

A mutation in the enamelin gene in a mouse model for co-dominant Amelogenesis imperfecta

SHORT TITLE: *ENAM MUTATION IN A MOUSE MODEL*

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Abstract

Amelogenesis imperfecta (AI) is an inherited disorder affecting tooth enamel formation. We previously isolated a mouse strain with an AI phenotype from a dominant ethylnitrosourea screen and mapped the disease causing defect to a 9 cM region of mouse chromosome 5. In the current study, we have sequenced two candidate genes, enamelin (Enam) and ameloblastin (Ambn), which are both located within the linkage region. Our data show that the AI phenotype is linked to a C > T transition in exon 8 of the Enam gene. The mutation predicts a C826T transition which is present in the Enam transcript and changes the Gln codon at position 176 into a premature stop codon (Gln176X). Conversely, no mutation could be detected in the Ambn gene. These results define the ATE1 mice as model for local hypoplastic autosomal dominant amelogenesis imperfecta (AIH2) which is caused by ENAM truncation mutations in humans.

Introduction

Tooth Enamel, the exterior coating of vertebrate teeth, is a biomineral with remarkable hardness and resistance to physical and biochemical attack (Schroeder, 1992). Its unique properties are related to large, highly organized hydroxyapatite crystals which contribute more than 90% to the total weight of mature enamel (Brudevold and Störenmark, 1967). The high grade of organization is the result of a complex biomineralization process which is controlled by a regulated expression of multiple genes (Paine et al., 2001). Disorders in this biomineralization process of enamel lead to diseases named Amelogenesis imperfecta (AI). To date, mutations in 5 Genes: amelogenin (AMELX), enamelin (ENAM), kallikrein-4 (KLK4), enamelysin (MMP-20) and DLX3 (Dong et al., 2005) have been found to cause AI. Ameloblastin (AMBN) which has been reported to have a modulating effect on calcium phosphate crystal morphology (Moradian-Oldak et al., 2003) is another candidate gene, but the involvement of AMBN in AI has not yet been proved (Stephanopoulos et al., 2005). We have previously introduced a mouse strain with an AI phenotype, which can serve as a new mouse model for hypoplastic autosomal co-dominant amelogenesis imperfecta (ATE1 mice). We mapped the defect to a 9 cM region of mouse chromosome 5, corresponding to human chromosome 4q21, the location of the human AMBN and ENAM genes (Hu et al., 2000) and hence considered *Ambn* and *Enam* to be the strongest candidates within this region to cause the disease in ATE1 mice (Seedorf et al., 2004).

AMBN (Ameloblastin, or Amelin, or Sheathlin) is part of the organic enamel-matrix. It is expressed at high levels by Ameloblasts during enamel formation, (Fong et al., 1996a) but also by odontoblasts (Nagano et al., 2003), in Hertwig's epithelial root

sheath (Fong et al., 1996b) and in odontogenic tumors (Toyosawa et al., 2000). The mature protein is composed of 447 amino acids. Soon after secretion the initial cleavages generate small polypeptides containing the N-terminus and relatively big polypeptides containing the C-terminus. The meaning of the protein for the mineralisation remains obscure. The murine gene consists of 11 exons and 10 introns. The putative start codon is at 82 base pairs in exon1. The transcript is alternatively spliced in men (Mardh et al., 2001) and mice (Simmons et al., 1998). The involvement of AMBN in AI has not yet been proved (Stephanopoulos et al., 2005), whilst several mutations causing an AI phenotype have been described for ENAM (Stephanopoulos et al., 2005) (Masuya et al., 2005).

