Target gene analyses of 39 amelogenesis imperfecta kindreds

Previously, mutational analyses identified six disease-causing mutations in 24 amelogenesis imperfecta (AI) kindreds. We have since expanded the number of AI kindreds to 39, and performed mutation analyses covering the coding exons and adjoining intron sequences for the six proven AI candidate genes [amelogenin (AMELX), enamelin (ENAM), family with sequence similarity 83, member H (FAM83H), WD repeat containing domain 72 (WDR72), enamelysin (MMP20), and kallikrein-related peptidase 4 (KLK4)] and for ameloblastin (AMBN) (a suspected candidate gene). All four of the X-linked AI families (100%) had disease-causing mutations in AMELX, suggesting that AMELX is the only gene involved in the aetiology of X-linked AI. Eighteen families showed an autosomal-dominant pattern of inheritance. Disease-causing mutations were identified in 12 (67%); eight in FAM83H, and four in ENAM. No FAM83H coding-region or splice-junction mutations were identified in three probands with autosomal-dominant hypocalcification AI (ADHCAI), suggesting that a second gene may contribute to the aetiology of ADHCAI. Six families showed an autosomal-recessive pattern of inheritance, and disease-causing mutations were identified in three (50%); two in MMP20, and one in WDR72. No disease-causing mutations were found in 11 families with only one affected member. We conclude that mutation analyses of the current candidate genes for AI have about a 50% chance of identifying the disease-causing mutation in a given kindred.

Dental enamel formation is a specialized process. Unravelling the mechanisms of dental enamel formation requires knowledge of all critical molecular participants. Determining the genes that cause syndromic and isolated AI provides this information. Identifying the genes that cause isolated AI narrows the focus to components that are most specialized for dental enamel formation. At the time of writing there were six proven candidate genes for AI: amelogenin (AMELX, Xp22.3-p22.1); enamelin (ENAM, 4q21); WD repeat containing domain 72 (WDR72, 15q21.3); family with sequence similarity 83, member H (FAM83H, 8q24.3); enamelysin (MMP20, 11q22.3-q23); and kallikrein-related peptidase 4 (KLK4, 19q13.4). The major secretory-stage enamel constituents (amelogenin, enamelin, ameloblastin, and MMP20) are thought to be specialized for tooth formation, as these genes degenerate in mammals that have lost, during evolution, the ability to make teeth or dental enamel (6–10).

At the Enamel VII conference (in 2005), we presented a paper that summarized the results of mutational analyses of 24 kindreds with isolated AI (11). At that time, only six of their disease-causing mutations had been identified. Since that report, new candidate genes for AI have been identified, and additional AI kindreds have been recruited. We now know the disease-causing mutations for 12 of the original 24 families and have ruled out the presence of coding region and splice junction mutations in the six known AI candidate genes plus ameloblastin (AMBN) in the families where the genetic cause could not be determined. Here we report the results of mutation analyses in
the original 24 and 15 additional AI kindreds and discuss them in the context of new information concerning normal and pathological enamel formation.

Material and methods

The human study protocol and patient consents were reviewed and approved by the Institution Review Boards at the University of Michigan.

Fifteen families with isolated enamel defects were recruited for genetic studies. Mutational analyses for AMBN were conducted in all families. Based upon the enamel phenotype and pattern of inheritance, the most likely of the six proven candidate genes for AI (AMELX, ENAM, FAM83H, WDR72, MMP20, and KLK4) was selected and the coding exons and nearby intron sequences were amplified, using genomic DNA from each proband as the template. The amplification products were characterized by DNA sequencing and then other family members were tested to determine their genotype with respect to each sequence variation; however, typically there were too few people in each family for haplotype analyses. All subjects received an oral examination, and intra-oral photographs and dental radiographs were obtained.

In most cases, 10 ml of peripheral whole blood was obtained from participating family members. Alternatively, buccal swabs were performed. Genomic DNA was isolated using the QIAamp DNA Blood Maxi Kit and protocol (Qiagen, Valencia, CA, USA). Genomic DNA (50 ng) from affected individuals was amplified using the Platinum PCR Supermix (Invitrogen, Carlsbad, CA, USA), and the amplification products were purified using the QIAquick PCR Purification Kit and protocol (Invitrogen). The concentration of purified amplimer was estimated by the intensity of its ethidium bromide-stained band on a 1% agarose gel. The DNA-sequencing reactions used 1.0 pmol/µl of oligonucleotide primer and 3 ng/µl for each 1000 bp of amplification product, and were analyzed using an ABI Model 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Michigan DNA sequencing core. The primer pairs and PCR conditions for the amplification of the coding regions were as previously described for AMBN, AMELX, ENAM, FAM83H, KLK4, and MMP20.

