A nonsense mutation in the enamelin gene causes local hypoplastic autosomal dominant amelogenesis imperfecta (AIH2)

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Amelogenesis imperfecta (AI) is an inherited tooth disorder affecting tooth enamel formation only. A gene for autosomal dominant AI, the local hypoplastic form, has been localized to a 4 Mb region on chromosome 4q (AIH2). The enamelin gene (ENAM), has been mapped to chromosome 4q21, to the same region as AIH2, and was recently shown to be mutated in patients with smooth and thin hypoplastic autosomal dominant AI (ADAI). In this study, we describe an ENAM mutation causing the local hypoplastic form of ADAI, a phenotype that accounts for 27% of the autosomal inherited cases in Northern Sweden. This nonsense mutation in the enamelin gene results in a truncated peptide of 52 amino acids as compared with 1142 amino acids of the normal protein. Our results show that while a splice site mutation is associated with smooth and thin hypoplastic AI, a base substitution resulting in a shorter peptide causes local hypoplasia of the enamel, a milder form of AI. These findings support ENAM as a disease gene, and shed new light on the molecular mechanism of the disease and to the function of the enamelin protein in enamel formation.

INTRODUCTION

Disturbances in the formation of enamel can lead to different tooth disorders. Amelogenesis imperfecta (AI) is a group of inherited tooth disorders that solely affect the enamel. This group is characterized by clinical and genetic heterogeneity and patients present with enamel that is thinner or softer than in normal teeth. Mutations in the amelogenin gene on the X chromosome are associated with different clinical forms of X-linked AI (1-4). We have previously shown that one gene for autosomal dominant AI, the local hypoplastic form, maps to a 4 Mb region on chromosome 4q11-q21 (AIH2). Further, the ameloblastin gene encoding an enamel matrix protein was localized to the critical region of AIH2 and was thereby appointed as a candidate gene (5). However, evaluation of the gene did not reveal any mutation(s) in the coding regions that were associated with the disorder (6).

Enamel matrix of developing teeth is rich in protein, the main proteins being amelogenin, ameloblastin and enamelin. The major component is amelogenin (90%), while enamelin is the largest protein in the enamel matrix. Enamelin was originally characterized by Fukae and Tanabe (7), who isolated the protein from unerupted pig teeth. Enamelin is a tooth-specific protein, as has been shown by northern blot analyses (8), and in situ hybridization experiments (9) on unerupted mouse mandibular incisors showed that enamelin transcripts were detected exclusively in mouse ameloblasts, with onset of enamelin expression being observed in pre-secretory ameloblasts. No signal was detected in pulp or bone (10). Immunohisto-chemistry experiments in bovine teeth indicated that enamelin is located with amelogenin in the prism cores (11).

The enamelin gene, ENAM, has been mapped with different techniques to the same region on chromosome 4q as AIH2 and AMBN (10,12), suggesting that this region could contain a cluster of genes encoding enamel proteins. A splice site mutation in ENAM was recently found to be associated with autosomal dominant AI, where the patients presented with smooth hypoplastic enamel (13). In this study, we demonstrate that a mutation in ENAM is causing local hypoplastic AI in the Swedish material of families (Fig. 1). The mutation is located in exon 4, where a base substitution in position 438 (cDNA) results in an amino acid change of lysine to a stop codon.

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RESULTS

A screen for larger rearrangements by Southern blotting did not reveal any genomic alterations associated with the local hypoplastic AI phenotype in ENAM. To screen for minor alterations in the ENAM gene, sequencing of PCR products or cloned PCR products was performed with automated sequencing. A haplotype common to all six families (families 7, 9, 11, 18, G and W) showing linkage to chromosome 4 was identified (14), and we therefore assumed that a single founder mutation was responsible for the AI in these families, and used DNA from one unaffected and one affected from a family with local hypoplastic AI to screen for mutations. A thymine-to-cytosine polymorphism was detected in intron 4 of the ENAM gene, IVS4 + 488T > C, and the cytosine variant was found in 21 of 72 chromosomes tested. Differences between the cDNA sequence (10) and the genomic sequence in our families were discovered. At position 1131 (cDNA), we observed a cytosine

Figure 1. Photographs showing the clinical manifestation of local hypoplastic AI. (A) The clinical manifestation of local hypoplastic AI shows horizontal rows of pits, grooves or a large hypoplastic area in the enamel. (B) Similar manifestation in an elderly relative of the individual in (A). Note that the maxillary medial and lateral incisors, left canine and premolar are crowned.