ENAM is the largest and least abundant of the three (Enamelin, Amelogenin and Ameloblastin) enamel matrix proteins, representing about 5% of the total proteins found in mature enamel (Uchida et al., 1991). It is a tooth-specific gene expressed by the enamel organ and, at a low level, in odontoblasts (Nagano et al., 2003). The mouse gene consists of 10 exons, while the human ENAM gene contains 9 exons, because the sequence corresponding to mouse exon 2 is absent in men, though the intron between the first and second exon in men shows a sequence homologous to mouse exon 2. This region is flanked by splice junctions, so that alternative splicing may occur (Hu et al., 2001b). Enamelin is expressed during all stages of enamel formation, and its expression ends prior to the expression of Amelogenin (Hu et al., 2001a). Like all enamel matrix proteins ENAM goes through proteolytic processing that leads to cleavage products of 155 kDa, 142 kDa, 89-kDa, 34 kDa, 32 kDa and 25 kDa (Fukae et al., 1996). This processing is believed to be of great importance for proper enamel biomineralization (Stephanopoulos et al., 2005).

In this study, we present the positional cloning of the ATE1 gene and identify a nonsense mutation in the Enam gene as the cause of the disease in ATE1 mice.

Materials & Methods

Mutation Screening and Genotyping

Genomic DNA was extracted from mouse tail tips using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the supplier's instructions. Each exon including the intron boundary regions of the *Ambn* and *Enam* genes were individually amplified by PCR with the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) as recommended by the supplier. The genomic nucleotide sequences used in the construction of primers were derived from a *Mus musculus* chromosome 5 genomic contig, strain C57BL/6J, containing the complete *Ambn* and *Enam* gene sequences (GenBank accession number NT 039308). All primers consisted of 20 nucleotides and had a G/C content of 50%. The following PCR conditions were used in all PCR amplifications: 94°C 15 min; 94°C 0:40 min followed by 19 cycles 93°C 0:40 min, 68°C 1:30 min (-0.5°C/cycle) and 19 cycles 93°C 0:40 min; 58°C 1:30 min (+1 sec./cycle). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and DNA sequencing was performed on an ABI 310 capillary sequencer (Applied Biosystems, Darmstadt, Germany) with sequencing reactions prepared with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) as described by the supplier.

Heterozygotes and homozygotes were categorized according to their phenotypes (see Fig. 1 for details) and three mice of each group were genotyped by DNA sequencing of exon 8.

Animals

The ATE1 mutants were obtained by intraperitoneally injection of ethylnitrosourea in wild-type C3HeB/FeJ animals. Isolation of the ATE1 strain and breeding conditions were described previously (Seedorf et al., 2004).

Results

The AI phenotype was previously mapped to a ~9 cM region of chromosome 5 between markers D5Mit10 and D5Mit18 (map position 45 – 54 Mb according to the Mouse Genome Informatics (MGI[®]) database of the Jackson Laboratory[®] gene map, version 3.5 (Seedorf et al., 2004). Two candidate genes, enamelin (Enam) and ameloblastin (Ambn) are located at map position 46 cM next to each other within the linkage region. In order to screen for AI causing mutations we sequenced all exons including flanking intron sequences and two overlapping DNA fragments of app. 500 bp located upstream of the transcription initiation sites which harbor the putative core promoters of the two genes. In case of the Ambn gene, identical sequences were obtained from homozygous AI mice and mice from the C3HeB/FeJ reference strain (data not shown). However, as shown in Fig. 2, a sequence deviation could be detected in the 8th exon of the Enam gene. The normally occurring cytosine at position 40 of the exon was replaced by thymine in the homozygote. This substitution corresponds to a C826T transition with respect to the cDNA sequence (numbering according to GenBank accession No. NM 017468) which is predicted to change the normally occurring Gln residue at position 176 of the precursor protein into a premature termination signal (Gln176X). As also shown in Fig. 2, detection of a G>A

transition mutation in the sequence of the reverse strand confirmed the presence of the mutation in exon 8. In order to further confirm that the Gln176X allele is associated with AI in ATE1 mice, we next genotyped three mice with the severe phenotype and three mice with the milder heterozygous form of the phenotype. As shown in Fig. 3, all three severely affected mice lacking enamel were homozygous for the mutated T allele whereas the less severely affected mice were heterozygous for the C826T mutation.

Discussion

In this manuscript, we present the positional cloning of the disease causing gene in the ATE1 mutant mouse strain which resulted from a dominant ENU mutagenesis screen (Seedorf et al., 2004). Our results demonstrate that abnormal tooth enamel formation is linked to a mutation in exon 8 of the Enam gene. The mutation, a C826T transition which is predicted to be present in the ENAM transcript, converts the Gln codon at position 176 into a premature stop codon (Gln176X).