Results

Original 24 AI kindreds

Disease-causing mutations in 12 of our original 24 AI kindreds (11) have been identified (Table 1). Two families showed an X-linked pattern of inheritance and both had AMELX mutations (12). Eleven families showed a dominant pattern of inheritance. Three of these families had ENAM mutations, and five had FAM83H defects. Five families showed recessive transmission. Six families had only a single affected individual (simplex pedigrees), A simplex inheritance pattern is when only one person in a kindred is affected, so the inheritance pattern appears to be recessive but could be a de novo or sporadic mutation. Note that one simplex case was found to be dominant and another was found to be recessive once the disease-causing mutation was identified.

AD, autosomal dominant; ADAI, autosomal-dominant amelogenesis imperfecta; AMELX, amelogenin; AR, autosomal recessive; ARAI, autosomal-recessive amelogenesis imperfecta; ENAM, enamelin; FAM83H, family with sequence similarity, member H; MMP20, enamelysin; WDR72, WD repeat containing domain 72.
so the pattern of transmission could not be determined. Simplex cases are usually recessive, but they can be the manifestations of de novo mutations with any inheritance pattern possible. Among the five families showing recessive transmission, we identified one disease-causing mutation in both alleles of MMP20 and one in both alleles of WDR72. Among the six simplex cases, a disease-causing mutation was identified in a single allele of FAM83H, so the inheritance pattern in this simplex case was actually autosomal dominant. In addition to the six proven AI candidate genes, mutational analyses for AMBN were performed for all of the probands, but no disease-causing mutations were identified in this gene.

Success in determining the causative mutation in these AI kindreds varied, depending upon the pattern of transmission. The disease-causing mutations were identified in both cases showing X-linked inheritance (100%). Among the 12 kindreds with dominant conditions (11 with a dominant pattern of inheritance and a dominant de novo mutation), disease mutations were characterized in eight (67%). Among the five recessive conditions, only two (40%) of the causative mutations were identified.

Additional AI kindreds
Fifteen additional AI kindreds were recruited and extensively, but not exhaustively, characterized. Of these additional kindreds, two were X-linked, six were dominant, and seven were simplex. Mutational analyses for the six AI candidate genes were prioritized based upon previously established genotype–phenotype correlations,

Fig. 1. Oral photographs of probands and pedigrees of eight amelogenesis imperfecta (AI) families of unknown aetiology. A dot on the pedigree marks subjects recruited in the study. Numbering starts with 25, as a continuation of previous work reporting mutational analyses of 24 AI kindreds (11). Pedigree analyses showed that families 25 and 26 have a dominant pattern of inheritance. Families 27–32 have either a recessive pattern of transmission or are de novo mutations.
and their transmission patterns were deduced from the pedigrees. Using this approach we were not able to identify a disease-causing mutation in eight of the 15 probands (Fig. 1). Among the seven remaining probands, four had novel mutations (Fig. 2), which included a nonsense mutation (p.W34X) in both alleles of enamelysin (MMP20) (18), a complete deletion of amelogenin (AMELX) (H-C. Chan, unpublished data). The proband of family 35 has autosomal-dominant hypoplastic AI caused by a p.Q398X mutation in one allele of family with sequence similarity 83, member H (FAM83H) (19). The proband of family 36 has a severe form of hypoplastic AI resulting from different mutations (p.422FsX448 and p.S216L) in both enamelin (ENAM) alleles (20). The father (p.422FsX448) and mother (p.S216L) had only one affected ENAM allele and both showed a very mild, but detectable, enamel phenotype.

Among the 39 AI kindreds characterized, disease-causing mutations were identified in 19, so screening a given AI kindred for mutations in the six proven candidate genes has about a 50% chance of success. AMELX mutations were identified in all four of the X-linked families (100%). Four ENAM and eight FAM83H mutations were identified among the 18 autosomal-dominant families (67%). One WDR72 and two MMP20 mutations were found among the six AI kindreds with known recessive conditions (50%). In addition, there were 11 simplex cases in which the disease-causing mutation could not be identified by mutational analyses of the known candidate genes.
Discussion

Screening the six proven AI candidate genes in kindreds with isolated AI has about a 50% chance of identifying the causative mutation, but the odds vary depending upon the pattern of inheritance. Next we discuss the findings of this study in the light of recent advances in our understanding of each form of AI according to its transmission pattern.