Figure 2. Molecular analysis of the enamelin gene. (A) Sequencing of DNA from individual IV:1 in family W revealed the mutation nt438 (A > T), which is not present in the unaffected individual IV:3. (B) Cleavage of normal and mutant alleles with MseI confirmed that the mutation cosegregated with the disease phenotype. (C) One of the AIH2 families (family W) used in this study. All individuals marked with an asterisk have been clinically examined and were included in the study. Filled symbols indicate affected individuals, open symbols with asterisks indicate unaffected individuals and open symbols without a asterisks mean that the individuals were not examined. The mutation leading to the amino acid change K53X is segregating with the disease in the family.
instead of a thymine. This proved to be due to an error in the originally reported cDNA sequence, which did not affect the deduced amino acid sequence of the enamelin protein (J.P.Simmer, unpublished data). Of the two previously reported nucleotide polymorphisms that affect the enamelin amino acid sequence (15), we observed adenosines (A) at positions 1138 and 3134 (cDNA), which translate to a glutamine at amino acid 286 and an aspartic acid at amino acid 948, respectively.

A mutation was found in exon 4 (Fig. 2A), where a base substitution corresponding to position 438 in the cDNA sequence alters adenosine to thymine in the nucleotide sequence, resulting in an amino acid change of lysine at 53 to a stop codon. The mutation creates an Msl cleavage site, which allowed us to set up an assay for genotyping the mutation in the families and controls (Fig. 2B). The mutation segregated with the disease in all six families with local hypoplastic Al previously shown to carry the disease-linked haplotype on chromosome 4q11–q13, and all affected individuals were heterozygous for the mutation (Fig. 2C). Furthermore, five small families with local hypoplastic enamel defects were also genotyped, and in one of these families the mutation was present in the in a heterozygous state in the affected individuals (Table 1). This family carried the disease-linked haplotype of markers centromeric of the enamelin gene (family 10). The remaining small local hypoplastic Al families, K, L, E and 20, did not share the haplotype (data not shown). The mutation was not detected in 184 control chromosomes from the same geographical region in Sweden.

**DISCUSSION**

Enamelin is a protein specific for enamel, since it has not been detected in any other tissues. This enamel matrix protein is secreted by ameloblasts and localized to the secretory face of Tomes processes. Cleavage products are found in the rod and interrod enamel (11). A possible role for enamelin is to control crystal formation together with amelogenin, the most abundant of the enamel matrix proteins, subsequently building up the highly organized structure of hydroxyapatite crystals.

Based on the recent chromosomal localization of ENAM to 4q21 (10,12) and the biological role of the protein that it encodes, this gene was considered an excellent candidate gene for the local hypoplastic type of Al. In this study, we discovered a novel mutation in exon 4 in the six families with local hypoplastic ADAI. Also five additional small families with local hypoplastic Al, not previously described, were genotyped for the mutation, and the patients in one of these families were

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<th>Family</th>
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found to be heterozygous for the mutation. The mutation that we observed in the affected individuals of our kindreds introduces a premature translation termination codon that limits the open reading frame to 52 codons, compared with 1142 codons in the wild-type mRNA. A enamelin has a signal peptide of 39 amino acids, the secreted enamelin protein would be reduced to 13 from 1103 amino acids. It therefore seems likely that the mutant allele is a loss-of-function allele causing the phenotype through haploinsufficiency. Interestingly, Rajpar et al. (13) recently reported of a splice site mutation associated with smooth and thin hypoplastic ADAI. Even though the functional consequences of this mutation are not known, one can speculate that the seemingly worse phenotype ‘smooth and thin hypoplastic enamel’ can be caused by an abnormal protein acting in a dominant negative way to disturb the cooperation of enamelin and amelogenin necessary to build up the enamel. Alternatively, the differences in phenotypes associated with the two ENAM mutations could be due to other genetic or non-genetic factors.