ENU is a powerful alkylating mutagen acting on spermatogonial stem cells in mice. Although the most commonly reported ENU-induced mutations are AT-to-TA transversions or AT-to-GC transitions, our finding of a GC-to-AT transition is consistent with the ENU mutagenesis mechanism and ENU-induced GC-to-AT transitions have been described previously in a number of cases (Jansen et al., 1994). In a study of ENU-induced mutations, Noveroske et al. (Noveroske et al., 2000) found that 63% of mutations were missense mutations, 26% caused abnormal splicing, 10% resulted in nonsense mutations, and ~1% caused "make-sense" mutations, which converted a stop codon into an amino-acid-coding codon.

Like all enamel matrix proteins enamelin goes through a complex pathway of proteolytic processing that involves multiple cleavage products (Fukae et al., 1996). This processing is believed to be crucial for proper enamel biomineralization (Stephanopoulos et al., 2005). Intact enamelin and the cleavage products containing the C-terminus are exclusively present at the superficial layers of the developing enamel matrix, whereas the downstream cleavage products accumulate predominantly in the deeper layers (Hu et al., 1997). In porcine enamel, enamelin is secreted by ameloblasts as a 186 kDa precursor, which accumulates along the secretory face of the ameloblast Tomes process. Proteolytic processing towards the C-terminus via a 155 kDa intermediate leads to the 142 kDa form of enamelin. Subsequent cleavage steps result in the 89 kDa fragment consisting of the first 627 amino acids of the enamelin precursor and a 34 kDa polypeptide starting at residue 632. Further processing of the 89 kDa fragment results in polypeptides of 32 kDa (containing amino acids 136–238) and 25 kDa, (containing amino acids 477-638) (Fukae et al., 1996). Since the Gln176X mutation leads to a truncated peptide containing only the first 175 amino acids of the 1,142 amino acid enamelin precursor, all functionally important enamelin processing products would either be absent or truncated significantly in the ATE1 mice. Thus, it seems plausible to us that the Gln176X variant would not be able to mediate proper enamel biomineralization and that the variant is essentially non-functional.

The AI phenotype has previously been mapped to a ~9 cM region between markers D5Mit10 and D5Mit18 at map position 45 – 54 Mb according to the Mouse Genome Informatics (MGI[®]) database of the Jackson Laboratory[®] gene map, version 3.5 (Seedorf et al., 2004). Since *Ambn* and *Enam* are located next to each other at map position 46 Mb in this map, our previous linkage data are consistent with our current

finding of a nonsense mutation in Enam. On the other hand, the most recent build of the mouse Ensemble gene map assigns the two markers to map positions 94.4 and 104.5 Mb, while the Ambn-Enam gene cluster is placed outside the linkage region at map positions 89.53 to 89.58 Mb. Dmp1 (dentin matrix protein 1) and the adjacent LOC666279, encoding a putative protein similar to dentin sialophosphoprotein precursor, which have been considered as candidates for dentinogenesis imperfecta, are located at 104.5 Mb which would be inside the linkage region according to the mouse Ensemble map. However, the MGI[®] gene map places Dmp1 at 56.0 Mb, clearly outside the linkage region, and mutation screening has so far not indicated disease-specific mutations in Dmp1 or other nearby candidate genes including osteopontin and integrin-binding sialoprotein, neither in patients with Amelogenesis imperfecta nor dentinogenesis imperfecta. Conversely, Enam mutations have previously been implicated in the pathogenesis of autosomal dominant AI in humans. The first Enam mutation was described by Rajpar (Rajpar et al., 2001) who found a splicing defect in a patient with autosomal dominant AI. Mardh et al. (Mardh et al., 2002) described an A>T nonsense mutation which was present in codon 53 (Lys73X) in a patient with the local hypoplastic form of autosomal dominant AI and resulted in a truncated peptide of 52 amino acids. Insertion of two nucleotides (AG) in codon 420 and a single base pair deletion in codon 195 was reported to result in truncated variants due to shifts in the normal reading frame in two other patients (Kida et al., 2002) (Hart et al., 2003). Thus, it seems highly likely that Enam and not Dmp1 is involved in the phenotype of the ATE1 mice.