X-linked AI

About 5–10% of all AI cases are X-linked (21). Currently, 16 different AMELX mutations have been reported in subjects with X-linked AI (Table 2). If the AI is X-linked, a mutation in AMELX is predictably found, suggesting that no other genes on the X-chromosome are involved in its aetiology. In all 16 AMELX mutations, the phenotype was limited to the enamel layer, excepting an open bite, which is probably a secondary effect as it is observed in all forms of AI, regardless of which gene is defective.

None of the reports that describe the dental phenotype resulting from defined AMELX mutations detected developmental abnormalities in the periodontium (cementum, periodontal ligament, and alveolar bone), despite the fact that such abnormalities would certainly have been diagnosed if they were part of the dental phenotype, given that dental examinations and radiographs are routinely used to characterize the dentition in patients with AI. In addition to there being no reports of developmental periodontal defects in individuals with AMELX mutations, there are solid data showing no, or only trace expression of amelogenin along developing tooth roots (22–24). These findings undermine the conclusions of an extensive and growing literature that describes amelogenin as a signalling molecule which serves important functions in the formation and healing of the periodontium.

As a rule, enamel malformations in X-linked AI are more severe in men than in women (who have a second copy of AMELX). Women often show vertical grooves in their enamel crowns. These are believed to result from alternating bands of ameloblasts secreting normal and
defective amelogenin during amelogenesis, depending upon which X-chromosome had been inactivated by the individual cells that later developed into ameloblasts (2, 25). The severity of the enamel phenotype in women can be influenced by skewed X-inactivation, where a disproportionate number of cells inactivate the X-chromosome carrying the mutant, or alternatively, the normal allele (26). The enamel phenotype in subjects with AMELX defects generally correlates with the nature of the AMELX mutation (27, 28). The phenotype in our family with the p.P70T defect in AMELX is consistent with previous descriptions of hypomineralization/hypomaturation enamel with brown discolouration (Figs 5, S1, and S2) (29–32).

Although amelogenins comprise 90% of the enamel matrix, only 5–10% of cases AI are X-linked. Abundance of the protein product is not a factor in determining the relative importance of AMELX in the aetiology of AI, which is a collection of many diseases exhibiting isolated enamel malformations. The number of AI-causing genes relates to the number of genes specialized for dental enamel formation (i.e. a loss of function would result in isolated enamel defects) plus the number of genes that function in multiple places, but enamel formation is most sensitive to their absence or alteration. Even if we knew the number of genes involved in the aetiology of AI, their individual contributions (in percentages of the total number of cases) would vary significantly, depending upon the number of mutations in each gene that could cause AI, the chances of these mutations occurring, and whether the resulting phenotypes would be dominant or recessive. Identifying the pantheon of genes that cause AI, and understanding their pathogenesis and mode of inheritance will allow us to appreciate the contributions of individual genes to the aetiology of AI as a whole.

Autosomal-dominant AI

About 46% of our AI kindreds showed a dominant pattern of inheritance, which is a lower proportion than found in Sweden (~61%) (33). It is likely, however, that a few of the remaining 11 simplex cases will prove to be dominant mutations. Most of the autosomal-dominant AI (ADAI) in our kindreds (12 out of 18 cases) was caused by defects in ENAM or FAM83H.

To date, 11 different disease-causing ENAM mutations have been reported (Table 3). ENAM defects show a dose effect (34). When one allele is defective the phenotype can range from non-penetrant (35), to minor...
well-circumscribed pits or horizontal grooves (13, 20, 34, 36), to pronounced horizontal grooves (37) with generally very thin enamel (38, 39), to virtually no enamel (13). When both ENAM alleles are defective, there is extreme enamel hypoplasia or no enamel (20, 34, 35, 40). In all four reported cases where both ENAM alleles are defective, one or both alleles had the p.V422PfsX448 mutation. In one case where the enamel was extremely thin and smooth, there was also defective eruption and coronal resorption (40), which we hypothesize was caused by secondary pathology in the enamel organ. The ameloblast layer becomes increasingly pathological as amelogenesis progresses in the absence of a true enamel layer in the Enam null mice (41, 42).