The human ENAM gene spans over 20 kb and has been found to consist of 9 exons and 8 introns (13,15). The murine ENAM gene consists of 10 exons and 9 introns (15). Exon 2 in mouse was absent in the human cDNA, but a sequence homologous to exon 2 is flanked by appropriate splice junctions, raising the possibility that there could be alternative splicing of the enamelin transcript. Still, this exon would not be translated, since the start codon is located downstream of this region. One polymorphism in the non-coding region of intron 4 was found and characterized when screening ENAM for mutations. Also, two divergences between the cDNA sequence (10) and the genomic sequence are reported and appear to be single-nucleotide polymorphisms (SNPs). The finding that both AMBN and ENAM are localized to the same chromosome region suggests that there is a cluster of enamel genes in the region on chromosome 4q. Dong et al. (12) localize ENAM centromeric of AMBN and AIH2. Furthermore, they compare BAC clone insert size and suggest that only 15 kb separates the genes for enamelin and ameloblastin. In the case of a cluster of genes in the AIH2 region encoding more players in enamel formation, genetic analysis of genomic sequence deposited in the databases could provide more candidate genes or a possible main regulatory unit for genes expressed in enamel formation. According to a recent revision of the AI patient material in Northern Sweden, the local hypoplastic phenotype constitutes 35%, (or 77 cases in 11 families) of the autosomal AI cases in northern Sweden are caused by the exon 4 mutation – a mutation that has only occurred once as judged by haplotype analysis. Preliminary data from mutation screening with a larger set of patients from the Swedish material of AI families have not revealed additional mutations in the enamelin gene associated with AI. We have previously shown that there is locus heterogeneity among the autosomal AI (16), demonstrating that there are additional genes outside the 4q region causing autosomal AI.

In summary, the discovery of an ENAM mutation in local hypoplastic AI demonstrates that different mutations in the enamelin gene can cause different clinical variants of ADAI - local and ‘smooth and thin’ hypoplastic enamel. Further, the mutation causing local hypoplastic AI in Northern Sweden is not associated with all cases of local hypoplastic AI. Taken together, this clearly shows that there is a genetic complexity behind the ADAI phenotypes, and indicates the need for further molecular investigations of ADAI.

MATERIALS AND METHODS

Family data

The families included in this study have been described elsewhere (16-19). In brief, six families, called 7, 9, 18, 11, G and W, with the local hypoplastic form of AI were included in the study. The enamel defects in these families were previously shown to be linked to chromosome 4q11-q21. The hypoplastic manifestation could be described as a horizontal row of pits, grooves or a large hypoplastic area in the enamel (Fig. 1). A part from the variations in number and localization of the hypoplastic defects, the phenotype was consistent within families and also between families. The inheritance pattern in all six families was clearly autosomal dominant. The families originate from the same geographical area in Västerbotten County in Northern Sweden and have the same haplotype of genetic markers.

Mutation analysis of the enamelin gene in AI patients

Southern blot analysis. To investigate ENAM for any major alteration in the patients with local hypoplastic AI, Southern blots were performed. Genomic DNA extraction from peripheral blood samples anticoagulated with EDTA was performed as described by Kärman et al. (16). Six micrograms of genomic DNA from one unaffected and one affected individual of the six families with local hypoplastic AI were digested with different restriction enzymes; EcoRI, HindIII, TaqI and BamHI (New England Biolabs, UK) in a total volume of 15 μl and in the temperature recommended by the supplier. The resulting fragments were separated by electrophoresis on a 0.8% agarose gel (SeaKemLE, FMC Bioproducts) and transferred to a hybridization membrane (GeneScreen Plus, NEN). The membranes were incubated for 4-6 h in a prehybridization solution and then hybridized with a [α-32P]dCTP-labeled probe. Fifty nanograms of a human ENAM cDNA clone was used as probe for the analyses. The hybridized nylon membranes were then placed on X-ray film (X-OMAT, AP Biotech) for 1-7 days.

