MMP-20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta

J-W Kim, J P Simmer, T C Hart, P S Hart, M D Ramaswami, J D Bartlett, J C-C Hu

**Key points**

- We identified a homozygous mutation in the gene encoding MMP-20 (enamelysin) in two affected members of a family with autosomal recessive pigmented hypomaturation amelogenesis imperfecta (AI) (MIM 204700). This is the first reported mutation in the MMP-20 gene that causes disease.
- The identified mutation was in the intron 6 splice acceptor (AG→TG).
- The enamel of the subjects’ teeth was pigmented, and its surface was mottled and rough. Radiographically, the enamel layer was usually more opaque than the underlying dentine, but not in all areas, and was never as radio-opaque as normal enamel.
- We present a standardised nomenclature for reporting MMP20 mutations that designates this mutation as g.30 561A→T, c.954-2A→T, or IVS6-2A→T.

**MATERIALS AND METHODS**

**Proband**

The proband first presented at 6 years of age at the Pediatric Dentistry Clinic at the University of Michigan (fig 1). The dental phenotype resembled the autosomal recessive pigmented hypomaturation type of amelogenesis imperfecta. The teeth appeared normal in size, with some crowding. The enamel layer was pigmented, showing an agar brown discoloration. The surface of the enamel was mottled and rough, but hard and brittle. Chunks of enamel had fractured away from several teeth, most notably on the lingual of the maxillary right canine (no. 6), and the buccals of the mandibular left canine (no. 22), teeth, most notably on the lingual of the maxillary right canine (no. 22), and right first bicuspid (no. 28). Radiographically, the enamel layer was usually more opaque than the underlying dentine, but not in all areas, and was never as radio-opaque as normal enamel. The proband had an anterior open bite. His teeth were generally not sensitive, except in response to extremes in temperature. The restoration of chipped teeth and correction of an anterior open bite. Family members received a complete dental and oral examination, including panographic radiographs. Oral histories were obtained from the grandmother and uncle by telephone interview.

**Polymerase chain reaction**

From participating family members, 10 ml of peripheral whole blood was obtained. Genomic DNA was isolated using...
the QIAamp DNA blood maxi kit (Qiagen Inc., Valencia, CA). Oligonucleotide primers for polymerase chain reaction are shown in table 1. The reactions had a 5 minute denaturation at 94˚C, followed by 40–50 cycles of denaturation at 94 ˚C for 30 seconds, primer annealing at the relevant temperature (table 1) for 30 seconds, and product extension at 72 ˚C for 30 seconds, with a final extension at 72 ˚C for 7 minutes. PCR amplification products were purified by the QIAquick PCR Purification kit and protocol (Qiagen Inc.).

MMP20 alleles were amplified using 200 random DNA samples taken from the general population and 50 samples from white subjects in the human variation panel of the Coriell Cell Repository (Camden, NJ, USA).

Haplotype analysis
Primers were designed to amplify five intronic regions of the MMP20 gene known to contain 17 single nucleotide polymorphisms (SNPs) (table 2). PCR was performed in a total volume of 25 µl containing 1 µmol/l each forward and reverse primers, 1 x PCR buffer, 1.5 mmol/l MgCl₂, 200 µmol/l dNTPs, 200 ng DNA, and 1 U of Taq DNA polymerase. Amplification conditions were an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 2 minutes, with a final extension at 68°C for 10 minutes. Following amplification, PCR products were gel purified and sequenced using the ABI Big Dye terminator kit and an ABI 3100 automated DNA sequencer. All products were sequenced from both directions to minimise sequencing artefacts. Haplotypes were generated based upon allele transmission.