Although only recently discovered to be part of the aetiology of inherited enamel defects, FAM83H accounts for more cases of ADAI than any other gene. This was true for this study and also for another study that performed mutational analyses on 91 families with inherited enamel defects, 71 of which satisfied the classical criteria for non-syndromic AI (32). FAM83H encodes an intracellular protein of unknown function that appears to be associated with the Golgi apparatus or trans-Golgi network (19), and is most strongly expressed by pre-ameloblasts (43). In the last few years, 18 different FAM83H disease-causing mutations have been reported (Table 4). All of the defects are missense or frameshift mutations located within a discrete region of the last exon that truncate the protein and apparently target it to the nucleus (44). The N-terminal region is the only part of the mutant proteins that is translated. This domain shares homology with the phospholipase D superfamily and is assumed to form dimers or to interact with another protein to cause dominant-negative effects in ameloblasts or their progenitors.

Defects in FAM83H cause autosomal-dominant hypocalcified AI (ADHCAI). The enamel layer has normal thickness in newly erupted teeth, but is rapidly lost by attrition following eruption and the teeth turn brown. Enamel that is not lost by attrition can sometimes persist indefinitely, such as islands of normal enamel. In this report we show the oral phenotypes of two new cases of hypocalcification AI caused by FAM83H defects. The first is the primary dentition of a Caucasian 3-yr-old boy with a p.Q677X mutation in FAM83H (Fig. 4). The second is a Caucasian family with a p.Q452X mutation (Figs 5, S2, and S3).

Some isolated cases of ADAI may come from defects in genes associated with syndromic AI. Collagen, type XVII, alpha 1 (COL17A1) contributes to the aetiology of junctional epidermolysis bullosa, a recessive disorder showing skin fragility, and AI (45). Mutations in a single allele of COL17A1 can cause isolated enamel defects (46, 47) or enamel defects combined with relatively minor skin fragility (48). Amelogenesis imperfecta can also occur along
Table 3

**Enamelin (ENAM) disease-causing mutations**

<table>
<thead>
<tr>
<th>Mutation no.</th>
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<th>cDNA</th>
<th>Protein</th>
<th>References</th>
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<td>p.W4S</td>
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<td>29–32 (here)</td>
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<td>13</td>
<td>g.(5421_5422)delC</td>
<td>c.(472_473)delC</td>
<td>p.P158H6X187</td>
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<td>14</td>
<td>g.(5466_5477)delC</td>
<td>c.(517_518)delC</td>
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<td>15</td>
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<td>c.(538_541)delC</td>
<td>p.L181CfsX187</td>
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<tr>
<td>16</td>
<td>g.5520G&gt;T</td>
<td>c.571G&gt;T</td>
<td>p.E191X</td>
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**AMELX gene structure:** numbered boxes indicate exons; introns are lines connecting the exons. Shaded exon regions are non-coding. The numbers below each exon show the range of amino acids encoded by it. Bold numbers indicate **AMELX** mutations. Mutation 5 is the deletion indicated by dashed lines. The gene numbers start from the first nucleotide of the **AMELX** reference sequence NG_012040.1. The cDNA numbers start from the translation initiation site of **AMELX** reference sequence NM_182680.1.

Table 2

**Amelogenin (AMELX) disease-causing mutations**

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Table 3

**Enamelin (ENAM) disease-causing mutations**

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Both **ENAM** alleles affected

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<th>Allele 2</th>
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<td>p.V422PfsX448</td>
<td>p.V422PfsX448</td>
<td>35, 40</td>
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</table>

**ENAM gene structure:** numbered boxes indicate exons; introns are lines connecting the exons. The numbers below each exon show the range of amino acids encoded by it. Shaded exon regions are non-coding. Bold numbers indicate **ENAM** mutations. The gene numbers start from the first nucleotide of the **ENAM** reference sequence NG_013024.1. The cDNA numbers start from the translation initiation site of **ENAM** reference sequence NM_031889.2.

*{g.13703_13704insAGTCAGTACCAGTACTGCTGTC; c.1020_1021insAGTCAGTACCAGTACTGCTGTC; p.V340_M341insSQYQYCVc*}
with nephrocalcinosis. Although nephrocalcinosis can be associated with impaired renal function, it is often asymptomatic and may go undiagnosed (49, 50). The genetic aetiology of this condition is currently unknown.

Autosomal-recessive AI

The diversity of enamel phenotypes in autosomal-recessive AI (ARAI) suggests that a large number of genes are involved in its aetiology (51). Only 10 mutations (in KLK4, MMP20, and WDR72) causing ARAI have been reported (Table 5). Consanguinity is a major contributor, as in all cases the same gene defect was observed in both alleles. Six of our 39 AI kindreds are known to have autosomal-recessive transmission patterns, and three of the causative mutations have been characterized in this group. We suspect that most of our simplex cases will turn out to be recessive conditions, as was determined for our kindred 33.