Sequencing of PCR products. Primers were designed from the human genomic sequence and used to amplify PCR fragments from one unaffected and one affected individual with local hypoplastic AI. Primers for amplifying and sequencing exon 1 were forward 5'-AAATGGAACTTGGCTGTG-3' and reverse 5'-GGAGAATTAACCAACATCTATG-3', and for exon 2, the forward primer was 5'-CATGTGCTGCCTTA-GAATTG-3' and reverse 5'-GAGACCTTGATGTATGGCC-3'. The forward primer 5'-TAGATAAGTTCAACATCTATG-3' and reverse 5'-GGTGCTCTACTCAGTACTAAGGC-3' were used to amplify exons 3-5, and the primers used to sequence the PCR products were forward 5'-GCATACCTTTTACAGAC-
CA-3', 5'-TGATGTTCATCGTCGAACTC-3', 5'-AACGGATTTTGGTGAAGTT-3' and reverse 5'-CAATTTGTTC-TAACGGAACAC-3', 5'-GGCTTCGAGGAGTCCTTATC-3' and 5'-AACGGATTTTGGTGAAGTT-3'. For amplifying and sequencing exon 6 the following primers were used: 5'-ATGGAGACAGCAGGTAGTAC-3', 5'-ACCACATTTCATTCATATTC-3' and 5'-TCCAGGCTCTTACGATA-3'. For amplifying and sequencing exon 7, primers 5'-GGCTACACAGG-CCTGGAAAT-3', 5'-TCAAGAATTTGCTGATATGAA-3', 5'-GCAAAAGGGAGATGACAG-3' and 5'-GGTTACTCATTATGTTT-3' were used. For amplifying and sequencing exon 8, primers 5'-ACGAATGGGATTTTCTCCTTGT-3' and 5'-GGCTACAGGG-TATGTGTTGG-3' were used. In the 5' end of exon 9, the following primers were used to amplify and sequence: 5'-ATCATGTGGCTGATAAGATCC-3', 5'-CAAGCAAGAAAGTTCACACC-3', 5'-CATGTTGGGAAAACAAAGGG-3' and 5'-GTGCCCCCCCATGACAGTACGTTAAG-3'. PCR was performed in a total volume of 25 μl containing 50 ng of genomic DNA, 1 x PCR buffer, 200 μM of each nucleotide (Merck, Germany), 1.5 μM of each primer, and 1 unit of Taq DNA polymerase (Roche, Germany). The PCR profile was 95°C/4 min followed by 33-35 cycles of 95°C/1 min, 52-62°C/1 min and 72°C/1 min, and then 72°C for 5 min, and the PCR reactions were performed on a PTC-200 (MJ Research). The PCR products were purified with Microcon PCR concentrators (Amicon, Millipore, Stockholm, Sweden). They were then sequenced by automated sequencing (Applied Biosystems, Model 3700) using Big Dye terminators (PE Biosystems, Foster City, CA) and the PCR primers or internal sequencing primers. The cycle sequencing reaction was run as follows: 96°C/5 min followed by 25 cycles of 96°C/10 s, 50°C/10 s, 60°C/4 min. Sequencing reactions were then purified with ethanol precipitation according to Perkin Elmer recommendations. Bidirectional sequencing using both forward and reverse primers was performed and all sequences were analyzed using the Sequence Navigator software version 1.0.1 and Factura 1.2 software (PE Biosystems).

In the 3' end of the ENAM gene, amplification of DNA from one unaffected and one affected from the local hypoplastic AI family, called 7, yielded PCR products of 1.2 kb and 2.8 kb. The primers used for amplifying these products were 5'-CACAGGGAGCCAGAAGTCAAT-3', 5'-ATCCGGGCTCAATGGGTAAAT-3' and 5'-GAAGATCCAGGGGAGGACA-3', 5'-AGGCGGAGCTTCCTCCTGCTT-3' respectively. These PCR products were ligated into a pCR2.1 vector and 10 independent clones were sequenced by automated sequencing using Big Dye terminators and ABI 377, as described.

Genotyping the mutation in exon 4. To confirm that the mutation cosegregated with the disease phenotype, PCR-amplified genomic DNA was digested with the restriction enzyme Msel and the resulting products were resolved on a 3% agarose gel, consisting of 2/3 Nusieve 3:1 agarose (BMA, Rockland, ME) and 1/3 LE agarose (SeaKem LE, FMC Bioproducts). Digestion of DNA from affected individuals produces bands of 223, 168, 74 and 19 bp, whereas the 168 and 74 band is not present in the unaffected individuals.

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