C826T homozygotes differed from heterozygotes based on their enamel abnormalities. Whereas the teeth in heterozygotes had a whitish, chalk-like appearance and microradiographs and toluidine-blue staining of non-decalcified

sections showed cracked enamel of reduced width (approx. 50%), homozygotes showed complete loss of enamel. Thus, the mutation is inherited as a co-dominant trait.

In summary, our results provide further support for Enam as prominent factor involved in the pathogenesis of AI and define the ATE1 mice as model for local hypoplastic autosomal dominant amelogenesis imperfecta (AIH2) which is caused by Enam truncation mutations also in humans.

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References

- Brudevold F, Störenmark R (1967). Chemistry of the mineral phase of enamel New York: Academic Press.
- Dong J, Amor D, Aldred M, Gu T, Escamilla M, MacDougall M (2005). DLX3 mutation associated with autosomal dominant amelogenesis imperfecta with taurodontism. *Am J Med Genet A* 133(138-41).
- Fong C, Hammarstrom L, Lundmark C, Wurtz T, Slaby I (1996a). Expression patterns of RNAs for amelin and amelogenin in developing rat molars and incisors. *Adv Dent Res* 10(195-200).
- Fong C, Slaby I, Hammarström L (1996b). Amelin: an enamel-related protein, transcribed in the cells of epithelial root sheath. *J Bone Miner Res.* 11(892-8).
- Fukae M, Tanabe T, Murakami C, Dohi N, Uchida T, Shimizu M (1996). Primary structure of the porcine 89-kDa enamelin. *10(Adv Dent Res):111-8.*
- Hart TC, Hart PS, Gorry MC, Michalec MD, Ryu OH, Uygur C, et al. (2003). Novel ENAM mutation responsible for autosomal recessive amelogenesis imperfecta and localised enamel defects. *J Med Genet.* 40(900-906).
- Hu CC, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, et al. (1997). Cloning and characterization of porcine enamelin mRNAs. *J Dent Res.* 76(1720-1729).
- Hu CC, Hart T, Dupont B, Chen J, Sun X, Qian Q, et al. (2000). Cloning human enamelin cDNA, chromosomal localization, and analysis of expression during tooth development. *J Dent Res* 79(912-9).
- Hu JC, Sun X, Zhang C, Simmer JP (2001a). A comparison of enamelin and amelogenin expression in developing mouse molars. *Eur J Oral Sci.* 109(125-32).
- Hu JC, Zhang CH, Yang Y, Karrman-Mardh C, Forsman-Semb K, Simmer JP (2001b). Cloning and characterization of the mouse and human enamelin genes. *J Dent Res* 80(898-902).
- Jansen J, Mohn G, Vrieling H, van Teijlingen C, Lohman P, van Zeeland A (1994). Molecular analysis of hprt gene mutations in skin fibroblasts of rats exposed in vivo to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. *Cancer Research* 54(2478-2485).
- Kida M, Ariga T, Shirakawa T, Oguchi H, Sakiyama Y (2002). Autosomal-dominant hypoplastic form of amelogenesis imperfecta caused by an enamelin gene mutation at the exon-intron boundary. *J Dent Res* 81(738-742).
- Mardh CK, Backman B, Simmons D, Golovleva I, Gu T, Holmgren G, et al. (2001). Human ameloblastin gene: genomic organization and mutation analysis in amelogenesis imperfecta patients. *Eur J Oral Sci* 109(8-13).