MMP20 and KLK4 are secreted proteases that cleave amelogenin, ameloblastin, and enamelin in developing teeth (52). Cleavage of amelogenin reduces its affinity for hydroxyapatite (53). Mmp20 cleaves enamel proteins during the secrectory stage and the cleavage products accumulate in the matrix. Klk4 is expressed in the maturation stage and degrades the accumulated organic matrix to facilitate its reabsorption into ameloblasts (54). The enamel produced by MMP20 and KLK4 null mice is soft and chips away after the teeth erupt into function (55–58). Based upon their known roles in enamel formation, it is not surprising that MMP20 and KLK4 defects cause AI.

WDR72 had no suspected role in enamel formation before it was shown, by genetic analyses, to cause AI (59). The gene is expressed by maturation-stage ameloblasts, and defects in both alleles cause hypomaturation AI. The teeth erupt with a creamy-brown colour, a rough texture, and reduced radiodensity when compared with normal enamel. Posteruptive changes vary, but the enamel can turn a deep orange-brown colour and undergo accelerated attrition, particularly on working surfaces. The five reported WDR72 disease-causing mutations were all truncation mutations in both alleles. Perhaps the deleted C-terminal domain performs an essential function in ameloblasts, although there are currently no data showing that the defective transcripts are translated into protein.

### Table 4

<table>
<thead>
<tr>
<th>No.</th>
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<th>cDNA</th>
<th>Protein</th>
<th>References</th>
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<tr>
<td>3</td>
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<td>c.1192C&gt;T</td>
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<td>c.1872_1873delCC</td>
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<td>c.2080G&gt;T</td>
<td>p.E694X</td>
<td>80</td>
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</tbody>
</table>

FAM83H gene structure: numbered boxes indicate exons; introns are lines connecting the exons. The numbers above each intron indicate the length of the intron in base pairs (bp). The numbers below each exon show the lengths of the exon in bp and below that the range of amino acids encoded by it. Shaded exon regions are non-coding. The 17 reported FAM83H missense or frameshift mutations are located between the sites marked 1 and 17 in bold. The gene numbers start from the first nucleotide of the FAM83H reference sequence NG_016652.1. The cDNA numbers start from the translation initiation site of FAM83H reference sequence NM_1984883.3.

null mice is soft and chips away after the teeth erupt into function (55–58). Based upon their known roles in enamel formation, it is not surprising that MMP20 and KLK4 defects cause AI.

WDR72 had no suspected role in enamel formation before it was shown, by genetic analyses, to cause AI (59). The gene is expressed by maturation-stage ameloblasts, and defects in both alleles cause hypomaturation AI. The teeth erupt with a creamy-brown colour, a rough texture, and reduced radiodensity when compared with normal enamel. Posteruptive changes vary, but the enamel can turn a deep orange-brown colour and undergo accelerated attrition, particularly on working surfaces. The five reported WDR72 disease-causing mutations were all truncation mutations in both alleles. Perhaps the deleted C-terminal domain performs an essential function in ameloblasts, although there are currently no data showing that the defective transcripts are translated into protein.

Aetiology of ARAI

The six proven candidate genes for AI account for about half of all AI cases (Table 1). Although the genes responsible for ADAI remain to be identified, less is known about the genetic aetiology of ARAI. So far, no AI-causing mutations have been identified in the genes encoding several proteins exported by ameloblasts, such
as AMBN (60), amelotin (AMTN) (61), and odontogenic ameloblast-associated protein (ODAM) (62). We suspect that these genes are involved in ARAI. The characterization of large consanguineous AI kindreds will continue to yield discoveries, but the application of whole-exome sequencing, which involves fragmenting genomic DNA and capturing coding sequences by hybridization to a chip, followed by next-generation DNA sequencing, is likely to yield important information concerning the diverse genetic aetiology of ARAI. This technique has recently been used to discover that FAM20A is defective in subjects with AI and gingival hyperplasia syndrome (63).

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Conflicts of interest – All authors declare that there are no potential conflicts of interest.

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Oral photographs and radiographs of the family 37 (AMELX, pP70T) proband at age 11.5.

Fig. S2. Oral photographs of the unaffected father (II:4) of family 37 (AMELX, pP70T).

Fig. S3. Oral photographs for family 39 (FAM83H, pQ452X). Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.