RESULTS
Mutational analyses were performed for the coding exons and intron–exon boundaries for five candidate genes (enamelin, ameloblastin, tuftelin, enamelysin, and kallikrein 4) believed to be critical for enamel development. The only gene found to differ from the wild type reference sequences was MMP20; the proband and his father were both homozygous for the same mutation (fig 2B). Clinical and pedigree analysis suggested a dominant pattern of inheritance of AI in this family; however, mutational analyses of the MMP20 gene was consistent with pseudodominance. Heterozygous carriers of the MMP20 mutation (I-2 and II-8) did not show clinical findings consistent with AI, while individuals homozygous for the MMP20 mutation (II-7 and III-11) demonstrated AI clinically and by history (fig 3). Similar findings have been reported for autosomal recessive AI caused by mutation of the ENAM gene.22 Current nomenclature recommendations...
for reporting sequence variations suggest using genomic and cDNA reference sequence.\textsuperscript{23–25} We used the MMP20 cDNA sequence for human MMP20 (NM_004771.2) and the human MMP20 gene sequence from human genome project chromosome 11 contig (NT_033899.6) as reference sequences. At the DNA level, the sequence variation that was present on both MMP20 alleles in the two affected members of our AI kindred is designated g.30 561A\textrightarrow{}T, c.954-2A\textrightarrow{}T or IVS6-2A\textrightarrow{}T. Sequence analysis did not reveal the g.30 561A\textrightarrow{}T mutation in any of the 200 random controls (400 alleles) nor the 100 alleles for the white human variation panel.

As this family was not known to be consanguineous, the finding of homozygosity in two individuals raises the possibility of a mutational hotspot or of inheritance of a common allele identical by descent. To answer this question, we haplotyped individuals for 18 SNPs by direct sequencing (Fig 3). The haplotype analysis demonstrates that II-5 and II-7 have inherited different parental haplotypes and are homozygous for all SNPs. Based upon previous haplotyping of white individuals, four predominant haplotypes were identified (data not shown). Three of these haplotypes (A, C, and D) were found to segregate within this nuclear family.

I-2 has haplotype AD. Her unaffected son, II-5, is homozygous for the A haplotype, while her affected son, II-7, and grandson, III-11, are homozygous for the D haplotype. As expected, the mother of the proband, II-8, was found to be a carrier of the mutation. In addition, her haplotype (D) associated with the mutation is identical to that of her affected husband. Her other allele is haplotype C. Thus, the mutation has arisen on the D haplotype. These results would argue against a mutational hotspot and for inheritance of the mutant alleles identical by descent.

**DISCUSSION**

This is the first report of a mutation in the matrix metalloproteinase-20 (MMP20) gene. The gene is only known to be expressed in developing tooth enamel, and is also known as enamelysin (OMIM \#604629). The mutation destroys the splice acceptor sequence at the 3' end of intron 6 (AG\textrightarrow{}TG). Intron 6 is a large intron (11 777 bp) that separates exons encoding the hemopexin domain (Fig 2). The hemopexin domain is not critical for catalytic activity, but is highly conserved among the derived MMP-20 amino acid sequences from pig,\textsuperscript{26} mouse,\textsuperscript{26} cattle,\textsuperscript{27} and human.\textsuperscript{28} The

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Pedigree and mutational analyses of the AI kindred. (A) Pedigree of the kindred with the (g.30 561A\textrightarrow{}T; c.954-2A\textrightarrow{}T or IVS6-2A\textrightarrow{}T) mutation. (B) DNA sequencing chromatograms are shown for wild-type (+/+, heterozygous (+/−) and homozygous (−/−) conditions. A→T transversion mutation site is indicated (arrows). (C) The structure of the human enamelysin gene marking the position of the mutation associated with AI is shown beneath the chromatograms. The exons are indicated by the 10 vertical lines; the introns are horizontal lines. Above each exon and below each intron are numbers indicating its length in base pairs. Below the gene structure, the 10 exons are aligned with a linear diagram of the MMP-20 structural domains. Above the exons are numbers indicating the range of amino acids encoded by each exon. Numbers beneath the protein structure indicate the range of amino acids in each structural domain. At the bottom of the figure is an alignment of the human MMP20 sequence with the two defective sequences that are most likely to result from the observed splice junction mutation. The wild type (wt) sequence is provided, beginning with the first amino acid (Cys296) of the hemopexin domain encoded in exon 6. A dash marks the end of the last amino acid (Arg318) encoded by exon 6. Under the wild type sequence is the defective amino acid sequence (p.I319fs338X) that would be expected by joining exon 6 to exon 8 (Ex6-8). At the bottom is the defective amino acid sequence (p.I319X) that would be expected if intron 6 is retained (+Int6).}
\end{figure}
function of the enamelysin hemopexin domain is not known, but studies of other matrix metalloproteinases indicate that the enamelysin C terminal domain (CTD) might be essential for defining substrate specificity, binding to tissue inhibitors of metalloproteinases (TIMPs), and could be required for activation of proMMP-20 at the cell surface. Additionally, hemopexin domains may be functionally important in localising the protein substrate or binding to facilitate cleavage.

