cDNAs were transiently transfected into HEK293 cells, and the culture-conditioned media were collected and subjected to electrophoresis. Western blot analysis with an MMP20-specific antibody revealed that mutant and wild-type *MMP20* cDNA produced a protein of approximately 45 kDa. This band was not

Figure 3. Clinical photographs and radiograph of the affected sibling (IV-2 in Fig. 1). (A) Frontal view. Proband is in mixed dentition, showing anterior open bite and hypomaturation phenotype. (B) Maxillary occlusal view. Caries is evident on posterior teeth. (C) Mandibular occlusal view. Caries is evident on posterior teeth. (D) Panoramic radiograph showing mixed dentition and reduced mineralization of the enamel.

detected in conditioned media from the negative control transfectant (data not shown). Casein zymograms performed to determine the protease activity of wild-type and mutant MMP20 revealed that the wild-type MMP20 protein cleaved the casein substrate (Fig. 1C), whereas the mutant protein was unable to cleave the substrate, consistent with a loss of proteolytic function.

DISCUSSION

These findings represent the second *MMP20* mutation, confirming that mutation of this gene is etiologic for some cases of autosomal-recessive hypomaturation AI (Table). Individuals homozygous for the exon 5 g.16250T>A mutation have hypomaturation AI and anterior open bite. Enamel anomalies were observed in both the primary and secondary dentitions. Heterozygous carriers of the mutation do not have an enamel phenotype.

MMP20, also called enamelysin (OMIM604629), is a member of the matrix metalloproteinase family, a group of zinc-dependent metalloproteinases involved in extracellular matrix remodeling (Nagase and Woessner, 1999; Simmer and Hu, 2002). MMP20 is unique among the MMP family because of its highly restricted expression. In developing teeth, *MMP20* is expressed primarily during the secretory to late-transition stages of amelogenesis (Bartlett *et al.*, 1998). With its specificity for amelogenin, the major organic component of the enamel matrix, MMP20 is considered a predominant enzyme for the processing of enamel matrix. Human MMP20 consists of 483 amino acids and shares extensive (89%) amino acid sequence identity with porcine MMP20 (Llano *et al.*, 1997; Bartlett *et al.*, 1998). MMP20 has the characteristic domain structure of most other MMPs, including a signal peptide, a propeptide that maintains enzyme latency, a catalytic domain

Table. Mutations Resulting in Autosomal-recessive Hypomaturation AI

Gene	Mutation Location	Genomic DNA ^a	cDNA ^b	Protein ^c	Reference
<i>KLK4</i>	exon 4	g.2142G>A	c.458G>A	p.W153X	Hart <i>et al.</i> , 2004
<i>MMP20</i>	intron 6	g.30561A>T	c.954-2A>T; IVS6-2A>T	p.O or p.I319X or p.I319fs338X	Kim <i>et al.</i> , 2005a
<i>MMP20</i>	exon 5	g.16250T>A	c.678T>A	p.H226Q	This report

^a Reference sequences **NT_033899.6** (*MMP20*) and **AF228497** (*KLK4*).

^b Reference sequences **NM_004771** and **NM_004917** (*KLK4*).

^c Reference sequences **O60882**.

with a zinc-binding site, and a hinge region that links the catalytic domain to the C-terminal hemoplexin-like domain (Llano *et al.*, 1997; Bartlett and Simmer, 1999). Computer modeling of pig enamelysin with the use of several crystal structures determined that 3 highly conserved amino acids—His226, His230, and His236—are the active sites of the zinc catalytic domain (Ryu *et al.*, 2000). The g.16250T>A substitution mutation identified in this report changes the conserved active site His226 residue of the zinc catalytic domain (p.H226Q). The glutamine substitution destroys this metal-binding site and abolishes catalytic activity. Results of the biochemical assays in which the g.16250T>A mutant *MMP20* demonstrates a complete lack of *MMP20* activity corroborate these computational predictions. A similar clinical phenotype (hypomaturation AI) is also seen with homozygous mutations in *KLK4* lacking the Ser207 active site of the catalytic triad (Hart *et al.*, 2004).

MMP20 processes amelogenin to produce biologically active fragments that function during the initial developmental stages of enamel (Ryu *et al.*, 1999). The complete loss of *MMP20* proteolytic activity may prevent proper processing and degradation of enamel matrix proteins, resulting in qualitative defects in enamel malformation. The mouse *mmp20* null model indicates that *MMP20* performs enamel matrix protein cleavages that are essential for processing and removal of enamel matrix proteins during the secretory and maturation stages of enamel development (Caterina *et al.*, 2002; Bartlett *et al.*, 2004). *mmp20* null mice do not process enamel matrix protein properly, yielding a severe AI phenotype, although hemizygous mice have no phenotype. Interestingly, the mature enamel in the *mmp20* null mouse contained less bulk mineral (53%) and less weight percent of mineral (range, 7-16%) compared with controls, and was significantly (~ 37%) softer than normal enamel. AI-affected teeth of *mmp20* null mice show attrition of the molar occlusal surfaces that is related to the time of eruption. A similar trend was observed in the affected siblings in the current report who showed such attrition on the primary molars (Figs. 2, 3). The murine knock-out model suggests that there is no compensation for *mmp20* function, indicating that it is necessary for proper enamel development. Clinical findings in individuals homozygous for the *MMP20* g.16250T>A mutation indicate an analogous situation in human enamel development. The finding that heterozygous carriers (*MMP20* g.16250T>A/wild-type) display no clinically apparent anomalies of enamel are similar to those observed in hemizygous mice (null/wild-type), suggesting that

one functional *MMP20* allele is sufficient for normal enamel development.

Similar to the g.16250T>A mutation, homozygosity of the previously identified *MMP20* g.30561A>T mutation responsible for recessive pigmented hypomaturation AI is also associated with anterior open bite (Kim *et al.*, 2005a). Whether this is a biologic or serendipitous relationship is unknown. The intron 6 g.30561A>T mutation is predicted to cause aberrant

splicing, but due to the restricted expression of *MMP20*, the effect at the mRNA level was not determined. The mutant mRNA generated by aberrant splicing likely undergoes nonsense-mediated decay, and individuals homozygous for the mutation would not produce functional *MMP20*, representing the equivalent of a null allele. Heterozygous carriers of the mutation did not show an altered enamel phenotype.

This study demonstrates that a mutation in the *MMP20* active site results in hypomaturation AI. These findings corroborate those of Kim *et al.* (2005a), that loss of function protease mutations underlie at least some forms of autosomal-recessive hypomaturation AI. However, 14 of the 15 families did not have detectable mutations in *KLK4* or *MMP20*, suggesting that other genes account for the majority of hypomaturation AI in this Turkish population. While it is possible that other types of *KLK4* and *MMP20* mutations may be involved, such as promoter or intronic mutations or deletions that encompass whole exons, results of homozygosity linkage mapping studies in these families were not consistent for linkage in the regions spanning either the *KLK4* (chromosome 16p13) or *MMP20* (chromosome 11q22.3-q23) locus (data not shown). Identification of these genes and the continued cataloging of mutations underlying different forms of AI will provide a greater understanding of the molecular basis of enamel anomalies. This understanding should facilitate the development of a classification scheme that relates clinical phenotypes and underlying molecular mechanisms that may aid dentists in determining the most appropriate treatment course.

ACKNOWLEDGMENTS

This work was supported by the intramural program of the NIDCR/NIH, Department of Health and Human Services, Bethesda, MD, USA.

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