- Mardh CK, Backman B, Holmgren G, Hu JC, Simmer JP, Forsman-Semb K (2002). A nonsense mutation in the enamelin gene causes local hypoplastic autosomal dominant amelogenesis imperfecta (AIH2). *Hum Mol Genet* 11(1069-1074).
- Masuya H, Shimizu K, Sezutsu H, Sakuraba Y, Nagano J, Shimizu A, et al. (2005). Enamelin (Enam) is essential for amelogenesis: ENU-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (AI). *Hum Mol Genet* 14(575-83).
- Moradian-Oldak J, Iijima M, Bouropoulos N, Wen H (2003). Assembly of amelogenin proteolytic products and control of octacalcium phosphate crystal morphology. *Connect Tissue Res* 44(58-64).
- Nagano T, Oida S, Ando H, Gomi K, Arai T, Fukae M (2003). Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J Dent Res* 82(982-6).
- Noveroske JK, Weber JS, Justice MJ (2000). The mutagenic action of N-ethyl-N-nitrosourea in the mouse. *Mamm. Genome* 11(478-483).
- Paine M, White S, Luo W, Fong H, Sarikaya M, Snead M (2001). Regulated gene expression dictates enamel structure and tooth function. *Matrix Biol.* 20(273-92).
- Rajpar MH, Harley K, Laing C, Davies RM, Dixon MJ (2001). Mutation of the gene encoding the enamel-specific protein, enamelin, causes autosomal-dominant amelogenesis imperfecta. *Hum Mol Genet* 10(1673-7).
- Schroeder H (1992). Oral structure biology New York: Thieme.
- Seedorf H, Springer IN, Grundner-Culemann E, Albers HK, Reis A, Fuchs H, et al. (2004). Amelogenesis imperfecta in a new animal model--a mutation in chromosome 5 (human 4q21). *J Dent Res* 83(608-612).
- Simmons D, Gu T, Krebsbach P, Yamada Y, MacDougall M (1998). Identification and characterization of a cDNA for mouse ameloblastin. *Connect Tissue Res* 39(3-12).
- Stephanopoulos G, Garefalaki M, Lyroudia K (2005). Genes and related proteins involved in amelogenesis imperfecta. *J Dent Res* 84(1117-26).
- Toyosawa S, Fujiwara T, Ooshima T, Shintani S, Sato A, Ogawa Y, et al. (2000). Cloning and characterization of the human ameloblastin gene. *Gene* 256(1-11).
- Uchida T, Tanabe T, Fukae M, M S (1991). Immunocytochemical and immunochemical detection of a 32 kDa nonamelogenin and related proteins in porcine tooth germs. *Arch Histol Cytol* 54(527-38).

Figure legends

Figure 1: Phenotype of the ATE1 mutant mice. Homozygotes and heterozygotes differ from wild-type mice based on the shown tooth abnormalities. Wild-type mice (left) have brown incisors, whereas teeth in heterozygous ATE1 mice (middle) have a whitish, chalk-like appearance. Homozygotes (right) show complete loss of enamel in the front and molar teeth.

Figure 2: Identification of a C826T (Gln176X) mutation in exon 8 of the ENAM gene. Shown are sequence tracings (left: plus strand, forward; right: minus strand, reverse) obtained for exon 8 of the ENAM gene from a homozygous ATE1 mouse (top) and a C3HeB/FeJ control (bottom). The amino acid sequence derived after translation of the ENAM cDNA is given below the nucleotide sequence. The mutated nucleotide is underlined; ###: translational termination signal.

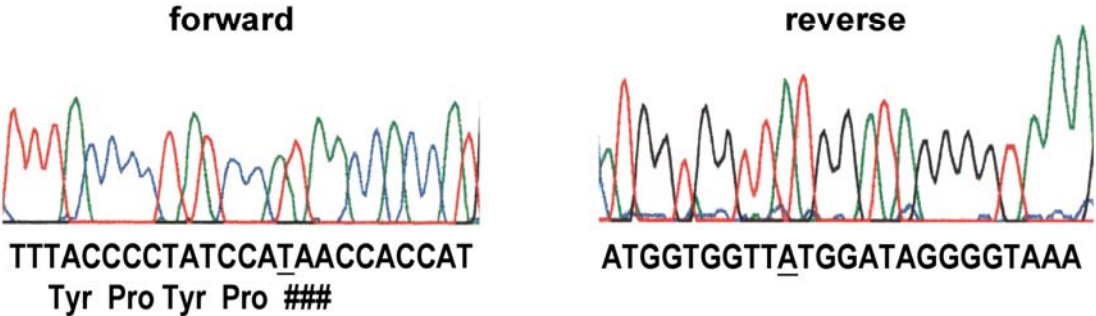
Figure 3: Comparison of sequence tracings obtained for wild-type and heterozygous or homozygous ATE1 mice. Mice were categorized according to their phenotypes (see Fig. 1) and three mice of each group were genotyped by DNA for the C826T allele as described in the Methods section. The mutated nucleotide is underlined.