Because enamelysin gene expression is restricted to developing teeth, it was not possible to determine experimentally the effect of this mutation on MMP-20 mRNA transcripts, but it is clear that the IVS6-2A→T mutation would preclude the specific removal of intron 6 by the splicing machinery. Two defective splicing outcomes seem to be most probable, both of which would introduce an upstream translation termination codon in the mRNA transcript, causing it to be degraded by the nonsense mediated decay (NMD) system.11–13 In the first splicing outcome, the retention of intron 6 in the mRNA would generate a large mRNA (over 13 000 nucleotides in length) with translation terminating in the first complete codon of the retained intron (p.1319X). In the second splicing scenario, exon 7 would not be recognised as an exon by the splicing machinery and the intron 6–exon 7–intron 7 segment would be deleted as a single intron, as has been shown to occur in other splice acceptor site mutations.14 Skipping exon 7 would shift the reading frame after R318 and introduce 19 extraneous amino acids before terminating translation. Additionally, the enamelysin C terminal domain (CTD) might be essential for processing and turnover of enamel matrix proteins during the secretory stage of amelogenesis and potentially a failure to activate KLK4 zymogen early in the maturation stage.

The principal functional deficits predicted from the g.30 561A→T mutation on the MMP-20 gene include (a) the reduction of MMP-20 activity caused by degradation of its mRNA, and (b) through the loss of a functional hemopexin domain. The major consequence of diminished MMP-20 activity would be a failure to process and turn over enamel proteins during the secretory stage of amelogenesis and potentially a failure to activate KLK4 zymogen early in the maturation stage.

Proteolytic processing is believed to be essential for proper enamel crystal elongation, which determines the thickness of the enamel layer as a whole. Enamel hypoplasia (abnormally thin enamel), is a major feature of the defective enamel deposited by the enamelysin knockout mouse. In contrast to the enamelysin knockout, the teeth of our proband were heavily pigmented and not exceptionally thin (hypoplastic), suggesting a problem with the removal of enamel proteins, which normally occurs during the early maturation stage of enamel development.15 We speculate that in humans, the processing and turnover of enamel matrix proteins during the secretory stage is necessary for the ultimate clearance of the organic matrix during enamel maturation.

Autosomal recessive pigmented hypomaturation A1 has recently been shown to have mutations in KLK4. A comparison of the dental phenotypes of the KLK4 and MMP20 probands shows that they share many similar features. The enamel crowns are normal in size and shape, have a rougher, duller, less reflective surface than normal enamel, appear to be more brittle in that they show a tendency to fracture or chip, but do not appear to be particularly susceptible to dental caries. The radiopacity of the defective enamel is generally less than that of normal enamel, but can still be distinguished from the underlying

Figure 3  Haplotype analysis of the AI kindred. Five intronic regions of the MMP20 gene known to contain SNPs were PCR amplified and sequenced. Haplotypes were generated based upon allele transmission. The left column shows the reference SNP (rs) number corresponding to each SNP while the right column shows the position in the MMP20 gene relative to the ATG start site, which is taken as +1. The mutation (g.30 561T) is shown in bold, which is part of the D haplotype (unshaded boxes). The A haplotype is indicated by dark grey boxes. The C haplotype is indicated by a light grey box.
dentine on radiographs. The coloration of the teeth is different, but extrinsic staining since tooth eruption could have contributed to the current appearance. The KLK4 teeth have a more homogenous dark yellow hue, while the MMP20 teeth have an irregular greyish brown discoloration and are a little more glossy. Overall, the dental phenotypes in the KLK4 and MMP20 probands are remarkably similar.

To date, mutations in four genes (AMELX, ENAM, KLK4, and MMP20) have been found to cause amelogenesis imperfecta. Of these, three (ENAM, KLK4, and MMP20) can cause ARAI. While the recessive mutation in ENAM causes hypoplastic Al, mutations in the proteases cause hypomaturation ARAI. As mutational analyses clarify the genetic bases of the various Al phenotypes, a standardised nomenclature system can be developed. In turn, this system will enable clinicians to better predict which candidate gene might be defective, giving the dental phenotype in their patients, and this knowledge will be useful when considering treatment alternatives, such as whether or not to attempt enamel bonding.

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