Figures



Figure 1

ATE1 homozygote



C3HeB/FeJ wild-type

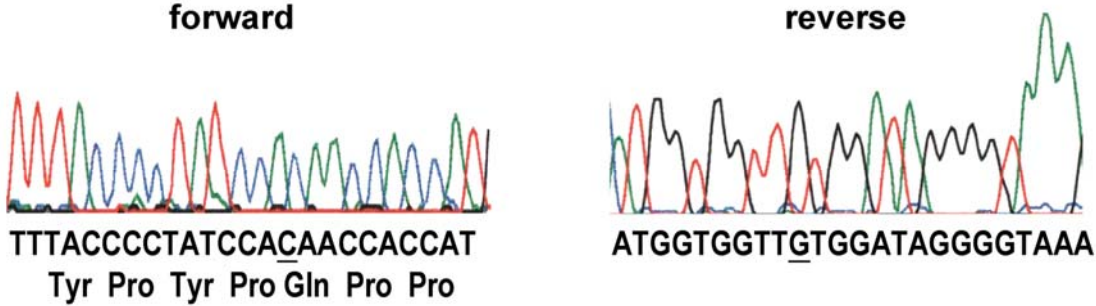
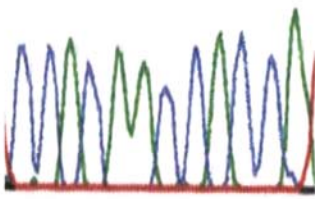
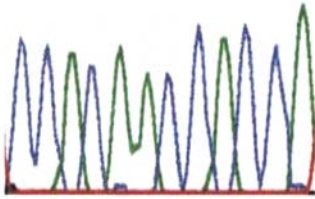
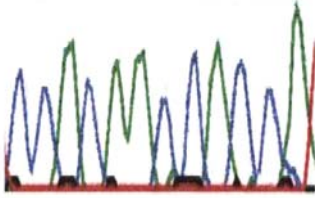


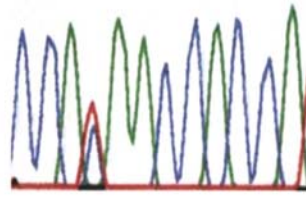
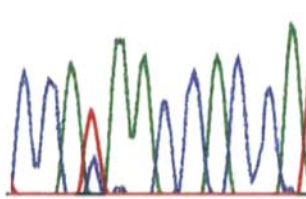
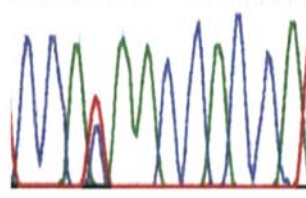
Figure 2

**C3HeB/FeJ
wild-type**



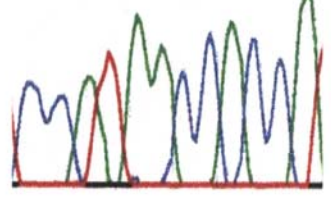
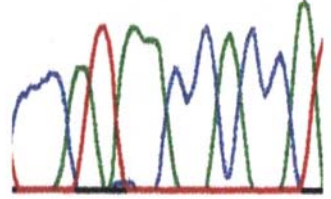
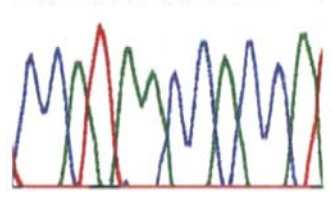
CCAAACCACCA

**ATE1
heterozygotes**



**CCAAACCACCA
C**

**ATE1
homozygotes**



CCAAACCACCA

Figure 3